Important neuronal toxicity of microtubule-bound Tau in vivo in Drosophila

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Received April 5, 2011; Revised and Accepted June 18, 2011

The microtubule-associated protein Tau is found in large amount in axons of neurons and is involved in human neurodegenerative diseases called tauopathies, which include Alzheimer’s disease. In these diseases, the Tau protein is abnormally hyperphosphorylated and one therapeutic strategy currently under consideration consists in inhibiting Tau phosphorylation. However, the consequences of an excess of hypophosphorylated Tau onto neuronal physiology have not been investigated in vivo. Here we studied how important is Tau phosphorylation for axonal transport and neurohormone release in vivo, using the Drosophila model. Surprisingly, our results demonstrate a stronger toxicity of hypophosphorylated Tau for neuronal function, when compared with normal or pseudophosphorylated Tau. This reveals a potential limit of the current therapeutic strategy aimed at inhibiting Tau phosphorylation.

INTRODUCTION

Tau is a protein enriched in axons discovered for its ability to bind and stabilize microtubules (MTs). The binding of Tau to MTs is regulated by phosphorylation on many Ser/Thr sites. These sites are the targets of different kinases, such as MARK and GSK-3 kinases (1). When phosphorylated on these sites, the affinity of Tau for MTs decreases (2). However, the functional consequences of a dysregulation of Tau phosphorylation, in particular, of an excess of MT-bound or MT-unbound Tau are still not clearly understood in vivo.

Indeed, in tauopathies and most animal models overexpressing the Tau protein, the neurodegenerative process is correlated to the accumulation of hyperphosphorylated Tau (3), indicating that cytosolic, MT-unbound Tau is toxic for neurons, and justifying therapeutic strategies aimed at inhibiting Tau phosphorylation.

On the other hand, many studies performed in cell culture and in vitro suggest that an excess of Tau bound to MTs can physically impair axonal transport, a physiological parameter of importance for neurons. When Tau was overexpressed in cell lines, there was a reduction in organelle movement, notably in the plus-end direction (4–6). In vitro experiments confirmed the negative effect of Tau bound to MTs onto molecular motors cooperation and binding to MTs (7–9). To date, these results obtained in vitro and in cell culture have not been confirmed in Tau transgenic animal models. Although some axonopathy was observed in Tau transgenic mice overexpressing wild-type human Tau (10–13), direct measurement of slow and fast transport rates in the optic nerve of tau overexpressing mice revealed no defect (14). Similarly, in Drosophila axons, the presence of human Tau induced axonal clogs, but no changes in vesicular motion (15). These negative results may be the consequence of the phosphorylation state of Tau that limits its MT binding in vivo. One way to circumvent this problem is to use a mutated Tau isoform that is partly insensitive to phosphorylation.

Here, we took advantage of the Drosophila model to compare in vivo the relative toxicity of an excess of Tau isoforms differing in their ability to be phosphorylated and to bind MTs. We focused on neuronal physiological parameters, such as vesicular motion in axons, neurohormone release and animal survival. Hypophosphorylatable MT-bound Tau strongly affected all these parameters, while the expression of wild-type or phosphomimetic MT-unbound Tau only had
minor effects. These results demonstrate the important neuronal toxicity of an excess of MT-bound Tau compared with hyperphosphorylated Tau in vivo.

RESULTS

Differential MT-binding properties of Tau isoforms

To investigate the in vivo consequences of Tau binding to MTs, we used three constructs of human Tau, which potentially bind differently to MTs when expressed in neurons: wild-type Tau (TauWT); hypophosphorylatable TauAP, which contains 14 alanine mutations of serine/threonine residues; and pseudophosphorylated TauE14, which displays glutamate residues at the same positions (16,17). Glutamate is commonly used to mimic phosphoserine or phosphothreonine residues because its negative charge is located three bonds from Cα compared with the phosphorous atom in phosphoserine and phosphothreonine (18). We overexpressed these different isoforms in a time- and tissue-specific manner, using the UAS/Gal4 system. We first verified the level of expression of the different constructs when expressed in adult neurons, by quantifying the amount of Tau protein using a polyclonal anti-Tau antibody (Fig. 1A). Our results show that there is no significant difference in the protein level of the three Tau isoforms. We could notice that, whereas there are many bands, probably corresponding to different phosphorylation states of Tau in the TauWT lane, the number of bands is reduced in the lanes with TauAP or TauE14, in accordance with the presence of mutations reducing the combination of phosphorylations in these isoforms. We also tested the MT-binding properties of these proteins: we purified the endogenous MTs from brains of flies expressing the different Tau isoforms in all neurons using the UAS/Gal4 driver. Control flies contain the Gal4 driver only. Two independent experiments were performed and gave the same result. The mean Tau enrichment on MTs (signal in pellet/signal in supernatant) normalized versus the actin signal (mean ± SEM, two independent repeats). There are no significant differences between the genotypes. Results are expressed relatively to the amount of TauWT. (B) Biochemical analysis of the amount of Tau protein interacting with MTs in vivo. Analysis by western blot of the amount of Tau present in the MT-bound (pellet:P) and -unbound (supernatant:S) fractions from heads of flies expressing the Tau isoforms in all neurons with an inducible Elav-Gal4 driver. Control flies contain the Gal4 driver only. Two different exposures of the Tau blot are shown. Two independent experiments were performed and gave the same result. The mean Tau enrichment on MTs (signal in pellet/signal in supernatant) normalized versus the AcTub (acetylated Tubulin) enrichment is shown in the accompanying histogram. Results are expressed relatively to the amount of TauWT enrichment. Asterisks indicate significant differences using t-test (∗∗P < 0.01). (C) Transmission electron microscopy micrographs of larval segmental nerves from flies expressing different Tau isoforms in motoneurons with the OK6-Gal4 driver. Three to four larvae were analyzed for each genotype. Scale bar is 500 nm. Arrows point to some of the MTs. A higher magnification of the squared area is shown in each panel for better visualization of MTs.

Figure 1. Different MT-binding properties of Tau isoforms. (A) Western blot of Drosophila head extracts to quantify the amount of the three Tau protein isoforms expressed in adult neurons with an inducible Elav-Gal4 driver. Control flies contain the Gal4 driver only. Histogram shows the quantification of the Tau signal normalized versus the actin signal (mean ± SEM, two independent repeats). There are no significant differences between the genotypes. Results are expressed relatively to the amount of TauWT. (B) Biochemical analysis of the amount of Tau protein interacting with MTs in vivo. Analysis by western blot of the amount of Tau present in the MT-bound (pellet:P) and -unbound (supernatant:S) fractions from heads of flies expressing the Tau isoforms in all neurons with an inducible Elav-Gal4 driver. Control flies contain the Gal4 driver only. Two different exposures of the Tau blot are shown. Two independent experiments were performed and gave the same result. The mean Tau enrichment on MTs (signal in pellet/signal in supernatant) normalized versus the AcTub (acetylated Tubulin) enrichment is shown in the accompanying histogram. Results are expressed relatively to the amount of TauWT enrichment. Asterisks indicate significant differences using t-test (∗∗P < 0.01). (C) Transmission electron microscopy micrographs of larval segmental nerves from flies expressing different Tau isoforms in motoneurons with the OK6-Gal4 driver. Three to four larvae were analyzed for each genotype. Scale bar is 500 nm. Arrows point to some of the MTs. A higher magnification of the squared area is shown in each panel for better visualization of MTs.
vesicles are not moving (Supplementary Material, Fig. S2).

Hypophosphorylatable Tau affects vesicle number in axons

To analyze the effects of the different Tau isoforms on axonal transport, we overexpressed these proteins in motoneurons. All motoneurons have axons oriented the same way within the segmental nerves, with their distal end innervating the peripheral muscles. We first looked at the amount of vesicular clogs, which are commonly considered as a correlate of disrupted axonal transport (19,20). We co-expressed a neuropeptide Y-GFP (NPY-GFP) or synaptotagmin-GFP (syt-GFP) construct to be able to visualize vesicles and obtained the same results with these two markers: for all Tau isoforms, we could find clogs of vesicles (Fig. 2A), which are more abundant in the distal part of nerves. In these clogs, the synaptic vesicles are not moving (Supplementary Material, Fig. S2).

Altogether, these results suggest that axonal transport may be somehow disrupted whatever the Tau isoform used.

To analyze in more detail the extent of transport disruption in the different cases, we quantified within the axons the number of individual vesicles that were not stuck in clogs and that were probably mobile (Fig. 2B). The expression of the isoforms TauE14 and TauWT that poorly bind to MTs, respectively, had no dramatic effect in the amount of such vesicles (99.9 ± 8.2% (n = 8, n.s. with TauE14) and 70.1 ± 5.1% (n = 9, P < 0.05 with TauWT compared with 100 ± 10.3%; n = 13 for wild-type larvae) (Fig. 2B and C). On the contrary, expression of MT-bound TauAP induced a strong decrease in the number of potentially mobile vesicles (41.1 ± 5.6%; n = 8; P < 0.001) (Fig. 2B and C). In conclusion, when considering the number of potentially mobile vesicles within axons, MT-bound TauAP is the most deleterious isoform.

Hypophosphorylatable Tau strongly affects vesicle motion in axons

We then tracked the motion of individual vesicles, through the cuticle, in anesthetized live animals. A representative kymograph is displayed for each condition in Figure 3A, where stationary vesicles appear as vertical bars. We measured different parameters of vesicle kinetics, such as the direction of movement, the pausing time, and the total net distance run in 1 s (Fig. 3B). When the Tau isoforms that do not strongly bind to MTs were overexpressed, we observed an increase in vesicle mean pausing time from 25.6 ± 8.0% in the control situation (n = 9) up to 32.8 ± 2.6% (n = 9, n.s.) and 43.9 ± 3.6% (n = 6, P < 0.001) in the presence of TauE14 and TauWT, respectively. The expression of MT-binding TauAP was much more deleterious, with the mean vesicle pausing time reaching 74.7 ± 9.3% (n = 9, P < 0.001) in the presence of this protein (Fig. 3B).

The directionality of the vesicular transport (anterograde versus retrograde transport) was only affected in the presence of TauAP, with a strong decrease in anterograde transport (5.5 ± 1.9%, n = 9, P < 0.001) compared with the control larvae (40.7 ± 4.6%, n = 9) (Fig. 3B). The instant velocity of vesicles was not decreased in any condition, but even slightly increased (Fig. 3B). Hence, the net distance run by a vesicle in 1 s was significantly decreased from 0.84 ± 0.10 μm in control larvae to 0.35 ± 0.14 μm (n = 9, P < 0.001) only when the TauAP construct was present (Fig. 3B).

Altogether, these data indicate a strong deleterious effect of the MT-binding Tau isoform on vesicular kinetics.

Hypophosphorylatable Tau and neurohormone release

The two previous sets of data indicate that an excess of hypophosphorylatable Tau alters both the number of potentially moving vesicles within nerves, as well as their motion. Hence, it is possible that such a strong defect in axonal transport results in altered release of neuropeptides or neurohormones at the synaptic terminal. We tested this hypothesis, using an easily scorable assay, based on the release of the Bursicon neurohormone. This release occurs just after fly eclosion and is required for cuticle tanning and wing expansion. In the absence of this neurohormone, the emerging flies have...
unexpanded wings, among other phenotypes (21) (Fig. 4A and B). This unexpanded wing phenotype is clearly visible and quantifiable. We first tested whether the inhibition of axonal transport per se could affect wing expansion. We inhibited the expression of the kinesin heavy or light chain or the dynein heavy chain with the OK6-Gal4 line that drives expression in type III terminals recently described as a release site for Bursicon (22). In all cases, we obtained flies with unexpanded wings at a very high penetrance (Fig. 4C). This demonstrates that axonal transport is indeed of importance for the Bursicon-dependent wing expansion. We then compared the effects of the three different Tau isoforms that differentially affect vesicular axonal transport. The Tau isoforms that do not strongly bind to MTs have no effect on wing expansion, whereas the TauAP isoform gives a very strong phenotype with 100% of the emerging flies having unexpanded wings (Fig. 4C). We obtained similar results, albeit with a lower penetrance, with the Burs12-Gal4 line (23), which is more specific to Bursicon-expressing neurons: 85% of TauAP expressing flies had impaired wing expansion ($n = 52$). Altogether, these results indicate that only the TauAP isoform is able to disrupt axonal transport to such an extent that affects Bursicon-dependent wing expansion. In addition, these data show that an excess of MT-bound hypophosphorylatable Tau impairs neuronal function much more drastically than an excess of wild-type or pseudophosphorylated Tau. We further confirmed this conclusion by expressing the different Tau isoforms in all neurons with the elav-Gal4 driver: the TauAP isoform led to a strong pupal lethality, which was not observed with the two other isoforms (Fig. 4D).

**DISCUSSION**

This study was aimed at understanding the consequences of an excess of MT-bound or MT-unbound Tau onto neuronal function in vivo. Many studies in cell culture or in vitro did show that MT-bound Tau impaired axonal transport by physically obstructing the molecular motors kinesin and dynein, resulting in an increase in motor pausing and its detachment from the MTs (5,7). However, these effects were not observed in vivo in several different studies that used wild-type Tau overexpression (14,15,24). Here, using a mutated human Tau that is hypophosphorylatable and that strongly binds to MTs, we could show that this Tau isoform affected vesicle motion in vivo, in a way similar to what was previously described in CHO cells (6) or N2a cells and rat primary neurons cultures (5): there was a decrease in the number of individual vesicles within the axon and an increase in the pausing rate of these vesicles. In addition, anterograde movement was more drastically impaired than retrograde movement, as previously described in neurons in culture (5) and in vitro (7). Altogether, these results show that the effects of Tau binding to MTs are similar in vertebrate and *Drosophila* neurons. Note that the defect in axonal transport observed here could not be the
The mutated residues are the serine proline/threonine proline (SP/TP) sites that lie within the proline-rich domain (11 residues) or within the C-terminal tail (3 residues), and the phosphorylation of subsets of these residues is known to alter MT assembly activity of Tau only to a limited extent (27,28). Here, we show that phosphorylation impairment (by mutation into alanine) of these sites altogether is sufficient to strongly increase the MT binding of Tau, independently of the serine residues within the MT-binding domain. Alternatively, the mutation of the 14 SP/TP sites into glutamate in TauE14 did not induce a more massive detachment of TauE14 from MTs compared with TauWT. One explanation is that most TauWT is actually not bound to MTs, probably because it is already phosphorylated on some of the critical sites that are mutated in TauE14.

In our set of data, we noticed that the TauWT construct was slightly more deleterious than TauE14 in axonal transport, although both TauWT and TauE14 bind MTs similarly. Contrarily to TauE14, the TauWT MT-bound and -unbound fractions have different phosphorylation state, with MT-bound TauWT being less phosphorylated (by comparing the migration of bands in pellet versus supernatant for each construct in Fig. 1A). Hence, it is possible that the phosphorylation state or the conformation of the Tau protein bound to MTs may play a role in inhibiting vesicular motion.

Because we show that most of TauWT overexpressed in the Drosophila nervous system does not bind endogenous MTs and because we show the strong deleterious effects of MT-bound Tau, our study shed light on the physiological importance of Tau phosphorylation in vivo to avoid an excess of MT-bound Tau. Notably, when overexpressed in vivo (at doses that are probably lower than in vitro), most wild-type Tau is probably rapidly phosphorylated, which would explain the previous negative results about Tau-induced changes on vesicular velocity in vivo. Then, the excess of cytosolic, phosphorylated, MT-unbound Tau would lead to a different type of toxicity. Indeed, the presence of a differential toxicity of hypophosphorylatable and pseudophosphorylated Tau is a striking conclusion drawn from our results. In the Drosophila model, phosphorylated Tau (TauWT and TauE14) was considered to be the most toxic species, mainly based on the eye phenotypes, such as eye roughness (17,29–32). This was based on the increase in Tau-induced eye roughness in the presence of kinases (33,34), and the fact that expression of TauE14 induced a much stronger eye phenotype than expression of TauWT, and that expression of TauAP induced no eye phenotype (17,35). However, a few discrepant results such as the expression of hypophosphorylatable TauS11A that induced a strong rough eye phenotype (36) suggested that not only phosphorylated Tau but also hypophosphorylated Tau can lead to cellular toxicity. Here, our data show that hypophosphorylatable Tau is more toxic than TauWT when considering vesicular motion in axons, neurohormone release and animal survival.

In different neurodegenerative diseases such as Alzheimer disease, Tau protein is abnormally hyperphosphorylated and one therapeutic strategy under consideration consists in inhibiting Tau phosphorylation. Because we show how deleterious is hypophosphorylatable Tau, our study highlights a potential limit of this strategy.
MATERIALS AND METHODS

Fly stocks

We used the transgenic lines UAS-Tau\textsuperscript{AP} and UAS-Tau\textsuperscript{E14} with the residues Thr\,111, Thr\,153, Ser\,175, Thr\,181, Ser\,199, Ser\,202, Thr\,205, Thr\,212, Thr\,217, Thr\,231, Ser\,235, Ser\,239, Ser\,404 and Ser\,422, respectively, mutated in alanine and glutamate and the wild-type UAS-Tau transgene (17). All Tau constructs correspond to the Human 0N4R isoform. For targeted expression of these constructs, we employed the Gal4 activator strain elav\textsuperscript{C155}-Gal4 (panneuronal, from the Bloomington stock center), the panneuronal inducible Gal4 GeneSwitch system (elav-Gal4GS, from H. Tricoire), OK6-Gal4 (which drives expression in motor neurons and bursicon positive neurons, from C. O’Kane) and Burs12-Gal4 (which drives expression in bursicon positive neurons, from B. White). Crosses were performed at 25°C for vesicle visualization, we used the UAS-syt-GFP construct (from the Bloomington stock center). For vesicle tracking, we used the UAS-NPY-GFP construct (kind gift from I. Robinson). UAS-RNAi lines directed against Kinesin Light Chain (CG5433), Kinesin Heavy Chain (CG7765) and Dynein Heavy Chain (CG7507) were obtained from the Vienna Drosophila Stock Center (respectively, stock numbers #22125, #44337, #28054).

Western blot

For each genotype, 40 adult fly heads were dissected in RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 20 mM EDTA, 1% Nonidet-P40) supplemented with 50 mM natrium fluoride, 10 mM sodium orthovanadate and a cocktail of protease inhibitors (Sigma-Aldrich). Samples were placed under agitation at 4°C for 15 min and then centrifuged at 11300 g for 20 min at 4°C to remove cellular debris. For each sample, 50 μg of proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were electrophoretically transferred onto nitrocellulose membrane (hybond C-Extra, Amersham Biosciences) prior to blotting and immunodetection by chemiluminescence (Amersham Biosciences). The following primary antibodies were used in this study: anti-acetylated tubulin (6-11B-1, 1:2000, Sigma-Aldrich) and polyclonal anti-human Tau (1:50,000, Dako). The secondary antibodies were peroxidase-labeled anti-mouse IgG and anti-rabbit IgG (1:10,000) from Jackson Immunoresearch Laboratories. For quantification, the signal intensity in each lane was quantified with the imageJ software and the ratio of Tau or acetylated tubulin signal in pellet versus supernatant was calculated for each genotype. The Tau ratio was divided by the acetylated tubulin ratio to give a relative enrichment of Tau in the MT pellet. Mean values were calculated for each genotype (Tau\textsuperscript{WT} being set at 1).

Transmission electron microscopy

Male third instar larvae were filleted in phosphate buffered saline (PBS) 1×, EDTA 1 mM, fixed overnight in 5% glutaraldehyde in 0.1 M PB (NaH\textsubscript{2}PO\textsubscript{4}, 2H\textsubscript{2}O and Na\textsubscript{2}HPO\textsubscript{4}, 2H\textsubscript{2}O), rinsed in PB and post-fixed in a 1% osmic plus 0.8% potassium ferrocianide for 2 h at dark and room temperature. After two rinses in PB, larvae were dehydrated in a graded series of ethanol solutions (30–100%) and embedded in EMBed 812 DER 736. Thin sections (85 nm; Leica-Reichert Ultracut E) were collected from segments A2-A3 just posterior to the brain. These sections were counterstained with uranyl acetate and lead citrate and observed using a Hitachi 7100 transmission electron microscope in the CRIC imaging facility (Montpellier, France). For quantification of MT density, seven axons per nerve were analyzed for each larva. Axonal area was measured with the imageJ software.

In vivo MT-binding assay and immunoblot analysis

This was based on the protocol described in reference (29). Fifty adult flies expressing the wild-type or mutant forms of Tau protein with the Elav-Gal4GS driver were collected (clusters of 25) in food tubes containing instant Drosophila Medium (Carolina Biological Supply Company, Burlington, NC, USA) with RU486 (mifepristone, Sigma-Aldrich) at a final concentration of 1% ethanol and 200 μg/ml RU486. Flies were incubated at 25°C for 6 days. Thereafter, adult flies’ heads were dissected and homogenized in 150 μl of Buffer-C+ [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.1, 50 mM NaF, 10 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 1 mM ethylene glycol tetraacetic acid, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich)] in the presence of taxol 20 μM (Sigma-Aldrich) diluted in dimethylsulfoxide. After centrifugation at 1000g for 10 min, the supernatant was layered onto a 2 volume cushion of buffer-C+ with 50% sucrose. After centrifugation at 100,000g for 30 min, the upper fraction, containing soluble tubulin, was collected as the soluble fraction and the pellet, containing MTs polymers, was resuspended in 150 μl of Laemmli sample buffer. Protein concentrations in the soluble fractions were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal volumes of the soluble fraction and pellet were analysed by 10% SDS–PAGE. Separated proteins were electrophoretically transferred onto nitrocellulose membrane (hybond C-Extra, Amersham Biosciences) prior to blotting and immunodetection by chemiluminescence (Amersham Biosciences). The following primary antibodies were used in this study: anti-acetylated tubulin (6-11B-1, 1:2000, Sigma-Aldrich) and polyclonal anti-human Tau (1:50,000, Dako). The secondary antibodies were peroxidase-labeled anti-mouse IgG and anti-rabbit IgG (1:10,000) from Jackson Immunoresearch Laboratories. For quantification, the signal intensity in each lane was quantified with the imageJ software and the ratio of Tau signal versus actin signal calculated for each genotype. The Tau ratio was divided by the acetylated tubulin ratio to give a relative enrichment of Tau in the MT pellet. Mean values were calculated for each genotype (Tau\textsuperscript{WT} being set at 1).

Larval dissection and confocal image acquisition

This was performed as in reference (37). Wandering male third instar larvae were dissected in PBS 1×, EDTA 1 mM and then fixed for 20 min in 4% paraformaldehyde in PBS 1×. Preparations were mounted in Vectashield media for observation (ABCYS, Paris, France). Confocal images were acquired from a BioRad MRC 1024 or a Zeiss LSM 510 Meta confocal microscope.
Non-invasive tracking of vesicles in segmental nerves

This was based on the protocol as described in reference (15). Male larvae, expressing NPY-GFP in their motor neurons, were anaesthetized with ether during 2 min and 30 s. There were mounted in polymerizing 1% agarose between a slide and a cover slip, with their ventral face up, and immediately placed under a 63× oil objective of an upright wide field fluorescent microscope to track vesicle motion within segmental nerves in segments A2–A3. A movie of 100 frames taken every 280 ms was recorded for one nerve of each larva. For each movie, we selected ~20 vesicles (on a fixed frame, i.e. not knowing if they were moving or not) and tracked them with the imageJ plugin ‘manual vesicle tracking’ developed by F. Cordelières (Institut Curie, Orsay, France). Percentage of pausing corresponds to the percentage of time during which vesicular instant velocity is inferior to 0.4 μm/s. Instant velocity is the velocity calculated between two consecutive time points: positive values correspond to anterograde movement, whereas negative values correspond to retrograde movement. Mean velocity is calculated as the total distance traveled whatever the direction of the vesicle divided by the total time interval during which the vesicle was tracked.

For each studied parameter, we analyzed the distribution of the mean value obtained for each larva and could not reject the hypothesis that this distribution is normal. Hence, we compared the results obtained for each genotype using the Student’s t-test.

For quantification of the number of individual vesicles, we used the first image of the movies and counted the number of vesicles in a fixed area of nerves. We expressed the data in percentage of the control genotype (OK6-Gal4/+;UAS-NPY/+).

In Figure 3, kymographs were built with the imageJ plugin ‘kymo’ developed by F. Cordelières (Institut Curie).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank M. Feany, C. O’Kane, I. Robinson and B. White for generously sharing fly stocks. We thank B. Franco, M. Laurans and C. Gamblin for technical help; J. Bockaert, F. Maschat and all lab members for helpful discussions about this manuscript. We thank the Montpellier CRIC and RIO (IGH) imaging facilities and the Bloomington and VDRC stock centers.

Conflict of Interest statement. None declare.

FUNDING

This work was supported by a France Alzheimer grant to M.-L.P., the CNRS, the INSERM, the French Ministry of Research and Technology and the FUI grant DiaTral to M.-L.P. This work was supported by the INSERM, the Région Haute-Normandie and France Alzheimer for M.L.

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