



HAL
open science

Metabolic regulations and neuron-glia interactions underlying long-term memory formation in *Drosophila melanogaster*

Eloise de Tredern

► **To cite this version:**

Eloise de Tredern. Metabolic regulations and neuron-glia interactions underlying long-term memory formation in *Drosophila melanogaster*. *Neurons and Cognition [q-bio.NC]*. Université Paris sciences et lettres, 2019. English. NNT : 2019PSLET009 . tel-03331628

HAL Id: tel-03331628

<https://pastel.hal.science/tel-03331628>

Submitted on 2 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée à l'ESPCI Paris

**Metabolic regulations and neuron-glia interactions
underlying long-term memory formation in
*Drosophila melanogaster***

**Régulations métaboliques et interactions glie-neurones
soutenant la formation de la mémoire à long terme chez
*Drosophila melanogaster***

Soutenue par

Éloïse de Tredern

Le 13 Septembre 2019

Ecole doctorale n° 158

**Cerveau,
Cognition,
Comportement**

Spécialité

Neurosciences

Composition du jury :

Dr. Nathalie Rouach Directrice de Recherche au Collège de France, PSL	<i>Présidente</i>
Pr. Ilona Grunwald Kadow Professeure à l'Université technique de Munich	<i>Rapportrice</i>
Dr. Jean-Maurice Dura Directeur de Recherche à l'Université de Montpellier	<i>Rapporteur</i>
Dr. Gilles Bonvento Directeur de Recherche à l'Université Paris Saclay	<i>Examineur</i>
Dr. Pierre-Yves Plaçais Chargé de Recherche à l'ESPCI, PSL	<i>Examineur</i>
Dr. Thomas Préat Directeur de Recherche à l'ESPCI, PSL	<i>Directeur de thèse</i>

Acknowledgements

J'aimerais remercier tout d'abord mes encadrants, Pierre-Yves et Thomas, tous les deux, pour leur présence constante, leur réactivité et leurs qualités scientifiques ; tous les deux, pour m'avoir offert des opportunités professionnelles uniques en m'envoyant à des congrès. Pierre-Yves, tu as été un véritable mentor, avec la mesure et l'attention que ce statut exige. Merci pour tes schémas et explications. Merci pour la qualité de tes critiques, toujours communiquées avec (légèreté,) tact et pertinence. Et merci pour les concerts ! Thomas, ton enthousiasme et ton soutien, ponctués avec talent de remarques cinglantes, m'ont à la fois motivée et beaucoup amusée. Merci d'insuffler autant d'énergie positive dans l'équipe. Je dois avouer qu'on ne s'est jamais ennuyés en réunion tous les trois, entre science et éclats de rire. Enfin, merci à tous les deux de m'avoir tant enrichie scientifiquement.

Merci aussi à mon jury de thèse de vous être mobilisés pour lire et commenter mon manuscrit, et pour assister à ma soutenance et discuter de mes résultats.

Si mes encadrants de thèse ont éveillé mon intérêt pour la recherche en neuroscience sur notre merveilleux modèle animal, d'autres scientifiques ont ouvert ma curiosité à la biologie. Les premiers à avoir stimulé mon intérêt pour les sciences de la vie, ce sont des professeurs de biologie de collège et de lycée, que je remercie profondément. En particulier, un certain Mr. T., dont j'aimais les cours et admirais la rigueur autant que je haïssais le caractère. Après le lycée, des professeurs du supérieur se sont évertués à me transmettre leur savoir et m'ont aidée à me structurer. Merci, Rémi Dejean, pour ta générosité en plus de ta déontologie. Merci chère équipe d'enseignants de l'ENS de ~~Cachan~~ Paris Saclay, pour vos cours de qualité, votre implication auprès de vos élèves, vos conseils, et bien sûr, merci de m'avoir donné l'opportunité de voir l'envers du décor - organiser 2h de cours en neurosciences en agrégation m'a fait prendre conscience de la précision de vos enseignements, et du temps que vous devez y consacrer. Merci aussi à Zsolt Lenkei de m'avoir fait confiance dans l'organisation des TDs de biologie en CPES. C'était une expérience unique au contact d'élèves de qualité, et qui m'a profondément touchée. Merci d'ailleurs particulièrement aux élèves, qui m'ont donné goût à l'enseignement, et dont les qualités m'ont impressionnée.

Merci à toutes mes autres rencontres scientifiques, qui m'ont apporté leurs questions, leurs réponses, leurs passions, leurs frustrations aussi (ça soulage de ne pas être seul). Vous rencontrer, et par conséquent, vouloir continuer à travailler avec vous est l'une des raisons qui m'ont convaincue de continuer en post-doctorat après ma thèse. En particulier, merci aux rencontres variées pendant les congrès auxquels j'ai eu la chance de participer, et dont j'ai pu apprécier les conseils techniques et les projets scientifiques dans des contextes exceptionnels.

Merci à tous les membres actuels et passés de l'équipe GDSM. Merci Valérie pour ta gentillesse, ton humanité et ton expertise scientifique que j'ai exploitée à plusieurs reprises. Merci Alice pour la pertinence de tes suggestions, et pour ces moments passés ensemble qui m'ont appris à découvrir une personnalité généreuse et enthousiaste, en particulier à Lausanne, en Normandie et en Bretagne. Merci Aurélie, ma première voisine, et non des moindres ! Merci pour tes conseils – en particulier en BM – et ton humour (quelques petites piques, ça évite de prendre la grosse tête n'est-ce pas). Malgré la présence de supères voisines après ton départ, je t'ai regrettée...

Merci à tous mes collègues-potes, avec qui travailler était à la fois agréable, épanouissant intellectuellement – et drôle. Je sais que l'approche historique n'est pas toujours pertinente (dixit l'un de mes encadrants qui se reconnaîtra), mais je trouve que finalement, c'est celle qui résonne avec ma sensibilité. Remontons donc quatre années en arrière. Merci à la dream-team du premier tiers-temps, les anciens, les vieux de la vieille, les fondateurs des viennoiseries du mercredi matin et des bières du jeudi soir. Pym, merci pour toutes ces discussions scientifiques à mon arrivée, elles me sont toujours utiles après quatre années à plancher sur le sujet. Merci pour ton abnégation à partager une table au bistrot, même après de longues journées. Merci d'avoir activement participé aux ragots du labo avant mon arrivée, car sinon, ç'aurait été le vide sidéral ! Merci Ghislain pour tes grands sourires et ta

gentillesse, j'aurais aimé profiter d'eux plus longtemps que 6 mois. Merci Lisa pour tout : ta bonne humeur, ta culture, ton intérêt, ta patience, ton anglais, tes encouragements, ton enthousiasme, et tous les supers moments passés ensemble. En particulier, merci pour ces jours en binôme pendant les congrès ; ce sont des moments uniques que je n'oublierai jamais, pendant lesquels j'ai autant aimé côtoyer l'humaine que la scientifique. Ma thèse aurait été complètement différente sans toi. Merci les autres « vieux » étudiants, en particulier Marie, pour les discussions intéressantes. Merci la dream-team du second tiers-temps : Laure – Noé – Yann. Vous avez teinté cette période de ma thèse de légèreté, et êtes tous les trois de vrais rayons de soleil dans un labo. Merci Gonzalo, resté parmi nous trop peu de temps, mais si sympa qu'on continue quand même à te recruter à nos heures perdues. Merci les filles de mon deuxième bureau, Julia – Laura – Yasmine, la dream-team du dernier tiers-temps ! Merci pour ces partages intempestifs de sucreries au moment de ma rédaction (ça donne du cœur à l'ouvrage), merci pour vos mots gentils. Yasmine, j'ai été très heureuse de passer ces quelques mois à ton voisinage. J'espère que tes futurs collègues de bureau ne t'imposeront pas d'entreposer des lampadaires, des chaises, des morceaux de bois qui puent dans notre bureau comme je l'ai fait. Merci Johann(a), toujours aussi frais depuis que tu es arrivé comme stagiaire, même si tu as pris quelques années d'expérience.

Merci aussi aux collègues de l'unité, (presque) toujours agréable à croiser au détour d'un couloir. (Enorme) merci à Khanh et Hyo-Jin, sans qui nous passerions tous bien plus de temps au bureau qu'à la paillasse. Vous faites un boulot incroyable, et en plus, vous êtes super chouettes ! Merci aux chercheur.e.s/enseignant.e.s-chercheur.e.s toujours ouverts aux discussions. Merci Sophie Pezet, toujours encourageante et prête à donner de bons conseils. Merci Karim, bienveillant et accueillant dès les premiers instants, le lanceur de débats (toujours intéressants). Merci Thierry pour ton grand sourire et tes blagues (même quand elles ne sont pas si drôles). Merci André pour ces multiples discussions intéressantes sur... absolument tout. Merci Serge pour les potins sur le domaine neuro droso ! Merci aux étudiants de l'unité. Merci (beaucoup) Sophie Bagur, modèle de rigueur, de culture, d'humour, d'ouverture d'esprit... Toutes ces discussions fascinantes que tu as le don de faire éclore autour de toi au quotidien vont profondément me manquer. Merci Mathilde, qui bien que manifestement brillante, est toujours restée humble, à la critique constructive et douce, et prompte à la remise en question personnelle. Merci les fanfarons, Karim et Thibault, le sourire aux lèvres, probablement une musique en tête, la créativité qui transpire, et une discussion riche. Merci Amina et Céline, pour ces entraides techniques et les bons moments en petit comité au student meeting. Merci les petits derniers du palmarès, trop tard rencontrés, Charles, Léa, Pauline, Rudy, pour ces moments de rire, notamment en réalité virtuelle. Merci à tous ! Travailler dans cette atmosphère a été pour moi exceptionnellement enrichissant scientifiquement et personnellement. Vous me manquerez.

Merci à tous mes potes-non-collègues, aussi. Certes, vous avez probablement moins collaboré à ce travail de thèse, mais votre présence à d'autres moments m'a été essentielle. Donc : merci mes chouchous biologistes, Jérémy et Benoît, Charlotte et Robin, pour les bonnes bouffes, les voyages, pour les moments de jeux de société, et même pour les discussions scientifiques ! Merci Judith pour ces rendez-vous du midi toujours agréables. Merci Oli pour ces quelques verres partagés au fil de la thèse, toujours intéressants et décalés. Merci les Weshs, alias les physiciens, les hippies de Cachan – Antoine, Antoine, Matou, Cavailou, Séverin, Bri, Momo, Gégé, Delphine, Greg, pleins d'imprévu et d'humour, pleins de vie, pour les vacances et voyages, les jeux (encore), les soirées, les verres, les nouvel-ans. Merci les autres ex-colocs non-physiciens, Max(ence) et Max(ime), les habitants de la cave. Merci les vieux copainings de CHAM/D, toujours au taquet, toujours décalés, toujours aussi cools.

Merci la smala. Merci Côme, papa, maman, pour votre originalité à chacun, vos influences variées, parfois incompréhensibles, le charme des moments passés ensemble. Merci chers parents pour votre sollicitude, votre présence à chaque moment de besoin. Merci maman pour ta cuisine ! Merci ma Jacqueline pour ton amour, ta présence constante et discrète, ton attention, ta bonne cuisine aussi. Merci ma sœur, ma jumelle, ma plus chère amie, ma plus violente adversaire, merci pour tes conseils, merci pour ta poésie, merci pour ta paix (souvent), merci pour tous les moments de complicité que nous vivons.

Enfin, merci Timothée(-Franklin). Ta stabilité a contré mon tempérament changeant, ta rigueur m'a inspirée, ton amour m'a rassurée, tes talents de cuisinier m'ont réconfortée. Big love.

PS. Merci à vous, très nombreuses mouches, aussi !



Success is not final, failure is not fatal, it is the courage to continue that counts.

Winston Churchill

Chez la *Drosophile*, la formation de la mémoire à long terme (MLT) est un processus énergétiquement coûteux qui est étroitement contrôlé par des paramètres exogènes et par le statut interne de l'organisme (Plaçais and Preat, 2013). Ce coût est spécifique à la formation de la MLT dépendante de la synthèse protéique *de novo*, ce qui pose la question de comment est régulé le métabolisme énergétique durant la formation de cette mémoire. Chez les vertébrés, plusieurs études ont déjà démontré que des voies métaboliques spécifiques soutenaient la formation de la MLT, comme l'hydrolyse du glycogène dans l'hippocampe (Gibbs et al., 2006; Suzuki et al., 2011). Cette voie catabolique se déroule dans les astrocytes, permettant la synthèse de lactate transféré par la suite aux neurones actifs (Gao et al., 2016; Suzuki et al., 2011). Le rôle des cellules gliales dans la formation de la MLT ou dans le métabolisme énergétique du cerveau n'est que peu documenté chez la drosophile (Matsuno et al., 2015). Cependant, *Drosophila melanogaster* est un organisme modèle très adapté à l'étude des mécanismes se déroulant dans des types cellulaires précis, voire même à des échelles subcellulaires. Les réseaux de neurones qui sous-tendent la formation de la mémoire y sont extrêmement bien décrits, en particulier au niveau des corps pédonculés, la structure associative principale chez la drosophile (Cognigni et al., 2018; Pascual and Preat, 2001), et des neurones spécifiques des corps pédonculés sont actifs durant le rappel de la MLT (Bouzaiane et al., 2015; Séjourné et al., 2011).

Durant ma thèse, nous avons étudié si les cellules gliales de la drosophile étaient impliquées dans la mémorisation à long terme d'une association aversive entre des odeurs et des chocs électriques. Grâce aux puissants outils génétiques disponibles chez ce modèle, nous avons démontré que les cellules gliales étaient précocement impliquées dans la consolidation de la MLT *via* un mécanisme moléculaire spécifique à ce type de mémoire. La drosophile, tout comme les mammifères, possède différents sous-types gliaux. Nous avons par conséquent disséqué les sous-types gliaux impliqués. De façon surprenante, nous avons mis en évidence que les cellules qui cernent les corps cellulaires des neurones, appelées cellules gliales corticales, étaient les seules cellules gliales impliquées dans le mécanisme étudié. Ceci était très inattendu, car la plupart des études qui portent sur les interactions fonctionnelles glie-neurones se focalisent au niveau des synapses.

Grâce à l'inhibition de l'expression de gènes dans des types cellulaires spécifiques et uniquement à l'âge adulte en association avec des expériences de comportement et d'imagerie *in vivo*, nous avons cherché les voies de signalisation mises en jeu dans la glie corticale durant la formation de la mémoire. Nos données mettent en évidence des interactions glie corticale – corps pédonculés spécifiquement durant la formation de la MLT. Pendant et/ou précocement après le conditionnement espacé, des neurones cholinergiques, probablement les neurones des corps pédonculés, libèrent de l'acétylcholine. Celle-ci active le récepteur cholinergique nAChR α 7 exprimé par la glie corticale. Cela induit une élévation du niveau de calcium intracellulaire et une libération du peptide proche de l'insuline Ilp4. Ilp4 agit de façon autocrine sur le récepteur à l'insuline à la membrane de la glie corticale, augmentant la synthèse de glucose à partir de tréhalose dans ces cellules. Le glucose peut ensuite être exporté vers les corps cellulaires des neurones des corps pédonculés pour soutenir leur consommation de glucose élevée.

Ces résultats expliquent pourquoi les défauts cognitifs observés durant le vieillissement et l'évolution de la maladie d'Alzheimer sont accompagnés de perturbations métaboliques telles que la résistance centrale à l'insuline et une diminution du catabolisme du glucose (Mullins et al., 2017; Mattson et al., 2008).

Différentes cellules sont présentes dans le cerveau : les neurones qui transportent l'information d'un endroit à un autre du cerveau, et les cellules gliales. Les neurones consomment beaucoup mais ne peuvent stocker de molécule énergétique comme les sucres. Les cellules gliales peuvent leur fournir ces sucres. Au cours de tâches cognitives exigeantes comme la formation de nouveaux souvenirs, il est probable que les besoins énergétiques des neurones augmentent. Le but de cette thèse a été de comprendre si les cellules gliales jouaient un rôle dans la formation de la mémoire en étudiant la mouche du vinaigre. Ce modèle est très pratique pour manipuler génétiquement des cellules précises avec un contrôle temporel. Nous avons montré que les cellules gliales étaient activées très tôt au cours de la formation de la mémoire par certaines molécules libérées par les neurones. Les cellules gliales impliquées entourent les corps cellulaires neuronaux et leur fournissent l'énergie nécessaire à leur activité. Ces résultats pourraient nous aider à mieux comprendre des pathologies dans lesquelles les neurones ont des problèmes énergétiques comme la maladie d'Alzheimer.

Two different kinds of cells populate the brain: neurons, the cells that convey information from one part of the brain to other areas; but also glial cells. Even though neurons need energy to maintain their activity, they are not able to store energetic molecules such as sugars. Glial cells can provide them with these sugars. During demanding tasks such as forming new memories, the energetic needs of neurons are expected to increase. The aim of this thesis was to understand if glial cells have a role during long term memory formation by studying *Drosophila*. This model is very convenient to genetically manipulate precise cells with a temporal control. We have showed that glial cells were activated very early during memory formation by molecules released by neurons. The glial cells implicated surround the neuronal cell bodies and provide them with energetic sugars. These results will help to understand diseases in which neurons have energetic abnormalities such as Alzheimer's disease.

Introduction

Chez l'humain, le système nerveux central consomme une part importante de l'énergie de l'organisme : environ 20% de sa consommation totale (Mink et al., 1981). La substance grise, qui contient les corps cellulaires neuronaux, les synapses ainsi qu'une grande partie des cellules gliales, constitue le principal puits d'énergie (Sokoloff et al., 1977). On estime actuellement que la transmission synaptique représente deux tiers de la consommation énergétique du système nerveux à l'état basal (Howarth et al., 2012). En contexte normal, le cerveau adulte des mammifères consomme essentiellement du glucose, et prélève ainsi 40 à 50% des sucres consommés quotidiennement (Shah et al., 2012). Ce glucose est transporté à-travers la barrière hématoencéphalique par des transporteurs GLUT1. Une fois dans le cerveau, il peut être prélevé par des neurones grâce à des transporteurs à haute affinité et haute capacité GLUT3, ou par des cellules gliales qui expriment quant à elles des transporteurs GLUT1 (Simpson et al., 2007). Le glucose, une fois dans ces cellules, peut ensuite être utilisé par différentes voies métaboliques. Une controverse existe actuellement sur le devenir du glucose dans les neurones et les cellules gliales. Certaines études tendent à montrer que les neurones prélèvent majoritairement du lactate qui leur est fourni par l'oxydation du glucose dans les astrocytes (Magistretti and Allaman, 2015), et prélèvent du glucose de façon minoritaire. Au contraire, d'autres chercheurs favorisent l'hypothèse d'un prélèvement direct de glucose par les neurones (Lundgaard et al., 2015). L'origine de l'incohérence entre certains résultats expérimentaux soutenant ces deux modèles n'est pour l'instant pas claire.

L'intensité et la durée d'activité des réseaux de neurones pourraient moduler les voies métaboliques mises en jeu dans les neurones et les cellules gliales. Chez les vertébrés, plusieurs études ont déjà démontré que des voies métaboliques spécifiques soutenaient la formation de la mémoire à long terme : l'hydrolyse du glycogène dans les astrocytes de l'hippocampe permet de synthétiser du lactate transmis aux neurones actifs (Gibbs et al., 2006; Suzuki et al., 2011). Mais quelle est la consommation énergétique précise du cerveau lors d'une activité cognitive complexe telle que la formation de la mémoire à long terme ? Quelles voies

métaboliques sont alors mises en jeu dans la glie et dans les neurones ? Durant ma thèse, j'ai tenté de répondre à ces questions grâce à l'étude de la mémoire à long terme chez l'organisme modèle *Drosophila melanogaster*.

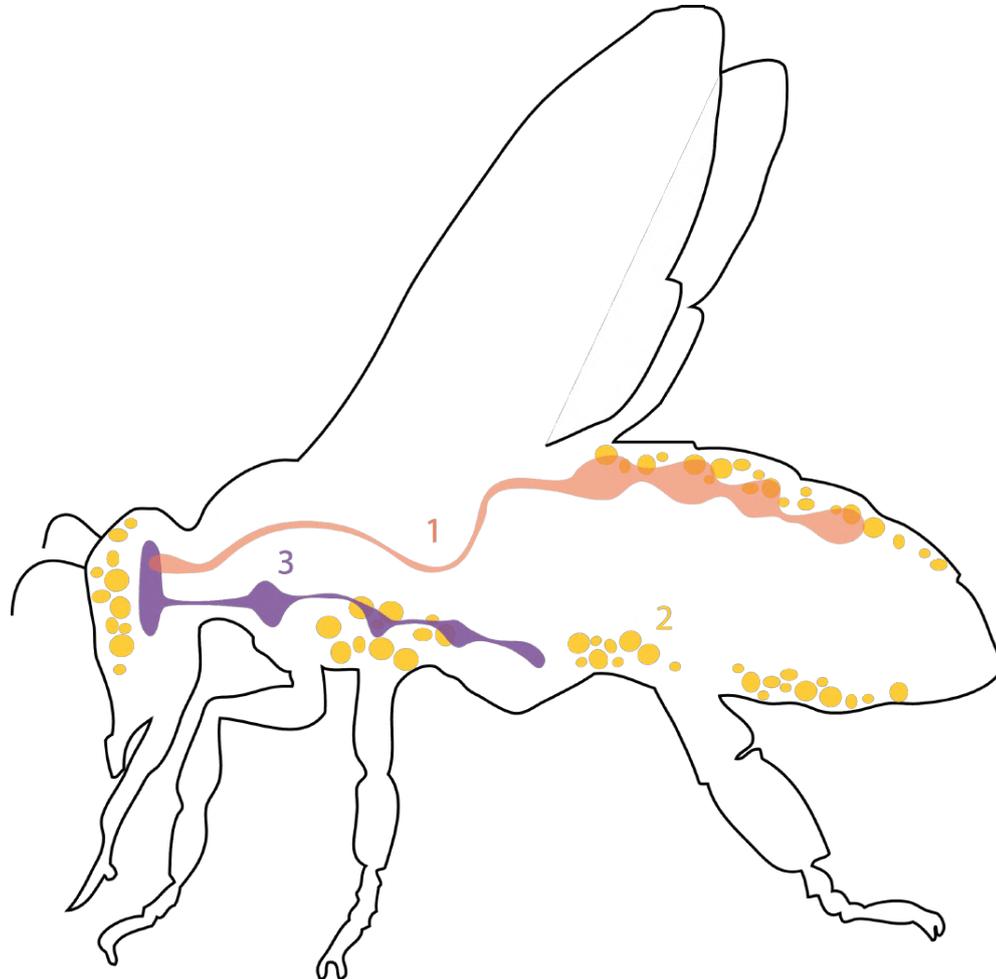


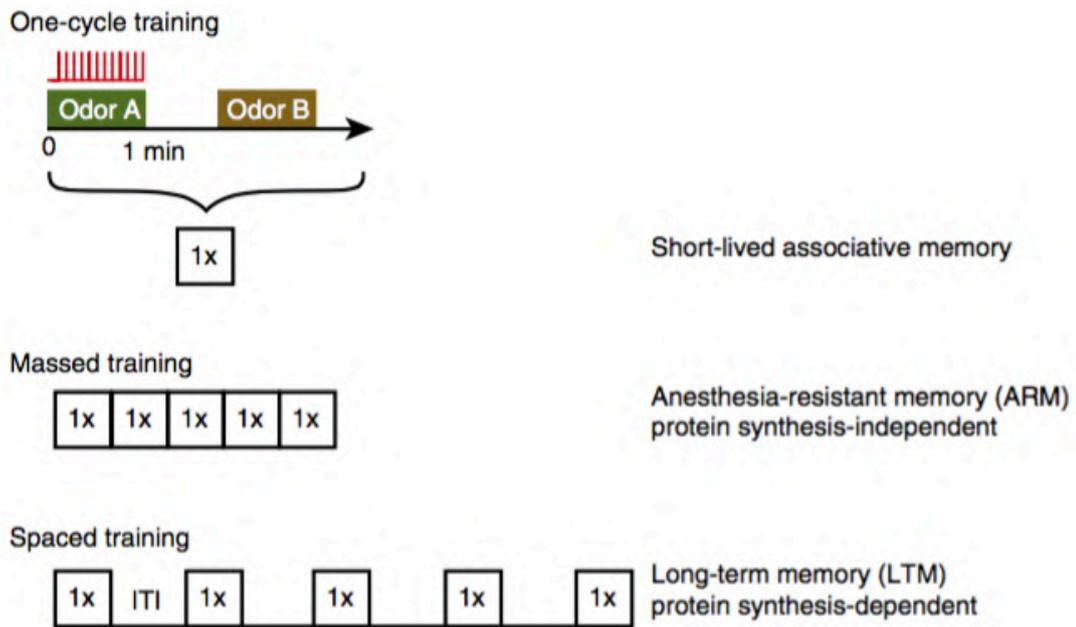
Figure A. Localisation de certains organes d'intérêt chez la drosophile. 1. L'hémolymphe est mise en mouvement grâce à un vaisseau dorsal contractile (rouge) au sein d'un système ouvert. 2. Les corps gras (jaunes) forment l'organe de stockage majoritaire des métabolites énergétiques sous forme de glycogène et d'acides gras. 3. Le système nerveux central (violet) est composé du cerveau et de la corde nerveuse ventrale subdivisée en différents ganglions. Adapté de (Lazareva et al., 2007; Tatar et al., 2014) et (Rittschof and Schirmeier, 2017).

Chez la drosophile, le principal sucre circulant n'est pas le glucose, mais le tréhalose, un dimère de glucose (Treherne, 1958). Il est hydrolysé en deux molécules de glucose sous l'action d'une enzyme, la tréhalase, dont l'activité est notamment régulée par l'insuline (Xu et al., 2013). L'entrée de tréhalose dans le système nerveux

de la drosophile est permise grâce à l'expression de son transporteur par les cellules formant un équivalent de la barrière hématoencéphalique : la glie de surface (Volkenhoff et al., 2015). Les neurones expriment le transporteur de glucose GLUT1 chez la drosophile également (Volkenhoff et al., 2018), mais il n'existe pas de vaisseaux sanguins (Figure A), et le mécanisme d'acheminement du glucose jusqu'aux neurones est pour l'instant inconnu. L'air contenant le dioxygène, quant à lui, arrive aux tissus par diffusion grâce à un réseau de trachées connectées directement avec l'air extérieur.

Grâce aux outils extrêmement résolutifs disponibles chez la mouche, des décennies de recherche ont permis de définir précisément les réseaux de neurones impliqués dans la formation de mémoires associatives (Cognigni et al., 2018; Pascual and Preat, 2001; Pavlowsky et al., 2018; Plaçais et al., 2012; Scheunemann et al., 2018; Séjourné et al., 2011; Yamagata et al., 2015). Au laboratoire, nous utilisons des protocoles de conditionnement associant des chocs électriques aversif à des odeurs (Tully and Quinn, 1985) (Figure B). Nous testons la mémoire formée dans un labyrinthe en T après le temps désiré. Une seule présentation du conditionnement permet de former une mémoire aversive qui s'éteint au bout de quelques heures (Quinn et al., 1974). La répétition de cinq cycles de conditionnement permet la formation de mémoires consolidées encore mesurables 24h après le conditionnement (Tully et al., 1994). Si ces répétitions sont répétées sans pause, un type de mémoire particulier appelé mémoire résistante à l'anesthésie à long terme (MRA-LT) est formé grâce à la mise en jeu de réseaux de neurones et de mécanismes moléculaires spécifiques (Tully et al., 1994). Au contraire, si ces répétitions sont espacées, un autre type de mémoire consolidée, la mémoire à long terme (MLT), définie par sa dépendance à la synthèse protéique *de novo* (Flexner et al., 1962; Tully et al., 1994), est formée grâce à la mise en jeu de réseaux de neurones et de mécanismes moléculaires différents. Ces deux types de mémoires consolidées sont antagonistes (Isabel et al., 2004; Plaçais et al., 2012).

A



B

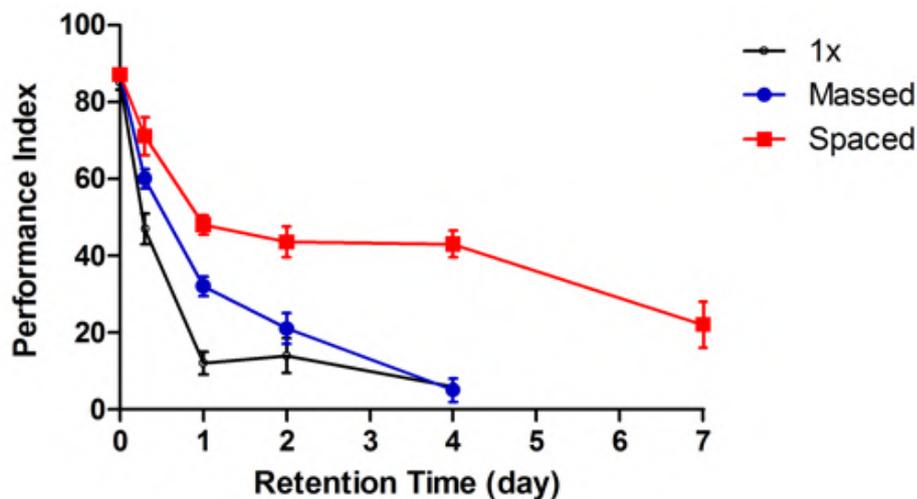


Figure B. Rétention de la mémoire formée après différents types de conditionnements associatifs aversifs chez la drosophile. A. Différents protocoles de conditionnement. B. Performance de mémoire au cours du temps après les différents conditionnements. Après un seul cycle de conditionnement associant chocs électriques et odeurs ("1x"), la mémoire formée est rapidement éteinte au bout de quelques heures. Au contraire, après 10 répétitions du même cycle de conditionnement, avec ("Spaced") ou sans ("Massed") pause de 15 minutes entre chaque cycle, la mémoire formée est plus robuste et dure plusieurs jours. Après un conditionnement espacé avec pauses, la mémoire formée est encore plus durable que sans pause. Adapté de (Tully et al., 1994).

Pour former ces mémoires, l'information olfactive est acheminée par des neurones olfactifs vers les lobes antennaires organisés en glomérules spécifiques d'un récepteur olfactif, tout comme chez les mammifères (Vosshall et al., 2000) (Figure C). Elle est ensuite transmise par des neurones de projection vers le centre intégratif du cerveau invertébré : les corps pédonculés (Heisenberg et al., 1985). Ceux-ci sont composés de neurones intrinsèques : les cellules de Kenyon, dont les axones forment les différents lobes des corps pédonculés dans la partie antérieure du cerveau (Figure D). Ces lobes sont contactés par deux types de neurones extrinsèques : une centaine de neurones dopaminergiques et 34 neurones de sortie cholinergiques, glutamatergiques ou GABAergiques (Aso et al., 2014a). Il a été récemment observé que chaque neurone dopaminergique et chaque neurone de sortie n'innervent en général qu'une zone d'un seul lobe (Aso et al., 2014a). Les lobes des corps pédonculés se subdivisent ainsi structurellement en 15 modules, chacun contacté par des neurones dopaminergiques et des neurones de sortie spécifiques. Il a par ailleurs été montré que l'information des chocs électriques était acheminée jusqu'aux corps pédonculés par les neurones dopaminergiques (Aso et al., 2010; Claridge-Chang et al., 2009; Riemensperger et al., 2005; Schwaerzel et al., 2003).

La détection de coïncidence entre l'information « odeur » et l'information « chocs électriques » au niveau des corps pédonculés active une voie de signalisation dépendant d'une synthèse d'AMPC (Gervasi et al., 2010). Cela enclenche les voies moléculaires impliquées dans la plasticité synaptique. Après le conditionnement, en particulier, le poids des synapses entre les neurones des corps pédonculés et les neurones de sortie en aval de ceux-ci est modifié (Hige et al., 2015; Séjourné et al., 2011). Cette modification de la force des synapses permet d'adapter le comportement de la drosophile à son expérience passée : après un conditionnement aversif, elle fuit l'odeur associée aux chocs.

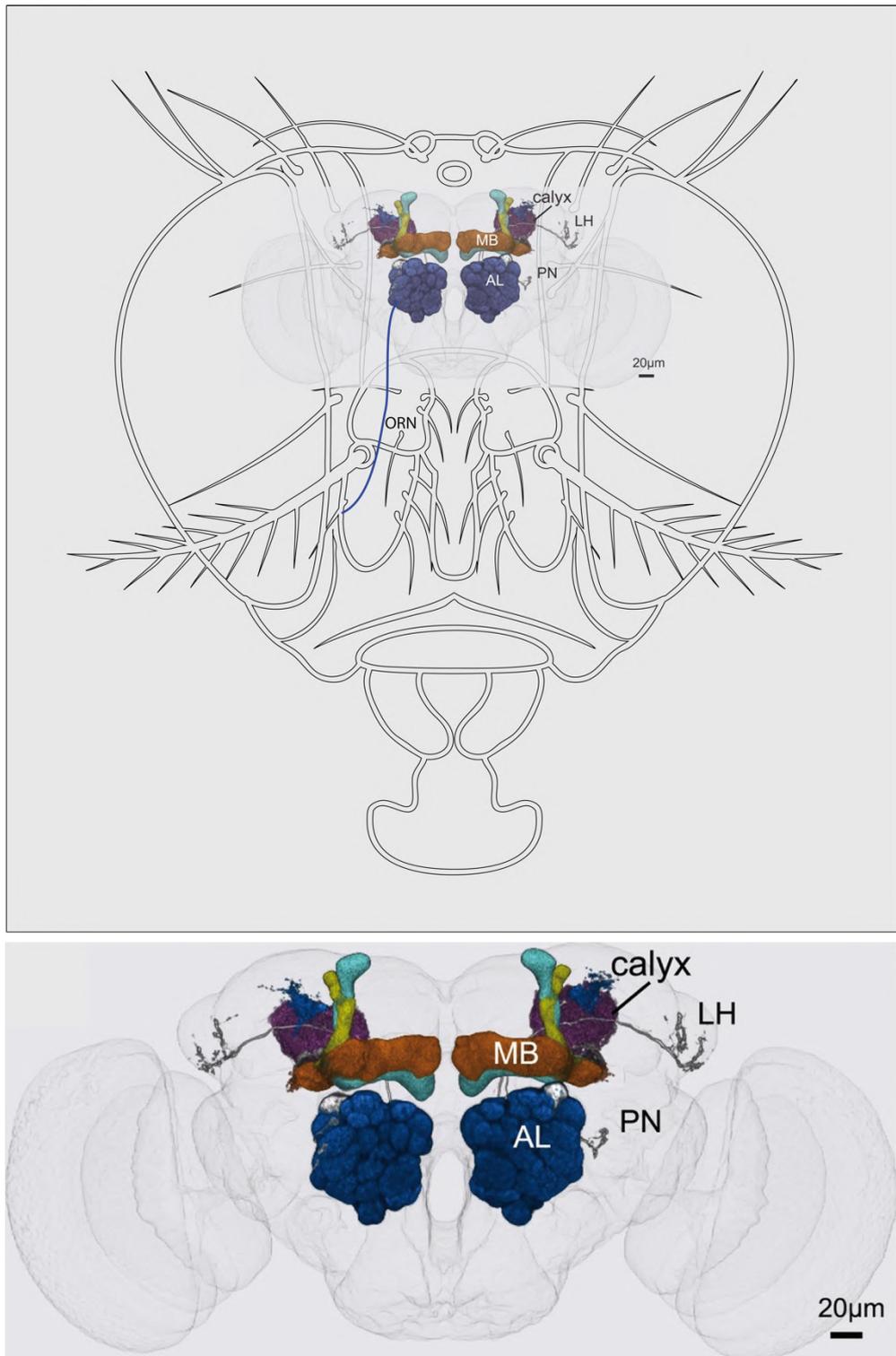


Figure C. Organisation du système olfactif dans le cerveau de la drosophile. Des neurones récepteurs olfactifs (ORN) présents dans les antennes et les palpes maxillaires reçoivent les molécules odorantes, et projettent au niveau des lobes antennaires (AL) subdivisés en glomérules. L'information y est transmise à des neurones de projection (PN) qui contactent les corps pédonculés (MB) et la corne latérale (LH). Adapté de (Aso et al., 2014a).

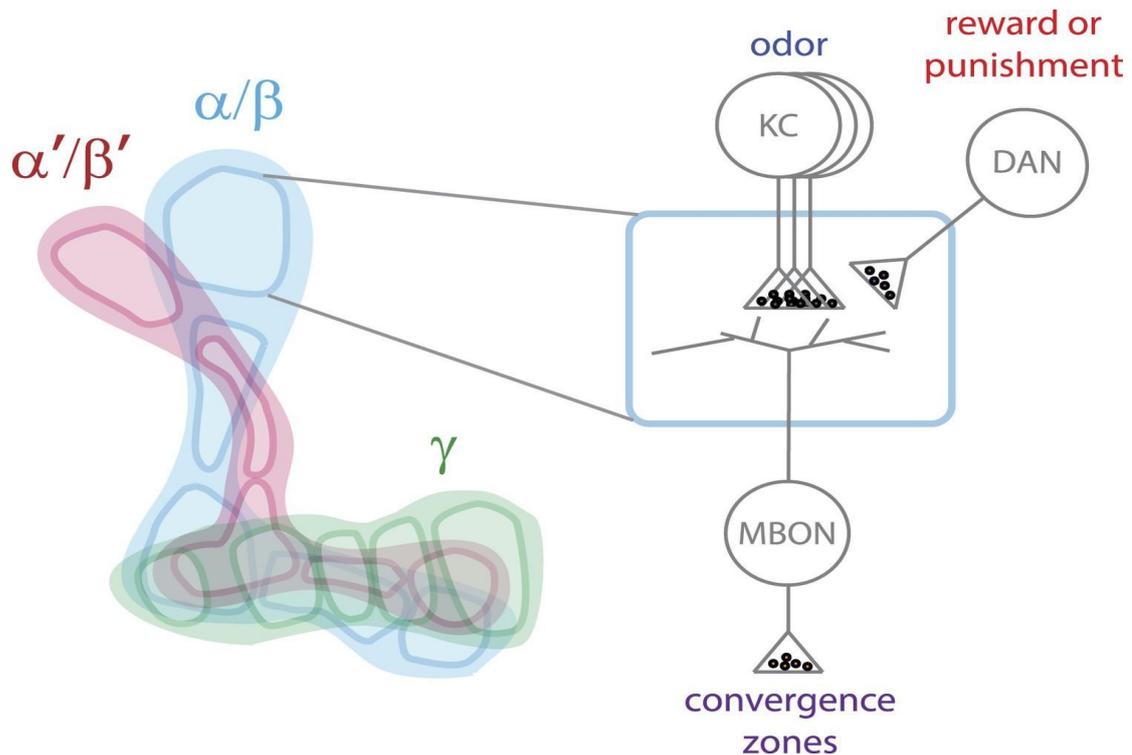


Figure D. Anatomie et organisation fonctionnelle des corps pédonculés en 15 compartiments. Les corps pédonculés sont composés des cellules de Kenyon (KC), neurones intrinsèques dont les axones forment les lobes verticaux α et α' , et les lobes médians β , β' et γ . Chaque compartiment reçoit l'information convergente des cellules de Kenyon (identité de l'odeur) et de neurones dopaminergiques (DAN) (valence du stimulus). La sortie des corps pédonculés est médiée par les neurones de sortie (MBON). D'après (Griffith, 2014).

Comme indiqué précédemment, la MRA-LT et la MLT sont antagonistes (Isabel et al., 2004). De plus, il a été montré au laboratoire que le statut métabolique des drosophiles influençait le type de mémoire qu'elles formaient : après un conditionnement espacé, des mouches mises à jeun pendant 21h avant et 24h après le conditionnement espacé ne forment plus de MLT apparemment trop coûteuse, mais forment de la MRA-LT (Plaçais and Preat, 2013). Des résultats indépendants suggéraient déjà que la signalisation à l'insuline, impliquée dans la régulation du métabolisme énergétique des cellules, était importante pour la formation de la mémoire, et en particulier de la mémoire à long terme (Chambers et al., 2015). Sur cette base, nous nous sommes demandé comment le métabolisme énergétique était modulé au cours de la formation de la MLT.

Tout comme les mammifères, la drosophile possède différents types de cellules gliales. Il est possible que les cellules gliales de tous les animaux possèdent une origine évolutive commune, bien que cette théorie soit controversée (Hartline, 2011). Le cerveau de la mouche est divisé en deux parties (Figure E) : la partie périphérique, appelée cortex, contient les corps cellulaires des neurones, tous entourés d'extensions de membrane provenant de cellules gliales corticales (Awasaki et al., 2008). Ce cortex est protégé par la glie de surface qui constitue l'équivalent de la barrière hémato-encéphalique (Stork et al., 2008). Les neurones possèdent un unique neurite susceptible de s'arboriser et qui s'étend vers le centre du cerveau, dans la neuropile. Dans cette seconde région, les axones et compartiment synaptiques entrent en contact avec deux types gliaux : les astrocytes et la glie engainante (Kremer et al., 2017). Les recherches menées jusqu'ici ont décrit de multiples rôles gliaux, qui sont spécifiques à chacun de ces sous-types cellulaires (Freeman, 2015). Il semblerait que la glie des invertébrés, tout comme la glie du mammifère, soit impliquée dans le métabolisme énergétique du cerveau. En effet, certains types gliaux contiennent des formes de stockages de molécules énergétiques tels que le glycogène ou les lipides (Wigglesworth, 1960). De plus, le métabolisme des sucres dans la glie est indispensable au développement et au bon fonctionnement du système nerveux (Volkenhoff et al., 2015). Différents types gliaux sont également susceptibles de libérer des peptides proches de l'insuline connus pour leur rôle de régulateurs du métabolisme énergétique, et d'être sensibles à ces peptides (Chell and Brand, 2010; Sousa-Nunes et al., 2011).

Le rôle des cellules gliales dans la formation de la mémoire ou dans le métabolisme énergétique du cerveau reste cependant pour l'instant très peu documenté chez les invertébrés. Très récemment, deux études publiées au début de ce travail de thèse ont montré que les cellules gliales de la mouche jouaient un rôle dans la formation de la mémoire à long terme (Matsuno et al., 2015) et dans les défauts de mémoire observés au cours du vieillissement (Yamazaki et al., 2014). À la suite de tous ces travaux, nous nous sommes demandé si la glie pouvait jouer un rôle dans les régulations métaboliques au cours de la formation de la mémoire la plus coûteuse, la mémoire à long terme, chez la drosophile.

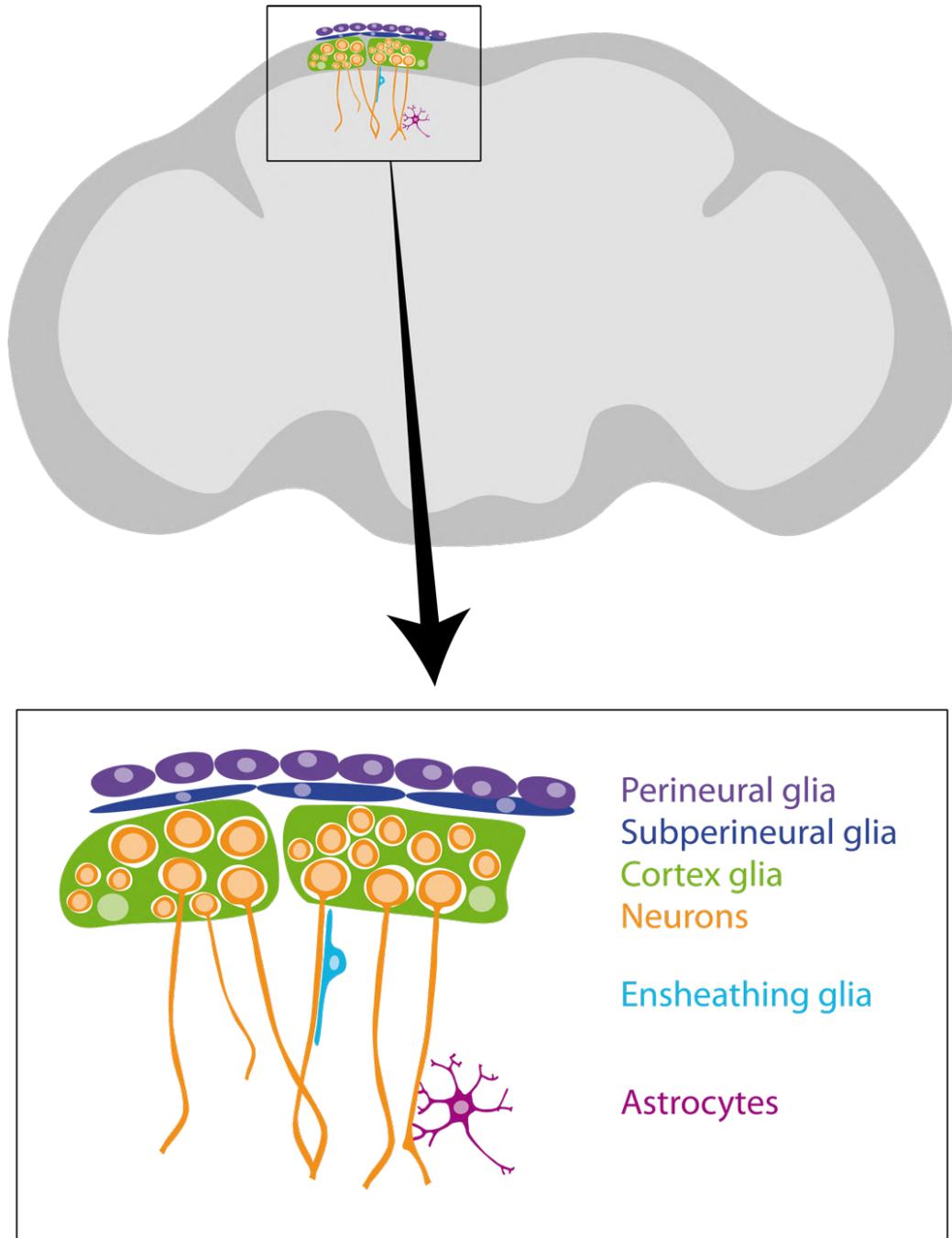


Figure E. Anatomie et localisation des cellules gliales dans le cerveau de la drosophile. Le cerveau de la mouche est divisé en deux régions : le cortex (gris foncé) contient les corps cellulaires des neurones, alors que la neuropile (gris clair) contient les régions axonales et dendritiques. La périphérie du cerveau est délimitée par les cellules gliales périneuriales et subpérineuriales, qui forment l'équivalent de la barrière hémato-encéphalique, et restreignent la diffusion de molécules de part et d'autre de la barrière. Dans le cortex, la glie corticale entoure individuellement les corps cellulaires des neurones. Dans la neuropile, la glie engainante (ensheathing) sépare les grandes structures du cerveau, tandis que les astrocytes étendent des digitations au plus proches des synapses.

Résultats

Durant ma thèse, nous avons étudié si les cellules gliales de la drosophile étaient impliquées dans la formation de la mémoire à long terme aversive formée après la présentation concomitante d'une odeur et de chocs électriques. Grâce au système d'expression binaire UAS-GAL4 disponible chez ce modèle, nous avons restreint l'expression du transgène *shibire^{ts}* uniquement dans des types cellulaires d'intérêt. Ainsi, uniquement en plaçant les drosophiles à haute température, nous avons inhibé le recyclage vésiculaire dans toute la glie, ou dans des sous-types de cellules gliales précis au moment désiré. Grâce à cette stratégie, nous avons démontré que les cellules gliales étaient précocement impliquées dans la consolidation de la mémoire à long terme, *via* un mécanisme moléculaire spécifique à ce type de mémoire. Nous avons ensuite disséqué les sous-types gliaux impliqués, et avons de façon surprenante mis en évidence que les cellules qui cernent les corps cellulaires des neurones, les cellules gliales corticales, étaient les seules cellules gliales impliquées dans le mécanisme étudié. Ceci était très inattendu, car la plupart des études qui portent sur les interactions fonctionnelles glie-neurones se focalisent au niveau des synapses ; la littérature sur les interactions glie-corps cellulaires de neurones du système nerveux central est en revanche quasi-inexistante, en particulier durant la formation de la mémoire.

Grâce à l'inhibition de l'expression de gènes dans des types cellulaires spécifiques et uniquement à l'âge adulte, nous avons cherché les voies de signalisation mises en jeu dans la glie corticale durant la formation de la mémoire à long terme. Nous avons pu montrer que dans la glie corticale adulte, l'expression d'un peptide proche de l'insuline, *Ilp4*, et du récepteur à l'insuline *InR*, étaient nécessaires à la formation de la mémoire à long terme. Cela suggère qu'une activation autocrine de la voie de signalisation de l'insuline intervient dans la glie corticale pour la formation de la mémoire à long terme. Nous avons également constaté que l'expression du récepteur cholinergique *nAChR α 7* était requise dans la glie corticale adulte, ce qui suggère qu'un signal cholinergique active la glie corticale durant la consolidation de la mémoire à long terme. Nous avons pu confirmer, grâce à des expériences d'imagerie *in vivo*, que la glie corticale était en effet sensible à une stimulation cholinergique, et

que celle-ci induisait une augmentation de la concentration calcique intracellulaire dans la glie corticale.

Nous nous sommes ensuite intéressés à une possible régulation du métabolisme des sucres par la voie de l'insuline en aval de l'activation cholinergique de la glie corticale. Nous avons mis en évidence un défaut spécifique de mémoire à long terme lors de la diminution artificielle de l'expression de la tréhalase dans la glie corticale adulte, et ainsi prouvé que la synthèse de glucose à partir de tréhalose, le sucre circulant principal chez les insectes, était indispensable dans la glie corticale pour la formation de la mémoire à long terme. De plus, grâce à l'expression d'une sonde FRET sensible au glucose et génétiquement encodée, nous avons mis en évidence que l'activation pharmacologique des récepteurs nicotiques induisait une augmentation de la concentration en glucose dans la glie corticale. Cette augmentation dépendait de l'expression du récepteur nAChR α 7 ainsi que du récepteur à l'insuline, confirmant que la voie de l'insuline se situe en aval de l'activation cholinergique de la glie. Enfin, nous avons démontré que l'expression du transporteur de glucose GLUT1 était nécessaire à la fois dans la glie corticale et dans les neurones des corps pédonculés pour la formation de la mémoire à long terme, mais pas pour des mémoires moins robustes. Comme nous avons pu par ailleurs montrer que la consommation de glucose augmentait dans les corps cellulaires des neurones des corps pédonculés durant la formation de la mémoire à long terme, ces résultats indiquent que le glucose synthétisé dans la glie corticale est transféré aux neurones durant leur activité particulièrement coûteuse énergétiquement.

Ainsi, nos données suggèrent qu'un signal cholinergique active la glie corticale qui encapsule les corps cellulaires des neurones. En réponse à ce signal, une élévation de la concentration en calcium intracellulaire induit la libération d'un peptide proche de l'insuline. Ce peptide active de façon autocrine son récepteur, ce qui augmente la synthèse de glucose à partir de tréhalose dans la glie corticale, et probablement son export vers les corps cellulaires des neurones des corps pédonculés (Figure F).

Enfin, dans un article où je figure en tant que seconde auteure (Plaçais et al., 2017), nous avons montré qu'au niveau des axones et des synapses des corps pédonculés, ce n'était pas la consommation de glucose, mais la consommation de pyruvate qui augmentait pour répondre aux besoins métaboliques des neurones.

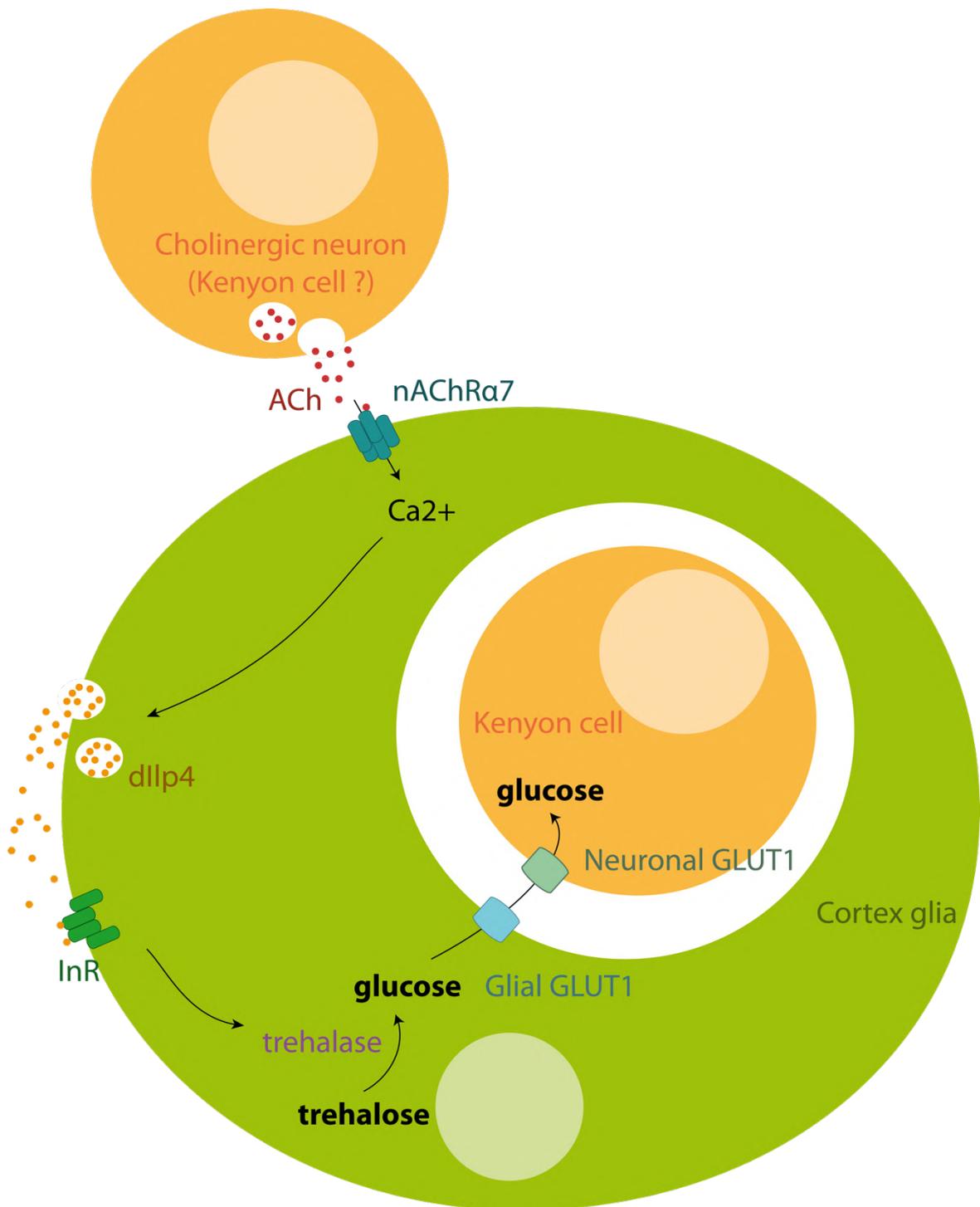


Figure F. Modèle de régulation du métabolisme des sucres dans la glie corticale et de leur export vers les cellules de Kenyon durant la formation de la mémoire à long terme.

Ainsi, nous avons mis en évidence que des voies métaboliques différentes étaient favorisées dans des compartiments neuronaux distincts durant la formation de la mémoire à long terme. La consommation de glucose par les corps cellulaires des neurones pourrait fournir le pyruvate nécessaire à l'activité synaptique. De plus, dans ce même article, nous avons montré qu'augmenter le métabolisme énergétique (la consommation de pyruvate) dans les neurones des corps pédonculés suffisait à induire la consolidation de l'association odeurs – chocs électrique en une mémoire à long terme, dépendant de la synthèse protéique. Ainsi, le métabolisme énergétique dans les neurones est instructeur dans le processus de consolidation de la mémoire.

Conclusion

Au cours de ma thèse, nous avons ainsi montré que des voies métaboliques spécifiques étaient mises en jeu dans les neurones des corps pédonculés impliqués dans la formation de la MLT. Étonnamment, le métabolisme énergétique peut être différemment régulé dans chaque compartiment neuronal : les corps cellulaires et les synapses. Cela pourrait expliquer le manque de cohérence entre résultats expérimentaux chez les mammifères, ces études n'ayant pas pris en compte les compartiments neuronaux dans lesquels le métabolisme énergétique était mesuré.

De plus, nous avons mis en évidence un mécanisme moléculaire original et inattendu qui relie pour la première fois l'activité neuronale à la régulation du métabolisme énergétique dans la glie de l'invertébré. Durant la formation de la mémoire à long terme, étonnamment, les cellules gliales au contact des corps cellulaires des neurones sont activées. Ce mécanisme est spécifique à la formation de la MLT et n'intervient pas dans la formation de la MRA-LT. Il a été suggéré indépendamment que la glie corticale modulait l'excitabilité des neurones (Melom and Littleton, 2013). Son action sur le métabolisme énergétique pourrait participer au contrôle de l'excitabilité des neurones durant la formation de la mémoire à long terme, en potentialisant une activité récurrente dans les réseaux de neurones.

Enfin, nos données montrent un lien entre signalisation de l'insuline dans la glie et métabolisme des sucres. Comme le récepteur à l'insuline est également très exprimé dans le cerveau des mammifères, y compris dans des structures impliquées dans la mémoire telles que le cortex ou l'hippocampe (Brüning et al., 2000), le rôle de cette

signalisation pourrait être conservé. De plus, certains patients atteints de la maladie d'Alzheimer présentent une résistance à l'insuline (Mullins et al., 2017), et un métabolisme du glucose diminué (Mattson et al., 2008). Ces dysfonctions pourraient expliquer les premiers symptômes de troubles cognitifs chez les malades. Si la voie de signalisation que nous avons mise en évidence chez la drosophile est conservée dans les cellules gliales chez l'homme, élaborer des molécules pharmacologiques capables d'activer le métabolisme spécifiquement dans les cellules gliales pourrait aider à contrecarrer les symptômes de la maladie.

ACKNOWLEDGEMENTS	0
RÉSUMÉ	4
RÉSUMÉS GRAND PUBLIC	5
RÉSUMÉ DETAILLÉ	6
TABLE OF CONTENTS	20
ABBREVIATIONS	25
INTRODUCTION	26
PART I: METABOLISM IN THE CENTRAL NERVOUS SYSTEM	28
I. 1. THE CENTRAL NERVOUS SYSTEM IS AN ENERGETICALLY DEMANDING STRUCTURE.....	29
<i>I. 1. a. CNS energy consumption</i>	29
<i>I. 1. b. Neuronal energetic sinks</i>	30
I. 1. b. i. The energy budget of the grey matter	30
I. 1. b. ii. The cost of housekeeping mechanisms in the CNS.....	33
I. 2. TAKING-UP SOME ENERGY: FROM BLOOD TO NEURONS	34
<i>I. 2. a. A diversity in the metabolites used by the CNS</i>	34
<i>I. 2. b. Glucose transport from BBB to brain cells</i>	36
I. 2. b. i. The GLUT family of glucose transporters.....	36
I. 2. b. ii. Expression of glucose transporters in the mammalian BBB	36
I. 2. b. iii. Expression of glucose transporters in mammalian brain cells	37
<i>I. 2. c. A brief overview of the main glucose catabolic pathways</i>	40
I. 2. c. i. Kinetics of ATP production.....	40
I. 2. c. ii. Anaplerotic precursors	40
I. 2. c. iii. Signaling properties.....	41
I. 3. METABOLIC COMPARTMENTATION.....	43
<i>I. 3. a. A discussed metabolic compartmentation between glia and neurons</i>	43
<i>I. 3. b. Advantages of a metabolic compartmentation</i>	47
I. 4. SPECIFICITIES OF DROSOPHILA METABOLISM	50
<i>I. 4. a. Trehalose is the main circulating sugar in the hemolymph</i>	50
<i>I. 4. b. Trehalose circulating level is regulated in particular by insulin signaling</i>	53
<i>I. 4. c. Trehalose can cross the Drosophila equivalent of the BBB</i>	54
<i>I. 4. d. Neuronal metabolism in Drosophila</i>	55

PART II: DROSOPHILA MEMORY.....	57
II. 1. LEARNING AND MEMORY THEORETICAL FRAMEWORK.....	57
II. 1. a. <i>A historical approach of memory</i>	57
II. 1. b. <i>Two independent long-lasting aversive memories exist in flies</i>	60
II. 1. c. <i>Ecological relevance of LTM and LT-ARM</i>	61
II. 2. BRAIN STRUCTURES INVOLVED IN MEMORY FORMATION.....	63
II. 2. a. <i>Diverse and precise genetic tools available in Drosophila</i>	63
II. 2. b. <i>Olfactory processing in Drosophila</i>	66
II. 2. c. <i>Olfactory integration in the Mushroom body</i>	68
II. 2. d. <i>Network encoding the unconditioned stimulus valence</i>	72
II. 2. e. <i>Mushroom body output neurons influence flies behavior</i>	75
II. 3. THE INTERPLAY BETWEEN MEMORY AND METABOLISM.....	78
II. 3. a. <i>LTM and LT-ARM mutual antagonism</i>	78
II. 3. b. <i>Insulin-like signaling modulation of memory</i>	82
II. 3. c. <i>Feeding status in appetitive LTM</i>	85
 PART III: DROSOPHILA GLIA AND THEIR INVOLVEMENT IN METABOLISM AND MEMORY	 87
III. 1. GLIAL CELLS ORIGIN AND DIVERSITY.....	88
III. 1. a. <i>Glial evolutionary origin</i>	88
III. 1. b. <i>Glial diversity, location and morphology in the Drosophila CNS</i>	89
III. 1. c. <i>The functions of glia in the Drosophila adult brain</i>	92
III. 1. c. i. <i>Cortex glia</i>	92
III. 1. c. ii. <i>Astrocytes</i>	95
III. 1. c. iii. <i>Ensheathing glia</i>	96
III. 2. INVERTEBRATE GLIA AND ENERGY METABOLISM.....	98
III. 2. a. <i>Anatomical considerations in diverse insects</i>	98
III. 2. b. <i>Glia contain metabolic stores</i>	100
III. 2. c. <i>Several glial subtypes can secrete peptides regulating energy metabolism</i>	102
III. 3. DROSOPHILA GLIA AND MEMORY.....	104
III. 3. a. <i>Drosophila glia are involved in LTM formation</i>	104
III. 3. b. <i>Glial metabolism is involved in age-related memory impairment</i>	105
 AIMS	 107

MATERIAL AND METHODS	109
I. FLY STRAINS AND RAISING CONDITIONS	109
II. CLONING.....	111
III. BEHAVIOR	111
<i>III. 1. Flies selection and preparation</i>	<i>111</i>
<i>III. 2. Aversive olfactory conditioning.....</i>	<i>112</i>
<i>III. 3. Memory test.....</i>	<i>114</i>
<i>III. 4. Olfactory acuity</i>	<i>115</i>
<i>III. 5. Shock response.....</i>	<i>115</i>
<i>III. 6. Locomotion assay</i>	<i>115</i>
<i>III. 7. Feeding assays.....</i>	<i>116</i>
III. 7. a. CAFÉ assay.....	116
III. 7. b. Sulforhodamine B experiments	117
IV. <i>IN VIVO</i> IMAGING.....	118
V. IMMUNOHISTOCHEMISTRY.....	121
VI. QUANTIFICATION AND STATISTICAL ANALYSES	121
RESULTS.....	123
I. AN INCREASE IN MUSHROOM BODY METABOLISM IS INSTRUCTIVE FOR LONG-TERM MEMORY	123
II. A NEURON-GLIA INTERACTION DURING LONG-TERM MEMORY CONSOLIDATION	139
<i>II. 1. Cortex glia participate in long-term memory consolidation early after the</i> <i>conditioning</i>	<i>140</i>
<i>II. 2. Cortex glia release insulin-like peptide 4 during long-term memory consolidation</i> <i>.....</i>	<i>144</i> <i>147</i>
<i>II. 3. Ilp4 acts autocrinally on cortex glia</i>	<i>148</i>
<i>II. 4. Cortex glia are activated by a cholinergic signal during memory formation.....</i>	<i>148</i>
<i>II. 5. Cortex glia metabolism is involved in long-term memory.....</i>	<i>153</i>
<i>II. 6. Cortex glia activation increases glucose intracellular concentration</i>	<i>155</i>
<i>II. 7. Cortex glia may support an increased glucose consumption in Kenyon cells soma..</i>	<i>158</i>

DISCUSSION	165
I. MODULATION OF KENYON CELLS METABOLISM DURING LONG-TERM MEMORY FORMATION.....	165
I. 1. <i>Mushroom-body neurons energy demand increases during LTM consolidation</i>	<i>165</i>
I. 1. a. At the level of the lobes	166
I. 1. b. At the level of the soma.....	166
I. 2. <i>Consequences of the metabolic switch in MB neurons on food intake.....</i>	<i>168</i>
I. 3. <i>Glucose memory enhancement</i>	<i>169</i>
II. CHOLINERGIC ACTIVATION OF CORTEX GLIA	170
II. 1. <i>Origin of the cholinergic input.....</i>	<i>170</i>
II. 1. a. Kenyon cells somatic transmission could activate cortex glia	170
II. 1. b. Other cholinergic sources that could underlie cortex glia cholinergic activation	172
II. 2. <i>Consequences on cortex glia intracellular signaling</i>	<i>173</i>
III. INSULIN SIGNALING AND MEMORY	174
III. 1. <i>Cortex glia release Ilp4 during memory formation</i>	<i>174</i>
III. 2. <i>Insulin-like signaling is involved in cognition in various species.....</i>	<i>176</i>
III. 2. a. Previous results on insulin signaling and cognition.....	176
III. 2. b. From insulin signaling in neurons to insulin signaling in glia.....	178
III. 3. <i>Insulin signaling in cortex glia regulates brain energy metabolism.....</i>	<i>180</i>
III. 3. a. Carbohydrate metabolism in cortex glia is regulated by neuronal inputs	180
III. 3. b. Metabolism in glia might support neuronal glucose consumption	184
III. 3. c. Insulin signaling in mammalian astrocytes also participates to CNS metabolism.....	186
GENERAL CONCLUSION AND PERSPECTIVES.....	187
ANNEX.....	193
GLUCOSE METABOLISM	193
1. The pentose phosphate pathway.....	193
2. Glycolysis.....	194
3. Glycolysis is followed by fermentation or by mitochondrial respiration	194
3.a. Fermentation	194
3. b. Mitochondrial respiration	195
REFERENCES.....	197
ABSTRACT	220

ACh	Acetylcholine
AD	Alzheimer's disease
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CREB	cAMP Response Element Binding-protein
CS	Canton Special
CS-	Neutral stimulus
CS+	Conditioned stimulus
CXM	Cycloheximide
DAN	Dopaminergic neuron
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescence protein
KC	Kenyon cell
LT-ARM	Long-term anesthesia-resistant memory
LTM	Long-term memory
MB	Mushroom body
MBON	Mushroom body output neuron
MCH	4-méthylcyclohexanol
MT-ARM	Middle-term anesthesia-resistant memory
MTM	Middle-term memory
OCT	3-octanol
ORN	Olfactory receptor neuron
OxPhos	Oxidative phosphorylation
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PN	Projection neuron
RNA	Ribonucleic acid
RNAi	Interfering RNA
ST-ARM	Short-term anesthesia-resistant memory
STM	Short-term memory
US	Unconditioned stimulus

A major advantage for an individual is to adapt quickly to environmental changes. One modality that allows this adaptation is learning and memory. An individual with learning capacities can remember new food locations, new threats... and adapt its behavior accordingly.

Memory is therefore an evolutionary advantage. But it also has a cost. Indeed, learning capacities imply that specific brain structures are formed during development, thanks to cellular division, neuronal pathfinding, differentiation and genes involved in these precise processes (Johnston, 1982; Burns et al., 2011; Moran et al., 2015). It also requires the metabolic expense of neuronal activity (Attwell and Laughlin, 2001; Howarth et al., 2012) and plasticity during memory formation, storage and retrieval. Moreover, it is well acknowledged that the central nervous system (CNS) consumes a large part of the whole-body energy (Mink et al., 1981). Thus, mechanisms controlling memory formation depending on the energy status of the organism might have been selected during evolution. The simplest mechanism would be that energy availability or energy metabolism themselves control the costliest brain activities such as memory formation. The recent discovery that long-term memory formation in *Drosophila* is not permitted under food restriction (Plaçais and Preat, 2013) asked if particular metabolic pathways intervene in neurons during long-term memory formation, and if indeed energy metabolism could have an instructive role in memory formation.

In my thesis, we studied *Drosophila* aversive olfactory memory in order to decipher the interplay between metabolism and long-term memory formation. Thanks to the extensive work previously performed on this model organism, the neural networks involved in long-term memory formation have been precisely described. We thus focused our study on the main associative structure involved in memory formation, the mushroom body and its intrinsic neurons the Kenyon cells. Our goal was to understand which metabolic pathways were involved in the different Kenyon cells neuronal compartments upon memory formation. Moreover, we aimed at assessing if neuronal energy metabolism was supported by glial cells during memory formation.

In order to answer these questions, it is first necessary to have an overview of what we know about neuronal metabolism. Central nervous system metabolism is peculiar as compared to other tissues. During the introduction, I will first discuss about these particularities and question the limits of our knowledge on the metabolic compartmentation between neurons and glial cells. I will then focus on memory formation and its relation to energy metabolism in the model organism I studied during my work, *Drosophila melanogaster*. Lastly in this introduction, I will describe *Drosophila* glia and discuss what is known about their involvement in brain metabolism and memory formation.

Energy metabolism in animals comprises all the chemical reactions occurring in a cell that liberate free energy from the breakdown of complex carbon macromolecules. It is a dynamic process that adapts constantly to meet the anabolic and energetic demand of the cells based on cell-intrinsic and environmental signals. Because energy constraints were prominent at the very beginning of life on earth, putting a strong pressure on the evolution of efficient energy producing systems, the cellular pathways involved in energy metabolism are almost universally shared among eukaryotes.

Still, some cells seem to require more energy to function properly and survive. During a stroke, for example, the most sensitive cells to blood flow deprivation are neurons (Lee et al., 2000). This prominent vulnerability of brain tissue to ischemic damage reflects its high metabolic rate.

Moreover, several CNS pathologies are linked to energy metabolism defects. For example, metabolic dysfunction is one candidate for initiating Alzheimer's Disease (AD) in humans (Lin and Beal, 2006; Mattson et al., 2008; Murray et al., 2011). The causal role of metabolic dysfunction in AD is supported by the increased epidemiological risk of AD associated with metabolic diseases such as diabetes, and by the fact that several metabolic enzymes involved in energy metabolism are downregulated in AD brains. In Parkinson's Disease (PD), the CNS metabolic profile is also modified (Mattson et al., 2008), and the main murine PD model, MPTP injection, causes degeneration of dopaminergic neurons and PD-like symptoms in animals by a mechanism involving the selective inhibition of mitochondrial complex I (Gerlach et al., 1991), suggesting that a metabolic impairment can be the cause of the disease.

As metabolic defects are strongly correlated to neuronal dysfunctions, we can expect that neurons have a high energy demand, and that the CNS activity must be energetically demanding.

I. 1. The central nervous system is an energetically demanding structure

The human CNS is composed of about 10^{11} neurons and the same number of glial cells (Herculano-Houzel, 2014), while the whole body contains 3.72×10^{13} cells (Bianconi et al., 2013). The nervous system thus hosts less than 1% of the body's cells. Nervous cells, as other cells, must get some energy to support their cellular works. Intuitively, one could think that they should consume 1% of the energy of the body; is it indeed the case? What energetic substrates do they use? Which cellular processes represent the main energetic cost during their activity? We will try to bring pieces of answer to these questions in this section.

I. 1. a. CNS energy consumption

It is often quoted in the literature that the brain accounts for 20% of energy expenditure while comprising less than 2% of the total body weight (Kety, 1957; Magistretti and Allaman, 2015; Barros et al., 2018), which sounds exceedingly high, but actually only represents 20 Watts. To my knowledge, the first documented attempt to measure brain energy consumption was done by Benedict, in 1938 (Benedict, 1938). He measured O_2 consumption and CO_2 production as an indirect measure of the metabolic rate in different organs. His data were subsequently used (Mink et al., 1981) and the comparison between the brain oxygen consumption and the whole body oxygen consumption yielded the conclusion that the human brain accounts for 20% of the whole body O_2 consumption. Besides, researchers found that the CNS at rest consumes 5.6 mg of glucose for 100 g of brain tissue per minute, which means in total for a brain weight of 1400 g, about 110 g glucose/day, or 440 kcal/day (Owen et al., 1967). As, on average, a human adult should absorb about 2000 kcal a day, and given that the recommended amount of carbohydrates intake is 55% of our caloric supply (according to the french Apports Nutritionnels Conseillés <https://www.anses.fr/fr/system/files/NUT2012SA0103Ra-2.pdf>), one should absorb the equivalent of 1100 calories in carbohydrates. As carbohydrates have a caloric content of about 4 kcal/g, it means that one should absorb 275 g of carbohydrates a day. Then, the glucose consumption of the brain represents either about 20% of the whole body energy intake, or 40% of the body total carbohydrates consumption (Shah

et al., 2012). Thus, the brain requires a lot of the whole-body energy. But what is this energy needed for?

I. 1. b. Neuronal energetic sinks

In mammals, we define two kinds of tissues in the CNS: the grey and the white matter, depending on their color after transversal section. The grey matter is located mostly in the periphery of the brain (cortex), in some internal brain structures, and in the middle of the spinal cord. It is composed of neuronal cell bodies, dendritic and post-synaptic compartments, capillaries and glial cells – mainly astrocytes and microglia. It also contains a small proportion of axons and myelin. On the contrary, the white matter is composed of axons aggregated in nervous tracts, and oligodendrocytes that enwrap the axons and form the myelin sheath. This myelin sheath gives the white color to the tissue. The pioneering neurobiologist Louis Sokoloff developed the 2-deoxyglucose tracer method in order to follow glucose uptake in these different brain regions, but also depending on cognitive tasks. This method revealed that basal rates of glucose metabolism vary between brain tissues, the most energetically costly compartment of the mammalian CNS being the grey matter (Sokoloff et al., 1977). White matter is supposed to consume only one third of the budget of grey matter.

Therefore, neuronal soma, dendrites, synapses and glia must be the main energetic wells in the CNS. Among these, which compartments are the main energy consumers? And, in these compartments, which cellular processes require energy?

I. 1. b. i. The energy budget of the grey matter

Brain glucose and O₂ consumption have been measured in humans and rats in different activity states (Figure 1), i.e. 0. no ion transport (injection of Na/K ATPase inhibitor), 1. no action potentials (such as after TTX treatment), 2. low functional activity without consciousness (such as coma or anesthesia), 3. functional activity and consciousness, and 4. high cortical activity with a concomitant high physiological activation (Lajtha et al., 2007, in chapter 3 written by Gjedde, Brain energy metabolism). The study showed that an increase in brain function correlated with an increase in glucose and O₂ consumption. This shows that brain metabolism increases with brain functional activity and cognition. It is thus supposed that neurons, as the

action potential-generating and information-encoding cells, consume most of the energy dedicated to the CNS. Thus, it was subsequently assumed that cerebral glucose and oxygen consumption could be used as indirect measures of local neuronal activity, and have been extensively used as methods for measuring local brain activity during different tasks (Barros et al., 2017).

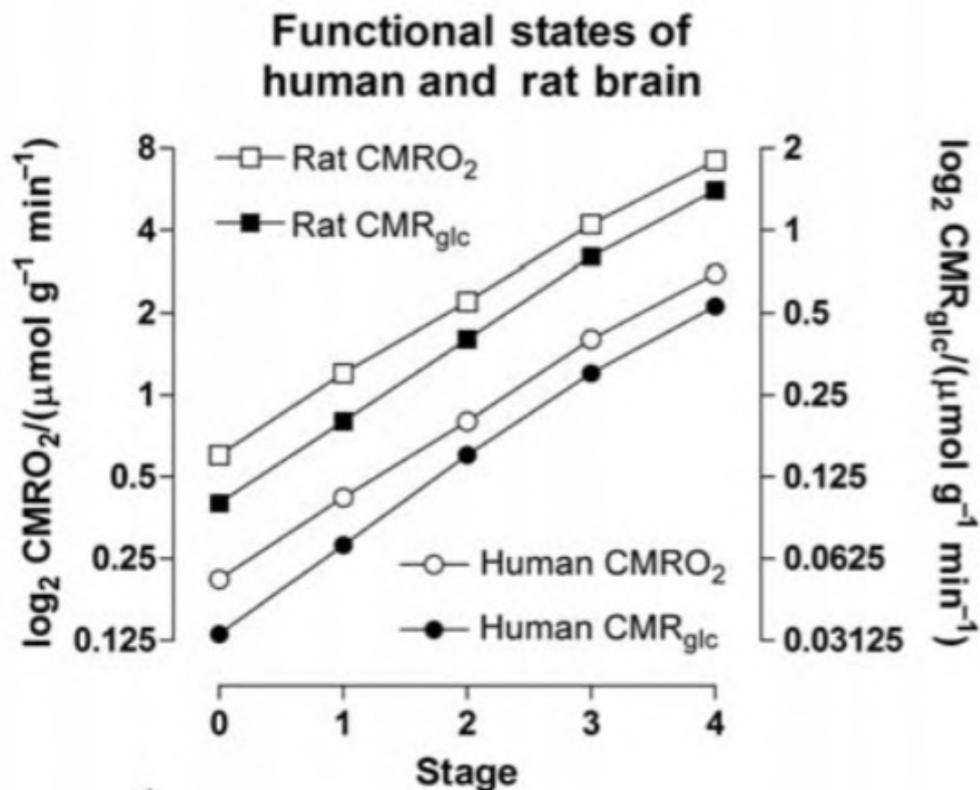


Figure 1. Relationship between functional activity of mammalian brain and estimates of rates of energy metabolism of oxygen and glucose. Reviewed in (Lajtha et al., 2007), chapter 3 written by Gjedde, Brain energy metabolism. CMR: Cerebral metabolic rate.

In modeling studies based on theoretical and experimental data (Attwell and Laughlin, 2001; Howarth et al., 2012), the contribution to energy consumption of the different molecular mechanisms occurring during signaling in the grey matter was estimated. About 25% of energy consumption in the cerebral cortex is supposed to be dedicated to housekeeping mechanisms (Howarth et al., 2012; Jolivet, 2009). Out of the remaining 75% of energy and considering only glutamatergic synapses, the authors calculated that generation of action potentials should account for 21% of energy consumption, postsynaptic receptors activation and the following ion fluxes 50%, maintaining the resting potential 20%. 4% of the energy would be consumed

by glutamate recycling, and 5% by presynaptic calcium entry. The corrected numbers relative to 100% energy consumption including housekeeping mechanisms are shown in Figure 2. This suggests that the cost of maintaining a resting potential (15% of the energy) is four times lower than the cost of neuronal transmission (about 60%).

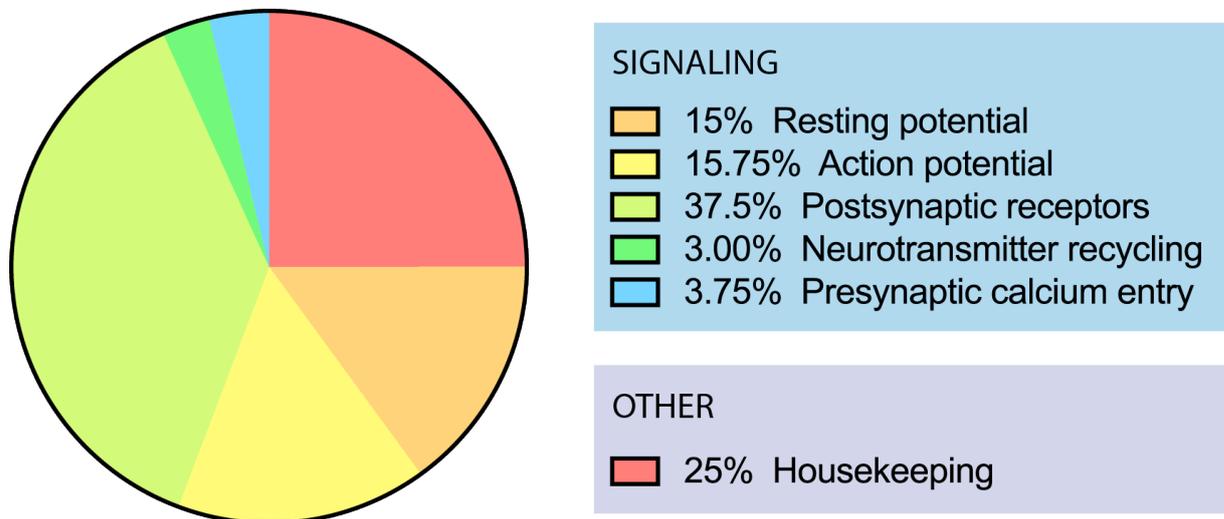


Figure 2. Predicted energy budget for the cerebral cortex. Adapted from (Howarth et al., 2012).

This modeling study was based on experimental data and assumptions. The amplitude of neuronal activity or its duration were not taken into account. It is therefore possible that the models are not relevant for *in vivo* neuronal activity that can vary in intensity and duration. Moreover, some experimental data contradict the model, so even if it gives clues about the cost of different processes, these data must not be naively applied to any condition. For instance, a study measured ATP consumption in presynaptic terminals from hippocampal neurons in culture following electrical stimulation (Rangaraju et al., 2014). Thanks to the use of pharmacological tools on cultured hippocampal neurons from wild type and mutant mice, the authors found that the primary source of activity-driven metabolic demand was the synaptic vesicle cycle, in contradiction with the previous models. If this is still true *in vivo* remains to be determined, as the measures were performed in hippocampal neurons primary cultures, and as no information was given in the study about the presence of glial cells in the culture. Neuronal signaling is thus a very energy-demanding process,

as it was first postulated. What accounts for the main energy burdens among housekeeping mechanisms, that consume 25% of brain total energy?

I. 1. b. ii. The cost of housekeeping mechanisms in the CNS

Housekeeping mechanisms correspond to the basal processes needed in the cells for their proper function, for instance the turnover of macromolecules, such as lipids and proteins in either neurons or glia, or to axonal transport (Attwell and Laughlin, 2001). The precise cost of all of these processes has only been evaluated in juvenile rat brain slices (Engl et al., 2017). Interestingly, blocking action potentials thanks to TTX in slices does not affect oxygen consumption, demonstrating that in slices, spontaneous activity does not impact brain metabolism, which allows furthermore to study the metabolic cost of different housekeeping mechanisms by using pharmacological tools. Na-K ATPase activity still accounts for 50% of oxygen consumption in slices, showing that Na-K ATPase highly contributes to brain energy consumption even in the absence of action potentials, probably to maintain the resting potential, which does not belong to housekeeping processes. Actin and microtubule treadmilling account for about 25% of oxygen consumption. Lipid synthesis surprisingly accounts for as much as 20% of the slice oxygen consumption, while protein synthesis is negligible, which is consistent with previous models (Engl and Attwell, 2015). It is still again important to mention that this study was performed in slices, and that the cost of housekeeping processes has not been measured *in vivo* in functional brain tissues.

Thanks to the above-mentioned studies, we can draw a global overview of the cost of the different cellular processes in the CNS. But these studies were based mainly on *ex vivo* data, did not take into account the cellular heterogeneity in the brain (neuronal populations, glial subtypes) and focused on baseline brain activity. It is now well acknowledged that a variety of stimuli trigger different neuronal activity states, for example neuronal spiking, with or without LTP or LTD, plasticity genes induction, protein synthesis... All these events can have a metabolic cost, but the study of neuronal energy consumption during memory formation as compared to baseline activity is still not well described in the literature. We will come back to this fundamental question over the following chapters.

I. 2. Taking-up some energy: from blood to neurons

Cells use the energy that derives from the breakdown of organic macromolecules to synthesize a universal chemical energy carrier in cells, ATP, from ADP and inorganic phosphate. Macromolecules such as fatty acids, amino acids, carbohydrates or ketone bodies are broken-down by different catabolic pathways. Under fed, physiological situations, the only metabolite that shows a difference in concentration between the blood provided to the CNS from arteries and the blood coming out of the CNS and conveyed in veins (“arteriovenous difference”) is glucose (Siesjö, 1978; Sibson et al., 1998). It has therefore been postulated that in normal conditions, the CNS only relies on glucose consumption to get the energy required for its function. Still, in certain circumstances, the CNS has been shown to use alternative fuels. We will give a very quick view of these, before coming back to the mechanism by which glucose reaches the CNS.

I. 2. a. A diversity in the metabolites used by the CNS

Electron micrographs of brain slices show that neurons and glia are densely filled with many mitochondria (Pysh and Khan, 1972). This observation is sufficient to hypothesize that brain tissues are highly oxidative, and that probably a majority of the metabolites consumed by the brain go through oxidative phosphorylation (OxPhos). Still, all the main energetic molecules, i.e. carbohydrates, lipids and amino acids, can be transformed into acetyl-coA, the metabolite used for mitochondrial OxPhos. This asks the question: which molecules are indeed consumed by the CNS in general, and in particular by neurons? Is glucose the only metabolite consumed by the CNS?

Siesjö, in 1978, showed that glucose was the obligate energetic fuel of the mammalian brain and the only substrate able to completely sustain neural activity. This view still dominates the field of neuroenergetics. The measured 1:6 ratio for glucose over oxygen consumption in the brain moreover supports the hypothesis that glucose is completely oxidized to CO₂ and H₂O through OxPhos, and that no other metabolite should be used by OxPhos in the brain.

Still, several other molecules can participate to CNS energy metabolism, depending on the energetic status of the whole organism, which allows humans to

survive many days of starvation while only storing 300g of carbohydrates maximum (Owen et al., 1967). Owen et al. showed in the 1960s' that in fasting humans, the brain can actually use a significant amount of ketone bodies (Owen et al., 1967), circulating metabolites that can be synthesized out of lipids.

Some other studies discussed about the possible direct utilization of lipids as energy metabolites for the brain in certain species (Pappenheimer and Setchell, 1973). In hypoglycemic sheep, glucose:O₂ consumption ratio decreases while the sheep brain has been reported not to efficiently take up ketone bodies (Lindsay and Setchell, 1976). The authors raised the possibility that fatty acids were used as energetic fuels in the CNS of starving sheep. In *Drosophila*, lipid droplets are stored in cortex glia, a glial subtype that unwraps all the neuronal soma (Wigglesworth, 1960; Bailey et al., 2015), and a decrease in beta-oxidation in glia can cause neurodegeneration (Schulz et al., 2015). Finally, thanks to the analysis of metabolites derived from ¹³C-labeled octanoate fatty acid perfused in living anesthetized rats, it has been suggested that fatty acids contribute as high as 20% to total brain oxidative energy production (Ebert et al., 2003). Interestingly, the analysis of metabolites containing the heavy isotope of carbon suggested that this beta-oxidation of fatty acids take place in the brain compartment where glutamine is synthesized, i.e. in glial cells. This implies that glial cells consume a much larger part of brain energy than previously expected, and that they can use fatty acid oxidation in addition to carbohydrate oxidation. Therefore, even if not widely accepted, fatty acids could support a significant proportion of the brain energy needs in certain conditions.

Finally, a study measured ¹⁴CO₂ produced from a variety of ¹⁴C-labeled compounds perfused in the brains of freely-moving rats (Zielke et al., 2007). The authors showed that glucose, lactate, pyruvate, glutamate, glutamine and acetate can all be oxidized by the mammalian brain to produce CO₂.

Still, even though other substrates can eventually be used as energy fuels for brain cells, the major metabolic substrate used in physiological conditions is glucose. How does this molecule reach the CNS?

I. 2. b. Glucose transport from BBB to brain cells

The CNS is separated from the rest of the body by a blood-brain barrier (BBB) in mammals, or its equivalent in insects (Obermeier et al., 2013). The BBB is necessary for maintaining an adequate microenvironment for neuronal survival and activity. It prevents immune cell infiltration and the paracellular diffusion of ions, small molecules, and macromolecules such as proteins thanks to the presence of tight, occlusive junctions between the cells forming the barrier. Therefore, it also blocks the entry of energy metabolites. How does glucose enter the CNS?

I. 2. b. i. The GLUT family of glucose transporters

Glucose belongs to the family of polar molecules, because of the presence of several alcohol and one aldehyde residues, and thus cannot freely diffuse through membranes. It is transported by the GLUT family of proteins, which are facilitative saturable membrane transporters (Mueckler and Thorens, 2013). GLUT proteins possess 12 transmembrane segments. They function according to the glucose concentration gradient and are therefore ATP-independent. Glucose binds at a specific extracellular site of the GLUT transporter, producing a conformational change in the transporter protein. After this change, the bound glucose is exposed to the intracellular compartment and may diffuse away (Vannucci et al., 1997). GLUT catalyze bi-directional transport. Thus, the presence of intracellular and/or extracellular glucose can affect the kinetics of glucose exit or entry.

I. 2. b. ii. Expression of glucose transporters in the mammalian BBB

Glucose concentration in the blood is supposed to be 5,5 mM, while brain glucose concentration is about 1,2 mM (Simpson et al., 2007). This gradient is favorable for glucose import into the brain if transporters are expressed by the cells forming the BBB. At the level of the capillaries, this BBB corresponds to endothelial cells forming the vessels and linked by tight junctions. They are covered by pericytes that control the vessels diameter and by astrocytic endfeet (Figure 3). Thus, to enter the CNS, glucose must go through the endothelial luminal and basal membranes at least. This transport is mediated by GLUT1, which is highly concentrated in both endothelial membranes (Vannucci et al., 1997; Simpson et al., 2007). Thanks to electron micrographs associated to immunogold labelling of GLUT1, it has been showed that

endothelial cells have an intracellular pool of GLUT transporters (Simpson et al., 2007). This pool was suggested as being possibly translocated to the luminal membrane upon stimulation in order to increase glucose transport, but such an effect has still not been demonstrated *in vivo*. Thanks to endothelial cells GLUT1 expression, glucose can enter the brain following its concentration gradient.

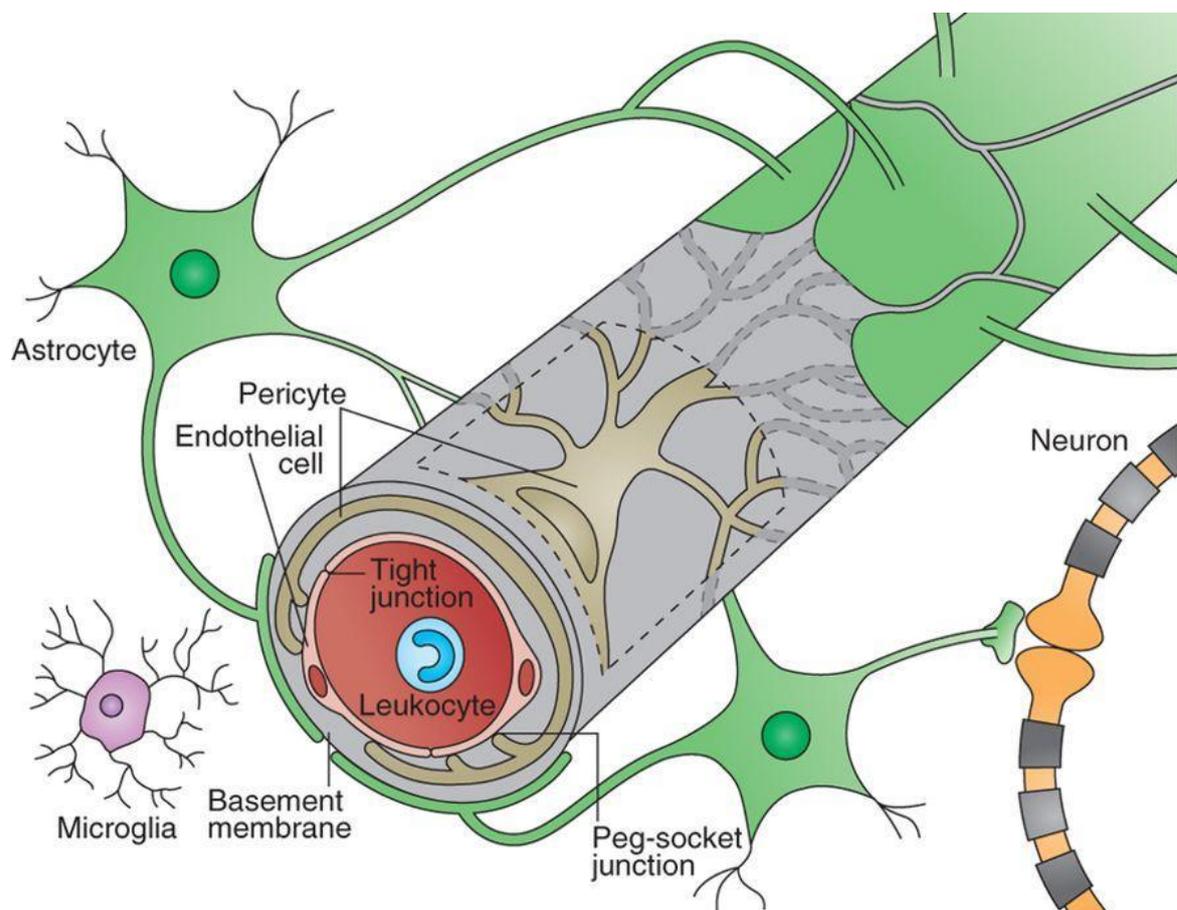


Figure 3. Organization of the blood brain barrier at the level of capillaries. Vascular endothelial cells sealed by tight junctions constitute the BBB. The endothelium abluminal surface is covered by a basement membrane in which pericytes reside. Astrocytic endfeet encircle the abluminal side of the vessel. From (Obermeier et al., 2013).

1. 2. b. iii. Expression of glucose transporters in mammalian brain cells

Once in the CNS, glucose can diffuse in the extracellular medium, where it can be taken-up by GLUT-expressing cells. Two glucose transporters are mainly expressed by brain cells in mammals. A 45 kDa light isoform of GLUT1 is expressed in all glia

and to a much lower extent in neurons. This light isoform of GLUT1 is reported to be mainly located in the grey matter, in astrocytic endfeet close to the capillaries, and in their processes adjacent to synaptic contacts (Morgello et al., 1995) (Figure 4). As astrocytic endfeet in contact with the capillaries express GLUT1, glucose can go through transglial route or diffuse paracellularly to reach neurons.



Figure 4. High magnification view of endothelial (e) and astrocytic (a) staining for GLUT1 in the monkey cortex. Electron micrograph showing GLUT1-immunoreactive processes that surround a region of synapses (s) in both endothelial (e) and astrocytic (a) cells. Magnification x23.240. From (Morgello et al., 1995).

GLUT2 is expressed in a small astrocytic subpopulation. The GLUT2 glucose transporter is the predominant glucose transporter in mammalian liver where it functions in bidirectional glucose transport. It is a high-capacity, low-affinity transporter, which contributes to the cells sensitivity to glucose. Astrocytes that express this transporter could therefore be involved in glucose sensing (Vannucci et al., 1997).

GLUT3, on the contrary, is expressed only in neurons. Interestingly, besides the CNS, GLUT3 is expressed in sperm, placenta, platelets, all cell types that display a high glucose metabolic rate (Simpson et al., 2007). In rat cerebellar granule cells

cultures, GLUT3 transports glucose at a six folds higher rate, with a higher affinity (about 3 mM) as compared to GLUT1 (8 mM) (Simpson et al., 2007). It is not clear whether GLUT3 is more present at the level of the neuronal processes or somata. Both have been described in the brain (Yu et al., 1995) as well as in cell cultures (Simpson et al., 2008). Thanks to this diversity of cell-type specific expression of GLUT proteins, all cell types - neurons and glial cells - are able to take-up glucose, likely with different kinetics and affinities.

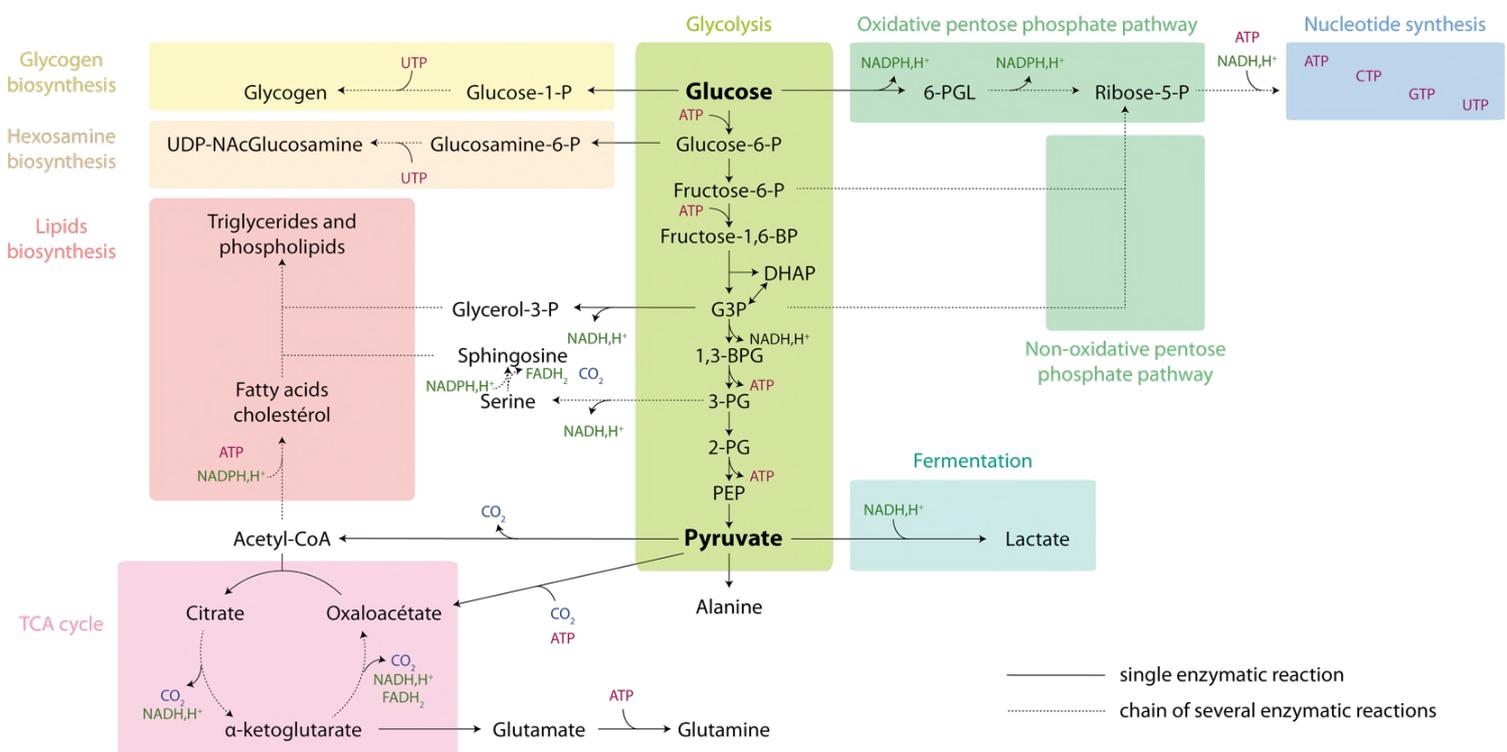


Figure 5. Glucose use by anabolic and catabolic pathways in animal cells. 1,3-BPG, 1,3-bisphosphoglycerate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 6PGL, 6-phosphogluconolactone; DHAP, dihydroxyacetone-phosphate; Fructose-6-P, fructose-6-phosphate; Fructose-1,6-BP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; Glucose-1-P, Glucose-1-phosphate; Glucose-6-P, Glucose-6-phosphate; Glucosamine-6-P, glucosamine-6-phosphate; PEP, phosphoenolpyruvate; Ribose-5-P, ribose 5-phosphate; UDP-NAcGlucosamine, uridine diphosphate-N-acetylglucosamine.

I. 2. c. A brief overview of the main glucose catabolic pathways

I will discuss the main concepts that are worth remembering about glucose cellular utilization in the context of the work done during this thesis. More information can be found about glucose metabolism in the Annex.

Glucose is a carbohydrate with 6 atoms of carbon. Its oxidation provides energy for the synthesis of ATP. It can be partially oxidized through glycolysis or the pentose phosphate pathway, providing precursors to fuel anabolic pathways, or subsequently fully oxidized through mitochondrial respiration (Figure 5). The main pathways providing substantial amounts of ATP are glycolysis and oxidative phosphorylation. These two pathways have different kinetics, anaplerotic properties and energetic yields. The flux of glucose and glucose derivatives through these pathways is modulated depending on the cellular needs. I will give a few considerations on the cellular consequences of the main glucose catabolic pathways and discuss their relevance in the case of neurons and glial cells.

I. 2. c. i. Kinetics of ATP production

Glucose is transformed into pyruvate by glycolysis, which yields 2 ATP (Figure 5). The transformation of pyruvate into lactate does not yield energy, while pyruvate oxidation by mitochondrial respiration provides a high additional ATP yield of about 15 ATP molecules. Thus, one 6-carbon glucose molecule will give only 2 ATP molecules through glycolysis, while the oxidation of each glucose equivalent (2 pyruvate molecules) through the TCA cycle will give about 30 ATP. Under high energy demand, one could think that respiration provides energy faster than glycolysis followed by lactate synthesis, but glucose flux through glycolysis is supposed to be ten to a hundred times quicker than pyruvate oxidation through the TCA cycle; thus, ATP synthesis rates are considered to be approximately the same between both (Liberti and Locasale, 2016). What can then be the respective advantages of these two pathways?

I. 2. c. ii. Anaplerotic precursors

Transcription, *de novo* protein synthesis and membrane remodeling are required for neuronal plasticity, such as during long-term memory formation (Yin et al., 1994).

Regarding their products, both glycolysis and the TCA cycle can provide protein precursors (Figure 5), but only the first step of glycolysis provides glucose-6-phosphate used by the pentose phosphate pathway to synthesize NADPH, lipids or nucleotides precursors.

Noteworthy, mitochondrial respiration from acetyl-coA alone is not able to fuel anabolic pathways. Indeed, in order to maintain the pool of metabolites needed for the TCA cycle to function, no intermediate can leave the cycle without decreasing its efficiency. Other 4-carbons metabolites, like succinate or oxaloacetate, must be provided to allow a subsequently synthesized metabolite to escape the TCA cycle. Interestingly, pyruvate carboxylase is a mitochondrial enzyme that can transform pyruvate into oxaloacetate. Pyruvate thus enters the TCA cycle via the activity of two enzymes: pyruvate dehydrogenase that synthesizes acetyl-coA and pyruvate carboxylase providing oxaloacetate, and can therefore support anabolic in addition to energetic needs.

Considering neuronal anabolic needs, both glycolysis (including subsequent pentose phosphate pathway) and the TCA cycle must thus be required for neuronal plasticity, with glycolysis and OxPhos providing amino-acids precursors and ATP, and pentose phosphate pathway providing NADPH in particular.

I. 2. c. iii. Signaling properties

It has been suggested that lactate production following glycolysis could have a signaling function. Indeed, lactate activates signaling pathways in muscle (Philp et al., 2005) and nervous cells (Barros, 2013; Proia et al., 2016; Magistretti and Allaman, 2018). For instance, during exercise, lactate is released from muscular cells, and this increase in lactate concentration activates vascular endothelial growth factor (VEGF) release (Philp et al., 2005) and signaling. Moreover, lactate can bind to GPR81 also called HCAR1, a G protein coupled receptor in mammals (Proia et al., 2016). This receptor is connected to a G_i protein that inhibits adenylate cyclase, thus decreasing the concentration of the second messenger cAMP. The mRNA encoding GPR81 and the protein itself localize to the hippocampus, neocortex and cerebellum, in somato-dendritic compartments, but also in endothelial cells forming the BBB and in astrocytic processes, suggesting a possible signaling role of lactate in these cells. In

cortical primary neurons cultures, HCAR1 activation by lactate induces a decrease in the spontaneous calcium spiking frequency (Bozzo et al., 2013), suggesting that lactate can affect neuronal excitability. Moreover, lactate, but not glucose or pyruvate, induces the expression of several plasticity related genes such as C-Fos and Bdnf in cultured mouse cortical neurons (Yang et al., 2014), strengthening the notion that lactate acts as a signaling cue.

In a nutshell, glycolysis and the TCA cycle have different energetic yields, produce different anabolic precursors, and have distinct consequences on the cellular microenvironment. Deciphering which pathways take place in the brain in diverse structures, diverse cell types, diverse subcellular compartments, and during diverse tasks can therefore give us clues about the molecular needs in these specific areas and conditions. Neuroenergetics emerged as a promising field of research, and different theories are now discussed about metabolism in the CNS. As stated earlier, glucose is the main metabolite that is used in the CNS and must therefore reach CNS cells. The proportion of glucose taken-up by neurons and glial cells is not clear during basal condition or during neuronal activity. Is there a difference in glucose uptake and metabolism between these cell types, as suggested by the difference in glucose transporters expression?

I. 3. Metabolic compartmentation

I. 3. a. A discussed metabolic compartmentation between glia and neurons

There is a well-documented metabolic compartmentation between neurons and glial cells, but the exact flux of molecules going through the different catabolic pathways, both in neurons and in glia, are still under debate. It is important to keep in mind that cellular metabolism is not static but rather is a dynamic process that functions to meet the bioenergetic demands of a given cell at a given time. It is very likely that all pathways occur to a certain extent in all cell types, and the current work carried in neuroenergetics aims at deciphering if the intensities of fluxes going through metabolic pathways differ in distinct cell types.

To get energy metabolites, neurons must either take-up glucose from their environment or be supported by glial-derived metabolites. As described above, neurons almost exclusively express the GLUT3 glucose transporter while glia express the GLUT1 light isoform. As GLUT3 transports glucose at a higher rate than GLUT1 (Maher et al., 1996) and as GLUT1 and GLUT3 are found at similar concentrations in the rat brain, it suggests that neurons transport glucose more efficiently than glial cells when energy demand increases. Several studies have focused on which cell types take-up more glucose in the CNS during basal state and neuronal activity. Unfortunately, the conclusions were not consistent, perhaps because of the heterogeneity between the protocols used. For example, two *in vivo* studies measured glucose uptake by neurons and astrocytes during the same specific brain activity, but had inconsistent conclusions: the first one (Chuquet et al., 2010) showed an enhanced glucose uptake in astrocytes as compared to neurons, while a more recent work (Lundgaard et al., 2015) showed more glucose uptake in neurons as compared to astrocytes.

Besides, neurons and glia express different metabolic enzymes (Magistretti and Allaman, 2015) which must influence the flux of metabolites going through the different catabolic pathways. It has been proposed that glia display an increased glycolytic rate potential as compared to neurons (Bélanger et al., 2011). Indeed, in primary cultures, they express high levels of Pfkfb3 while neurons are devoid of this

enzyme. Pfkfb3 catalyzes the synthesis of fructose-2,6-bisphosphate, an activator of the glycolytic enzyme PFK (Almeida et al., 2004; Herrero-Mendez et al., 2009). Consistently, astrocytes express many glycolytic enzymes at higher levels than neurons (Lovatt et al., 2007).

Finally, neurons do not seem to contain any energy store such as lipid droplets or glycogen, likely as the result of an evolutionary constraint. Indeed, glycogen inclusions are deleterious for neurons in various species (Magistretti and Allaman, 2007; Vilchez et al., 2007; Duran et al., 2012; Sinadinos et al., 2014). On the other hand, glia such as astrocytes in mammals and different glial subtypes in *Drosophila* store glycogen granules (Freeman and Doherty, 2006; Brown and Ransom, 2007; Suzuki et al., 2011) and express the enzymes necessary for both glycogen synthesis and degradation (Lovatt et al., 2007). In insects, glial cells also store fatty acid droplets (Kis et al., 2015). Thus, neurons display a high energy demand but only glia actually store energy metabolites. This organization favors the view that glia support neuronal energy metabolism by providing chemicals to neurons. Accordingly, several lines of evidence, including anatomical and functional data, strengthen this idea. For example, an elegant approach has been employed to study the metabolic coupling between neurons and astrocytes in slices (Rouach et al., 2008). Glucose diffusion through gap junctions inside the astrocytic network is stimulated when inducing epileptic or evoked neuronal activity. As this effect is blocked by glutamate receptor antagonists, it depends on glutamate signaling, suggesting a metabolic support from glia to neurons “on demand”.

Noteworthy, when degrading their energy stores – fatty acids or glycogen – glia can provide neurons with glucose only if they express glucose-6-phosphatase, the enzyme that catalyzes the dephosphorylation of glucose-6-phosphate. Indeed, glucose-6-phosphate cannot be transported by GLUTs, and thus cannot get out of a cell. Glucose-6-phosphatase has been scarcely reported as expressed in glia in mammals. In *Drosophila*, its coding mRNA is not much concentrated in glia (Davie et al., 2018). Therefore, it is unlikely that glycogen-derived glucose is exported from glia to neurons. On the contrary, different other metabolites could be transferred to neurons following the degradation of these stores.

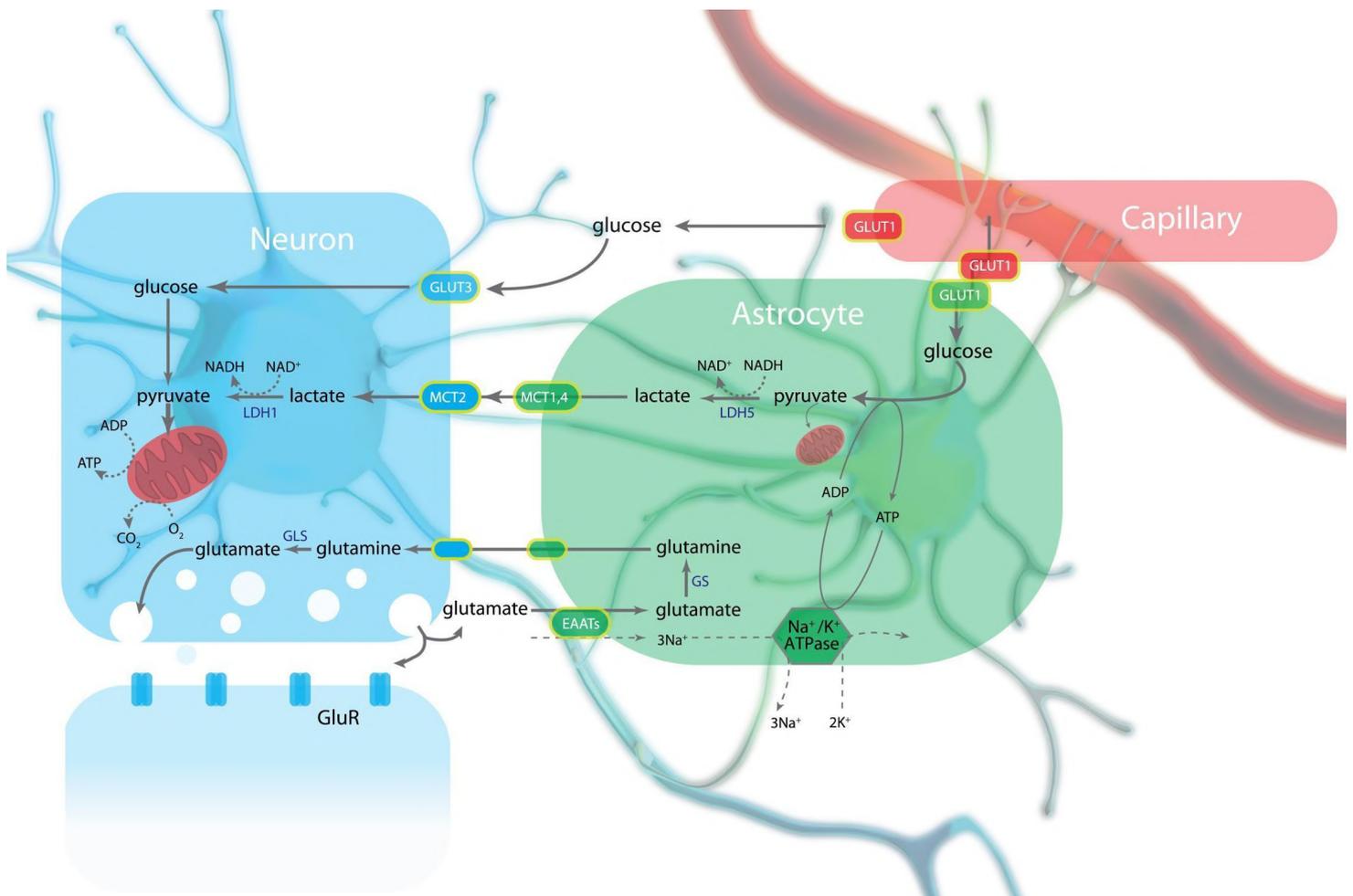


Figure 6. The Astrocyte-Neuron Lactate Shuttle model. A large proportion of the glutamate released by excitatory neurons at the synapse is taken up by astrocytes via excitatory amino acid transporters EAATs using the electrochemical gradient of Na^+ as a driving force, with a stoichiometry of one glutamate to three sodium ions. Na^+ concentration is maintained by the action of the Na^+/K^+ ATPase, an ATP-consuming process. This energetic cost triggers glycolysis in astrocytes followed by the conversion of pyruvate into lactate. Lactate is shuttled to neurons through monocarboxylate transporters. In neurons, lactate can be used as an energy substrate following its conversion to pyruvate by lactate dehydrogenase. This model describes a mechanism by which glia provide an energetic fuel to neurons on demand. From Magistretti and Allaman, 2015.

As astrocytes are thought to metabolically support neurons, one well-supported theory is that glia, being more glycolytic than neurons and expected to consume less energy, provide a glycolytic end-product – lactate – to fuel mitochondrial OxPhos in neurons (Pellerin and Magistretti, 1994) (Figure 6). In particular, lactate can be produced out of glycogen degradation. Accordingly, intracellular application of oxamate, an inhibitor of the lactate degrading enzyme lactate dehydrogenase,

hyperpolarizes neurons in slices, and decreases spiking frequency in CA1 hippocampal region *in vivo* (Sada et al., 2015). Pyruvate co-application rescued the effect of oxamate, showing that neuronal excitability is directly modulated by intracellular pyruvate availability, this pyruvate originating from lactate oxidation.

This Astrocyte-Neurons Lactate Shuttle (ANLS) theory has been supported by numerous studies but is still debated. I will give an example of contradictory data regarding this model. In a first study on brain slices, Kasischke and colleagues measured the intrinsic fluorescence properties of NADH/NAD⁺ with 2-photon excitation to evaluate oxidative metabolism over time in neurons and astrocytes in hippocampal slices (Kasischke et al., 2004). After neuronal excitation of the Schaffer collateral, a first dip in NADH thought to happen in neuronal mitochondria is followed by a NADH overshoot localized in astrocytes, in the stratum radiatum of CA1 region. This suggested an elevated mitochondrial activity in neurons, and a subsequent increase in glycolysis in astrocytes. In addition, the authors measured elevated cytoplasmic NADH levels by about 60% in astrocytes relative to the neighboring neurons, suggesting elevated glycolysis over mitochondrial activity in astrocytes as compared to neurons. Thus, this study supports the ANLS hypothesis.

A second study used the Peredox NADH:NAD⁺ fluorescent probe in neurons from acute hippocampal slices. Cytosolic NADH:NAD⁺ transients were measured in the dentate gyrus neuronal soma (Díaz-García et al., 2017). After 60 brief electrical pulses, cytosolic NADH:NAD⁺ ratio increased in neuronal soma, suggesting elevated glycolysis in this compartment. Blocking glycolysis at the GAPDH step by brief treatments with iodoacetic acid reduced the NADH:NAD⁺ transients, while blocking lactate import or lactate dehydrogenase did not block the increase, indicating that glycolysis is the source of NADH instead of OxPhos or lactate oxidation. The authors reproduced these results *in vivo* in the mice barrel cortex after sensory stimulation, showing that this effect is physiologically relevant. These data suggest that neuronal stimulation triggers neuronal glycolysis instead of lactate uptake, which contradicts the above-mentioned study (Kasischke et al., 2004).

Interestingly, the main difference between these studies is the neuronal compartment on which they focus. The first one studies NADH:NAD⁺ transients in a

CA1 region that contains mainly dendrites and astrocytes, while the second one focuses on neuronal cell bodies. Therefore, it is possible that the inconsistency in the results originate from the fact that metabolism is not the same between neuronal compartments, with a favored glycolysis in neuronal soma, and increased TCA cycle and OxPhos in synaptic compartments.

Thus, metabolism is still currently viewed as being compartmentalized between cell types, but controversy remains, and no clear rule has been demonstrated. It is possible that heterogeneity exists between brain regions or between different neuronal subtypes or compartments, even though both glycolysis and mitochondrial respiration should occur to a certain extent in all neuronal compartments and in glia. What constrains shaped this division of labor between cell types in the CNS? We will briefly discuss about the putative advantages of such a metabolic compartmentation in the next section.

I. 3. b. Advantages of a metabolic compartmentation

As it was mentioned before, glycogen storage is deleterious for neurons as it triggers apoptotic signaling (Vilchez et al., 2007) and neuronal loss (Duran et al., 2012), and is associated to reduced locomotion and lifespan in flies and mice. Moreover, glycogen accumulates in neurons during ageing, and preventing this accumulation improves neurological function with age (Sinadinou et al., 2014). The biological underpinnings of glycogen inclusions deleterious effects in neurons, but not in glia, remain to be elucidated. It is possible that glycogen storage implies structural constraints or chemical by-products, such as reactive oxygen species, that are more particularly toxic for neurons.

For the time being, differences in transporters and catabolic enzymes location between the different compartments of a single neuron have not been deeply studied. Would it be possible that neurons, which are highly organized and polarized cells, have a compartmentalized intracellular metabolism? We can expect that at the synapse, the huge energy burden associated with synaptic activity will be met thanks to high-yield, fast metabolic pathways, while the longer-term energetic cost of

housekeeping mechanisms, mainly in the soma, would be supported by slower metabolic pathways. It is then possible that all the neuronal compartments are not equipped with the same enzymatic content and the same mitochondrial density, and that biochemical compartments exist in neurons.

The possibility that neurons display metabolites intracellular compartments has been theoretically ruled-out for many chemicals (Barros and Martínez, 2007). Indeed, metabolites such as glucose, ATP, pyruvate or lactate are too highly concentrated (> mM) and diffuse too fast to allow the existence of metabolites concentration gradients in the cytosol. Still, with no need for concentration gradients, it is likely that local fluctuations in metabolic rates occur in different cytosolic compartments. Indeed, it is already known that neuronal enzymes and organelles are not evenly distributed in all compartments, and some recent evidence support this idea of an intracellular compartmentation of metabolism (Saks et al., 2008; Jang et al., 2016; Dienel et al., 2017).

In a paper aiming at describing the different compartments in unipolar *Drosophila* neurons, the authors described that the ribosomal protein synthesis machinery was mainly located in the neuronal cell bodies (Rolls et al., 2007; Rolls and Jegla, 2015), and suggested that there should be an active mechanism that restricts the protein machinery to this compartment. Similarly, endoplasmic reticulum and lysosomes are highly concentrated in the cell bodies of the neurons (Maday and Holzbaur, 2016). Besides, mitochondria are distributed in all neuronal compartments, but they are very densely located in the neuronal soma, and the soma seems to be the main compartment where they are synthesized and degraded even though some synthesis and degradation can occur at distal sites (Misgeld and Schwarz, 2017).

In *C. elegans*, the formation of metabolic compartments *in vivo* near synapses was recently described (Jang et al., 2016). These metabolic compartments, called “metabolons”, contain several glycolytic enzymes responding to local energy demands and synaptic function.

Altogether, these data support the view that metabolism could be heterogeneous between the neuronal functional domains.

Whether neuronal metabolism is not homogenous between compartments is a technically difficult question to answer in mammals. Indeed, neuronal soma, dendrites and synaptic compartments are often intermingled and very close to each other in the mammalian brain. Moreover, a single astrocyte can potentially contact all these three compartments. On the contrary, the *Drosophila* brain contains unipolar neurons. If presynaptic and postsynaptic compartments are difficult to distinguish and are not always well polarized along the neurite (Takemura et al., 2017), neuronal soma are clearly separated from the neuropil. Indeed, all the neuronal soma are located in the periphery of the brain, in a region called the cortex. There, they are individually encapsulated by astrocyte-like cells called cortex glia. *Drosophila* neurons extend a single process towards the neuropil located in the center of the brain, where their neurites are in contact with neuropil glia subtypes. It is thus a very convenient model to study, first, the metabolic pathways taking place in each neuronal compartment, and second, the interactions between the different neuronal compartments and glial cells.

In order to have a better understanding of the work done during the thesis, it is crucial to appreciate the particularities of *Drosophila* metabolism, and what we currently know about energy metabolism in the *Drosophila* brain.

I. 4. Specificities of *Drosophila* metabolism

Contrary to mammals, *Drosophila* do not have closed blood vessels, or hemoglobin or erythrocytes equivalents. Instead, an open circulatory system driven by a contractile dorsal vessel allows the transport of metabolites in the hemolymph to the different organs (Pereanu et al., 2007). Oxygen reaches internal tissues thanks to a branched network of air-filled tubes called tracheae, which are independent from the dorsal vessel (Figure 7 and Figure 9).

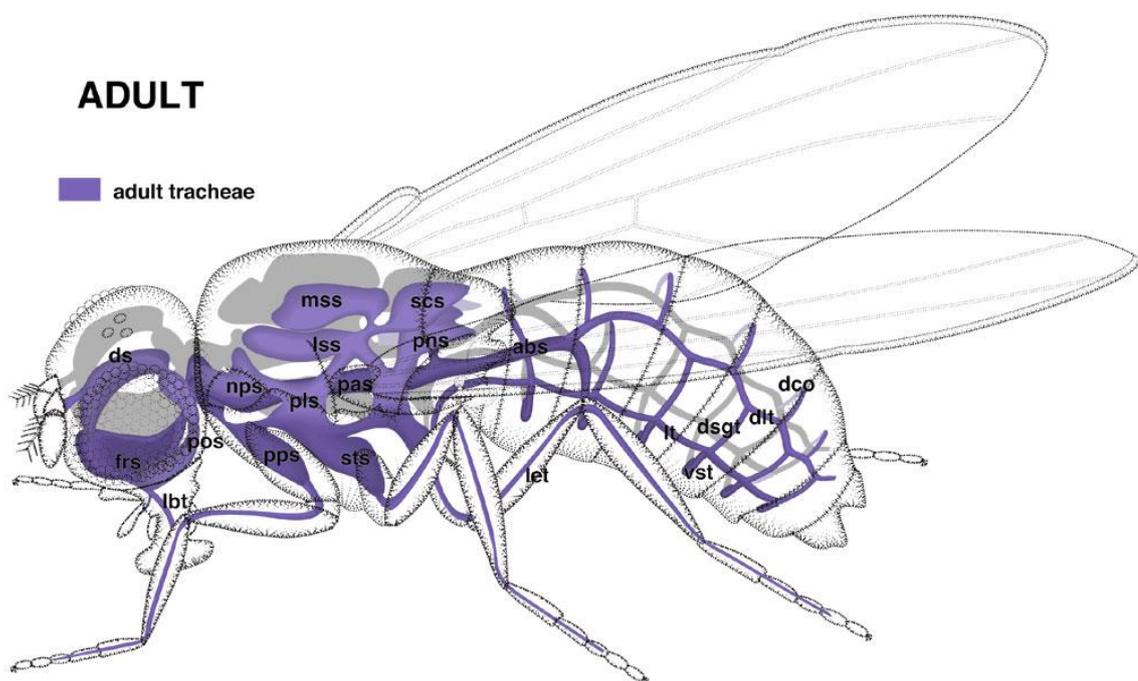


Figure 7. The main tracheal system of adult *Drosophila*. Flies tracheal system (purple) consists of sacs and tubular structures called tracheae, opened at one end at the body surface, and branching out into the body at the other end, directly connecting tissues. From <http://www.sdbonline.org/>, Volker Hartenstein.

I. 4. a. Trehalose is the main circulating sugar in the hemolymph

The composition of the invertebrate hemolymph is different from the mammalian blood. For example, potassium concentration is about 3 mM in human blood but 15 mM in *Drosophila* (Olsson et al., 2016). Insects hemolymph contains high amounts of peptides, free amino acids, and proteins (Reyes-DelaTorre et al., 2012). Other

important differences are the levels and identities of circulating carbohydrates. In mammals, a circulating D-glucose level of 5,5 mM is tightly maintained thanks to different hormones such as insulin and glucagon that act on various remote tissues. This is fundamental for the physiological integrity of proteins and lipids, as glucose is a very reactive reducing carbohydrate, that reacts in particular through a glycation reaction that is non-enzymatically controlled. Glycation can alter proteins or lipids properties, decreasing their functionality, and is a health issue in diabetic patients with high glycaemia.

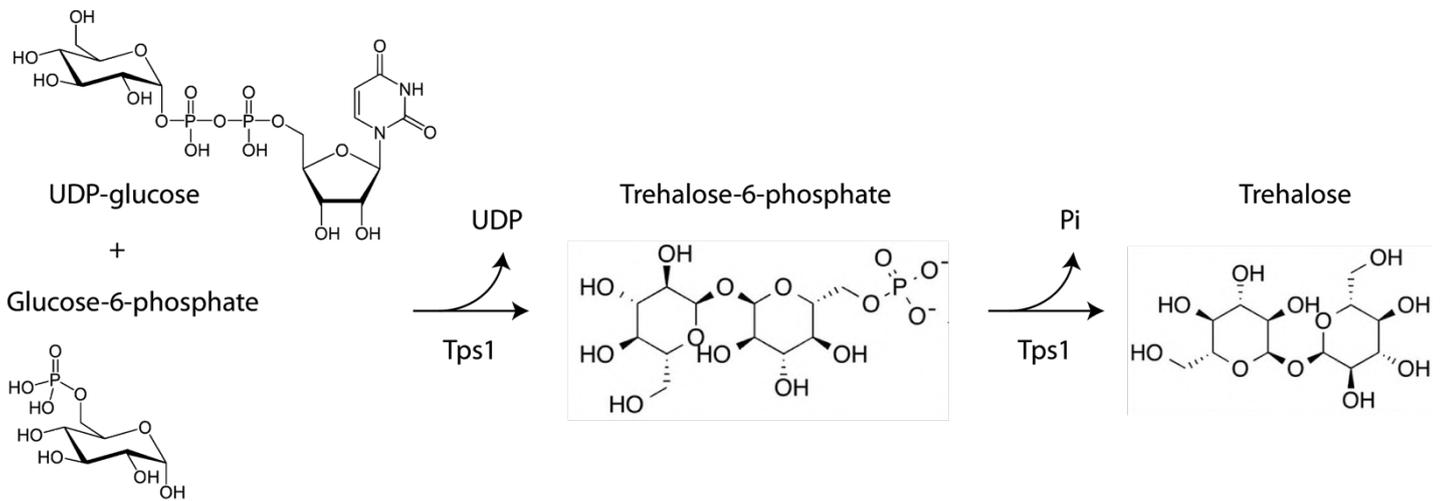


Figure 8. Trehalose synthesis is catalyzed by Trehalose-6-phosphate synthase 1 (Tps1) in *Drosophila*. Tps1 N-terminal trehalose-6-phosphate (T6P) synthase domain catalyzes the production of T6P using glucose-6-phosphate and UDP-glucose. Tps1 C-terminal T6P phosphatase domain dephosphorylates T6P to generate trehalose.

Many diverse species among algae, plants, fungi and invertebrates fila, use trehalose as a go-between between carbohydrate storage and the availability of glucose for cellular needs (Reyes-DelaTorre et al., 2012) (Figure 8). Trehalose constitutes the major hemolymph sugar in particular in most arthropods. Indeed, the absorption of radioactive glucose and other carbohydrates in the gut of *Schistocerca*, a locust, rapidly gives rise to the appearance of radioactive trehalose in the hemolymph (Treherne, 1958). Accordingly, in the cockroach, 30 min after the injection of radiolabeled glucose into the hemolymph, only radiolabeled trehalose is detected (Treherne, 1960). Trehalose levels increase after glucose ingestion while body glucose

does not change, and trehalose concentration drops rapidly during insects flight (Evans and Dethier, 1957), suggesting a strong implication of trehalose in their energy metabolism.

Trehalose is synthesized by the insect functional equivalent of the liver, the fat body (Figure 9), a diffuse organ that extends in the thorax, the abdomen and the head and which is in strong contact with in the hemolymph (Candy and Kilby, 1959; 1961). Fat bodies also store glycogen, fat and proteins.

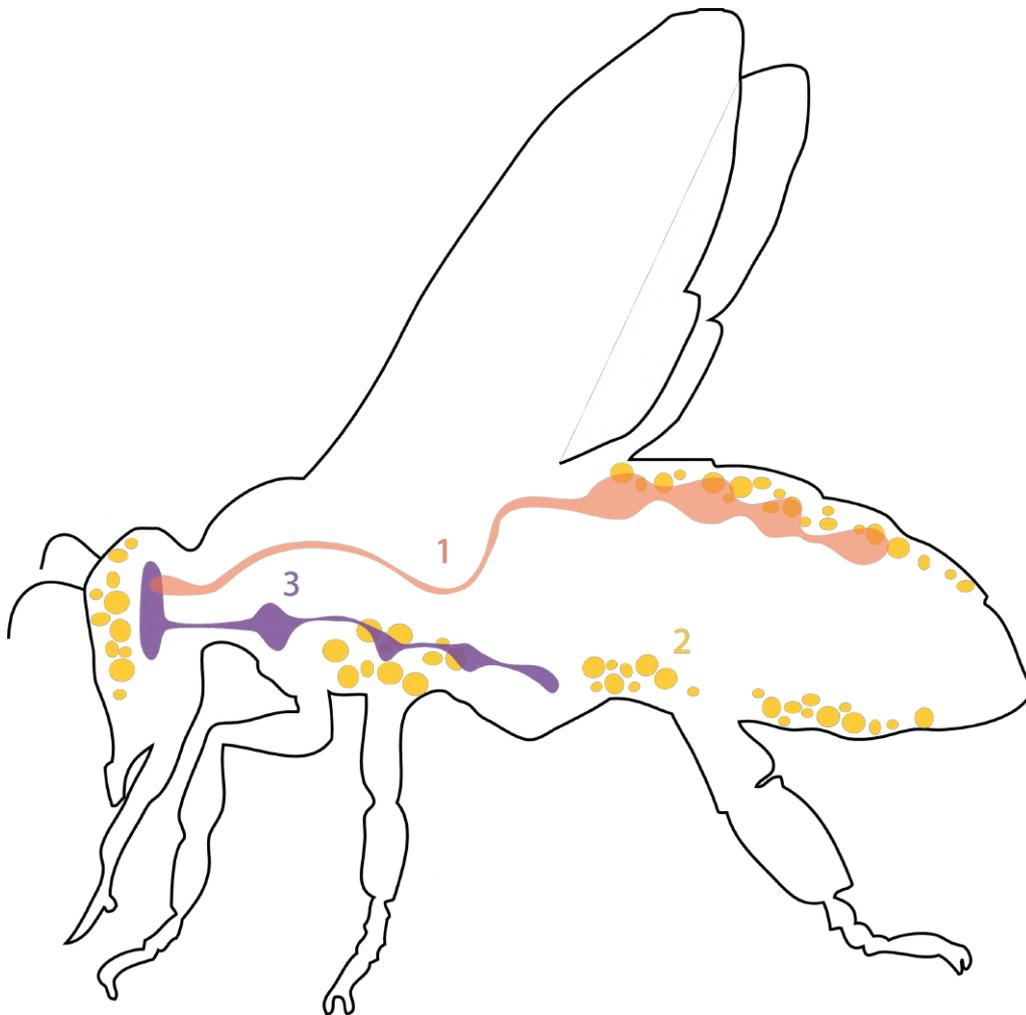


Figure 9. Schematic of the location of the major systems associated with brain metabolism in *Drosophila*. 1. Hemolymph circulation is driven by the contraction of a dorsal heart. 2. The fat body is a diffuse tissue found in the head, thorax and abdomen. It is composed of fat cells located right underneath the cuticle, and is here shown in yellow. 3. The CNS is composed of a brain and ventral nerve cord with segmented ganglia. Adapted from (Lazareva et al., 2007; Tatar et al., 2014) and (Rittschof and Schirmeier, 2017).

Trehalose is synthesized from the condensation of two glucose equivalents, yielding a non-reducing sugar far less reactive than glucose (Figure 8), and with better stability at high temperature and in extreme pHs. This stability allows no reaction with amine groups, which is particularly relevant for insects which hemolymph contains high concentrations of amino-acids and proteins. In addition, trehalose also functions as a stabilizer in solutions for other organic molecules, like lipids and proteins.

Thanks to trehalose chemical properties, its level in insects hemolymph is not as low and tightly regulated as glucose level in mammals. Indeed, trehalose levels can vary by an order of magnitude between conditions (Becker et al., 1996), from 3,5 to 70 mM in the blowfly (Evans and Dethier, 1957), 40 mM in the cockroach (Treherne, 1960), 20 mM in the locust (Treherne, 1958) and up to 2% in the desert locust (Howden and Kilby, 1956), which means around 60 mM. Glucose and fructose are also present, generally in lower and more variable concentrations that depend on the nutritional state of the animal (Rittschof and Schirmeier, 2017); for instance, glucose concentration in the hemolymph is about 2 mM in the cockroach (Treherne, 1960).

I. 4. b. Trehalose circulating level is regulated in particular by insulin signaling

In mammals, blood glucose level is maintained constant in particular by the action of the insulin hormone on several peripheral tissues as muscles, liver and fat tissues (Reyes-DelaTorre et al., 2012). In *Drosophila*, eight different insulin-like peptides, orthologous to mammalian insulin and IGF proteins, and only one insulin receptor are encoded in the genome (Nässel et al., 2015). Insulin-like signaling deficiency during development leads to a developmental delay, a reduced body size and high levels of carbohydrates and lipids (Rulifson et al., 2002; Broughton et al., 2008) accompanied with extended longevity. The same deficiency only at adulthood increases circulating carbohydrates and lipid levels, similarly to what is observed in diabetic patients or diabetic mice when there is generalized insulin resistance (Teleman, 2010). Thus, the regulation of circulating metabolites concentration by insulin signaling is conserved among species.

There are also direct evidences that insulin regulates carbohydrates, and in particular trehalose metabolism in different invertebrates. The injection of bovine insulin in adult males beetles *Tribolium castaneum* increases trehalase mRNA

expression in the head and fat bodies, but does not change trehalose transporter expression (Xu et al., 2013). On the contrary, in the same species, after Insulin-like peptide 2 knock-down, trehalase mRNA expression decreases in the head and fat bodies. Finally, InR knockdown in the fat body produces a significant delay in the restoration of fat body glycogen after starvation and a decrease in glycogen synthase mRNA level, showing that insulin signaling also plays on glycogen stores in insects (Yamada et al., 2018).

I. 4. c. Trehalose can cross the *Drosophila* equivalent of the BBB

Drosophila CNS is protected by several sheaths of cells forming an equivalent of the mammalian blood-brain barrier. It is composed by surface glia: perineural cells and subperineural cells (Figure 27). Dye injection experiments showed the barrier property of this two-layer structure (Stork et al., 2008). Subperineural cells are tightly linked by septate junctions, preventing any paracellular diffusion of polar or charged molecules, while perineural cells contributes to the barrier selectivity on larger molecules thanks to the secretion of a carbohydrate-rich lamella (Stork et al., 2008).

In order to enter the brain, trehalose must go through the BBB. Treherne demonstrated that the cockroach CNS can take-up circulating trehalose (Treherne, 1960). He measured glucose and trehalose concentrations in the hemolymph following radiolabeled glucose injections. Glucose is rapidly transformed into trehalose in the hemolymph, probably by the fat bodies, and 7 times more trehalose than glucose is taken-up by the ventral nerve cord. Thus, both glucose and to a larger extend trehalose are able to cross the BBB and enter the CNS.

Knocking-down the expression of the two characterized trehalose transporters Tret1-1 and Tret1-2 in glial cells induces a locomotion defect, showing that trehalose transport through glial cells membranes is necessary for normal CNS activity (Volkenhoff et al., 2015). Oppositely, knocking-down these transporters in neurons does not affect viability or locomotion, suggesting that trehalose transport only occurs in glial cells. Therefore, a metabolic compartmentation between glia and neurons is thought to exist in *Drosophila* CNS as it exists in mammals, and neurons should not directly consume trehalose to support their activity.

I. 4. d. Neuronal metabolism in *Drosophila*

If not using trehalose, what do *Drosophila* neurons take-up as energy metabolites? It was recently shown that GLUT1 is expressed in neurons (Volkenhoff et al., 2018). Besides, glial cultures secrete alanine and lactate made out of trehalose break-down, two molecules that could potentially be used by neurons (Volkenhoff et al., 2015). Glia could also potentially provide glucose thanks to trehalose breakdown into two molecules of glucose. It is likely that, as in mammals, *Drosophila* neurons use different substrates to fulfil their energy and anabolic requirements.

Thus, energy metabolism in the *Drosophila* and in mammalian CNS seem to share many properties. Indeed, metabolites entering the CNS are controlled by a physical and chemical barrier comprising glial cells, and metabolism is compartmentalized between CNS cell types. The relevance of this metabolic compartmentation and its dynamics during neuronal activity are not fully understood in mammals and just begin to be studied in *Drosophila*.

For some particularly demanding cognitive tasks, the metabolic cost of brain activity may change. Synaptic plasticity is associated with specific molecular mechanisms that take place in different neuronal compartments and are expected to be associated with a high energetic cost. This makes memory formation a good model to study how neuronal activity levels may be supported by specific metabolic pathways with a behavioral significance. As *Drosophila* offers a convenient model for the study of metabolism in different neuronal compartments and of their interaction with glia, we studied energy metabolism during long-term memory formation in this model species.

II. 1. Learning and memory theoretical framework

II. 1. a. A historical approach of memory

Some of the first organisms used to study the molecular mechanisms underlying memory were invertebrate species. Invertebrate animals, as *Aplysia*, *Caenorhabditis elegans* or *Drosophila melanogaster*, own a relatively limited number of neurons, allowing an efficient reductionist approach, but still exhibit complex behavioral outputs. Researchers first focused on simple habituation or sensitization protocols, and on associative “classical” and operant conditionings. For instance, Nobel Prize laureate Eric Kandel studied the defensive gill-withdrawal reflex of *Aplysia* (Kupfermann and Kandel, 1969). Experiments on these simple models validated the synaptic plasticity theory (Zucker et al., 1971; Castellucci and Kandel, 1976), but also raised new questions. Early in the 70s’, Eric Kandel’s team demonstrated that the number of repetitions of a training could impact the dynamics of memory formation and extinction by involving specific molecular pathways (Carew et al., 1972). Indeed, in *Aplysia*, while a single training produces only minutes-lasting short-term memory, a repeated spaced training triggers the formation of long-term memories lasting for days.

In our laboratory, we study memory by conditioning flies thanks to an olfactory Pavlovian conditioning (Pavlov, 1927). We associate odors to an unconditioned stimulus: electric shocks during olfactory aversive conditioning, or sugar during olfactory appetitive conditioning (see Methods for more details, Figure 39). After one cycle of aversive training, flies exhibit a high memory performance when immediately tested, but their memory rapidly decays within a few hours. Interestingly, some genetic mutants as the *amnesiac* mutant were found to be defective in middle-term memory when tested at 3 h, while exhibiting a normal short-term memory (Quinn et al., 1979). This suggested that middle-term memory does not directly derive from short-term aversive memories, and does not rely on the exact same molecular mechanisms. Moreover, middle-term memory, and later short-term memory (Knappek et al., 2011; Bouzaiane et al., 2015), could be divided into two components: one

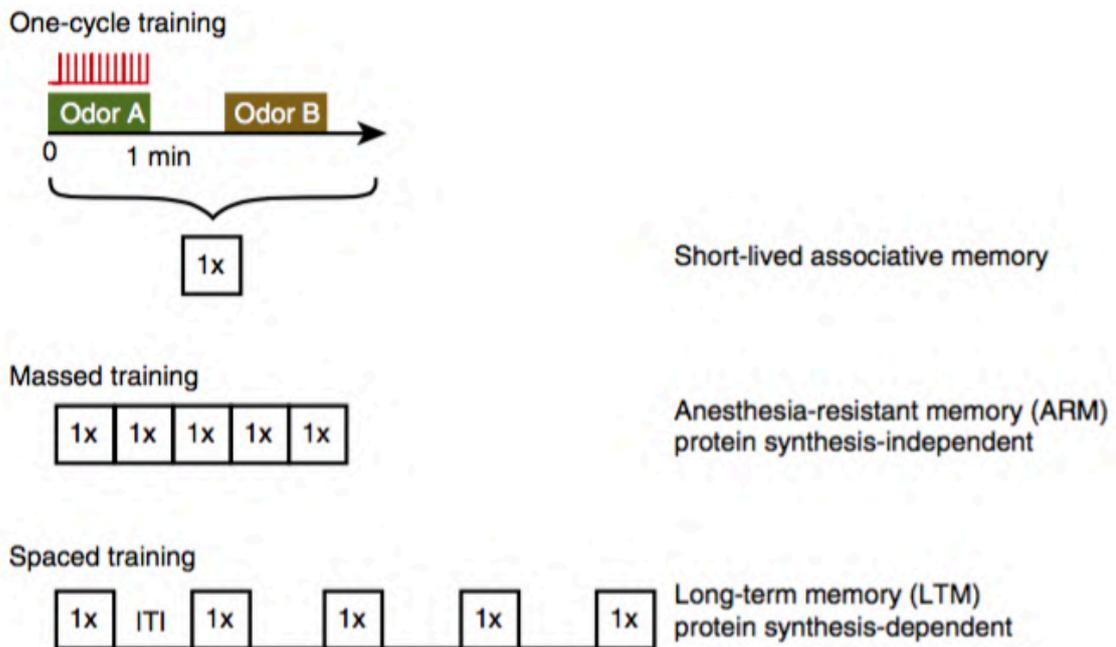
component that relies on specific genes, for example *rutabaga*, and which is sensitive to cold anesthesia, named Anesthesia-Sensitive Memory (ASM), and another component that relies on other genes such as *radish*, and which is not sensitive to cold anesthesia and is thus called Anesthesia-Resistant Memory (ARM).

In *Drosophila*, research first focused on shorter-lasting memories, but 40 years ago came the first demonstration that consolidated memories could also be formed: 4 repetitions of aversive training cycles with 2-h intervals allowed the formation of a memory that lasted for 24 h, much longer than after a single training (Quinn et al., 1974). Subsequent studies described with better details the properties of long-lasting memories in flies (Tully et al., 1994) (Figure 10) and showed that two different long-lasting memories can be formed depending on the training protocol. A spaced training, corresponding to the repetition of training sessions with a 15-min rest interval between each, produces a stronger and longer-lasting memory called “long-term memory” (LTM) than a massed training i.e. without pause between the repetitions (Tully et al., 1994), that produces a memory that was named “long-term anesthesia-resistant long-term memory” (LT-ARM). Noteworthy, both memories are resistant to anesthesia, which is hallmark of consolidated memories.

Their names came from analogies with shorter-lasting memory phases earlier described, as some molecular features are shared. Still, it is unlikely that long-lasting memories are the direct consequence of the consolidation of shorter-lasting memories. Indeed, in appetitive and courtship memories, genetically manipulated flies that cannot form a proper short-term or middle-term memory can still form a normal long-term memory (Trannoy et al., 2011; Redt-Clouet et al., 2012). Thus, memories measured at different time points after the conditioning can be formed independently of each other and may coexist at different time points while being expressed or not.

Before going into more details about the main structures involved in memory encoding in *Drosophila*, I will discuss more about the mechanistic differences between LTM and LT-ARM.

A



B

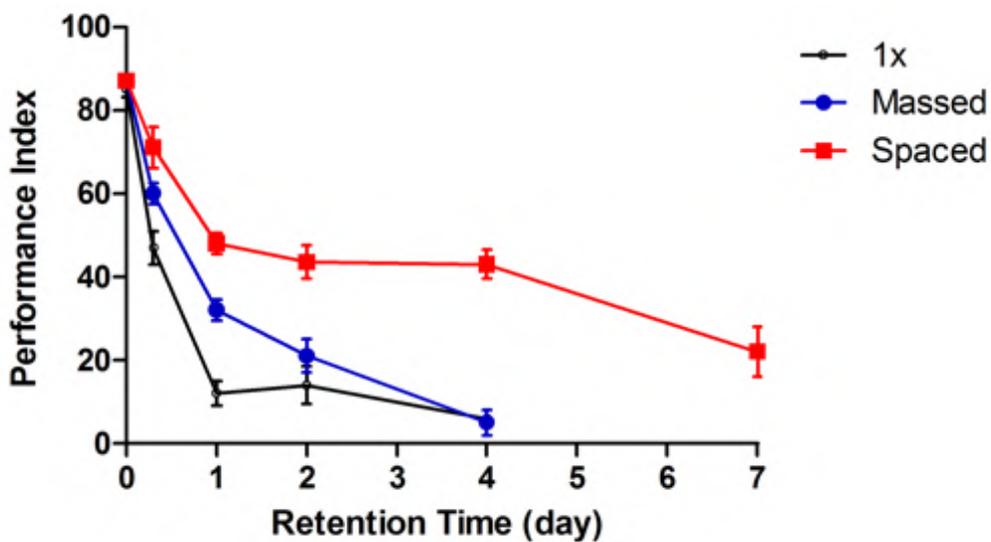


Figure 10. Memory retention of *Drosophila* conditioned thanks to different training protocols. A. Conditioning protocols used, consisting of one cycle or 5 repetitions of training cycles that associate an odor with electric shocks with or without 15 min intervals of rest. From Plaçais et al., 2017. B. Lifetime of memories formed after the different training protocols. After a single association between odors and electric shocks (“1x”), flies form a memory that rapidly extinguishes. On the contrary, repeating 10 times the association with (“Spaced”) or without (“Massed”) 15-min intervals of rest allows the formation of consolidated memories that last for several days. Notice the difference in memory scores between spaced-trained flies and massed-trained flies, 1 to 4 days after the conditioning. Adapted from (Tully et al., 1994).

II. 1. b. Two independent long-lasting aversive memories exist in flies

LTM and LT-ARM do not rely on the same neuronal networks and molecular cascades. While after a spaced training, memory depends on *de novo* protein synthesis (Flexner et al., 1962; Tully et al., 1994), memory after massed training is independent of *de novo* protein synthesis and requires the *radish* gene to be functional (Tully et al., 1994). Besides, LTM, but not LT-ARM, requires for instance *amnesiac* or CREB transcription factor (Yin et al., 1994), while LT-ARM requires serotonin synthesis (Lee et al., 2011; Plaçais and Preat, 2013) (Figure 11). It was first shown that *radish* mutants were defective only in LT-ARM and not LTM (Tully et al., 1994), but this result could not be reproduced in the lab.

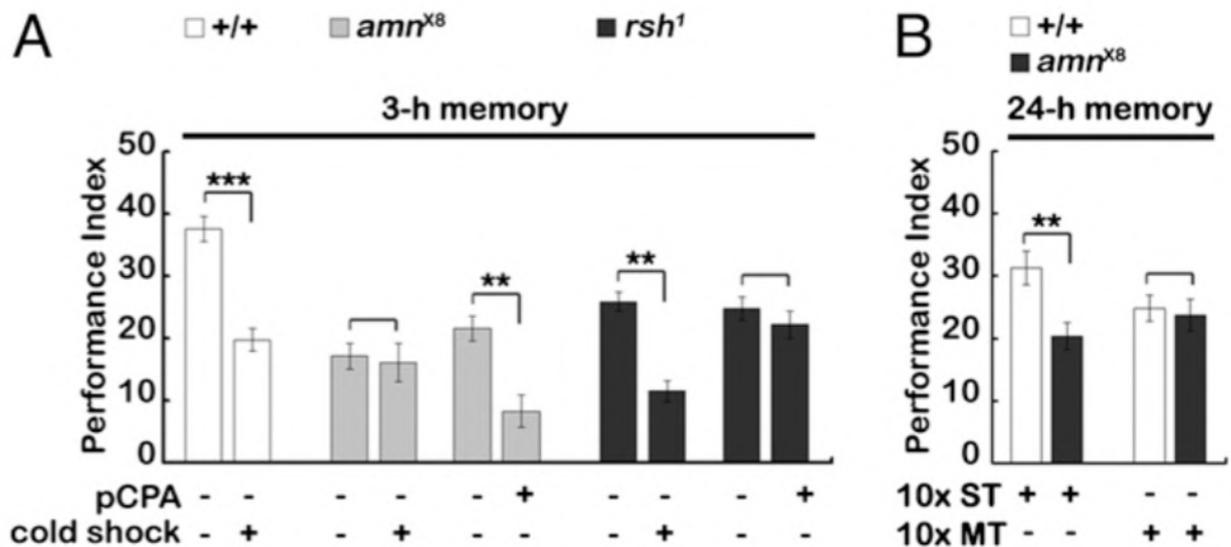


Figure 11. Different memory phases depend on distinct molecular pathways. A. After a single training cycle and when flies are tested at 3 h post-training, *amnesiac* mutants show a defect in anesthesia-sensitive memory and thus are not sensitive to cold anesthesia. Oppositely, *radish* mutants show a defect in pCPA-sensitive anesthesia-resistant memory and therefore display a memory that is not disturbed by pCPA treatment, a serotonin synthesis inhibitor. B. After a spaced training (10x ST), *amnesiac* mutants display a LTM defect, but show a normal LT-ARM after a massed training (10x MT). From (Lee et al., 2011).

This finding asked, first, why evolution selected the ability to form long-lasting memories that depend on different molecular mechanisms in flies, meaning, what was the ecological relevance of these two kinds of long-lasting memories. Second, it asked what were the precise mechanistic distinctions between these two memory

phases. Finally, it asked what control mechanism would gate memory consolidation towards one or the other memory phase, and if both could be consolidated at the same time or be mutually exclusive. These two consolidated memories will interest us further in the thesis, therefore it is worth describing them in more details.

II. 1. c. Ecological relevance of LTM and LT-ARM

Importantly, LTM seems more reliable, stronger, and longer-lasting than LT-ARM (Tully et al., 1994). Indeed, memory performance at 1 day is higher after a spaced training, and is still high after 7 days, while memory is lower at 1 day and is extinguished within 4 days after a massed training (Figure 10). Therefore, LTM should be favored by evolution for a longer-lasting adaptation of the animal to its environment. It may ensure that only information acquired over several independent events—that should therefore be highly predictive—is stored in a costly but long-lasting protein synthesis-dependent memory.

Alternatively, consolidating a memory for a significant part of the animal's life is detrimental if memory cannot be efficiently erased when not relevant. It seems that, when the conditioned stimulus (odor) is presented again without the unconditioned stimulus (electric shocks), LT-ARM is extinguished more readily than LTM (Qin and Dubnau, 2010). Therefore, LTM and LT-ARM could have been selected by evolution in response to two different constraints: either long-lasting, frequently encountered associations, which are meaningful to be remembered and consolidated for almost the whole life of the organism, or acute but strong associations that can change over time and for which memories must be rapidly extinguished. A study carried on parasitoid wasps showed that LTM is more easily formed in species that may adapt more because they have a broader host spectrum as compared to another species that do not have to adapt to their hosts (Smid et al., 2007). Interestingly, the species with an increased LTM performance does not exhibit any LT-ARM, suggesting that genetic factors favoring LTM formation disfavored LT-ARM formation.

Besides, flies exhibit a better memory performance when tested alone after a spaced training, but a decreased memory performance when tested alone after a massed training (Chabaud et al., 2009). This indicates that these memories also have differential social relevance, which might explain the emergence of both memories.

Another explanation for the existence of these two memories is the energetic cost associated with each one. Indeed, when flies are subjected to a spaced training and are then placed in empty vials with no food, they die earlier than controls that do not form LTM but received odors and shocks or than flies conditioned following a massed training (Mery and Kawecki, 2005). Thus, LTM formation must have dramatic consequences on the flies energy consumption as compared to LT-ARM, and could be unfavored under food shortage, or in species which frequently face food restriction. This hypothesis was one basis for subsequent studies of the interaction between metabolism and memory formation in our laboratory.

Not only specific molecular mechanisms, but also specific neuronal networks support each of the above-mentioned memory phases. I will give a broad picture of the main neural circuits involved in memory formation in *Drosophila*.

II. 2. Brain structures involved in memory formation

The development and refinement of genetic tools for the precise functional analysis of neuronal circuits (Pfeiffer et al., 2008) gave rise to an abundant literature resulting in a detailed description of the circuits involved in sensory perception and memory. In the following I will describe some of these tools that I used in my work.

II. 2. a. Diverse and precise genetic tools available in *Drosophila*

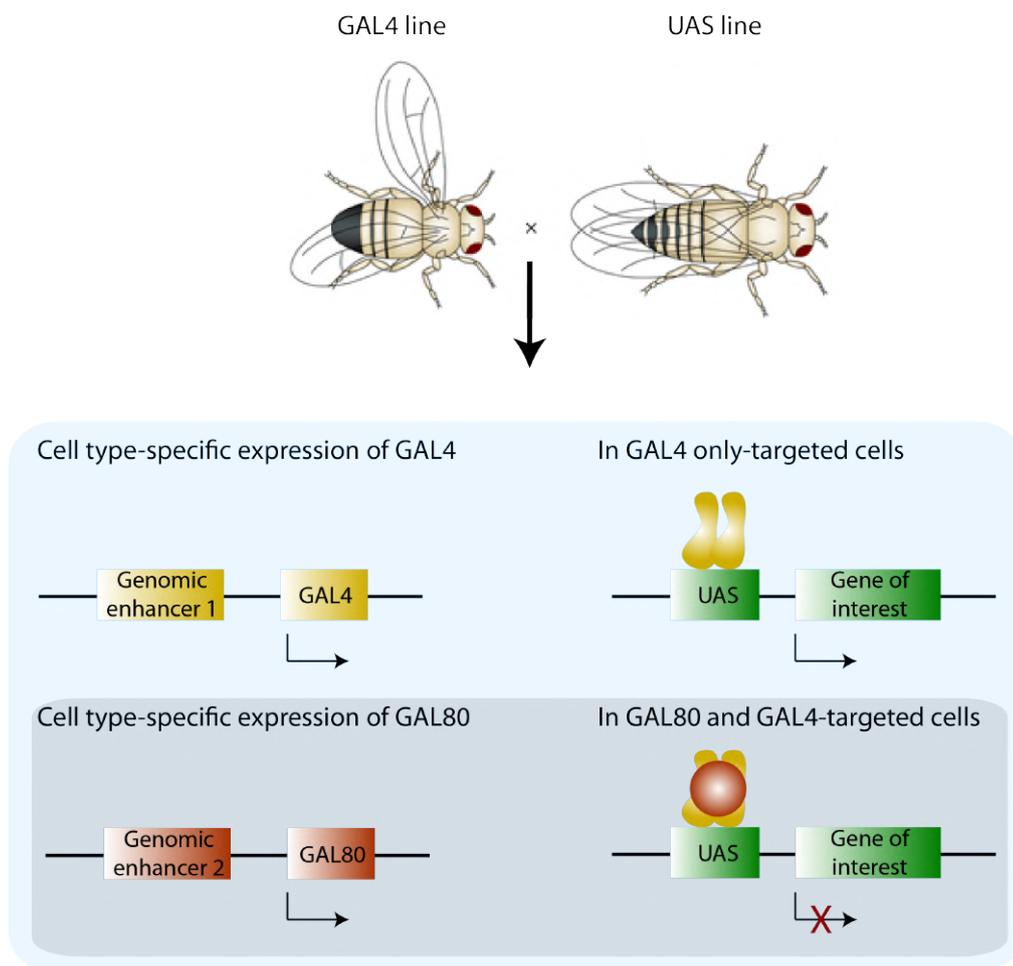


Figure 12. Principle of the GAL4/UAS dual expression system. In the progeny of a cross between a GAL4 and a UAS line, the gene of interest placed downstream of the UAS sequence is expressed only in GAL4-targeted cells. As GAL80 is a GAL4 inhibitor, if GAL80 is also present in the progeny, it will restrict GAL4 activity to the subset of cells that do not express GAL80. If the variant of GAL80 used is the thermosensitive GAL80^{ts}, switching flies at high temperature will allow the time-restricted expression of the gene of interest in the cells of interest. Adapted from (St Johnston, 2002).

A major strength of molecular genetics is to allow the precise and reproducible spatial control of transgene expression. The use of binary expression systems, such as the well-known UAS/GAL4 system (Brand and Perrimon, 1993), enables such a control with high versatility regarding the targeted cells (Figure 12). After the generation of thousands of GAL4 lines (Pfeiffer et al., 2008), even more precise tools have been designed: the split-GAL4 lines (Luan et al., 2006). The split-GAL4 strategy consists in splitting GAL4 into two halves that are expressed under the control of distinct upstream enhancers; GAL4 is thus functional only in cells where both enhancers are active, which can restrict the GAL4-targeted cells to only a few cells, for example a pair of neurons.

Moreover, it is possible to further restrict GAL4 activity by the additional expression of GAL80, its inhibitor, in precise cellular subset (Figure 12). In addition to the GAL4/UAS system, that allows a spatially controlled transgene expression, it is possible to temporally control this expression. As all invertebrates, flies do not regulate their body temperature. This allows the use of thermogenetic tools, among which the thermosensitive version of GAL80, GAL80^{ts}. It is usually expressed under an ubiquitous driver, classically ubiquitin (McGuire et al., 2003; 2004). This GAL80^{ts} is active at permissive temperatures - 18 to 25°C - but is not functional at 30°C. We can thus express it ubiquitously and then induce the expression of the transgene of interest for only one to three days before performing any experiments, by only switching flies to a high temperature. This allows to prevent any developmental defect during gene knockdown.

In addition to the UAS/GAL4 dual expression system, LexA/LexA-OP and QF/QUAS dual expression systems allow the manipulation of two or even three different cell types independently (Pfeiffer et al., 2010). In the laboratory, we commonly use a combination of GAL4/UAS and LexA/LexA-OP systems, typically for manipulating gene expression in some cells of interest while expressing a fluorescent probe in other cells.

Finally, another thermogenetic tool, the thermosensitive *shibire* allele (*shi^{ts}*), enables the time-controlled blockade of neurotransmission. This tool, combined with the GAL4/UAS system, has been instrumental in determining the neuronal circuits involved in behaviors.

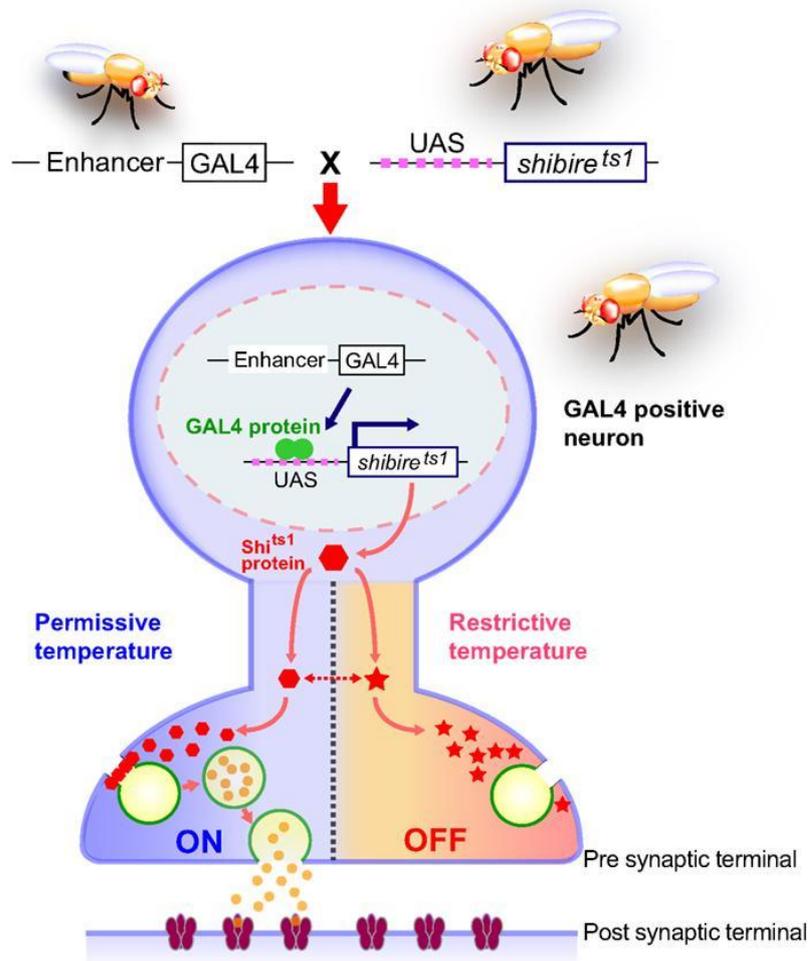


Figure 13. The *shibire^{ts}* transgene. A GAL4 line specific to the neuronal subset of interest is crossed to the *UAS-shi^{ts}* line. Progeny ectopically expressing *shi^{ts}* in GAL4-positive neurons are raised at permissive temperature. When the temperature is shifted from permissive to restrictive, the thermosensitive Dynamin is rapidly inactivated and synaptic vesicle recycling is interrupted. As a result, the GAL4-positive neurons are depleted of synaptic vesicles and synaptic transmission is rapidly and reversibly blocked. From (Kasuya, 2009).

Shi^{ts} is a thermosensitive dominant negative isoform of the Dynamin, a protein necessary for the formation of vesicles during endocytosis (Figure 13). At permissive temperature (18-25°C), *shibire^{ts}* adopts a normal conformation, but when flies are switched to a high temperature (33°C), vesicular endocytosis and exocytosis are no longer functional.

Besides the *UAS-shi^{ts}* line, diverse interesting UAS lines have been constructed by the fly community and are now stored in libraries and easily accessible. For instance,

libraries of UAS-RNAi lines have been extensively generated. Their use allows the temporally and spatially restricted knock-down of endogenous genes expression. Now, thousands of these lines can be ordered from different libraries over the world (Bloomington in the states, NIG in Japan, VDRC in Europe...). Moreover, many other UAS lines have been constructed, allowing the overexpression of *Drosophila* genes, human genes, portions of proteins, reporters, fluorescent probes...

These tools offer for instance the possibility to inhibit chemical synapses from specific neurons, and thus to establish causality between single neuron activity and behaviours.

During olfactory conditioning, different brain structures are involved in the detection and the integration of sensory stimuli. I will first describe the olfactory network involved in memory: the conditioned stimulus pathway, and the networks conveying unconditioned stimulus information; then, the integrative structures where coincidence detection occurs: the mushroom body. Finally, I will describe some recent evidence about the output neurons which guide consequent behavior.

II. 2. b. Olfactory processing in *Drosophila*

The organization of the *Drosophila* olfactory network, at least for the first synapses after sensory reception, is very similar to what is found in mammals. First, in the periphery, olfactory receptor neurons can be activated by chemical stimuli from the environment. The dendritic processes of these 1300 neurons are located in sensory hairs in antennae and maxillary palps (Figure 14). These cholinergic neurons project directly to the CNS, targeting bilateral glomerular brain structures called antennal lobes. Information from neurons expressing the same olfactory receptor - plus an invariable co-receptor Or83b - is conveyed mostly to only one glomerulus, and conversely one glomerulus receives information from neurons expressing only one receptor (Jefferis et al., 2001; Couto et al., 2005). 40 functional genes encode olfactory receptors in flies, and 41 glomeruli are dedicated to sensory inputs (Vosshall et al., 2000). In each glomerulus, about 5 cholinergic olfactory projection neurons integrate the information. Different local interneurons also receive and modulate glomerular activation (Vosshall et al., 2000). The location of the different glomeruli is stereotyped (Vosshall et al., 2000).

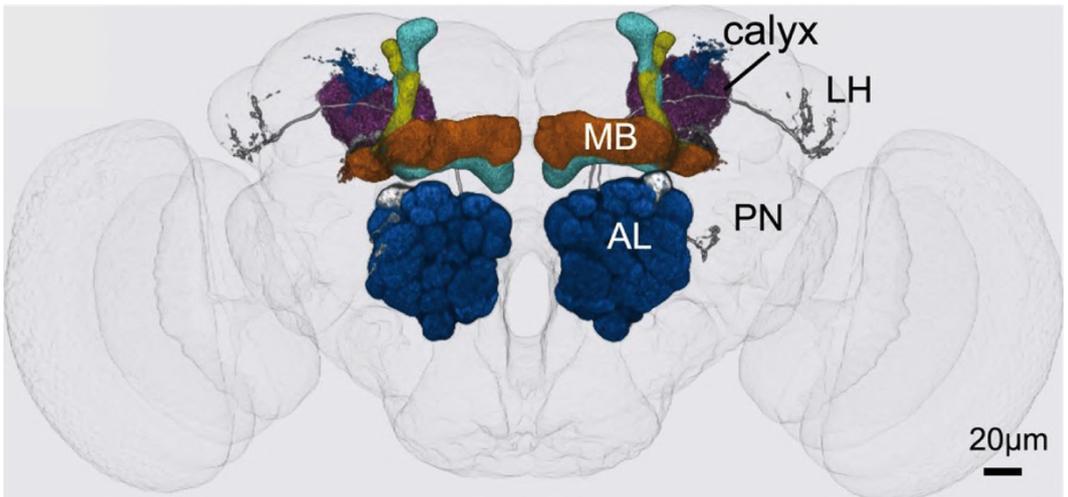
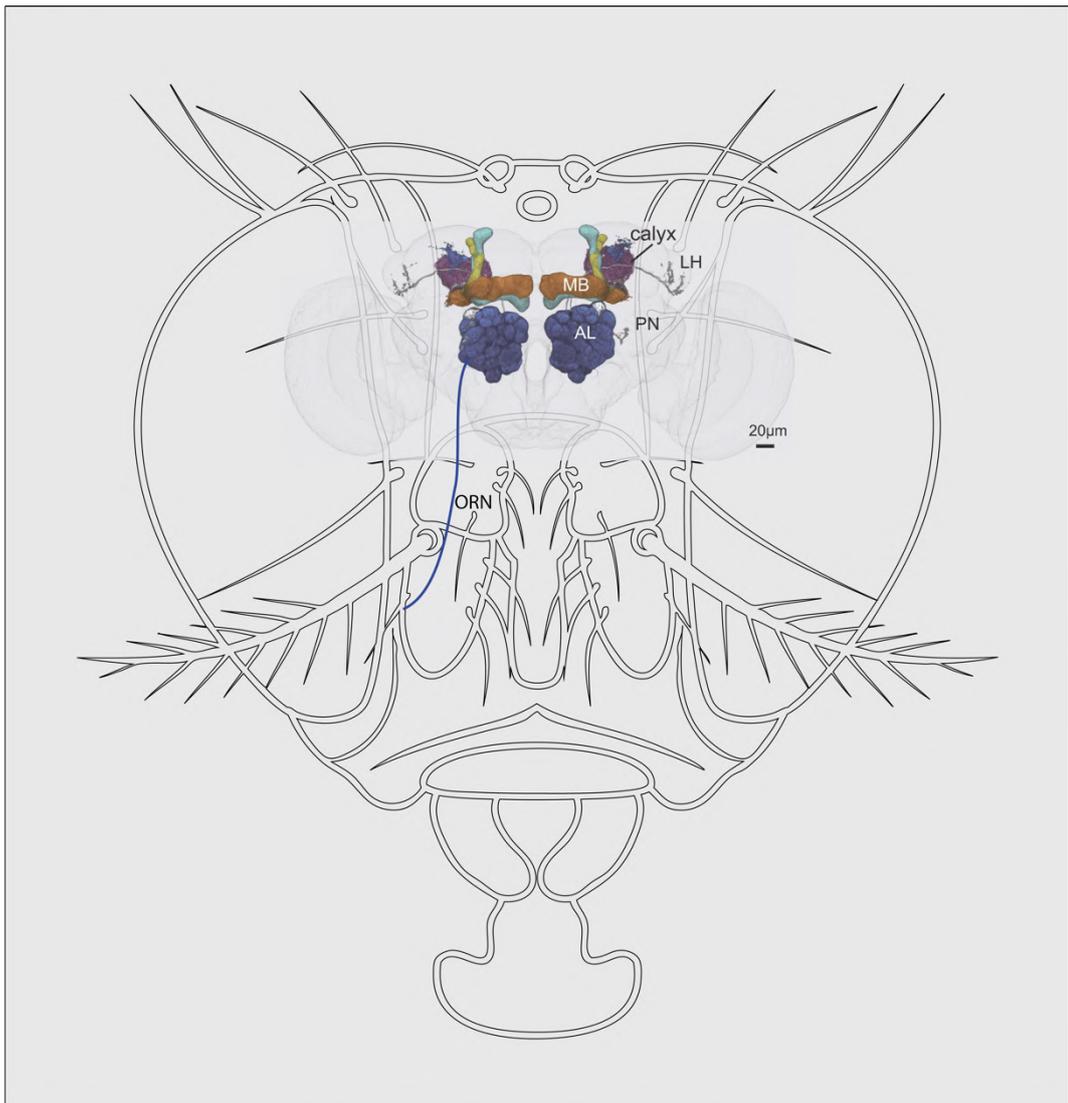


Figure 14. Global organization of the olfactory system in *Drosophila*. Olfactory receptor neurons (ORN) present in the antennae and maxillary palps project to the antennal lobes (AL) composed of several neuropil subdomains called glomeruli. Information is transmitted to olfactory projection neurons (PN) which send processes to the mushroom body (MB) at the level of the calyx, and to the lateral horn (LH). Adapted from (Aso et al., 2014a).

One odor at a given concentration triggers the activation of a specific combination of olfactory receptors, thus activating a few given glomeruli. This creates a combinatorial spatial map of activation that is odor and concentration-specific (Vosshall et al., 2000). This spatial map may then be treated in higher sensory centers within the brain to translate stimuli features into meaningful neural information.

The cholinergic olfactory projection neurons send their processes towards two regions in the brain: the lateral horn and the dendritic region of the mushroom body neurons called the calyx (Figure 14).

II. 2. c. Olfactory integration in the mushroom body

Mushroom bodies are bilateral, symmetrical neuropil structures found in annelids and all arthropods. They do not only receive olfactory, but also visual and mechanosensory information, making it an integrative structure (Barth and Heisenberg, 1997; Ito et al., 1998; Vogt et al., 2014). Besides, flies missing the mushroom body by genetic mutation or by chemical deletion, or flies lacking mushroom body output during memory retrieval, do not form memory (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Dubnau et al., 2001; McGuire et al., 2001; Pascual and Preat, 2001; Trannoy et al., 2011). Thus, it has been early hypothesized that the mushroom body was the center for memory association and storage in flies. The mushroom body was first discovered in 1850 by Félix Dujardin (Dujardin, F. 1850. Mémoire sur le système nerveux des insectes. Ann. Sci. Nat. Zool. 14: 195-206). He compared its anatomy in different invertebrate species, and already hypothesized that it supported acquired behaviors, as compared to instincts, hard-wired behaviors. Surprisingly, the mushroom body and hippocampus are similarly organized regarding their chemosensory afferents and feedback networks, which suggests that they could share a common evolutionary origin (Wolff and Strausfeld, 2016).

In *Drosophila melanogaster*, each mushroom body contains about 2 000 intrinsic neurons out of the 100 000 neurons of the *Drosophila* brain (Aso et al., 2014a). They are called Kenyon cells. Their cell bodies form a cluster in the dorsal and posterior part of the brain (Tanaka et al., 2008). Kenyon cells are only two synapses away from

olfactory reception, as they receive direct synaptic inputs from olfactory projection neurons in the calyx (Figure 14). Interestingly, each Kenyon cell receives inputs from several olfactory projection neurons originating from multiple glomeruli (Caron et al., 2013), and therefore integrates the information coming from different olfactory receptors. Contrary to olfactory glomeruli, the spatial distribution of Kenyon cells activated by a given olfactory receptor is not conserved across individuals (Caron et al., 2013). Kenyon cells that are relevant for memory formation are cholinergic (Barnstedt et al., 2016).

Kenyon cells are unipolar neurons. Their neurites first display a dendritic compartment in a region called the calyx of the mushroom body as stated above, before projecting towards the anterior of the brain and converging in an axonal tract called the pedunculus that extends until the most anterior part of the brain. There, neurites may or not branch and form five different lobes (Figure 15).

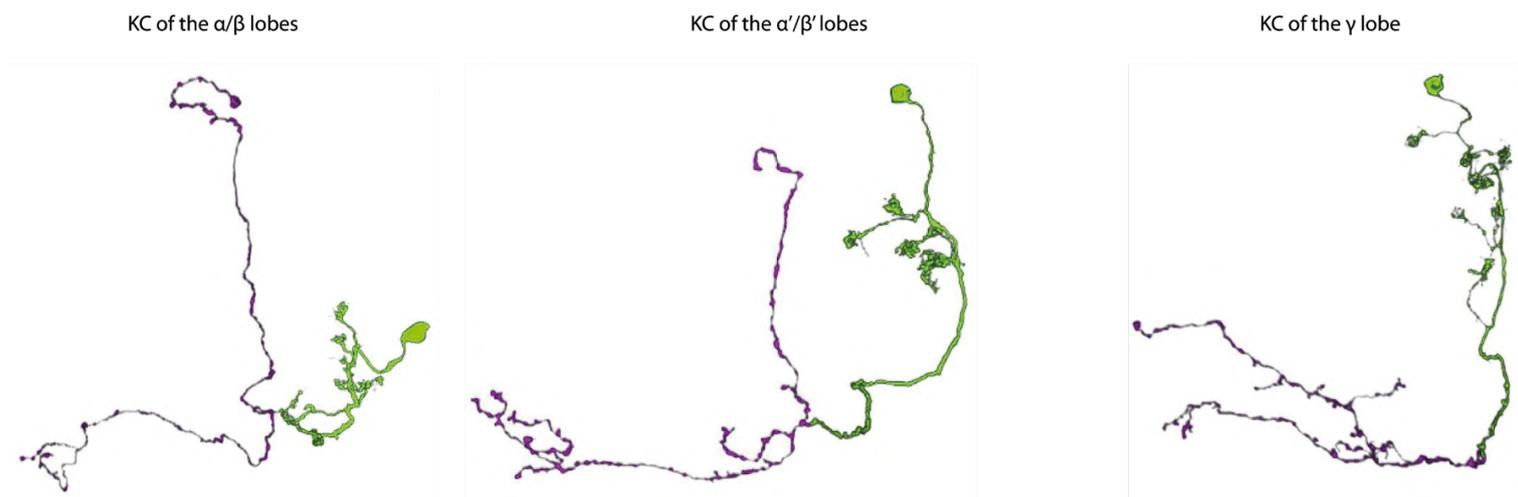


Figure 15. Anatomy of single Kenyon cells constituting the 5 lobes of the mushroom body. The cell body, primary neurite, dendrites, and axon in the pedunculus are displayed in green and axons in the lobes are magenta. From (Aso et al., 2014a).

Three main different Kenyon cell types have been defined according to the branching of their axons at the distal end of the pedunculus (Figure 15). The α/β neurons and α'/β' neurons axons bifurcate after the pedunculus and give rise to two processes, each of them forming either a medial (β and β') or a vertical lobe (α and

α'). The third cell type, γ neurons, has an axonal process that does not bifurcate, and forms only a medial lobe (γ lobe).

Two different types of neurons contact the mushroom body lobes: dopaminergic neurons (DAN) and mushroom body output neurons (MBON).

Out of the seven dopaminergic neurons clusters in the central fly brain, only one targets the calyx region: PPL2a, and two target domains of the mushroom body lobes: PPL1 and PAM clusters (Figure 16). These clusters contain in total about 100 neurons of 20 types (Aso et al., 2014a), whose axon terminals project to only one or two specific compartments that tile the entire mushroom body lobes. 17 of the 20 DAN types project to only one single compartment. PPL1 neurons soma sit immediately lateral to the mushroom body calyx and project a bundle of neurites medially between the peduncle and the vertical lobes, while the PAM cluster extends its projections in the horizontal lobes (Claridge-Chang et al., 2009).

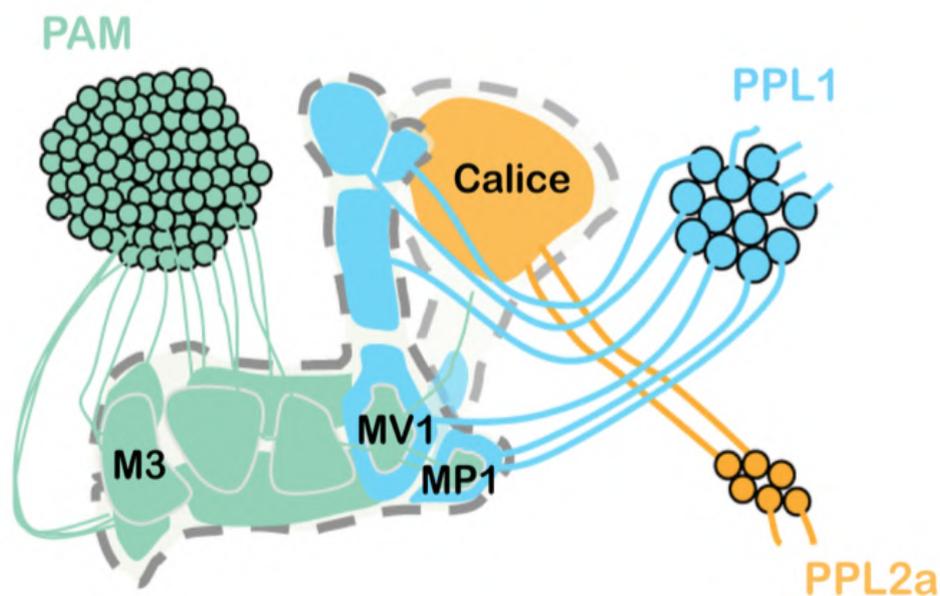


Figure 16. Location of mushroom body-projecting dopaminergic neurons. A subset of PPL1 neurons targets both the vertical lobes and the pedunculus of the mushroom body. A few PAM neurons project mainly to the medial lobes, and some of them contact the beginning of the alpha lobe (Aso et al., 2014a). Finally, a subset of PPL2a neurons targets the mushroom body calyx.

Besides olfactory inputs coming from projection neurons at the level of the calyx and dopaminergic neurons inputs at the level of the lobes, Kenyon cells make synaptic connections with mushroom body output neurons (MBON) at the level of the lobes. Like dopaminergic neurons, MBON also have dendrites that connect to a few of the mushroom body discrete compartments. Two-color labeling experiments revealed an overlap between the axon termini of dopaminergic neurons types and the dendritic arbors of MBON types that innervate the same compartment (Tanaka et al., 2008; Aso et al., 2014a) (Figure 17), suggesting a possible communication between both.

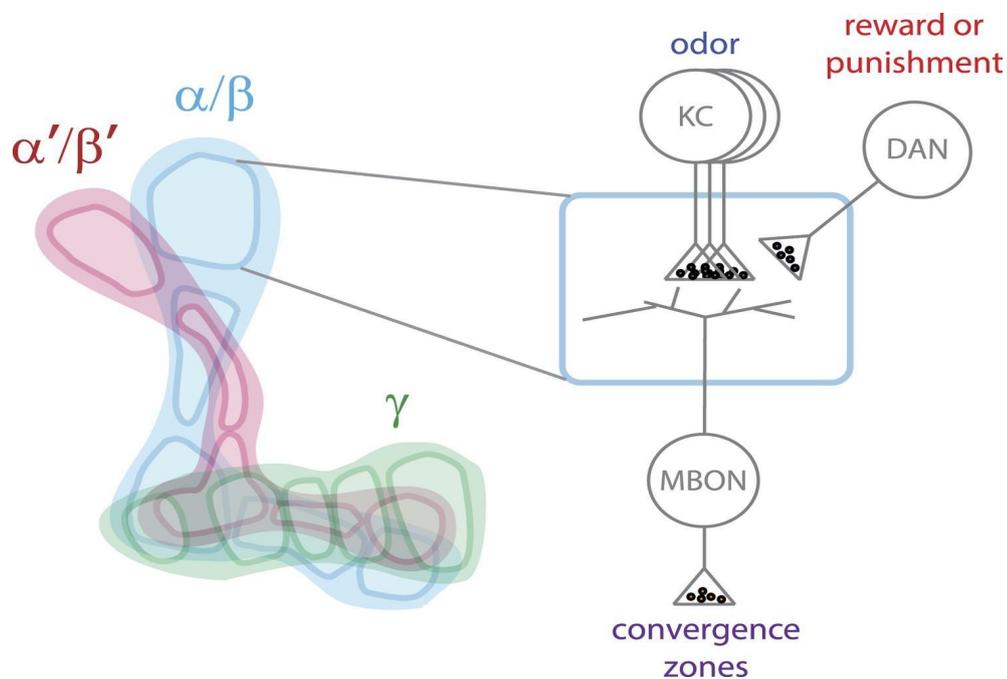


Figure 17. Anatomical and functional organization of the mushroom body into 15 individual compartments. Each compartment receives the converging information from Kenyon cells, coding the odor, and specific dopaminergic neurons. Output from these 15 compartments are mediated by specific output neurons. From (Griffith, 2014).

The mushroom body was early thought to mediate memory association. For this to be true, information of the odor but also about electric shocks should converge on this structure.

II. 2. d. Network encoding the unconditioned stimulus valence

Two main kinds of unconditioned stimuli have been used in the lab and by others for olfactory conditioning in flies: sugar and electric shocks. How electric shocks are perceived by sensory organs and conveyed to the central brain is not precisely known. Still, work on the neurons encoding the valence of the unconditioned stimulus and conveying it to the mushroom body has been fruitful. In mammals, it is thought that unexpected rewards are encoded by an increase in the activity of dopaminergic neurons located in the ventral tegmental area and substantia nigra, while the absence of a predicted reward causes a dip in their activity (Schultz et al., 1997). In *Drosophila*, dopaminergic neurons are also involved in valence encoding. First, it is possible to induce memory formation by associating an odor not with coincident real electric shocks but with a concomitant activation of a subset of PPL1 cluster dopaminergic neurons (Claridge-Chang et al., 2009). Later studies identified one PAM neuron - MB-M3, and three neurons belonging to the PPL1 cluster - MB-MP1, MB-V1 and MB-MV1 - which separate activation during olfactory stimulation was sufficient to induce aversive memory formation with different temporal dynamics (Aso et al., 2010; 2012). Oppositely, blocking these neurons during the conditioning impaired the formation of electric-shock reinforced aversive memory. Interestingly, specific dopaminergic neurons are involved preferentially for some memory phases, such as MB-M3 in middle-term anesthesia sensitive memory (Aso et al., 2010; 2012). Contrary to what was first proposed (Schwaerzel et al., 2003), during appetitive memory formation, a few dopaminergic neurons also encode the appetitive valence of the unconditioned stimulus (Burke et al., 2012; Liu et al., 2012; Waddell, 2013; Lin et al., 2014). Moreover, specific dopaminergic neurons also encode valence for short-term and long-term appetitive memory (Yamagata et al., 2015).

Direct evidence showed that both acetylcholine-mediated olfactory conditioned stimulus and dopamine-mediated unconditioned stimulus valence information converge on the MBs during memory formation, where their coincident detection activates a particular signaling pathway. The calcium activated adenylate cyclase Rutabaga is required for memory formation in the mushroom body, highlighting the importance of cAMP signaling in this structure (Levin et al., 1992; Blum et al., 2009). The co-application of 10 μ M dopamine and 10 mM acetylcholine on the fly brain

results in rutabaga-dependent PKA activation in mushroom body vertical lobes (Gervasi et al., 2010), while the application of only one of these neurotransmitters does not elicit any PKA activation (Figure 18). This experiment confirmed rutabaga adenylate cyclase as the coincidence detector in mushroom body intrinsic neurons.

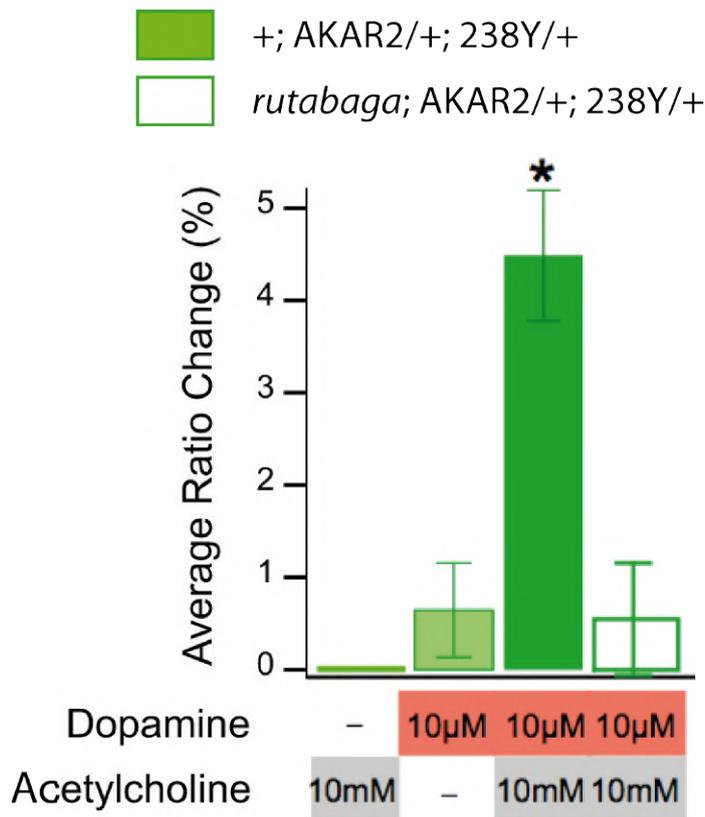


Figure 18. Pairing dopamine to acetylcholine stimulation induces a PKA activation in the vertical lobes of the mushroom body. Mean FRET ratio changes of the PKA-sensitive AKAR probe in response to 10 mM acetylcholine, 10 μM dopamine, or a paired application of acetylcholine and dopamine. The increase in PKA response following dopamine and acetylcholine co-application is not displayed in *rutabaga* adenylate cyclase mutants. From (Gervasi et al., 2010).

Thus, the mushroom body mediates the coincident detection of odors and aversive/appetitive stimuli. In agreement with a role of this structure in memory encoding, odor-evoked calcium signals increase in Kenyon cells axons upon aversive memory formation: in γ lobes (Boto et al., 2014) and in α'/β' lobes (Wang et al., 2008) after only one cycle of conditioning, and in α lobes specifically after a repeated spaced conditioning leading to LTM (Yu et al., 2006). Still, these results were recently

contradicted by a study that measured an increased KC response to the unconditioned stimulus-associated odor as compared to the neutral one only after an appetitive training, but not after aversive conditioning (Louis et al., 2018).

Finally, the activity of specific Kenyon cells subtypes is necessary for the retrieval of each memory phase, confirming the importance of this structure in memory formation but also in memory recall. In particular, the activity of α/β neurons is necessary for long-term memory retrieval (Séjourné et al., 2011; Bouzaiane et al., 2015) (Figure 19).

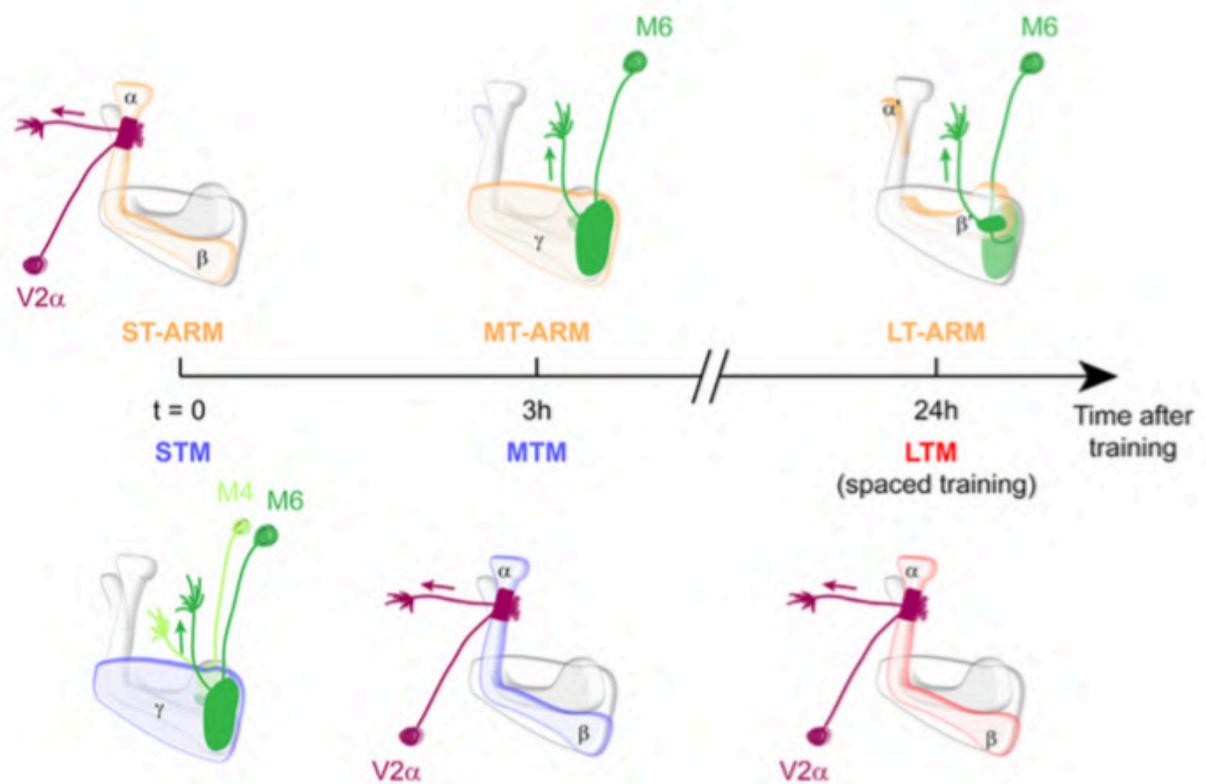


Figure 19. Mushroom body neurons are differentially involved in memory retrieval. α/β neurons and MB-V2 neurons activities are required for long-term memory retrieval, and specific neuronal networks are involved in each memory phase. From (Bouzaiane et al., 2015; Séjourné et al., 2011).

To sum up, the mushroom body associates information coming from both unconditioned and conditioned stimuli and encodes a memory trace of this association. How is this information transferred to downstream brain areas and translated into the appropriate behavior?

II. 2. e. Mushroom body output neurons influence flies behavior

Interestingly, only activating several MBON induces a specific behavioral response, either attraction or repulsion (Aso et al., 2014b). Moreover, several papers have demonstrated that neurotransmission from specific MBON was necessary for the retrieval of different memory phases, but not for memory acquisition or consolidation. MB-V2 mushroom body output neurons, for instance, are involved in aversive long-term memory retrieval (Séjourné et al., 2011; Bouzaiane et al., 2015) (Figure 19). The MB-V2 cholinergic MBON connect the α/α' vertical lobes, which is consistent with the fact that α/β Kenyon cells are involved in LTM retrieval (Bouzaiane et al., 2015). They project to the lateral horn. A very recent study from the laboratory demonstrated that MB-V2 projections to the lateral horn are integrated by a network also involved in innate behavioral response to odors (Dolan et al., 2018). It thus showed that learned and innate olfactory information can interact in identified neurons to produce appropriate behavior. MBON are therefore thought to bias the fly's behavior depending on previous experience. Ultimately, MBON connect to five regions outside the mushroom bodies that are thought to guide behavior (Aso et al., 2014a).

Besides, the studies describing MBON involved in memory retrieval asked if plasticity occurred at the level of the KC - MBON synapses. Indeed, these MBON responses to odor stimulations are changed after the conditioning. The first study to tackle this question revealed that MB-V2 neurons decrease their responses to the trained odorant as compared to the neutral odor after aversive conditioning, suggesting a synaptic depression of KC - MBON synapse (Séjourné et al., 2011) (Figure 20). Oppositely, MB-M4/M6 MBON, involved in aversive and appetitive short-term memories retrieval, show a potentiated activation to the conditioned odor by aversive learning (Bouzaiane et al., 2015; Oswald et al., 2015) and are oppositely depressed by reward learning (Oswald et al., 2015).

All these works suggest that memory is encoded at least at the KC - MBON synapses in a specific way: potentiation or depression, in order to adapt the behavioral output to the relevant odorant. But what mechanisms underlie these changes? DAN provide information about the valence of the stimuli, and MBON are involved in influencing the behavior towards approach or escape. As most DAN only

contact one mushroom body compartment and, in general, one MBON, DAN are ideally located to modulate Kenyon cells output synapses onto a specific MBON partner. This anatomy therefore suggests a logic for associative learning. Can dopaminergic input change the synaptic strength of odor-specific KC - MBON synapse, in a way that would bias behavior to adapt it to the external world?

Interestingly, in naïve flies, activating dopamine release by MP1 DAN concomitantly to an odor presentation induces odor-specific long-term depression of the KC - γ 1 MBON MVP2 synapse, without changing Kenyon cells odor response (Hige et al., 2015) (Figure 21). This depression was measured by voltage-clamp in the MB compartment that was specifically connected by MP1 dopaminergic neurons. This work thus supports the theory stating that dopaminergic inputs change KC - MBON synaptic strength to adapt the fly behavior according to its experience, here according to the salience of stimuli associated to the encountered odorants.

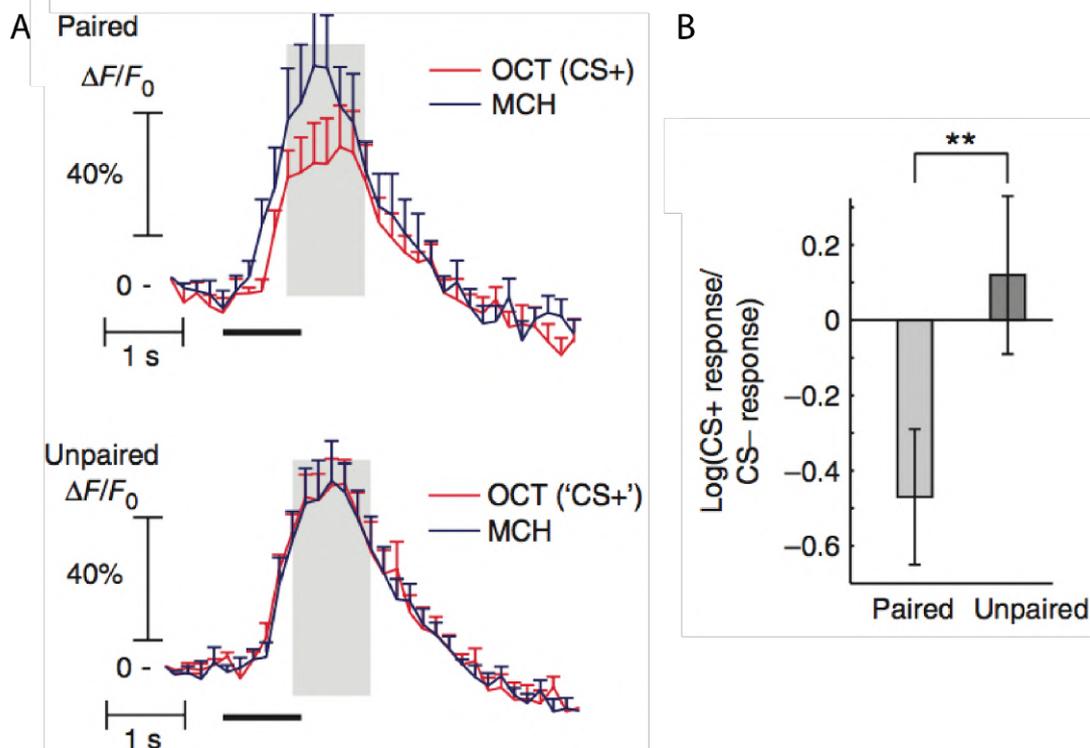


Figure 20. MB-V2 neuron response to the conditioned odor is decreased after conditioning. A. After one cycle of aversive conditioning “Paired” or after a control non-associative training “Unpaired”, MB-V2 neurons calcium response is measured during the presentation of the conditioned stimulus (CS+, OCT) and the unconditioned stimulus (MCH). B. In paired flies, during the time window shaded in gray, the MB-V2 calcium response to CS+ presentation is decreased relative to CS- presentation in flies forming memory. From (Séjourné et al., 2011).

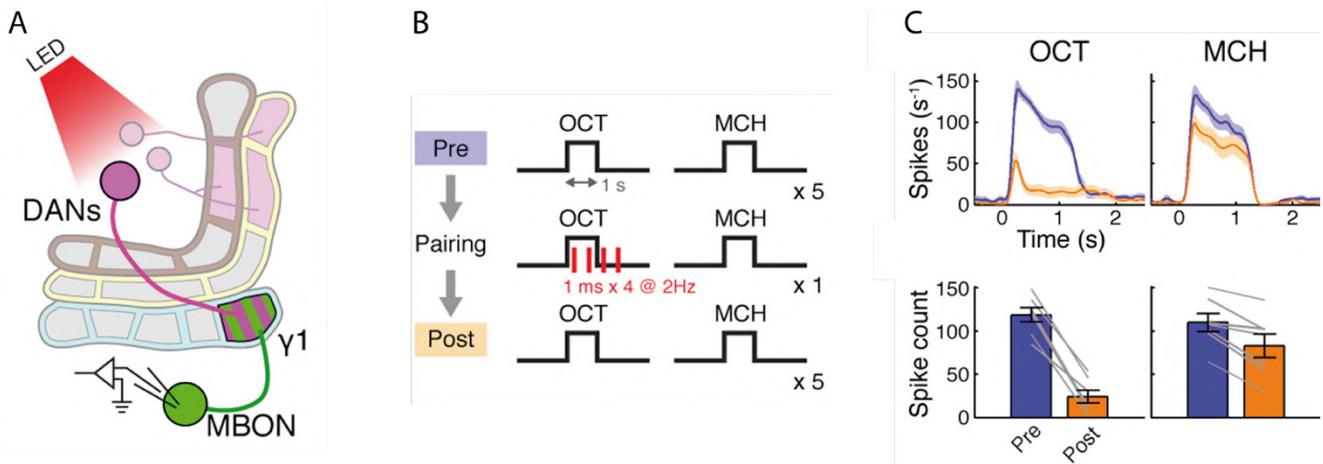


Figure 21. Pairing of odor and DAN activation induces depression of KC - MBON synapse. A. Expression of CsChrimson in MP1 dopaminergic neuron (DAN) allowed for the light-controlled activation of this neuron, in parallel of spike recording in $\gamma 1$ mushroom body output neuron (MBON) MVP2. B. Scheme showing the protocol used for measuring spiking response in $\gamma 1$ MBON in response to odor delivery, before and after the pairing between MP1 DAN activation and octanol (OCT) delivery. C. $\gamma 1$ MBON response to OCT is specifically reduced after the pairing between MP1 DAN activation and octanol but is not changed to methylcyclohexanol (MCH). From (Hige et al., 2015).

Thus, there is now strong evidence that the critical changes that store information in the brain occur at specific synapses within the circuit, in particular at the KC – MBON synapses. But in the case of long-term memory, synaptic plasticity depends on gene regulations and *de novo* protein synthesis that occur in particular in the neuronal cell body. Therefore, if synaptic plasticity is a local, synapse-specific mechanism, it is crucial that regulations occur in the neuronal soma.

Now that I described the main neuronal networks involved in memory formation, I will discuss what is known of the interaction between memory formation and energy metabolism in flies.

II. 3. The interplay between memory and metabolism

II. 3. a. LTM and LT-ARM mutual antagonism

It was first suggested that both LTM and LT-ARM were formed at once after a spaced training (Tully et al., 1994), but further work showed that LTM formation leads to the extinction of LT-ARM (Isabel et al., 2004). Moreover, treating LTM mutants with the protein inhibitor cycloheximide completely abolished their residual memory after a spaced training (Plaçais et al., 2012).

Besides, oscillatory activity increases in specific dopaminergic neurons, MV1 and MP1, after a spaced training, and is required for LTM formation as blockade of these neurons during the inter-trial intervals of a spaced training precludes LTM formation (Plaçais et al., 2012). On the opposite, the blockade of these neurons after a massed training enhances LT-ARM formation, and activating these neurons after a massed training decreases LT-ARM. This phenomenon supports that LT-ARM and LTM are mutually exclusive, and that a gating mechanism involving MV1/MP1 neurons oscillations allows for the consolidation of one or the other long-lasting memory (Figure 22).

Antagonism between LTM and LT-ARM formation was further confirmed in an evolutionary framework (Mery et al., 2007; Lagasse et al., 2012). Indeed, it is possible to select natural *Drosophila* populations over 28 generations exhibiting a better LT-ARM or LTM performance independently. The progeny of LTM-selected populations performs better in LTM tested 24 h after a spaced training, but strikingly, their LT-ARM performance after massed training is substantially decreased. Oppositely, LT-ARM selected populations form a better LT-ARM but a decreased LTM. This experiment again argues in favor of the view that LTM and LT-ARM formation rely on antagonistic mechanisms that can be selected in a mutually exclusive fashion.

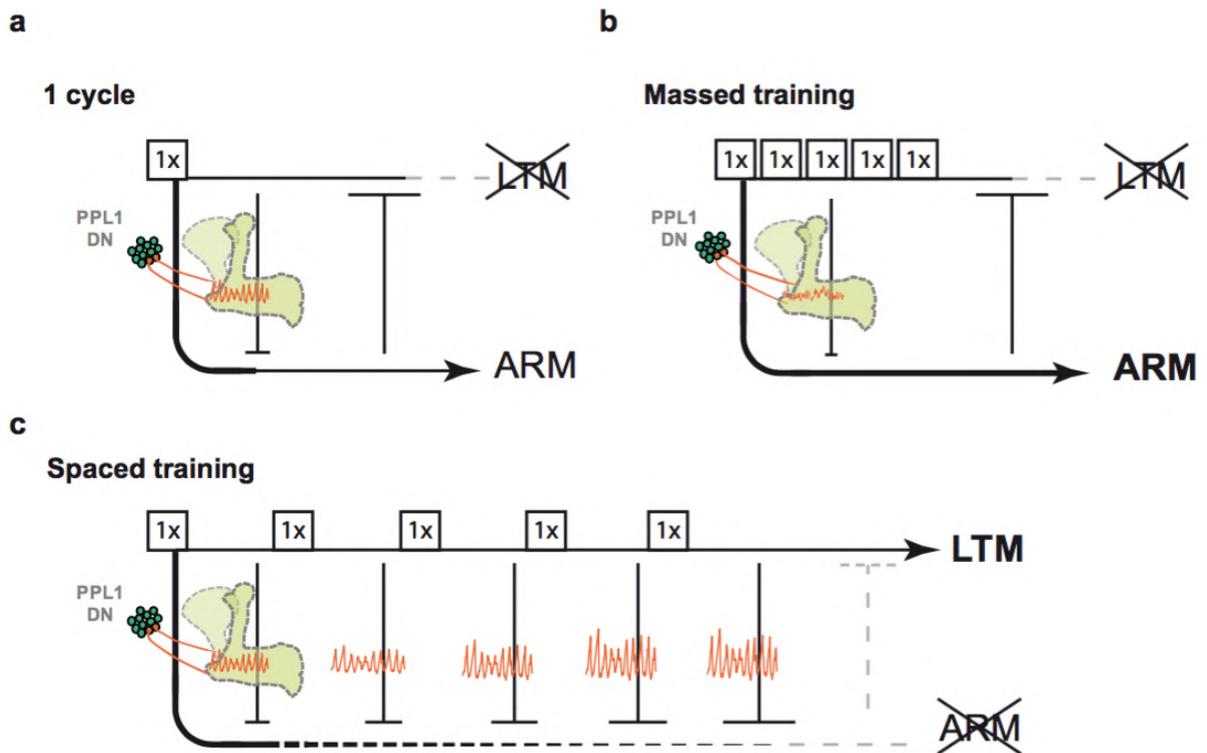


Figure 22. Model explaining the gating between LTM and LT-ARM formation thanks to dopaminergic activity. (a) After a single-cycle training, oscillatory activity in MV1/MP1 is not sustained. (b) Massed training reduces the activity of MV1 and MP1 neurons, which leads to robust ARM consolidation and represses the LTM pathway. (c) Only during the rest intervals of a spaced training can oscillatory neurons repeatedly and fully inhibit ARM consolidation, allowing the energetically costly LTM pathway. From (Plaçais et al., 2012).

As these two consolidated memories coexist in flies, which parameters influence the formation of one or the other, i.e. what parameters control the gating between these memories? LTM and LT-ARM do not have the same energetic cost (Mery and Kawecki, 2005), and it was hypothesized that flies feeding status could impact the formation of one or the other memory. Indeed, flies starving for 21 h before and 24 h after the conditioning do not form protein synthesis-dependent LTM anymore. Instead, they form pCPA-sensitive LT-ARM (Plaçais and Preat, 2013) (Figure 23). The metabolic status of the flies is actually encoded in the activity of the previously studied dopaminergic neurons MV1/MP1. When the flies are fed, these neurons exhibit a high amplitude of slow spiking activity at 0.15Hz. This activity is almost completely erased after food deprivation (Plaçais and Preat, 2013). These works led the way to several studies on the impact of the energetic status of the organism on memory formation.

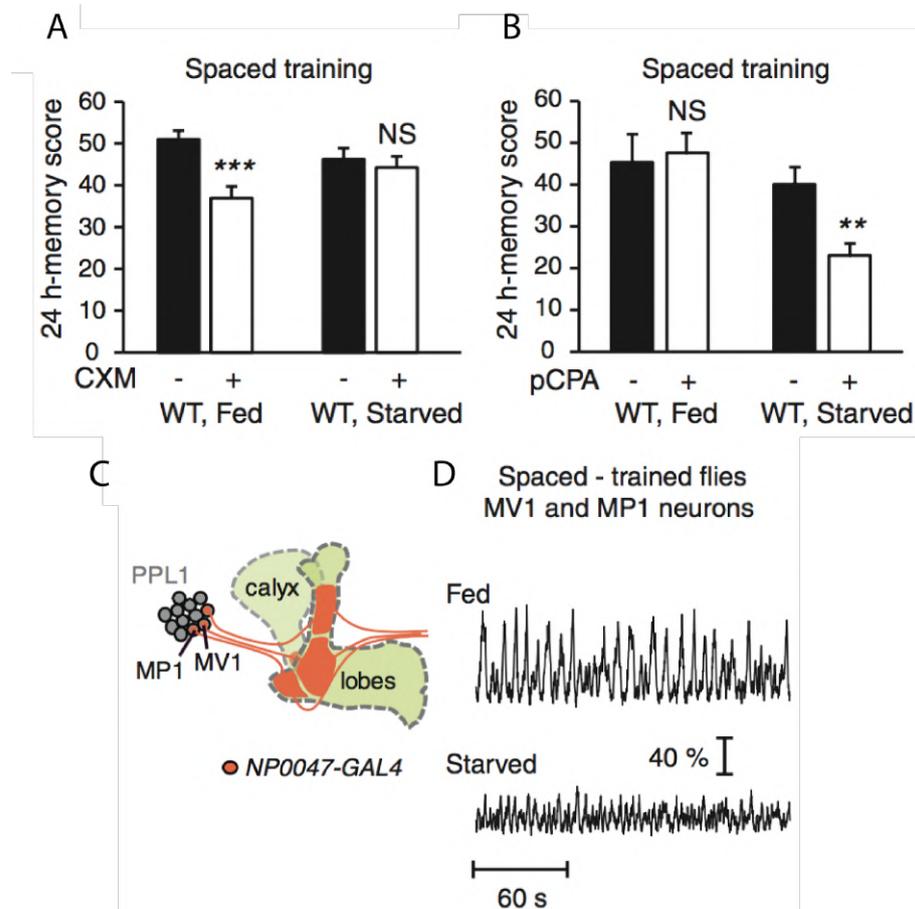


Figure 23. Flies feeding status gates memory formation. A. Inhibiting protein synthesis with a cycloheximide (CXM) treatment precludes memory formation in fed flies after a spaced training, but does not affect it in starved flies, showing that starved flies do not form LTM. B. Oppositely, preventing serotonin synthesis with pCPA does not affect memory formation in fed flies after a spaced training, but does disturb it in starved flies, showing that starved flies form LT-ARM. C. Scheme of MP1 and MV1 dopaminergic neurons projecting at the level of the mushroom body lobes. D. Two illustrative examples of MV1 and MP1 neurons activity after spaced training in a fed fly, featuring large, regular oscillations, and in a starved fly, where the signal amplitude is greatly reduced. From (Plaçais and Preat, 2013).

In parallel to the work conducted in our lab, a study asked the effect of fasting on LTM formation (Hirano et al., 2013). The authors showed that a moderate fasting, only before the conditioning, facilitated protein synthesis-dependent LTM formation after only one cycle of conditioning (Figure 24). This facilitation occurred only if flies were fasted for 9 to 16 h before and not after the conditioning, a time-period that is shorter than the period of 21 h of fasting before and 24 h after the conditioning that induces

the switch from LTM to LT-ARM formation earlier mentioned (Plaçais and Preat, 2013). LTM formation after spaced training requires CREB and CREB-binding protein coactivator CBP in Kenyon cells. Surprisingly, this fasting-LTM (Hirano et al., 2013) was shown to also depend on CREB phosphorylation, but on CRTC (cAMP-regulated transcriptional co-activator) dephosphorylation and translocation to the nucleus in these neurons instead of CBP. In mammalian metabolic tissues, CRTC is phosphorylated by insulin signaling, which is suppressed by fasting. Interestingly, fed flies carrying only one mutant allele of the insulin receptor substrate Chico, *Chico*¹, exhibited CRTC translocation to the nucleus in MB neurons, while fed wild type flies did not. Accordingly, LTM was facilitated after one cycle of training in *Chico*^{1/+} flies (Figure 24) (Hirano et al., 2013).

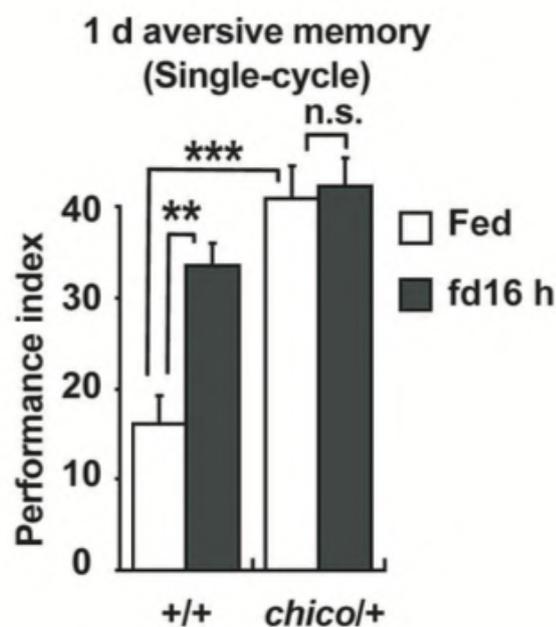


Figure 24. Fasting prior to learning increases 24 h memory, which is already facilitated in flies carrying one mutant allele of Chico. Wild-type (+/+) flies with *ad libitum* feeding exhibit a poor 24 h aversive memory performance after only one cycle of training, which is doubled after 16 h of food deprivation prior to conditioning (fd16h). Flies carrying a loss-of function mutant allele of Chico (*chico*/+) exhibit an enhanced 24 h memory after only one cycle of conditioning that is not further increased by 16 h of food deprivation (fd16h). From Hirano et al., 2013.

Still, other results, including results from the same laboratory that conducted the last mentioned study, oppositely suggested that insulin signaling was necessary for memory formation, both in mammals and in *Drosophila*.

II. 3. b. Insulin-like signaling modulates memory

Several studies showed a link between insulin signaling and cognitive functions in various species (Stockhorst et al., 2004; Dou et al., 2005; Lin et al., 2010; Chen et al., 2011; Costello et al., 2012). The mammalian brain is an insulin-sensitive organ that expresses the insulin receptor (Havrankova et al., 1978) and insulin-like growth factors (IGF) receptors (Bondy et al., 1992). Insulin signaling in the brain decreases food intake and body weight (Brüning et al., 2000). Besides its effect on feeding, it also modulates cognition. Indeed, intranasal insulin administration improves memory in healthy humans (Stockhorst et al., 2004; Benedict et al., 2004). Moreover, insulin signaling levels are decreased in patients displaying Alzheimer's disease (Mullins et al., 2017), and this correlates with an altered memory performance; intranasal insulin injections also improve memory in AD patients (Craft, 2012). As the hippocampus expresses the insulin receptor, all these data point to a role of insulin signaling in memory formation. Recently, several papers have confirmed that insulin signaling was necessary for memory formation in rodents (Chen et al., 2011; Costello et al., 2012; Soto et al., 2019).

At first, insulin signaling was not suspected to alter brain glucose uptake or energy metabolism, as peripheral hyperinsulinemia did not affect the BBB transport or net metabolism of glucose (Hasselbalch et al., 1999; Seaquist et al., 2001), but this view has been recently challenged. In cultures of mice astrocytes, the cooperation of insulin and IGF-1 triggers the translocation of GLUT1 at the cell membrane and an increased glucose uptake; and *in vivo* in the barrel cortex, insulin and IGF-1 signaling are both necessary for the recovery of hypoglycemia-induced depression of synaptic transmission (Fernandez et al., 2017). Moreover, InR knockout in astrocytes results in a decreased GLUT1 expression, a decreased glucose intake in astrocytes and in the whole brain (García-Cáceres et al., 2016). Interestingly, InR KO astrocytes exhibit a parallel increased expression of carnitine palmitoyltransferase 1C, shifting astrocytic fuel preference from glucose to lipids (García-Cáceres et al., 2016). Thus, insulin

signaling actually regulates carbohydrate metabolism in mammals, both in the periphery and in the brain.

While only one Insulin Receptor (InR) exists in *Drosophila* (Brogiolo et al., 2001), eight insulin-like peptides (Ilp), Ilp1 to Ilp8, are encoded in the fly genome (Grönke et al., 2010). Overexpressing each of them during development triggers an increased body size, demonstrating their functionality (Ikeya et al., 2002). Ilps are expressed in a tissue- and time-specific manner to allow multiple functions during development and at adulthood, such as the regulation of energy metabolism and storage. Ilp2, 3 and 5 are secreted by a few Insulin-Producing Cells (IPCs) which are neurosecretory cells located in the medial part of the *Drosophila* brain. These IPCs regulate systemic trehalose levels and energy stores (Broughton et al., 2005; 2008). *Drosophila* glia also secrete insulin-like peptides. We will come back on this topic in the chapter focusing on glia.

In flies, a role of insulin signaling in learning and memory was also recently demonstrated. It was first showed that homozygous flies mutant for the insulin receptor substrate Chico, Chico¹, exhibit a defect in middle-term memory due to a mushroom body development impairment (Naganos et al., 2012). In a mutant background, the expression of Chico in Kenyon cells is sufficient to restore normal MB development and aversive middle-term memory, showing that this developmental defect is cell autonomous. Still, the study did not tackle if insulin signaling could intervene in memory formation at adulthood in longer-lasting memories.

Interestingly, a subsequent study identified that insulin signaling was acutely required at adulthood for LTM formation, but not for immediate memory or LT-ARM (Chambers et al., 2015) (Figure 25). As the authors only used an inducible ubiquitous driver in order to express an InR dominant negative transgene, it is still uncertain in which cells insulin signaling is necessary for long-term memory formation. Knocking down insulin signaling in the mushroom body during the whole life induced a memory defect; thus, it was suggested in this study that insulin signaling might be acutely required in the mushroom body, but developmental and compensatory effects were not avoided.

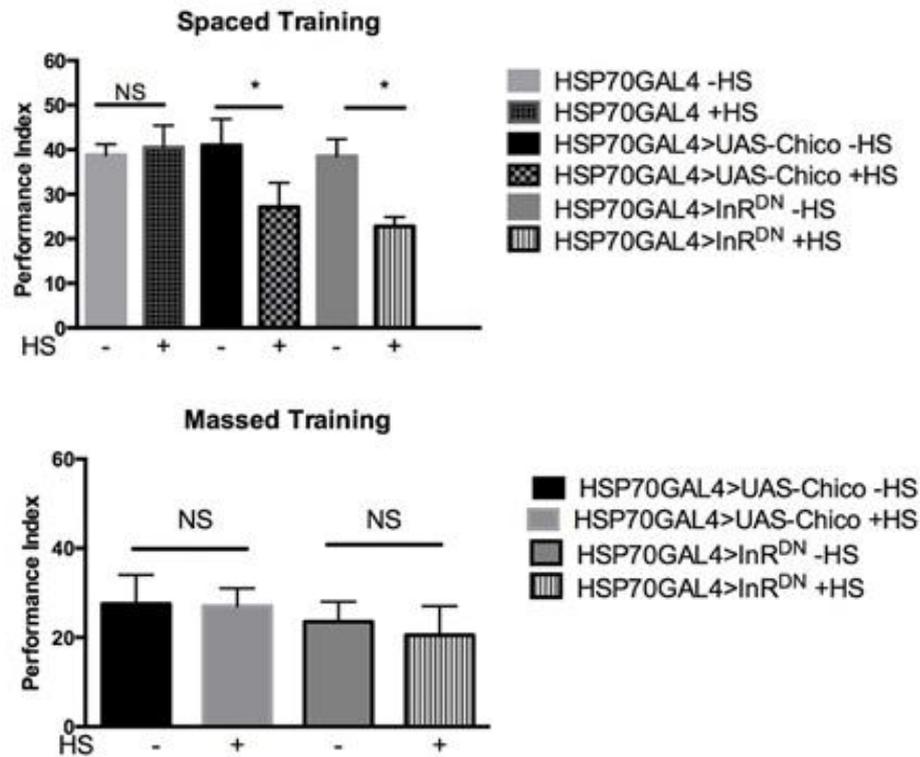


Figure 25. Insulin signaling is acutely required for memory formation. Acute expression of UAS-chico [HSP70GAL4>UAS-chico +HS] or a dominant negative isoform of insulin receptor InR^{DN} [HSP70GAL4>InR^{DN} +HS] disrupts 24 h memory after spaced training, but not after massed training. From (Chambers et al., 2015).

Strikingly, two publications originating from the same laboratory thus showed that chico¹ mutants exhibit a defect in 1 h middle-term memory (Naganos et al., 2012), but chico^{1/+} heterozygous flies display a facilitated long-term memory after only one cycle of conditioning (Figure 24) (Hirano et al., 2013). This suggests that precise levels of insulin signaling are required for proper cognition, and that insulin signaling might differentially influence memory formation depending on the memory phase and on the internal state of the flies. The conditional knockdown of insulin receptor in specific cells and only at adulthood is needed to better decipher the role of insulin signaling in memory formation, by circumventing developmental defects and possible compensatory mechanisms.

More recently, a defect in 3 to 9 h memory - defined in the paper as MTM - was observed by ablating the IIP producing cells (IPCs) in 10 days old flies (Tanabe et al., 2017). This defect was not seen in 30-day old flies, suggesting that IPCs do not

function properly upon ageing. The age-related memory impairment was rescued by the overexpression of an insulin-like peptide, *Ilp3*, in IPCs. Conversely, 3 h memory was impaired in young flies following *Ilp3* knock-down in adult IPCs. Interestingly, flies expressing a dominant-negative variant of *InR* in all neurons or all glial cells exhibited no deficit in 3 h memory after one cycle of conditioning, suggesting that the insulin-like peptide target is not neuronal or glial for 3 h memory formation. On the opposite, the authors showed that *InR* was required in the fat bodies for 3 h memory. Noteworthy, a role of *InR* in LTM has not been addressed in this study.

To conclude, in both mammals and invertebrates, different studies described that disturbing insulin signaling altered memory formation. Moreover, it seems that 3 h memory as well as long-term memory, tested at 24 h, require acute insulin signaling. If the origin and the targets of this signal are the same remain to be determined.

II. 3. c. Feeding status in appetitive LTM

Interestingly, flies form appetitive memory only if they have been food-deprived prior to the conditioning (Tempel et al., 1983). It was suggested to be caused by the absence of motivation in fed flies for feeding on sugar during the conditioning, but recent work also demonstrated that both the energetic status of flies and the energetic value of the sugar are encoded in the fly brain and impact memory formation.

Indeed, associating a sweet non-caloric sugar to an odor leads to a significant appetitive short-term memory (Burke and Waddell, 2011). On the contrary, it does not allow long-term memory formation. Sweet taste must actually be associated to energy intake to trigger long-term memory (Colomb et al., 2009; Burke and Waddell, 2011; Musso et al., 2015). Strikingly, similarly to their involvement in aversive long-lasting memories gating, MP1 dopaminergic neurons exhibit a higher spiking frequency after feeding with a nutritious sugar as compared to feeding with a non-caloric sugar, i.e. in fed flies as compared to starved flies. Blocking MP1 activity in flies conditioned with a nutritious sugar blocks long-term appetitive memory formation, while activating MP1 after feeding with a non-nutritious sugar is sufficient to drive long-term appetitive memory formation. Thus, MP1 neuron activity might also encode the caloric content

of the food ingested by the flies during conditioning and gate appetitive long-term memory formation (Musso et al., 2017).

As the metabolic status of the organism but also insulin signaling influence memory formation, our lab aimed at better understanding how metabolism was modulated during learning and memory. In mammals, it is well acknowledged that glial cells are involved in brain metabolism. As explained before, this is not well known in *Drosophila*, while the fly brain contains different well-defined glial subtypes that are characterized by specific morphologies, but also diverse functions. I will describe the various *Drosophila* glia briefly, and what is known about their involvement in memory.

Part III: Drosophila glia and their involvement in metabolism and memory

Glia are usually defined as non-excitabile cells that reside in the nervous system exclusively and are associated with neurons. Usually, researchers exclude from that definition cells of mesodermal origin, except for vertebrate microglia.

Glial cells are present in many animals, invertebrates as well as vertebrates. Although they were first described as cellular nervous system glue that would only provide a mechanic support to the highly complex and fragile neurons, four decades of research have firmly established that this initial view of glial function is far too simplified. Glial cells actually include very diverse cell types, endowed with a broad spectrum of physiological functions that are crucial for adequate brain function (Allen and Lyons, 2018; Dallérac et al., 2018).

I will first focus on the debated evolutionary origin of glia and describe Drosophila brain glia. Then, I will develop Drosophila glia involvement in metabolism, and finally present what is known about their role in memory.

III. 1. Glial cells origin and diversity

The *Drosophila* brain contains different glial subtypes, which described functions appear to be similar to those of mammalian glia. Still, it is currently under debate whether mammalian and insect glia share a common evolutionary origin.

III. 1. a. Glial evolutionary origin

As they share structural and functional commonalities between taxa from invertebrates to vertebrates, glia could either share a common evolutionary origin, or have been selected because of shared adaptive pressures (Hartline, 2011). The Metazoa basal to the Bilateria do not seem to possess glia, even when they do have a nervous system, indicating that glia have evolved after the apparition of nervous systems (Hartline, 2011) (Figure 26). Still, the common ancestor between vertebrates and invertebrates is supposedly a primitive flatworm belonging to Acoelomorpha that does possess glia. It would therefore be likely that all glial cells share a common origin.

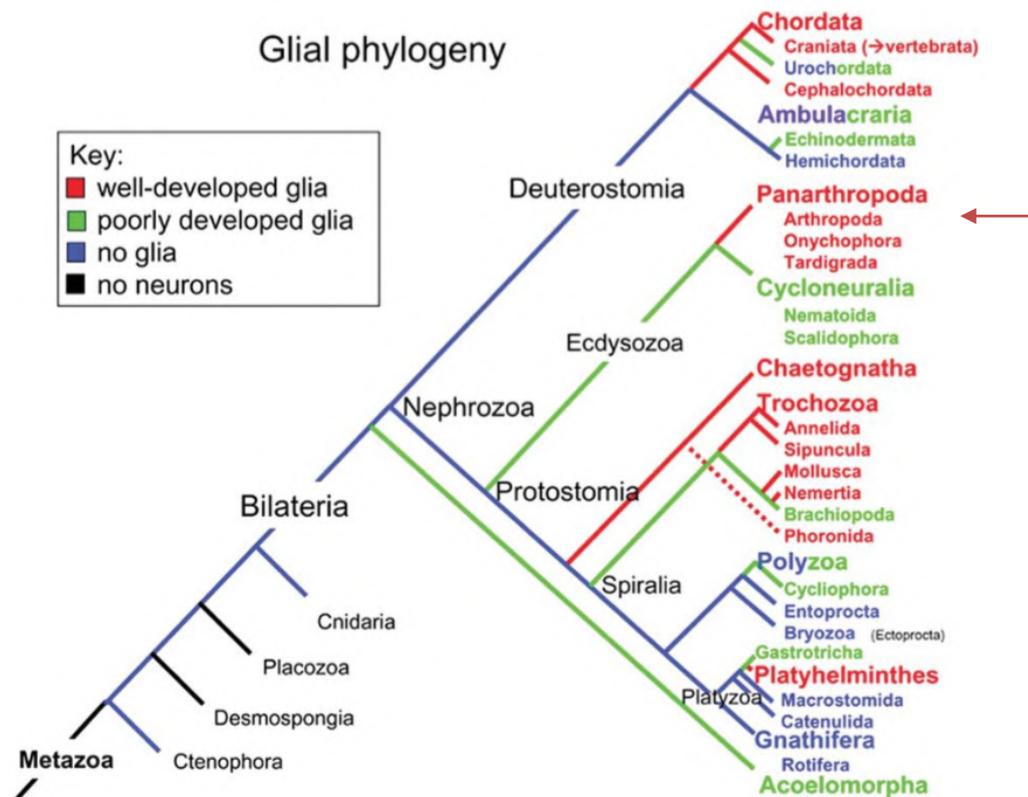


Figure 26. Glial phylogeny. Phylogenetic tree of various living bilaterian taxa. *Drosophila melanogaster* belongs to phylum Arthropoda, indicated by the red arrow. From (Hartline, 2011).

III. 1. b. Glial diversity, location and morphology in the *Drosophila* CNS

The mature adult *Drosophila* CNS contains about 15 000 glial cells (Kremer et al., 2017), meaning that they represent about 10% of CNS cells. In the brain, these cells divide into several subtypes (Figure 27).

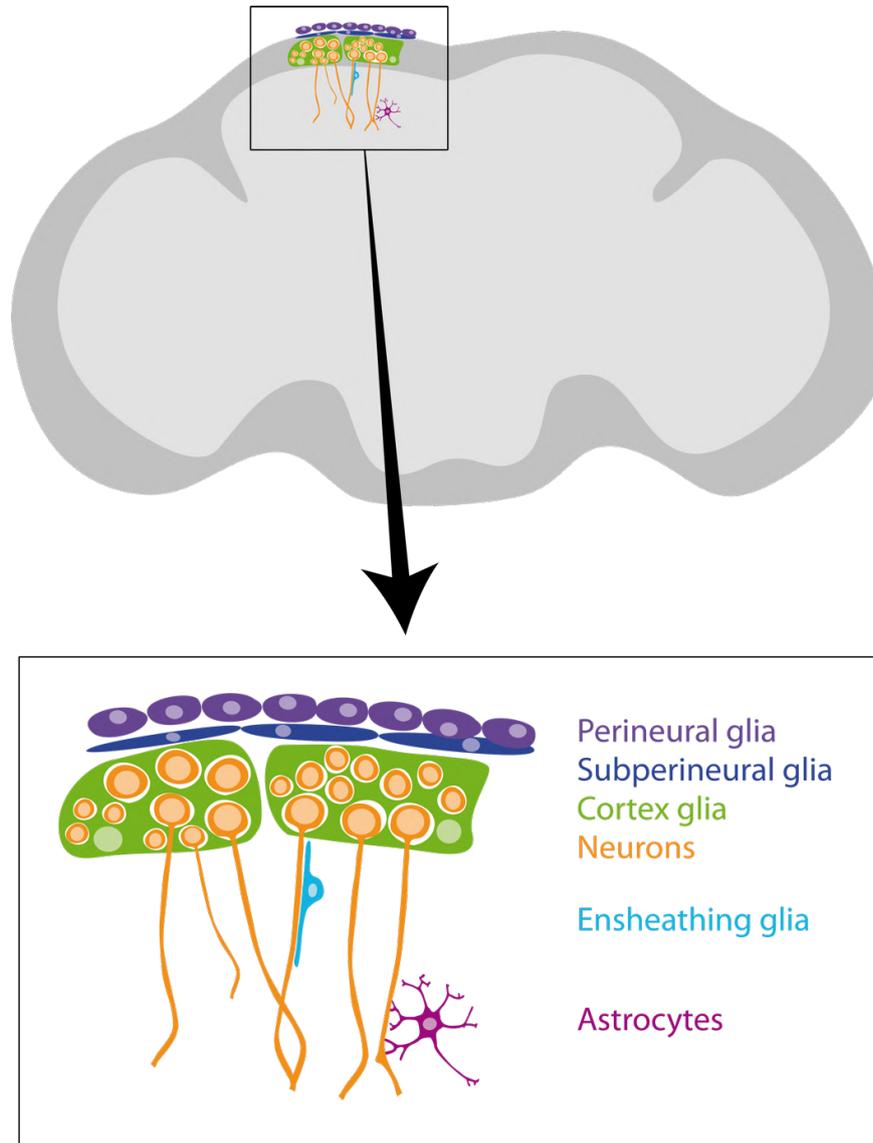


Figure 27. Anatomy and location of *Drosophila* brain glia. The fly brain is divided into the cortex (dark grey), where the neuronal soma reside, and the neuropil (light grey) that contains neurites and synapses. The outermost periphery of the brain is delimited by perineural and subperineural glia together forming surface glia. Right underneath, cortex glia enwrap individually each neuronal soma. Distal neurites gather into nervous tracts and form brain structures separated by layers of ensheathing glia. Neuropil compartments are invaded by astrocytic processes.

Perineural, subperineural and cortex glia are located in the most superficial layers of the brain called the cortex (Figure 27). 2250 perineural and 300 subperineural cells together form the blood brain barrier (Kremer et al., 2017), a 2–3 μm thick double layer that contiguously covers the entire CNS (Figure 28). Subperineural cells establish intercellular septate junctions that prevent free paracellular diffusion from the periphery towards the CNS (Bainton et al., 2005).

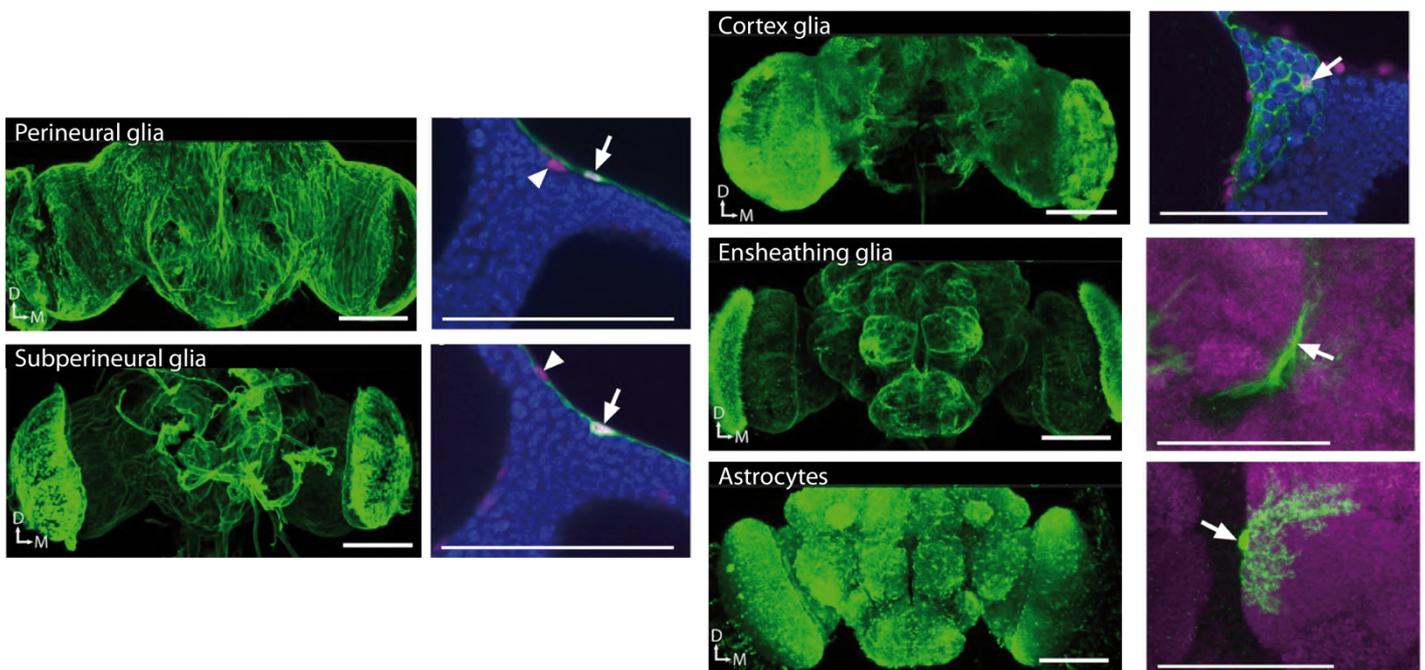


Figure 28. Immunolabeling of the different glial subtypes in the fly brain. Different glial subtypes are visualized by crossing flies carrying subtype-specific drivers with a membrane-tagged reporter (UAS-mCD8GFP); brains from the progeny are then dissected and immunostained. For each subtype, on the left, the morphology of one GFP-tagged glial subtype in the whole brain is presented, and on the right, the staining of an individual cell is shown. For images of single cells: for perineural, subperineural, cortex glia, blue: neuronal nuclei-specific Elav immunostaining, purple: glial nuclei-specific Repo immunostaining; for ensheathing glia and astrocytes, purple: nc82 neuropile-specific marker. 130–160 μm projections are shown. Adapted from (Kremer et al., 2017) and (Awasaki et al., 2008).

2600 cortex glia are located in the periphery of the brain, insulating individually all the neuronal soma in a honeycomb-like network of glial processes (Figure 28) (Kremer et al., 2017). Cortex glial processes between neighboring neurons can be as thin as 50 nm (Coutinho-Budd et al., 2017). Each cortex glial cell enwraps up to 100 somas

(Freeman, 2015). The enclosure of one neuronal cell body can be shared by two different cortex glial cells (Kremer et al., 2017). Cortex glia also enwrap the proximal neurites traveling across the cortex towards the neuropil. Two neighboring glial cells segregate with little overlap, minimizing the area of contact with each other while maximizing the surface of contact with neurons (Kremer et al., 2017). In the peripheral part of the brain, cortex glia occasionally fail to envelop the most distal part of neuronal cell bodies, but superineural glial cells then complete neuronal soma enclosure (Freeman, 2015). Interestingly, during development, the daughter cells from the same neuroblast are usually encapsulated by one cortex glial cell, maybe authorizing the orchestrated control of their activity (Spéder and Brand, 2018).

Two other glial subtypes, astrocytes and ensheathing glia, are located in the central part of the brain, in contact with the neuropil. 3700 ensheathing glial cells insulate neuropil regions and nerve tracts (Kremer et al., 2017), subdividing brain lobes into anatomically discrete compartments. Their cell bodies are located between the cortex and neuropil regions but are sometimes found in-between neuropil regions. They show little overlap (Kremer et al., 2017).

About 4600 astrocytes elaborate a dense meshwork of a highly ramified morphology formed by processes of varying thickness and long filopodia projecting into adjacent tract regions (Kremer et al., 2017). A substantial portion of the neuropil synaptic space is close to at least one of these processes (about 5%) which can sit in average about 1 μm away and as close as 0.1 μm away from a synapse (Stork et al., 2014). Astrocytes do not delimitate their projection to a single neuropil region so they must not be dedicated to specific synaptic units. Surprisingly, the density of astrocytic processes is inversely proportional to the density of synapses in the respective neuropil regions (Kremer et al., 2017).

A few specific glial subtypes have been described in the retina, but as this brain area is *a priori* not involved in olfactory memory formation, it is out of the focus of this thesis.

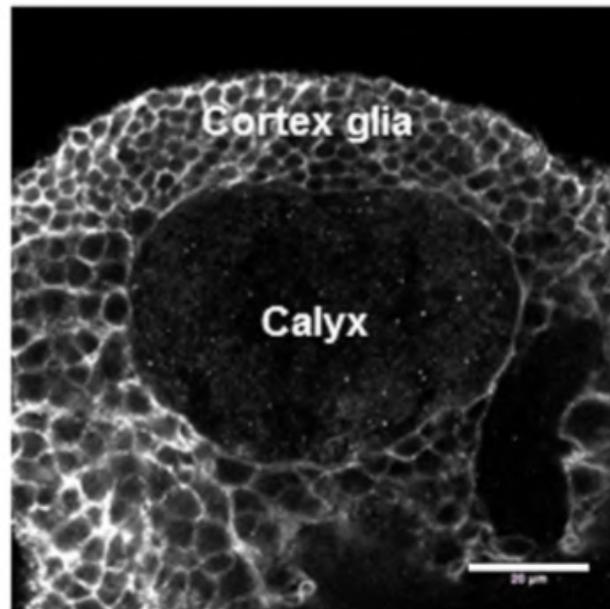
III. 1. c. The functions of glia in the *Drosophila* adult brain

As I first mentioned, surface glia establish the blood-brain barrier. I will therefore focus on the three other glial subtypes present in the *Drosophila* brain that are in direct contact with neurons: cortex, astrocyte and ensheathing glia. They likely mediate key events that allow them to modulate neural circuit assembly, function, plasticity, or homeostasis (Freeman, 2015). Data demonstrating their function is still scarce, but several studies recently reported multiple functions for these glial subtypes. We will focus on their functions at adulthood.

III. 1. c. i. *Cortex glia*

Based on their location and morphology, cortex glia were thought to regulate the microenvironment of neuronal soma in a homeostatic way. Now, recent data support this hypothesis. It has been first shown that cortex glia, as well as other glial subtypes, constrain tracheal development (Pereanu et al., 2007), suggesting a role in controlling oxygen delivery to neurons. Interestingly, it has been suggested that cortex glia could participate in establishing the diffusion barrier in addition to the BBB (Stork et al., 2008). Even though it has been often suggested in the literature that cortex glia could provide a trophic support to neurons (Freeman, 2015), evidence about such a metabolic interaction is lacking.

Cortex glia express Innexin 2 (*Inx2*), a transmembrane protein that forms gap junctions in insects and is heterologous to pannexins in mammals (Farca Luna et al., 2017) (Figure 29). Downregulating both *Inx2* and glutamine synthase in cortex glia increases total sleep, while their overexpression has the opposite effect (Farca Luna et al., 2017). Thus, cortex glia influence sleep, but the precise mechanism involved has not been described. Mammalian astrocytes also express connexins that are essential for their function, such as the metabolic support of neurons or the modulation of neuronal networks activity (Giaume et al., 1997; Rouach et al., 2008). It is thus possible that cortex glia directly modulate neuronal activity level.



Inx2^{BG}-Gal4 > mCD8::GFP

Figure 29. Cortex glia express Inx2. Inx2BG-GAL4 drives the expression of membrane-bound mCD8::GFP in cortex glia. From (Farca Luna et al., 2017).

A very interesting study indeed showed that mutating Zydeco, a NCKX Na/K/Ca exchanger highly expressed in cortex glia (Figure 30), cancelled near-membrane calcium transients in these cells in larvae, and increased seizure susceptibility at adulthood (Melom and Littleton, 2013). This seizure-prone phenotype is rescued by expressing Zydeco only in cortex glia in a mutant Zydeco background.

Interestingly, cortex glia intracellular calcium is constitutively elevated in *zyd1* mutants (Melom and Littleton, 2013), and TRPA1-mediated cortex glia activation induces seizures, suggesting that elevating calcium levels in cortex glia might increase neuronal excitability. Calmodulin expression in cortex glia is necessary for seizure initiation, suggesting that a signaling pathway in cortex glia must control neuronal excitability, but the precise mechanism remains unknown. Thus, calcium transients in cortex glia that surround neuronal soma may normally regulate the activity state of neural networks.

Mammalian astrocytic networks are also important to set neuronal basal resting potential (Chever et al., 2016), but their role at the cell body level has not yet been investigated. This original work from Troy Littleton's laboratory demonstrates the functional importance of the neuronal soma and proximal neurite's environment in a

framework where most of the studies on neuronal firing focus at the synaptic level. Confirming this work, a very recent study also showed that an altered cortex glial morphology underlies light-induced epilepsy in flies (Kunduri et al., 2018).

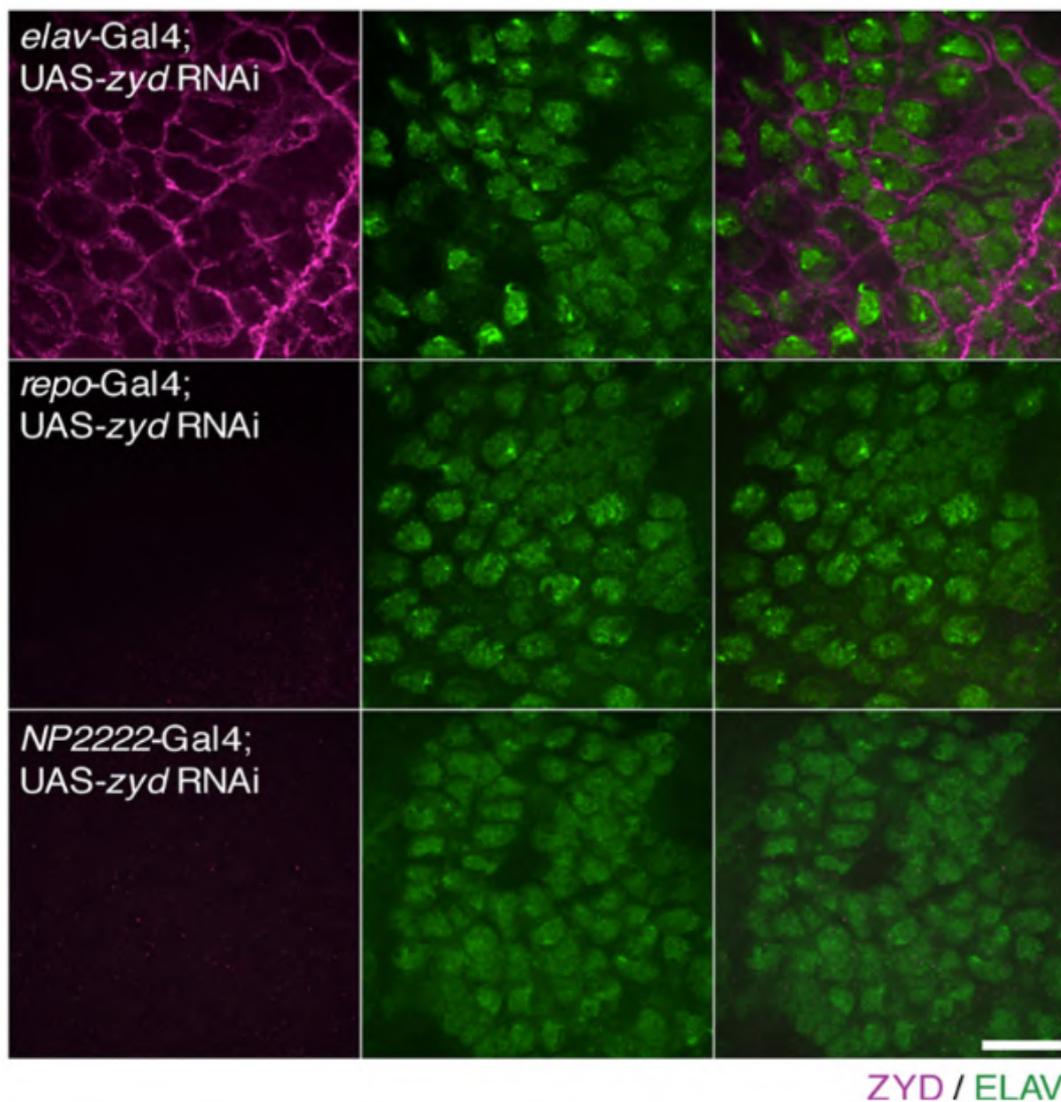


Figure 30. Zydeco is expressed in cortex glia. Optical section of the CNS of *Drosophila* larvae expressing pan-neuronal (*elav-Gal4*), pan-glial (*repo-Gal4*), or cortex glial (*NP2222-Gal4*) zydeco (*zyd*) RNAi. Elav is a neuron-specific nuclear molecular marker. From (Melom and Littleton, 2013).

Finally, cortex glia express the molecular machinery necessary for engulfing degenerating neurons, i.e. the scavenger receptor Draper and downstream targets (Doherty et al., 2009), and these cells actually participate to the elimination of degenerating neurons (Nakano et al., 2019). They are also involved in brain remodeling during development (Awasaki et al., 2011b).

III. 1. c. ii. Astrocytes

As they are in quite close contact with synapses, it was early postulated that astrocytes could modulate neurotransmission and synaptic plasticity also in *Drosophila*. Accordingly, they express several proteins involved in neurotransmitter recycling, such as Eaat1 transporters (Freeman et al., 2003) that are necessary for glutamate buffering to prevent excitotoxicity (Rival et al., 2004), or GABA transporters GAT (Muthukumar et al., 2014; Stork et al., 2014; Zhang et al., 2017) that modulate GABA levels at the synapse. A first evidence suggested that astrocytes might be involved in modulating synaptic strength at the level of the olfactory receptor neuron/projection neuron synapse (Liu et al., 2014). Indeed, elevating their calcium level is sufficient to decrease the strength of this synapse (Figure 31). It is interesting to notice that the opposite effect is found in cortex glia: increasing calcium levels in cortex glia seems to increase neuronal excitability as it induces seizures (Melom and Littleton, 2013).

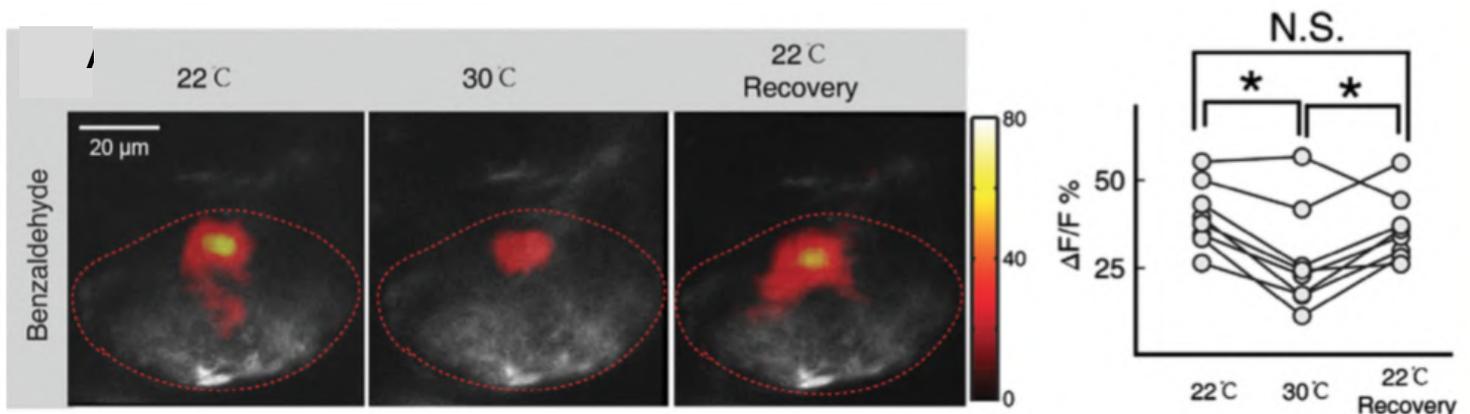


Figure 31. Astrocyte-like glial activation inhibits odor-evoked projection neurons responses. A. The calcium response to benzaldehyde was measured thanks to the targeted expression of GCaMP3 in benzaldehyde-specific responding projection neurons. The expression of TrPA1 in astrocytes allowed their activation at high temperature (30°C). B. Projection neurons odor response was significantly reduced when astrocytes were activated. From (Liu et al., 2014).

In addition, in a pathological hyperexcitability context, depleting focal adhesion molecules in astrocytes ameliorates recovery from seizures in *Drosophila* (Cho et al., 2018). This can be compared to what has been shown in mammals (Chever et al., 2016) in which astrocytic connexins 30/43 deficiency increases neurons resting

potential, and increases seizure frequency while decreasing their duration and severity. Thus, decreasing cell adhesion as well as connectivity in astrocytic networks is beneficial for epileptic recovery both in mammals and *Drosophila*.

At the behavioral level, astrocytes are involved in the circadian rhythm. Indeed, the *Ebony* gene, involved in the circadian cycle, is expressed in glia (Suh and Jackson, 2007). In an *ebony* mutant background, expression of *ebony* in glial cells is sufficient to restore a normal circadian rhythm. Recent data point out that *ebony* is indeed mostly expressed in glia and predominantly in astrocytes (Croset et al., 2018; Davie et al., 2018). Interestingly, *Ebony* codes for an N- β -alanyl-biogenic amine synthetase, an enzyme that links beta-alanine to biogenic amines like dopamine or histamine. It is thus possible that astrocytes play on the level of free dopamine, a neurotransmitter that is involved in rhythmicity and other behaviors. Besides, disturbing calcium levels or vesicle dynamics in astrocytes alters circadian motor output (Ng et al., 2011).

Altogether, in *Drosophila*, astrocytes can regulate neuronal excitability at the level of the distal neurites and synapses, while cortex glia influence neuronal excitability by acting at the level of the soma and proximal neurite. Thus, the fruit fly is a useful model to study the interaction between specific neuronal compartments and glial cells.

III. 1. c. iii. Ensheathing glia

Ensheathing glia clear neuronal debris after axonal injury. Indeed, ensheathing glia express *Draper*, a scavenger receptor (MacDonald et al., 2006; Doherty et al., 2009), and are activated during neuronal injury, allowing the proper phagocytosis of degenerating neurons at adulthood (Doherty et al., 2009). Insulin signaling through *InR* and *Akt* is activated locally in ensheathing glia after nerve injury (Musashe et al., 2016), and downstream *PI3K* signaling increases *Draper* levels (Doherty et al., 2014; Lu et al., 2017). Conversely, degenerating axons are cleared less efficiently in aged flies due to a decrease in *PI3K* signaling in glia associated to reduced levels of *Draper* (Purice et al., 2016).

A role of ensheathing glia in circadian behavior has also been demonstrated. *Eaat2* mutants display increased daytime sleep. This effect is phenocopied by the blockade

of vesicular trafficking in ensheathing glia (Stahl et al., 2018), and rescued by Eaat2 expression only in ensheathing glia.

Altogether, these works demonstrate that each glial subtype in the fly brain is involved in specific molecular processes that allow brain homeostasis and function, including its behavioral outputs. If research in mammals early shed light on the metabolic interaction between neurons and glia, this relationship just begins to be discussed in insects.

III. 2. Invertebrate glia and energy metabolism

The anatomical description of the relationship between invertebrate glia and neurons first suggested that these cell types could exchange metabolites. In addition, it has been shown that glia can integrate and release molecular cues that signal the whole body metabolic status during development, and recent evidence suggest that glia control metabolite supply to neurons in flies.

III. 2. a. Anatomical considerations in diverse insects

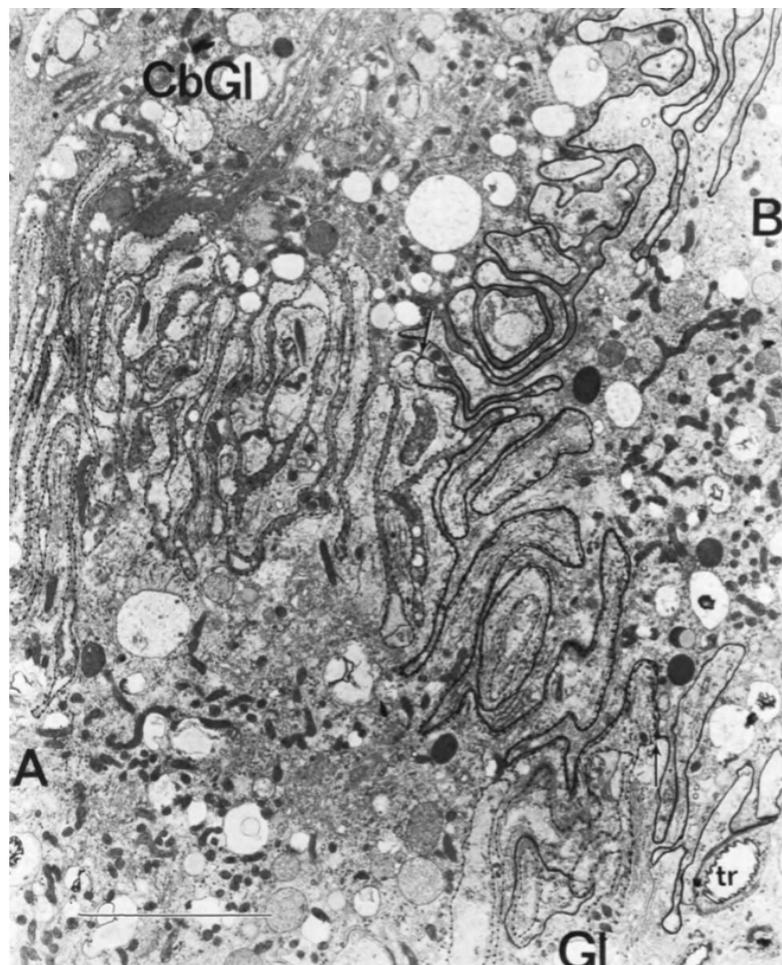


Figure 32. Glia extend processes into neuronal soma in the locust. Membrane interactions between the neighboring motor neurons A and B and the cell-body glial cell (CbGl) they share shown on an electron micrograph. The membrane of neuron A has been emphasized by dots, and that of neuron B by a continuous line. Scale bar 5 μ m. From (Hoyle et al., 1986).

Historical studies observed in various invertebrate species the equivalent of cortex glia in *Drosophila*: glia surrounding the neuronal soma, often called “satellite glia” or “cell-body glia”. As early as in 1900, Holmgren described such satellite glia in the snail. Thanks to light microscopy, he showed in the large gastropod cells that glia penetrate the neurons and send finger-like processes towards the neuronal cytoplasm, naming this structure the trophospongium (Figure 32 and Figure 33). This up to 12-folds increase (Hoyle et al., 1986) in the surface of contact between the neuronal soma and cortex glia suggested important chemical exchanges. As neuronal soma-surrounding glia are located between blood-brain barrier, trachea and neurons, forming an apparent uninterrupted layer that is linked by gap junctions, they could limit the diffusion of substances that reach the neuronal soma, and thus provide a controlled neuronal trophic support. Indeed, the time for a molecule to diffuse across a gap of about 20 nm could be negligible compared to the time to escape to the outside along the long cleft caused by this circumvolved morphology (Wolfe and Nicholls, 1967).

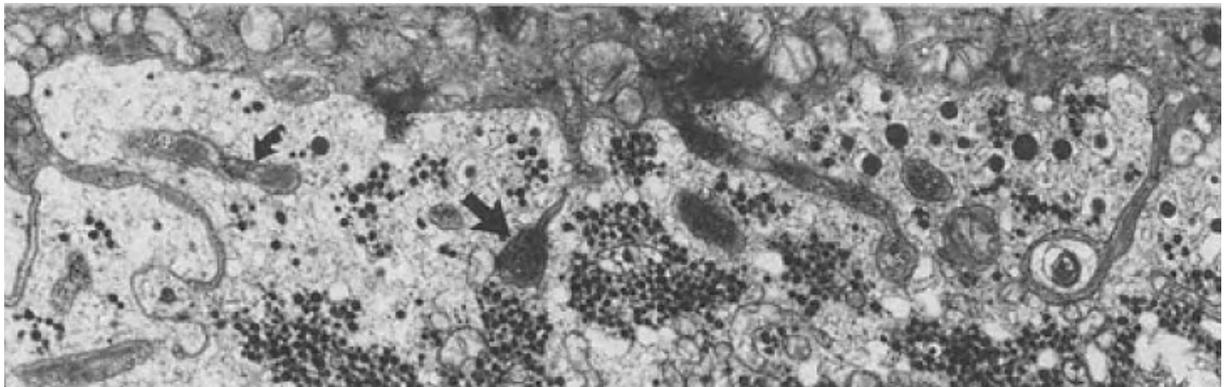


Figure 33. Electron micrograph of the interaction between a neuronal soma and its neighboring glia in a ganglion of the leech. Arrows indicate processes from glia into the neuron. Magnification x5000. From *Neuron—Glia Interrelations During Phylogeny: II. Plasticity and Regeneration*, Antonia Vernadakis, Betty I. Roots.

Interestingly, prominent adherens junctions strengthen the contact in between glial layers and between cortex glia and neurons (Pereanu et al., 2005; Kis et al., 2015) (Figure 34).

In the cockroach, these glial processes are invaded with mitochondria and vesicles (Hess, 1958). It has also been described in scorpions and crustaceans. This very conserved mechanism during evolution of invertebrate species must thus be of crucial importance for invertebrate brain or ganglia function. In mammals, glial invaginations have in general not been described; on the opposite, perikaryal projections originating from neurons can invade glial cells, for example in satellite glial cells that reside in the peripheral nervous system, in the rachidian dorsal root ganglia (Pannese, 2002).

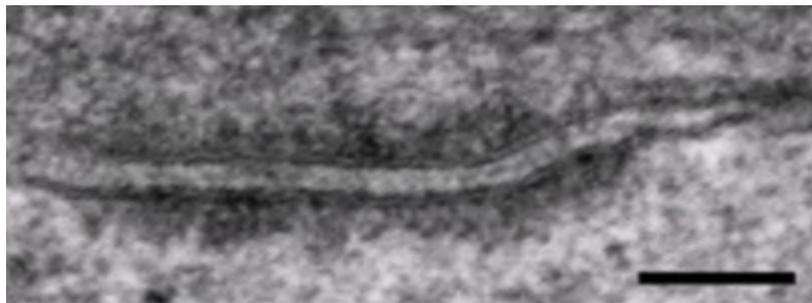


Figure 34. A subperineural glia (above) and a superficial cortex glia (down) are connected with adherens junctions. Scalebar 100nm. From (Kis et al., 2015).

Not only their morphology, but also their cytoplasmic content, suggest that glia can provide a metabolic support to neurons in insects.

III. 2. b. Glia contain metabolic stores

In 1960, Wigglesworth showed in the cockroach that surface glia and cortex-glia-like cells unwrapping neuronal soma contain glycogen granules (Wigglesworth, 1960) (Figure 35), which was further confirmed in cortex glia in *Drosophila* (Kis et al., 2015). Ensheathing glia has also been reported to contain glycogen granules (Oland et al., 2008). These glycogen reserves in insect neuroglia are depleted upon starvation (Wigglesworth, 1960) (Figure 35) and rapidly re-synthesized after feeding.

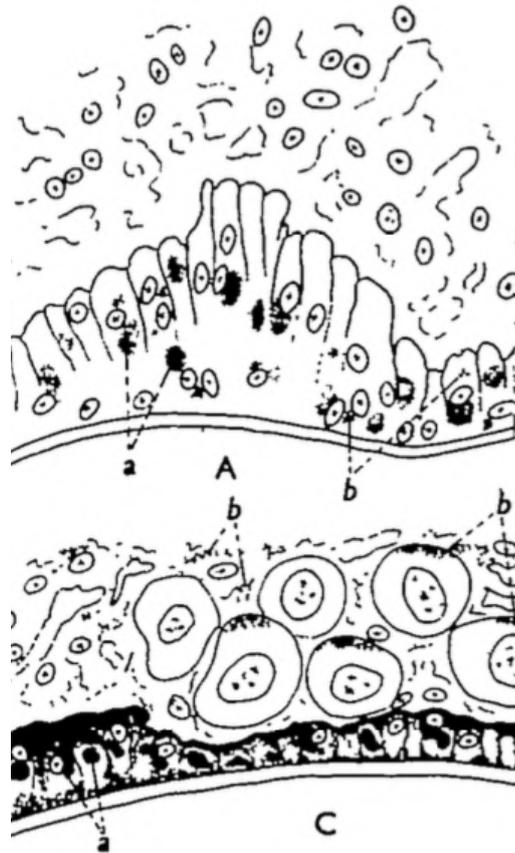


Figure 35. Schematic horizontal section of the anterior end of the terminal ganglion from a cockroach starved for 3 weeks. A. Drawing of an electron micrograph of a ganglion of a starved cockroach; perineural glia contain clumps of mitochondria and traces of glycogen. B. Drawing of an electron micrograph of a ganglion of a starved cockroach 3 hours after feeding on honey and serum, showing glycogen in perineural and in cortex glial cells. a: mitochondria; b: glycogen inclusions. From (Wigglesworth, 1960).

Moreover, in the cockroach, cortex glia accumulate lipid droplets following the injection of a high amount of carbohydrates (Wigglesworth, 1960). These droplets actually localize in the glial processes around the neuronal soma, suggesting again a metabolic support from glia to neurons (Figure 36). In *Drosophila*, lipid droplets are also stored in cortex glia (Bailey et al., 2015; Kis et al., 2015), and a decrease in beta-oxidation in glia decreases flies longevity (Schulz et al., 2015).

As glia accumulate metabolic stores and are in such close apposition to neurons, it should favor communication and chemical exchanges between both cell types. Recent advances in the study of flies CNS development showed that metabolic signals can be released between neurons and glial cells.

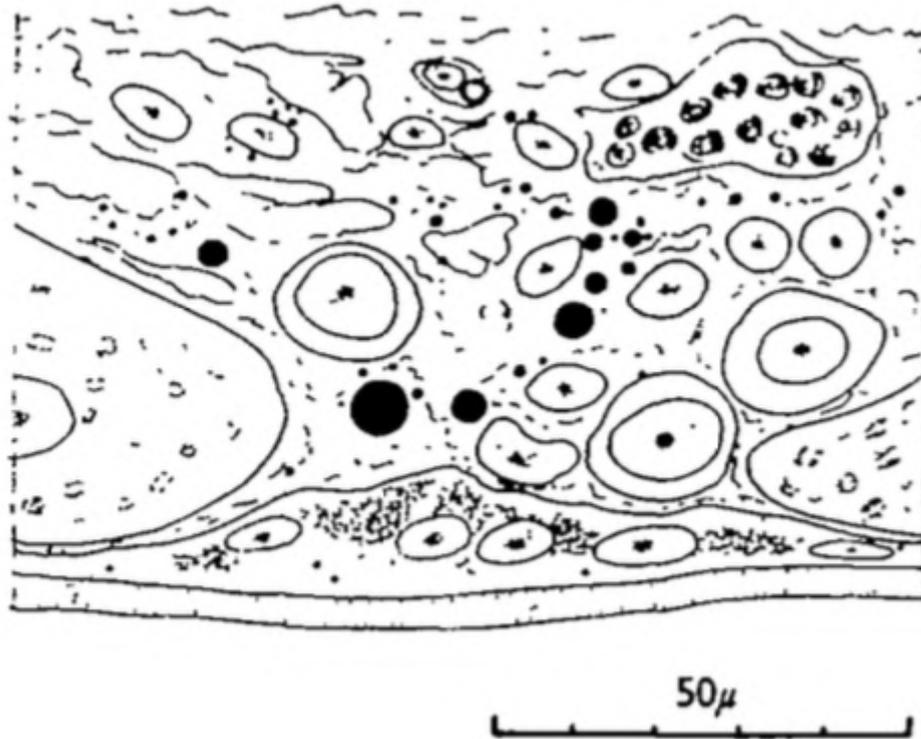


Figure 36. Schematic of a ganglion 20 hr after the injection of 0.2 mL of a solution of 10% glucose into a normally nourished male cockroach. A few fat droplets are located in the perineural cells (lower cells in the periphery), while numerous droplets are located in cortex glia cytoplasm (above). From (Wigglesworth, 1960).

III. 2. c. Several glial subtypes can secrete peptides regulating energy metabolism

In flies, glial cells are sensitive to nutrient-derived hormonal signals coming from the fat bodies and secrete insulin-like peptides in response. Indeed, neuroblast divisions are stopped between embryonic development and larval stage. Following a fat-body derived signal, surface and cortex glia release insulin-like peptide 6 (Chell and Brand, 2010) that activates InR and downstream signaling in neuroblasts, allowing neuroblast exit from quiescence (Chell and Brand, 2010; Sousa-Nunes et al., 2011; Spéder and Brand, 2014).

Moreover, after neuroblasts exit from quiescence, gliogenesis restarts from a pool of neuroglioblast precursors. The proliferation of cortex and perineural glia also depends on insulin signaling (Avet-Rochex et al., 2012). In addition, cortex glial proper ensheathment of neuronal cell bodies depends on nutritional cues that activate insulin-like signaling in these cells (Spéder and Brand, 2018) (Figure 37).

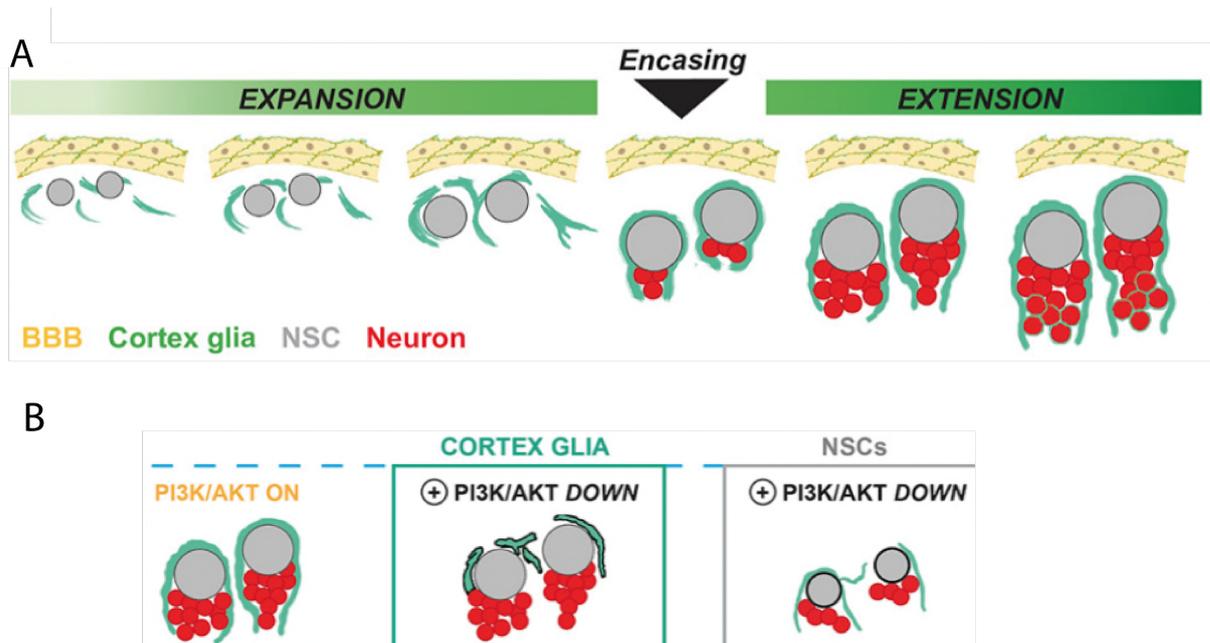


Figure 37. Insulin-like signaling in cortex glia is necessary for the proper ensheathment of neuronal soma during development. A. Sketch representing the timeline of cortex glial chamber formation. B. During development, if PI3K/Akt signaling is deficient in cortex glia, cortex glia do not extend the processes allowing neuroblast chamber formation. If PI3K/Akt signaling is deficient in neural stem cells (NSCs), neuroblasts do not efficiently divide, and cortex glia do not properly ensheath them neither. From (Spéder and Brand, 2018).

In a nutshell, this shows that surface and cortex glia can both emit and be sensitive to insulin-like peptides, at least during larval stages. The consequences of insulin signaling on cellular energy metabolism during development has not been studied, as only proliferation was analyzed in the above-mentioned works.

Glia thus store energetic molecules, are sensitive to nutritional cues and can emit insulin-like peptides in flies. As mentioned earlier, we postulate that during memory formation, the high energy demand of neuronal activity could be supported by glia. Before illustrating the work done during my PhD, I will finally give an overlook of the current knowledge we have about glial involvement in memory formation.

III. 3. *Drosophila* glia and memory

In vertebrates, it was first reported that glia were involved in the metabolic support of neuronal activity during memory formation in chicken (Gibbs et al., 2006). The authors showed that glycogenolysis in the brain was necessary for memory tested 2 h after an aversive training. The metabolic support of neurons during long-term memory formation was further investigated in rats (Newman et al., 2011; Suzuki et al., 2011). Blocking glycogenolysis in the hippocampus right before or immediately after the training disrupted long-term memory formation, but not shorter-term memory (Suzuki et al., 2011). As this effect was phenocopied by monocarboxylate transporter knock-down and rescued by lactate perfusion, the study nicely showed that astrocyte to neurons glycogen-derived lactate shuttle was necessary for long-term memory formation. Moreover, blocking noradrenaline signaling right before the conditioning prevents long-term memory specifically, which is rescued by exogenous lactate injection (Gao et al., 2016). Actually, adrenergic signaling through β 2 adrenergic receptors is necessary in hippocampal astrocytes for fear memory; this adrenergic signaling is coupled to an astrocytic lactate release during fear memory formation (Gao et al., 2016). Thus, strong evidence points to a role of lactate supply to neurons during long-term memory formation in vertebrates.

In comparison, in *Drosophila*, a glial involvement in memory has not been thoroughly investigated. Some punctual observations have been reported, though, that are detailed hereafter.

III. 3. a. *Drosophila* glia are involved in LTM formation

Back in 2004, a mutant that exhibited a specific LTM defect was characterized in T. Preat's group. The mutated gene, *crammer*, encodes a cysteine proteinase expressed predominantly in glia (Comas et al., 2004). The overexpression of *crammer* exclusively in glia induces a LTM defect. Thus, the role of glia in LTM formation was early suggested, but the precise glial subtype involved and the underlying mechanisms could not at that time be easily determined.

More recently, it was shown that LTM formation requires *Klingon* expression in neurons as well as in glia, which allows an increase in *Repo* expression in glia

following training (Matsuno et al., 2015). Klingleon is a cell adhesion molecule, and Repo is a transcription factor essential for glial differentiation. This suggests that neuron-glia interactions are essential for maintaining a glial identity or function, which is necessary for LTM formation. On the contrary, LT-ARM formation was not affected by neuronal or glial Klingleon knockdown, suggesting again that LTM and LT-ARM rely on drastically different cellular mechanisms. The authors replicated a long-term memory defect when knocking down Repo only in astrocytes, demonstrating that astrocytes are involved in long-term memory formation.

III. 3. b. Glial metabolism is involved in age-related memory impairment

During ageing, middle-term 1 h aversive memory and long-term memory are significantly altered (Tamura et al., 2003; Mery, 2007), a process referred to as age-related memory impairment (AMI). 1 h AMI can be rescued by decreasing pyruvate carboxylase expression in glia, while conversely, memory is impaired in younger flies with pyruvate carboxylase overexpressed in glia (Yamazaki et al., 2014). Interesting to notice, the pyruvate carboxylase inhibition did not rescue AMI for long-term memory. The study showed that 1 h AMI was linked to a decrease in D-serine levels, and rescued by D-serine feeding to the flies. The authors concluded that D-serine release from glia was decreased during ageing, and that this was the cause of 1 h AMI. Alternatively, if this has not been investigated in the original study, it is worth noting that pyruvate carboxylase is one of the enzyme involved in gluconeogenesis, a pathway that antagonizes pyruvate entry into the mitochondria and pyruvate subsequent oxidation. Thus, an altered pyruvate metabolism in glia could be a cause of AMI. Moreover, D-serine can be transformed into pyruvate, which would be consistent with the rescue of 1 h AMI in flies feeding on D-serine. Finally, the glial subpopulation involved was not further analyzed, as the authors used the Nrv2-GAL4 pan-glia driver, and Repo-Gal4 pan-glia driver.

In a nutshell, these results provided the first cues about a glial involvement in memory formation, with a possible link to energy metabolism.

Altogether, the recent works conducted in *Drosophila* suggest that glial cells play an essential role in memory, as they do in mammals.

Several clues indicate that brain metabolism is essential for its proper function: metabolic dysfunctions often affect primarily the brain (Lee et al., 2000), and brain metabolism is altered in many neurodegenerative disease as well as during ageing (Mattson et al., 2008). It is therefore crucial to better understand brain metabolism and its modulation in various activity and physiological states.

Memory is an energy-consuming process that relies on neuron-glia interactions and metabolic regulations in mammals (Suzuki et al., 2011; Gao et al., 2016). The size of vertebrates central nervous systems and the complexity of neuronal and glial morphologies make the study of metabolism in memory-relevant neuronal and glial networks difficult. The genetically tractable *Drosophila* model offers a nice compromise between the complexity of the behaviors it displays and the sophisticated experiments that can be conducted, from precise genetic manipulations to *in vivo* imaging of the brain. Thus, we used this model organism to study metabolism during memory formation.

During my thesis, I first asked whether long-term memory formation had a significant, measurable cost, as suggested in the literature (Mery and Kawecki, 2005; Plaçais and Preat, 2013). Answering this question led the way to the study of the neuronal metabolic pathways involved in memory formation (Plaçais et al., 2017). Based on this first work that evidenced the prominent role of metabolism in controlling long-term memory, and given the importance of neuron-glia coupling in vertebrate brain energy metabolism, I questioned the requirement of glial cells mechanisms in *Drosophila* long-term memory. Thanks to time-restricted gene knockdown in specific cell populations, immunohistochemistry and *in vivo* imaging experiments, I further aimed at deciphering the molecular pathways underlying neuron-glia interactions during long-term memory formation.

I. Fly strains and raising conditions

Fly strains were obtained from various sources. The GAL4 driver lines targeting glial cells were obtained from Marc Freeman (Alrm-GAL4, 54H02-GAL4, 54H02-GAL80), Serge Birman (MZ0709-GAL4, NP6293-GAL4), Laurent Seugnet (Moody-GAL4) and VDRC (NP2222-GAL4).

Name of the line (chromosome)	Target (GAL4 lines)	Reference
Repo-GAL4 (III)	All glia	(Awasaki and Lee, 2011; Lai and Lee, 2006)
MZ0709-GAL4 (III)	Ensheathing glia	(Ito et al., 1995)
Alrm-GAL4 (III)	Astrocytes	(Doherty et al., 2009)
Moody-GAL4 (III)	Subperineural glia	(Bainton et al., 2005; Schwabe et al., 2005)
NP6293-GAL4 (II)	Perineural glia	(Awasaki and Lee, 2011)
NP2222-GAL4 (II)	Cortex glia	(Awasaki and Lee, 2011)
54H02-GAL4 (III)	Cortex glia	(Coutinho-Budd et al., 2017)
54H02-GAL80 (III)		(Coutinho-Budd et al., 2017)
UAS-Shi ^{ts} (III)		(Kitamoto, 2002; Plaçais et al., 2012; Séjourné et al., 2011)
UAS-mCD8::GFP (III)		(Scheunemann et al., 2018)
Mi{PT-GFSTF.2}Treh ^{MI05512-GFSTF.2}		Bloomington #59825
tubulin-GAL80 ^{ts} (II)		(Musso et al., 2015)

To check the expression pattern of GAL4 drivers, each line was crossed to UAS-mCD8::GFP, and brains from the progeny were immunostained with anti-GFP

antibody (see immunohistochemistry section). We also used the MiMiC line #59825 (Bloomington), in which GFP is inserted in the endogenous locus of Trehalase gene.

54H02-LexA was generated in the lab with a protocol described in the next section. We used the following UAS-RNAi lines ordered from the Bloomington and VDRC libraries:

Targeted gene	CG	Reference
nicotinic Acetylcholine Receptor $\alpha 7$ (nAChRa7)	CG32538	TRiP JF02570, Bloomington 27251
		VDRC KK100756
Insulin-like peptide 4 (Ilp4)	CG6736	TriP HMS02660 Bloomington 43288
		TriP HMS00547 Bloomington 33682
Insulin Receptor (InR)	CG18402	TRiP HMS03166 Bloomington 51518
		VDRC GD992
Trehalase	CG9364	TriP HMC03381 Bloomington 51810
Glucose transporter 1 (Glut1)	CG43946	TriP HMS02152 Bloomington 40904
		VDRC GD13326
Lactate dehydrogenase (LDH)	CG10160	VDRC KK110190
		TriP HMS00039 Bloomington 33640
Putative lactate dehydrogenase	CG13334	VDRC GD15007

All flies, obtained from libraries or received after the injection of transgenes, except flies from the TriP RNAi collection, were outcrossed for 5 generations to a reference strain carrying the w^{1118} mutation in an otherwise Canton Special (CS) genetic background. Because TriP RNAi transgenes are labeled by a $y+$ marker, these lines were outcrossed to a y^1w^{67c23} strain in an otherwise Canton S background.

We raised flies on a standard medium. Flies used for behavior were raised in a 12:12 light-dark cycle, at 18°C, with 60% of humidity. Crosses for imaging

experiments were raised at 25 °C to increase the expression level of genetically-encoded sensors through the UAS/GAL4 system.

For crosses used in behavior experiments, we selected between 80 and 110 females, and 30 males of the appropriate genotype, that we placed for mating in the same bottles. Parents were transferred to fresh medium twice a week. This allowed the flies of the progeny to grow under food abundance and to hatch regularly for being used in behavioral experiments. The progeny from a cross was gathered during maximum five weeks after the cross was launched.

II. Cloning

Cloning was performed using the Gateway Technology (Life Technologies), following the manufacturer's instructions. Briefly, a 990pb PCR fragment resulting from the amplification of the GMR GAL4 driver line 54H02-GAL4 was cloned into the pBPnlsLexA::p65Uw vector for 54H02-LexA (Pfeiffer et al., 2010). To amplify the 54H02 enhancer fragment, we used the following primers:

AttB1-54H02-Forward

5' *GGGGACAAGTTTGTACAAAAAAGCAGGCTGAACGATACAGTGCAGCTTCCGTGC* 3'

and AttB2-54H02-Reverse

5' *GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCAACGGACGTAGCGGAAGGGAG* 3'.

Gateway-specific sequences AttB1 and AttB2 are shown in italics letters, and 54H02 specific sequences are shown in normal letters.

For amplification, we used a high-fidelity polymerase Pfu Ultra II Fusion (Agilent Technologies) with proofreading activity. Vectors were checked by restriction. Transgenic fly strains were obtained by site-specific embryonic injection of the resulting vectors, which was outsourced to Rainbow Transgenic Flies (CA, USA). Insertion site was VK00027 (3rd chromosome).

III. Behavior

III. 1. Flies selection and preparation

For behavior experiments, one to three days-old flies were selected one to three days before the experiment. Each group of flies used for behavior contained about 40

to 80 flies. They were kept in clean bottles and at the appropriate temperature in incubators dedicated for behavior.

To restrict UAS/GAL4-mediated expression exclusively to the adult stage, we used the TARGET system (McGuire et al., 2003): GAL4 activity was inhibited at the 18 °C raising temperature by a thermosensitive version of GAL80 expressed ubiquitously under the control of the tubulin promoter tubulin-GAL80^{ts}. Flies were selected two to three days before the experiment and transferred to a 30.5°C, 60% humidity incubator until the conditioning in order to induce the expression of the RNAi. For control, non-induced experiments, flies were selected the day before the experiment and transferred to 18°C, 60% humidity incubator until the conditioning.

30 to 60 min before the experiment started, flies were transferred from the incubators to the experimental room, at 25°C, 80% humidity, in order to have the time to adapt to these new environmental conditions.

III. 2. Aversive olfactory conditioning

Odorants used for olfactory conditioning were 3-octanol (99% purity Sigma-Aldrich 218405) and 4-methylcyclohexanol (99% purity Sigma-Aldrich 153095) diluted in paraffin oil (VWR) at a final concentration of $2,79 \cdot 10^{-1} \text{ g.L}^{-1}$, as previously described. At these concentrations, both odorants are equally aversive to the flies.

We conditioned flies thanks to an automated aversive setup (Pascual and Preat, 2001). An apparatus called a barrel contained 6 holes covered with a copper grid (Figure 38). Thus, up to 6 groups of flies were placed in each barrel. 6 barrels could be used in each experiment, so 36 groups of flies could be conditioned in parallel. The copper grids were linked to a generator that automatically delivered electric shocks, and the barrel was connected to an air flow that allowed odor delivery as determined by a set of electro-valves. Valves and the generator were computer-controlled.

Different conditioning protocols were used. A single cycle of conditioning (Figure 39 A) consisted in the presentation of an air flux without odor during 90 s, then 1 min of the conditioned odor A (CS⁺) presentation during which 12 60-volt electric shocks were given to the flies, followed by 45 s of pause with neutral air flux, 1 min of odor B (CS⁻) presentation without any other stimulus, and 45 s of final pause with neutral air.

Massed training consisted in five consecutive training cycles, and spaced training consisted in five cycles spaced by 15-minute rest intervals.

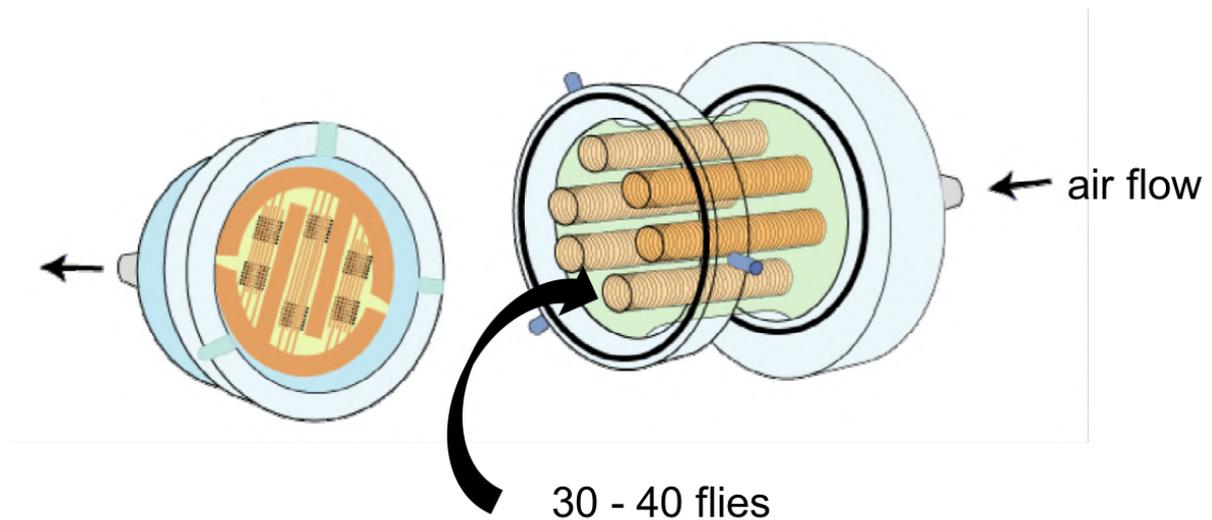


Figure 38. A barrel used for aversive conditioning. The copper grids are linked to a generator that automatically delivers electric shocks, and the barrel is connected to an air flow that allows simultaneous odor delivery.

Before the conditioning, each group of 40-80 flies were separated into two halves. The first half was conditioned with 3-octanol as odor A (CS⁺) and 4-methylcyclohexanol as odor B (CS⁻), while the other half was subsequently conditioned with 4-methylcyclohexanol as odor A and 3-octanol as odor B. This procedure allowed us to accurately measure memory scores independently of the biases in air flux carrying the two odors that could happen during conditioning, and, to a bigger extent, in the T-maze during testing.

In experiments using Shi^{ts}, we aimed at performing a sharp blockade after olfactory conditioning. Therefore, flies were conditioned at permissive temperature (25°) and placed in pre-warmed bottles at 33°C immediately after the end of the last training cycle. After spending the appropriate time period at 33°C (2, 6 or 14h), flies were then returned to an incubator set at the permissive temperature of 18°C. Permissive-temperature control experiments were performed at 18°C. The time course of the temperature shifts employed in an experiment are displayed above the graph of memory performance in each figure.

III. 3. Memory test

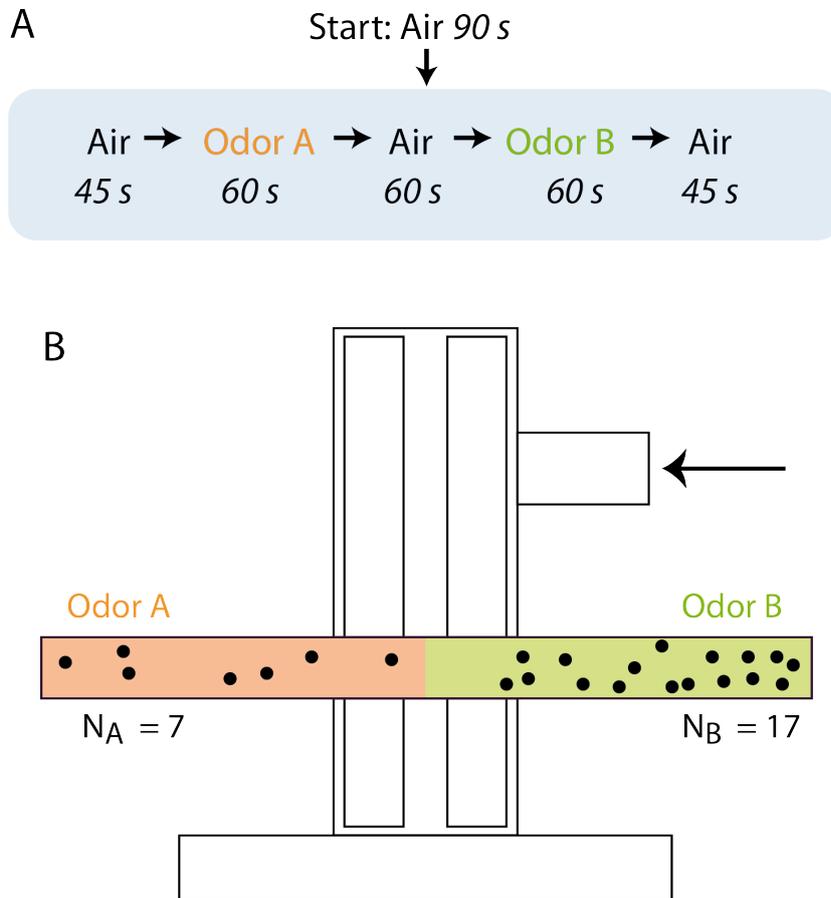


Figure 39. Conditioning and testing aversive memory. A. One cycle of aversive conditioning. Odor A is associated to electric shocks delivery; then, a control odor B is presented. B. Memory is tested in a T-maze, within which each arm is perfused with one of the two odors used during the conditioning. The arrow indicates the emplacement where flies are introduced in the T-maze, in an elevator that is put down during the test.

The memory test was performed by exposing the flies to both odors simultaneously in a T-maze as described before (Tully and Quinn, 1985) (Figure 39 B). Each arm of the maze was connected to a bottle in which one odor was diluted in mineral oil at the same concentrations as for the conditioning. Each arm thus delivered a distinct odor. Flies were given 1 min in darkness to choose between the two arms. Half of a memory score was calculated as the number of flies that avoided the conditioned odor minus the number of flies that went towards the conditioned odor, divided by the total number of flies in the experiment.

A single memory score value was the average of the performance from two groups of flies of the same genotype trained with either octanol or methylcyclohexanol as the conditioning stimulus: $memory\ score = \langle \frac{N_B - N_A}{N_B + N_A} \times 100 \rangle$. Therefore, it ranged from -100 (all flies going towards the conditioned odor) to 100 (all flies avoiding the conditioned odor).

In our experiments, flies were either tested 3 h after a single-cycle training, or 24 h after a massed or a spaced training.

III. 4. Olfactory acuity

Because the delivery of electric shocks can modify olfactory acuity (Preat, 1998), our olfactory avoidance tests were performed on flies that had been first presented another odor paired with electric shocks. Hence, flies were presented one odor – octanol or methylcyclohexanol – at the same concentration as during conditioning, associated to electric shocks in the conditioning barrels, and immediately tested in a T-maze for avoidance to the other odor. Each odor was tested for 1 min in darkness against its solvent (paraffin oil). The odor was delivered alternatively through the right or left arm of the maze. The olfactory avoidance was calculated as for the memory response test and then used as a score. An olfactory avoidance of 100 indicates complete behavioral repulsion.

III. 5. Shock response

The shock response tests were performed at 25°C, by placing flies in two connected barrels; electric shocks were provided in only one of the barrels. Flies were allowed to move freely between the two sides: one side with shocks, the other side without shock. After 1 min, flies were trapped in each barrel, collected and counted. The barrel where the electric shocks were delivered was switched between two groups. Shock avoidance was calculated as for the memory test and then used as a score. A shock avoidance of 100 indicates complete behavioral repulsion.

III. 6. Locomotion assay

Two- to three-day-old female flies were selected 24 h before the assay and placed in regular food vials at 18 °C. They were then conditioned with a spaced training or a

spaced unpaired protocol. In each experiment, 32 flies of each condition were assayed. Each fly was transferred to Trikinetics (www.trikinetics.com) glass tubes sealed with fly food on one side and a plug on the other side. Tubes were put in Trikinetics *Drosophila* Activity Monitors. These monitors were housed in a temperature-controlled incubator at 18 °C under constant light. Beam breaks were recorded over 1-min intervals. Activity scores were calculated by averaging the total number of beam breaks during a 1-h or 4-h period after conditioning.

III. 7. Feeding assays

Measurements of food intake (CAFE assay and dye-feeding assay) were conducted on flies of a single sex to minimize variability. We chose to work on female flies to match imaging experiments. Female flies were selected without anesthesia 24 h before the experiment and kept on plain food at 18 °C and 60% humidity, except when the experiment involves thermal induction of RNAi, in which case female flies were collected 2 days before the experiment and then kept at 30 °C on plain food.

III. 7. a. CAFÉ assay

Our CAFÉ assay was adapted from (Ja et al., 2014) (Figure 40). The experiment was performed in 2 ml microtubes. Three holes were drilled in the lid of the tube: two 0.5 mm- diameter holes for air exchange and one 2.0 mm-diameter hole in which a truncated 200 μ l pipette tip was inserted. The pipette tip was used to maintain a calibrated glass micropipette (5 μ l, catalogue no. 53432-706; VWR). The glass micropipette was filled with a 5% sucrose solution diluted in mineral water (Evian). A vegetal oil layer of about 0.3 ml was superposed to minimize evaporation. In addition, a piece of cotton soaked with 0.4 ml mineral water was placed in the bottom of each tube, in order to allow *ad libitum* drinking, and to minimize evaporation. Immediately after each conditioning protocol, single flies were transferred to the prepared microtubes; capillaries pre-filled with sucrose solution were introduced once all flies were transferred.

The initial length l_0 of sucrose solution was measured in each capillary. In all experiments, unoccupied microtubes were included as evaporation controls. Then, the microtubes were kept at 18 °C and 60% humidity. After the desired period of time t , the length of solution remaining in each capillary l_t was measured. The length of

sucrose solution consumed by each fly was calculated as $l_0 - l_t - l_e$, where l_e was the average length of solution that had evaporated in unoccupied control tubes. A single experiment typically included eight flies per genotype and per condition. The indicated 'n' is the number of single flies measured for each condition.

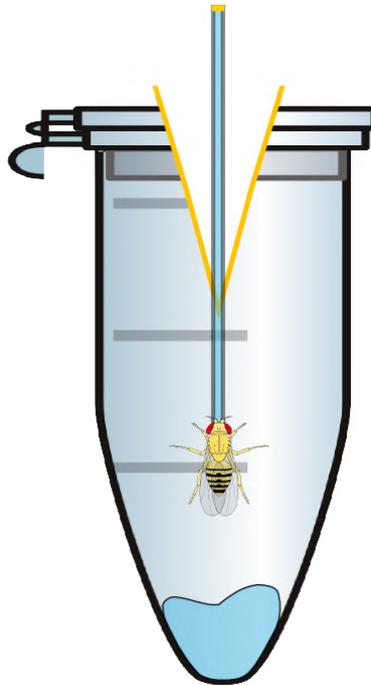


Figure 40. Schematic of the CAFÉ assay. The fly could walk on the capillary and reach the sucrose solution that it contains. The cotton soaked with water in the bottom of the microtube and the oil layer on top of the capillary prevented an excessive evaporation. Food consumption could be evaluated over time by measuring the length of food present in the capillary.

III. 7. b. Sulforhodamine B experiments

For the colorimetric experiments, we adapted a previously described protocol (Riemensperger et al., 2011) (Figure 41). After conditioning, flies were collected in bottles containing a cotton soaked with 3mL of a 5% sucrose – 100mM sulforhodamine B solution in mineral water (Evian). Flies were then kept at 18°C, 70% humidity. After 1 h, they were transferred to a 2 mL microtube and frozen in liquid nitrogen. Groups of 5 flies were grinded in 1.5 mL microtubes with 200 μ L PBS. Microtubes were then centrifuged at 12000 rpm for 7 minutes. Supernatants were collected and centrifuged again for 7 minutes at 12000 rpm. Then, 100 μ l of each

supernatant was transferred to a 96-wells plate, and absorbance was measured at 570 nm. As a blank absorbance control, naive flies placed on a 5% sucrose solution without dye were treated in parallel. The mean absorbance of these controls was subtracted from the absorbance of the other samples. A single experiment typically included 3–4 groups of five flies per condition. The complete dataset for a given associative protocol was normalized to the control unpaired protocol.

For measurements on naive flies, naive flies of the indicated genotype were placed at 30 °C for 2 days to induce RNAi expression in relevant groups, or kept at 18 °C for 2 days (non-induced flies). Sucrose intake was then measured for 1 h with the dye-feeding assay. Sucrose intake was normalized to the non-induced flies of the same genotype, to cancel inter-genotype variations that were important in this assay. The indicated 'n' is the number of groups of 5 flies measured for each condition.

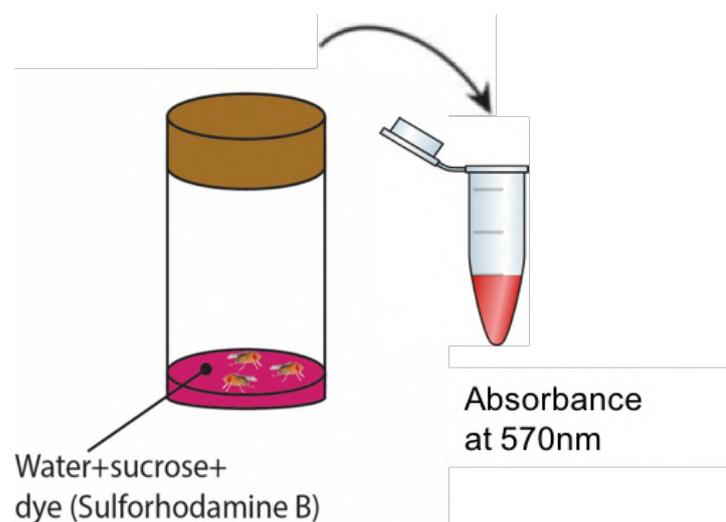


Figure 41. Schematic of the colorimetric Sulforhodamine B feeding assay. The colored food is absorbed by the flies and its amount can be evaluated by grinding flies and measuring the absorbance of the supernatant at the proper wavelength.

IV. *In vivo* imaging

For *in vivo* imaging experiments, female flies were preferred because their bigger size made surgery easier, as in all previous imaging work from our lab. Flies were kept

alive and selected without anesthesia, then glued on a plastic coverslip (Figure 42) thanks to a non-toxic dental glue (Protemp II 3M ESPE). The plastic coverslip was fixed to an imaging cell and 90 μ L of saline solution was added on top of the plastic located above the head. The plastic coverslip was then cut to allow access to the fly head, and the cuticle above the head was gently removed from the area of interest. Finally, trachea and fat were pushed aside from the imaging field, and a fresh drop of saline was added above the head.

For calcium imaging, we used the following saline solution that was used over multiple experiments in the laboratory (Bouzaiane et al., 2015; Musso et al., 2015):

NaCl	130 mM	(ref Sigma S9625)
KCl	5mM	(ref Sigma P3911)
MgCl ₂	2mM	(ref. Sigma M9272)
CaCl ₂	2mM	(ref. Sigma C3881)
Sucrose	36 mM	(ref. Sigma S9378)
Hepes-hemisodium salt	5 mM	(ref.Sigma H7637)

The experiments involving the glucose sensor, which are the most recent, were performed using a slightly modified solution in which the 36mM sucrose was replaced with D-Glucose 5 mM (ref. Sigma G8270), D-Trehalose 20 mM (ref. Sigma T9531) and Sucrose 10 mM. This modification was set in the laboratory, independently of the sensor used, to avoid the criticism that metabolizable sugars would not be available for the brain during *in vivo* imaging experiments.

For nicotine experiments, nicotine was freshly diluted from a commercial liquid (Sigma aldrich, N3876) into the saline used for imaging on each experimental day. A perfusion setup at a flux of 2.5mL/min allowed for the time-restricted application of 50 μ M nicotine on top of the brain. Baseline recording was performed during 1 min, then saline supply was switched to drug supply. The solution reached the *in vivo* preparation within 30 seconds. The stimulation was maintained for 30 seconds, before switching back the saline perfusion for an additional 5 min.

For metabolic experiments, Validamycin A (Sigma 32347), a trehalase competitive inhibitor, was directly diluted into saline, at a final concentration of 40 mM, aliquoted and stored at -20°C. A freshly unfrozen aliquot was used for every new fly. After 1 min of baseline acquisition, 10 μ L of the solution was added to the 90 μ L saline droplet on top of the brain, bringing Validamycin to a final concentration of 4 mM. The signal was further acquired for 12 min.

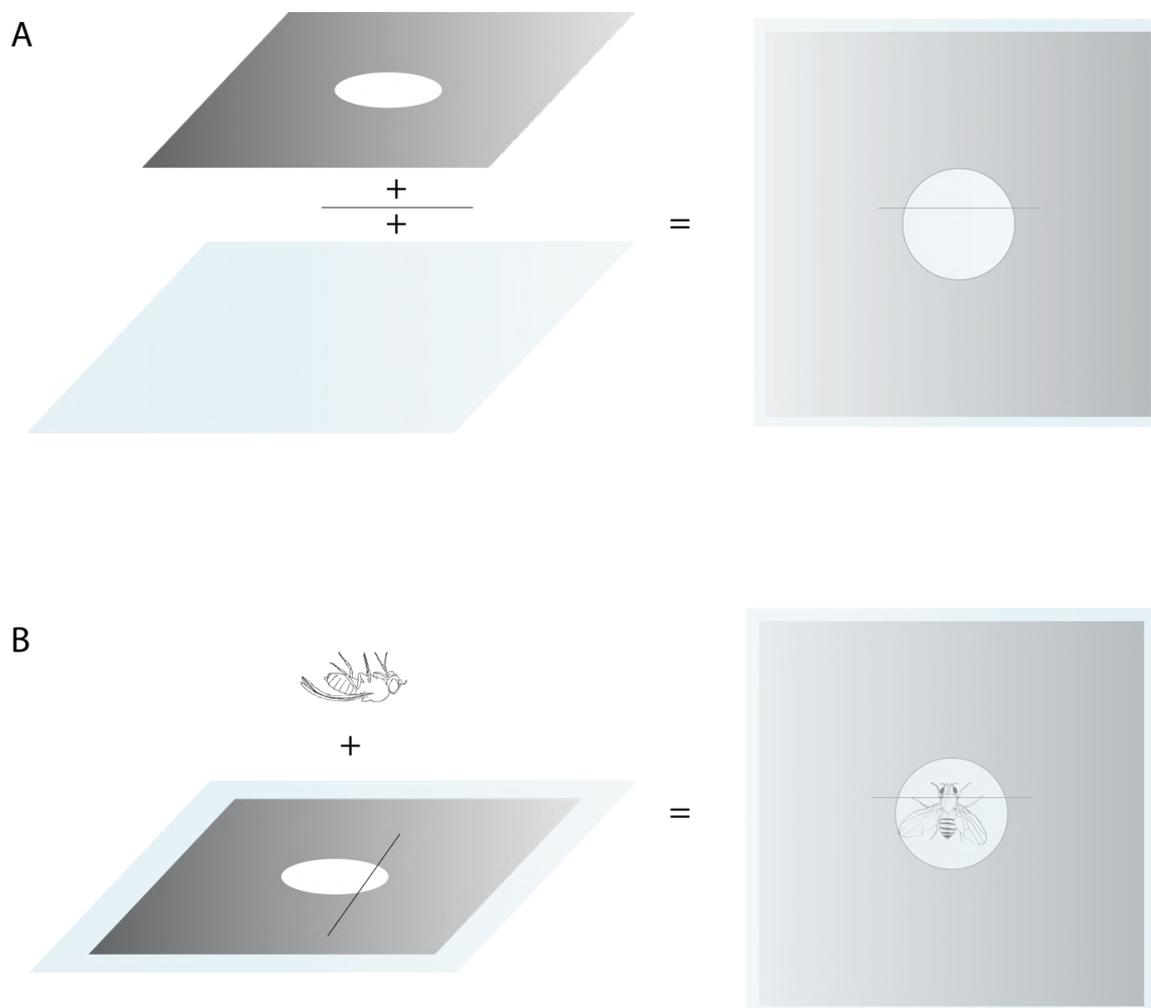


Figure 42. Procedure of flies gluing for *in vivo* imaging. A. A coverslip is prepared by gluing an upper thick plastic coverslip holed in its center, a thin metal stick in between, and a lower thin layer of plastic sheet. B. A drosophila is handled by its leg and glued on the plastic coverslip with its neck at the level of the metal stick. This allows the correct dissection of living flies, and subsequent brain imaging.

V. Immunohistochemistry

Anti-IIP4 antibody was obtained from Linda Partridge and Susan Broughton, anti-InR antibody from Jaakko Mattila and Oscar Puig, and anti-VACHT from Toshihiro Kitamoto.

Before dissection, whole female flies of F1 progenies (3–4 days after eclosion at 25 °C) were fixed in 4% formaldehyde in PBST (PBS containing 1% Triton X-100) at 4 °C overnight. Brains were dissected in PBST solution and rinsed three times for 20 min in PBST, blocked with 2% bovine serum albumin in PBST for 2 h and incubated with primary antibodies at 1:400 (rabbit anti-GFP (catalogue number: A11122), Invitrogen Molecular Probes), 1:1000 (rabbit anti-InR), 1:200 (rabbit anti-IIP4) and 1:100 (mouse anti-nc82, DSHB, catalogue reference: nc82) in the blocking solution at 4 °C overnight.

After rinsing, brains were incubated with secondary antibodies at 1:400 (anti-rabbit conjugated to Alexa Fluor 488 (A11034), anti-mouse conjugated to Alexa Fluor 594 or 647 (A11005 and A21245, Invitrogen Molecular Probes) in the PBST-BSA 2% solution for 3 h at room temperature. After rinsing, brains were mounted in Prolong Mounting Medium (Lifetechnology) for imaging. Images were acquired with a Nikon A1R confocal microscope. Confocal Z-stacks were imported into NIH Image J for analyses.

VI. Quantification and statistical analyses

For behavior experiments, 2 groups of about 20-60 flies were conditioned and tested for the two odors used in this study reciprocally. From these two groups the memory score was calculated as previously described, which represents an experimental replicate (Pascual and Preat, 2001). For imaging experiments, one replicate corresponds to one fly brain. All data are presented as mean \pm SEM.

Comparisons of the data series between two conditions were achieved by a two-tailed unpaired t test. Comparisons between more than two distinct groups were

made using a one-way ANOVA, followed by Newman-Keuls pairwise comparisons between the experimental groups and their controls. ANOVA results are presented as the value of the Fisher distribution $F(x,y)$ obtained from the data, where x is the number of degrees of freedom between groups and y is the total number of degrees of freedom for the distribution. Statistical tests were performed using the GraphPad Prism 7 software. In the figures, asterisks illustrate the significance level of the t test, or of the least significant pairwise comparison following an ANOVA, with the following nomenclature: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant, $p > 0.05$.

I. An increase in mushroom body metabolism is instructive for long-term memory

As it was already shown in the literature that long-term memory formation decreases the flies resistance to starvation (Mery and Kawecki, 2005) and that long-term memory is no longer formed in starved flies (Plaçais and Preat, 2013), we aimed at evaluating the energetic cost associated with long-term memory formation in flies.

For this purpose, I developed in our team two already published food consumption assays: the CAFÉ assay (Ja et al., 2014) and a colorimetric food consumption assay (Riemensperger et al., 2011). I could measure that food intake dramatically increased during the first hours after long-term memory conditioning (article, Figure 1). I additionally showed that locomotor activity was not differentially affected by a spaced conditioning that allows long-term memory formation, as compared to an unpaired conditioning. We further evaluated whether energy metabolism was modulated in the mushroom body in conditioned flies, and showed that pyruvate consumption was indeed increased specifically in Kenyon cells axons and synapses early during long-term memory consolidation. As long-term memory formation was facilitated when artificially increasing pyruvate catabolism in Kenyon cells, I assessed how this manipulation affected food consumption in naïve flies. Strikingly, I showed that only increasing or decreasing Kenyon cells energy metabolism consistently influenced the flies' food consumption (article, Figure 4F and G). Finally, as the dopaminergic receptor DAMB was necessary in Kenyon cells for the increased pyruvate metabolism, I evaluated food consumption of flies expressing DAMB receptor RNAi in Kenyon cells, and measured no increase in food consumption after a spaced conditioning as compared to controls (article, Figure 6E). Interestingly, several mutant lines unable to form LTM still displayed an increased food consumption after training (article, Figure 6F), suggesting that the mutant genes act downstream of dopaminergic signaling in Kenyon cells for memory formation. This work was published in 2017 in Nature communication, in which I figure as second author.

ARTICLE

Received 31 Jul 2016 | Accepted 4 Apr 2017 | Published 5 Jun 2017

DOI: 10.1038/ncomms15510

OPEN

Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory

Pierre-Yves Plaçais¹, Éloïse de Tredern^{1,*}, Lisa Scheunemann^{1,*}, Séverine Trannoy^{1,†}, Valérie Goguel¹, Kyung-An Han², Guillaume Isabel^{1,†} & Thomas Preat¹

Efficient energy use has constrained the evolution of nervous systems. However, it is unresolved whether energy metabolism may resultantly regulate major brain functions. Our observation that *Drosophila* flies double their sucrose intake at an early stage of long-term memory formation initiated the investigation of how energy metabolism intervenes in this process. Cellular-resolution imaging of energy metabolism reveals a concurrent elevation of energy consumption in neurons of the mushroom body, the fly's major memory centre. Strikingly, upregulation of mushroom body energy flux is both necessary and sufficient to drive long-term memory formation. This effect is triggered by a specific pair of dopaminergic neurons afferent to the mushroom bodies, via the D5-like DAMB dopamine receptor. Hence, dopamine signalling mediates an energy switch in the mushroom body that controls long-term memory encoding. Our data thus point to an instructional role for energy flux in the execution of demanding higher brain functions.

¹Genes and Dynamics of Memory Systems, Brain Plasticity Unit, CNRS, ESPCI Paris, PSL Research University, 10 rue Vauquelin, Paris 75005, France.

²Department of Biological Sciences, Border Biomedical Research Center, University of Texas at El Paso, El Paso, Texas 79968, USA. † Present addresses: Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115, USA (S.T.); Research Center on Animal Cognition, Université Paul Sabatier, Toulouse, France (G.I.). * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to P.-Y.P. (email: pierre-yves.placais@espci.fr) or to T.P. (email: thomas.preat@espci.fr).

Energy fluxes, that is, the sequential catabolism of energy-carrying molecules, involve substrates that are extremely conserved across the animal kingdom, even more so than genes or signalling pathways. This is especially striking for central nervous systems, which use glucose as their main energy source¹. Energy efficiency is indeed put forward as a major factor of the selective pressure driving the evolution of nervous systems². Moreover, efficient power use is stated as a design principle of neuronal network architecture³; this underlies for example the widespread occurrence of sparse coding in sensory systems^{3–5}. Beyond network architecture, one pertinent question is how energy fluxes intervene in brain function. The predominant view in the field of neuroenergetics is that energy is supplied ‘on demand’ to neurons in support of their activity⁶. However, some data also suggest active regulation of brain function, especially memory, by glucose⁷. Yet, our knowledge about the interplay between energy metabolism and memory formation at the molecular and cellular levels remains very limited. Several studies documented the importance of astrocytic lactate production for memory^{8,9}, especially long-term memory¹⁰, but there has been much evidence as well that lactate may have signalling roles aside from being an energy substrate^{11–15}. Hence, the major question remains open whether the magnitude of energy flux could be informative for neurons, wherein it would control and not only support memory processes or other brain functions.

Drosophila is a genetically tractable organism, and as such it is a powerful animal model to address these questions. Flies can form olfactory memory as a result of the association between an odorant and electric shocks. Experiencing a single cycle of olfactory training is not sufficient for the flies to form long-lasting memory (Fig. 1a). A repeated massed training protocol yields a 24 h-memory called anaesthesia-resistant memory (ARM), which does not rely on *de novo* protein synthesis¹⁶. The most stable long-term memory (LTM), which is protein synthesis-dependent, requires rest intervals between the multiple associations (spaced training)¹⁶. Therefore, the LTM formation does not simply result from accumulating knowledge through repeated trials, but rather relies on specific mechanisms that are triggered upon the particular temporal pattern of spaced training¹⁷. We previously showed that the trigger for LTM formation involves rhythmic signalling on mushroom body (MB) neurons from specific dopaminergic neurons, which occurs during and immediately after spaced training¹⁸. The MBs are the major integrative brain center that supports learning and memory in insects, and a putative functional homologue of the mammalian hippocampus^{19,20}. *Drosophila* MBs are paired structures including ~2,000 intrinsic neurons per brain hemisphere. These neurons receive dendritic input from the antennal lobes through projection neurons²¹ in the calyx area on the posterior part of the brain. Their axons form a fascicle that traverses the brain to the anterior part, where they branch to form horizontal and vertical lobes according to three major branching patterns²². MB lobes are covered by compartmentalized dopaminergic innervation from 20 cell types, subsets of which provide reinforcement signalling during olfactory conditioning^{23–25}, through the dopR/dDA1 receptor^{26,27}. MB neurons can therefore detect the coincident onset of olfactory and electric shock stimuli^{28,29}.

We previously showed that LTM formation is blocked when flies are starved before and after training, which is beneficial to their survival to food restriction³⁰. This prompted us to investigate the interplay between LTM formation and energy metabolism in *Drosophila*, through a combination of behavioral experiments involving genetic knock-down or neural circuit manipulation in adult flies, *in vivo* imaging of pyruvate metabolism at the cellular level, and feeding assays. Our results

establish that following spaced training, flies strongly increase their energy intake. This reflects an elevation of energy flux in MB neurons, which we show is a necessary, but also a sufficient condition to consolidate memory into LTM. This upregulation is initiated by dopamine signalling through a specific receptor, the D5-like DAMB receptor. Dopamine signalling thus mediates an energy-based gating that is instructive to initiate LTM formation in the *Drosophila* MB.

Results

Flies eat more sucrose upon LTM formation. First, we sought to evaluate whether LTM formation is a costly process, and if so, to what extent. Flies were subjected to a spaced training protocol, and their subsequent intake of a sucrose solution was measured using two distinct methods: a dye-feeding assay and a Capillary Feeding (CAFE) assay (see Experimental Procedures for details). Immediately after spaced training, flies consumed twice as much sucrose solution as control flies that had undergone a non-associative unpaired protocol (Fig. 1b,c). This effect did not occur following the massed training protocol (Fig. 1b,c). The CAFE assay additionally demonstrated that the increased feeding behaviour specifically was observed only during the 4 h following spaced training (Fig. 1c). Flies had direct and *ad libitum* access to pure mineral water in this assay, suggesting that their increased feeding behaviour after spaced training results from a search to increase their energy intake, and not from elevated thirst. To confirm this, we conducted a control dye-feeding assay without sucrose. In this case, there was no difference in dye ingestion between flies that underwent a spaced training or an unpaired protocol (Fig. 1d). Finally, we asked if the increased feeding could be the result of higher locomotor activity. We monitored the locomotor activity of flies for 4 h after either of these two protocols (see Experimental Procedures for details). During the first hour, and also during the whole period of measurement, we observed no difference between the two conditions (Fig. 1e), which indicates that the increased energy intake after spaced training does not stem from an elevated energy demand due to increased activity, but is actually due to LTM formation itself. This series of experiments, performed on flies with unlimited access to food before conditioning, strongly suggests that spaced training has a dramatic impact on the energy budget of the whole fly. Surprisingly, regulation of energy metabolism starts at an early stage in LTM formation.

MB energy metabolism is increased upon LTM formation. To confirm the impact of LTM formation on energy balance, we aimed to detect whether an upregulation in energy consumption actually occurred in the fly brain following spaced training, and more specifically in MB neurons, which play a prominent role in olfactory LTM^{31–33}. *In vivo* imaging of energy metabolism with cellular resolution has become possible only recently, thanks to the development of genetically-encoded fluorescent indicators that are sensitive to the intracellular concentrations of various energy metabolites³⁴. Both glucose and lactate can act as neuronal energy sources, but in either case the energy-producing pathways converge to yield pyruvate, the input energy substrate for oxidative phosphorylation in mitochondria. We introduced the pyruvate sensor Pyronic³⁵ into *Drosophila*, which was expressed in MB neurons using the UAS/GAL4 system, by means of the VT30559 GAL4 driver, which is very specific to MB neurons within the central brain (Supplementary Fig. 1A, Supplementary Movies 1 and 2). The Pyronic ratio (see Experimental Procedures), which increases with pyruvate concentration, was recorded by two-photon imaging from the MB vertical lobes, an established site of LTM encoding^{31,32} (Fig. 2a). Although FRET

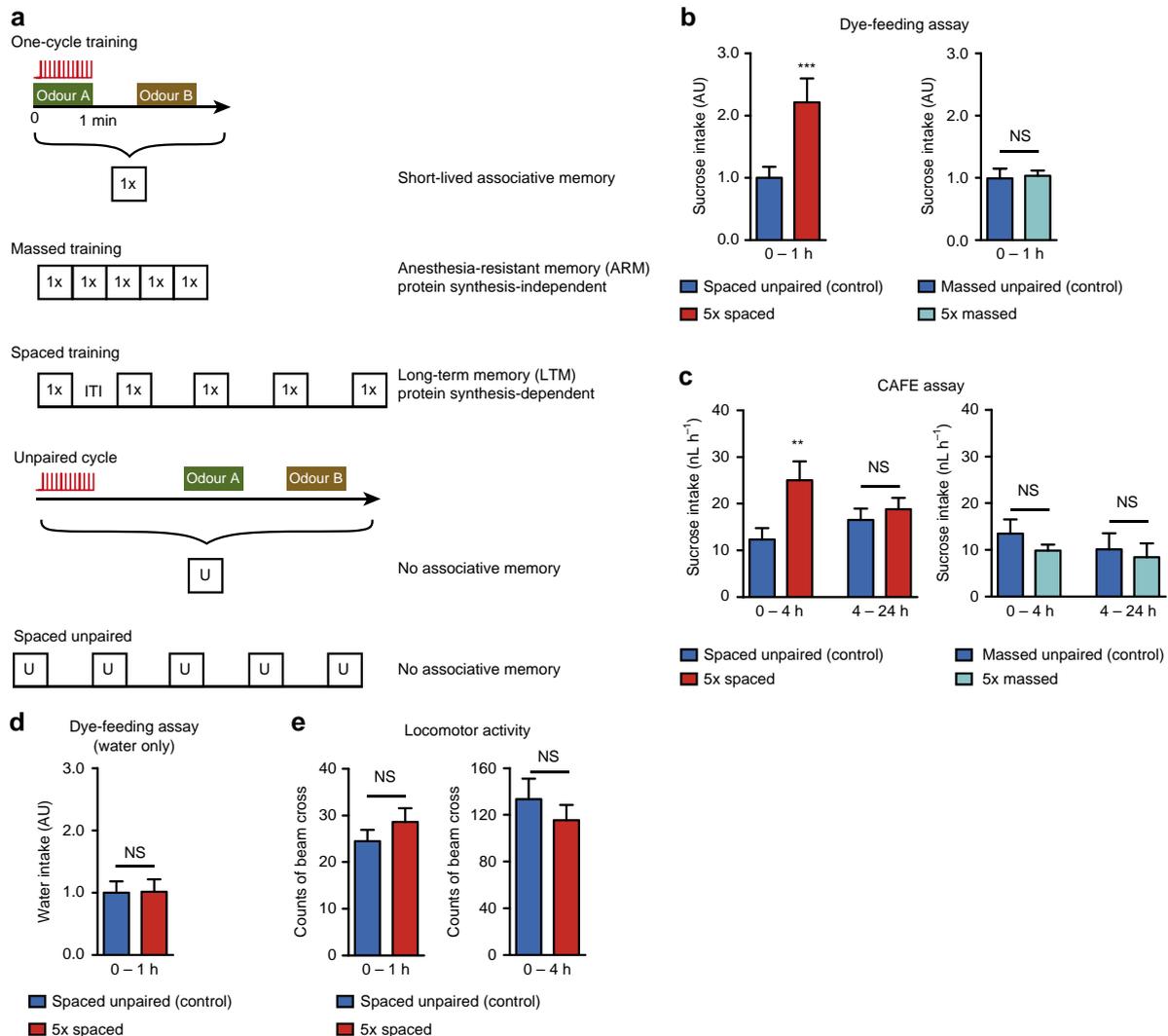


Figure 1 | Spaced training causes increased sucrose intake. (a) Diagram illustrating the different olfactory aversive conditioning protocols used in this study (ITI: inter-trial intervals, 15-min duration). For the so-called massed unpaired control (b,c), a reduced ITI of 2 min was applied to avoid association between shocks and the odour B of the previous cycle. (b) Flies were transferred onto a dyed 5% sucrose solution for 1 h immediately after the indicated conditioning procedure. Following a spaced associative training, flies ingested twice as much sucrose as the non-associative spaced unpaired control ($n = 17$ groups of five flies; $t_{32} = 4.18$; $P = 0.0002$). This was not the case following a massed training ($n = 12$; $t_{22} = 0.19$; $P = 0.85$). (c) Sucrose intake by single flies was measured 4 and 24 h after the indicated conditioning procedure in a CAFE assay. During the first 4 h (but not after) flies ingested twice as much sucrose after their spaced training as after the unpaired control protocol (0–4 h: $n = 111$ –112; $t_{221} = 2.69$; $P = 0.007$. 4–24 h: $n = 110$ –112; $t_{220} = 0.65$; $P = 0.51$). No difference was observed at any time point following massed protocols (0–4 h: $n = 32$; $t_{62} = 1.11$; $P = 0.27$, 4–24 h: $n = 31$ –32; $t_{61} = 0.37$; $P = 0.71$). (d) In a dye-feeding assay without sucrose, there were no difference between flies of the spaced and unpaired conditions ($n = 16$ groups of 5 flies; $t_{30} = 0.058$; $P = 0.95$). (e) The locomotor activity of flies was measured as the number of beam crosses in a Trikinetics device during 4 h following conditioning. Results are displayed for the first hour after conditioning, as a control for the dye-feeding assay or for the whole period, as a control for the CAFE assay. In both cases, there was no difference between flies that had a spaced training or an unpaired control protocol ($n = 130$ –131; 0–1 h: $t_{253} = 1.08$; $P = 0.28$; 0–4 h: $t_{253} = 0.87$; $P = 0.39$).

sensors are sensitive to the intracellular concentration, what is physiologically significant is the energy flux. Hence the relevant measurement is the rate of pyruvate consumption or production, rather than its concentration. Inspired by a previously published protocol³⁵, we used sodium azide (a potent inhibitor of mitochondrial complex IV) to block pyruvate mitochondrial uptake and measure the resulting pyruvate accumulation. In naive flies, the addition of 5 mM azide to the solution bathing the brain during live imaging induced a strong and rapid increase in the Pyronic ratio (Fig. 2b, Supplementary Fig. 1B), until saturation of the sensor. We performed similar experiments in flies that had been subjected to different conditioning protocols, in a time

window (30 min to 2.5 h after the end of conditioning) when flies display increased sucrose intake after spaced training (see Fig. 1c). Compared to an unpaired spaced protocol or to a massed training, the spaced training resulted in a marked enhancement of pyruvate consumption in MB neurons (Fig. 2c,d, Supplementary Fig. 1C). After unpaired or massed protocols, pyruvate consumption rates were similar to those measured in naive flies.

On cultured mammalian astrocytes, it was shown that treatment with rotenone, another inhibitor of oxidative phosphorylation, causes an activation of glycolysis in reaction to mitochondrial blocking³⁶. Thus, the pyruvate accumulation following azide

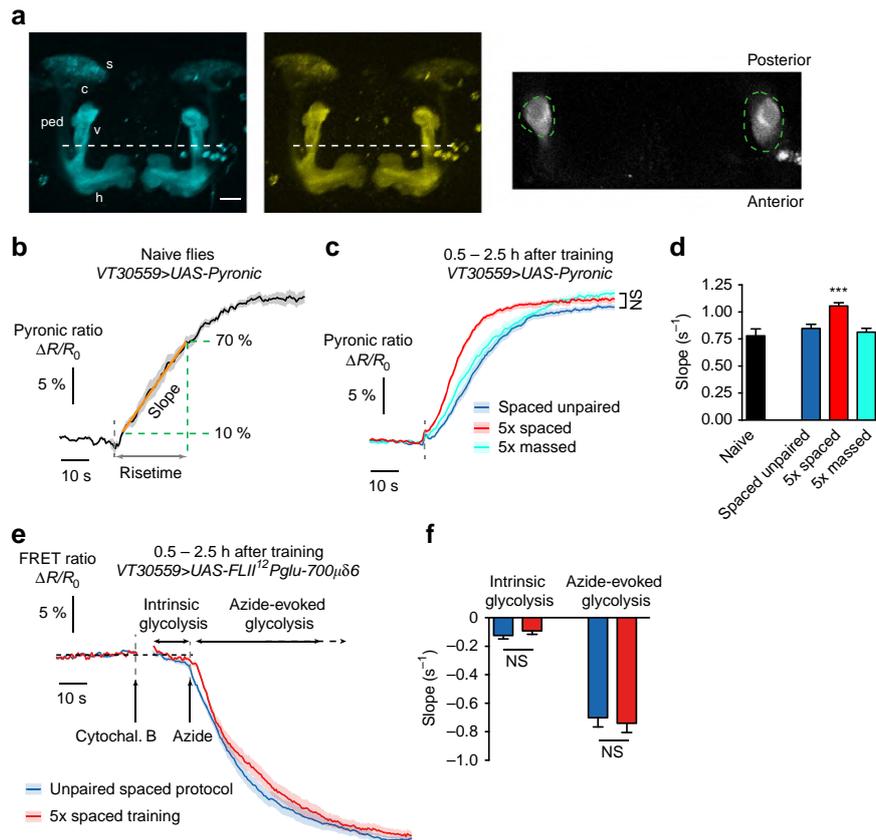


Figure 2 | The energy flux in MB neurons is increased following spaced training. (a) Images of the Pyronic sensor expressed in MB neurons through the VT30559 driver, obtained by 2-photon microscopy. The two images on the left show the 3D reconstruction of image stacks in the mTFP and Venus channels in the conditions that were used for live recordings (scale bar: 20 μ m). MB neurons have their soma (s) in the posterior part of the brain; their dendrites form the calyx (c), and axons are bundled into the peduncle (ped) that traverses the brain posteriorly to anteriorly, where axons ramify into vertical (v) and horizontal (h) lobes. Live recordings presented thereafter were obtained from horizontal planes located halfway to the top of the vertical lobes (dashed white line). An illustrative example is shown on the right, with the corresponding regions of interest (green dashed lines). (b) Application of 5 mM sodium azide (grey dashed line) results in an increased Pyronic ratio in the MB vertical lobes of naive flies ($n = 6$). The rise was linear up to 70% of the plateau. The slope and risetime were used to quantify the kinetics of pyruvate accumulation during this linear phase (see Experimental Procedures). (c) Measurement of pyruvate accumulation with the same procedure after different conditioning protocols (unpaired: $n = 22$; 5 \times spaced $n = 26$; 5x massed $n = 16$). There was no difference in the plateau value between the three conditions ($F_{2,121} = 3.05$, $P = 0.051$). (d) Average slope from data shown in b,c. The slope after spaced training was higher than after a spaced unpaired protocol or after massed training ($F_{2,121} = 15.37$, $P < 0.0001$). (e) The FRET glucose sensor FLII¹²Pglu-700 μ δ 6 was expressed in MB neurons to monitor the azide-induced activation of glycolysis in flies that had undergone spaced training or unpaired control protocol ($n = 15$ per condition). Intrinsic glycolysis was measured following the application 20 μ M cytochalasin B, a pharmacological blocker of glucose transporter (see Experimental Procedures for details). A subsequent application of 5 mM azide strongly enhanced glucose consumption. (f) There was no difference between flies of the two conditions either for intrinsic glycolysis rate ($t_{56} = 0.99$; $P = 0.32$) or for azide-evoked glycolysis ($t_{56} = 0.43$; $P = 0.67$).

treatment that we measured in MB neurons may originate not only from the stop of mitochondrial uptake, but also from the increase of pyruvate production by glycolysis. To clarify the interpretation of our Pyronic data, we expressed a FRET glucose sensor FLII¹²Pglu-700 μ δ 6 in MB neurons to monitor the effect of azide treatment on glycolysis, in a protocol similar to ref. 36. We observed that azide treatment indeed instantly boosted glucose consumption (Fig. 2e). Interestingly, comparing flies after spaced training or after unpaired protocol, we observed that both intrinsic and azide-evoked glycolytic rates were similar between the two conditions (Fig. 2e,f). This indicates that the accelerated azide-evoked pyruvate accumulation observed in flies after spaced training does not come from an upregulated glycolytic capacity, but from an increased pyruvate production from another source, that is not any more consumed by mitochondria. Altogether, this series of experiments points to an upregulation of mitochondrial flux in MB neurons shortly after spaced training.

Energy metabolism is not globally upregulated. The upregulation of energy metabolism that we evidenced in MB neurons could be part of a more global shift in brain energy consumption. To address this point, we measured the rate of pyruvate accumulation induced by azide in other brain structures. We expressed the Pyronic sensor through the *Feb170* driver, which was used previously to target the ellipsoid body (EB)³⁷, and which also labels a cluster of median neurosecretory cells (mNSC) in the pars intercerebralis (Fig. 3a). In these two structures, azide application resulted in an accumulation of pyruvate (Fig. 3b). Noticeably, the plateau value reached by the Pyronic ratio was consistently lower in EB neurons than in mNSC (Fig. 3b) (see Discussion). Interestingly, in both structures, the rate of pyruvate accumulation after spaced training or after an unpaired protocol was similar (Fig. 3c–e). This shows that energy metabolism is not upregulated in the whole brain upon LTM formation, and that it might be an MB-specific phenomenon.

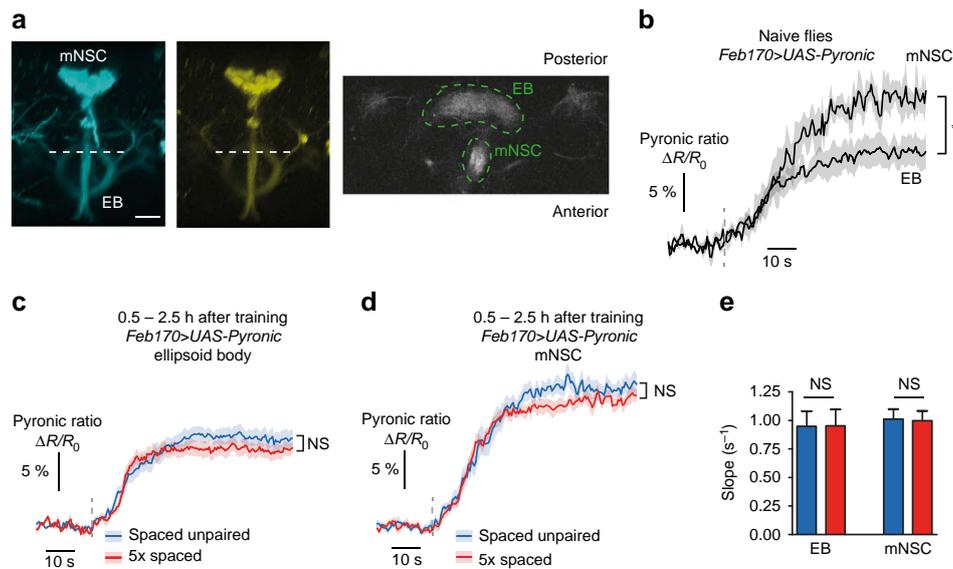


Figure 3 | The energy flux in ellipsoid body neurons or in median neurosecretory cells is not increased following spaced training. (a) Images of the Pyronic sensor expressed through the *Feb170* driver, obtained by two-photon microscopy. The two images on the left show the 3D reconstruction of image stacks in the mTFP and Venus channels in the conditions that were used for live recordings (scale bar, 20 μm). This driver is commonly used to label ellipsoid body (EB) neurons, but it also strongly labels a cluster of ~ 20 median neurosecretory cells (mNSC). Live recordings were obtained from a horizontal plane where both the upper part of the EB and a section of the descending branch from mNSC were visible (dashed white line). An illustrative example is shown on the right, with the corresponding regions of interest (green dashed lines). (b) Application of 5 mM sodium azide (grey dashed line) caused an increase in the Pyronic ratio in the EB and the mNSC ($n=5$). The plateau value was significantly lower in the EB than in the mNSC ($t_9=2.62$; $P=0.028$). (c) Measurement of pyruvate accumulation in EB neurons following azide application after spaced training or control unpaired protocol. The plateau value was not statistically different between the two conditions ($n=12-13$; $t_{23}=1.47$; $P=0.15$). (d) Measurement of pyruvate accumulation in mNSC following azide application after spaced training or control unpaired protocol. The plateau value was not statistically different between the two conditions ($n=13$; $t_{24}=1.37$; $P=0.18$). (e) Average slope from data shown in c,d. The slope after spaced training was not different from after a spaced unpaired protocol in either structure (EB: $t_{23}=0.06$; $P=0.96$; mNSC: $t_{24}=0.12$; $P=0.90$).

Increased energy flux in MB is sufficient for LTM formation.

The mitochondrial pyruvate dehydrogenase (PDH) complex catalyses the first step of pyruvate metabolism for oxidative phosphorylation. The activity of the E1 enzyme of this complex (PDHE1) is regulated, being activated by the pyruvate dehydrogenase phosphatase (PDP)^{38,39}, and inhibited by phosphorylation through pyruvate dehydrogenase kinase (PDK; Fig. 4a)³⁹. To investigate whether enhanced energy flux actually underlies LTM formation, we aimed to manipulate the level of mitochondrial activity in MB neurons. We expressed exclusively at the adult stage (see Experimental Procedures) RNAi against the β subunit of PDHE1 (PDHE1 β), PDP or PDK in MB neurons together with the Pyronic sensor. Following azide application, the rate of pyruvate accumulation was decreased in naive flies expressing either PDHE1 β or PDP RNAi compared to control flies, and was conversely increased in flies expressing PDK RNAi (Fig. 4b). The plateau value of the Pyronic ratio was also lower with PDHE1 and PDP RNAi (Fig. 4b), betraying higher steady-state pyruvate level (Supplementary Fig. 2A), probably because glycolysis was more activated in these flies as a result of the reduction of mitochondrial activity. These results are thus consistent with PDHE1 and PDP knockdown powering down mitochondrial flux, and on the contrary PDK knockdown boosting it.

Interestingly, the expression of RNAi against PDHE1 β or PDP in MB neurons at adult stage impaired LTM formation following spaced training (Fig. 4c,d), without altering the naive odour or electric shock avoidance (Supplementary Table 1). No defect was observed in the absence of RNAi induction (Supplementary Fig. 2B,C). Importantly, the memory induced by massed training was unaffected by the expression of either RNAi (Fig. 4c,d).

Hence, spaced training specifically triggers an increase in MB energy consumption, which is pivotal for LTM formation.

We next asked whether the increased energy consumption could act as a trigger for LTM encoding. If so, we reasoned that forced enhancement of MB energy flux should facilitate the consolidation of the learned experience into LTM. On the contrary, it should not facilitate LTM formation if the increased energy flux is only supportive of LTM but does not constitute a signal that triggers the LTM pathway. LTM formation normally requires at least five spaced cycles¹⁶⁻¹⁸. We trained flies with only two spaced cycles of associative conditioning and measured the memory score 24 h later. Flies that expressed RNAi against PDK in MB neurons at the adult stage exhibited higher memory than their controls (Fig. 4e, Supplementary Fig. 2D), but normal odour or shock avoidance (Supplementary Table 1). Moreover, the memory formed by these flies was sensitive to the ingestion of cycloheximide (CXM), a protein synthesis inhibitor, before conditioning, which is a hallmark of LTM formation (Fig. 4e). These results demonstrate that increased energy metabolism in MB neurons is not only necessary, but also sufficient for LTM formation. The expression of PDK RNAi had no effect on memory formed after massed training (Supplementary Fig. 2E), highlighting that the facilitative effect is specific to LTM. It had no effect either on the LTM score after regular five spaced cycles-training (Supplementary Fig. 2E), which is consistent with the increased energy metabolism having a gating effect at an early stage of LTM process (see Discussion).

Finally, we wondered if manipulating the level of mitochondrial activity in MB neurons had an effect on the feeding behaviour. In the dye-feeding assay, naive flies expressing the RNAi against PDHE1 β , which downregulates mitochondrial

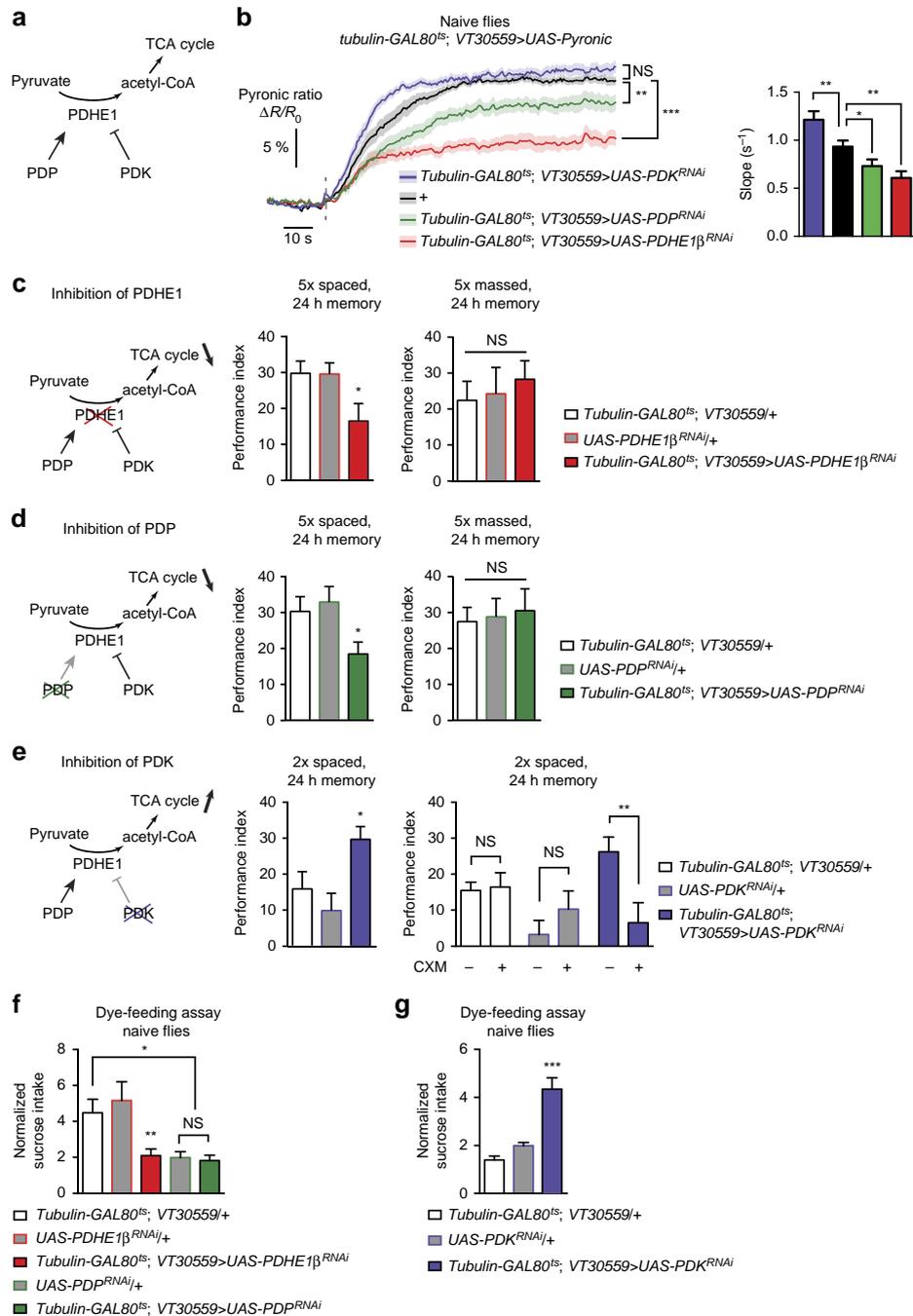


Figure 4 | Enhanced energy flux in MB neurons following spaced training is necessary and sufficient for LTM formation. (a) Representation of the action of PDHE1, PDP and PDK. In mitochondria, PDHE1 catalyzes the conversion of pyruvate to acetyl-coA, which enters the tricarboxylic acid cycle (TCA). PDHE1 can be inactivated through phosphorylation by PDK. In contrast, PDP can activate a phosphorylated PDHE1. (b) Imaging of pyruvate accumulation following azide application in naive flies co-expressing Pyronic and RNAi against either the β subunit of PDHE1 (PDHE1 β), PDP or PDK in MB neurons at adult stage. The plateau value corresponding to sensor saturation was significantly different between the different conditions ($n = 11-17$; $F_{3,100} = 26.8$, $P < 0.0001$). The slope of accumulation was also significantly different: pyruvate accumulation was faster with PDK inhibition, and slower with PDP and PDHE1 inhibition, compared to control flies ($F_{3,100} = 12.24$, $P < 0.0001$). (c) Inhibition of PDHE1 β in adult MB neurons impaired 24-h memory after 5 \times spaced training ($n = 15-19$; $F_{2,49} = 3.89$, $P = 0.028$), but not after 5 \times massed training ($n = 10-11$; $F_{2,31} = 0.26$, $P = 0.77$). (d) Inhibition of PDP in adult MB neurons impaired 24-h memory after 5 \times spaced training ($n = 14-15$; $F_{2,43} = 3.95$, $P = 0.027$), but not after 5 \times massed training ($n = 10-11$; $F_{2,31} = 0.088$, $P = 0.92$). (e) Flies expressing an RNAi against PDK in MB neurons exclusively at the adult stage showed increased memory after two spaced training cycles as compared to their genotypic controls ($n = 10-12$; $F_{2,31} = 5.16$, $P = 0.012$). The memory of these flies, but not of their genotypic controls, was sensitive to CXM treatment, which is a hallmark of LTM (*tubulin-GAL80^{ts}; VT30559/+*: $n = 17$ and 14; $t_{29} = 0.22$; $P = 0.83$; +/*UAS-PDK^{RNAi}*: $n = 12$ and 13; $t_{23} = 1.09$; $P = 0.29$; *tubulin-GAL80^{ts}; VT30559/UAS-PDK^{RNAi}*: $n = 18$ and 19; $t_{35} = 2.87$; $P = 0.007$). (f,g) Inhibition of PDHE1 β in adult MB neurons of naive flies resulted in a marked decrease of sucrose intake compared to the two genotypic controls (f). For PDP inhibition, one of the genotypic controls showed also low sucrose intake, which hampers the interpretation of the data. ($n = 18$ groups of 5 flies; $F_{4,89} = 6.24$, $P = 0.0002$). Inhibition of PDK induced a strong increase in sucrose intake compared to the genotypic controls (g; $n = 12$ groups of five flies; $F_{2,34} = 28.7$, $P < 0.0001$).

pyruvate uptake, displayed a strong decrease in sucrose intake compared to their genotypic controls (Fig. 4f). Strikingly, flies expressing the RNAi against PDK, which increases pyruvate uptake by mitochondria, showed an opposite behaviour, having a much higher sucrose intake than their controls (Fig. 4g). Remarkably, these results evidence that feeding behaviour is directly regulated by the level of energy flux in the MB. Moreover, the effect observed with PDK RNAi could explain why normal flies show an increased sucrose intake after spaced training, since this protocol in itself upregulates MB energy metabolism (Figs 1 and 2).

Defined dopaminergic input neurons activate MB energy flux.

The LTM-gating dopaminergic neurons we previously identified¹⁸ intervene at an early stage of LTM formation. Therefore, we investigated the putative link between dopamine signalling and the observed increase in MB energy flux. First, we asked whether LTM-gating dopamine signalling is also critical in the same time window. We used the thermosensitive mutant dynamin *Shibire^{ts}* (*Shi^{ts}*)⁴⁰, which is dysfunctional at an elevated temperature, to block the output of genetically targeted dopaminergic neurons precisely during this time window. For this, two genetic drivers were used (Fig. 5a): the first one is a split-GAL4 line (*MB438B*, ref. 22) whose labelling is highly specific to two dopaminergic neurons from the paired posterior lateral 1 (PPL1) cluster, known as the V1 and MP1 neurons (respectively designated as PPL1- α 2 α 2 and PPL1- γ 1pedc in the systematic nomenclature proposed in ref. 22). The second one is a classical GAL4 driver (*NP2758*) that has been used in several studies to label only MP1 neurons within dopaminergic neurons^{41,42}. Flies expressing *Shibire^{ts}* through either of these drivers displayed an LTM defect when transferred to the restrictive temperature for 3 h immediately after spaced training (Fig. 5b), but not when kept at the permissive temperature (Supplementary Fig. 3A) or after massed training (Supplementary Fig. 3B). MP1 dopaminergic neurons thus play a master role in controlling LTM formation.

We postulated that signalling from MP1 neurons could trigger the upregulation of MB energy flux after spaced training. To test this hypothesis, we aimed to artificially activate these neurons in naive flies using the heat-sensitive cation channel dTrpA1 (refs 18,43), and to measure the resulting energy flux in MB neurons. On the basis of the genetic enhancers that were used to build the *MB438B* split-GAL4 line, we identified a LexA driver (*30E11-LexA*) that allows targeting of a small set of dopaminergic neurons, including MP1 neurons, independently of the GAL4/UAS system (Supplementary Fig. 3C). We then used this driver to express dTrpA1, while expressing the Pyronic sensor in MB neurons as before. When flies were subjected to thermal treatment to activate dTrpA1-expressing neurons, the rate of pyruvate consumption measured immediately afterward in MB neurons was strongly increased as compared to flies that received a similar treatment but did not express dTrpA1 (Fig. 5c, Supplementary Fig. 3D). Without thermal treatment, no difference was observed between the two conditions (Supplementary Fig. 3E). Altogether, these data suggest that following spaced training, dopamine signalling from MP1 neurons triggers increased energy consumption in MB neurons. In this case, and given the observed facilitative effect of PDK inhibition on LTM formation (Fig. 4e), the activation of MP1 neurons should facilitate LTM formation. We trained flies with only two spaced cycles of associative conditioning, interspersed by periods of heat-activation. The 24 h-memory of flies expressing dTrpA1 through either *MB438B* or *NP2758* driver was increased as compared to their genetic controls (Fig. 5d,e). As was the case for PDK inhibition, the memory formed in flies expressing

dTrpA1 through *MB438B* driver was sensitive to CXM treatment (Fig. 5d), which reveals actual LTM formation with this protocol. No increase occurred in the absence of activation periods (Supplementary Fig. 3F). These results reveal that dopamine signalling from MP1 neurons, which upregulates MB energy flux, is sufficient to engage LTM formation.

DAMB couples dopamine with MB energy flux and LTM. It is known that flies mutant for the D1 receptor *dDA1* are defective for all forms of aversive olfactory memories, including LTM²⁷. Here, we expressed RNAi against *dDA1* in MB neurons at adult stage and confirmed that LTM after spaced training was impaired (Supplementary Fig. 4A, Supplementary Table 1). However, when this RNAi was expressed in MB neurons and MP1 neurons were activated in naive flies, the activation of MB energy consumption was still observed (Supplementary Fig. 4B). This indicates that the action of MP1 neurons on MB energy state is mediated by a distinct receptor that specifically controls LTM formation. Two experimental observations implicate DAMB, a type 1 dopamine receptor that is highly expressed in the MB (ref. 44) and is functionally similar to mammalian D5 (ref. 45). First, *damb* mutant flies were defective in LTM formation (Fig. 6a), but displayed normal olfactory acuity and shock perception (Supplementary Table 1). To confirm the physiological involvement of DAMB in LTM, we expressed RNAi against DAMB in MB neurons exclusively at the adult stage. This impaired LTM performance (Fig. 6b, Supplementary Fig. 4C), but not the 24 h-memory after massed training (Fig. 6b), nor the naive odour or shock avoidance (Supplementary Table 1). Second, we observed that when DAMB expression was knocked-down in MB neurons using an RNAi construct, MP1 activation failed to increase MB energy consumption (Fig. 6c, Supplementary Fig. 4D). Furthermore, expressing RNAi against DAMB in adult MB neurons prevented the increase in neuronal energy consumption after spaced training (Fig. 6d, Supplementary Fig. 4E) that otherwise occurred in flies not expressing this RNAi (Supplementary Fig. 4F). These results establish that DAMB mediates the promoting effect of dopamine signalling from MP1 neurons on energy flux in MB neurons. Consistent with these cellular data, we also observed that DAMB knock-down in adult MB neurons failed to elicit the increased sucrose intake that is normally observed after spaced training (Fig. 6e, Supplementary Fig. 4G). This result links the extra feeding behaviour with the upregulation of energy flux in MB neurons, but it could still be that this behaviour is due to the presence of LTM itself, rather than early upstream process leading to its formation. To discriminate between these two alternatives, using the dye-feeding assay, we tested two other genetic mutants that we identified as specifically defective for LTM: *crammer*⁴⁶ and *debra*⁴⁷. Interestingly, while as expected the *damb* mutant did not show increased feeding following spaced training, the two other mutants still showed the extra feeding phenotype (Fig. 6f). This is consistent with the idea that the increased sucrose intake specifically following spaced training originates from the early upregulation of MB energy metabolism, and may not be affected by manipulations that hampers LTM at later stages.

Discussion

In this study we investigated whether and how energy metabolism in the *Drosophila* MB regulates LTM formation. Our results evidence that LTM formation involves rapid and major alterations of the energy balance, measurable both at the cellular and whole body levels. Using cell-type specific knock-down of enzymes that regulate mitochondrial energy consumption, we showed that the increased energy flux in MB neurons was

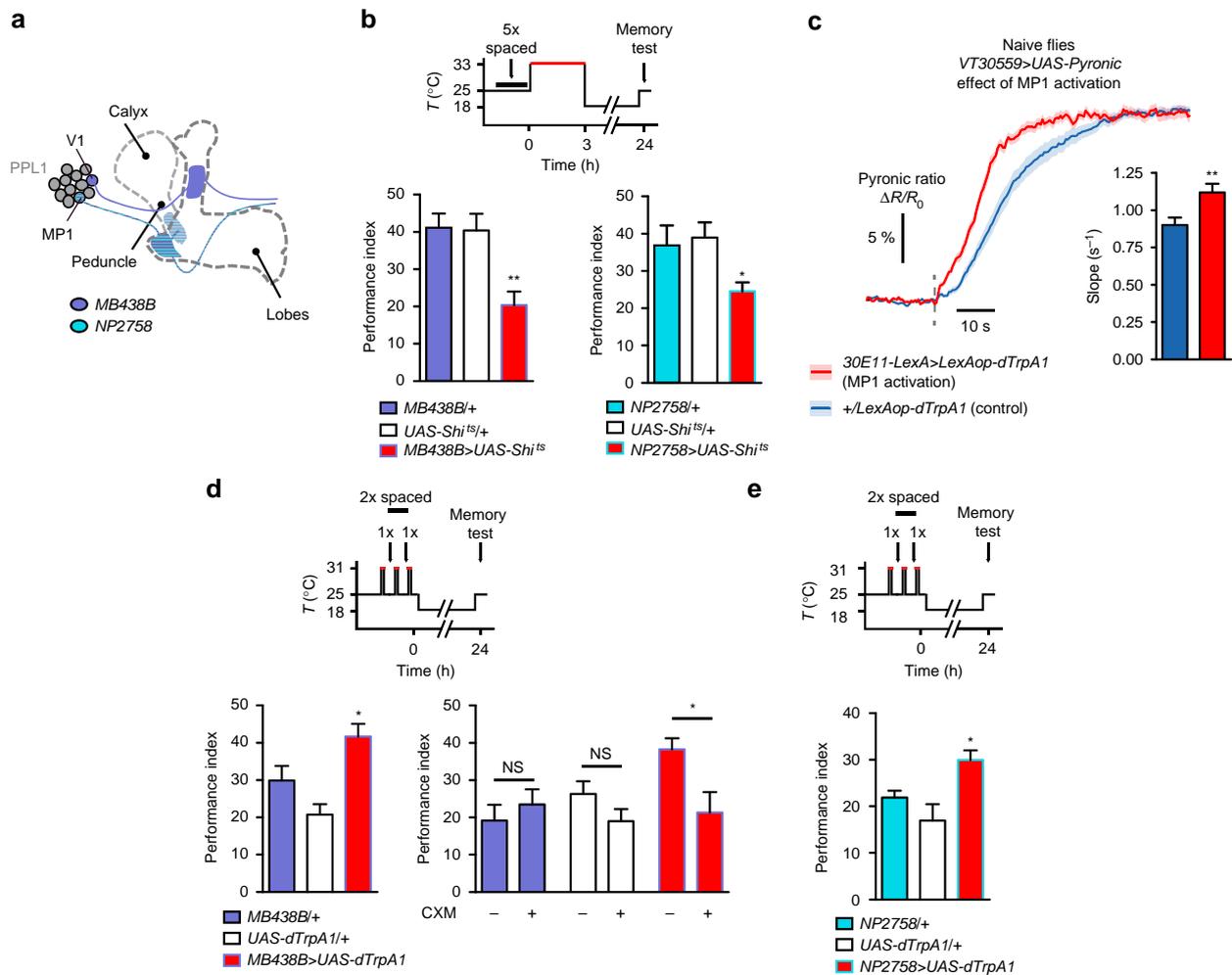


Figure 5 | Dopamine input on MB neurons triggers increased energy flux and LTM formation. (a) Illustration of the dopaminergic neurons in the PPL1 cluster that are targeted by the *MB438B* and *NP2758* drivers. Both drivers target MP1 neurons. Expression patterns of the driver lines used in this study are available online on several public databases. See Experimental Procedures for details. (b) Flies were submitted to a 3 h period at 33 °C following spaced training to block the output of *Shi^{ts}*-expressing neurons. Flies expressing *Shi^{ts}* through either the *MB438B* or *NP2758* driver showed an LTM defect as compared to their genotypic controls (*MB438B*: $n = 7-11$, $F_{2,26} = 9.23$, $P = 0.0011$; *NP2758*: $n = 8-11$, $F_{2,26} = 4.40$, $P = 0.023$). (c) Flies were subjected to a thermal treatment that consisted of two 3-min periods at 33 °C separated by 5 min. Pyruvate accumulation following azide application was measured 2-3 min later. *30E11-LexA > LexAop-dTrpA1* flies ($n = 10$) were compared to *+/-LexAop-dTrpA1* genotypic control flies ($n = 13$). The slope of pyruvate accumulation was higher in flies with activated MP1 neurons ($t_{44} = 2.87$; $P = 0.0063$). (d) Flies were subjected to two cycles of spaced training, interspersed by 1-min periods at 31 °C to activate *dTrpA1*-expressing neurons. Flies expressing *dTrpA1* through the *MB438B* driver showed enhanced 24 h-memory compared to their genotypic controls (*MB438B*: $n = 8-10$, $F_{2,26} = 10.19$, $P = 0.0006$). The memory of these flies, but not of their genotypic controls, was sensitive to cycloheximide (CXM) treatment, which is a hallmark of LTM (*MB438B/+*: $n = 12$; $t_{22} = 0.73$; $P = 0.47$; *+/-UAS-dTrpA1*: $n = 12$; $t_{22} = 1.56$; $P = 0.13$; *MB438B/UAS-dTrpA1*: $n = 12$; $t_{22} = 2.72$; $P = 0.012$). (e) Same training protocol as in d. Flies expressing *dTrpA1* through the *NP2758* driver showed enhanced 24 h-memory compared to their genotypic controls (*NP2758*: $n = 12$, $F_{2,35} = 6.89$, $P = 0.0032$).

necessary, but also sufficient for labile memory to be consolidated into LTM. Accordingly, we established that specific dopaminergic neurons (MP1 neurons) which are required early after spaced training for LTM formation actually activate MB energy flux, through a specific dopamine receptor (the DAMB receptor). Hence, our data demonstrate an instructional role of energy metabolism pathways in LTM formation and bring forward a functional significance to dopamine signalling in the control of LTM.

We used sodium azide as an inhibitor of oxidative phosphorylation to block mitochondrial pyruvate uptake and measured the resulting intracellular pyruvate accumulation. Because glycolysis is upregulated to compensate for blocked mitochondrial activity³⁶, the pyruvate accumulation does not simply mirror the rate of pyruvate consumption before azide application, which

complicates the interpretation of the results. For *in vitro* applications of similar protocols, this difficulty was bypassed by depleting extracellular medium from glucose, which prevents the feedback activation of glycolysis upon azide treatment³⁵, but this technique cannot be used *in vivo*. However, two arguments support that our measurements indeed evaluate the mitochondrial flux. First, in naive flies, the rate of azide-evoked pyruvate accumulation in MB neurons matched the expected decreased or increased mitochondrial flux upon knock-down of regulatory enzymes in the same neurons, either activating (PDHE1, PDP) or inhibitory (PDK). Second, using a genetically-encoded glucose sensor, we observed that the following azide treatment in flies after spaced training or unpaired conditioning was increased to the same level, although the rate of pyruvate accumulation was higher in the

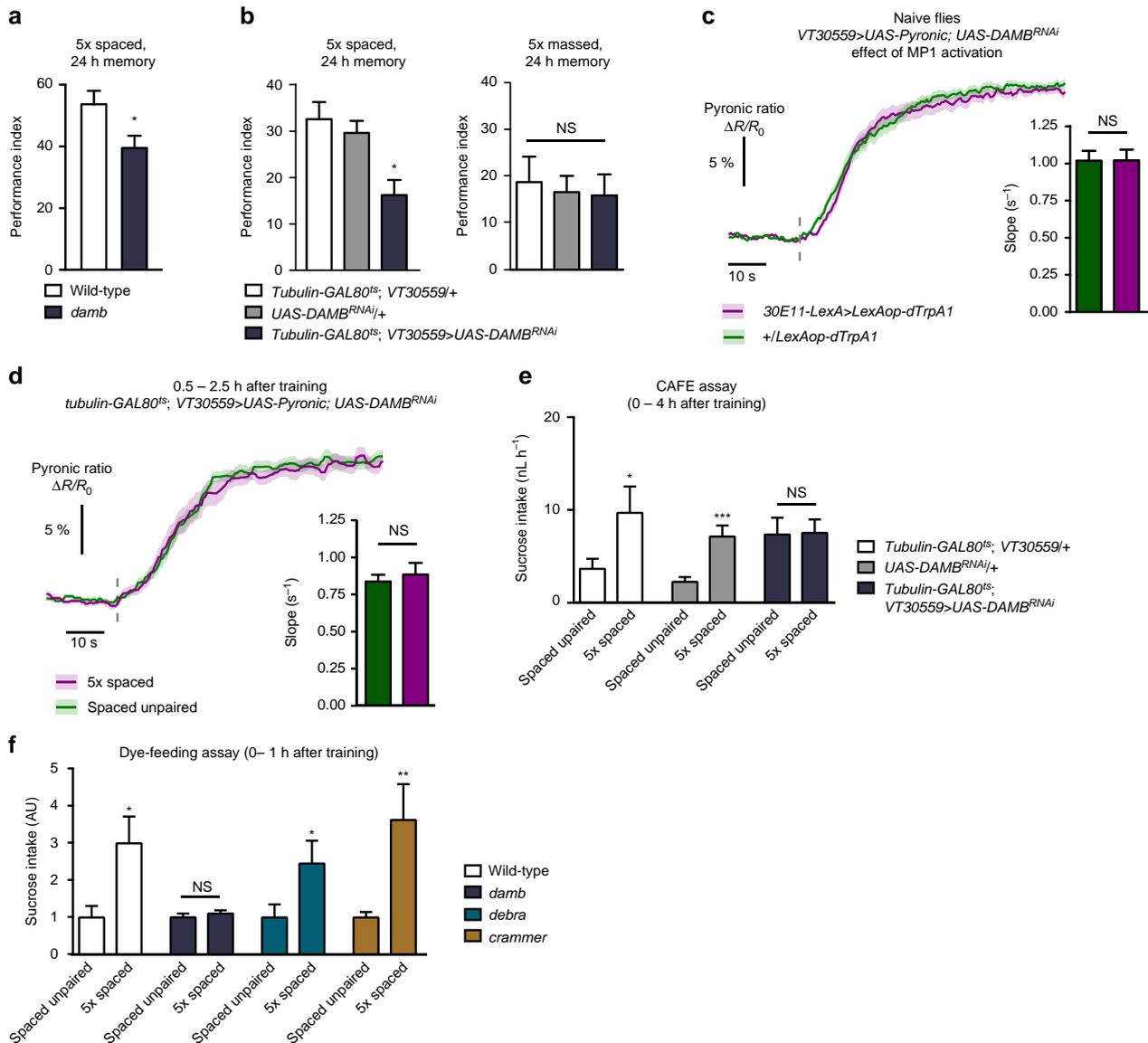


Figure 6 | DAMB in MB neurons mediates the effect of dopamine on energy flux and LTM formation. (a) *damb* mutant flies displayed an LTM defect in comparison to wild-type flies ($n = 12-13$; $t_{23} = 2.41$, $P = 0.024$). (b) Flies expressing RNAi against DAMB in MB neurons exclusively at the adult stage showed an LTM defect as compared to their genotypic controls ($n = 19-21$; $F_{2,60} = 7.39$, $P = 0.0014$), but exhibited normal 24 h-memory after massed training in comparison to their genotypic controls ($n = 14$; $F_{2,41} = 0.10$, $P = 0.90$). (c) An RNAi construct against DAMB was expressed in MB neurons. In this context, thermal treatment failed to activate MB energy flux in *30E11-LexA > LexAop-dTrpA1* flies ($n = 13$) as compared to *+ / LexAop-dTrpA1* flies ($n = 15$). No difference was measured in the slope of pyruvate accumulation between the two conditions ($t_{54} = 0.023$; $P = 0.98$). (d) The expression of the RNAi against DAMB at the adult stage in MB neurons suppressed the upregulated energy flux in MB neurons following spaced training (5 × spaced; $n = 10$; unpaired: $n = 10$; slope: $t_{34} = 0.54$; $P = 0.59$). (e) Following spaced training, flies expressing RNAi against DAMB at the adult stage in MB neurons did not present the increase in sucrose intake observed in their genotypic controls ($n = 69-72$; driver control: $t_{141} = 2.01$; $P = 0.046$; effector control: $t_{138} = 3.77$; $P = 0.0002$; flies expressing RNAi: $t_{141} = 0.085$; $P = 0.93$). (f) Several mutations leading to specific LTM defects were tested in the dye-feeding assay. Following spaced training, the *damb* mutant did not show an increased sucrose intake compared to the unpaired control protocol ($n = 9$; $t_{16} = 0.77$; $P = 0.45$). However, the *debra* ($n = 16-17$; $t_{31} = 2.07$; $P = 0.047$) and *crammer* ($n = 15-16$; $t_{29} = 2.77$; $P = 0.0096$) mutants still showed increased feeding behaviour like wild-type flies ($n = 24-25$; $t_{47} = 2.63$; $P = 0.012$).

former condition. This suggests that the additional pyruvate accumulation measured after spaced training does not come from an increased glycolytic flux in MB neurons. In addition, our behavioral experiments established that LTM formation is sensitive to genetic manipulations that tend to decrease mitochondrial flux, and on the contrary is facilitated when this flux is stimulated. Thus our results altogether point to a pivotal role of neuronal oxidative phosphorylation in LTM formation. The evidence reported here are consistent with an upregulation of

oxidative energy metabolism in MB neurons being an instructive signal to trigger LTM formation. Noteworthy, PDK knockdown in MB neurons did not increase memory after the regular LTM-forming five spaced cycles training protocol. On the same line, we showed in a previous study that activating a broader subset of dopaminergic neurons, including MP1 neurons, did not increase LTM after spaced training¹⁸. These results, together with the fact that energy metabolism is upregulated at an early stage of memory consolidation, are also consistent with the fact that the

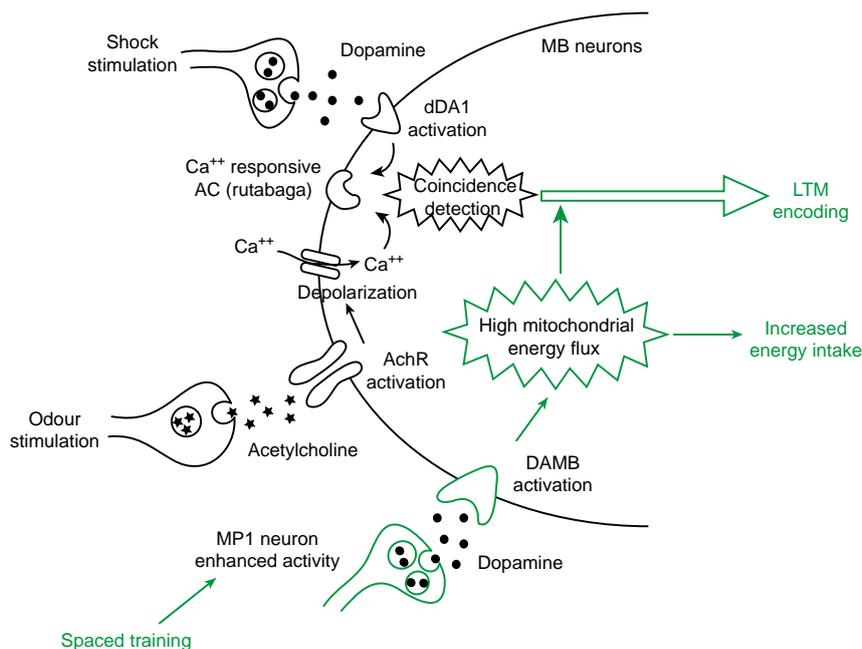


Figure 7 | Model of energy-based gating of LTM in *Drosophila*. During spaced training, the shock perception is relayed to MB neurons by dopamine neurons through the activation of the dDA1/dopR (D1) receptor (46), and olfactory input is delivered from olfactory projection neurons through cholinergic signaling. The simultaneous perception of these two stimuli activates the calcium-dependent adenylyl-cyclase (AC) rutabaga. In parallel, the enhanced activity of MP1 neurons activates the DAMB (D5) receptor, which triggers an energy flow into MB neurons. This increased energy both launches and sustains processes that lead to the formation of LTM.

metabolic shift functions as a gate to control whether LTM formation is enabled or not, but is independent from the downstream mechanisms that regulate the strength or robustness of the LTM. Hence, once the gate is open, that is, either after five spaced cycles in normal flies or after two spaced cycles in flies with stimulated energy metabolism, LTM is formed at similar magnitude, yielding similar memory scores.

The measurements of pyruvate accumulation we conducted in other structures of the fly's brain than MB did not reveal an upregulation following spaced training. Hence, the metabolic activation that we report in MB neurons is not a local manifestation of a whole brain effect, and the possibility that it constitutes an MB-specific phenomenon cannot be ruled out. Interestingly, these experiments also showed that plateau value reached by pyruvate accumulation varied between brain structures: although of similar magnitude in MB neurons and mNSC, it was much lower in EB neurons. Under the reasonable assumption that this plateau value corresponds to the saturation of the sensor, this observation betrays a higher steady-state pyruvate concentration in EB neurons. The other situation in which we observed such an effect is upon decreased PDH activity in MB neurons, which is expected to decrease mitochondrial energy metabolism and in turn activate glycolysis. Although it is difficult to draw firm conclusions with pyruvate data only, these observations suggest that distinct parts of the brain may rely on different metabolic profiles—here EB neurons being for example more glycolytic than MB and mNSC. This is an illustration of the exciting perspectives the *in vivo* use of energy metabolism sensors opens up in *Drosophila*.

Overall, our results support the existence of an energy switch in the MB that is triggered by dopamine signalling through the DAMB receptor to launch LTM formation (Fig. 7). In this model, dopamine acts via two receptors on MB neurons for LTM formation with distinct functional involvement: dDA1, a D1 receptor, signals the unconditioned stimulus to MB neurons for all forms of aversive memories²⁷, and DAMB is specifically

required to trigger LTM encoding. In mammals many studies have shown a prominent role of dopamine signalling in hippocampal-dependent memory tasks including LTM^{48,49}, through D1-class receptors^{50–52}, but without discriminating the involvement of D1 or D5 receptors. A couple of recent reports have started to investigate the role of either receptor type separately, revealing that both may actually be involved, but their respective functional roles remains to be clarified^{52–54}.

Importantly, the present study highlights an unsuspected role of DAMB-mediated dopamine signalling that drives MB neurons into a high-energy state. Although the downstream intracellular processes that mediate this effect remain to be unveiled, it can be expected that they have been evolutionarily conserved, so that dopamine signalling may also upregulate the fuelling of specific brain structures in mammals. Two well-established findings suggest that the mammalian hippocampus could likewise be functionally controlled by energy flux. First, dopaminergic neurons from the ventro-tegmental area and hippocampus form a functional loop that controls the formation of LTM⁴⁸. Second, it was previously demonstrated that fluctuations in hippocampal extracellular glucose levels occur during the execution of demanding tasks and modulate memory performance^{55,56}.

In *Drosophila*, like in mammals, metabolic compartmentalization was reported, wherein neuronal oxidative metabolism is fuelled by glial glycolysis⁵⁷. It would be interesting to investigate whether glial aerobic glycolysis is activated upon LTM formation in register with neuronal oxidative phosphorylation. Alternatively, because it is the major energy source for the fly brain, it may be that glial glycolysis operates close to its maximum capacity at steady state, and that LTM formation mobilizes additional energy stores such as glucose released from glial glycogenolysis¹⁰. Interestingly, it was proposed that the increased lactate transfer to neurons from aerobic glycolysis could serve to spare some glucose for other purpose than energy production, such as synaptic creation or remodelling⁵⁸.

Our results in *Drosophila* demonstrate that spaced training induces increased energy flux in MB neurons and the doubling of sucrose uptake over identical time windows, both processes involving DAMB signalling. Strikingly, we observed that bidirectional modulation of MB mitochondrial metabolism with PDHE1 or PDK knockdown, respectively, had a bidirectional effect of sucrose intake by the flies. Since DAMB signalling in MB neurons stimulates energy metabolism, it is therefore likely that the increased feeding behaviour following spaced training is a direct consequence of the upregulation of energy metabolism in MB neurons. It was shown that in *Drosophila* larvae, feeding behaviour is regulated by insulin signalling on MB neurons and synaptic output from MB neurons⁵⁹. The present study suggests that this property may be extended to adult *Drosophila* as well.

The extra feeding being triggered by MB high-energy state, and being independent from LTM formation itself, the question of what the extra ingested food is used for and how it correlates to the amount of extra energy consumed in MB neurons remains unanswered. But the fact that LTM formation represents such a high demand provides a straightforward explanation for the groundbreaking observation that flies trained to form LTM and deprived from food and water after training exhibit premature death⁶⁰. Previously, we also demonstrated that LTM formation is prevented when flies are starved before and after training, which is beneficial to their survival³⁰. It must be stressed that the direct and strong impact of LTM formation on the need for energy intake was probably a powerful drive for the selection of such an adaptive plasticity mechanism. From a mechanistic point of view, it is noteworthy that adaptive plasticity under starvation occurs through the downregulation of MP1 neuron activity³⁰. Indeed, the same neurons were recently shown to signal the ingestion of a caloric content by starved flies through the DAMB receptor in MB neurons, thereby enabling the consolidation of a labile reward memory trace into LTM⁴². These findings provide valuable food for thought regarding how the selective pressure on energy efficiency has shaped the evolution of taste and nutrient-signalling circuits on the one hand and memory networks on the other hand to result in such an integrated and protective mechanism that controls both aversive and appetitive LTM in *Drosophila*.

Our results show that LTM formation is sensitive to an impairment of mitochondrial flux, but notably that ARM formed after massed training is not, suggesting that specific forms of aversive memory rely on distinct brain metabolic states. In flies and in honeybees, it was shown that another behavioral trait, namely aggression, is controlled by the metabolic state of the brain, increased aggressive behaviour being causally linked to a decrease of neuronal oxidative phosphorylation⁶¹ and a shift towards aerobic glycolysis⁶². These works and the present study altogether outline a new conceptual frame implying a tight coupling between behaviour and brain metabolic profile. There is much to find out about the functional outcomes of local metabolic plasticity⁶³, but in light of these pioneer studies insect research could be illuminating in the near future on these topics.

Methods

Fly strains. Flies (*Drosophila melanogaster*) were raised on standard medium containing yeast, cornmeal and agar at 18 °C and 60% humidity under a 12 h:12 h light-dark cycle (with the exception of imaging experiments: see the corresponding paragraph below). The *UAS-Shi^{ts}* (inserted on chromosome III) and *UAS-dTrpA1* (chr. II) lines have previously been used in multiple studies from our laboratory^{18,30,64}. The *LexAop-dTrpA1* line was used in a previous study⁶⁵. The *UAS-Pyronic* line (chr. III) and the *UAS-FLII¹²Pglu-700μd6* line were generated for the purpose of this study (see below). The *damb*, *crammer* and *debra* mutant lines were already published^{46,47,66}, the *MB438B* split-GAL4 line was used in a previous study²², and the *30E11-LexA* line was obtained from Y. Aso (Janelia Research

Campus). The *UAS-DAMB^{RNAi}*, *UAS-dDA1^{RNAi}*, *UAS-PDHE1β^{RNAi}*, *UAS-PDP^{RNAi}* and *UAS-PDK^{RNAi}* lines correspond respectively to KK110947, KK102341, KK104022, GD31661 and KK106641 from the Vienna *Drosophila* Resource Center (VDRC); the *VT30559* GAL4 line also came from the VDRC. The *Feb170* line was obtained from Francois Bolduc (University of Alberta). All driver lines were verified by immunostainings of mCD8::GFP expression. Expression patterns are available online (<http://brainbase.imp.ac.at> for the VT lines, <http://fweb.janelia.org/cgi-bin/flew.cgi> for the JFRC lines).

To restrict UAS/GAL4-mediated expression exclusively to the adult stage, we used the TARGET system⁶⁷: GAL4 activity was inhibited at the 18 °C raising temperature by a thermosensitive version of GAL80 expressed ubiquitously under the control of the tubulin promoter *tubulin-GAL80^{ts}* (inserted on chr. II), as previously reported⁴²; GAL4 activity was released by transferring adult flies at 30 °C for 2–3 days.

Olfactory conditioning and memory test. The behaviour experiments, including the sample sizes, were conducted similarly to other studies from our research group^{17,18,31}. Experimental flies were transferred to fresh bottles containing standard medium on the day before conditioning. Groups of 40–50 flies were subjected to one of the following olfactory conditioning protocols: five consecutive associative training cycles (5 × massed training), five associative cycles spaced by 15-min inter-trial intervals (5 × spaced conditioning), or two cycles spaced by a 15-min interval (2 × spaced training). Non-associative control protocols (unpaired protocols) were also employed for imaging and feeding assays. Conditioning was performed using previously described barrel-type machines that allow parallel training of up to 6 groups³¹. Throughout the conditioning protocol, each barrel was attached to a constant air flow at 21 min⁻¹. For a single cycle of associative training, flies were first exposed to an odorant (the CS+) for 1 min while 12 pulses of 5 s-long 60 V electric shocks were delivered; flies were then exposed 45 s later to a second odorant without shocks (the CS-) for 1 min. Odour and shocks were delivered separately during unpaired cycles, with shocks occurring 3 min before the first odorant. For the massed unpaired protocol, unpaired cycles were separated by 2-min intervals to avoid the association of shocks with the second odorant of the previous cycle. The odorants 3-octanol and 4-methylcyclohexanol, diluted in paraffin oil at 0.360 and 0.325 mM respectively, were alternately used as conditioned stimuli.

For experiments involving neuronal blockade with Shi^{ts}, flies were transferred to preheated bottles in a 33 °C room, immediately after the end of the last cycle of the training protocol. For experiments involving neuronal activation with dTrpA1, the barrels containing flies were attached to a 31 °C air flow at 21 min⁻¹. For experiments involving CXM feeding, flies were transferred to vials containing filter paper soaked with 35 mM CXM in mineral water and 5% sucrose for 14–16 h before training (Fig. 4e, flies at 30 °C) or for 16–18 h before training (Fig. 5d, flies at 18 °C). After training and until memory test, flies were kept on regular food.

The memory test was performed in a T-maze apparatus. When tested 24 h after training, flies were kept at 18 °C between training and testing. This procedure is standard in our laboratory to obtain robust and reproducible memory scores on a 24-h range. A single performance index value is the average of two scores obtained from two groups of genotypically identical flies conditioned in two reciprocal experiments, using either odorant as CS+. The indicated 'n' is the number of independent performance index values for each genotype.

Feeding assays. Measurements of food intake (CAFÉ assay and dye-feeding assay) were conducted on flies of a single sex to minimize variability. We chose to work on female flies to match imaging experiments (see below). Female flies were selected without anaesthesia 24 h before the experiment and kept on plain food at 18 °C and 60% humidity, except when the experiment involved thermal induction of RNAi, in which case female flies were collected 2 days before the experiment and then kept at 30 °C on plain food.

Our CAFÉ assay was adapted from ref. 68. The experiment was performed in 2 ml microtubes. Three holes were drilled in the lid of the tube: two 0.5 mm-diameter holes for air exchange and one 2.0 mm-diameter hole in which a truncated 200-μl pipette tip was inserted. The pipette tip was used to maintain a calibrated glass micropipette (5 μl, catalogue no. 53432-706; VWR). The glass micropipette was filled with a 5% sucrose solution in mineral water (Evian). A vegetal oil layer of ~0.3 μl was superposed to minimize evaporation. In addition, a piece of cotton soaked with 0.4 ml mineral water was placed in the bottom of each tube, in order to allow *ad libitum* drinking, and to minimize evaporation. After each conditioning protocol, single flies were transferred to the prepared microtubes; capillaries pre-filled with sucrose solution were introduced once all flies were transferred. The initial length l_0 of sucrose solution was measured for each capillary. In each experiment, unoccupied microtubes were included as evaporation controls. Then, the microtubes were kept at 18 °C and 60% humidity. After the desired period of time, the length of solution remaining (l_t) in each capillary was measured. The length of sucrose solution consumed by each fly was calculated as $l_0 - l_t - l_e$, where l_e is the average length of solution that had evaporated in unoccupied control tubes. A single experiment typically included eight flies per genotype and per condition. The indicated 'n' is the number of single flies measured for each condition.

For the colorimetric experiment (dye-feeding assay), we used a previously described protocol⁶⁹. After conditioning, flies were collected in bottles containing a cotton disk soaked with 3 ml of a 5% sucrose–100 mM sulforhodamine B solution in mineral water. Flies were then kept at 18 °C and 60% humidity. After 1 h, flies were transferred to a 2 ml microtube and frozen in liquid nitrogen. Groups of five flies were deposited in a 1.5 ml microtube and ground in 200 μ l PBS. Microtubes were then centrifuged at 12,000 r.p.m. for 7 min. The supernatants were collected and centrifuged again at 12,000 r.p.m. for 7 min. Then, 100 μ l of each supernatant was aliquoted to 96-well plate, and absorbance was measured at 570 nm. As a blank absorbance control, naive flies placed on a 5% sucrose solution without sulforhodamine B were treated in parallel. The mean absorbance of these controls was subtracted from the absorbance of the other samples. A single experiment typically included 3–4 groups of five flies per condition. The complete dataset from a given associative protocol was normalized to the control unpaired protocol. For measurements on naive flies (Fig. 4f,g), naive flies of the indicated genotype were placed at 30 °C for 2 days to induce RNAi expression in relevant groups, or kept at 18 °C for 2 days (non-induced flies). Sucrose intake was then measured for 1 h with the dye-feeding assay. Sucrose intake was normalized to the non-induced flies of the same genotype, to cancel inter-genotype variations that are important in this assay. The indicated ‘*n*’ is the number of groups of 5 flies measured for each condition.

Measurements of locomotor activity. Two to three day old female flies were selected 24 h before the assay and placed in regular food vials at 18 °C. They were then conditioned with a spaced training or a spaced unpaired protocol. In each experiment, 32 flies of each condition were assayed. Each fly was transferred to Trikinetics (www.trikinetics.com) glass tubes sealed with fly food on one side and a plug on the other side. Tubes were put in Trikinetics Drosophila Activity Monitors. These monitors were housed in a temperature-controlled incubator at 18 °C under constant light. Beam breaks were recorded at 1-min intervals. Activity scores were calculated by averaging the total number of beam breaks during a 1-h or 4-h period after conditioning.

Immunohistochemistry experiments. Female flies carrying the driver transgene were crossed to *UAS-mCD8::GFP* or *LexAop-mCD8::GFP* males. Before dissection, whole flies of female F1 progenies (3–4 days after eclosion at 25 °C) were fixed in 4% formaldehyde in PBT (PBS containing 1% Triton X-100) at 4 °C overnight. Brains were dissected in Drosophila Ringer solution and fixed for 1 h at room temperature (RT) in 4% formaldehyde in PBT. Samples were then rinsed three times for 20 min in PBT, blocked with 2% bovine serum albumin in PBT for 2 h and incubated with primary antibodies at 1:400 (rabbit anti-GFP (catalogue number: A11122), Invitrogen Molecular Probes) and 1:100 (mouse anti-nc82, DSHB, (catalogue reference: nc82)) in the blocking solution at 4 °C overnight. After rinsing, brains were incubated with secondary antibodies at 1:400 (anti-rabbit conjugated to Alexa Fluor 488 (A11034), anti-mouse conjugated to Alexa Fluor 594 (A11005), Invitrogen Molecular Probes) in the blocking solution for 3 h at RT. After rinsing, brains were mounted in Prolong Mounting Medium (Lifetechnology) for microscopy analysis. Images were acquired with a Nikon AIR confocal microscope. Confocal Z-stacks were acquired in 1 μ m slices and imported into NIH ImageJ for analyses.

Generation of transgenic flies. The 2545_{pcDNA3.1(-)}Pyronic plasmid³⁵ (obtained from G. Bonvento) was digested by BamHI and BclI. The resulting 2,287 bp fragment was purified by electrophoresis and cloned into the pUAST vector digested by BglII. The correct orientation of the insert was verified by restriction.

The 2250 SIN-cPPT-PGK-FLIIP-glu700-WHV plasmid (obtained from G. Bonvento) was digested by BamHI and XbaI. The resulting 2,395 bp fragment was purified by electrophoresis and cloned into the pUAST vector digested by BglII and XbaI. The resulting construct was verified by restriction.

Transgenic fly strains were obtained by embryonic injection of the resulting vector, which was outsourced to Rainbow Transgenic Flies, Inc (CA, USA).

In vivo imaging of energy metabolism. All imaging experiments were performed on female flies expressing Pyronic or the glucose sensor in MB neurons through the *VT30559* GAL4 driver or in EB neurons and mNSC using the *Feb170* GAL4 driver. Female flies were preferred because their bigger size makes surgery easier, as in all previous imaging work from our lab. Other transgenes that were used in addition to *VT30559* and *UAS-Pyronic* are indicated in the figure panels. Crosses for imaging experiments were raised at 25 °C to increase the expression level of genetically-encoded sensors through the UAS/GAL4 system. However, flies were raised at 18 °C for the experiments involving GAL80⁴⁵ (Fig. 4d,e), in order to avoid any leak of GAL4 activity. The two-day induction period at 30 °C drove sufficient expression of Pyronic to perform imaging. For experiments on conditioned flies, data were collected indiscriminately from 30 min to 3 h after training.

Flies were prepared for *in vivo* imaging, as described previously³⁰. The proboscis was not glued, and no agarose was applied on the brain. At the end of surgery, a 90 μ l-droplet of physiological solution (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 36 mM sucrose, 5 mM HEPES-hemisodium salt, pH 7.3–7.4)

was applied on the preparation. Two-photon imaging was performed on a Leica TCS-SP5 upright microscope, equipped with a $\times 25$, 0.95 NA water-immersion objective. Two-photon excitation of mTFP was achieved using a Mai Tai DeepSee laser tuned to 825 nm. 512 \times 250 images were acquired at a rate of two images per second. In general, the image encompassed the vertical lobes of both brain hemispheres, although only one hemisphere was visible in some preparations. The emission channels for mTFP and Venus were the same, as described in ref. 29. One minute after the beginning of image acquisition, 10 μ l of a 50 mM sodium azide solution (prepared in the same physiological solution) were injected into the 90 μ l droplet bathing the fly’s brain. Being a weak base, 50 mM azide had no detectable effect on the pH of the solution (pH = 7.42 \pm 0.03 measured on three different solutions).

Image analysis was performed using a custom-written Matlab script. Regions of interest (ROI) were delimited by hand around each visible vertical lobe, and the average intensity of both mTFP and Venus channels over each ROI were calculated over time after background subtraction. The Pyronic sensor was designed so that FRET from mTFP to Venus decreases when pyruvate concentration increases³⁵. To obtain a signal that positively correlates with pyruvate concentration, the inverse FRET ratio was computed, that is, mTFP intensity divided by Venus intensity. This ratio was normalized by a baseline value calculated over the 30 s preceding azide injection to give a normalized ‘Pyronic ratio’. The increase in the Pyronic ratio typically had a bilevel waveform, with a linear increase from ~ 10 to $\sim 70\%$ of the plateau. The risetime (from $t = 0$ until reaching 70% of the plateau) and the slope (between 10 and 70% of the plateau) were determined automatically using the *statelevels*, *risetime* and *slewrate* functions in the Matlab signal processing toolbox. Traces from all hemispheres were pooled. The indicated ‘*n*’ is the number of animals that were assayed for each condition. All acquisition and analysis parameters were determined during preliminary experiments that are not included in the study.

For experiments with the FLII¹²Pglu-700 μ 66 glucose sensor, the modifications to the protocol followed with Pyronic were the following: the images were acquired at 850 nm. The FRET ratio (YFP/CFP) was computed to obtain a signal positively correlated to glucose concentration. An application of cytochalasin B was made 20 s before azide application. For cytochalasin B application, cytochalasin B (Sigma Aldrich) was aliquoted in DMSO at a concentration of 2 mM. After a ten times dilution in physiological solution, 10 μ l were injected into the 90 μ l droplet bathing the brain to reach a final concentration of 20 μ M. For unexplained reasons, the application of cytochalasin B produced a transient but marked drop of fluorescence in both channels. This caused artifactual twitches in the FRET ratio signals, which were masked on Fig. 2e. Glycolytic rates were measured as the slope of a linear fit to the data 12 s before azide injection for intrinsic glycolysis, and 20 s after for azide-evoked glycolysis.

Statistical analyses. All data are presented as mean \pm s.e.m. The different experimental groups are determined by their genotypes, so no randomized allocation was performed, and the investigators were not blinded to the group allocation during experiments. Comparisons of the data series between two conditions were achieved by a two-tailed unpaired *t*-test. Results of *t*-tests are given as the value t_x of the *t* distribution with x degrees of freedom obtained from the data. Comparisons between more than two distinct groups were made with a one-way ANOVA, followed by Newman–Keuls pairwise comparisons between the experimental group and its controls. The *t*-test and ANOVA are robust against slight deviations from normal distributions or the inequality of variances if the sample sizes are similar between groups⁷⁰, which was the case in our experiments. Therefore, we did not systematically test our data for normality or verify variance homogeneity before statistical tests. Instead, we adopted a uniform analysis strategy for all our data, as advised in ref. 70. ANOVA results are given as the value of the Fisher distribution $F_{(x,y)}$ obtained from the data, where x is the number of degrees of freedom between groups and y is the total number of degrees of freedom of the distribution. Statistical tests were performed using the GraphPad Prism 5 software. In the figures, asterisks illustrate the significance level of the *t*-test, or of the least significant pairwise comparison following an ANOVA, with the following nomenclature: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: not significant, $P > 0.05$.

Data availability. All relevant data are available from the authors.

References

- Magistretti, P. J. & Allaman, I. A cellular perspective on brain energy metabolism and functional imaging. *Neuron* **86**, 883–901 (2015).
- Niven, J. E. & Laughlin, S. B. Energy limitation as a selective pressure on the evolution of sensory systems. *J. Exp. Biol.* **211**, 1792–1804 (2008).
- Sengupta, B. & Stemmler, M. B. Power consumption during neuronal computation. *Proc. IEEE* **102**, 738–750 (2014).
- Laughlin, S. B. & Sejnowski, T. J. Communication in neuronal networks. *Science* **301**, 1870–1874 (2003).
- Olshausen, B. A. & Field, D. J. Sparse coding of sensory inputs. *Curr. Opin. Neurobiol.* **14**, 481–487 (2004).
- Magistretti, P. J., Pellerin, L., Rothman, D. L. & Shulman, R. G. Energy on demand. *Science* **283**, 496–497 (1999).

7. McNay, E. C. & Gold, P. E. Food for thought: fluctuations in brain extracellular glucose provide insight into the mechanisms of memory modulation. *Behav. Cogn. Neurosci. Rev.* **1**, 264–280 (2002).
8. Gibbs, M. E., Anderson, D. G. & Hertz, L. Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* **54**, 214–222 (2006).
9. Newman, L. A., Korol, D. L. & Gold, P. E. Lactate produced by glycogenolysis in astrocytes regulates memory processing. *PLoS ONE* **6**, e28427 (2011).
10. Suzuki, A. *et al.* Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* **144**, 810–823 (2011).
11. Bozzo, L., Puyal, J. & Chatton, J.-Y. Lactate modulates the activity of primary cortical neurons through a receptor-mediated pathway. *PLoS ONE* **8**, e71721 (2013).
12. Barros, L. F. Metabolic signaling by lactate in the brain. *Trends Neurosci.* **36**, 396–404 (2013).
13. Tang, F. *et al.* Lactate-mediated glia-neuronal signalling in the mammalian brain. *Nat. Commun.* **5**, 3284 (2014).
14. Lauritzen, K. H. *et al.* Lactate receptor sites link neurotransmission, neurovascular coupling, and brain energy metabolism. *Cerebral Cortex* **24**, 2784–2795 (2014).
15. Yang, J. *et al.* Lactate promotes plasticity gene expression by potentiating NMDA signaling in neurons. *Proc. Natl Acad. Sci. USA* **111**, 12228–12233 (2014).
16. Tully, T., Preat, T., Boynton, S. C. & Vecchio, M. D. Genetic dissection of consolidated memory in *Drosophila*. *Cell* **79**, 35–47 (1994).
17. Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in *Drosophila*. *Science* **304**, 1024–1027 (2004).
18. Plaças, P.-Y. *et al.* Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat. Neurosci.* **15**, 592–599 (2012).
19. Wolff, G. H. & Strausfeld, N. J. Genealogical correspondence of mushroom bodies across invertebrate phyla. *Curr. Biol.* **25**, 38–44 (2015).
20. Wolff, G. H. & Strausfeld, N. J. Genealogical correspondence of a forebrain centre implies an executive brain in the protostome-deuterostome bilaterian ancestor. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **371**, 20150055 (2016).
21. Masse, N. Y., Turner, G. C. & Jefferis, G. S. X. E. Olfactory information processing in *Drosophila*. *Curr. Biol.* **19**, R700–R713 (2009).
22. Aso, Y. *et al.* The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife* **3**, e04577 (2014).
23. Schwarzel, M. *et al.* Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* **23**, 10495–10502 (2003).
24. Claridge-Chang, A. *et al.* Writing memories with light-addressable reinforcement circuitry. *Cell* **139**, 405–415 (2009).
25. Aso, Y. *et al.* Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* **8**, e1002768 (2012).
26. Kim, Y.-C., Lee, H.-G. & Han, K.-A. D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. *J. Neurosci.* **27**, 7640–7647 (2007).
27. Qin, H. *et al.* Gamma Neurons Mediate Dopaminergic Input during Aversive Olfactory Memory Formation in *Drosophila*. *Curr. Biol.* **22**, 608–614 (2012).
28. Heisenberg, M. Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* **4**, 266–275 (2003).
29. Gervasi, N., Tchénio, P. & Preat, T. PKA dynamics in a *Drosophila* learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. *Neuron* **65**, 516–529 (2010).
30. Plaças, P.-Y. & Preat, T. To favor survival under food shortage, the brain disables costly memory. *Science* **339**, 440–442 (2013).
31. Pascual, A. & Preat, T. Localization of long-term memory within the *Drosophila* mushroom body. *Science* **294**, 1115–1117 (2001).
32. Yu, D., Akalal, D.-B. G. & Davis, R. L. *Drosophila* α/β mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron* **52**, 845–855 (2006).
33. Hirano, Y. *et al.* Fasting launches CRTRC to facilitate long-term memory formation in *Drosophila*. *Science* **339**, 443–446 (2013).
34. San Martín, A. *et al.* Single-cell imaging tools for brain energy metabolism: a review. *Neurophotonics* **1**, 011004 (2014).
35. San Martín, A. *et al.* Imaging mitochondrial flux in single cells with a FRET sensor for pyruvate. *PLoS ONE* **9**, e85780 (2014).
36. Bittner, C. X. *et al.* High resolution measurement of the glycolytic rate. *Front. Neuroenerget.* **2**, 26 (2010).
37. Chambers, D. B. *et al.* Insulin signaling is acutely required for long-term memory in *Drosophila*. *Front. Neural Circuits* **9**, 8 (2015).
38. Chen, J. *et al.* Identification of novel modulators of mitochondrial function by a genome-wide RNAi screen in *Drosophila melanogaster*. *Genome Res.* **18**, 123–136 (2008).
39. Lavington, E. *et al.* A small system–high-resolution study of metabolic adaptation in the central metabolic pathway to temperate climates in *Drosophila melanogaster*. *Mol. Biol. Evol.* **31**, 2032–2041 (2014).
40. Kitamoto, T. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81–92 (2001).
41. Krashes, M. J. *et al.* A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. *Cell* **139**, 416–427 (2009).
42. Musso, P.-Y., Tchénio, P. & Preat, T. Delayed dopamine signaling of energy level builds appetitive long-term memory in *Drosophila*. *Cell Rep.* **10**, 1023–1031 (2015).
43. Hamada, F. N. *et al.* An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature* **454**, 217–220 (2008).
44. Han, K. A., Millar, N. S., Grotewiel, M. S. & Davis, R. L. DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* **16**, 1127–1135 (1996).
45. Kim, Y.-C., Lee, H.-G., Seong, C.-S. & Han, K.-A. Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of *Drosophila melanogaster*. *Gene Exp. Patterns* **3**, 237–245 (2003).
46. Comas, D., Petit, F. & Preat, T. *Drosophila* long-term memory formation involves regulation of cathepsin activity. *Nature* **430**, 460–463 (2004).
47. Kottler, B., Lampin-Saint-Amaux, A., Comas, D., Preat, T. & Goguel, V. Debra, a protein mediating lysosomal degradation, is required for long-term memory in *Drosophila*. *PLoS ONE* **6**, e25902 (2011).
48. Lisman, J. E. & Grace, A. A. The hippocampal-VTA loop: controlling the entry of information into long-term memory. *Neuron* **46**, 703–713 (2005).
49. Rossato, J. I., Bevilacqua, L. R. M., Izquierdo, I., Medina, J. H. & Cammarota, M. Dopamine controls persistence of long-term memory storage. *Science* **325**, 1017–1020 (2009).
50. Lemon, N. & Manahan-Vaughan, D. Dopamine D1/D5 receptors gate the acquisition of novel information through hippocampal long-term potentiation and long-term depression. *J. Neurosci.* **26**, 7723–7729 (2006).
51. O’Carroll, C. M., Martin, S. J., Sandin, J., Frenguelli, B. & Morris, R. G. M. Dopaminergic modulation of the persistence of one-trial hippocampus-dependent memory. *Learn. Mem.* **13**, 760–769 (2006).
52. Sariñana, J., Kitamura, T., Künzler, P., Sultzman, L. & Tonegawa, S. Differential roles of the dopamine 1-class receptors, D1R and D5R, in hippocampal dependent memory. *Proc. Natl Acad. Sci. USA* **111**, 8245–8250 (2014).
53. Moraga-Amaro, R. *et al.* Dopamine receptor D5 deficiency results in a selective reduction of hippocampal NMDA receptor subunit NR2B expression and impaired memory. *Neuropharmacology* **103**, 222–235 (2016).
54. Sariñana, J. & Tonegawa, S. Differentiation of forebrain and hippocampal dopamine 1-class receptors, D1R and D5R, in spatial learning and memory. *Hippocampus* **26**, 76–86 (2016).
55. McNay, E. C., Fries, T. M. & Gold, P. E. Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. *Proc. Natl Acad. Sci. USA* **97**, 2881–2885 (2000).
56. Smith, M. A., Riby, L. M., Eekelen, J. A. & Foster, J. K. Glucose enhancement of human memory: a comprehensive research review of the glucose memory facilitation effect. *Neurosci. Biobehav. Rev.* **35**, 770–783 (2011).
57. Volkenhoff, A. *et al.* Glial glycolysis is essential for neuronal survival in *Drosophila*. *Cell Metabolism* **22**, 437–447 (2015).
58. Goyal, M. S. & Raichle, M. E. Gene expression-based modeling of human cortical synaptic density. *Proc. Natl Acad. Sci. USA* **110**, 6571–6576 (2013).
59. Zhao, X. L. & Campos, A. R. Insulin signalling in mushroom body neurons regulates feeding behaviour in *Drosophila* larvae. *J. Exp. Biol.* **215**, 2696–2702 (2012).
60. Mery, F. & Kawecki, T. J. A cost of long-term memory in *Drosophila*. *Science* **308**, 1148 (2005).
61. Li-Byarlay, H., Rittschof, C. C., Massey, J. H., Pittendrigh, B. R. & Robinson, G. E. Socially responsive effects of brain oxidative metabolism on aggression. *Proc. Natl Acad. Sci. USA* **111**, 12533–12537 (2014).
62. Chandrasekaran, S. *et al.* Aggression is associated with aerobic glycolysis in the honey bee brain. *Genes Brain Behav.* **14**, 158–166 (2015).
63. Barros, L. F., Sierralta, J. & Weber, B. How doth the little busy bee: unexpected metabolism. *Trends Neurosci.* **38**, 1–2 (2015).
64. Plaças, P.-Y., Trannoy, S., Friedrich, A. B., Tanimoto, H. & Preat, T. Two pairs of mushroom body efferent neurons are required for appetitive long-term memory retrieval in *Drosophila*. *Cell Rep.* **5**, 769–780 (2013).
65. Liu, C. *et al.* A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* **488**, 512–516 (2012).
66. Selcho, M., Pauls, D., Han, K.-A., Stocker, R. F. & Thum, A. S. The role of dopamine in *Drosophila* larval classical olfactory conditioning. *PLoS ONE* **4**, e5897 (2009).
67. McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K. & Davis, R. L. Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* **302**, 1765–1768 (2003).
68. Ja, W. W. *et al.* Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl Acad. Sci. USA* **104**, 8253–8256 (2007).

69. Riemensperger, T. *et al.* Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl Acad. Sci. USA* **108**, 834–839 (2011).
70. Motulsky, H. *Intuitive Biostatistics*, 2nd edition (Oxford University Press, New York, 2010).

Acknowledgements

We thank Honorine Lucchi and Aurélie Lampin-Saint-Amaux for assistance in behavioral experiments and molecular biology. We thank Gilles Bonvento and L. Felipe Barros for sharing the Pyronic and FLII¹²Pglu-700μδ6 plasmids, and François Bolduc for providing the *Feb170* GAL4 line. Research in the T.P. laboratory was funded by the Agence Nationale pour la Recherche and the Labex MemoLife. E.D.T. was funded by a doctoral fellowship from the French Ministry of Research. L.S. was funded by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (SCHE 1884/1-1).

Author contributions

P.-Y.P., G.I. and T.P. designed the experiments; P.-Y.P., E.d.T., L.S., S.T. and G.I. performed experiments; V.G., K.-A.H. contributed new fly lines; P.-Y.P. wrote the original draft of the manuscript, which was commented and edited by all authors. P.-Y.P. and T.P. supervised the work. T.P. obtained funding for the project.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Plaçais, P.-Y. *et al.* Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory. *Nat. Commun.* **8**, 15510 doi: 10.1038/ncomms15510 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017

This article describes an increased pyruvate consumption in Kenyon cells axonal and synaptic compartments after spaced conditioning. The extra pyruvate can originate from an internal production (neuronal glycolysis or amino-acid oxidation) or from an external source (increased glycolysis in other neurons, or more likely, in glial cells). We thus decided to study whether glia were involved in long-term memory formation, and, if so, which signaling or metabolic pathways were required in glial cells to support long-term memory.

II. A neuron-glia interaction during long-term memory consolidation

II. 1. Cortex glia participate in long-term memory consolidation early after the conditioning

To reveal if glia were involved in long-term memory formation, we chose to use a broad tool that targets many cellular mechanisms called *shibire^{ts}* (Figure 13) (van der Bliek and Meyerowitz, 1991). By only switching flies at a high temperature of 33°C, this transgene allows to block vesicular recycling in the cells where it is expressed with a temporal control. Thus, it is a very efficient tool to temporally dissect the involvement of the cells of interest in signaling mechanisms. It has been useful to extensively study various behaviors and has already been used to block glial cells function in previous published works (Ng et al., 2011; Sousa-Nunes et al., 2011; Melom and Littleton, 2013; Artiushin et al., 2018; Weiss et al., 2019).

We expressed *shibire^{ts}* in all glial cells thanks to the pan-glia driver Repo-Gal4 (Awasaki et al., 2008). Flies were subjected to a spaced training protocol that allows the formation of long-term memory and transferred at 33°C for 14 h right after the conditioning. Blocking vesicular recycling in glial cells in the flies of interest (Repo-Gal4>UAS-Shi^{ts} flies) impaired long-term memory performance as compared to their genotypic controls (Figure 43 A). No defect was observed in the same genotype in the absence of blockade, i.e. without restrictive temperature treatment (Figure 43 A).

Our next aim was to study which glial subtypes were involved in this mechanism. We thus repeated the 14 h of blockade experiment on restricted glial subtypes thanks to specific GAL4 drivers. The drivers used are summarized in Table 1. We expected that one of the glial subtypes in contact with the neuropile and therefore in quite close association with synapses, astrocytes or ensheathing glia, would be implicated, and less likely other glial subtypes. Surprisingly, we phenocopied the long-term memory defect previously observed only when blocking cortex glia (Figure 43 B). Thus, vesicular recycling in cortex glia is necessary for long-term memory consolidation. To confirm this result, we aimed at rescuing the long-term memory defect obtained after pan-glia blockade by preventing Gal4 activity in cortex glia thanks to the genetic construct R54H02-Gal80 (Coutinho-Budd et al., 2017). Indeed, when blocking all glial subtypes except cortex glia after the conditioning, we rescued the long-term memory

defect (Figure 43 C), showing that cortex glia are the only glial cells participating to the mechanism blocked by *shibire^{ts}*.

As mentioned above, *shibire^{ts}* is a genetic tool that allows the temporal dissection of the involvement of cells of interest. Thus, we further aimed at defining the time window during which cortex glia were involved in long-term memory consolidation. We repeated the same experimental strategy, but blocked cortex glia for shorter time periods. Blocking cortex glia for 6 h and even for only 2 h after the conditioning significantly decreased long-term memory performance (Figure 43 D), while flies expressing *shibire^{ts}* in cortex glia but not subjected to a switch at restrictive temperature displayed a normal long-term memory.

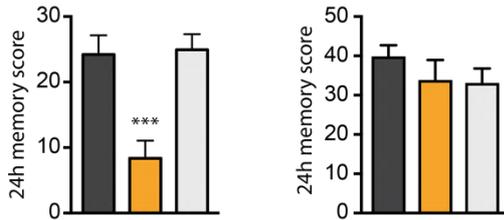
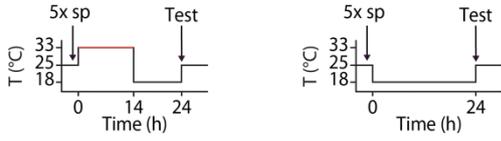
We further replicated this memory impairment by blocking cortex glial cells for 6 h after the conditioning using a second cortex-glia specific Gal4 driver, NP2222-Gal4 (Figure 43 D). Finally, this involvement of cortex glia was specific for long-term memory, as long-term anesthesia resistant memory was not affected by blocking cortex glia during 6 h after a 5 x massed conditioning (Figure 43 E). Moreover, when flies were subjected to a single cycle of training, blocking all glia during the whole experiment did not impair 3 h memory (Figure 43 F). These results show, first, that cortex glial cells are involved early in memory consolidation, and second, that the process inhibited by *shibire^{ts}* is not involved in other memories than long-term memory. Naturally, our results do not rule out that cellular mechanisms that are not blocked by *shibire^{ts}* can be involved in glial cells for these other memory phases.

Targeted cells	Driver name	References
All glia	Repo-Gal4	(Awasaki and Lee, 2011; Lai and Lee, 2006)
Perineural glia	NP6293-Gal4	(Awasaki et al., 2008)
Subperineural glia	Moody-Gal4	(Bainton et al., 2005)
Ensheathing glia	MZ0709-Gal4	(Ito et al., 1995)
Astrocytes	Alrm-Gal4	(Doherty et al., 2009)
Cortex glia	R54H02-Gal4 NP2222-Gal4	(Coutinho-Budd et al., 2017) (Awasaki et al., 2008)

Table 1. List of the glial subtypes-specific GAL4 drivers used in this study

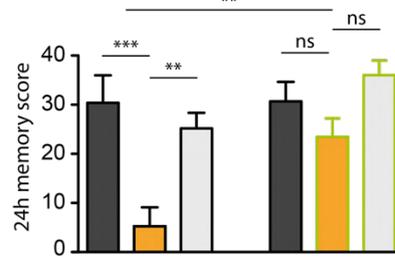
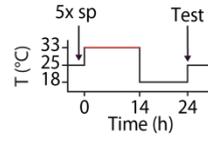
A

■ Repo-GAL4/+
 ■ Repo-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}

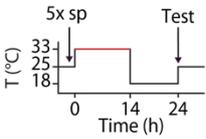


C

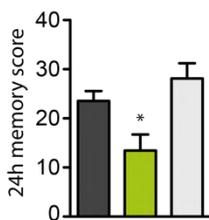
■ Repo-GAL4/+
 ■ Repo-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}
 ■ Repo-GAL4/54H02-Gal80, UAS-Shi^{ts}
 ■ +/54H02-Gal80, UAS-Shi^{ts}



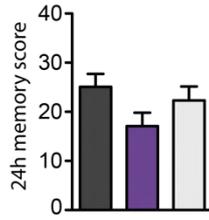
B



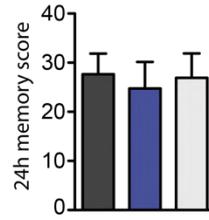
■ 54H02-GAL4/+
 ■ 54H02-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}
 ■ NP6293-GAL4/+
 ■ NP6293-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}
 ■ Moody-GAL4/+
 ■ Moody-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}
 ■ MZ0709-GAL4/+
 ■ MZ0709-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}
 ■ Alrm-GAL4/+
 ■ Alrm-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}



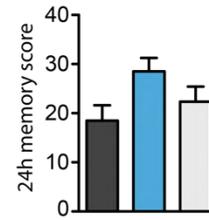
Cortex glia



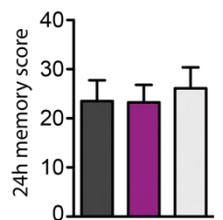
Surface glia
Perineural



Surface glia
Subperineural



Ensheathing glia

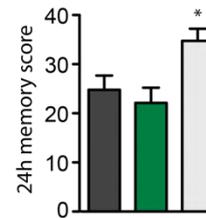
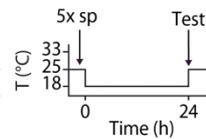
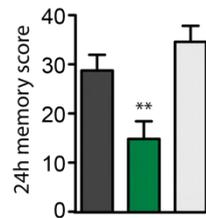
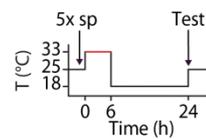
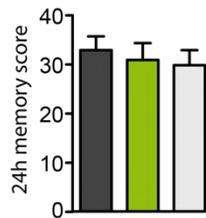
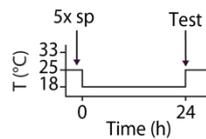
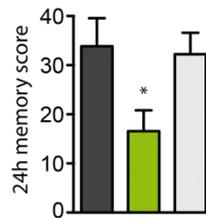
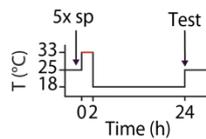
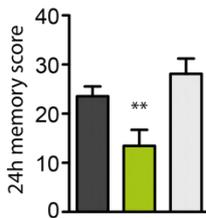
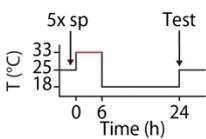


Astrocytes

D

■ 54H02-GAL4/+
 ■ 54H02-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}

■ NP2222-GAL4/+
 ■ NP2222-GAL4/UAS-Shi^{ts}
 □ +/UAS-Shi^{ts}



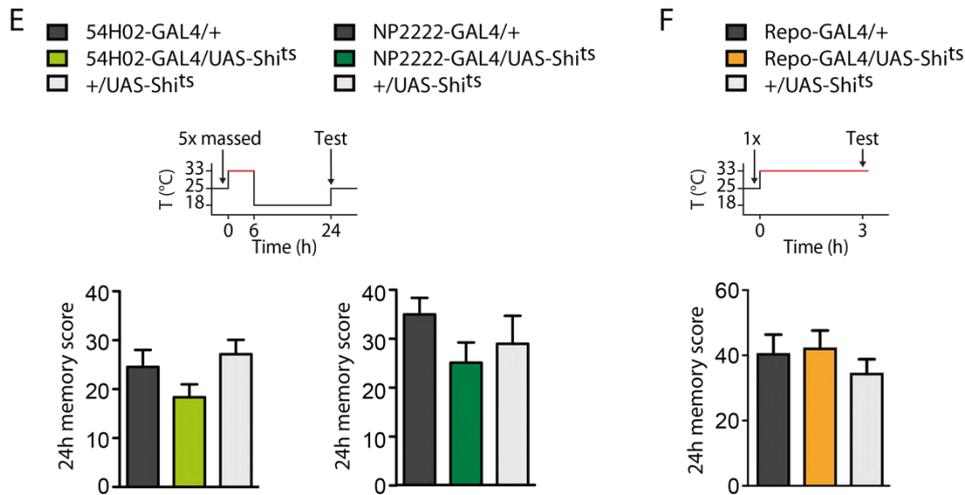


Figure 43. Cortex glia is involved very early during long-term memory formation. A. Long-term memory was impaired when blocking glial cells, in Repo-Gal4>UAS-Shi^{ts} flies subjected to a 14 h heat treatment at restrictive temperature (33°C) after the 5 x spaced conditioning as compared to the genotypic controls (n = 28, $F_{2,83} = 0.9315$, $P < 0.0001$). Flies from the same genotype displayed a normal long-term memory when not subjected to restrictive temperature treatment (n = 8, $F_{2,23} = 0.7395$, $P = 0.4894$). B. Flies memory performance after specific glial subtypes blockade for 14 h after the long-term memory conditioning. Only when blocking cortex glia, flies displayed a long-term memory defect. Blocking each other single glial subtype thanks to a 14 h heat treatment did not significantly affect long-term memory (perineural glia NP6293-Gal4: n = 28, $F_{2,83} = 2.208$, $P = 0.1165$; subperineural glia Moody-Gal4: n = 14, $F_{2,41} = 0.09845$, $P = 0.9065$; ensheathing glia MZ0709-Gal4: n = 18, $F_{2,53} = 2.868$, $P = 0.0660$; Alrm-Gal4: n = 16, $F_{2,47} = 0.1552$, $P = 0.8567$). C. When blocking all glia for 14 h after the 5 x spaced conditioning, flies exhibited a long-term memory defect, that was rescued by preventing cortex glial blockade thanks to the R54H02-Gal80 transgene (n = 16/17; $F_{5,96} = 7.195$, $P < 0.0001$). D. While flies expressing *shibire*^{ts} in cortex glia but not subjected to a switch at restrictive temperature displayed a normal long-term memory (n = 14; $F_{2,41} = 0.2530$, $P = 0.7778$), blocking cortex glia for 6 h (n = 19; $F_{2,56} = 8.645$, $P = 0.0006$) and even for only 2 h (n = 14; $F_{2,41} = 3.930$, $P = 0.0279$) after the conditioning yielded a long-term memory defect. Similarly, blocking cortex glia thanks to a second specific Gal4 driver, NP2222-Gal4, during 6 h after the conditioning, gave a similar long-term memory defect after a 5 x spaced training (n = 17/18; $F_{2,51} = 9.135$, $P = 0.0004$). This defect was not displayed by the same flies which were not subjected to restrictive temperature treatment (n = 19/20; $F_{2,58} = 5.361$, $P = 0.0074$, post-hoc test not significant between one control and the genotype of interest). The scheme above each memory graph indicates the time-course of temperature treatments. E. Blocking cortex glia for 6 h after a 5 x massed training did not affect long-term anesthesia resistant memory formation (54H02-Gal4: n = 27; $F_{2,74} = 2.239$, $P = 0.1139$; NP2222-Gal4: n = 18; $F_{2,53} = 1.213$, $P = 0.3059$), demonstrating that this mechanism is involved specifically during long-term memory formation. F. Repo-Gal4>UAS-Shi^{ts} flies trained with a one-cycle protocol (1x) at 33°C and tested at the restrictive temperature of 33°C after 3h at 33°C did not display middle-term memory defect (n = 12, $F_{2,35} = 0.5619$, $P = 0.5755$).

Altogether, these results demonstrate that cortex glia are involved specifically in long-term memory formation, during a time window as short as 2 h after the conditioning, likely through a mechanism involving vesicular release such as the exocytosis of signaling molecules, or the targeting of transporters at the membrane.

II. 2. Cortex glia release insulin-like peptide 4 during long-term memory consolidation

From a morphological perspective, the involvement of cortex glia can be surprising, as these cells only interact with neurons at the level of the neuronal soma. Noteworthy, during long-term memory consolidation, specific events are triggered in neuronal soma, such as *de novo* protein synthesis (Flexner et al., 1962; Tully et al., 1994), but also genetic and epigenetic regulations (Yin et al., 1994). Moreover, long-term memory is energetically costly (Mery and Kawecki, 2005), and its consolidation is gated by the metabolic state of the organism (Plaçais and Preat, 2013). It is thus possible that a metabolic signal is provided broadly to neurons at the somatic level, in order to gate costly long-term memory formation. We hypothesized that cortex glia could be involved either by providing a metabolic signal, or in the direct metabolic support of Kenyon cells soma.

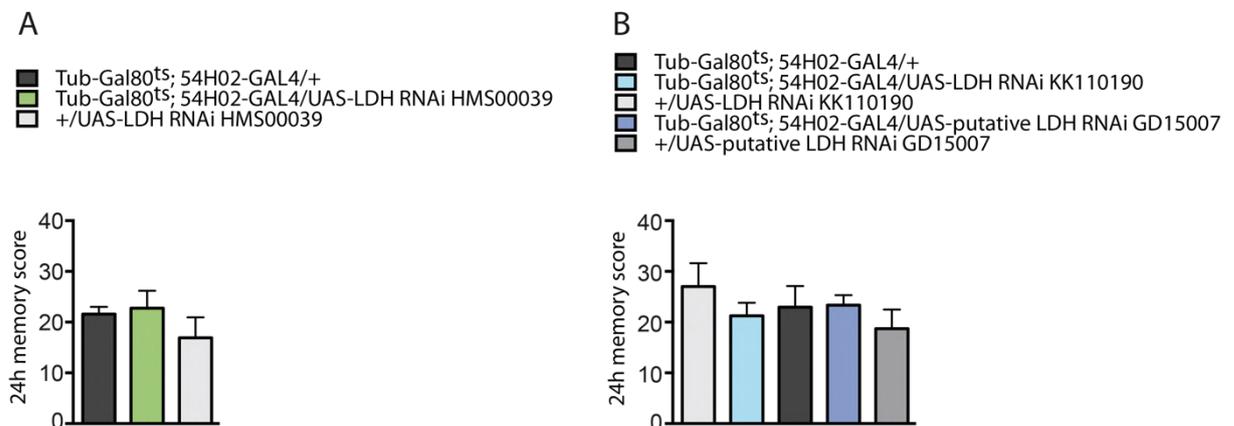


Figure 44. LDH and putative LDH (CG13334) knockdown in the adult cortex glia did not affect long-term memory performance. A. $n = 12$; $F_{2,35} = 0.947$, $P = 0.3982$. B $n = 17$; $F_{4,84} = 0.7279$, $P = 0.5755$.

We first investigated if cortex glia could support Kenyon cells through a lactate supply, as already known in mammals for astrocytes (Suzuki et al., 2011; Gao et al., 2016). Thus, we decided to knock-down lactate dehydrogenase, the enzyme that catalyzes the conversion of pyruvate into lactate - and the other way around - in the

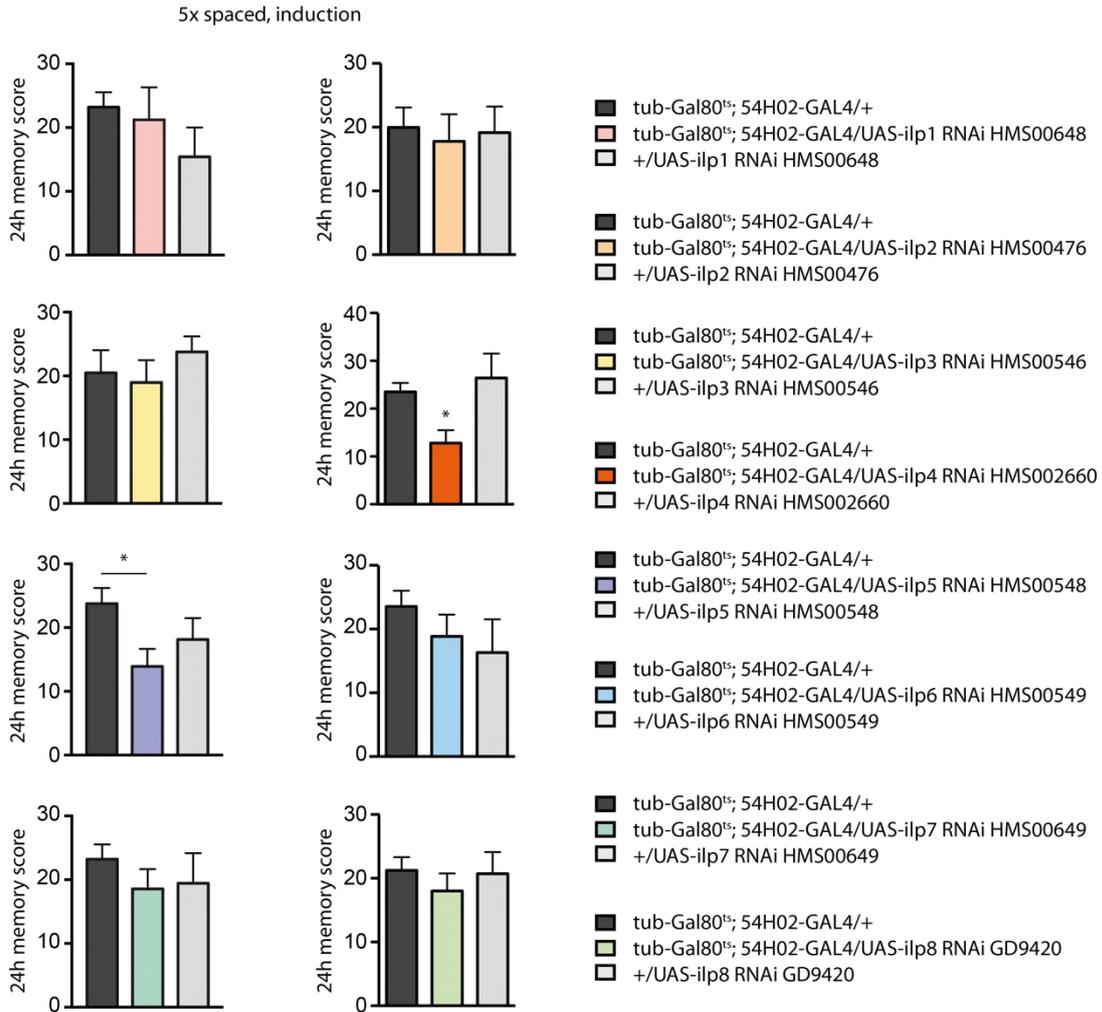
adult cortex glia. We found that knocking-down the gene that encodes *Drosophila* LDH (CG10160) in the adult cortex glia thanks to two non-overlapping RNAi did not affect flies long-term memory performance (Figure 44 A). We additionally knocked-down a putative lactate dehydrogenase (CG13334) in the adult cortex glia, but it did not disturb long-term memory formation either (Figure 44 B).

Alternatively, cortex glia could release a signal necessary for long-term memory formation. Not much had been described in the literature about cortex glia. These cells release the neurotrophin Spätzle 3 that is required for their morphology in larval stages (Coutinho-Budd et al., 2017) and insulin-like peptide 6, a signal necessary for neuroblasts exit from quiescence during development (Sousa-Nunes et al., 2011).

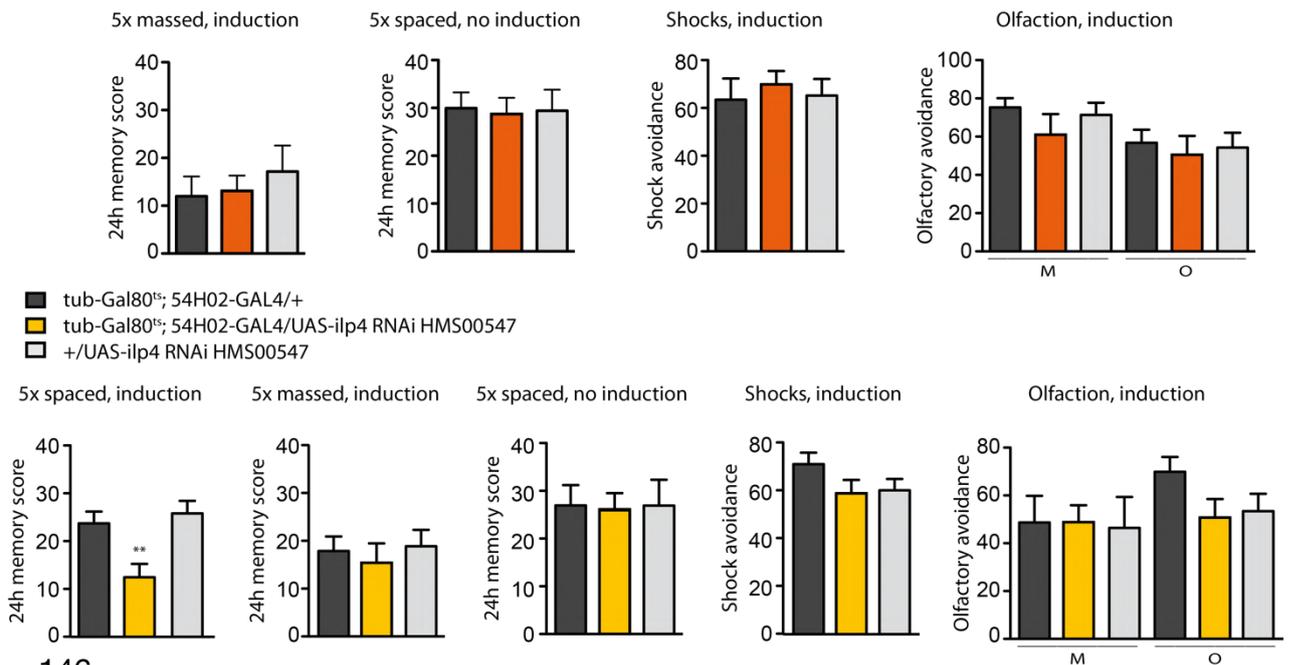
As we hypothesized that cortex glia were directly or indirectly involved in energy metabolism regulation, we asked if cortex glia released an insulin signal during long-term memory formation and decided to knock-down, specifically in cortex glia and at adulthood, the eight different genes encoding insulin-like peptides (Ilp) in the flies genome (Grönke et al., 2010). Interestingly, we found that knocking-down Ilp4 in the adult cortex glia phenocopied the previous cortex glial blockade, while the knockdown of other Ilps did not affect long-term memory formation (Figure 45 A). We confirmed this result with a second specific, non-overlapping RNAi directed against Ilp4 (Figure 45 B). Ilp4 was specifically necessary for long-term memory in the adult cortex glia, as its knockdown did not affect memory formed after a massed training (Figure 45 B). Long-term memory in the genotypic controls, as well as olfactory and shock acuity after RNAi induction were normal (Figure 45 B). Thus, these data suggest that cortex glia release Ilp4 during long-term memory formation.

Interestingly, Ilp4 was described as one of the two most conserved insulin-like peptides among *Drosophila* species (Grönke et al., 2010), but until now, no role was attributed to the gene during development or in adults. We examined its distribution in the adult brain thanks to a specific antibody that was previously published (Grönke et al., 2010). Indeed, Ilp4 distribution co-localized with cortex glia (Figure 45 C), and knocking-down Ilp4 in cortex glia decreased Ilp4 staining in the adult brain (Figure 45 D), supporting our behavioral data. Together with the previous *shibire^{ts}* experiment, these results show that cortex glia release an insulin-like signal, Ilp4, early during memory consolidation, a process that is required for long-term memory formation.

A



B



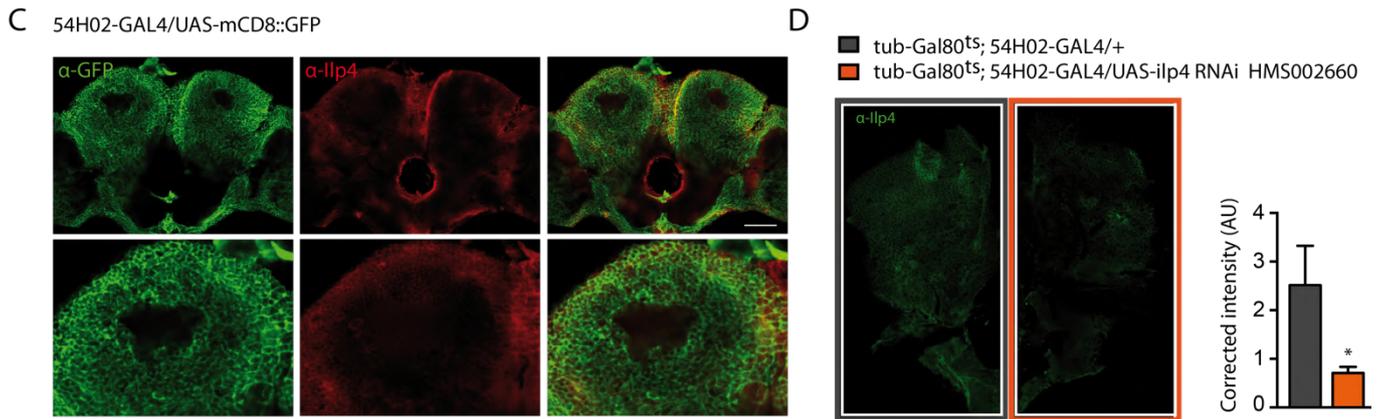


Figure 45. Ilp4 expression in cortex glia is necessary for long-term memory. A. Knocking-down all insulin-like peptides - except Ilp4 - in the adult cortex glia did not affect long-term memory formation after a 5 x spaced training (Ilp1: n = 13/14, $F_{2,40} = 0.9332$, $P = 0.4021$; Ilp2: n = 21/22, $F_{2,63} = 0.08445$, $P = 0.9191$; Ilp3: n = 21, $F_{2,72} = 0.696$, Ilp4: n = 18, $F_{2,53} = 4.244$, $P = 0.0197$, $P = 0.5020$; Ilp5: n = 31, $F_{2,92} = 2.957$, $P = 0.0570$; Ilp6: n = 17, $F_{2,50} = 0.5651$, $P = 0.5721$). B. Ilp4 knockdown in the adult cortex glia disturbed long-term memory formation after a 5 x spaced training specifically (RNAi HMS00547: n = 23, $F_{2,68} = 7.585$, $P = 0.0011$), but did not affect memory formation after a 5 x massed training (RNAi HMS02660: n = 16, $F_{2,47} = 0.3839$, $P = 0.6834$; RNAi HMS00547: n = 19, $F_{2,56} = 0.2522$, $P = 0.7780$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls (RNAi HMS02660: n = 17, $F_{2,50} = 0.02743$, $P = 0.9730$; RNAi HMS00547: n = 12, $F_{2,35} = 0.01572$, $P = 0.9844$). Shock reactivity (RNAi HMS02660: n = 12, $F_{2,35} = 0.2077$, $P = 0.8135$; RNAi HMS00547: n = 12, $F_{2,35} = 1.758$, $P = 0.1882$) and olfactory acuity (RNAi HMS02660: M: n = 12, $F_{2,35} = 0.9113$, $P = 0.4119$, O: n = 12, $F_{2,35} = 0.1483$, $P = 0.8628$; RNAi HMS00547: M: n = 12, $F_{2,35} = 0.01595$, $P = 0.9842$, O: n = 12, $F_{2,35} = 2.138$, $P = 0.1340$) after Ilp4 knockdown were normal. B. Brains from 54H02-Gal4>UAS-mCD8::GFP were dissected and immunostained thanks to anti-GFP (green) and anti-IIP4 (red) antibodies. Ilp4 colocalized with cortex glia. Lower images show higher magnification of the calyx region in the whole-brain images showed above. Scale bar: 40 μm . C. Representative images of brains from the above-mentioned genotypes and immunostained thanks to an anti-IIP4 antibody. Ilp4 knockdown significantly reduced the corrected fluorescence intensity in the brains as compared to controls (two-tailed unpaired t test, n = 5, $t_8 = 2.283$, $P = 0.0259$).

II. 3. Ilp4 acts autocrinally on cortex glia

Insulin-like peptides are known to have neuronal (Zhao and Campos, 2012; Tsao et al., 2018) and glial (Avet-Rochex et al., 2012; Spéder and Brand, 2018) targets in the fly brain. Interestingly, acute insulin signaling is required specifically for long-term memory formation (Chambers et al., 2015). Moreover, the expression of the insulin receptor, InR, in the mushroom body, is required for normal learning and memory (Chambers et al., 2015). Still, the consequences of an acute InR knockdown in the adult mushroom body has never been studied on long-term memory. As suggested in the literature, we hypothesized that Ilp4 released by cortex glia would activate insulin signaling in the mushroom body. Thus, we expressed an InR RNAi in the adult mushroom body and checked for long-term memory performance (Figure 46 A). Unexpectedly, we did not measure any memory defect, suggesting that the mushroom body was not the target of Ilp4, and that contrary to what was stated in the literature without clear demonstration, insulin signaling might not be acutely necessary in the mushroom body for long-term memory.

In order to find candidate targets of Ilp4, we analyzed InR expression in the adult brain (Figure 46 B). InR was surprisingly highly expressed in cortex glial cells, a data further confirmed by single-cell transcriptomic results (Davie et al., 2018) (Figure 46 C). Therefore, we checked if Ilp4 could autocrinally activate InR in cortex glia. Indeed, InR knockdown in the adult cortex glia led to a specific long-term memory defect (Figure 46 D). We further confirmed this effect thanks to a second, non-overlapping InR RNAi. Together, these data strongly suggest that Ilp4 acts autocrinally to activate cortex glial cells during long-term memory consolidation.

II. 4. Cortex glia are activated by a cholinergic signal during memory formation

Our results so far indicated that cortex glia is engaged in a self-sustained state after spaced training, but we did not know at this stage how glia is initially activated. We expected that neuronal activity, in particular in the mushroom body intrinsic neurons Kenyon cells, may activate cortex glia. As Kenyon cells that are relevant for memory formation are cholinergic (Barnstedt et al., 2016), and, more generally, as acetylcholine is the main excitatory neurotransmitter in *Drosophila*, we sought if an acetylcholine receptor was required in cortex glia for long-term memory. Noteworthy,

the nicotinic receptor nAChR α 7 is expressed in mammalian astrocytes (Shen and Yakel, 2012; Liu et al., 2015; Papouin et al., 2017) and microglia (Sadigh-Eteghad et al., 2016). Thus, we investigated if the expression of its orthologous gene in *Drosophila* was necessary in cortex glia for long-term memory formation. Indeed, knocking-down nAChR α 7 in the adult cortex glia strongly impaired long-term memory performance (Figure 47 A). This phenotype was mimicked when using a second, non-overlapping RNAi. The same genetic manipulation did not affect long-term anesthesia resistant memory or 3 h memory, demonstrating that nAChR α 7 is involved specifically in long-term memory formation. Finally, long-term memory was not impaired when RNAi expression was not induced, and sensory acuity was not affected by nAChR α 7 knockdown. All these data evidence that cortex glia must be activated by a cholinergic signal acting on nAChR α 7 receptors during long-term memory formation.

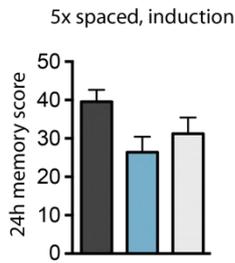
The nAChR α 7 subunit exhibits remarkably high calcium permeability in mammals (Shen and Yakel, 2012). As an insight into the consequence of the receptor activation in cortex glia, we measured calcium levels in cortex glia in response to a nicotinic stimulation, *in vivo* in the fly brain. Following a 30 s nicotine perfusion at 50 μ M, we measured a sustained increase in GCaMP6f fluorescence, demonstrating an increase in calcium concentration in cortex glia (Figure 47 B). Importantly, we observed a significantly reduced calcium burst when nAChR α 7 was knocked-down. This provides a functional evidence that nAChR α 7 can indeed mediate an increased calcium signaling in cortex glia upon its activation.

As we expected that this cholinergic activation could originate from Kenyon cells, we inhibited neurotransmission in these neurons during the same 2 h time window as cortex glia involvement (Figure 43 D) thanks to the *shibire^{ts}* transgene. Indeed, blocking Kenyon cells for 2 h after 5 x spaced conditioning significantly decreased long-term memory formation (Figure 47 C) but did not affect long-term anesthesia resistant memory.

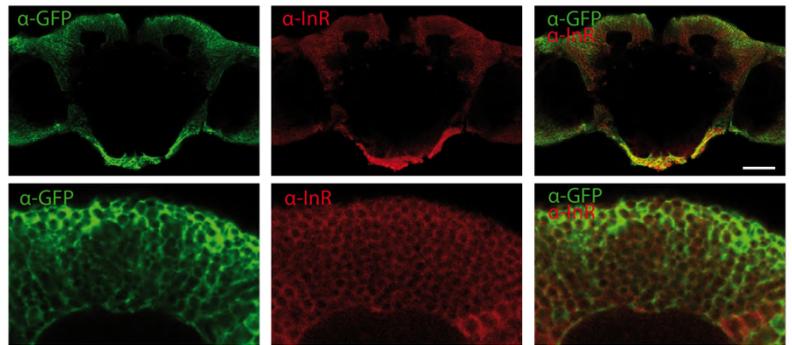
In a nutshell, these data indicate that a cholinergic signal, maybe released from Kenyon cells, activates cortex glia during long-term memory formation through a calcium burst. In mammals, beta-pancreatic cells release insulin following an increase in intracellular calcium concentration (Wollheim and Sharp, 1981). It is thus conceivable that a calcium elevation in cortex glia triggers insulin-like peptide release.

A

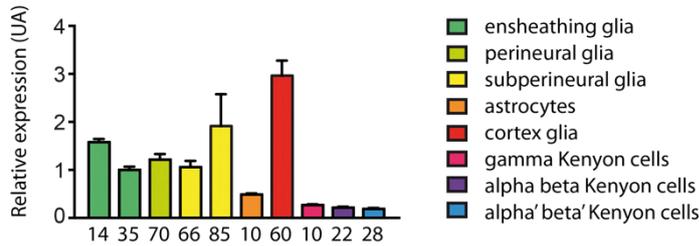
- tub-Gal80^{ts}; VT30559-GAL4/+
- tub-Gal80^{ts}; VT30559-GAL4/UAS-InR RNAi HMS03166
- +/UAS-InR RNAi HMS03166



B 54H02-Gal4>UAS-mCD8::GFP

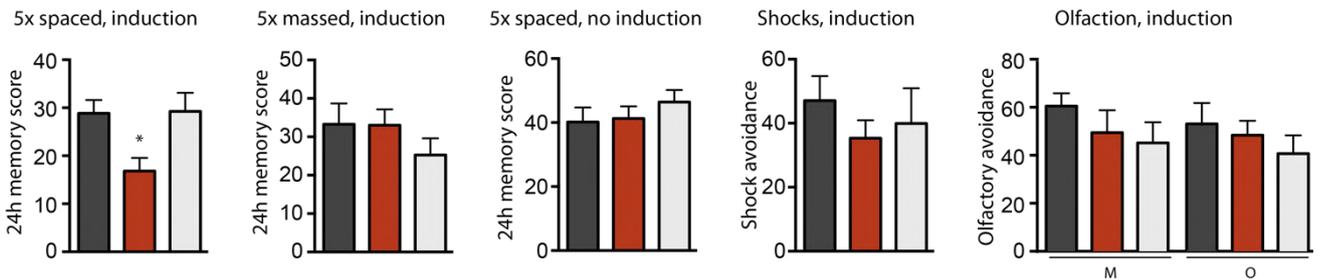


C



D

- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-InR RNAi HMS03166
- +/UAS-InR RNAi HMS03166



- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-InR RNAi GD992
- +/UAS-InR RNAi GD992

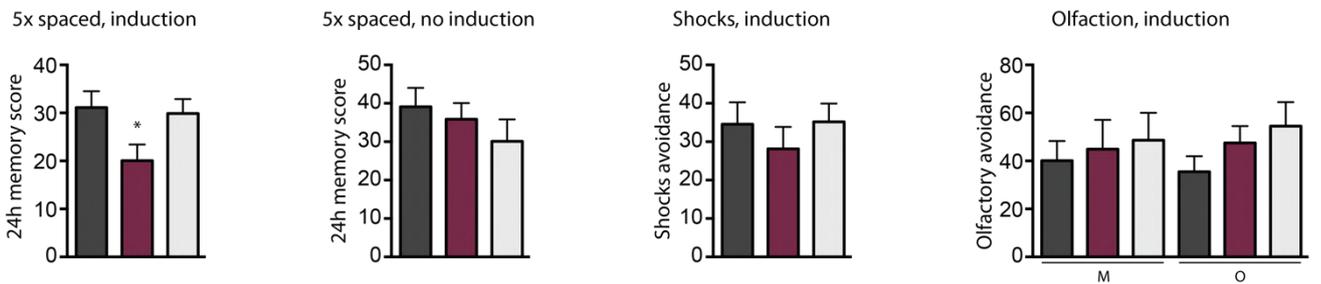
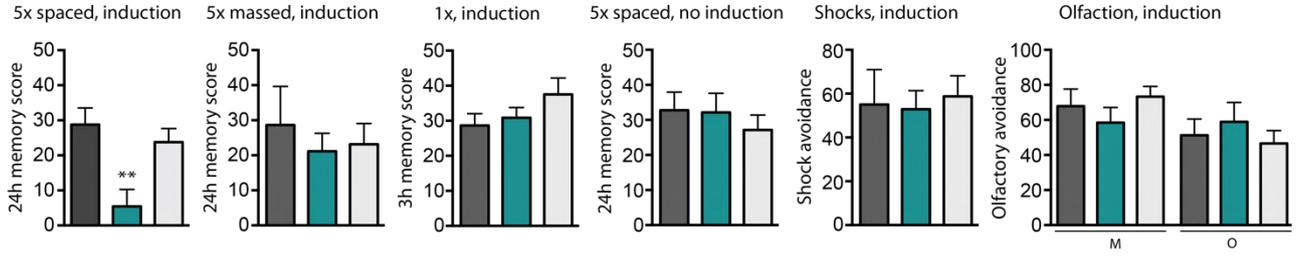


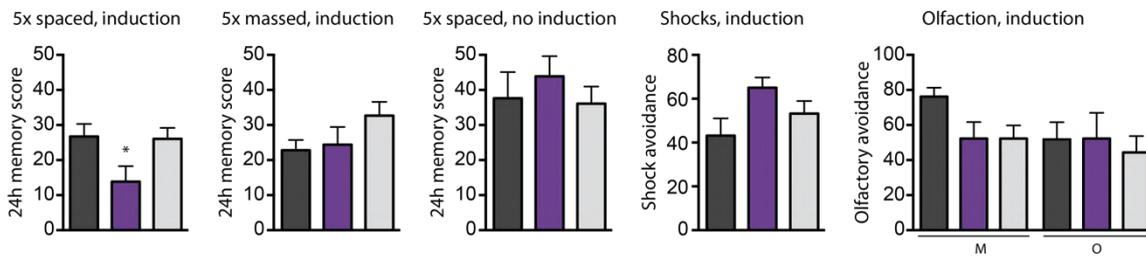
Figure 46. InR expression in the adult cortex glia is necessary for long-term memory. A. InR knockdown in the adult mushroom body did not affect long-term memory performance ($n = 22$, $F_{2,65} = 3.049$, $P = 0.0545$). B. Brains from 54H02-Gal4>UAS-mCD8::GFP were dissected and immunostained thanks to anti-GFP (green) and anti-InR (red) antibodies. InR strongly colocalized with cortex glial membrane. Lower images show higher magnification of the region above the calyx in the whole-brain images showed above. Scale bar: 40 μm . C. Relative expression of InR in various cell clusters according to Davie et al., 2018. The number of the cluster in the initial study is indicated under each cluster. D. InR knockdown in the adult cortex glia disrupted long-term memory as compared to the genotypic controls after a 5 x spaced training (RNAi HMS03166: $n = 24$, $F_{2,71} = 4.951$, $P = 0.0098$; RNAi GD992: $n = 30$, $F_{2,85} = 3.417$, $P = 0.0375$), but did not affect long-term anesthesia-resistant memory after a 5 x massed training (RNAi HMS03166: $n = 16$, $F_{2,47} = 0.9415$, $P = 0.3976$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls (RNAi HMS03166: $n = 18$, $F_{2,53} = 0.6859$, $P = 0.5082$; RNAi GD992: $n = 12$, $F_{2,35} = 0.8274$, $P = 0.4461$). Shock reactivity (RNAi HMS03166: $n = 12$, $F_{2,35} = 0.5004$, $P = 0.6108$; RNAi GD992: $n = 16$, $F_{2,47} = 0.521$, $P = 0.5974$) and olfactory acuity (RNAi HMS03166: M: $n = 12$, $F_{2,35} = 0.9956$, $P = 0.3803$, O: $n = 12$, $F_{2,35} = 0.623$, $P = 0.5425$; RNAi GD992: M: $n = 8$, $F_{2,23} = 0.6661$, $P = 0.4868$, O: $n = 8$, $F_{2,23} = 1.451$, $P = 0.2702$) after InR knockdown were normal.

A

- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-nAChRa7 RNAi JF02570
- +/UAS-nAChRa7 RNAi JF02570

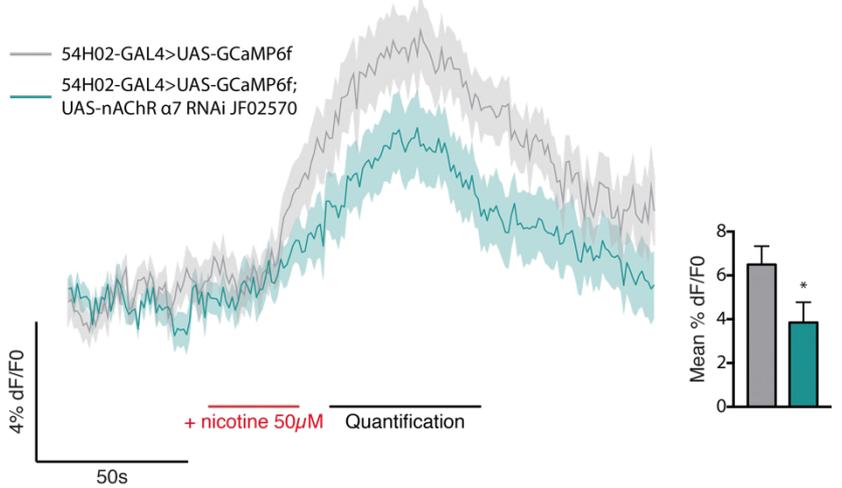
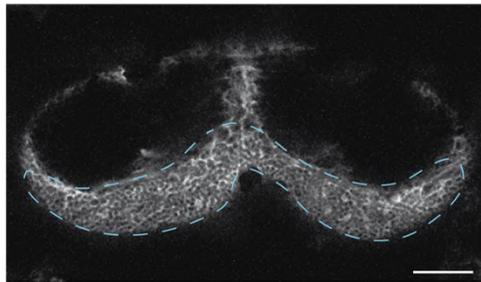


- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-nAChRa7 RNAi KK100756
- +/UAS-nAChRa7 RNAi KK100756



B

In vivo GCaMP6f imaging in cortex glia



C

- VT30559-GAL4/+
- VT30559-GAL4/UAS-Shi^{ts}
- +/UAS-Shi^{ts}

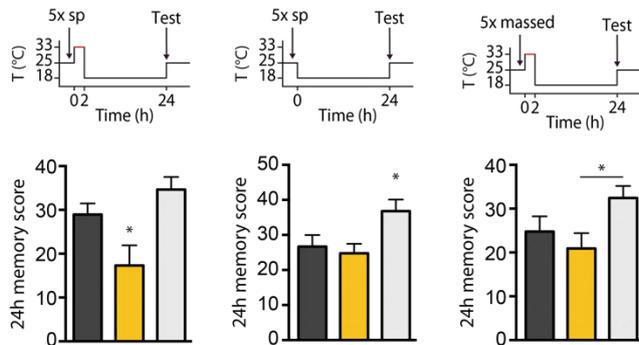


Figure 47. nAChR α 7 activation in cortex glia during memory formation triggers a calcium burst. A. nAChR α 7 knockdown in the adult cortex glia disrupted long-term memory as compared to the genotypic controls after a 5 x spaced training (RNAi JF02570: n = 8, $F_{2,23} = 7.461$, $P = 0.0030$; RNAi KK100756: n = 16, $F_{2,44} = 3.762$, $P = 0.0314$), but did not affect long-term anesthesia-resistant memory after a 5 x massed training (RNAi JF02570: n = 8, $F_{2,23} = 0.2475$, $P = 0.7830$; RNAi KK100756: n = 10, $F_{2,29} = 1.721$, $P = 0.1980$), or 3 h memory after only one cycle of training (RNAi JF02570: n = 16, $F_{2,46} = 1.522$, $P = 0.2295$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls (RNAi JF02570: n = 8, $F_{2,23} = 0.3796$, $P = 0.6888$; RNAi KK100756: n = 11, $F_{2,32} = 0.459$, $P = 0.6363$). Shock reactivity (RNAi JF02570: n = 8, $F_{2,23} = 0.06377$, $P = 0.9384$; RNAi KK100756: n = 16, $F_{2,47} = 3.091$, $P = 0.0552$) and olfactory acuity (RNAi JF02570: M: n = 12, $F_{2,35} = 0.8186$, $P = 0.4498$, O: n = 12, $F_{2,35} = 0.4401$, $P = 0.6477$; RNAi KK100756: M: n = 10, $F_{2,29} = 3.355$, $P = 0.05$, O: n = 10, $F_{2,29} = 0.1474$, $P = 0.8636$) after nAChR α 7 knockdown were normal. B. *In vivo* imaging of calcium dynamics in cortex glia following nicotine perfusion at 50 μ M for 30 s. Left: Representative image of the GCaMP6f signal in cortex glia in one focal plane, with the corresponding regions of interest in which calcium dynamics are analyzed (blue dashed lines). Right: Average traces of calcium response triggered by nicotine perfusion *in vivo* (n = 27/29). The 30 s period during which nicotine was perfused is indicated by a red line; the time window used for quantification is indicated by a black line. Quantification of the mean response during the 10 - 60 s after the end of nicotine perfusion for both genotypes is shown on the right. The calcium response following nicotine perfusion was decreased significantly with nAChR α 7 knockdown in cortex glia (two-tailed unpaired t test, n = 5, $t_{54} = 2.121$, $P = 0.0385$). C. Blocking neurotransmission in Kenyon cells in the flies of interest (VT30559-Gal4/UAS-Shi^{ts} flies) for 2 h after 5 x spaced conditioning decreased significantly long-term memory as compared to the controls (n = 22/23, $F_{2,66} = 6.402$, $P = 0.0029$). Long-term memory in the flies of interest was normal when not subjected to the restrictive temperature (n = 25/26, $F_{2,76} = 4.349$, $P = 0.0164$, post-hoc test not significant against one of the two controls). Blocking neurotransmission in Kenyon cells in the flies of interest after a massed training did not affect memory (n = 25/26, $F_{2,76} = 3.165$, $P = 0.0480$, post-hoc test not significant against one of the two controls).

II. 5. Cortex glia metabolism is involved in long-term memory

In *Drosophila* as in mammals, the major metabolic pathway regulated by insulin signaling is carbohydrate catabolism (Mattila and Hietakangas, 2017). We asked if indeed, insulin signaling in cortex glia triggered a regulation of carbohydrate metabolism. In insects, the main circulating carbohydrate is trehalose, a nonreducing disaccharide (Reyes-DelaTorre et al., 2012). Its degradation is catalyzed by trehalase, an enzyme that transforms trehalose into two molecules of glucose. It was previously described that in the fly brain, trehalase was mainly expressed in glial cells (Volkenhoff et al., 2015). We thus asked if indeed trehalase was expressed in cortex glia. We used a transgenic line (Mi{PT-GFSTF.2}Treh^{MI05512-GFSTF.2}) in which a protein trap cassette

was inserted in an intron of the endogenous Trehalase locus (Nagarkar-Jaiswal et al., 2015). This cassette contains a EGFP-FIAsH-StrepII-TEV-3xFlag ("GFSTF") construction. The cassette has been inserted in the appropriate coding frame with the Trehalase gene to allow for the expression of a EGFP-fused trehalase. Indeed, the enzyme showed an expression pattern that corresponded to cortex glia (Figure 48 A).

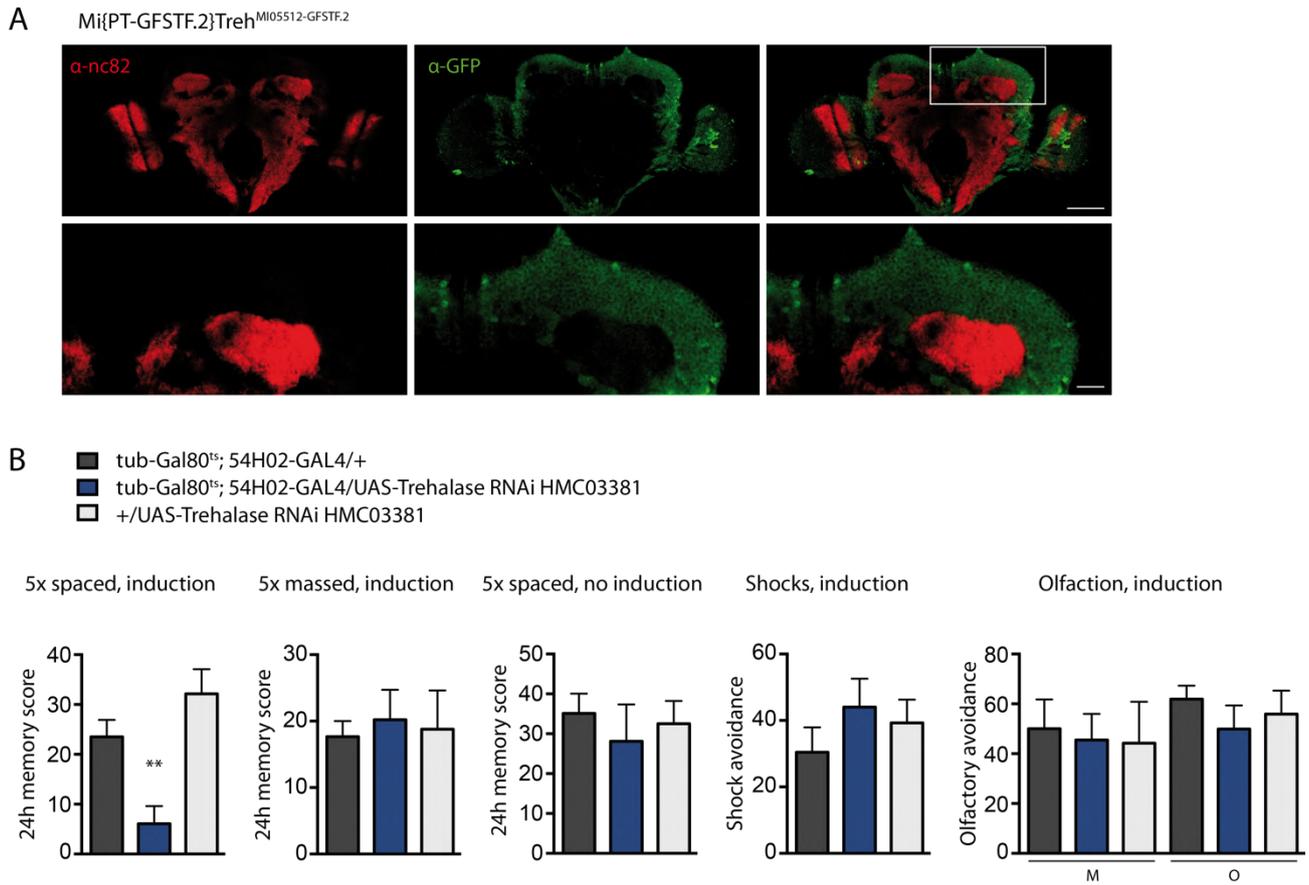


Figure 48. Trehalase is required in cortex glia for long-term memory. A. Analysis of GFP distribution pattern in dissected and stained brains from the Mi{PT-GFSTF.2}Treh^{MI05512-GFSTF.2} MiMiC line. GFP localized mainly to the cortex of the brain, in a pattern that strongly resembled that of cortex glia. The lower images show a zoom of the region boxed on the merge image of the entire brain. Scale bars: up, 40 μ m; down 10 μ m. B. Trehalase knockdown in the adult cortex glia disrupted long-term memory as compared to the genotypic controls after a 5 x spaced training (n = 10, $F_{2,35} = 10.98$, $P = 0.0002$), but did not affect long-term anesthesia-resistant memory after a 5 x massed training (n = 9, $F_{2,26} = 0.08254$, $P = 0.921$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls (n = 10, $F_{2,28} = 0.2702$, $P = 0.7653$). Shock reactivity (n = 16-18, $F_{2,51} = 0.8001$, $P = 0.4550$) and olfactory acuity (M: n = 8, $F_{2,23} = 0.05249$, $P = 0.9490$, O: n = 8, $F_{2,23} = 0.5195$, $P = 0.6023$) were not altered in the flies of interest.

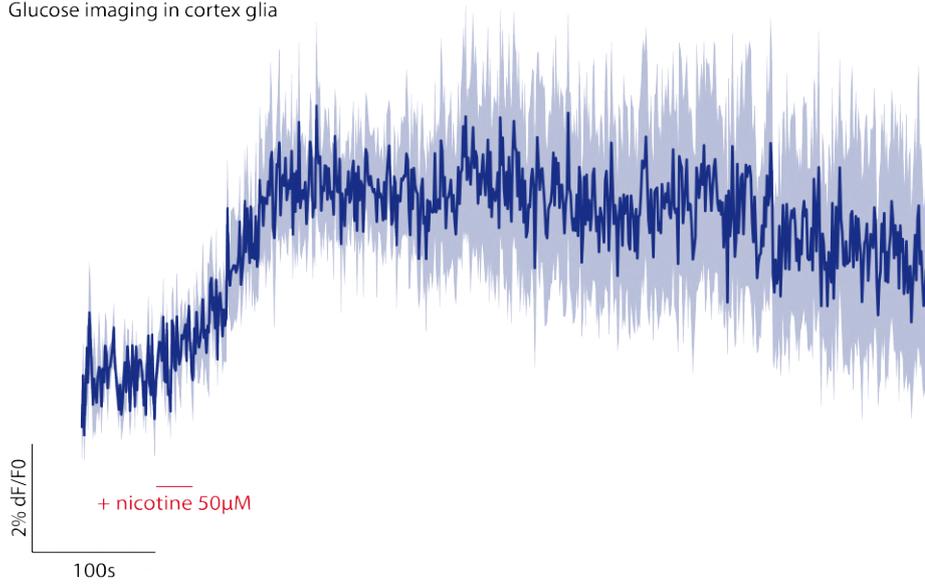
As insulin signaling can regulate trehalase expression and activity in insects (Satake et al., 1997; Xu et al., 2013), we expected that *Ilp4* autocrine signaling could target a modulation of trehalase activity in cortex glia during long-term memory formation. Indeed, knocking down trehalase in the adult cortex glia decreased long-term memory performance (Figure 48 B), while it did not affect long-term anesthesia-resistant memory. Finally, long-term memory was not impaired in the flies of interest when RNAi expression was not induced, and sensory acuity was not affected by the RNAi induction. All these data indicate that trehalase activity might be regulated by insulin signaling in cortex glia for proper long-term memory formation.

II. 6. Cortex glia activation increases glucose intracellular concentration

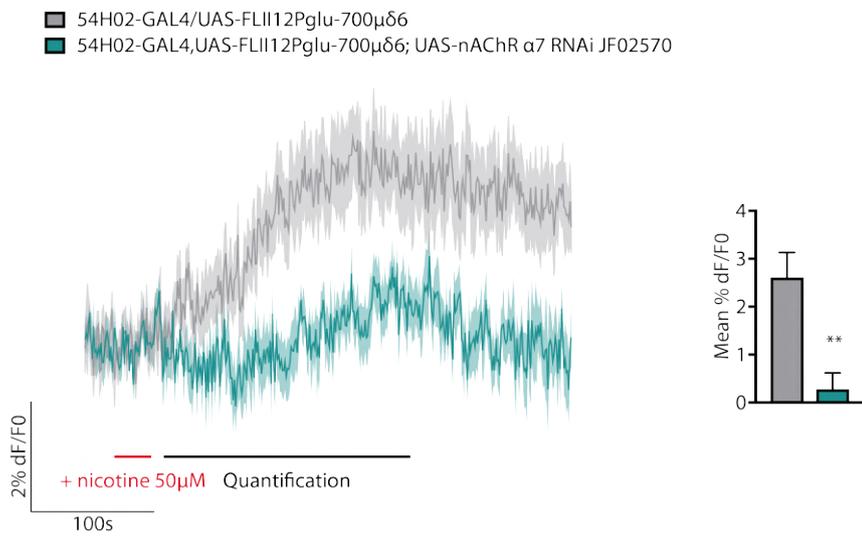
On the basis of all above-mentioned data, we drew a model of cortex glia activation. During long-term memory formation, a cholinergic signal activates cortex glia, which triggers calcium entry and calcium induced release of *Ilp4*. *Ilp4* then acts autocrinally on *InR*, which activates trehalase and results in increased glucose synthesis. If this model is true, then by activating cortex glia thanks to a cholinergic signal, one should observe an increased intracellular glucose concentration in glia. To test this hypothesis, we expressed in cortex glia a glucose sensor (Takanaga et al., 2008) which functionality was already validated in the fly brain in neurons (Plaçais et al., 2017) and glia, including cortex glia (Volkenhoff et al., 2018). In living flies, we applied the same nicotine treatment as for calcium imaging: we perfused nicotine in the brain bathing solution at 50 μ M for 30 s, and imaged glucose concentration over time. After nicotine stimulation, glucose concentration strikingly increased in cortex glia during the whole recording (Figure 49 A).

Remarkably, the increase in glucose concentration in cortex glia following nicotine perfusion was significantly lowered when knocking-down *nAChR α 7* or *InR* in the same cells (Figure 49 B and C). In agreement with our model, these results show that *InR* and the nicotinic receptors are involved in the same pathway, and that *InR* acts downstream of acetylcholine signaling for upregulating glucose synthesis.

A Glucose imaging in cortex glia



B Glucose imaging in cortex glia



C Glucose imaging in cortex glia

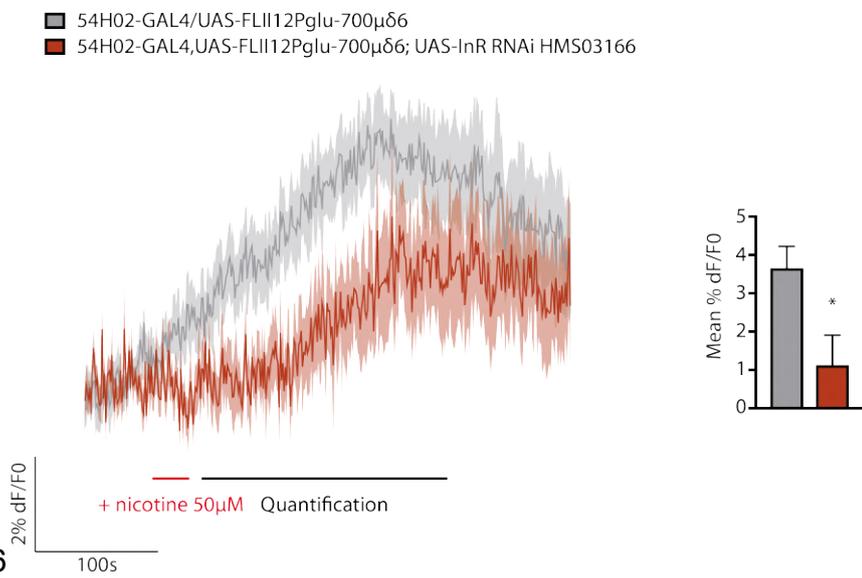


Figure 49. Activating cortex glia triggers an intracellular glucose elevation that depends on insulin-like signaling. A. We expressed a genetically-encoded glucose sensor in cortex glia, in 54H02-GAL4/UAS-FLII12Pglu-700 $\mu\delta$ 6 flies. After 30 s of 50 μ M nicotine stimulation, glucose concentration was sustainably increased in cortex glia for at least 10 min (n = 9). B. nAChR α 7 knockdown in cortex glia significantly lowered the nicotine-induced glucose elevation during the 200 s quantification window, starting 10 s after the end of nicotine perfusion (two-tailed unpaired t test, controls n = 13 flies, RNAi n = 8 flies, $t_{19} = 3.066$, P = 0.0064). C. InR knockdown in cortex glia significantly lowered the nicotine-induced glucose elevation during the 200 s quantification window (two-tailed unpaired t test, n = 10 flies, $t_{18} = 2.483$, P = 0.0231). B, C. The 30 s period during which nicotine is perfused is indicated by a red line; the 200 s time window (10 to 210 s after the end of the stimulation) used for quantification is indicated by a black line.

Altogether, our data support that during long-term memory formation, acetylcholine triggers nicotinic receptors activation in cortex glia and a subsequent sustained calcium transient. This could activate a calcium-induced release of IIP4, which autocrinally acts on cortex glia *via* InR. This insulin signaling ultimately upregulates trehalase, which activity supports an increase in glucose concentration in cortex glia.

II. 7. Cortex glia may support an increased glucose consumption in Kenyon cells soma

What are the consequences of such an increase in glucose synthesis in cortex glia? Two main hypotheses can be formulated: glucose synthesis in cortex glia supports 1/ an autonomous energy or anabolic demand, or 2/ the energy or anabolic demand of other cells. As pyruvate consumption in Kenyon cells axons was already shown to be increased during long-term memory formation (Plaçais et al., 2017), and as cortex glia enwrap the neuronal soma, we asked if cortex glia could provide glucose to neurons involved in long-term memory formation, i.e. to Kenyon cells soma.

First, we asked if glucose consumption was changed in Kenyon cells soma during long-term memory formation. We expressed the same glucose probe but this time in Kenyon cells, thanks to the VT30559-Gal4 driver. In order to measure the net glucose consumption in these cells, we blocked its synthesis and measured glucose concentration decrease over time which directly represents glucose consumption rate. As trehalose is the main circulating sugar and the main metabolite used by the brain, we chose to block glucose synthesis out of trehalose thanks to Validamycin A, a specific competitive inhibitor of trehalase that has previously been characterized as a potential pesticide in various insects (Zhao et al., 2016; Tang et al., 2017; Adhav et al., 2018). We thus blocked trehalase activity in the brain thanks to the injection of Validamycin A at 4 mM in the brain bathing solution during *in vivo* imaging of glucose concentration in Kenyon cells soma. Treatment with Validamycin A resulted in a decrease in intracellular glucose concentration over time. The slope of the decrease represented the glucose consumption rate that occurred in the cells right before inhibiting trehalase. Strikingly, after a 5 x spaced training, glucose concentration in

Kenyon cells soma decreased faster than after an unpaired conditioning that did not allow memory formation (Figure 50 A), suggesting that glucose was consumed at a higher rate in Kenyon cells soma during long-term memory formation.

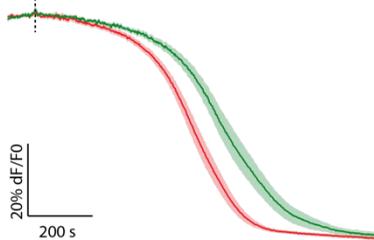
As Kenyon cells soma are tightly and completely enclosed by cortex glia, and as trehalase expression is necessary in cortex glia for long-term memory, we hypothesized that a glucose shuttle from cortex glia to neurons occurred during long-term memory formation. Consistently, knocking down the only described GLUT glucose transporter in the *Drosophila* genome, GLUT1, with Trip HMS02152 RNAi, in both Kenyon cells and cortex glia, resulted in long-term memory defects (Figure 50 B and D). We tried to reproduce this effect with a second non-overlapping RNAi, VDRC GD13326. Long-term memory formation was prevented when this RNAi was expressed in the adult cortex glia (Figure 50 D), but not in Kenyon cells (Figure 50 C). Interestingly, the two RNAis do not target the same Glut1 isoforms: HMS02152 targets all isoforms except isoform U, while GD13326 only targets isoforms L, R, S, T, U, V, W, and X. Thus, our data suggest that Kenyon cells and cortex glia do not express the same GLUT1 isoforms. Moreover, decreasing GLUT1 expression in cortex glia and Kenyon cells did not disturb anesthesia-resistant long-term memory or sensory acuity. Finally, long-term memory was normal when RNAi expression was not induced.

Overall, our results point out that during long-term memory formation, cortex glia synthesize glucose out of trehalose following a neuronal signal that triggers an autocrine amplifying mechanism (Figure 51). Glucose can then be shuttled to the demanding Kenyon cells soma.

A Glucose imaging in Kenyon Cells soma after training

- 5x spaced, unpaired
- 5x spaced, paired

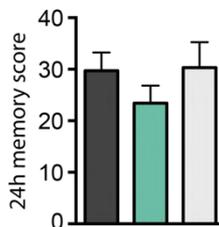
+ Validamycin A



C

- tub-Gal80^{ts}; VT30559-GAL4/+
- tub-Gal80^{ts}; VT30559-GAL4/UAS-GLUT1 RNAi GD13326
- +/UAS-GLUT1 RNAi GD13326

5x spaced, induction



B

- tub-Gal80^{ts}; VT30559-GAL4/+
- tub-Gal80^{ts}; VT30559-GAL4/UAS-GLUT1 RNAi HMS02152
- +/UAS-GLUT1 RNAi HMS02152

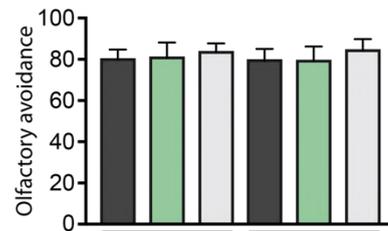
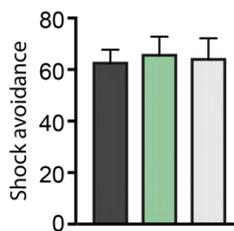
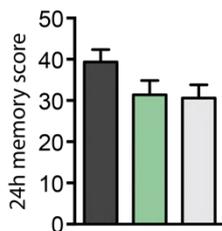
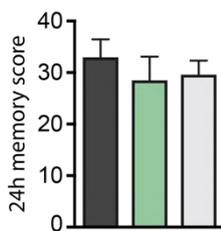
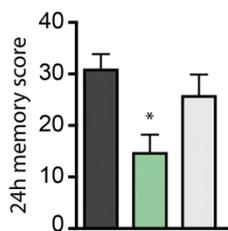
5x spaced, induction

5x massed, induction

5x spaced, no induction

Shocks, induction

Olfaction, induction



D

- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-GLUT1 RNAi GD13326
- +/UAS-GLUT1 RNAi GD13326

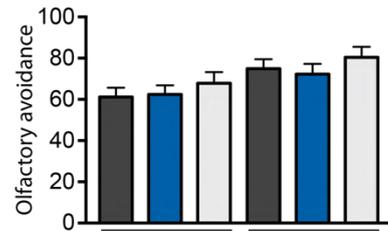
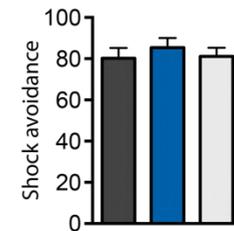
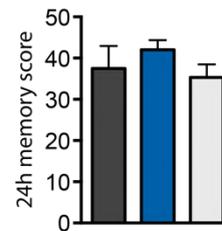
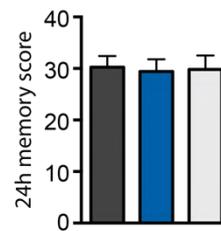
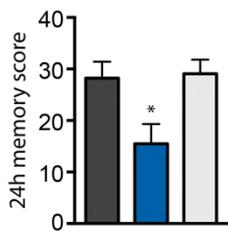
5x spaced, induction

5x massed, induction

5x spaced, no induction

Shocks, induction

Olfaction, induction



- tub-Gal80^{ts}; 54H02-GAL4/+
- tub-Gal80^{ts}; 54H02-GAL4/UAS-GLUT1 RNAi HMS02152
- +/UAS-GLUT1 RNAi HMS02152

5x spaced, induction

5x massed, induction

5x spaced, no induction

Shocks, induction

Olfaction, induction

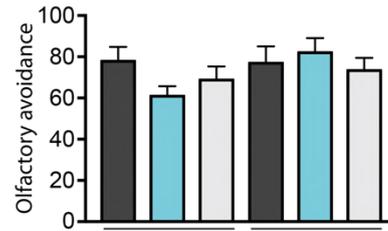
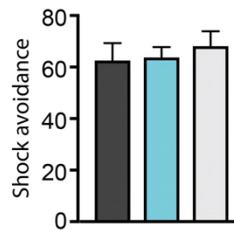
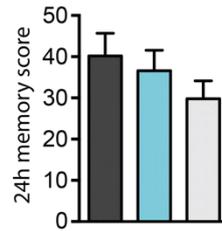
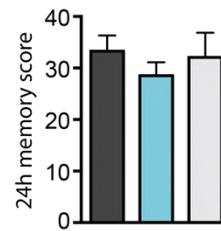
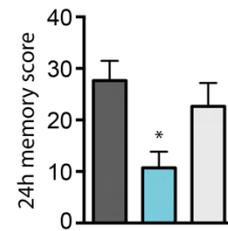


Figure 50. Glucose is shuttled from cortex glia to Kenyon cells soma during long-term memory formation. A. Glucose concentration in Kenyon cells soma decreased faster following Validamycin A injection (4 mM) in flies after a 5 x spaced, paired conditioning, that leads to long-term memory formation, as compared to flies conditioned with an unpaired training where electric shocks and odors are not simultaneously presented, showing that glucose consumption in Kenyon cells soma increased during long-term memory formation. B. GLUT1 knockdown of all isoforms (except isoform U) in the adult Kenyon cells disrupted long-term memory as compared to the genotypic controls after a 5 x spaced training ($n = 14/15$, $F_{2,43} = 5.012$, $P = 0.0113$), but did not affect long-term anesthesia-resistant memory after a 5 x massed training ($n = 10$, $F_{2,29} = 0.3506$, $P = 0.7074$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls ($n = 15$, $F_{2,44} = 2.223$, $P = 0.1209$). Shock reactivity ($n = 10$, $F_{2,29} = 0.04953$, $P = 0.9518$) and olfactory acuity (M: $n = 10$, $F_{2,29} = 0.1038$, $P = 0.9018$, O: $n = 10$, $F_{2,29} = 0.2154$, $P = 0.8076$) were not altered in the flies of interest. C. GLUT1 knockdown of isoforms L, R, S, T, U, V, W, and X in the adult Kenyon cells did not disrupt long-term memory ($n = 13/14$, $F_{2,40} = 0.9327$, $P = 0.4023$). D. GLUT1 knockdown of all isoforms (except isoform U) (RNAi HMS02152) and of isoforms L, R, S, T, U, V, W, and X (RNAi GD13326) in the adult cortex glia disrupted long-term memory as compared to the genotypic controls after a 5 x spaced training (RNAi HMS02152: $n = 17$, $F_{2,50} = 5.041$, $P = 0.0103$; RNAi GD13326: $n = 13/14$, $F_{2,39} = 5.397$, $P = 0.0088$), but did not affect long-term anesthesia-resistant memory after a 5 x massed training (RNAi HMS02152: $n = 10$, $F_{2,29} = 0.487$, $P = 0.6198$; RNAi GD13326: $n = 12$, $F_{2,35} = 0.03001$, $P = 0.9705$). Without RNAi induction, no long-term memory defect was displayed by the flies of interest as compared to the genotypic controls (RNAi HMS02152: $n = 10$, $F_{2,29} = 0.487$, $P = 0.6198$; RNAi GD13326: $n = 10$, $F_{2,29} = 0.7763$, $P = 0.4701$). Shock reactivity (RNAi HMS02152: $n = 10$, $F_{2,29} = 0.2285$, $P = 0.7972$; RNAi GD13326: $n = 10$, $F_{2,29} = 0.362$, $P = 0.6996$) and olfactory acuity (RNAi HMS02152: M: $n = 10$, $F_{2,29} = 1.877$, $P = 0.1725$, O: $n = 10$, $F_{2,29} = 0.3735$, $P = 0.6918$; RNAi GD13326: M: $n = 10$, $F_{2,29} = 0.5371$, $P = 0.5906$, O: $n = 10$, $F_{2,29} = 0.7249$, $P = 0.4935$) were not altered in the flies of interest.

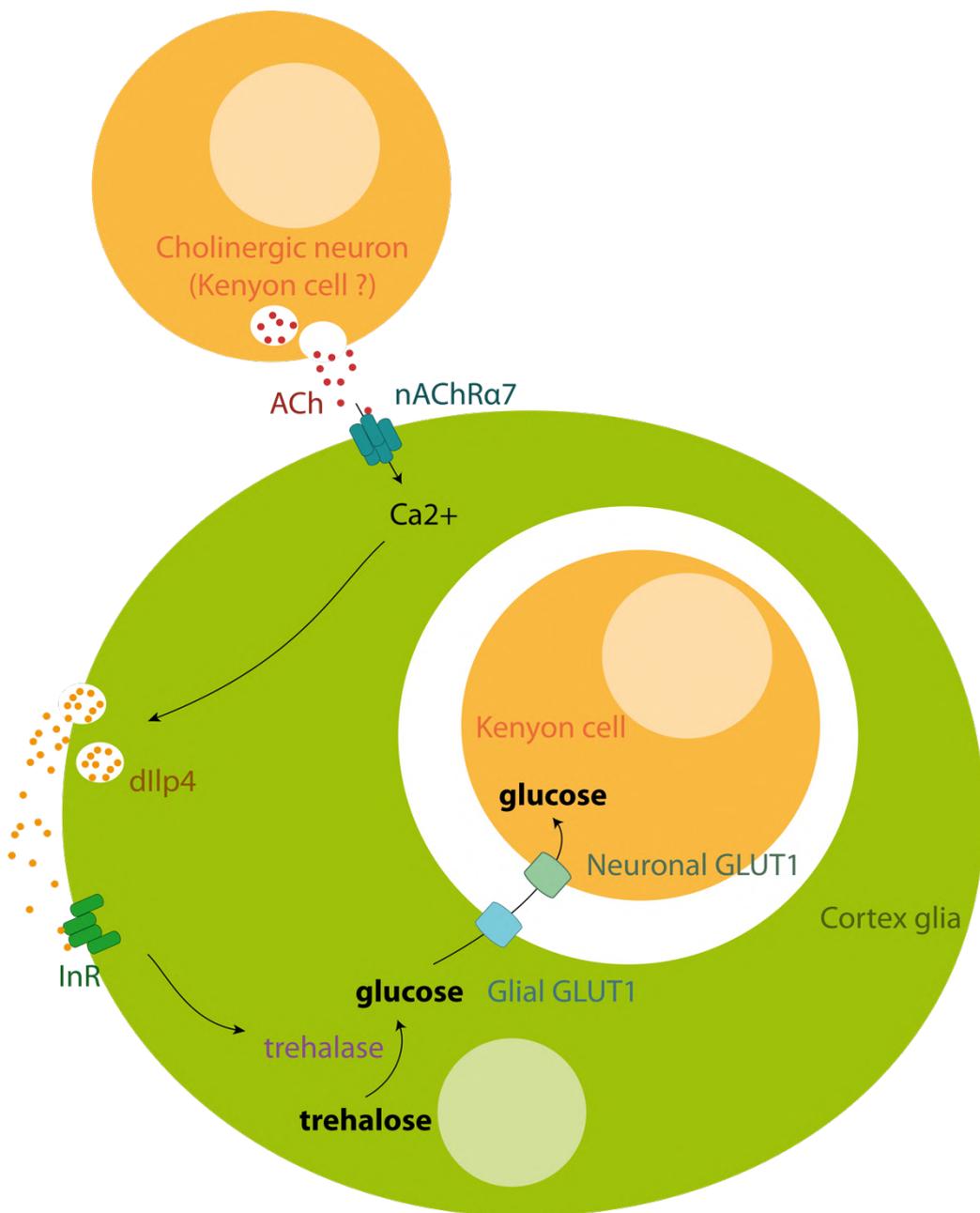


Figure 51. Model of cortex glia activation and of the resulting glucose shuttle to Kenyon cells soma during long-term memory formation. During long-term memory formation, a cholinergic signal that might originate from Kenyon cells activity activates cortex glia. This results in an intracellular calcium burst which triggers Ilp4 release that autocrinally acts on cortex glia through InR. Insulin signaling then activates trehalase, resulting in an increased glucose synthesis, and in a facilitated glucose shuttle from cortex glia to the demanding Kenyon cells soma.

Even though the neural circuits that convey the CS+ and US information towards the Mushroom body have been extensively described in the past few years, the neuronal events that gate the consolidation of an association in the long term are less understood. The work carried on here provides new evidence that metabolic fluxes in Kenyon cells are predictive of long-term memory consolidation, and that glial cells are actively involved in the molecular processes underlying long-term memory formation.

I. Modulation of Kenyon cells metabolism during long-term memory formation

I. 1. Mushroom-body neurons energy demand increases during LTM consolidation

Disrupting glucose import or pyruvate catabolism in Kenyon cells specifically disrupted long-term memory consolidation, demonstrating the role of carbohydrate metabolism in long-term memory formation. In line with our work, in the context of song learning in birds, mitochondrial complexes I and IV activities increase after one day of training experience (Dong et al., 2009). Accordingly, after a classical conditioning in honey bees, the expression of enzymes involved in oxidative metabolism are modulated in the brain (da Silva Menegasso et al., 2017). Moreover, we showed that artificially increasing mitochondrial metabolism in Kenyon cells triggers the consolidation of long-term memory after a training that, in regular conditions, does not allow the efficient formation of long-lasting protein-synthesis dependent memory (Article, Fig. 4e). Thus, it seems that energy metabolism in Kenyon cells not only supports but also drives memory consolidation.

Interestingly, it seems that the modulation of energy metabolism in Kenyon cells is not homogenous in all compartments. On the contrary, specific variations in metabolic fluxes can be measured in the mushroom body lobes, i.e. in Kenyon cells synaptic and axonal compartments, as compared to their soma.

1. 1. a. At the level of the lobes

In the Mushroom body lobes, we measured an increased pyruvate consumption in flies forming long-term memory early after the conditioning as compared to controls. On the other hand, we did not measure a difference in glucose utilization. This indicates that glycolysis is not modulated in Kenyon cells axons upon long-term memory formation, while mitochondrial metabolism is substantially increased through pyruvate consumption.

Noteworthy, in humans, pyruvate dehydrogenase deficiency induces epileptic seizures in a context of reduced basal cerebral activity measured by electroencephalogram. Moreover, in a mouse model with pyruvate dehydrogenase knockdown in the CNS, neurons exhibit decreased evoked local field potentials associated to a decreased spiking rate of fast-spiking neurons (Jakkamsetti et al., 2019). Thus, pyruvate metabolic rate in neurons likely restricts their excitability. For the time being, we did not assess if only enhancing metabolism in Kenyon cells increased their excitability. We could for example measure the amplitude of their calcium response, or the number of Kenyon cells that respond to an odor presentation, when artificially increasing their mitochondrial metabolism. If indeed they exhibit an enhanced calcium response to odors or if more Kenyon cells are recruited upon odor presentation when their energy metabolism is up-regulated, this could provide one basis for explaining the facilitation of long-term memory formation by allowing a recurrent activity in these neurons.

This enhanced metabolism may otherwise provide the energy needed to support synaptic activity and action potential firing, and/or an increased demand of neurotransmitter synthesis.

1. 1. b. At the level of the soma

Contrary to the mushroom body lobes, we measured an increase in glucose utilization in Kenyon cells soma early after long-term memory conditioning. What glucose is needed for is unknown, and further experiments will be needed to answer this question, but we can draw first hypotheses.

During memory consolidation, different events take place in the soma: the regulation of gene expression involving for instance CREB transcription factor (Yin et

al., 1994), *de novo* protein synthesis (Flexner et al., 1962; Tully et al., 1994), protein targeting and transport... Noteworthy, transcription and translation are energetically costly. Indeed, the transcription of each mRNA codon requires the hydrolysis of 8 ATP equivalents, and translation of each codon requires at least 4 additional ATP equivalents (Engl and Attwell, 2015). Moreover, during translation, several proteins are glycosylated, which also involves glucose utilization, but *a priori* to a far lower extent than transcription and translation. Accordingly, it has been reported that the machineries involved in gene regulation, transcription, protein synthesis, protein targeting and carbohydrates metabolism were enriched in the mice hippocampus at the moment when spatial memory performance drastically increases, i.e. at the steep-phase of their learning curve (Borovok et al., 2016). Thus, part of the glucose could be consumed by glycolysis followed or not by OxPhos in the soma, which would provide ATP to support these costly events.

Besides, specific anabolic needs in the soma could constrain the metabolic fluxes during long-term memory consolidation. For example, *de novo* protein synthesis occurs mainly in the soma and therefore, the need for extra pyruvate-derived amino acids (alanine...) or 3-phosphoglycerate derived amino acids (serine) could speed up glycolysis as its products are quickly consumed, while not accordingly speeding-up OxPhos in this compartment. Alternatively, glucose could fuel the pentose phosphate pathway and give rise to NADPH and nucleotides synthesis. NADPH is necessary in particular for lipid synthesis and antioxidant mechanisms. Interestingly, in cell cultures, it was suggested that the artificial increase in glycolytic rate in neurons decreased the pentose-phosphate metabolic rate, and that this triggered apoptosis (Herrero-Mendez et al., 2009). It was therefore suggested that a limited glycolysis, but a high pentose-phosphate pathway, occurred in neurons. Noteworthy, in this *in vitro* model, increasing the expression of GLUT1 in neurons increased glucose flux through the pentose phosphate pathway. It is thus possible that in our case, memory formation induces reactive oxygen species production, which has to be dampen by reducing reactions thanks to the use of NADPH synthesized *via* the pentose phosphate pathway.

Finally, it is also possible that glycolysis in the soma fuels the axon, and ultimately the synapse, with pyruvate, in a model where both compartments do not share the

same anabolic or energy needs but collaborate and display a complementary metabolism. Indeed, as astrocytic processes density and synaptic density are inversely correlated in the fly brain, in particular at the level of the calyx and mushroom body lobes (Kremer et al., 2017), it is probable that astrocytes do not directly support the localized energetic consumption of synaptic activity.

Thus, our data consistently show that glycolysis increases only in the soma during long-term memory consolidation, while pyruvate consumption increases in the mushroom body lobes, meaning in axons and synapses. If our hypothesis of an intracellular compartmentation of energy metabolism in Kenyon cells is true, we expect to measure a two-fold increase in pyruvate consumption in the mushroom body lobes as compared to glucose consumption in the neuronal soma. Unfortunately, the main limitation of our pyruvate and glucose imaging techniques is that we cannot quantify absolute metabolic rates. We can only compare if metabolic rates differ significantly between two conditions. The information of glucose or pyruvate absolute concentrations and consumption would be accessible with a fluorescence live-imaging microscope. This very recently developed setup is not currently accessible in the laboratory, even though the team is asking for a specific funding in order to acquire this device. Such information could be used to evaluate if the measured glucose and pyruvate consumptions fit to the theoretical energy needs of different cellular processes: protein synthesis, increased neuronal excitability... Moreover, we could then calculate if indeed a correlation links glucose consumption in the soma and pyruvate consumption in the lobes, which would favor the hypothesis of a complementary metabolism between these two compartments.

I. 2. Consequences of the metabolic switch in MB neurons on food intake

In conditioned flies, the increase in mushroom body lobes mitochondrial activity is correlated to an increase in food consumption. It is unlikely that this behavior is only driven by the energetic cost of Kenyon cells activity, which should not represent such a huge burden. Interestingly, in naive flies, only boosting mitochondrial metabolism in Kenyon cells induces an increase in food consumption (Article, Figure 4 F and G). It has already been reported that different mushroom body output neurons drive approach behavior (Aso et al., 2014b). Moreover, the activation of a subset of

mushroom body output neurons has recently been shown to trigger food consumption (Al-Anzi and Zinn, 2018). Consistently, blocking Kenyon cells activity disrupts feeding behavior in starved adult flies and in larvae, showing that their activity is necessary for food-seeking behaviors (Zhao and Campos, 2012; Tsao et al., 2018). Taken together, these results indicate that mushroom body activity is necessary and sufficient for driving feeding behavior, and that only enhancing mushroom body metabolism is sufficient for driving feeding. The capacity of the mushroom body integrating structure to directly modulate feeding upon memory formation might have been selected by natural selection. Indeed, an elevated mushroom body activity is a signature of contexts associated with food intake or potential energetic costs: during appetitive memory formation, which also engages mushroom body activity, the fly encounters food that can be immediately consumed. On the other hand, during long-term aversive memory formation, the CS+ becomes highly predictive of recurrent punishment, and the fly should increase its energetic stores in order to prepare its escape in case this aversive stimulus is presented again.

1. 3. Glucose memory enhancement

A few decades of research already described an effect called “glucose memory enhancement” (Messier, 2004). Glucose ingestion in humans (Benton et al., 1994) as well as injection in mice right after the conditioning, but not after a 1 h delay (Messier, 1997), increases memory performance. The ingestion of a similarly pleasurable but not energetic saccharin solution does not improve memory formation (Messier, 2004). Still, glucose injection rapidly increases insulin levels. As insulin injection with a normoglycemic clamp increases memory (Craft et al., 1999), it is not easy to determine if the increase in memory performance is only the consequence of insulin burst or of an additive effect of glucose and insulin. Still, directly injecting glucose into the mice hippocampus right before a spatial memory task enhances memory acquisition as compared to control mice (Canal, 2005). It suggests that local glucose supply to the brain may limit neuronal activity in certain brain areas such as the hippocampus during costly cognitive tasks. Our data provide a mechanism explaining how glucose providing is physiologically regulated to support long-term memory formation, although we still do not know by which pathways exactly this glucose is consumed.

II. Cholinergic activation of cortex glia

II. 1. Origin of the cholinergic input

Our study demonstrates that functional nAChR α 7-containing nicotinic receptors are expressed in cortex glial cells and are acutely required for long-term memory formation in *Drosophila* (Figure 47). Still, we could not identify with certainty the neurons from which acetylcholine is released.

As acetylcholine is readily broken-down by acetylcholinesterase in the extracellular medium, it is likely that this neurotransmitter is locally released close to cortex glia. Cortex glia are located in the periphery of the brain, in contact with neuronal soma and proximal neurites. Thus, acetylcholine acting on cortex glia could be released 1. by cholinergic neuronal soma, 2. by their proximal neurites, 3. by their synaptic terminals that target the periphery of the brain. Acetylcholine is the main excitatory neurotransmitter in the insect brain, and thus many neurons could activate cortex glia.

II. 1. a. Kenyon cells somatic transmission could activate cortex glia

Mushroom body neurons that are involved in memory formation are cholinergic (Barnstedt et al., 2016). As cortex glia are in direct contact with Kenyon cells soma, it is possible that cortex glia are activated by a somatic release of acetylcholine from Kenyon cells.

Such a somatic release of neurotransmitter has been studied in the leech central nervous system, where Retzius neurons release serotonin at the somatic level. This signal can then depolarize neighboring glia (De-Miguel et al., 2015). Moreover, in mammals, it has been shown that glial cells in contact with neuronal soma also express nicotinic receptors. In the mammalian dorsal root ganglia, satellite glial cells enwrap sensory neurons soma as do cortex glia in flies (Hanani, 2005). Interestingly, these peripheral neurons display the same morphology as *Drosophila* neurons, i.e. a unique neurite that extends from the soma. It then divides into two branches, one that forms the sensory endings in the periphery and the other one that extends towards the CNS. As their membranes are separated by only 20 nm, sensory neurons and

satellite glia can establish close interactions with reciprocal signaling. Interestingly, even though sensory neurons are not cholinergic, they express choline acetyl transferase as well as vesicular acetylcholine transporter, and putative cholinergic vesicles have been identified in sensory neurons soma; on the other hand, satellite glia express acetylcholine receptors, which might allow them to communicate (Hanani, 2005). Moreover, carbachol perfusion on *ex vivo* dorsal root ganglia preparations elicits a calcium response in satellite glial cells, confirming the functionality of nicotinic receptors at their surface (Yasmine Rabah, personal communication). Finally, in the parasympathetic ganglia, the postsynaptic neurons which soma are enwrapped by satellite glia can release acetylcholine at the somatic level through a synapse-independent pathway (Johnson and Pilar, 1980). It is possible that a similar mechanism supports neuronal soma communication with cortex glia in *Drosophila*.

Consistent with this hypothesis, blocking Kenyon cells neurotransmission during the time window of cortex glia involvement induces a long-term memory defect (Figure 47 C). Still, we did not know any protein that was involved specifically in neurotransmitter somatic release and thus could not knock it down; our manipulation blocked all types of neurotransmission from Kenyon cells, not only acetylcholine release that could occur in the soma. It is likely that beside a putative somatic neurotransmission, affecting Kenyon cells synaptic activity right after the conditioning disturbs memory formation by preventing a recurrent activity in the network.

In the near future, different experiments might help to answer if indeed, mushroom body neurons could activate cortex glia. For instance, it would be possible to express the temperature sensitive TrPA1 channel in Kenyon cells and GCaMP6f in cortex glia. Thanks to these genetic manipulations, one could activate Kenyon cells by only switching flies at high temperature, and record calcium levels in cortex glia. If the cholinergic signal originates from Kenyon cells soma, and if TrPA1 is sufficient to activate a somatic release of acetylcholine, we would expect that calcium level increase locally around Kenyon cells soma. Still, such a manipulation could also fail to elicit a calcium elevation in cortex glia if it does not trigger the appropriate neurotransmission such as a somatic neurotransmitter release.

II. 1. b. Other cholinergic sources that could underlie cortex glia cholinergic activation

Alternatively, cortex glia could be activated by acetylcholine released from cholinergic neurons at the level of the proximal neurite or from synapses located close enough to the cortical region. Interestingly, DmSLC22A knockdown in Kenyon cells enhances 3 h memory, and therefore DmSLC22A has recently been described as a memory suppressor gene (Gai et al., 2016). This gene encodes an acetylcholine transporter and is detected in Kenyon cells with immunohistochemistry, in particular at the level of their dendrites located in the calyx, quite close to cortex glia. It is thought that this transporter limits acetylcholine action from olfactory projection neurons to Kenyon cells, but it could also prevent a local diffusion of projection neurons-released acetylcholine towards the cortex where it could activate cortex glia. A single cycle of conditioning causes new projection neuron synapses to respond to the odor along with those normally activated prior to conditioning (Yu et al., 2004). After a spaced training, a consequently enhanced acetylcholine release from projection neurons at the level of the calyx might overcome DmSLC22A activity, and acetylcholine might then diffuse from the calyx to the cortex where it could activate cortex glia. Alternatively, DmSLC22A could be internalized, or its activity might be repressed after a spaced training, authorizing acetylcholine diffusion towards cortex glia. Actually, DmSLC22A is also highly expressed in cortex glia according to RNA seq data (Davie et al., 2018), which provides further support for the hypothesis that acetylcholine can reach the cortex.

Besides, mushroom body vertical lobes extend high enough in the brain to cross the cortex region on the top of the brain. By observing electron microscopy images, it appears that the mushroom body alpha lobe is surrounded with ensheathing glia in the cortex, and should not directly interact with cortex glia. It does not rule out that Kenyon cells might activate cortex glia at this level. As explained above for the somatic transmission, we will be able to express TrPA1 in Kenyon cells in order to activate them by transferring flies at high temperature while recording calcium dynamics in cortex glia located around the vertical lobes and explore this hypothesis.

To sum up, it is for the time being not possible to drive any conclusion about the source of the acetylcholine signal that activates cortex glia during memory formation, but projection neurons and Kenyon cells are two potential sources.

II. 2. Consequences on cortex glia intracellular signaling

We found that acetylcholine can activate cortex glia by acting on receptors containing the nAChR α 7 nicotinic acetylcholine subunit. Earlier evidence already suggested that insect glia express nicotinic receptors, as glial cells nicotinic activation is necessary for their proper development in the olfactory system (Heil et al., 2007). Our results are also in agreement with the work conducted on the leech which showed that neuropil glial cells exhibit a calcium influx after nicotinic stimulation, showing that nicotinic acetylcholine receptors are expressed and functional in these cells (Ballanyi and Schlue, 1989; Hochstrate and Schlue, 1995).

Similarly to what we have found in cortex glia, mammalian astrocytes in the hippocampus express the α 7 nicotinic receptor subunit (Shen and Yakel, 2012; Papouin et al., 2017). nAChR α 7 paralogous proteins share almost 50% of identity between humans and flies. Interestingly, activating nAChR α 7 in hippocampal astrocytes induces the vesicular release of D-serine, which is necessary as a co-agonist for activating hippocampal NMDAR involved in long-term potentiation and for long-term memory formation (Papouin et al., 2017). In this model, acetylcholine is thought to be released from cholinergic neurons in the medial septum that send projections to cortical regions where acetylcholine is involved in long-range and long-lasting effects (Teles-Grilo Ruivo and Mellor, 2013). Thus, it seems that acetylcholine-mediated communication from neurons to glia and activation of glial vesicular release are shared between species, in particular during memory formation. Still, divergence exists in the mechanisms occurring in these two species – a possible somatic transmission in flies as compared to synaptic release in mice, and the induction of the release of an insulin-like peptide in *Drosophila* as compared to D-serine in mice. These divergences must originate from differences in morphological and/or physiological constraints between both species. It is also possible that both mechanisms exist in flies as well as mammals, but the study in mice did not investigate any impact of astrocytes activation by acetylcholine on their carbohydrate uptake or catabolism; and, during my PhD, we did not inhibit D-serine synthesis in cortex glia. It would be possible, in the near future, to knock-down serine racemase, the rate-limiting enzyme for D-serine synthesis, in the adult cortex glia, and check if long-term memory performance is affected. This would be consistent with the fact that functional

NMDAR are also required for aversive learning and long-term memory in flies (Xia et al., 2005).

Mammalian nAChR α 7 subunit exhibits a substantial permeability to calcium, as demonstrated in astrocytes (Shen and Yakel, 2012). Here, we showed that this subunit shares the same property in *Drosophila*, as nicotinic stimulation resulted in a temporally sustained calcium increase in cortex glia, which was sensitive to nAChR α 7 knock-down in these cells. Although the pharmacological stimulation may occur brainwide in this experiment, the fact that a knock-down of nAChR α 7 receptor in cortex glia decreases the response argues in favor of a direct stimulation of these cells (Figure 47 C). Our data, in addition to works performed by others (Ballanyi and Schlue, 1989; Hochstrate and Schlue, 1995), thus confirms that glial cells are sensitive to neurotransmitters in insects. These neurotransmitters trigger specific downstream signaling pathways that are relevant for brain activity and cognition, such as insulin signaling in *Drosophila*.

III. Insulin signaling and memory

III. 1. Cortex glia release Ilp4 during memory formation

As explained in the introduction, cortex glia and surface glia release an insulin-like peptide, Ilp6, at least during development (Chell and Brand, 2010; Sousa-Nunes et al., 2011). We showed by immunohistochemistry that cortex glia additionally express Ilp4. Moreover, Ilp4 acute expression as well as vesicular trafficking in cortex glia right after the conditioning are required for long-term memory formation. Together, these results suggest that Ilp4-containing vesicles are released by cortex glia early during long-term memory formation.

Ilp4 is the second most highly conserved insulin-like peptide after Ilp7, among the eight Ilps encoded in the fly genome (Grönke et al., 2010). It was suggested that its higher amino acid sequence conservation reflected its essential functions that should be different from the other Ilps and therefore could not be compensated (Grönke et al., 2010). Still, Ilp4 function was never reported during development or at adulthood. Indeed, Ilp4 loss of function mutants did not display any evident phenotype (Grönke

et al., 2010). So far, its expression was only detected in the midgut during development (Brogiolo et al., 2001). Thus, our results provide the first evidence for Ilp4 function in *Drosophila*.

Interestingly, in the fly genome, the closest peptide to Ilp4 is Ilp6 (Grönke et al., 2010), the one that has been shown to be expressed in glia, and in particular in cortex glia, during development (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Thus, their shared expression pattern could originate from a shared evolutionary origin, probably through the duplication of one of the two genes with the associated promoter/enhancer sequences. If Ilp6 has a role only during development and Ilp4 only acts in adults is unclear. During development, dedicated insulin-like peptides act either in the periphery or in the central nervous system. Indeed, Ilp6 is expressed by surface and cortex glia, released during development, and activates insulin-like signaling in neuroblasts which triggers their reactivation and divisions; but Ilp6 released by glia does not affect the peripheral body growth (Sousa-Nunes et al., 2011). In starved conditions, Ilp6 is not released and neuroblasts are not reactivated. Overexpressing Ilp6 in canonical insulin producing cells (IPCs) does not rescue this effect of starvation (Sousa-Nunes et al., 2011), suggesting that insulin released by these neurosecretory cells cannot reach the central nervous system and thus only acts in the periphery. Together, these results show that insulin released by glial cells must target the central nervous system, while insulin released by neurosecretory cells targets the periphery (Sousa-Nunes et al., 2011). This suggests that metabolism in the periphery and in the brain might be independently regulated. It might be relevant for keeping appropriate cognitive functions in extreme starving conditions.

In the introduction, I discussed about a recent study that showed that Ilp3 production in insulin producing cells was decreased upon ageing, and that overexpressing Ilp3 in IPCs rescued age-related middle-term memory impairment by acting on the fat body (Tanabe et al., 2017). In this work, we additionally showed that Ilp4 autocrinally activates the insulin receptor expressed on cortex glia membranes during long-term memory formation, which was not expected in the literature. Thus, insulin signaling is involved in both middle-term and long-term memories, but their cellular source and targets are distinct.

III. 2. Insulin-like signaling is involved in cognition in various species

III. 2. a. Previous results on insulin signaling and cognition

Before investigating its role in cognition, many studies focused on the role of insulin signaling in longevity. Indeed, in *Caenorhabditis elegans* and *Drosophila melanogaster*, among the first described mutants had increased lifespan (Johnson, 1990; Wolkow et al., 2000). These strains actually carry mutations in genes involved in insulin-like signaling, such as InR or its receptor substrate Chico. Interestingly, populations of flies selected for their increased learning rates display a 15% decrease in longevity, associated with a minor increase in dry adult body mass. Oppositely, flies with an expanded longevity exhibit a 40% reduction in their memory (Burger et al., 2008). Consistently, in populations artificially selected for better long-term memory performance, males exhibit a significantly decreased longevity (Lagasse et al., 2012). The authors did not decipher which molecular pathways were altered in these strains, but the phenotypes correlate well with phenotypes due to respective increase or decrease in insulin signaling. This asks an interesting question about the link between neuronal activity and longevity. It is possible that by acting on the metabolic state of the brain or the metabolic substrates used by neurons, i.e. glucose or ketone bodies for example, hormones such as insulin influence the longevity of the whole organism. As insulin receptor is expressed in the brain, both in invertebrates and vertebrates, and that for instance IGF-1 receptors in the brain affect lifespan in mice (Kappeler et al., 2008), it would be interesting to clarify how InR expression in different cell types, i.e. neurons and glia, could impact lifespan.

Besides, in pathological conditions, altered insulin signaling correlates with cognitive dysfunctions (Mullins et al., 2017). Type 2 diabetic patients display an increased circulating insulin level, but a decrease in insulin sensitivity and signaling including in the brain. They exhibit low cognitive decrements, for example in verbal and visual memory (Biessels and Reagan, 2015). Their average cognitive performance is around the thirty-fifth to forty-fifth percentile of normative data (Biessels and Reagan, 2015). This cognitive defect could originate from the observed altered insulin signaling, but this causal link is currently not demonstrated. Still, it is supported by

the fact that a similar cognitive impairment in non-diabetic middle-aged and older adults is associated with insulin resistance (Bruehl et al., 2010).

Alzheimer's disease patients also display insulin resistance and altered brain metabolism (Mullins et al., 2017). Indeed, brain glucose import is impaired in Alzheimer's disease brains, and glucose metabolism is decreased (Murray et al., 2011); this decrease precedes brain atrophy and neurodegeneration (Engler et al., 2006; Murray et al., 2011). Estimates of brain metabolism correlate positively with measures of cognition (Engler et al., 2006; Gejl et al., 2017), which encourages fundamental and clinic research on glucose metabolism in Alzheimer's disease models. As earlier mentioned, brains from AD patients display decreased insulin-like signaling. GLP-1 (glucagon-like peptide 1) is a hormone that stimulates glucose-induced insulin release. A six-month treatment of AD patients with a GLP-1 analog, liraglutide, raises brain glucose transport capacity, and reverts the effect of the disease duration on brain glucose hypometabolism (Gejl et al., 2017). Thus, it seems that the alteration of glucose metabolism in AD could originate from insulin resistance and could be reversed by insulin-stimulating treatments. Intranasal insulin injections in these patients attenuate the decrease in cerebral glucose metabolism; moreover, they also improve memory performance (Craft, 2012). Together, these results suggest that insulin resistance is linked to the altered metabolism and cognitive dysfunctions in AD. Investigating the role of insulin signaling in normal cognition could thus reveal potential targets for treatments against the pathology.

In mammals, it has been shown that insulin signaling can influence memory formation also in normal brains. Indeed, intranasal insulin injections in healthy humans, and direct insulin injection in the mammalian brain, both improve memory performance (Stockhorst et al., 2004; Benedict et al., 2004; Chen et al., 2011). The insulin receptor is accordingly expressed in discrete CNS regions in mammals, including the hippocampus (Brüning et al., 2000). Noteworthy, hippocampal IGF2 expression is up-regulated 20 h to 36 h after aversive inhibitory avoidance conditioning in mice (Chen et al., 2011). Knocking-down IGF2 early after the conditioning decreases long-term memory performance, while oppositely, IGF2 bilateral injections in the hippocampus improve memory performance and extend

memory retention over time (Chen et al., 2011). On the contrary, injecting insulin or IGF-2 in the amygdala does not improve hippocampus-dependent memory performance (Stern et al., 2014). Besides, in mice, selectively inactivating both insulin and IGF-1 receptors in all cell types in the hippocampus and amygdala induces metabolic abnormalities and cognitive - including memory - impairments (Soto et al., 2019). This supports the hypothesis that insulin signaling in the brain is important for learning and memory. The studies did not investigate the respective contributions of the different cell types to the observed cognitive defects, but independently, it was shown that inactivating InR only in astrocytes disturbs the mice behavior (Cai et al., 2018). Here, we described that insulin signaling is required in glia for normal long-term memory in flies, contrary to what has been first suggested.

III. 2. b. From insulin signaling in neurons to insulin signaling in glia

Insulin receptor expression and functionality have already been demonstrated in diverse glial subtypes in *Drosophila* larvae. Insulin-like signaling within glia has been shown to be important for diverse mechanisms, such as the control of neuroblasts exit from quiescence (Sousa-Nunes et al., 2011), gliogenesis (Avet-Rochex et al., 2012) or neuroblasts encapsulation during larval development (Spéder and Brand, 2018). Here, we additionally demonstrate that adult cortex glia express high levels of insulin receptors, and that acute insulin signaling is required for long-term memory formation with a different spatial resolution than what has been demonstrated earlier. As explained in the introduction, several lines of evidence suggested that insulin signaling was necessary in the mushroom body for learning and memory. First, the expression of InR is required during development in the mushroom body for normal learning and memory; second, acute ubiquitous insulin signaling is necessary specifically for long-term memory (Chambers et al., 2015). Still, the acute knockdown of InR in the mushroom body or in all glia did not impair immediate or 3-h memory (Tanabe et al., 2017). Before our work, the consequences of an acute InR knockdown in the adult mushroom body or in glia had not been studied on long-term memory. Our data demonstrate that insulin signaling is acutely required in cortex glial cells instead of mushroom body neurons (Chambers et al., 2015) for proper long-term memory formation.

Altogether, we provide evidence that cortex glia release Ilp4 that acts autocrinally on the insulin receptors they express predominantly. It may be the need for a spatial or temporal amplification of cortex glia activation that has led to the selection of such a complicated autocrine mechanism. Indeed, a simpler mechanism could have evolved with the direct modulation of glucose metabolism by a cholinergic stimulation; still, insulin signaling must have been selected as it broadly regulates carbohydrate metabolism through evolutionary conserved canonical pathways. It is moreover possible that cortex glia integrate insulin-like signals originating from different sources, which could turn on their metabolism depending on environmental or internal parameters.

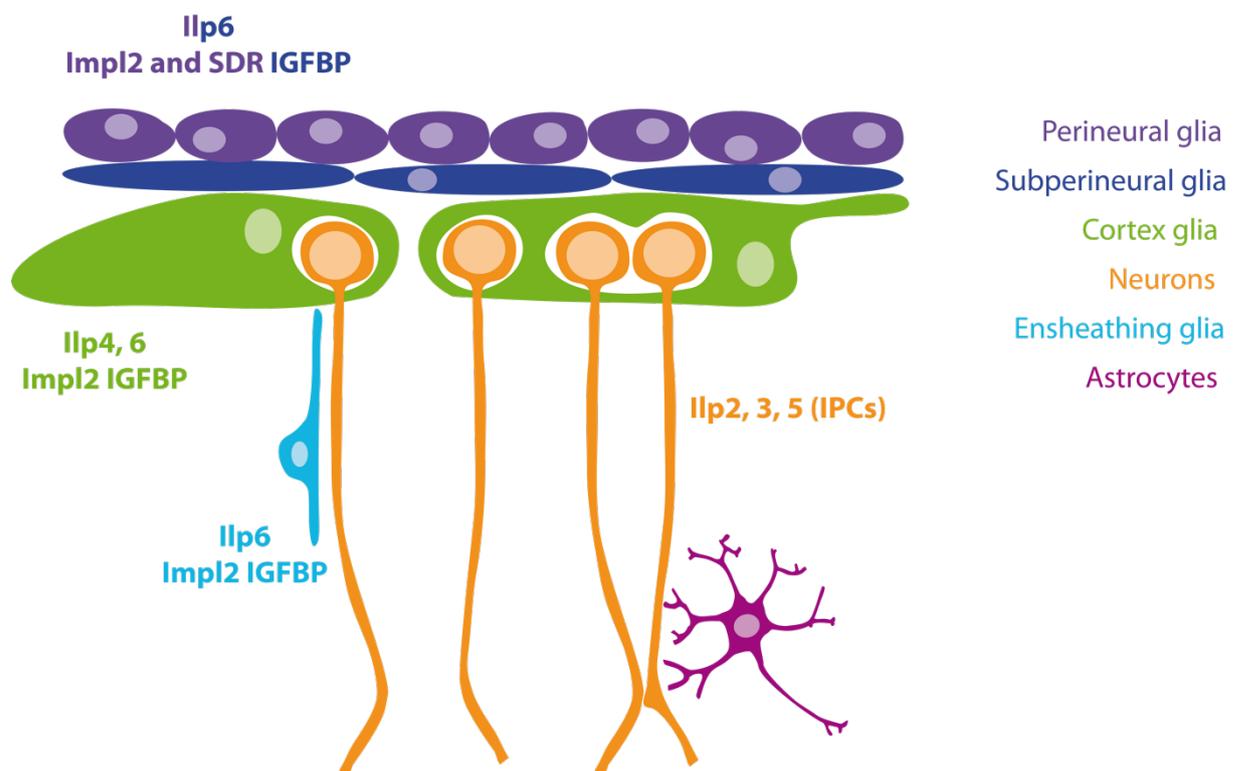


Figure 52. Cell type-specific expression of insulin-like peptides and IGFBP in the Drosophila brain. IPCs: insulin producing cells. Data from (Davie et al., 2018) and this study.

Remarkably, according to recent RNA-seq data (Davie et al., 2018), cortex, surface and ensheathing glia also express Impl2, an insulin growth factor binding protein (IGFBP) homolog (Figuroa-Clarevega and Bilder, 2015), and surface glia express

SDR, another IGFBP (Figure 52). When secreted, IGFBPs bind to insulin-like peptides and decrease their activity. Thus, these glial subtypes should be able to both activate or inhibit insulin signaling. Noteworthy, ImpL2 binds to Iip4 (Okamoto et al., 2013), and is expressed in cortex glia (Davie et al., 2018). In contrast, SDR binds predominantly to Iip3, but not to Iip4 (Okamoto et al., 2013), and is expressed in surface glia (Davie et al., 2018). Thus, it is possible that cortex and surface glia differentially modulate insulin signaling. As Iip3 is expressed by insulin-producing cells and targets the periphery such as the fat body (Tanabe et al., 2017), surface glia – which are in contact with the outside of the brain – could modulate insulin action in the periphery, while cortex glia could modulate insulin action in the central nervous system, reinforcing the notion of independent insulin regulations between periphery and CNS.

In a nutshell, cortex glia and other glial subtypes should be able to 1/ release insulin signals, 2/ release IGFBP that can modulate insulin signals activity and 3/ be sensitive to insulin signals.

Our work, along with previous studies, point that insulin signaling is essential for cognitive functions that are not only related to feeding or foraging, but also for higher-level cognitive functions such as memory formation in an evolutionary-conserved fashion. Moreover, our data link neuronal acetylcholine signaling to a glial release of insulin, which has not been investigated before.

III. 3. Insulin signaling in cortex glia regulates brain energy metabolism

The description of cortex glia anatomy early suggested that they were a potential source of neuronal trophic support (Wolfe and Nicholls, 1967). The discovery that adult cortex glia can both release and be activated by insulin-like peptides is the first non-anatomical clue that strengthens this hypothesis.

III. 3. a. Carbohydrate metabolism in cortex glia is regulated by neuronal inputs

Acetylcholine is the main excitatory neurotransmitter in the fly brain. As a consequence of an acute cholinergic activation of cortex glia in naive flies, we have measured a quick and sustained increase in intracellular glucose concentration that depends on both nAChR α 7 and InR (Figure 49). Accordingly, in mice, stimulating cultured hippocampal astrocytes with glutamate, the main excitatory neurotransmitter

in mammals, triggers an increase in astrocytic glucose uptake through GLUT1 within 10 seconds (Loaiza et al., 2003). In both species, neuronal excitatory activity thus triggers a fast metabolic regulation in the neighboring glia that results in an intracellular glucose concentration elevation; but, contrary to what we showed, this effect is thought to be mediated by an increase in glutamate uptake in mammals according to the ANLS model, and not by glutamate direct action on its receptors (Pellerin and Magistretti, 1994).

Still, a more recent study elegantly showed that glucose diffusion through gap junctions inside the astrocytic network was stimulated when inducing epileptic or evoked neuronal activity, and that this effect was blocked by AMPA glutamate receptor antagonists. It must thus depend on glutamate signaling (Rouach et al., 2008), but it was further indicated in this paper that astrocytes that express gap junctions do not express AMPA receptors, suggesting that this effect must be mediated by glutamate acting on AMPA receptors in astrocytes that are not part of the gap-junction network, or in neurons.

In flies, circulating glucose levels are maintained relatively low, and glucose is mainly synthesized out of the intracellular breakdown of circulating trehalose thanks to the intracellular enzyme trehalase. The glucose elevation in cortex glia could thus originate from an increased glucose uptake from an already existing extracellular glucose pool that might have been synthesized by surface glia, or from the synthesis of glucose inside cortex glia themselves. As knocking-down trehalase in cortex glia disrupts long-term memory, cortex glia intracellular glucose must at least partly originate from an intracellular trehalose breakdown. Still, it is possible that both pathways, i.e. glucose import via GLUT1 and glucose synthesis via trehalase activity, contribute to the overall intracellular glucose elevation.

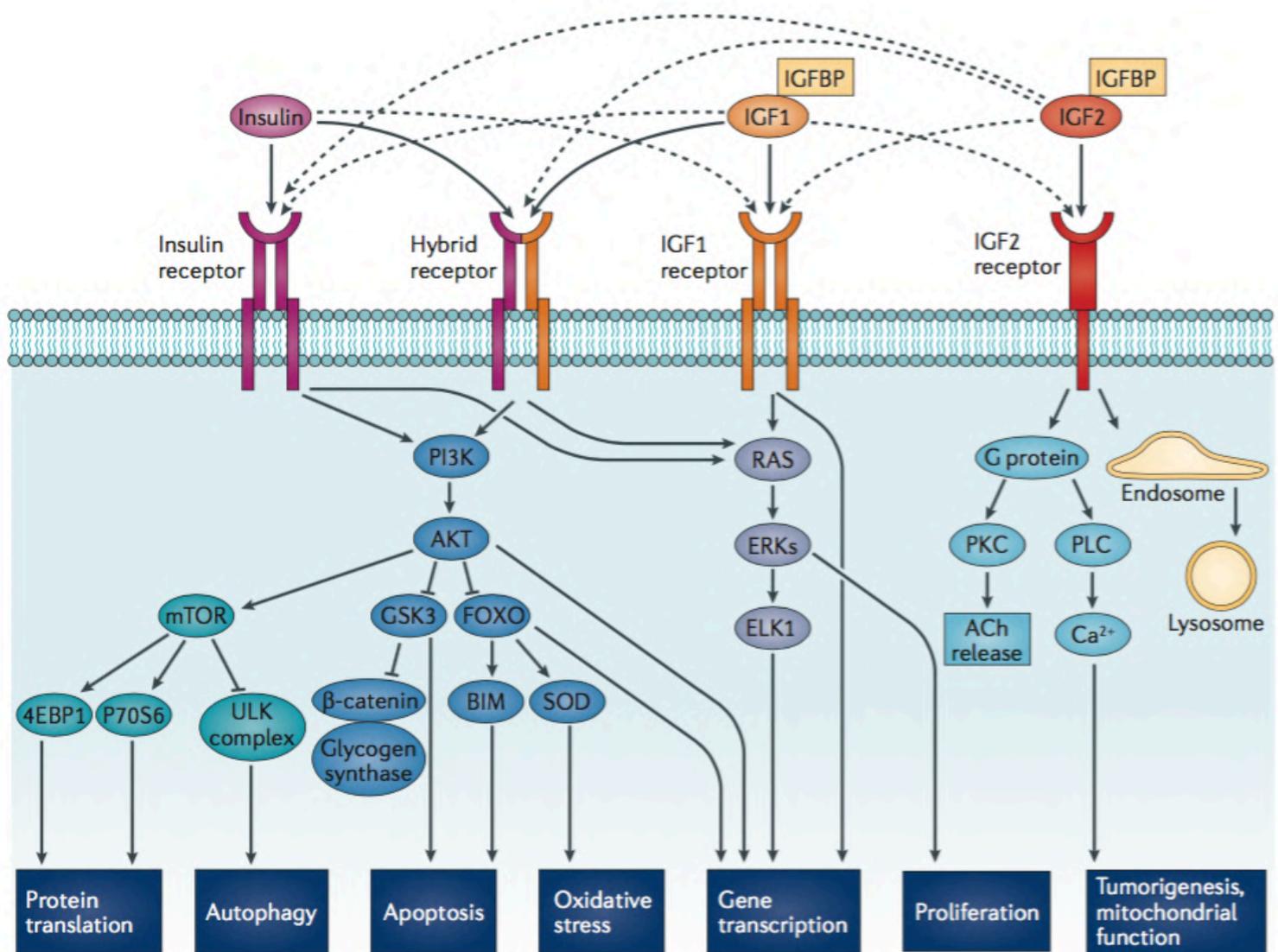


Figure 53. Canonical insulin-like signaling pathways in metazoan. From (Fernandez and Torres-Alemán, 2012).

As the nicotine-induced glucose elevation depends on InR expression in cortex glia (Figure 49 C), it is likely that *Ilp4* release, activation of InR and subsequent insulin-like signaling trigger the activation of trehalase. In agreement with this hypothesis, in the silkworm *Bombyx mori*, the injection of an insulin-like peptide called Bombyxin increases midgut and muscle trehalase activity, but the effect of the peptide on the brain was not evaluated (Satake et al., 1997). Moreover, the injection of bovine insulin in adult males beetles *Tribolium castaneum* increases trehalase mRNA expression in the head and fat bodies (Xu et al., 2013). The precise regulation of metabolic enzymes

by insulin-like peptides is scarcely known in *Drosophila*, but we can expect that trehalase regulation is conserved among invertebrates. Thus, it is possible that insulin signaling in cortex glia activates trehalase activity and maybe expression, which results in an increased glucose synthesis. Insulin signaling indeed activates various intracellular targets that could activate trehalase (Figure 53).

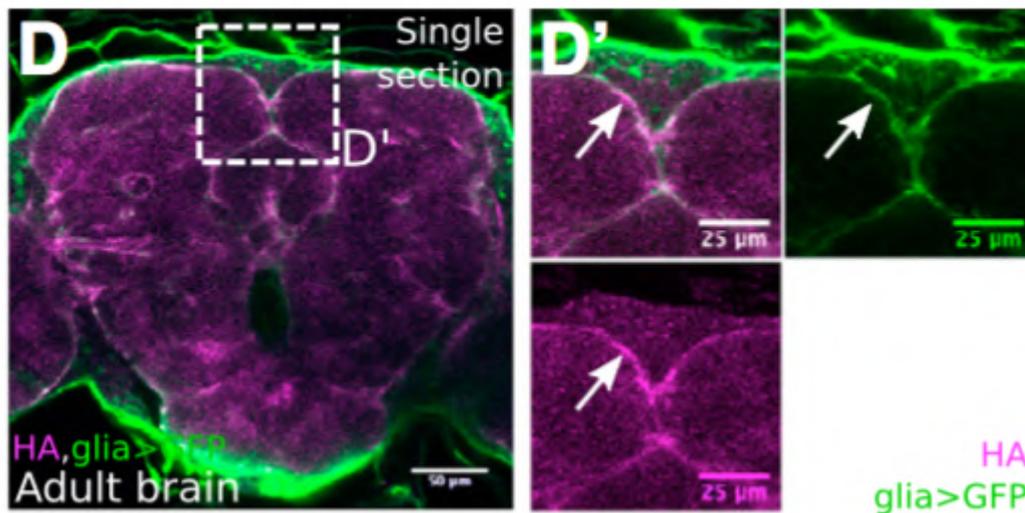


Figure 54. Trehalase is expressed throughout the brain including in cortex glia. Immunolabeling of HA (fuchsia) in flies carrying a HA-tagged trehalase genomic construct and expressing GFP in glia. The white arrow indicates the boundary between the cortex and the neuropile, where different glial membranes are located. In the cortex, trehalase expression is visible. From (Volkenhoff et al., 2015).

If cortex glia can process trehalose, they must be able to take-up this molecule. Two different trehalose transporters have been described in the fly genome: Tret1-1 and Tret1-2. Tret1-1 expression was reported in perineural glia in larvae (Volkenhoff et al., 2015), but it is also visible in the rest of the brain, which favors the hypothesis that trehalose can be imported into other brain cells. In addition, trehalase was also reported as mainly expressed in surface glia (Volkenhoff et al., 2015), but it is possible to visualize its expression in the whole CNS in the figure published in this study (Figure 54) and in particular in cortex glia on their and our own images (Figure 48). According to single-cell RNA-seq data (Davie et al., 2018 and see <http://scope.aertslab.org/>), all glial subtypes express trehalase in the adult CNS to a much larger extent than neurons, in particular cortex glia, but also ensheathing glia and astrocytes to a lower extent. It suggests that other glial subtypes can take-up circulating trehalose and

could be involved in the supply of energy metabolites such as glucose to neurons. If long-term memory is impaired by trehalose transporters knockdown in the adult cortex glia, it would further confirm our model of increased trehalose transport and hydrolysis in cortex glia for long-term memory support.

III. 3. b. Metabolism in glia might support neuronal glucose consumption

As cortex glia intracellular glucose increase after a cholinergic activation, we first investigated if, as in the ANLS model in mammals, cortex glia could supply neurons with lactate to support their high metabolic activity. We favored this hypothesis according to the result we published that showed an increased pyruvate, but not glucose, consumption in the mushroom body lobes specifically during long-term memory formation (Plaçais et al., 2017). Still, knocking-down lactate dehydrogenase enzymes in the adult cortex glia did not suppress long-term memory formation (Figure 44). This result led us to investigate other metabolic pathways that could be regulated by insulin signaling in cortex glia, in particular trehalose hydrolysis to glucose. The simplest consequence of an elevation of glucose concentration in cortex glia following their activation is an increase in the glucose gradient from the intracellular space to the extracellular medium and to the neuronal cytosol, which is even further accentuated in the case of increased glucose consumption in neurons. Thus, without any other regulation, the increase in cortex glia intracellular glucose concentration could support an elevation of glucose transfer from glia to neuronal soma through GLUT1. Consistently, we measured an increase in glucose consumption in Kenyon cells neuronal soma during long-term memory formation (Figure 50 A). We did not demonstrate that this increase in glucose consumption relies on cortex glia glucose release. Still, GLUT1 expression is necessary in both Kenyon cells and cortex glia for long-term memory (Figure 50 B and D). As Kenyon cells glucose consumption increases during long-term memory early consolidation, GLUT1 must allow glucose import in this cell type. On the opposite, cortex glia can intracellularly process trehalose into glucose, it is thus more likely that GLUT1 allows glucose export from – rather than import in – cortex glia during long-term memory formation. This would support a model in which glucose is shuttled from cortex glia to the highly active neuronal soma during long-term memory consolidation. We still lack any direct

evidence of this shuttle, and further experiments are needed to confirm this hypothesis. In particular, we plan to measure if the increased glucose consumption in Kenyon cells is abolished when GLUT1 – or the other genes involved in the signaling pathway occurring in cortex glia, such as *Ilp4* or *InR* – is knocked-down in cortex glia.

Even if it is not currently widely studied, it was already suggested that neuronal activity implies a particular energetic cost at the level of the soma. Indeed, in the mammalian dorsal root ganglia, the number of satellite glial cells per neuron increases in proportion to the neuronal volume, consistent with the idea that satellite glial cells metabolically support the neurons (Hanani, 2005). If cortex glia were early suggested as potential supportive cells responding to the neuronal energy demand, it is to my knowledge the first data to support this claim. Interestingly, cortex glia express *Inx2*, an innexin protein that forms gap junctions in insects (Farca Luna et al., 2017). These junctions are permeable to molecules that have a molecular mass inferior to 1100g/mol (Loewenstein, 1966), such as, in particular, glucose and trehalose. Therefore, in case of a high neuronal energy demand, trehalose break-down into glucose in the whole cortex glial network and glucose diffusion in this network, similarly to what was described in the mammalian brain (Rouach et al., 2008), could contribute to provide glucose to a localized energy sink.

As the brain activity is highly costly, reducing neuronal activity when food is not accessible is an efficient way to reduce the whole-body energy consumption. In the wild, most animals do not have a permanent access to food, thus it is likely that control processes prevent an excessive brain energy expenditure under basal conditions. If so, rapid mechanisms must allow the modulation of the brain energy supply so that relevant information can still be processed and stored while access to food is restricted; upregulating metabolism by itself could actually trigger long-term memory formation, as suggested in our article (Plaçais et al., 2017). Indeed, when animals start feeding, metabolites concentrations - glucose, amino acids, trehalose... - increase quickly in the circulating fluids, and can readily be taken up by the brain (Treherne, 1958). For the formation of appetitive associative memory during which an odor is associated to food ingestion and thus to energy intake, neural networks activity energetic need might be directly supported. Indeed, this increase in circulating

metabolites could drive an elevation of neuronal excitability, favoring their recurrent activity that might be required for memory consolidation. On the contrary, meeting a strongly aversive cue is not related to energy intake, but neuronal networks activity must be supported to allow an efficient long-term memory formation. IIP4 release from cortex glia may provide an insulin signal that drives an increased carbohydrate metabolism that otherwise would not be sufficient in the absence of energy intake during a spaced aversive conditioning, and that is necessary for switching on neuronal activity in order to efficiently form long-term memory. As discussed in the introduction, several lines of evidence suggest that cortex glia can modulate neuronal excitability by acting at the level of the soma (Melom and Littleton, 2013; Kunduri et al., 2018); it is thus possible that cortex glia actively increase Kenyon cells excitability during memory consolidation through a glucose shuttle, either to authorize their recurrent activity, or to recruit more neurons if a sensory stimulus is encountered again, so that relevant information is even more efficiently stored.

III. 3. c. Insulin signaling in mammalian astrocytes also participates to CNS metabolism

Interestingly in mammals, as explained in the introduction, InR knockout in astrocytes results in a decreased GLUT1 expression, and a decreased glucose intake in astrocytes but also in the whole brain (García-Cáceres et al., 2016). Therefore, it seems that insulin signaling in glial cells is conserved to regulate the brain carbohydrate metabolism.

In immune cells, the signaling pathways involved in the detection of pathogens converge onto PI3K/Akt signaling, i.e. the pathway downstream insulin-like signaling (Ganeshan and Chawla, 2014). This signaling triggers a metabolic switch in dendritic cells and lymphocytes with an increase in GLUT1 expression. Thus, insulin and insulin-like peptides regulate carbohydrate metabolism in diverse cell types that do not belong to the canonical targets of insulin, liver, muscles or adipose tissue.

The results I obtained during my thesis support the view that specific metabolic pathways are required in memory-relevant brain structures during long-term memory formation in *Drosophila*, as it has been demonstrated in vertebrates (Gibbs et al., 2006; Suzuki et al., 2011). Indeed, we showed that metabolic fluxes increase both in Kenyon cells soma and synaptic compartments early after long-term memory conditioning. While in Kenyon cells soma, glycolysis is up-regulated, glycolytic flux remains identical in their axons and presynaptic compartments - the mushroom body lobes; instead, mitochondrial pyruvate consumption increases. Thus, we describe for the first time that distinct metabolic pathways are enhanced in specific cellular compartments in a memory-relevant brain structure. It is possible that glycolysis in Kenyon cells soma provides the pyruvate used by axonal and synaptic mitochondrial activities. This could explain some inconsistency in the results obtained on glucose consumption rates in neurons and astrocytes in mammalian brains (Chuquet et al., 2010; Jakoby et al., 2013; Patel et al., 2014; Lundgaard et al., 2015; Díaz-García et al., 2017). Indeed, the studies conducted until now did not specifically measure glucose uptake in distinct neuronal compartments. It is possible that neuronal soma uptake more glucose than synapses, and thus, that glucose consumption rate is higher in neurons over astrocytes at the somatic level, but lower at the synaptic level.

The enhanced metabolism in Kenyon cells is specific for long-term memory consolidation, as it is not measured after a single training or after a massed training that do not lead to *de novo* protein synthesis-dependent long-term memory formation. This asks if increased metabolism in Kenyon cells is a consequence of long-term memory consolidation, or is the main factor that controls it. Our experiments evidence that decreasing glucose import or pyruvate metabolism in Kenyon cells impairs long-term memory, and oppositely, enhancing pyruvate metabolism in Kenyon cells facilitates memory consolidation into *de novo* protein synthesis-dependent long-term memory. This suggests that indeed, metabolism in Kenyon cells restricts memory consolidation in the long-term, and that overcoming this restriction is sufficient to form long-term memory. Our data thus provide a mechanism explaining glucose memory enhancement observed in mammals

(Messier, 2004): glucose supply is needed in memory relevant circuits for long-term memory formation, and conversely, only enhancing metabolism in memory-encoding circuits, either by genetic manipulation or by direct glucose injection, increases memory consolidation.

If the increased metabolic consumption in Kenyon cells soma, axons and synapses is dedicated to ATP synthesis is still unknown. It is possible that these pathways also provide precursors for the biosynthesis of amino acids both in the soma and synaptic compartments, for neurotransmitter synthesis at the synapse (for instance, acetyl-CoA is needed for acetylcholine synthesis), or for NADPH synthesis in the soma and subsequent NADP-dependent antioxidant mechanisms or lipid biosynthesis.

It will be interesting to clarify what metabolic pathways are required for shorter-lasting memories. Indeed, even if such a strong energy demand in Kenyon cells is not occurring during the formation of shorter-term memories as compared to long-term memory, it is much likely that Kenyon cells still need energy supply, maybe through other pathways. This would also support the hypothesis that the regulation of the metabolic fluxes in Kenyon cells contributes itself to regulate the robustness and molecular pathways involved in the memory formed.

Moreover, we describe a molecular pathway that links neuronal activity to metabolic regulation in glial cells for the first time in *Drosophila melanogaster*. Indeed, during long-term memory formation, a cholinergic signal activates the nicotinic receptors nAChR α 7 expressed by neuronal soma-enwrapping glia: cortex glia. Even though our data is consistent with a release of acetylcholine from Kenyon cells soma, we did not provide an evidence of the origin of the cholinergic input that activates cortex glia. Indeed, tools to locally manipulate glial cells around a given structure are lacking, and we thus could not dissect in which region cortex glia was activated for memory formation. As extracellular acetylcholine is readily catabolized by acetylcholine esterase, this neurotransmitter is expected to be locally delivered to its targets; thus, a neuronal somatic release of acetylcholine is the most likely way to activate cortex glia. Still, the molecular mechanisms involved in neuronal somatic release of neurotransmitter are not well studied. Thus, it is for the time being

complicated to answer the question about the origin of the cholinergic input to cortex glia, and further experiments are needed to confirm this model.

nAChR α 7 activation induces a calcium burst in cortex glia, which then release IIP4, an insulin-like peptide with no described function until now, but very conserved among *Drosophila* species. In addition, thanks to immunohistochemistry experiments, we describe that the insulin receptor InR is highly expressed in cortex glia, and that this expression is necessary for proper long-term memory formation. We show that insulin signaling regulates energy metabolism in cortex glia. Immunolabeling images show trehalase expression in cortex glia, which is necessary for long-term memory. Moreover, thanks to *in vivo* imaging experiments in naïve flies, we provide evidence that a cholinergic activation triggers a glucose concentration elevation in cortex glia, which depends on nAChR α 7 and InR expression. Altogether, these results point that nAChR α 7 activation triggers IIP4 release, that acts autocrinally on InR expressed by cortex glia, which results in the up-regulation of trehalase activity, and in an increase in cortex glia intracellular glucose concentration. Surprisingly, lactate dehydrogenase activity seems to be dispensable in cortex glia for memory formation (Figure 44), which suggests that a lactate shuttle from cortex glia to neuron is unlikely to occur. Instead, the expression of the glucose transporter GLUT1 is needed in both cortex glia and Kenyon cells, which suggests that the signaling mechanism we describe in cortex glia might enhance a cortex glia-neuronal soma glucose shuttle to support the elevated neuronal soma glucose consumption during memory formation. We still lack a direct evidence of this shuttle, and future experiments will hopefully confirm this interaction, such as glucose imaging in Kenyon cells while concomitantly inhibiting the signaling pathway we describe in cortex glia. If Kenyon cells are indeed the source of acetylcholine that activates cortex glia, a positive loop between Kenyon cells activity to cortex glia glucose could enhance the long-lasting neuronal circuit activity during long-term memory consolidation (Figure 55).

As all the above-described mechanisms are necessary specifically for long-term memory but not for shorter-lasting memories, it is likely that this metabolic regulation in cortex glia only occurs when long-lasting neuronal activity must be supported to allow memory consolidation through long-term plasticity changes.

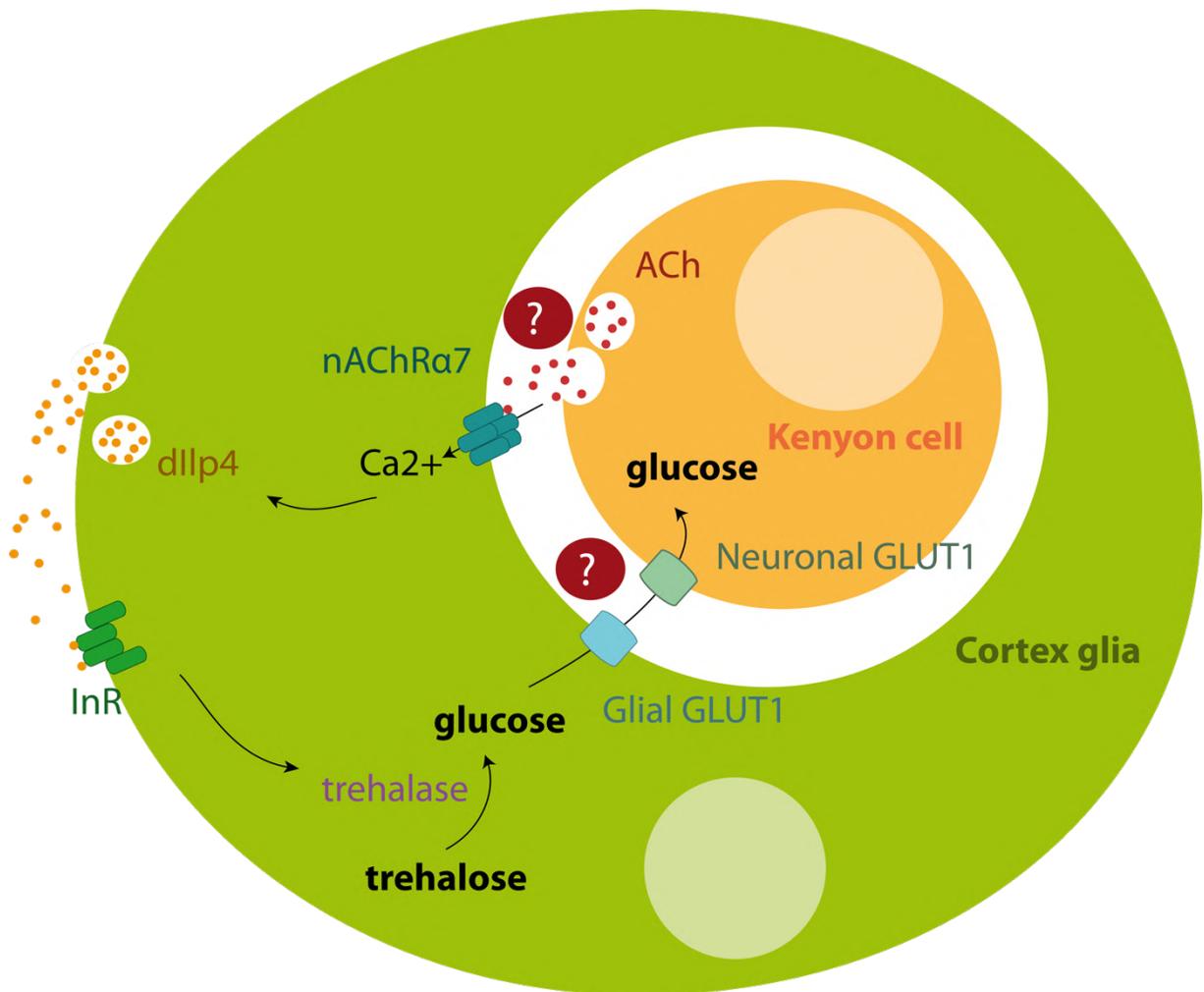


Figure 55. Currently hypothetical model of cortex glia metabolic support of Kenyon cells soma during long-term memory formation. Question marks indicate the main points that remains to be solved: is the cholinergic signal coming from Kenyon cells? If yes, at which level (soma or dendrites)? Does the increased glucose synthesis in cortex glia support glucose consumption in Kenyon cells soma?

Finally, it is to our knowledge the first evidence of a cortex glia – neuron signaling and metabolic interaction at adulthood. It is possible that cortex glia regulate both neuronal excitability and energy metabolism at the level of the neuronal soma. Such mechanisms might have many functional consequences on animal behavior, such as sleep and arousal, and remain to be more deeply investigated.

Our work therefore links a neuronal activity-dependent activation of cortex glia to local insulin release and energy metabolism regulation during long-term memory

formation. It is of particular importance as it is becoming increasingly evident that metabolic changes, among which increased central insulin resistance and lower brain glucose metabolism, accompany cognitive decrements during aging and Alzheimer's disease in humans (Mattson et al., 2008; Mullins et al., 2017). Investigating if glial sensitivity to insulin or carbohydrates metabolism is altered in aged flies or in animal models of Alzheimer's disease would be interesting in order to design therapeutic treatments that precisely target glia with hopefully few side effects.

Glucose metabolism

Glucose complete oxidation in the presence of O₂ into CO₂ and H₂O is energetically favored, with a Gibbs free energy of -2870 kJ/mol (Figure 56). Therefore, glucose is a highly energetic molecule. In the cells, its favorable oxidation can be coupled to other reactions that are unfavorable endergonic reactions.

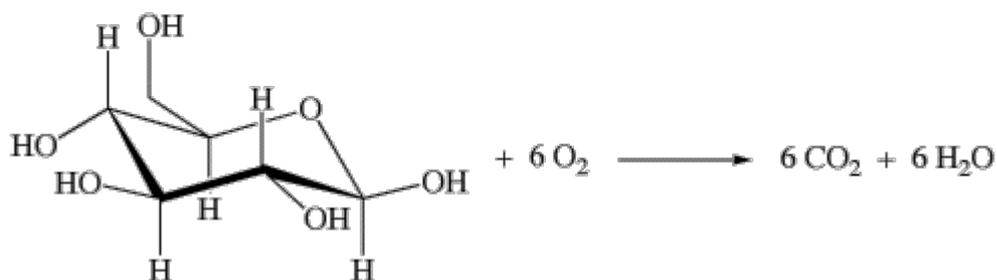


Figure 56. Glucose chemical structure and reaction of complete oxidation into CO₂ and H₂O.

Glucose is catabolized in eukaryotic cells by several pathways (Figure 5) including glycolysis and the pentose phosphate pathway, followed by fermentation or mitochondrial oxidation. These pathways provide different ATP yields and metabolites for the cells.

1. The pentose phosphate pathway

The pentose phosphate pathway uses glucose-6-phosphate generated thanks to the first glycolytic enzyme, hexokinase (see after). It is composed of two steps.

First, the oxidation of glucose-6-phosphate into ribulose-5-phosphate and CO₂ allows the reduction of NADP⁺ into NADPH. Second, several reactions convert ribulose-5-phosphate into different phospho-carbohydrates. Among the generated molecule, this pathway gives rise to glyceraldehyde-3-phosphate and fructose-6-phosphate that can be further broken-down by glycolysis.

Importantly, several anabolic precursors essential for biosynthesis are produced through this pathway. For instance, ribose-5-phosphate is essential for nucleotides synthesis, and erythrose-4-phosphate is needed for aromatic amino acids synthesis.

On the other hand, this pathway is the only way to reduce NADP⁺ into NADPH. NADPH is the unique electron supplier for several redox and anabolic reactions, as glutathione reduction or fatty acids biosynthesis.

This pathway occurs in the cytosol of the cells, and is independent of oxygen availability as it uses H₂O to oxidize glucose.

2. Glycolysis

Glycolysis is a sequence of 10 catalyzed reactions. It allows the transformation of one molecule of glucose (6 carbons) into two molecules of pyruvate (3 carbons). For every molecule of glucose going through glycolysis, 2 molecules of ATP are synthesized, and 2 molecules of NAD⁺ are reduced to NADH.

More than only providing energy, glycolysis is an anaplerotic pathway that provides different precursors for biosynthesis, for example, dihydroxyacetone phosphate that is involved in glycerophospholipid synthesis.

This pathway occurs in the cytosol of the cells, and is independent of oxygen availability.

To allow glycolysis to go on, the regeneration of NAD⁺ from NADH is necessary. This regeneration is allowed by either fermentation or mitochondrial respiration.

3. Glycolysis is followed by fermentation or by mitochondrial respiration

Under physiological conditions with a normal oxygen supply, glycolysis can be followed either by lactate synthesis or by the TCA cycle plus oxidative phosphorylation that consumes O₂, which is called mitochondrial respiration.

3.a. Fermentation

In animal cells, fermentation corresponds to the direct reduction of pyruvate into lactate thanks to the catalytic action of lactate dehydrogenase, allowing the parallel regeneration of NAD⁺ from NADH (Figure 5). Lactate can be exported by the cell into the extracellular medium following its gradient thanks to monocarboxylate transporters, but is not used by any biosynthetic pathway. It can only be converted back to pyruvate when taken up by another cell.

Aerobic fermentation takes place in the cytosol and does not require oxygen.

3. b. Mitochondrial respiration

Pyruvate can alternatively be used in mitochondria *via* a two-step mechanism, with the generation of NADH and FADH₂ electron carriers by the TCA cycle followed by the oxidation of these electron carriers by the electron transport chain to generate ATP.

Pyruvate enters the mitochondrial matrix and is decarboxylated into Acetyl-CoA by the pyruvate dehydrogenase complex (Figure 57). This complex comprises different enzymes including E1 that catalyzes the first step: pyruvate decarboxylation. Importantly, two antagonistic enzymes modulate pyruvate dehydrogenase E1 activity. Its phosphorylation by pyruvate dehydrogenase kinase (PDK) inhibits E1, while this inhibition is released by its dephosphorylation by pyruvate dehydrogenase phosphatase (PDP).

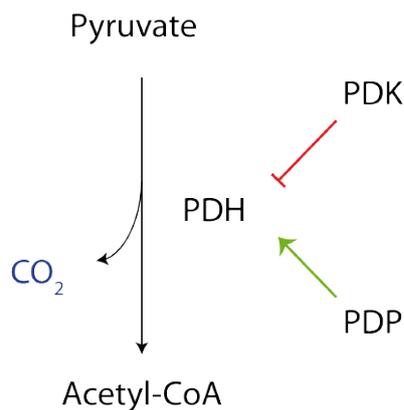


Figure 57. Pyruvate dehydrogenase activity is modulated by two antagonistic enzymes. PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase.

Mitochondrial respiration uses Acetyl-CoA to fuel the TCA cycle. The TCA cycle completely oxidizes this 2-carbons molecule into 2 CO₂, generating NADH and FADH₂ in the process of carbon oxidation.

NADH and FADH₂ transfer their electrons to the mitochondrial electron transport chain (Figure 58). Thanks to the sequential transfer of electrons between different complexes, a proton gradient is created between the intermembrane space and the matrix of the mitochondria. Ultimately, the electrons participate to the reduction of O₂ into H₂O. The dissipation of the mitochondrial proton gradient through the ATP

synthase complex provides the energy needed for the phosphorylation of ADP into ATP.

Mitochondrial respiration takes place in the mitochondria and requires oxygen.

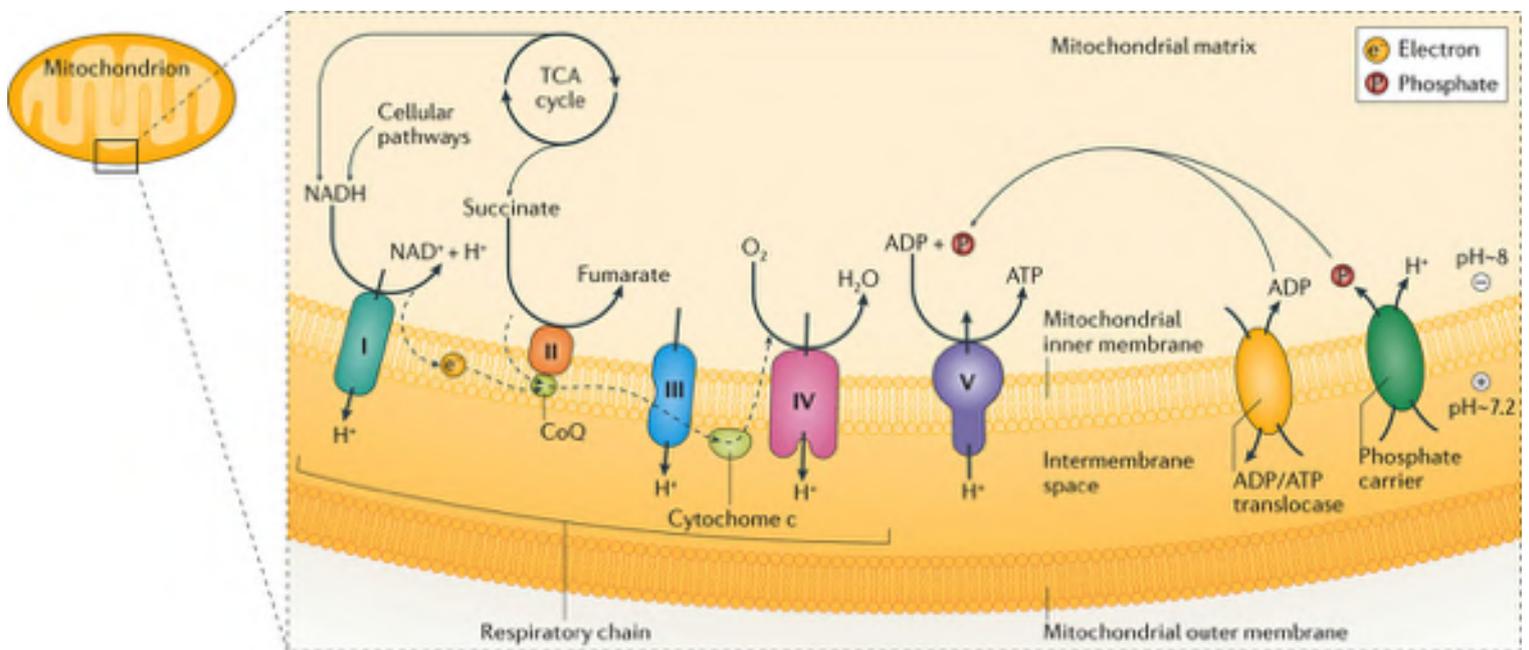


Figure 58. Organization of the mitochondrial electron transport chain and ATP synthase. Electrons are transferred from NADH and succinate to mitochondrial complexes I and II respectively. The subsequent transfer of electrons to successive mitochondrial complexes allows the formation of a proton gradient, from the intermembrane space towards the mitochondrial matrix. Dissipating the gradient through complex V provides the energy needed for the phosphorylation of ADP into ATP. From (Gorman et al., 2016).

- Adhav, A.S., Kokane, S.R., and Joshi, R.S. (2018). Functional characterization of *Helicoverpa armigera* trehalase and investigation of physiological effects caused due its inhibition by Validamycin A formulation. *International Journal of Biological Macromolecules* *112*, 638–647.
- Al-Anzi, B., and Zinn, K. (2018). Identification and characterization of mushroom body neurons that regulate fat storage in *Drosophila*. *Neural Dev* *13*, 18.
- Allen, N.J., and Lyons, D.A. (2018). Glia as architects of central nervous system formation and function. *Science* *362*, 181–185.
- Almeida, A., Moncada, S., and Bolanos, J.P. (2004). Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol* *6*, 45–51.
- Artiushin, G., Zhang, S.L., Tricoire, H., and Sehgal, A. (2018). Endocytosis at the *Drosophila* blood-brain barrier as a function for sleep. *eLife* *7*, 523.
- Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.-T., Dionne, H., Abbott, L.F., Axel, R., Tanimoto, H., et al. (2014a). The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* *3*, e10806.
- Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A.B., Ito, K., Scholz, H., and Tanimoto, H. (2012). Three Dopamine Pathways Induce Aversive Odor Memories with Different Stability. *PLoS Genet* *8*, e1002768–17.
- Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K.R., Vogt, K., Belliart-Guérin, G., Plaçais, P.-Y., Robie, A.A., Yamagata, N., Schnaitmann, C., et al. (2014b). Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *eLife* *3*, e04580.
- Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific Dopaminergic Neurons for the Formation of Labile Aversive Memory. *Current Biology* *20*, 1445–1451.
- Attwell, D., and Laughlin, S.B. (2001). An energy budget for signaling in the grey matter of the brain. *Journal of Cerebral Blood Flow & Metabolism* *21*, 1133–1145.
- Avet-Rochex, A., Kaul, A.K., Gatt, A.P., McNeill, H., and Bateman, J.M. (2012). Concerted control of gliogenesis by InR/TOR and FGF signalling in the *Drosophila* post-embryonic brain. *Development* *139*, 2763–2772.
- Awasaki, T., and Lee, T. (2011). New tools for the analysis of glial cell biology in *Drosophila*. *Glia* *59*, 1377–1386.
- Awasaki, T., Huang, Y., O'Connor, M.B., and Lee T. (2011b). Glia instruct developmental neuronal remodeling through TGF- β signaling. *Nat Neurosci.* *14*(7), 821-3.
- Awasaki, T., Lai, S.-L., Ito, K., and Lee, T. (2008). Organization and postembryonic development

of glial cells in the adult central brain of *Drosophila*. *J. Neurosci.* 28, 13742–13753.

Bailey, A.P., Koster, G., Guillermier, C., Hirst, E.M.A., MacRae, J.I., Lechene, C.P., Postle, A.D., and Gould, A.P. (2015). Antioxidant Role for Lipid Droplets in a Stem Cell Niche of *Drosophila*. *Cell* 163, 340–353.

Bainton, R.J., Tsai, L.T.Y., Schwabe, T., DeSalvo, M., Gaul, U., and Heberlein, U. (2005). *moody* Encodes Two GPCRs that Regulate Cocaine Behaviors and Blood-Brain Barrier Permeability in *Drosophila*. *Cell* 123, 145–156.

Ballanyi, K., and Schlue, W.R. (1989). Electrophysiological characterization of a nicotinic acetylcholine receptor on leech neuropile glial cells. *Glia* 2, 330–345.

Barnstedt, O., Oswald, D., Felsenberg, J., Brain, R., Moszynski, J.-P., Talbot, C.B., Perrat, P.N., and Waddell, S. (2016). Memory-Relevant Mushroom Body Output Synapses Are Cholinergic. *Neuron* 89, 1237–1247.

Barros, L.F. (2013). Metabolic signaling by lactate in the brain. *Trends in Neurosciences* 36, 396–404.

Barros, L.F., and Martínez, C. (2007). An Enquiry into Metabolite Domains. *Biophysj* 92, 3878–3884.

Barros, L.F., Bolanos, J.P., Bonvento, G., Bouzier-Sore, A.-K., Brown, A., Hirrlinger, J., Kasparov, S., Kirchhoff, F., Murphy, A.N., Pellerin, L., et al. (2017). Current technical approaches to brain energy metabolism. *Glia* 66, 1138–1159.

Barros, L.F., Brown, A., and Swanson, R.A. (2018). Glia in brain energy metabolism: A perspective. *Glia* 66, 1134–1137.

Barth, M., and Heisenberg, M. (1997). Vision affects mushroom bodies and central complex in *Drosophila melanogaster*. *Learning & Memory* 4, 219–229.

Becker, A., Schlöder, P., Steele, J.E., and Wegener, G. (1996). The regulation of trehalose metabolism in insects. *Experientia* 52, 433–439.

Benedict, C., Hallschmid, M., Hatke, A., Schultes, B., Fehm, H.L., Born, J., and Kern, W. (2004). Intranasal insulin improves memory in humans. *Psychoneuroendocrinology* 29, 1326–1334.

Benedict, F.G. (1938). *Vital Energetics*.

Benton, D., Owens, D.S., and Parker, P.Y. (1994). Blood glucose influences memory and attention in young adults. *Neuropsychologia* 32, 595–607.

Bélanger, M., Allaman, I., and Magistretti, P.J. (2011). Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. *Cell Metabolism* 14, 724–738.

Bianconi, E., Piovesan, A., Facchin, F., Beraudi, A., Casadei, R., Frabetti, F., Vitale, L., Pelleri, M.C., Tassani, S., Piva, F., et al. (2013). An estimation of the number of cells in the human body. *Annals of Human Biology* 40, 463–471.

- Biessels, G.J., and Reagan, L.P. (2015). Hippocampal insulin resistance and cognitive dysfunction. *Nat Rev Neurosci* *16*, 660–671.
- Blum, A.L., Li, W., Cressy, M., and Dubnau, J. (2009). Short- and Long-Term Memory in *Drosophila* Require cAMP Signaling in Distinct Neuron Types. *Current Biology* *19*, 1341–1350.
- Bondy, C., Werner, H., Roberts, C.T., and LeRoith, D. (1992). Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. *Nsc* *46*, 909–923.
- Borovok, N., Neshet, E., Levin, Y., Reichenstein, M., Pinhasov, A., and Michaelovski, I. (2016). Dynamics of Hippocampal Protein Expression During Long-term Spatial Memory Formation. *Mol Cell Proteomics* *15*, 523–541.
- Boto, T., Louis, T., Jindachomthong, K., Jalink, K., and Tomchik, S.M. (2014). Dopaminergic Modulation of cAMP Drives Nonlinear Plasticity across the *Drosophila* Mushroom Body Lobes. *Current Biology* *24*, 822–831.
- Bouzaiane, E., Trannoy, S., Scheunemann, L., Plaçais, P.-Y., and Preat, T. (2015). Two Independent Mushroom Body Output Circuits Retrieve the Six Discrete Components of *Drosophila* Aversive Memory. *CellReports* *11*, 1280–1292.
- Bozzo, L., Puyal, J., and Chatton, J.-Y. (2013). Lactate Modulates the Activity of Primary Cortical Neurons through a Receptor-Mediated Pathway. *PLoS ONE* *8*, e71721–e71729.
- Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* *118*, 401–415.
- Broggiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology* *11*, 213–221.
- Broughton, S.J., Piper, M.D.W., Ikeya, T., Bass, T.M., Jacobson, J., Driège, Y., Martinez, P., Hafen, E., Withers, D.J., Leivers, S.J., et al. (2005). Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proc Natl Acad Sci USA* *102*, 3105–3110.
- Broughton, S., Alic, N., Slack, C., Bass, T., Ikeya, T., Vinti, G., Tommasi, A.M., Driège, Y., Hafen, E., and Partridge, L. (2008). Reduction of DILP2 in *Drosophila* triages a metabolic phenotype from lifespan revealing redundancy and compensation among DILPs. *PLoS ONE* *3*, e3721.
- Brown, A.M., and Ransom, B.R. (2007). Astrocyte glycogen and brain energy metabolism. *Glia* *55*, 1263–1271.
- Bruehl, H., Sweat, V., Hassenstab, J., Polyakov, V., and Convit, A. (2010). Cognitive impairment in nondiabetic middle-aged and older adults is associated with insulin resistance. *Journal of Clinical and Experimental Neuropsychology* *32*, 487–493.
- Brüning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone,

- W., Müller-Wieland, D., and Kahn, C.R. (2000). Role of brain insulin receptor in control of body weight and reproduction. *Science* 289, 2122–2125.
- Burger, J.M.S., Kolss, M., Pont, J., and Kawecki, T.J. (2008). LEARNING ABILITY AND LONGEVITY: A SYMMETRICAL EVOLUTIONARY TRADE-OFF IN DROSOPHILA. *Evolution* 62, 1294–1304.
- Burke, C.J., and Waddell, S. (2011). Remembering Nutrient Quality of Sugar in Drosophila. *Current Biology* 21, 746–750.
- Burke, C.J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M.J., Das, G., Gohl, D., Silies, M., Certel, S., and Waddell, S. (2012). Layered reward signalling through octopamine and dopamine in Drosophila. *Nature Publishing Group* 492, 433–437.
- Burns, J.G., Foucaud, J., and Mery, F. (2011). Costs of memory: lessons from “mini” brains. *Proceedings of the Royal Society B: Biological Sciences* 278, 923–929.
- Cai, W., Xue, C., Sakaguchi, M., Konishi, M., Shirazian, A., Ferris, H.A., Li, M.E., Yu, R., Kleinriders, A., Pothos, E.N., et al. (2018). Insulin regulates astrocyte gliotransmission and modulates behavior. *J. Clin. Invest.* 128, 2914–2926.
- Canal, C.E. (2005). Glucose injections into the dorsal hippocampus or dorsolateral striatum of rats prior to T-maze training: Modulation of learning rates and strategy selection. *Learning & Memory* 12, 367–374.
- Candy, D.J., and Kilby, B.A. (1959). Site and mode of trehalose biosynthesis in the locust. *Nature* 183, 1594–1595.
- Candy, D.J., and Kilby, B.A. (1961). The biosynthesis of trehalose in the locust fat body. *Biochem. J.* 78, 531–536.
- Carew, T.J., Pinsker, H.M., and Kandel, E.R. (1972). Long-term habituation of a defensive withdrawal reflex in aplysia. *Science* 175, 451–454.
- Caron, S.J.C., Ruta, V., Abbott, L.F., and Axel, R. (2013). Random convergence of olfactory inputs in the Drosophila mushroom body. *Nature* 497, 113–117.
- Castellucci, V., and Kandel, E.R. (1976). Presynaptic facilitation as a mechanism for behavioral sensitization in Aplysia. *Science* 194, 1176–1178.
- Chabaud, M.-A., Isabel, G., Kaiser, L., and Preat, T. (2009). Social Facilitation of Long-Lasting Memory Retrieval in Drosophila. *Current Biology* 19, 1654–1659.
- Chambers, D.B., Androschuk, A., Rosenfelt, C., Langer, S., Harding, M., and Bolduc, F.V. (2015). Insulin signaling is acutely required for long-term memory in Drosophila. *Front. Neural Circuits* 9, 8.
- Chell, J.M., and Brand, A.H. (2010). Nutrition-Responsive Glia Control Exit of Neural Stem Cells from Quiescence. *Cell* 143, 1161–1173.

- Chen, D.Y., Stern, S.A., Garcia-Osta, A., Saunier-Rebori, B., Pollonini, G., Bambah-Mukku, D., Blitzer, R.D., and Alberini, C.M. (2011). A critical role for IGF-II in memory consolidation and enhancement. *Nature* *469*, 491–497.
- Chever, O., Dossi, E., Pannasch, U., Derangeon, M., and Rouach, N. (2016). Astroglial networks promote neuronal coordination. *Science Signaling* *9*, ra6–ra6.
- Cho, S., Muthukumar, A.K., Stork, T., Coutinho-Budd, J.C., and Freeman, M.R. (2018). Focal adhesion molecules regulate astrocyte morphology and glutamate transporters to suppress seizure-like behavior. *Proc Natl Acad Sci USA* *9*, 201800830–201800836.
- Chuquet, J., Quilichini, P., Nimchinsky, E.A., and Buzsáki, G. (2010). Predominant enhancement of glucose uptake in astrocytes versus neurons during activation of the somatosensory cortex. *J. Neurosci.* *30*, 15298–15303.
- Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell* *139*, 405–415.
- Cognigni, P., Felsenberg, J., and Waddell, S. (2018). Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*. *Current Opinion in Neurobiology* *49*, 51–58.
- Colomb, J., Kaiser, L., Chabaud, M.A., and Preat, T. (2009). Parametric and genetic analysis of *Drosophila* appetitive long-term memory and sugar motivation. *Genes, Brain and Behavior* *8*, 407–415.
- Comas, D., Petit, F., and Preat, T. (2004). *Drosophila* long-term memory formation involves regulation of cathepsin activity. *Nature* *430*, 460–463.
- Costello, D.A., Claret, M., Al-Qassab, H., Plattner, F., Irvine, E.E., Choudhury, A.I., Giese, K.P., Withers, D.J., and Pedarzani, P. (2012). Brain Deletion of Insulin Receptor Substrate 2 Disrupts Hippocampal Synaptic Plasticity and Metaplasticity. *PLoS ONE* *7*, e31124–13.
- Coutinho-Budd, J.C., Sheehan, A.E., and Freeman, M.R. (2017). The secreted neurotrophin Spätzle 3 promotes glial morphogenesis and supports neuronal survival and function. *Genes & Development* *31*, 2023–2038.
- Couto, A., Alenius, M., and Dickson, B.J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Current Biology* *15*, 1535–1547.
- Craft, S., Asthana, S., Newcomer, J.W., Wilkinson, C.W., Matos, I.T., Baker, L.D., Cherrier, M., Lofgreen, C., Latendresse, S., Petrova, A., et al. (1999). Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose. *Arch. Gen. Psychiatry* *56*, 1135–1140.
- Craft, S. (2012). Intranasal Insulin Therapy for Alzheimer Disease and Amnesic Mild Cognitive Impairment. *Arch Neurol* *69*, 29–10.
- Croset, V., Treiber, C.D., and Waddell, S. (2018). Cellular diversity in the *Drosophila* midbrain revealed by single-cell transcriptomics. *eLife* *7*, 312.

da Silva Menegasso, A.R., Pratavieira, M., de Saldanha da Gama Fischer, J., Carvalho, P.C., Roat, T.C., Malaspina, O., and Palma, M.S. (2017). Profiling the proteomics in honeybee worker brains submitted to the proboscis extension reflex. *J Proteomics* *151*, 131–144.

Dallérac, G., Zapata, J., and Rouach, N. (2018). Versatile control of synaptic circuits by astrocytes: where, when and how? *Nat Rev Neurosci* 1–15.

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, Ł., Aibar, S., Makhzami, S., Christiaens, V., Bravo González-Blas, C., et al. (2018). A Single-Cell Transcriptome Atlas of the Aging *Drosophila* Brain. *Cell* *174*, 982–998.e20.

de Belle, J.S., and Heisenberg, M. (1994). Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* *263*, 692–695.

De-Miguel, F.F., Leon-Pinzon, C., Noguez, P., and Mendez, B. (2015). Serotonin release from the neuronal cell body and its long-lasting effects on the nervous system. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* *370*, 20140196–20140199.

Dienel, G.A., Cruz, N.F., Sokoloff, L., and Driscoll, B.F. (2017). Determination of Glucose Utilization Rates in Cultured Astrocytes and Neurons with [14C]deoxyglucose: Progress, Pitfalls, and Discovery of Intracellular Glucose Compartmentation. *Neurochem Res* *42*, 50–63.

Díaz-García, C.M., Mongeon, R., Lahmann, C., Koveal, D., Zucker, H., and Yellen, G. (2017). Neuronal Stimulation Triggers Neuronal Glycolysis and Not Lactate Uptake. *Cell Metabolism* *26*, 361–374.e364.

Doherty, J., Logan, M.A., Tasdemir, O.E., and Freeman, M.R. (2009). Ensheathing Glia Function as Phagocytes in the Adult *Drosophila* Brain. *Journal of Neuroscience* *29*, 4768–4781.

Doherty, J., Sheehan, A.E., Bradshaw, R., Fox, A.N., Lu, T.-Y., and Freeman, M.R. (2014). PI3K Signaling and Stat92E Converge to Modulate Glial Responsiveness to Axonal Injury. *PLoS Biol* *12*, e1001985–16.

Dolan, M.-J., Belliard-Guérin, G., Bates, A.S., Frechter, S., Lampin-Saint-Amaux, A., Aso, Y., Roberts, R.J.V., Schlegel, P., Wong, A., Hammad, A., et al. (2018). Communication from Learned to Innate Olfactory Processing Centers Is Required for Memory Retrieval in *Drosophila*. *Neuron* *100*, 651–668.e658.

Dong, S., Replogle, K.L., Hasadsri, L., Imai, B.S., Yau, P.M., Rodriguez-Zas, S., Southey, B.R., Sweedler, J.V., and Clayton, D.F. (2009). Discrete molecular states in the brain accompany changing responses to a vocal signal. *Proc. Natl. Acad. Sci. U.S.a.* *106*, 11364–11369.

Dou, J.-T., Chen, M., Dufour, F., Alkon, D.L., and Zhao, W.-Q. (2005). Insulin receptor signaling in long-term memory consolidation following spatial learning. *Learning & Memory* *12*, 646–655.

Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* *411*, 476–480.

- Duran, J., Tevy, M.F., Garcia-Rocha, M., Calbó, J., Milán, M., and Guinovart, J.J. (2012). Deleterious effects of neuronal accumulation of glycogen in flies and mice. *EMBO Mol Med* 4, 719–729.
- Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by ¹³C nuclear magnetic resonance spectroscopy. *J. Neurosci.* 23, 5928–5935.
- Engl, E., and Attwell, D. (2015). Non-signalling energy use in the brain. *J. Physiol. (Lond.)* 593, 3417–3429.
- Engl, E., Jolivet, R., Hall, C.N., and Attwell, D. (2017). Non-signalling energy use in the developing rat brain. *Journal of Cerebral Blood Flow & Metabolism* 37, 951–966.
- Engler, H., Forsberg, A., Almkvist, O., Blomquist, G., Larsson, E., Savitcheva, I., Wall, A., Ringheim, A., Langstrom, B., and Nordberg, A. (2006). Two-year follow-up of amyloid deposition in patients with Alzheimer's disease. *Brain* 129, 2856–2866.
- Evans, D.R., and Dethier, V.G. (1957). The regulation of taste thresholds for sugars in the blowfly. *Journal of Insect Physiology* 1–15.
- Farca Luna, A.J., Perier, M., and Seugnet, L. (2017). Amyloid Precursor Protein in *Drosophila* Glia Regulates Sleep and Genes Involved in Glutamate Recycling. *J. Neurosci.* 37, 4289–4300.
- Fernandez, A.M., and Torres-Alemán, I. (2012). The many faces of insulin-like peptide signalling in the brain. *Nat Rev Neurosci* 13, 225–239.
- Fernandez, A.M., Hernandez-Garzón, E., Perez-Domper, P., Perez-Alvarez, A., Mederos, S., Matsui, T., Santi, A., Trueba-Saiz, A., García-Guerra, L., Pose-Utrilla, J., et al. (2017). Insulin Regulates Astrocytic Glucose Handling Through Cooperation With IGF-I. *Diabetes* 66, 64–74.
- Figueroa-Clarevega, A., and Bilder, D. (2015). Malignant *Drosophila* Tumors Interrupt Insulin Signaling to Induce Cachexia-like Wasting. *Developmental Cell* 33, 47–55.
- Flexner, J.B., Flexner, L.B., Stellar, E., DEe la Haba, G., and Roberts, R.B. (1962). Inhibition of protein synthesis in brain and learning and memory following puromycin. *Journal of Neurochemistry* 9, 595–605.
- Freeman, M.R. (2015). *Drosophila* Central Nervous System Glia. *Cold Spring Harb Perspect Biol* 7.
- Freeman, M.R., and Doherty, J. (2006). Glial cell biology in *Drosophila* and vertebrates. *Trends in Neurosciences* 29, 82–90.
- Freeman, M.R., Delrow, J., Kim, J., Johnson, E., and Doe, C.Q. (2003). Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. *Neuron* 38, 567–580.
- Gai, Y., Liu, Z., Cervantes-Sandoval, I., and Davis, R.L. (2016). *Drosophila* SLC22A Transporter

Is a Memory Suppressor Gene that Influences Cholinergic Neurotransmission to the Mushroom Bodies. *Neuron* 90, 581–595.

Ganeshan, K., and Chawla, A. (2014). Metabolic Regulation of Immune Responses. *Annu. Rev. Immunol.* 32, 609–634.

Gao, V., Suzuki, A., Magistretti, P.J., Lengacher, S., Pollonini, G., Steinman, M.Q., and Alberini, C.M. (2016). Astrocytic β 2-adrenergic receptors mediate hippocampal long-term memory consolidation. *Proc. Natl. Acad. Sci. U.S.A.* 113, 8526–8531.

García-Cáceres, C., Quarta, C., Varela, L., Gao, Y., Gruber, T., Legutko, B., Jastroch, M., Johansson, P., Ninkovic, J., Yi, C.-X., et al. (2016). Astrocytic Insulin Signaling Couples Brain Glucose Uptake with Nutrient Availability. *Cell* 166, 867–880.

Gejl, M., Brock, B., Egefjord, L., Vang, K., Rungby, J., and Gjedde, A. (2017). Blood-Brain Glucose Transfer in Alzheimer's disease: Effect of GLP-1 Analog Treatment. *Sci. Rep.* 7, 17490.

Gerlach, M., Riederer, P., Przuntek, H., and Youdim, M.B. (1991). MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *European Journal of Pharmacology* 208, 273–286.

Gervasi, N., Tchenio, P., and Preat, T. (2010). PKA Dynamics in a *Drosophila* Learning Center: Coincidence Detection by Rutabaga Adenylyl Cyclase and Spatial Regulation by Dunce Phosphodiesterase. *Neuron* 65, 516–529.

Giaume, C., Taberero, A., and Medina, J.M. (1997). Metabolic trafficking through astrocytic gap junctions. *Glia* 21, 114–123.

Gibbs, M.E., Anderson, D.G., and Hertz, L. (2006). Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* 54, 214–222.

Gorman, G.S., Chinnery, P.F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., Suomalainen, A., Thorburn, D.R., Zeviani, M., and Turnbull, D.M. (2016). Mitochondrial diseases. *Nature Publishing Group* 2, 16080.

Griffith, L.C. (2014). A big picture of a small brain. *eLife* 3, e04577–4.

Grönke, S., Clarke, D.-F., Broughton, S., Andrews, T.D., and Partridge, L. (2010). Molecular Evolution and Functional Characterization of *Drosophila* Insulin-Like Peptides. *PLoS Genet* 6, e1000857–18.

Hanani, M. (2005). Satellite glial cells in sensory ganglia: from form to function. *Brain Research Reviews* 48, 457–476.

Hartline, D.K. (2011). The evolutionary origins of glia. *Glia* 59, 1215–1236.

Hasselbalch, S.G., Knudsen, G.M., Videbaek, C., Pinborg, L.H., Schmidt, J.F., Holm, S., and Paulson, O.B. (1999). No effect of insulin on glucose blood-brain barrier transport and cerebral metabolism in humans. *Diabetes* 48, 1915–1921.

Havrankova, J., Roth, J., and Brownstein, M. (1978). Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* 272, 827–829.

Heil, J.E., Oland, L.A., and Lohr, C. (2007). Acetylcholine-mediated axon-glia signaling in the developing insect olfactory system. *Eur J Neurosci* 26, 1227–1241.

Heisenberg, M., Borst, A., Wagner, S., and Byers, D. (1985). *Drosophila* mushroom body mutants are deficient in olfactory learning. *J Neurogenet* 2, 1–30.

Herculano-Houzel, S. (2014). The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. *Glia* 62, 1377–1391.

Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., and Bolanos, J.P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C–Cdh1. *Nat Cell Biol* 11, 747–752.

Hess, A. (1958). The fine structure of nerve cells and fibers, neuroglia, and sheaths of the ganglion chain in the cockroach (*Periplaneta americana*). *J Biophys Biochem Cytol* 4, 731–742.

Hige, T., Aso, Y., Modi, M.N., Rubin, G.M., and Turner, G.C. (2015). Heterosynaptic Plasticity Underlies Aversive Olfactory Learning in *Drosophila*. *Neuron* 88, 985–998.

Hirano, Y., Masuda, T., Naganos, S., Matsuno, M., Ueno, K., Miyashita, T., Horiuchi, J., and Saitoe, M. (2013). Fasting Launches CRTC to Facilitate Long-Term Memory Formation in *Drosophila*. *Science* 339, 1–5.

Hochstrate, P., and Schlue, W.R. (1995). Ca²⁺ influx into leech neuropile glial cells mediated by nicotinic acetylcholine receptors. *Glia* 15, 43–53.

Howarth, C., Gleeson, P., and Attwell, D. (2012). Updated energy budgets for neural computation in the neocortex and cerebellum. *Journal of Cerebral Blood Flow & Metabolism* 32, 1222–1232.

Hoyle, G., Williams, M., and Phillips, C. (1986). Functional morphology of insect neuronal cell-surface/glia contacts: the trophospongium. *J. Comp. Neurol.* 246, 113–128.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Current Biology* 12, 1293–1300.

Isabel, G., Pascual, A., and Preat, T. (2004). Exclusive consolidated memory phases in *Drosophila*. *Science* 304, 1024–1027.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., and Strausfeld, N.J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learning & Memory* 5, 52–77.

Ito, K., Urban, J., and Technau, G.M. (1995). Distribution, classification, and development of

Drosophila glial cells in the late embryonic and early larval ventral nerve cord. *Roux Arch. Dev. Biol.* *204*, 284–307.

Ja, W.W., Carvalho, G.B., Benzer, S., Brummel, T., Mak, E.M., la Rosa, de, N.N., Fang, A.Y., and Liong, J.C. (2014). Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. U.S.A.* *104*, 1–4.

Jakkamsetti, V., Marin-Valencia, I., Ma, Q., Good, L.B., Terrill, T., Rajasekaran, K., Pichumani, K., Khemtong, C., Hooshyar, M.A., Sundarajan, C., et al. (2019). Brain metabolism modulates neuronal excitability in a mouse model of pyruvate dehydrogenase deficiency. *Sci Transl Med* *11*, eaan0457.

Jakoby, P., Schmidt, E., Ruminot, I., Gutierrez, R., Barros, L.F., and Deitmer, J.W. (2013). Higher Transport and Metabolism of Glucose in Astrocytes Compared with Neurons: A Multiphoton Study of Hippocampal and Cerebellar Tissue Slices. *Cerebral Cortex* *24*, 222–231.

Jang, S., Nelson, J.C., Bend, E.G., Rodríguez-Laureano, L., Tueros, F.G., Cartagena, L., Underwood, K., Jorgensen, E.M., and Colón-Ramos, D.A. (2016). Glycolytic Enzymes Localize to Synapses under Energy Stress to Support Synaptic Function. *Neuron* *90*, 278–291.

Jefferis, G.S., Marin, E.C., Stocker, R.F., and Luo, L. (2001). Target neuron prespecification in the olfactory map of *Drosophila*. *Nature* *414*, 204–208.

Johnson, D.A., and Pilar, G. (1980). The release of acetylcholine from post-ganglionic cell bodies in response to depolarization. *J. Physiol. (Lond.)* *299*, 605–619.

Johnson, T.E. (1990). Increased life-span of age-1 mutants in *Caenorhabditis elegans* and lower Gompertz rate of aging. *Science* *249*, 908–912.

Johnston, T.D. (1982). *Advances in the Study of Behavior* (Academic Press).

Jolivet, R. (2009). Deciphering neuron-glia compartmentalization in cortical energy metabolism. *Front Neuroenergetics* *1*, 1–10.

Kappeler, L., Filho, C.D.M., Dupont, J., Leneuve, P., Cervera, P., Périn, L., Loudes, C., Blaise, A., Klein, R., Epelbaum, J., et al. (2008). Brain IGF-1 Receptors Control Mammalian Growth and Lifespan through a Neuroendocrine Mechanism. *PLoS Biol* *6*, e254–10.

Kasischke, K.A., Vishwasrao, H.D., Fisher, P.J., Zipfel, W.R., and Webb, W.W. (2004). Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* *305*, 99–103.

Kasuya, J. (2009). Neuronal mechanisms of learning and memory revealed by spatial and temporal suppression of neurotransmission using shibirets1, a temperature-sensitive dynamin mutant gene in *Drosophila melanogaster*. *Front. Mol. Neurosci.* *2*, 1–6.

Kety, S.S. (1957). *METABOLISM OF THE NERVOUS SYSTEM* (Pergamon Press Ltd).

Kis, V., Barti, B., Lippai, M., and Sass, M. (2015). Specialized Cortex Glial Cells Accumulate Lipid

Droplets in *Drosophila melanogaster*. *PLoS ONE* 10, e0131250–19.

Kitamoto, T. (2002). Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila*. *Proc Natl Acad Sci USA* 99, 13232–13237.

Knappek, S., Sigrist, S., and Tanimoto, H. (2011). Bruchpilot, A Synaptic Active Zone Protein for Anesthesia-Resistant Memory. *Journal of Neuroscience* 31, 3453–3458.

Kremer, M.C., Jung, C., Batelli, S., Rubin, G.M., and Gaul, U. (2017). The glia of the adult *Drosophila* nervous system. *Glia* 65, 606–638.

Kunduri, G., Turner-Evans, D., Konya, Y., Izumi, Y., Nagashima, K., Lockett, S., Holthuis, J., Bamba, T., Acharya, U., and Acharya, J.K. (2018). Defective cortex glia plasma membrane structure underlies light-induced epilepsy in *cpe* mutants. *Proc. Natl. Acad. Sci. U.S.a.* 68, 201808463–10.

Kupfermann, I., and Kandel, E.R. (1969). Neuronal controls of a behavioral response mediated by the abdominal ganglion of *Aplysia*. *Science* 164, 847–850.

Lagasse, F., Moreno, C., Preat, T., and Mery, F. (2012). Functional and evolutionary trade-offs co-occur between two consolidated memory phases in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 279, 4015–4023.

Lai, S.-L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in *Drosophila*. *Nat Neurosci* 9, 703–709.

Lajtha, A., Gibson, G.E., Dienel, G.A., and Dienel, G.A. (2007). *Handbook of Neurochemistry and Molecular Neurobiology* (Boston, MA: Springer Science & Business Media).

Lazareva, A.A., Roman, G., Mattox, W., Hardin, P.E., and Dauwalder, B. (2007). A role for the adult fat body in *Drosophila* male courtship behavior. *PLoS Genet* 3, e16.

Lee, J.M., Grabb, M.C., Zipfel, G.J., and Choi, D.W. (2000). Brain tissue responses to ischemia. *J. Clin. Invest.* 106, 723–731.

Lee, P.-T., Lin, H.-W., Chang, Y.-H., Fu, T.-F., Dubnau, J., Hirsh, J., Lee, T., and Chiang, A.-S. (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.a.* 108, 13794–13799.

Levin, L.R., Han, P.L., Hwang, P.M., Feinstein, P.G., Davis, R.L., and Reed, R.R. (1992). The *Drosophila* learning and memory gene *rutabaga* encodes a Ca²⁺/Calmodulin-responsive adenylyl cyclase. *Cell* 68, 479–489.

Liberti, M.V., and Locasale, J.W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in Biochemical Sciences* 41, 211–218.

Lin, C.H.A., Tomioka, M., Pereira, S., Sellings, L., Iino, Y., and van der Kooy, D. (2010). Insulin signaling plays a dual role in *Caenorhabditis elegans* memory acquisition and memory retrieval. *J. Neurosci.* 30, 8001–8011.

- Lin, M.T., and Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* *443*, 787–795.
- Lin, S., Oswald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. *Nat Neurosci* *17*, 1536–1542.
- Lindsay, D.B., and Setchell, B.P. (1976). The oxidation of glucose, ketone bodies and acetate by the brain of normal and ketonaemic sheep. *J. Physiol. (Lond.)* *259*, 801–823.
- Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B.D., Aso, Y., Friedrich, A.B., Siwanowicz, I., Rubin, G.M., Preat, T., and Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* *488*, 512–516.
- Liu, H., Zhou, B., Yan, W., Lei, Z., Zhao, X., Zhang, K., and Guo, A. (2014). Astrocyte-like glial cells physiologically regulate olfactory processing through the modification of ORN-PN synaptic strength in *Drosophila*. *Eur J Neurosci* *40*, 2744–2754.
- Liu, Y., Zeng, X., Hui, Y., Zhu, C., Wu, J., Taylor, D.H., Ji, J., Fan, W., Huang, Z., and Hu, J. (2015). Activation of $\alpha 7$ nicotinic acetylcholine receptors protects astrocytes against oxidative stress-induced apoptosis: implications for Parkinson's disease. *Neuropharmacology* *91*, 87–96.
- Loaiza, A., Porras, O.H., and Barros, L.F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *J. Neurosci.* *23*, 7337–7342.
- Loewenstein, W.R. (1966). Permeability of membrane junctions. *Ann. N. Y. Acad. Sci.* *137*, 441–472.
- Louis, T., Stahl, A., Boto, T., and Tomchik, S.M. (2018). Cyclic AMP-dependent plasticity underlies rapid changes in odor coding associated with reward learning. *Proc. Natl. Acad. Sci. U.S.A.* *115*, E448–E457.
- Lovatt, D., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., He, W., Lin, J.H.-C., Han, X., Takano, T., Wang, S., Sim, F.J., et al. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *J. Neurosci.* *27*, 12255–12266.
- Lu, T.-Y., MacDonald, J.M., Neukomm, L.J., Sheehan, A.E., Bradshaw, R., Logan, M.A., and Freeman, M.R. (2017). Axon degeneration induces glial responses through Draper-TRAF4-JNK signalling. *Nature Communications* *8*, 14355–14359.
- Luan, H., Peabody, N.C., Vinson, C.R., and White, B.H. (2006). Refined Spatial Manipulation of Neuronal Function by Combinatorial Restriction of Transgene Expression. *Neuron* *52*, 425–436.
- Lundgaard, I., Li, B., Xie, L., Kang, H., Sanggaard, S., Haswell, J.D.R., Sun, W., Goldman, S., Blekot, S., Nielsen, M., et al. (2015). Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. *Nature Communications* *6*, 6807.
- MacDonald, J.M., Beach, M.G., Porpiglia, E., Sheehan, A.E., Watts, R.J., and Freeman, M.R.

(2006). The Drosophila Cell Corpse Engulfment Receptor Draper Mediates Glial Clearance of Severed Axons. *Neuron* 50, 869–881.

Maday, S., and Holzbaur, E.L.F. (2016). Compartment-Specific Regulation of Autophagy in Primary Neurons. *J. Neurosci.* 36, 5933–5945.

Magistretti, P.J., and Allaman, I. (2007). Glycogen: a Trojan horse for neurons. *Nat Neurosci* 10, 1341–1342.

Magistretti, P.J., and Allaman, I. (2015). A cellular perspective on brain energy metabolism and functional imaging. *Neuron* 86, 883–901.

Magistretti, P.J., and Allaman, I. (2018). Lactate in the brain: from metabolic end-product to signalling molecule. *Nature Publishing Group* 19, 235–249.

Maher, F., Davies-Hill, T.M., and Simpson, I.A. (1996). Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons. *Biochem. J.* 315 (Pt 3), 827–831.

Matsuno, M., Horiuchi, J., Yuasa, Y., Ofusa, K., Miyashita, T., Masuda, T., and Saitoe, M. (2015). Long-term memory formation in Drosophila requires training-dependent glial transcription. *J. Neurosci.* 35, 5557–5565.

Mattila, J., and Hietakangas, V. (2017). Regulation of Carbohydrate Energy Metabolism in Drosophila melanogaster. *Genetics* 207, 1231–1253.

Mattson, M.P., Gleichmann, M., and Cheng, A. (2008). Mitochondria in Neuroplasticity and Neurological Disorders. *Neuron* 60, 748–766.

McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of Drosophila mushroom body signaling in olfactory memory. *Science* 293, 1330–1333.

McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal Gene Expression Targeting with the TARGET and Gene-Switch Systems in Drosophila. *Science Signaling* 2004, pl6–pl6.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* 302, 1765–1768.

Melom, J.E., and Littleton, J.T. (2013). Mutation of a NCKX Eliminates Glial Microdomain Calcium Oscillations and Enhances Seizure Susceptibility. *Journal of Neuroscience* 33, 1169–1178.

Mery, F. (2007). Aging and its differential effects on consolidated memory forms in Drosophila. *Experimental Gerontology* 42, 99–101.

Mery, F., and Kawecki, T.J. (2005). A cost of long-term memory in Drosophila. *Science* 308, 1148–1148.

Mery, F., Pont, J., Preat, T., and Kawecki, T.J. (2007). Experimental evolution of olfactory memory in Drosophila melanogaster. *Physiol. Biochem. Zool.* 80, 399–405.

- Messier, C. (1997). Object Recognition in Mice: Improvement of Memory by Glucose. *Neurobiology of Learning and Memory* 67, 1–4.
- Messier, C. (2004). Glucose improvement of memory: a review. *European Journal of Pharmacology* 490, 33–57.
- Mink, J.W., Blumenschine, R.J., and Adams, D.B. (1981). Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. *Am. J. Physiol.* 241, R203–R212.
- Misgeld, T., and Schwarz, T.L. (2017). Mitostasis in Neurons: Maintaining Mitochondria in an Extended Cellular Architecture. *Neuron* 96, 651–666.
- Moran, D., Softley, R., and Warrant, E.J. (2015). The energetic cost of vision and the evolution of eyeless Mexican cavefish. *Science Advances* 1, e1500363–e1500363.
- Morgello, S., Uson, R.R., Schwartz, E.J., and Haber, R.S. (1995). The human blood-brain barrier glucose transporter (GLUT1) is a glucose transporter of gray matter astrocytes. *Glia* 14, 43–54.
- Mueckler, M., and Thorens, B. (2013). The SLC2 (GLUT) family of membrane transporters. *Molecular Aspects of Medicine* 34, 121–138.
- Mullins, R.J., Diehl, T.C., Chia, C.W., and Kapogiannis, D. (2017). Insulin Resistance as a Link between Amyloid-Beta and Tau Pathologies in Alzheimer’s Disease. *Front. Aging Neurosci.* 9, 1531–16.
- Murray, I.V.J., Proza, J.F., Sohrabji, F., and Lawler, J.M. (2011). Vascular and metabolic dysfunction in Alzheimer's disease: a review. *Exp Biol Med (Maywood)* 236, 772–782.
- Musashe, D.T., Purice, M.D., Speese, S.D., Doherty, J., and Logan, M.A. (2016). Insulin-like Signaling Promotes Glial Phagocytic Clearance of Degenerating Axons through Regulation of Draper. *CellReports* 16, 1838–1850.
- Musso, P.-Y., Lampin-Saint-Amaux, A., Tchenio, P., and Preat, T. (2017). Ingestion of artificial sweeteners leads to caloric frustration memory in *Drosophila*. *Nature Communications* 1–10.
- Musso, P.-Y., Tchenio, P., and Preat, T. (2015). Delayed Dopamine Signaling of Energy Level Builds Appetitive Long-Term Memory in *Drosophila*. *CellReports* 10, 1023–1031.
- Muthukumar, A.K., Stork, T., and Freeman, M.R. (2014). Activity-dependent regulation of astrocyte GAT levels during synaptogenesis. *Nat Neurosci* 17, 1340–1350.
- Naganos, S., Horiuchi, J., and Saitoe, M. (2012). Mutations in the *Drosophila* insulin receptor substrate, CHICO, impair olfactory associative learning. *Neuroscience Research* 73, 49–55.
- Nagarkar-Jaiswal, S., DeLuca, S.Z., Lee, P.-T., Lin, W.-W., Pan, H., Zuo, Z., Lv, J., Spradling, A.C., and Bellen, H.J. (2015). A genetic toolkit for tagging intronic MiMIC containing genes. *eLife* 4.
- Nakano, R., Iwamura, M., Obikawa, A., Togane, Y., Hara, Y., Fukuhara, T., Tomaru, M., Takano-

- Shimizu, T., and Tsujimura, H. (2019). Cortex glia clear dead young neurons via Drpr/dCed-6/Shark and Crk/Mbc/dCed-12 signaling pathways in the developing *Drosophila* optic lobe. *Developmental Biology* 1–54.
- Nässel, D.R., Liu, Y., and Luo, J. (2015). Insulin/IGF signaling and its regulation in *Drosophila*. *General and Comparative Endocrinology* 221, 255–266.
- Newman, L.A., Korol, D.L., and Gold, P.E. (2011). Lactate Produced by Glycogenolysis in Astrocytes Regulates Memory Processing. *PLoS ONE* 6, e28427.
- Ng, F.S., Tangredi, M.M., and Jackson, F.R. (2011). Glial Cells Physiologically Modulate Clock Neurons and Circadian Behavior in a Calcium-Dependent Manner. *Current Biology* 21, 625–634.
- Obermeier, B., Daneman, R., and Ransohoff, R.M. (2013). Development, maintenance and disruption of the blood-brain barrier. *Nature Medicine* 19, 1584–1596.
- Okamoto, N., Nakamori, R., Murai, T., Yamauchi, Y., Masuda, A., and Nishimura, T. (2013). A secreted decoy of InR antagonizes insulin/IGF signaling to restrict body growth in *Drosophila*. *Genes & Development* 27, 87–97.
- Oland, L.A., Biebelhausen, J.P., and Tolbert, L.P. (2008). Glial investment of the adult and developing antennal lobe of *Drosophila*. *J. Comp. Neurol.* 509, 526–550.
- Olsson, T., MacMillan, H.A., Nyberg, N., Staerk, D., Malmendal, A., and Overgaard, J. (2016). Hemolymph metabolites and osmolality are tightly linked to cold tolerance of *Drosophila* species: a comparative study. *J. Exp. Biol.* 219, 2504–2513.
- Owald, D., Felsenberg, J., Talbot, C.B., Das, G., Perisse, E., Huetteroth, W., and Waddell, S. (2015). Activity of Defined Mushroom Body Output Neurons Underlies Learned Olfactory Behavior in *Drosophila*. *Neuron* 86, 417–427.
- Owen, O.E., Morgan, A.P., Kemp, H.G., Sullivan, J.M., Herrera, M.G., and Cahill, G.F. (1967). Brain metabolism during fasting. *J. Clin. Invest.* 46, 1589–1595.
- Pannese, E. (2002). Perikaryal surface specializations of neurons in sensory ganglia. *Int. Rev. Cytol.* 220, 1–34.
- Papouin, T., Dunphy, J.M., Tolman, M., Dineley, K.T., and Haydon, P.G. (2017). Septal Cholinergic Neuromodulation Tunes the Astrocyte-Dependent Gating of Hippocampal NMDA Receptors to Wakefulness. *Neuron* 94, 840–854.e847.
- Pappenheimer, J.R., and Setchell, B.P. (1973). Cerebral glucose transport and oxygen consumption in sheep and rabbits. *J. Physiol. (Lond.)* 233, 529–551.
- Pascual, A., and Preat, T. (2001). Localization of long-term memory within the *Drosophila* mushroom body. *Science* 294, 1115–1117.
- Patel, A.B., Lai, J.C.K., Chowdhury, G.M.I., Hyder, F., Rothman, D.L., Shulman, R.G., and Behar,

K.L. (2014). Direct evidence for activity-dependent glucose phosphorylation in neurons with implications for the astrocyte-to-neuron lactate shuttle. *Proc Natl Acad Sci USA* *111*, 5385–5390.

Pavlov, I.P. (1927). Conditioned Reflexes.

Pavlovsky, A., Schor, J., Plaçais, P.-Y., and Preat, T. (2018). A GABAergic Feedback Shapes Dopaminergic Input on the *Drosophila* Mushroom Body to Promote Appetitive Long-Term Memory. *Current Biology* *28*, 1783–1793.e1784.

Pellerin, L., and Magistretti, P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA* *91*, 10625–10629.

Pereanu, W., Shy, D., and Hartenstein, V. (2005). Morphogenesis and proliferation of the larval brain glia in *Drosophila*. *Developmental Biology* *283*, 191–203.

Pereanu, W., Spindler, S., Cruz, L., and Hartenstein, V. (2007). Tracheal development in the *Drosophila* brain is constrained by glial cells. *Developmental Biology* *302*, 169–180.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.-T.B., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Lavery, T.R., et al. (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* *105*, 9715–9720.

Pfeiffer, B.D., Ngo, T.-T.B., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in *Drosophila*. *Genetics* *186*, 735–755.

Philp, A., Macdonald, A.L., and Watt, P.W. (2005). Lactate--a signal coordinating cell and systemic function. *Journal of Experimental Biology* *208*, 4561–4575.

Plaçais, P.-Y., and Preat, T. (2013). To favor survival under food shortage, the brain disables costly memory. *Science* *339*, 440–442.

Plaçais, P.-Y., de Tredern, E., Scheunemann, L., Trannoy, S., Goguel, V., Han, K.-A., Isabel, G., and Preat, T. (2017). Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory. *Nature Communications* *8*, 1–14.

Plaçais, P.-Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliart-Guérin, G., Vernier, P., Birman, S., Tanimoto, H., and Preat, T. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat Neurosci* *15*, 592–599.

Preat, T. (1998). Decreased odor avoidance after electric shock in *Drosophila* mutants biases learning and memory tests. *Journal of Neuroscience* *18*, 8534–8538.

Proia, P., Di Liegro, C.M., Schiera, G., Fricano, A., and Di Liegro, I. (2016). Lactate as a Metabolite and a Regulator in the Central Nervous System. *Ijms* *17*.

Purice, M.D., Speese, S.D., and Logan, M.A. (2016). Delayed glial clearance of degenerating

axons in aged *Drosophila* is due to reduced PI3K/Draper activity. *Nature Communications* 7, 12871–13.

Pysh, J.J., and Khan, T. (1972). Variations in mitochondrial structure and content of neurons and neuroglia in rat brain: an electron microscopic study. *Brain Research* 36, 1–18.

Qin, H., and Dubnau, J. (2010). Genetic disruptions of *Drosophila* Pavlovian learning leave extinction learning intact. *Genes, Brain and Behavior* 9, 203–212.

Quinn, W.G., Harris, W.A., and Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 71, 708–712.

Quinn, W.G., P, S., and R, B. (1979). The *Drosophila* memory mutant amnesiac. *Nature* 1–3.

Rangaraju, V., Calloway, N., and Ryan, T.A. (2014). Activity-driven local ATP synthesis is required for synaptic function. *Cell* 156, 825–835.

Redt-Clouet, C., Trannoy, S., Boulanger, A., Tokmatcheva, E., Savvateeva-Popova, E., Parmentier, M.-L., Preat, T., and Dura, J.-M. (2012). Mushroom body neuronal remodelling is necessary for short-term but not for long-term courtship memory in *Drosophila*. *Eur J Neurosci* 35, 1684–1691.

Reyes-DelaTorre, A., Teresa, M., and Rafael, J. (2012). Carbohydrate Metabolism in *Drosophila*: Reliance on the Disaccharide Trehalose. In *Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology*, C.-F. Chang, ed. (InTech), pp. 1–22.

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., Iche-Torres, M., Cassar, M., Strauss, R., Preat, T., et al. (2011). Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl. Acad. Sci. U.S.a.* 108, 834–839.

Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment Prediction by Dopaminergic Neurons in *Drosophila*. *Current Biology* 15, 1953–1960.

Rittschof, C.C., and Schirmeier, S. (2017). Insect models of central nervous system energy metabolism and its links to behavior. *Glia* 106, 15400–15416.

Rival, T., Soustelle, L., Strambi, C., Besson, M.-T., Iché, M., and Birman, S. (2004). Decreasing Glutamate Buffering Capacity Triggers Oxidative Stress and Neuropil Degeneration in the *Drosophila* Brain. *Current Biology* 14, 599–605.

Rolls, M.M., and Jegla, T.J. (2015). Neuronal polarity: an evolutionary perspective. *Journal of Experimental Biology* 218, 572–580.

Rolls, M.M., Satoh, D., Clyne, P.J., Henner, A.L., Uemura, T., and Doe, C.Q. (2007). Polarity and intracellular compartmentalization of *Drosophila* neurons. *Neural Dev* 2, 7–15.

Rouach, N., Koulakoff, A., Abudara, V., Willecke, K., and Giaume, C. (2008). Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science* 322, 1551–1555.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies:

- growth and diabetic phenotypes. *Science* 296, 1118–1120.
- Sada, N., Lee, S., Katsu, T., Otsuki, T., and Inoue, T. (2015). Epilepsy treatment. Targeting LDH enzymes with a stiripentol analog to treat epilepsy. *Science* 347, 1362–1367.
- Sadigh-Eteghad, S., Majdi, A., Mahmoudi, J., Golzari, S.E.J., and Talebi, M. (2016). Astrocytic and microglial nicotinic acetylcholine receptors: an overlooked issue in Alzheimer's disease. *Journal of Neural Transmission* 123, 1359–1367.
- Saks, V., Beraud, N., and Wallimann, T. (2008). Metabolic Compartmentation – A System Level Property of Muscle Cells. *Ijms* 9, 751–767.
- Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., Suzuki, A., and Mizoguchi, A. (1997). Bombyxin, an insulin-related peptide of insects, reduces the major storage carbohydrates in the silkworm *Bombyx mori*. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* 118, 349–357.
- Scheunemann, L., Plaçais, P.-Y., Dromard, Y., Schwärzel, M., and Preat, T. (2018). Dunce Phosphodiesterase Acts as a Checkpoint for *Drosophila* Long-Term Memory in a Pair of Serotonergic Neurons. *Neuron* 98, 350–365.e355.
- Schultz, W., Dayan, P., and Montague, P.R. (1997). A neural substrate of prediction and reward. *Science* 275, 1593–1599.
- Schulz, J.G., Laranjeira, A., Van Huffel, L., Gärtner, A., Vilain, S., Bastianen, J., Van Veldhoven, P.P., and Dotti, C.G. (2015). Glial β -Oxidation regulates *Drosophila* Energy Metabolism. *Sci. Rep.* 5, 7805.
- Schwabe, T., Bainton, R.J., Fetter, R.D., Heberlein, U., and Gaul, U. (2005). GPCR Signaling Is Required for Blood-Brain Barrier Formation in *Drosophila*. *Cell* 123, 133–144.
- Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* 23, 10495–10502.
- Seaquist, E.R., Damberg, G.S., Tkac, I., and Gruetter, R. (2001). The effect of insulin on in vivo cerebral glucose concentrations and rates of glucose transport/metabolism in humans. *Diabetes* 50, 2203–2209.
- Séjourné, J., Plaçais, P.-Y., Aso, Y., Siwanowicz, I., Trannoy, S., Thoma, V., Tedjakumala, S.R., Rubin, G.M., Tchenio, P., Ito, K., et al. (2011). Mushroom body efferent neurons responsible for aversive olfactory memory retrieval in *Drosophila*. *Nat Neurosci* 14, 903–910.
- Shah, K., DeSilva, S., and Abbruscato, T. (2012). The Role of Glucose Transporters in Brain Disease: Diabetes and Alzheimer's Disease. *Ijms* 13, 12629–12655.
- Shen, J.-X., and Yakel, J.L. (2012). Functional $\alpha 7$ Nicotinic ACh Receptors on Astrocytes in Rat Hippocampal CA1 Slices. *J Mol Neurosci* 48, 14–21.

- Sibson, N.R., Dhankhar, A., Mason, G.F., Rothman, D.L., Behar, K.L., and Shulman, R.G. (1998). Stoichiometric coupling of brain glucose metabolism and glutamatergic neuronal activity. *Proc Natl Acad Sci USA* *95*, 316–321.
- Siesjö, B.K. (1978). Brain energy metabolism and catecholaminergic activity in hypoxia, hypercapnia and ischemia. *J. Neural Transm. Suppl.* 17–22.
- Simpson, I.A., Carruthers, A., and Vannucci, S.J. (2007). Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *Journal of Cerebral Blood Flow & Metabolism* *27*, 1766–1791.
- Simpson, I.A., Dwyer, D., Malide, D., Moley, K.H., Travis, A., and Vannucci, S.J. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. *American Journal of Physiology-Endocrinology and Metabolism* *295*, E242–E253.
- Sinadinos, C., Valles-Ortega, J., Boulan, L., Solsona, E., Tevy, M.F., Marquez, M., Duran, J., Lopez-Iglesias, C., Calbó, J., Blasco, E., et al. (2014). Neuronal glycogen synthesis contributes to physiological aging. *Aging Cell* *13*, 935–945.
- Smid, H.M., Wang, G., Bukovinszky, T., Steidle, J.L.M., Bleeker, M.A.K., van Loon, J.J.A., and Vet, L.E.M. (2007). Species-specific acquisition and consolidation of long-term memory in parasitic wasps. *Proceedings of the Royal Society B: Biological Sciences* *274*, 1539–1546.
- Sokoloff, L., Reivich, M., Kennedy, C., Rosiers, Des, M.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O., and Shinohara, M. (1977). The [¹⁴C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *Journal of Neurochemistry* *28*, 897–916.
- Soto, M., Cai, W., Konishi, M., and Kahn, C.R. (2019). Insulin signaling in the hippocampus and amygdala regulates metabolism and neurobehavior. *Proc. Natl. Acad. Sci. U.S.a.* *116*, 6379–6384.
- Sousa-Nunes, R., Yee, L.L., and Gould, A.P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in *Drosophila*. *Nature* *471*, 508–512.
- Spéder, P., and Brand, A.H. (2014). Gap Junction Proteins in the Blood-Brain Barrier Control Nutrient-Dependent Reactivation of *Drosophila* Neural Stem Cells. *Developmental Cell* *30*, 309–321.
- Spéder, P., and Brand, A.H. (2018). Systemic and local cues drive neural stem cell niche remodelling during neurogenesis in *Drosophila*. *eLife* *7*, 2763.
- St Johnston, D. (2002). The art and design of genetic screens: *Drosophila melanogaster*. *Nat Rev Genet* *3*, 176–188.
- Stahl, B.A., Peco, E., Davla, S., Murakami, K., Caicedo Moreno, N.A., van Meyel, D.J., and Keene, A.C. (2018). The Taurine Transporter Eaat2 Functions in Ensheathing Glia to Modulate Sleep and Metabolic Rate. *Curr. Biol.* *28*, 3700–3708.e3704.

- Stern, S.A., Chen, D.Y., and Alberini, C.M. (2014). The effect of insulin and insulin-like growth factors on hippocampus- and amygdala-dependent long-term memory formation. *Learning & Memory* 21, 556–563.
- Stockhorst, U., de Fries, D., Steingrueber, H.-J., and Scherbaum, W.A. (2004). Insulin and the CNS: effects on food intake, memory, and endocrine parameters and the role of intranasal insulin administration in humans. *Physiology & Behavior* 83, 47–54.
- Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R.J., and Klämbt, C. (2008). Organization and function of the blood-brain barrier in *Drosophila*. *J. Neurosci.* 28, 587–597.
- Stork, T., Sheehan, A., Tasdemir-Yilmaz, O.E., and Freeman, M.R. (2014). Neuron-glia interactions through the Heartless FGF receptor signaling pathway mediate morphogenesis of *Drosophila* astrocytes. *Neuron* 83, 388–403.
- Suh, J., and Jackson, F.R. (2007). *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447.
- Suzuki, A., Stern, S.A., Bozdagi, O., Huntley, G.W., Walker, R.H., Magistretti, P.J., and Alberini, C.M. (2011). Astrocyte-Neuron Lactate Transport Is Required for Long-Term Memory Formation. *Cell* 144, 810–823.
- Takanaga, H., Chaudhuri, B., and Frommer, W.B. (2008). GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochimica Et Biophysica Acta (BBA) - Biomembranes* 1778, 1091–1099.
- Takemura, S.-Y., Aso, Y., Hige, T., Wong, A., Lu, Z., Xu, C.S., Rivlin, P.K., Hess, H., Zhao, T., Parag, T., et al. (2017). A connectome of a learning and memory center in the adult *Drosophila* brain. *eLife* 6, 5643.
- Tamura, T., Chiang, A.-S., Ito, N., Liu, H.-P., Horiuchi, J., Tully, T., and Saitoe, M. (2003). Aging specifically impairs amnesiac-dependent memory in *Drosophila*. *Neuron* 40, 1003–1011.
- Tanabe, K., Itoh, M., and Tonoki, A. (2017). Age-Related Changes in Insulin-like Signaling Lead to Intermediate-Term Memory Impairment in *Drosophila*. *CellReports* 18, 1598–1605.
- Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. *J. Comp. Neurol.* 508, 711–755.
- Tang, B., Yang, M., Shen, Q., Xu, Y., Wang, H., and Wang, S. (2017). Suppressing the activity of trehalase with validamycin disrupts the trehalose and chitin biosynthesis pathways in the rice brown planthopper, *Nilaparvata lugens*. *Pesticide Biochemistry and Physiology* 137, 81–90.
- Tatar, M., Post, S., and Yu, K. (2014). Nutrient control of *Drosophila* longevity. *Trends Endocrinol. Metab.* 25, 509–517.
- Teleman, A.A. (2010). Molecular mechanisms of metabolic regulation by insulin in *Drosophila*. *Biochem. J.* 425, 13–26.

- Teles-Grilo Ruivo, L.M., and Mellor, J.R. (2013). Cholinergic modulation of hippocampal network function. *Front Synaptic Neurosci* 5, 2.
- Tempel, B.L., Bonini, N., Dawson, D.R., and Quinn, W.G. (1983). Reward learning in normal and mutant *Drosophila*. *Proc Natl Acad Sci USA* 80, 1482–1486.
- Trannoy, S., Redt-Clouet, C., Dura, J.-M., and Preat, T. (2011). Parallel Processing of Appetitive Short- and Long-Term Memories In *Drosophila*. *Current Biology* 21, 1647–1653.
- Treherne, J.E. (1958). The Absorption and Metabolism of Some Sugars in the Locust, *Schistocerca Gregaria*. *Journal of Experimental Biology* 1–15.
- Treherne, J.E. (1960). The Nutrition of the Central Nervous System in the Cockroach, *Periplaneta americana* L. 1–21.
- Tsao, C.-H., Chen, C.-C., Lin, C.-H., Yang, H.-Y., and Lin, S. (2018). *Drosophila* mushroom bodies integrate hunger and satiety signals to control innate food-seeking behavior. *eLife* 7, 44.
- Tully, T., and Quinn, W.G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol A* 157, 263–277.
- Tully, T., Preat, T., Boynton, S.C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79, 35–47.
- van der Blik, M., and Meyerowitz, E.M. (1991). Dynamin-like protein encoded by the *Drosophila* shibire gene associated with vesicular traffic. 1–4.
- Vannucci, S.J., Maher, F., and Simpson, I.A. (1997). Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia* 21, 2–21.
- Vilchez, D., Ros, S., Cifuentes, D., Pujadas, L., Vallès, J., García-Fojeda, B., Criado-García, O., Fernández-Sánchez, E., Medraño-Fernández, I., Domínguez, J., et al. (2007). Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. *Nat Neurosci* 10, 1407–1413.
- Vogt, K., Schnaitmann, C., Dylla, K.V., Knappek, S., Aso, Y., Rubin, G.M., and Tanimoto, H. (2014). Shared mushroom body circuits underlie visual and olfactory memories in *Drosophila*. *eLife* 3, e02395.
- Volkenhoff, A., Hirrlinger, J., Kappel, J.M., Klämbt, C., and Schirmeier, S. (2018). Live imaging using a FRET glucose sensor reveals glucose delivery to all cell types in the *Drosophila* brain. *Journal of Insect Physiology* 106, 55–64.
- Volkenhoff, A., Weiler, A., Letzel, M., Stehling, M., Klämbt, C., and Schirmeier, S. (2015). Glial Glycolysis Is Essential for Neuronal Survival in *Drosophila*. *Cell Metabolism* 22, 437–447.
- Vosshall, L.B., Wong, A.M., and Axel, R. (2000). An olfactory sensory map in the fly brain. *Cell* 102, 147–159.
- Waddell, S. (2013). Reinforcement signalling in *Drosophila*; dopamine does it all after all.

Current Opinion in Neurobiology 23, 324–329.

Wang, Y., Mamiya, A., Chiang, A.S., and Zhong, Y. (2008). Imaging of an Early Memory Trace in the *Drosophila* Mushroom Body. *Journal of Neuroscience* 28, 4368–4376.

Weiss, S., Melom, J.E., Ormerod, K.G., Zhang, Y.V., and Littleton, J.T. (2019). Glial Ca²⁺ signaling links endocytosis to K⁺ buffering around neuronal somas to regulate excitability. *eLife* 8, 932.

Wigglesworth, V.B. (1960). THE NUTRITION OF THE CENTRAL NERVOUS SYSTEM IN THE COCKROACH. 1–15.

Wolfe, D.E., and Nicholls, J.G. (1967). Uptake of radioactive glucose and its conversion to glycogen by neurons and glial cells in the leech central nervous system. *J. Neurophysiol.* 30, 1593–1609.

Wolff, G.H., and Strausfeld, N.J. (2016). Genealogical correspondence of a forebrain centre implies an executive brain in the protostome-deuterostome bilaterian ancestor. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 371, 20150055–15.

Wolkow, C.A., Kimura, K.D., Lee, M.S., and Ruvkun, G. (2000). Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system. *Science* 290, 147–150.

Wollheim, C.B., and Sharp, G.W. (1981). Regulation of insulin release by calcium. *Physiological Reviews* 61, 914–973.

Xia, S., Miyashita, T., Fu, T.-F., Lin, W.-Y., Wu, C.-L., Pyzocha, L., Lin, I.-R., Saitoe, M., Tully, T., and Chiang, A.-S. (2005). NMDA Receptors Mediate Olfactory Learning and Memory in *Drosophila*. *Current Biology* 15, 603–615.

Xu, J., Sheng, Z., and Palli, S.R. (2013). Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, *Tribolium castaneum*. *PLoS Genet* 9, e1003535.

Yamada, T., Habara, O., Kubo, H., and Nishimura, T. (2018). Fat body glycogen serves as a metabolic safeguard for the maintenance of sugar levels in *Drosophila*. *Development* 145.

Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A.B., Sima, R.J., Preat, T., Rubin, G.M., and Tanimoto, H. (2015). Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proc Natl Acad Sci USA* 112, 578–583.

Yamazaki, D., Horiuchi, J., Ueno, K., Ueno, T., Saeki, S., Matsuno, M., Naganos, S., Miyashita, T., Hirano, Y., Nishikawa, H., et al. (2014). Glial dysfunction causes age-related memory impairment in *Drosophila*. *Neuron* 84, 753–763.

Yang, J., Ruchti, E., Petit, J.M., Jourdain, P., Grenningloh, G., Allaman, I., and Magistretti, P.J. (2014). Lactate promotes plasticity gene expression by potentiating NMDA signaling in neurons. *Proc Natl Acad Sci USA* 111, 12228–12233.

Yin, J.C., Wallach, J.S., Del Vecchio, M., Wilder, E.L., Zhou, H., Quinn, W.G., and Tully, T. (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory in

Drosophila. *Cell* 79, 49–58.

Yu, D., Akalal, D.-B.G., and Davis, R.L. (2006). *Drosophila* α/β Mushroom Body Neurons Form a Branch-Specific, Long-Term Cellular Memory Trace after Spaced Olfactory Conditioning. *Neuron* 52, 845–855.

Yu, D., Ponomarev, A., and Davis, R.L. (2004). Altered representation of the spatial code for odors after olfactory classical conditioning; memory trace formation by synaptic recruitment. *Neuron* 42, 437–449.

Yu, S., Tooyama, I., Ding, W.G., Kitasato, H., and Kimura, H. (1995). Immunohistochemical localization of glucose transporters (GLUT1 and GLUT3) in the rat hypothalamus. *Obes. Res.* 3 *Suppl* 5, 753S–76S.

Zhang, Y.V., Ormerod, K.G., and Littleton, J.T. (2017). Astrocyte Ca^{2+} Influx Negatively Regulates Neuronal Activity. *eNeuro* 4.

Zhao, L., Yang, M., Shen, Q., Liu, X., Shi, Z., Wang, S., and Tang, B. (2016). Functional characterization of three trehalase genes regulating the chitin metabolism pathway in rice brown planthopper using RNA interference. *Sci. Rep.* 6, 27841.

Zhao, X.L., and Campos, A.R. (2012). Insulin signalling in mushroom body neurons regulates feeding behaviour in *Drosophila* larvae. *J. Exp. Biol.* 215, 2696–2702.

Zielke, H.R., Zielke, C.L., and Baab, P.J. (2007). Oxidation of ^{14}C -labeled compounds perfused by microdialysis in the brains of free-moving rats. *Journal of Neuroscience Research* 85, 3145–3149.

Zucker, R.S., Kennedy, D., and Selverston, A.I. (1971). Neuronal circuit mediating escape responses in crayfish. *Science* 173, 645–650.

In flies, long-term memory (LTM) formation is an energetically costly mechanism that is tightly controlled by both external parameters and the internal state (Plaçais and Preat, 2013). This energetic cost is specific for *de novo* protein synthesis-dependent LTM, which questions the role and the regulation of energy metabolism during memory formation. In vertebrates, several studies already described that specific metabolic pathways support LTM, such as glycogen hydrolysis (Gibbs et al., 2006; Suzuki et al., 2011). This catabolic pathway actually occurs in astrocytic glia, which then provide glycogen-derived lactate to the active neurons (Gao et al., 2016; Suzuki et al., 2011). The involvement of glial cells in LTM is, for the time being, poorly documented in flies (Matsuno et al., 2015). Still, *Drosophila melanogaster* is a well-suited model organism to study molecular mechanisms in precise cell types, or even at the subcellular level. The neural networks underpinning LTM formation are now quite well understood in *Drosophila*, in particular at the level of the mushroom body, the main associative structure in the fly brain (Cognigni et al., 2018; Pascual and Preat, 2001), and specific mushroom body neurons participate in aversive LTM retrieval (Séjourné et al., 2011; Bouzaiane et al., 2015).

In this thesis, we studied if and how glial cells were involved in the formation of aversive LTM following an association between odors and electric shocks in flies. Thanks to the use of several powerful genetic tools that are available in this model, we demonstrated that glial cells were early involved in the consolidation of memory, specifically for LTM. *Drosophila*, as mammals, possess various glial subtypes. We dissected which glial subtype was involved, and surprisingly, we found that the glial cells enwrapping the neuronal soma, named cortex glia, were involved in LTM consolidation. This was highly unexpected, as the work conducted until now focused on neuron-glia interactions at the level of the synapse, leaving glia-neuronal soma interactions understudied, in particular during memory formation.

By using cell type specific gene knockdown restricted to adulthood associated to behavioral and *in vivo* imaging experiments, we investigated the signaling pathway occurring in cortex glia during LTM formation. Our data evidence a model of cortex glia – mushroom body neurons interactions that occur specifically during LTM

formation. During and/or early after the spaced conditioning, cholinergic neurons, probably mushroom body neurons, release acetylcholine that activates the cholinergic receptor nAChR α 7 expressed in cortex glia. This triggers a calcium elevation in cortex glia that induces the release of the insulin-like peptide IIP4, which function was unknown until now. IIP4 autocrinally activates the insulin receptor expressed in cortex glia, increasing glucose synthesis from trehalose in these cells, and glucose export towards the mushroom body neuronal soma to support their increased glucose demand.

These results are of particular importance as it is becoming increasingly evident that metabolic changes, among which increased central insulin resistance and lower brain glucose metabolism, accompany cognitive decrements during aging and Alzheimer's disease in humans (Mullins et al., 2017; Mattson et al., 2008).

Chez la *Drosophile*, la formation de la mémoire à long terme (MLT) est un processus énergétiquement coûteux qui est étroitement contrôlé par des paramètres exogènes et par le statut interne de l'organisme (Plaçais and Preat, 2013), ce qui pose la question de comment est régulé le métabolisme énergétique durant la formation de cette mémoire. Chez les vertébrés, plusieurs études ont déjà démontré que des voies métaboliques spécifiques soutenaient la formation de la MLT, comme l'hydrolyse du glycogène dans les astrocytes de l'hippocampe (Gibbs et al., 2006; Suzuki et al., 2011), permettant la synthèse de lactate transféré par la suite aux neurones actifs (Gao et al., 2016). Le rôle des cellules gliales dans la formation de la MLT ou dans le métabolisme énergétique du cerveau n'est que peu documenté chez la *Drosophile* (Matsuno et al., 2015). Cependant, *Drosophila melanogaster* est un organisme modèle très adapté à l'étude des mécanismes se déroulant dans des types cellulaires précis, voire même à des échelles subcellulaires. Durant ma thèse, nous avons étudié si les cellules gliales de la *Drosophile* étaient impliquées dans la mémorisation à long terme d'une association aversive entre des odeurs et des chocs électriques. Grâce aux puissants outils génétiques disponibles chez ce modèle, nous avons démontré que les cellules gliales étaient précocement impliquées dans la consolidation de la MLT via un mécanisme moléculaire spécifique à ce type de mémoire. La *Drosophile*, tout comme les mammifères, possède différents sous-types gliaux. Nous avons par conséquent disséqué les sous-types gliaux impliqués. De façon surprenante, nous avons mis en évidence que les cellules qui cernent les corps cellulaires des neurones, appelées cellules gliales corticales, étaient les seules cellules gliales impliquées dans le mécanisme étudié. Ceci était très inattendu, car la plupart des études qui portent sur les interactions fonctionnelles glie-neurones se focalisent au niveau des synapses.

Grâce à l'inhibition de l'expression de gènes dans des types cellulaires spécifiques et uniquement à l'âge adulte en association avec des expériences de comportement et d'imagerie *in vivo*, nous avons cherché les voies de signalisation mises en jeu dans la glie corticale durant la formation de la mémoire. Nos données mettent en évidence des interactions glie corticale – corps pédonculés spécifiquement durant la formation de la MLT. Pendant et/ou précocement après le conditionnement espacé, des neurones cholinergiques, probablement les neurones des corps pédonculés, libèrent de l'acétylcholine. Celle-ci active le récepteur cholinergique nAChR α 7 exprimé par la glie corticale. Cela induit une élévation du niveau de calcium intracellulaire et une libération du peptide proche de l'insuline Ilp4. Ilp4 agit de façon autocrine sur le récepteur à l'insuline à la membrane de la glie corticale, augmentant la synthèse de glucose à partir de tréhalose dans ces cellules. Le glucose peut ensuite être exporté vers les corps cellulaires des neurones des corps pédonculés pour soutenir leur consommation de glucose élevée.

Mots clés : Mémoire, glie, *Drosophile*, métabolisme, soma

ABSTRACT

In flies, long-term memory (LTM) formation is an energetically costly mechanism that is tightly controlled by both external parameters and the internal state (Plaçais and Preat, 2013), which questions the role and the regulation of energy metabolism during memory formation. In vertebrates, several studies already described that specific metabolic pathways support LTM, such as glycogen hydrolysis (Gibbs et al., 2006; Suzuki et al., 2011) in astrocytic glia, which then provide glycogen-derived lactate to the active neurons (Gao et al., 2016). The involvement of glial cells in LTM is, for the time being, poorly documented in flies (Matsuno et al., 2015). Still, *Drosophila melanogaster* is a well-suited model organism to study molecular mechanisms in precise cell types, or even at the subcellular level. In this thesis, we studied if and how glial cells were involved in the formation of aversive LTM following an association between odors and electric shocks in flies. Thanks to the use of several powerful genetic tools that are available in this model, we demonstrated that glial cells were early involved in the consolidation of memory, specifically for LTM. *Drosophila*, as mammals, possess various glial subtypes. We dissected which glial subtype was involved, and surprisingly, we found that the glial cells enwrapping the neuronal soma, named cortex glia, were involved in LTM consolidation. This was highly unexpected, as the work conducted until now focused on neuron-glia interactions at the level of the synapse, leaving glia-neuronal soma interactions understudied, in particular during memory formation.

By using cell type specific gene knockdown restricted to adulthood associated to behavioral and *in vivo* imaging experiments, we investigated the signaling pathway occurring in cortex glia during LTM formation. Our data evidence a model of cortex glia – mushroom body neurons interactions that occur specifically during LTM formation. During and/or early after the spaced conditioning, cholinergic neurons, probably mushroom body neurons, release acetylcholine that activates the cholinergic receptor nAChR α 7 expressed in cortex glia. This triggers a calcium elevation in cortex glia that induces the release of the insulin-like peptide Ilp4, which function was unknown until now. Ilp4 autocrinally activates the insulin receptor expressed in cortex glia, increasing glucose synthesis from trehalose in these cells, and glucose export towards the mushroom body neuronal soma to support their increased glucose demand.

Keywords : memory, glia, *Drosophila*, metabolism, soma