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Microtubule stabilising peptides rescue tau phenotypes in-vivo

Shmma Quraishe, Megan Sealey, Louise Cranfield & Amritpal Mudher

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The microtubule cytoskeleton is a highly dynamic, filamentous network underpinning cellular structure and function. In Alzheimer's disease, the microtubule cytoskeleton is compromised, leading to neuronal dysfunction and eventually cell death. There are currently no disease-modifying therapies to slow down or halt disease progression. However, microtubule stabilisation is a promising therapeutic strategy that is being explored. We previously investigated the disease-modifying potential of a microtubule-stabilising peptide NAP (NAPVSIPO) in a well-established *Drosophila* model of tauopathy characterised by microtubule breakdown and axonal transport deficits. NAP prevented as well as reversed these phenotypes even after they had become established. In this study, we investigate the neuroprotective capabilities of an analogous peptide SAL (SALLRSIPA). We found that SAL mimicked NAP's protective effects, by preventing axonal transport disruption and improving behavioural deficits, suggesting both NAP and SAL may act via a common mechanism. Both peptides contain a putative 'SIP' (Ser-Ile-Pro) domain that is important for interactions with microtubule end-binding proteins. Our data suggests this domain may be central to the microtubule stabilising function of both peptides and the mechanism by which they rescue phenotypes in this model of tauopathy. Our observations support microtubule stabilisation as a promising disease-modifying therapeutic strategy for tauopathies like Alzheimer's disease.

Alzheimer's disease (AD) is the commonest cause of dementia in the elderly. It is characterised by progressive cognitive decline associated with neuronal dysfunction and death. Extracellular plaques made up of A β peptide and intraneuronal filaments/tangles composed of abnormal, highly phosphorylated forms of tau, neuropathologically define AD. Though this disease was first described over 100 years ago, disease-modifying therapies are still elusive and AD is on the rise. It is estimated that 65.7 million people worldwide will be living with dementia by 2030¹. Clearly, there is an urgent, unmet need for disease-modifying therapies to treat AD^{2,3}.

Tau is a microtubule binding protein that is important for the assembly, maintenance and stability of microtubules (MT). Hyper-phosphorylation of tau, as found in AD, decreases its affinity for tubulin, compromising its ability to stabilise the MTs and thus disrupting cytoskeletal integrity and axonal transport⁴⁻⁶. These phospho-tau mediated phenotypes are evident in many *in-vivo* models of tauopathy⁷⁻¹⁰ including our own *Drosophila* model in which a wild-type, highly phosphorylated isoform of human tau (htau^{ON3R}) is expressed¹¹.

Drosophila is an established model system for analysing the cellular and molecular mechanisms that underlie a variety of neurodegenerative diseases, particularly tau-associated diseases^{12,13}. Htau^{ON3R}-expression in this model causes neuronal dysfunction, characterised by MT destabilisation⁶, axonal transport disruption¹¹, synaptic defects¹² and behavioural impairments¹¹. This model has been used to explore the effectiveness of disease-modifying interventions to either reduce tau phosphorylation or enhance MT stabilisation^{6,14}. Treatment with NAP (NAPVSIPO also known as 'davunetide'), a small octapeptide derived from activity dependent neuroprotective protein (ADNP)¹⁵ effectively restores MT integrity^{14,16} and protects MT-dependent axonal transport in both rodent¹⁷ and *Drosophila*¹⁴ models of disease. Moreover, in the *Drosophila* model, NAP-mediated protection against htau^{ON3R} phenotypes spans cellular and molecular dysfunction through to behavioural defects *in-vivo*¹⁴.

NAP is reported to modulate MT dynamics in a fashion similar to MT plus-end tracking proteins (+TIPS)¹⁸. +TIPS target the dynamic ends of MTs, catalysing immediate changes in MT stability, directionality and growth^{19,20}. However, the majority of +TIPs do not interact directly with the MT plus-end and/or MT lattice. Instead, this interaction occurs through end-binding proteins (EBs), which recognise and bind conserved Ser-x-Ile-Pro (SxIP) polypeptide motifs within +TIPs²¹. In addition to the classical 'SxIP' motif, 'SIP' and 'IP' sequences are also reported to mediate EB interaction with its binding partners²². NAP contains a 'SIP' motif

Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, Building 85, University of Southampton, Southampton, SO17 1BJ, UK. Correspondence and requests for materials should be addressed to S.Q. (email: s.quraishe@soton.ac.uk)

within its amino acid sequence (NAPVSIPQ). It interacts *in-vitro*¹⁸ with both EB1, a key regulator of MT dynamics and polymerisation^{23,24} and EB3 a central component in dendritic spine formation²⁵. Silencing of either EB1 or EB3 abolishes NAP's protective activity in PC12 cells. Furthermore, silencing of EB3 in primary cortical neurons inhibits NAP-mediated dendritic spine formation¹⁸. A novel NAP analogue, SKIP, is reported to bind NAP and enhance axonal transport in ADNP-deficient mice²⁶. It is therefore conceivable that NAP restores MT integrity and function in our *Drosophila* model of tauopathy by interacting with EB's via its SIP domain. The data presented here tests this hypothesis by exploring the MT stabilising potential of another analogous peptide called SAL (SALLRSIPA also termed ADNF-9), which also contains a SIP domain. SAL is derived from the glial precursor protein, activity dependent neurotrophic factor (ADNF). It exhibits similar neuroprotective capabilities to NAP in numerous animal and cell models of injury and disease^{27–31}.

In this study we investigated whether SAL, like NAP could also protect against htau^{ON3R}-mediated neuronal dysfunction in our *Drosophila* model of tauopathy. The phenotypes that arise in this model occur as a direct or indirect consequence of MT breakdown. This model was therefore ideally suited to test SAL's ability to modulate MT integrity and thus determine the importance of the SIP domain in MT stabilising therapeutic approaches.

Results

SAL prevents htau^{ON3R}-mediated locomotor impairment. Expression of htau^{ON3R} within motor neurons of *Drosophila* manifests in a number of distinct phenotypes including crawling defects in larvae¹¹. Larvae expressing htau^{ON3R} exhibit a restricted and non-continuous crawling behaviour indicative of impaired neuronal function^{14,32}. Using the image-tracking software Ethovision, crawling parameters were quantified including velocity, meander (turning rate per distance travelled) and angular velocity (turning rate per time elapsed). Meander and angular velocity are presented on a negative measurement scale in Ethovision. As previously shown¹⁴, 2.5 µg/ml NAP treatment significantly improved velocity (Fig. 1a, red bar, $p = 0.0003$, $n = 14$) compared to untreated htau^{ON3R}-expressing larvae (Fig. 1a, black bar, $n = 39$). Meander and angular velocity were also significantly improved in NAP-treated larvae (Fig. 1b, red bar, $p = 0.017$, $n = 12$ and Fig. 1c, red bar, $p = 0.0426$, $n = 12$, respectively) compared to untreated htau^{ON3R}-expressing larvae (Fig. 1b and c, black bar, $n = 44$ and 39, respectively). Larvae treated with SAL displayed a dose dependent improvement in all parameters tested. Treatment with 1.25 µg/ml and 2.5 µg/ml of SAL (Fig. 1a, pale green bar, $n = 16$ and light green bar, $n = 39$, respectively) did not improve velocity compared to untreated htau^{ON3R}-expressing larvae (Fig. 1a, black bar, $n = 39$). However, 5 µg/ml SAL (Fig. 1a, green bar, $p = 0.0001$, $n = 29$) and 10 µg/ml SAL (Fig. 1a, dark green bar, $p < 0.0001$, $n = 40$) significantly improved velocity compared to untreated htau^{ON3R} larvae (Fig. 1a, black bar, $n = 39$). Meander of htau^{ON3R}-expressing larvae treated with 1.25 µg/ml and 2.5 µg/ml of SAL (Fig. 1b, pale green bar, $n = 17$ and light green bar, $n = 40$, respectively) did not improve compared to untreated htau^{ON3R}-expressing larvae (Fig. 1b, black bar, $n = 44$). However, 5 µg/ml SAL (Fig. 1b, green bar, $p = 0.0061$, $n = 31$) and 10 µg/ml SAL (Fig. 1b, dark green bar, $p = 0.0022$, $n = 49$) significantly improved meander compared to untreated htau^{ON3R} larvae (Fig. 1b, black bar, $n = 44$). Similar to the results for the previous crawling parameters assessed, angular velocity of htau^{ON3R}-expressing larvae treated with 1.25 µg/ml and 2.5 µg/ml of SAL (Fig. 1c, pale green bar, $n = 17$ and light green bar, $n = 38$, respectively) did not improve compared to untreated htau^{ON3R}-expressing larvae (Fig. 1c, black bar, $n = 39$). However, as also demonstrated for velocity and meander, 5 µg/ml SAL (Fig. 1c, green bar, $p = 0.0044$, $n = 31$) and 10 µg/ml SAL (Fig. 1c, dark green bar, $p = 0.0165$, $n = 46$) significantly improved angular velocity compared to untreated htau^{ON3R} larvae (Fig. 1b, black bar, $n = 39$). Treatment with SAL (2.5 µg/ml and 10 µg/ml) did not alter the crawling performance of controls compared to untreated controls (Supplementary Figure 1b–d). Data were analysed by unpaired Student's two-tailed t-test.

SAL prevents htau^{ON3R}-mediated disruption of axonal transport. In this model, axonal transport is disrupted because of MT breakdown^{6,11}. This can be visualised *in-vivo* and in real time through the expression of vesicular neuropeptide-Y-GFP (vGFP) in the motor neurons of living intact larvae¹¹. Efficient axonal transport in untreated wild-type (wt) control larvae was evident by a homogeneous distribution of vGFP in peripheral nerves (Fig. 2a, $n = 10$). In htau^{ON3R}-expressing larvae, large vGFP accumulates were distributed within the axons of peripheral nerves, illustrating profound axonal transport disruptions (Fig. 2b and e, black bar, $p < 0.0001$, $n = 9$). As previously demonstrated, 2.5 µg/ml of NAP prevented axonal transport deficits in htau^{ON3R}-expressing larvae, thus restoring MT integrity (Fig. 2c and e, red bar, $p < 0.0001$, $n = 5$)¹⁴. Given that SAL improved crawling behaviour at higher doses (5 µg/ml and 10 µg/ml) compared to the more efficacious NAP (2.5 µg/ml), we assessed axonal transport deficits in htau^{ON3R}-expressing larvae treated with 10 µg/ml of SAL. We found that 10 µg/ml of SAL prevented axonal transport deficits as effectively as 2.5 µg/ml of NAP (Fig. 2d and e, dark green bar, $p < 0.0001$, $n = 9$). Quantification of the total axonal area occupied by vGFP accumulates confirmed these results (Fig. 2e). Data were analysed by one-way ANOVA with Bonferroni's correction.

SAL does not alter tau phosphorylation. The MT binding and stabilising ability of htau is reduced by hyper-phosphorylation^{5,6}, leading to compromised cytoskeletal integrity. Reduction of tau phosphorylation through genetic and chemical manipulation is known to improve htau^{ON3R}-mediated phenotypes^{6,33}. However, we previously reported that NAP does not alter the phosphorylation status of htau^{ON3R} and was conferring protection by another mechanism¹⁴. We therefore investigated whether SAL was preventing the emergence of the htau^{ON3R}-mediated phenotypes by reducing tau phosphorylation or whether it too, was bypassing the pathogenic phospho-htau^{ON3R} protein. We examined a number of phospho-tau epitopes associated with AD in both SAL-treated and untreated htau^{ON3R}-expressing 1–3 day old flies. Each phospho-tau antigen (intensity of signal, pixels/mm²) was normalised to total htau levels on the same blot. This enabled assessment of changes in the phosphorylation status of tau, independent of changes in total tau levels. The AT180 monoclonal antibody detects phosphorylation of tau at Thr231. This site is important for MT binding, and phosphorylation, as occurs in AD

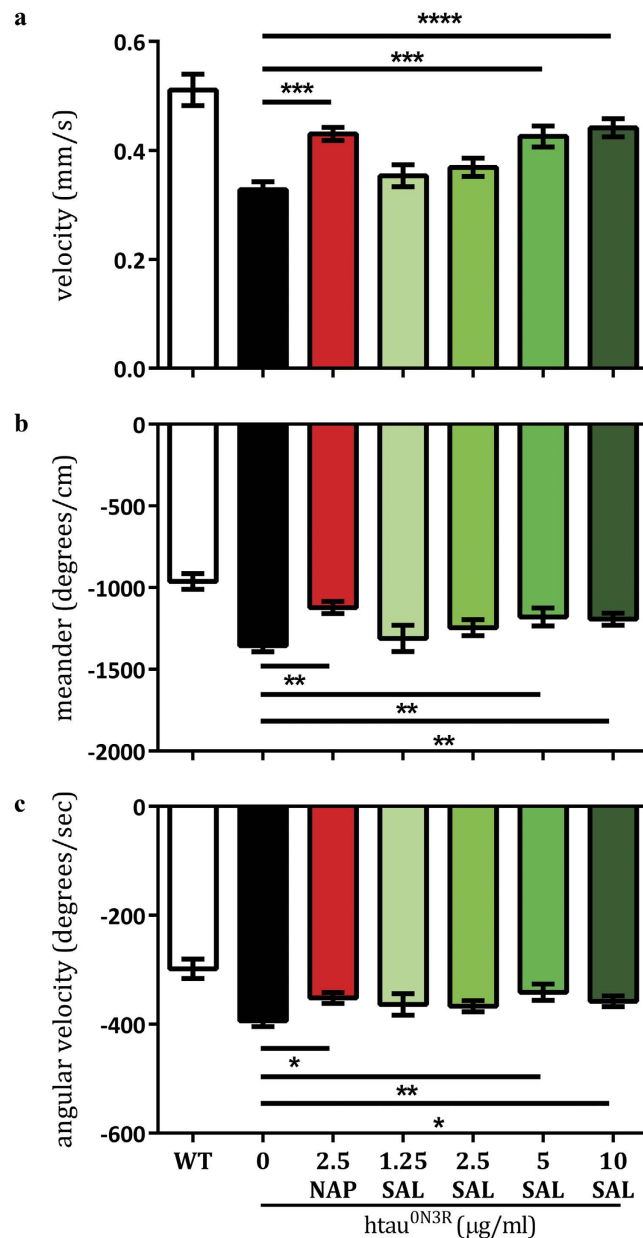


Figure 1. SAL improves the locomotor phenotype in $htau^{ON3R}$ -expressing *Drosophila* larvae. Crawling performance (velocity, meander (turning rate per distance travelled) and angular velocity (turning rate per time elapsed)) was quantified using the tracking-software Ethovision. NAP treatment significantly improved all crawling parameters (a–c, red bar) compared to untreated $htau^{ON3R}$ -expressing larvae (a–c, black bar). A dose dependent improvement in all crawling parameters was observed in SAL-treated larvae. 5 $\mu\text{g/ml}$ SAL (green bars) and 10 $\mu\text{g/ml}$ SAL (dark green bars) significantly improved crawling performance: velocity (a), meander (b) and angular velocity (c) compared to untreated $htau^{ON3R}$ -expressing larvae (black bars). Data were analysed with an unpaired Student's *t*-test. Error bars represent mean \pm S.E.M., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as determined by unpaired Student's two-tailed *t*-test, $n = \text{wt}$ (21–27), $htau^{ON3R}$ (39–44), 2.5 $\mu\text{g/ml}$ NAP (12–14), 1.25 $\mu\text{g/ml}$ SAL (16–17), 2.5 $\mu\text{g/ml}$ SAL (38–40), 5 $\mu\text{g/ml}$ SAL (29–31) and 10 $\mu\text{g/ml}$ SAL (40–49).

at this site, inhibits binding of tau to MTs³⁴. $Htau^{ON3R}$ -expressing flies treated with SAL at 5 or 10 $\mu\text{g/ml}$ did not show changes in tau phosphorylation at the phospho-tau epitope detected by the AT180 antibody compared to untreated $htau^{ON3R}$ larvae (Fig. 3a, $n = 5$). The AT8 monoclonal antibody detects phosphorylation of tau at Ser202/Thr205. $Htau^{ON3R}$ -expressing flies treated with SAL at 5 or 10 $\mu\text{g/ml}$ did not show changes in tau phosphorylation at the phospho-tau epitope detected by the AT8 antibody compared to untreated $htau^{ON3R}$ larvae (Fig. 3b, $n = 5$). Another phospho-tau epitope, Ser396/Ser404, that is abnormally phosphorylated in AD can be detected by the PHF-1 antibody³⁵. Similar to AT180 and AT8, $htau^{ON3R}$ -expressing flies treated with SAL at 5 or 10 $\mu\text{g/ml}$ did not show changes in tau phosphorylation at the phospho-tau epitope detected by the PHF-1 antibody compared to

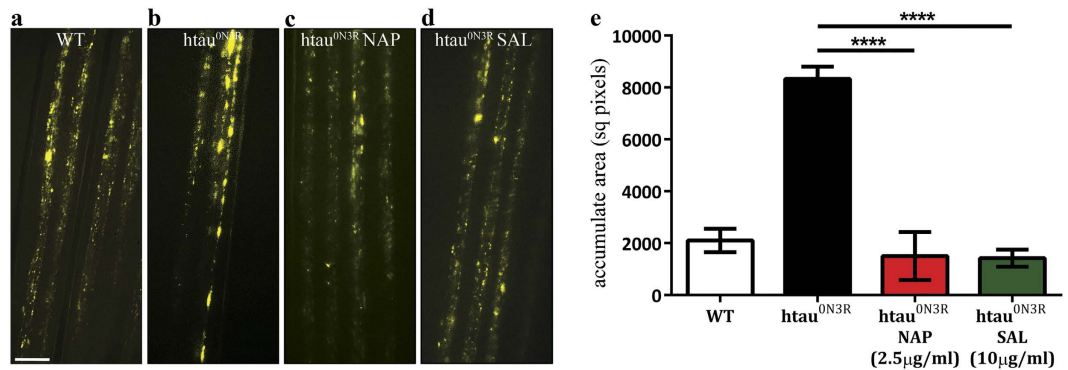


Figure 2. SAL prevents axonal transport deficits in *htau*^{ON3R}-expressing *Drosophila* larvae. Wild-type, control untreated larvae exhibited a homogeneous distribution of vGFP fluorescence in peripheral nerves indicative of an efficient axonal transport system (a). *Htau*^{ON3R} larvae exhibited accumulation of vGFP, indicative of disrupted axonal transport (b). 2.5 μg/ml NAP prevented accumulation of vGFP in *htau*^{ON3R} larval motor neurons (c). 10 μg/ml SAL also prevented accumulation of vGFP in *htau*^{ON3R} larval motor neurons (d). The total area of axons (within a defined region) encompassed by vGFP accumulates was greater in *htau*^{ON3R} larvae compared to controls. 2.5 μg/ml NAP and 10 μg/ml SAL reduced the area covered by vesicular accumulates back to control levels (e). Data were analysed by one-way ANOVA with Bonferroni's correction. Error bars represent mean ± S.E.M., $P^{****} < 0.0001$; $n = \text{wt} (10), \text{htau}^{\text{ON3R}} (9), 2.5 \mu\text{g/ml NAP} (5), 10 \mu\text{g/ml SAL} (9)$. Scale bar: 10 μm.

untreated *htau*^{ON3R} larvae (Fig. 3c, $n = 5$). Total dtau and htau levels were not significantly different in SAL-treated compared to untreated *htau*^{ON3R}-expressing flies (Fig. 3d and e). Actin was used as an additional protein loading control for all samples (Fig. 3d,e and Supplementary Figure 2). We did not include wt control animals in this analysis as they do not express *htau*^{ON3R} (Supplementary Figure 1a). Wide view panels of all the blots are presented in Supplementary Figure 2. Data were analysed by one-way ANOVA with Bonferroni's correction.

Discussion

In this study, we demonstrate that the neuroprotective peptide SAL (SALLRSIPA) provides protection against *htau*^{ON3R}-mediated phenotypes as also demonstrated for an analogous neuroprotective peptide NAP (NAPVSIPQ), albeit at higher doses (5 and 10 μg/ml for SAL compared to 2.5 μg/ml for NAP). SAL significantly improved *htau*^{ON3R}-mediated phenotypes *in-vivo*, including axonal transport disruption and behavioural defects. SAL was able to rescue *htau*^{ON3R} phenotypes without altering phosphorylation at key disease-associated epitopes. These results are reminiscent of those observed with NAP-treatment in this model of tauopathy¹⁴. SAL is reported to exhibit a similar neuroprotective profile compared to NAP^{36,37}. However, several studies have also shown that NAP is more efficacious than SAL^{15,29}. This is consistent with our observations in the present study.

SAL and NAP – two neuroprotective peptides. SAL and NAP are short peptides derived from two secreted astroglial parent proteins, ADNF and ADNP (respectively)^{15,38,39}. NAP and SAL were identified as the essential regions of their respective parent proteins for conferring neuroprotection³⁹. In subsequent studies, SAL and NAP were found to protect against a variety of cellular insults including neurotoxic drugs such as NMDA⁴⁰ and ethanol^{28,41}. They were also found to be protective in models of Alzheimer's disease^{7,27,42,43}, diabetic neuropathy⁴⁴, amyloid lateral sclerosis and ADNP induced tauopathy^{10,17,45}. The molecular mechanism underpinning NAP's neuroprotective ability is thought to occur by MT stabilisation⁴⁶, which counters axonal transport defects^{14,17}. It is conceivable that like NAP, SAL also has MT stabilising effects as it has been shown to displace NAP in an *in-vitro* MT binding assay⁴⁷. SAL also promotes neurite outgrowth in rat hippocampal cultures, a function reliant on MT stabilisation and plasticity⁴⁸. The data presented herein supports this further by showing that like NAP, SAL also protects against *htau*^{ON3R}-mediated behavioural and axonal transport defects, which arise due to cytoskeletal destabilisation^{6,14}. These *htau*^{ON3R}-phenotypes could be attributed to neurodevelopmental effects given the drivers used in this study (D42- and Elav-Gal4) are not exclusively post-mitotic and motor-neuron specific^{49,50}. D42-Gal4 is expressed in all post-mitotic motor neurons and in some sensory neurons in the peripheral nervous system⁴⁹. Elav-Gal4 is also expressed in post-mitotic neurons, but is transiently expressed in embryonic glial cells and neuroblasts⁵⁰. Importantly, expression of *htau*^{ON3R} with these drivers results in robust phenotypes, including MT destabilisation and disrupted axonal transport within the motor neurons of *Drosophila*. We have previously shown rescue of *htau*^{ON3R}-mediated axonal transport deficits after just 24 hours NAP treatment¹⁴. This implies that the protective effects of NAP and SAL target *htau*^{ON3R}-phenotypes that arise in this model due to MT-associated neuronal dysfunction¹¹. We previously demonstrated by EM that cytoskeletal integrity in wt (OreR) controls was unaffected by NAP treatment¹⁴. Unsurprisingly, no effect of NAP was evident in axonal transport or behavioural assays in these wt controls. In contrast, the same ultrastructural analysis showed a disrupted cytoskeleton, which was restored by NAP-treatment in *htau*^{ON3R}-expressing animals¹⁴. Likewise, in the current study, wt controls treated with SAL did not show any statistically significant differences in locomotor performance compared to untreated controls, as illustrated in Supplementary Figure 1b–d. An important point to note is that wt strains such as OreR are more robust when compared to isogenised, Gal4/UAS strains which may be susceptible to Gal4 titrations. It would have been ideal to confirm the tau-specific, neuroprotective effect of these peptides, by assessing

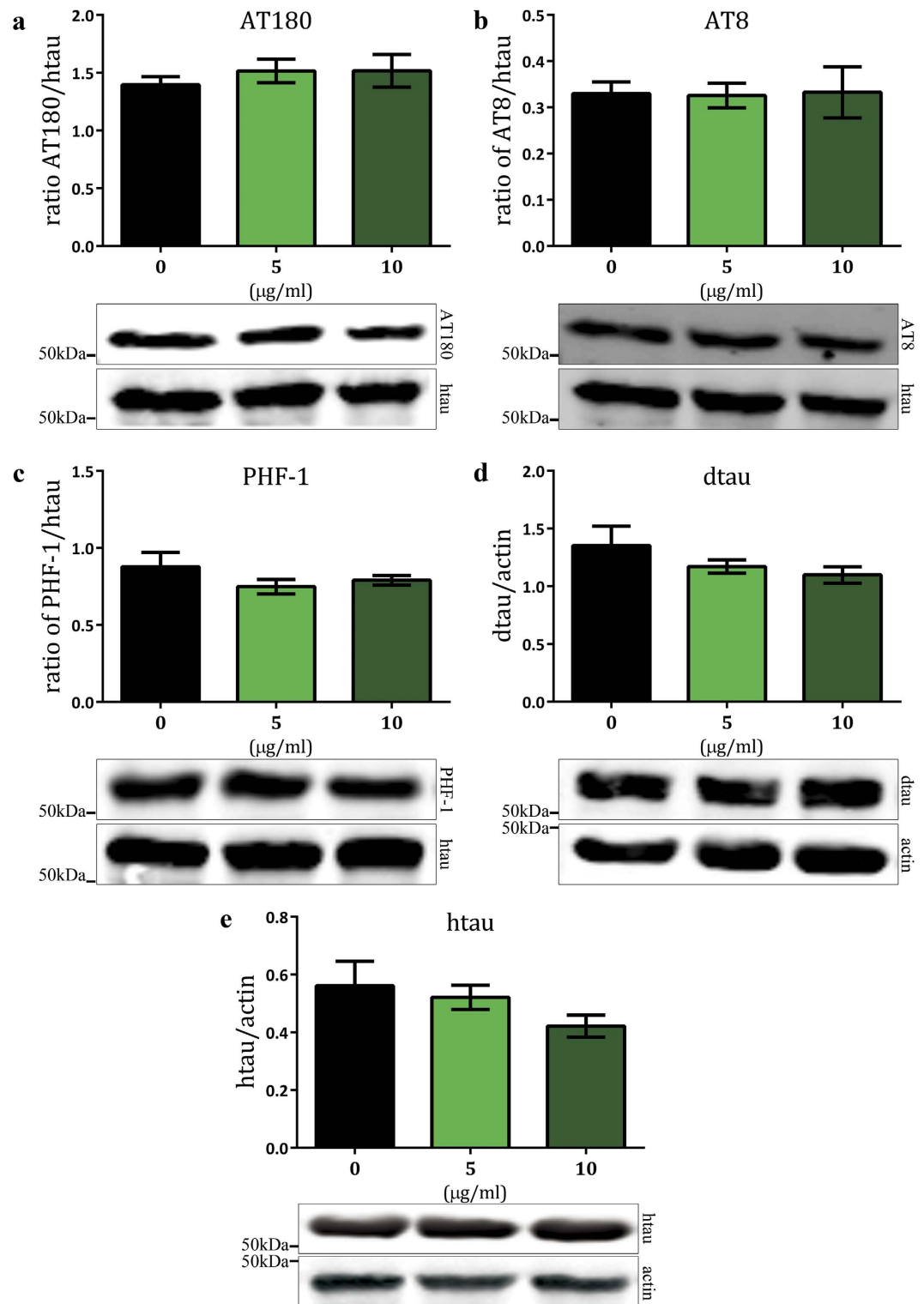


Figure 3. SAL does not alter total htau^{ON3R} levels or phosphorylation at a number of sites relevant to AD.

For each phospho-tau antigen, intensity of signal (pixels/mm²) was normalised to total tau levels. There was no significant change in the levels of the phospho-tau epitopes detected by AT180 (a), AT8 (b), and PHF-1 (c) after treatment with 5 $\mu\text{g/ml}$ SAL (green bars) and 10 $\mu\text{g/ml}$ SAL (dark green bars) SAL. Total dtau levels were not altered by SAL treatment (d). Representative blots are shown (a-d). All lanes were run on the same gel. Data were analysed by one-way ANOVA with Bonferroni's correction. Error bars represent mean \pm S.E.M; $n = \text{htau}^{\text{ON3R}}$ (5), 5 $\mu\text{g/ml}$ SAL (5), 10 $\mu\text{g/ml}$ SAL (5).

their impact on transgenic control lines with a UAS background (e.g. UAS-LacZ). However, our primary aim in this study was to determine if SAL-treatment significantly affected htau^{0N3R}-phenotypes arising because of MT destabilisation. As such, treated and untreated *Drosophila* expressing htau^{0N3R} were reared and tested alongside each other to minimise any titration and genetic artefacts. Interestingly, we found that axonal transport deficits could be prevented and efficient axonal transport maintained in htau^{0N3R}-expressing animals at a comparable level to robust wt OreR control larvae.

Mode of action of SAL and NAP. Hyper-phosphorylated tau is considered to be the toxic species in tauopathies. It is believed to cause degeneration both due to loss of MT-binding function and accumulation of toxic tau aggregates^{51,52}. Tau-centric disease-modifying strategies rescue tau phenotypes by reducing tau phosphorylation^{11,53}, increasing MT stabilisation^{9,14,46,54} or reducing tau aggregation⁵⁵. Our data imply neuroprotective effects independent of reductions in tau phosphorylation, but whether these peptides impact on tau aggregation remains to be determined. Interestingly, we have previously shown that inhibition of GSK-3 β rescues tau phenotypes and restores MT integrity, by reducing tau phosphorylation with a consequent increase in tau protein levels and insoluble granular tau oligomers⁵⁶. In the present study, we did not observe any significant changes in total htau^{0N3R} protein and phosphorylation levels after treatment with SAL and as demonstrated for NAP¹⁴. Previous findings from our lab, as well as other studies conducted in rodent and cell culture models of tauopathy, strongly suggest that NAP neuroprotects by fortification of MTs^{14,16–18,57}.

In this study, we assessed the neuroprotective capabilities of both NAP and SAL peptides individually. SAL showed dose-dependent neuroprotective effects, consistent with our previous observations for NAP¹⁴. Other studies have also reported dose-dependent neuroprotective effects for both peptides²⁹. In a rat model of cholinotoxicity, NAP was more efficacious compared to SAL in cholinergic protection²⁹. NAP has also been shown to be more effective than SAL in providing long term protection against loss of spatial memory in apolipoprotein E-deficient mice¹⁵ and AF64A-treated animals²⁹. A combinatorial peptide approach would also have been interesting but is beyond the scope of the current study. However, previous studies have investigated the protective effects of combining both NAP and SAL. A few studies have shown that both peptides are more efficacious together, than either alone^{58–61}. These peptides do not exhibit stereo-selectivity⁵⁹. The more stable, all D-amino acid SAL (D-SAL) showed efficacy *in-vivo* and *in-vitro* models of disease^{30,31}. In a model of fetal alcohol syndrome (FAS), administration of both D-NAP and D-SAL reduced fetal demise, however, no significant differences between combination and individual drug treatments were seen. In the same study, apolipoprotein E knockout mice treated with both D-NAP and D-SAL showed improved performance in the Morris water maze⁵⁹. In another study, NAP alone was effective in preventing alcohol-induced fetal death, whereas SAL at the same dose was not protective. However, a combinatorial treatment with NAP and SAL was more effective in preventing growth restriction due to prenatal alcohol treatment⁶⁰. These studies suggest a dose-dependent, synergistic effect rather than an additive effect. The differences reported in the literature and the differences in efficacious dose that we too observe may be attributable to the non-homologous amino acids either side of the SIP motif in the two peptides.

Interestingly, both peptides are derived from parent proteins that are secreted by glial cells in response to vasoactive intestinal peptide (VIP)^{15,38}. VIP is expressed under conditions of stress and one of the early events that occurs during stress or insult mediated injury is a dynamic reorganisation of the cytoskeleton⁶². Both NAP and SAL (NAPVSIPQ and SALLRSIPA) contain a SIP motif⁶³. The 'SIP' motif within NAP has been implicated in protection against ethanol and tetrodotoxin toxicity in cortical neurons⁴⁰. Substitution of proline (P) with alanine (A) abolishes neuroprotection against oxidative stress (H₂O₂) in pheochromocytoma (PC12) cells⁶⁴. Indeed, the SIP motif of NAP and SAL is essential for neuroprotection and interaction with key MT end-binding proteins EB1 and EB3, promoting MT assembly and neuronal plasticity^{18,26}. Collectively, our data and the studies discussed imply that SAL acts in a similar manner as NAP to confer neuroprotection. However, since the molecular mode of action of SAL has not been explored as comprehensively as that of NAP, this cannot be concluded unequivocally without further investigations.

The data presented here supports the use of SIP containing neuropeptides like NAP and SAL for protection against MT destabilisation such as that seen in tauopathies. Importantly, this work highlights MT stabilisation as a disease-modifying therapeutic strategy that holds great promise for tauopathies like AD where abnormal tau-mediated MT dysfunction is evident.

Methods

***Drosophila* genotypes and drug treatments.** Transgenic expression of htau^{0N3R} (y¹w¹¹¹⁸; P{UAS-MAPT.A}59 A; Bloomington Stock Centre, stock no. 181) was directed to *Drosophila melanogaster* motor neurons using either D42-Gal4, or the D42-Gal4 driver fused to vesicular GFP-tagged neuropeptide-Y (D42-GAL4.UAS-NPY:GFP) as previously described¹¹. Pan-neural expression was established with the Elav-Gal4 driver. Female virgin flies homozygous for the D42 or Elav driver were crossed to male flies homozygous for htau^{0N3R} under the UAS promoter (+;+; UAS-htau^{0N3R}), or with Oregon-R (OreR) wt, control males. Stocks and transgenic crosses were maintained at 23 °C on a 12 h light/dark cycle. Flies were raised on basic food consisting of malt extract, maize meal, soya flour, agar, granulated sugar, yeast, and propionic acid. NAP (NAPVSIPQ) and SAL (SALLRSIPA) (*L*-isoforms synthesised by Peptide Protein Research Ltd, UK) were delivered to basic fly food at a final concentration of 2.5 μ g/ml (NAP) and 1.25 μ g/ml, 2.5 μ g/ml, 5 μ g/ml or 10 μ g/ml (SAL). Late L3-stage larvae were selected for by size and wandering behaviour.

Larval locomotion assay. Larval locomotion analysis was conducted using a semi-quantitative assay as previously described³². Briefly, crawling behaviour was analysed on 1% agarose plates dyed with 0.1% w/v Alcian blue (Hopkin and Williams, UK). L3 larvae were positioned in the centre of each plate and allowed to acclimatise for 2 min prior to testing. Open field activity was recorded for 2 min (trial 1). This was further repeated for 2

more trials. Wherever possible, genotypes and treatments were randomised between adjacent plates. Videos of larval locomotion were analysed in Ethovision 3.0 software (Noldus) to determine velocity, angular velocity and meander.

In-vivo axonal transport analysis. All treatment groups were subjected to a 3–4 hour timed lay on apple juice agar plates. F1 eggs were transferred to either basic, NAP or SAL treated food. Larvae were left to develop to L3 wandering stage (day 5). Axonal transport analysis was conducted as previously described^{11,65}. Briefly, L3 larvae were anaesthetised in diethylether vapour for 15 min, immobilised on glass slides in 1% agarose ventral face up and mounted under coverslips. Peripheral nerves were analysed between the 2nd and 4th denticle bands. For total area acquisition, vGFP accumulates were imaged at x63 on an Axioplan2 Epifluorescence Microscope (Zeiss), and thresholded in Metamorph software (Molecular Devices, CA, USA).

Western blotting. 1–3 day old adult fly heads were homogenised in buffer (150 mM NaCl, 50 mM MES pH6.8, 1% triton-X, protease inhibitor cocktail and 1% SDS). For phospho-tau epitope detection, the following cocktail of phosphatase and kinase inhibitors was also added: 30 mM sodium fluoride, 20 mM sodium pyrophosphate, 40 mM 2-glycerophosphate, 3.5 mM sodium orthovanadate and 10 μ M staurosporin. Samples were spun for 2 mins at 3,000 g; the supernatant was removed and heated for 5 mins at 95 °C in Laemmli buffer. Samples were subjected to standard 10% SDS-PAGE and transferred to Protran Nitrocellulose Membrane (Whatman, UK). Blots were probed with the following primary antibodies: anti-human tau (1:15,000, Dako, UK), anti-dtau (1:500, gifted by Prof. St. Johnston, University of Cambridge, UK), anti-actin (1:5000, Abcam, UK) and anti-phospho-tau: PHF-1 (1:2000, gifted by Dr. Peter Davies, Albert Einstein College of Medicine; Bronx, NY), AT180 (1:100, Source, Biosciences, UK), AT8 (1:800, Source, Biosciences, UK). Signal was detected using fluorescently-conjugated secondary antibodies; goat anti-mouse (Alexa-Fluor, Invitrogen, UK) and goat anti-rabbit (IRDye, Licor, UK), used at 1:20,000, and quantified with an Odyssey Infrared Imaging Scanner (LiCor) at 700 nm and 800 nm to give intensity values in pixels/mm².

Statistics. Statistical analysis was carried out in Prism 5.0 (GraphPad, University of Southampton, Southampton, UK), using a *T*-test or one-way ANOVA with a *post hoc* Bonferroni's Multiple Comparison Test. All values are reported as means \pm S.E.M. *P* < 0.05 was considered significant.

References

- Banerjee, S. The macroeconomics of dementia—will the world economy get Alzheimer's disease? *Archives of medical research* **43**, 705–709, doi: 10.1016/j.arcmed.2012.10.006 (2012).
- Ghezzi, L., Scarpini, E. & Galimberti, D. Disease-modifying drugs in Alzheimer's disease. *Drug design, development and therapy* **7**, 1471–1478, doi: 10.2147/DDDT.S41431 (2013).
- Corbett, A. & Ballard, C. New and emerging treatments for Alzheimer's disease. *Expert opinion on emerging drugs* **17**, 147–156, doi: 10.1517/14728214.2012.675327 (2012).
- Alonso, A. C., Zaidi, T., Grundke-Iqbal, I. & Iqbal, K. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 5562–5566 (1994).
- Lovestone, S. & Reynolds, C. H. The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. *Neuroscience* **78**, 309–324 (1997).
- Cowan, C. M., Bossing, T., Page, A., Shepherd, D. & Mudher, A. Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau *in vivo*. *Acta neuropathologica* **120**, 593–604, doi: 10.1007/s00401-010-0716-8 (2010).
- Shiryaev, N. *et al.* NAP protects memory, increases soluble tau and reduces tau hyperphosphorylation in a tauopathy model. *Neurobiology of disease* **34**, 381–388, doi: 10.1016/j.nbd.2009.02.011 (2009).
- Zhang, B. *et al.* Microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 227–231, doi: 10.1073/pnas.0406361102 (2005).
- Zhang, B. *et al.* The microtubule-stabilizing agent, epothilone D, reduces axonal dysfunction, neurotoxicity, cognitive deficits, and Alzheimer-like pathology in an interventional study with aged tau transgenic mice. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **32**, 3601–3611, doi: 10.1523/JNEUROSCI.4922-11.2012 (2012).
- Vulih-Shultzman, I. *et al.* Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. *The Journal of pharmacology and experimental therapeutics* **323**, 438–449, doi: 10.1124/jpet.107.129551 (2007).
- Mudher, A. *et al.* GSK-3beta inhibition reverses axonal transport defects and behavioural phenotypes in Drosophila. *Molecular psychiatry* **9**, 522–530, doi: 10.1038/sj.mp.4001483 (2004).
- Chee, F. C. *et al.* Over-expression of tau results in defective synaptic transmission in Drosophila neuromuscular junctions. *Neurobiology of disease* **20**, 918–928, doi: 10.1016/j.nbd.2005.05.029 (2005).
- Cowan, C. M. *et al.* Modelling tauopathies in Drosophila: insights from the fruit fly. *International journal of Alzheimer's disease* **2011**, 598157, doi: 10.4061/2011/598157 (2011).
- Quraishe, S., Cowan, C. M. & Mudher, A. NAP (davunetide) rescues neuronal dysfunction in a Drosophila model of tauopathy. *Molecular psychiatry* **18**, 834–842, doi: 10.1038/mp.2013.32 (2013).
- Bassan, M. *et al.* Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *Journal of neurochemistry* **72**, 1283–1293 (1999).
- Oz, S., Ivashko-Pachima, Y. & Gozes, I. The ADNP derived peptide, NAP modulates the tubulin pool: implication for neurotrophic and neuroprotective activities. *PLoS One* **7**, e51458, doi: 10.1371/journal.pone.0051458 (2012).
- Jouroukhin, Y. *et al.* NAP (davunetide) modifies disease progression in a mouse model of severe neurodegeneration: protection against impairments in axonal transport. *Neurobiology of disease* **56**, 79–94, doi: 10.1016/j.nbd.2013.04.012 (2013).
- Oz, S. *et al.* The NAP motif of activity-dependent neuroprotective protein (ADNP) regulates dendritic spines through microtubule end binding proteins. *Molecular psychiatry* **19**, 1115–1124, doi: 10.1038/mp.2014.97 (2014).
- Bearce, E. A., Erdogan, B. & Lowery, L. A. TIPs tour guides: how microtubule plus-end tracking proteins (+TIPs) facilitate axon guidance. *Frontiers in cellular neuroscience* **9**, 241, doi: 10.3389/fncel.2015.00241 (2015).
- Akhmanova, A. & Steinmetz, M. O. Microtubule +TIPs at a glance. *Journal of cell science* **123**, 3415–3419, doi: 10.1242/jcs.062414 (2010).

21. Honnappa, S. *et al.* An EB1-binding motif acts as a microtubule tip localization signal. *Cell* **138**, 366–376, doi: 10.1016/j.cell.2009.04.065 (2009).
22. Stout, J. R. *et al.* Kif18B interacts with EB1 and controls astral microtubule length during mitosis. *Molecular biology of the cell* **22**, 3070–3080, doi: 10.1091/mbc.E11-04-0363 (2011).
23. Komarova, Y. *et al.* Mammalian end binding proteins control persistent microtubule growth. *The Journal of cell biology* **184**, 691–706, doi: 10.1083/jcb.200807179 (2009).
24. Vitre, B. *et al.* EB1 regulates microtubule dynamics and tubulin sheet closure *in vitro*. *Nature cell biology* **10**, 415–421, doi: 10.1038/ncb1703 (2008).
25. Gu, J., Firestein, B. L. & Zheng, J. Q. Microtubules in dendritic spine development. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **28**, 12120–12124, doi: 10.1523/JNEUROSCI.2509-08.2008 (2008).
26. Amram, N. *et al.* Sexual divergence in microtubule function: the novel intranasal microtubule targeting SKIP normalizes axonal transport and enhances memory. *Molecular psychiatry*, doi: 10.1038/mp.2015.208 (2016).
27. Gozes, I., Divinski, I. & Piltzer, I. NAP and D-SAL: neuroprotection against the beta amyloid peptide (1–42). *BMC neuroscience* **9** Suppl 3, S3, doi: 10.1186/1471-2202-9-S3-S3 (2008).
28. Parnell, S. E. *et al.* Concurrent dietary administration of D-SAL and ethanol diminishes ethanol's teratogenesis. *Alcoholism, clinical and experimental research* **31**, 2059–2064, doi: 10.1111/j.1530-0277.2007.00524.x (2007).
29. Gozes, I., Giladi, E., Pinhasov, A., Bardea, A. & Brenneman, D. E. Activity-dependent neurotrophic factor: intranasal administration of femtomolar-acting peptides improve performance in a water maze. *J Pharmacol Exp Ther* **293**, 1091–1098 (2000).
30. Gozes, I., Sragovich, S., Schirer, Y. & Idan-Feldman, A. D-SAL and NAP: Two Peptides Sharing a SIP Domain. *Journal of molecular neuroscience: MN* **59**, 220–231, doi: 10.1007/s12031-015-0701-8 (2016).
31. Shiryayev, N., Pikman, R., Giladi, E. & Gozes, I. Protection against tauopathy by the drug candidates NAP (davunetide) and D-SAL: biochemical, cellular and behavioral aspects. *Current pharmaceutical design* **17**, 2603–2612 (2011).
32. Sinadinos, C., Cowan, C. M., Wyttenbach, A. & Mudher, A. Increased throughput assays of locomotor dysfunction in *Drosophila* larvae. *Journal of neuroscience methods* **203**, 325–334, doi: 10.1016/j.jneumeth.2011.08.037 (2012).
33. Jackson, G. R. *et al.* Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila*. *Neuron* **34**, 509–519 (2002).
34. Sengupta, A. *et al.* Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. *Archives of biochemistry and biophysics* **357**, 299–309, doi: 10.1006/abbi.1998.0813 (1998).
35. Mondragon-Rodriguez, S., Perry, G., Luna-Munoz, J., Acevedo-Aquino, M. C. & Williams, S. Phosphorylation of tau protein at sites Ser(396–404) is one of the earliest events in Alzheimer's disease and Down syndrome. *Neuropathology and applied neurobiology* **40**, 121–135, doi: 10.1111/nan.12084 (2014).
36. Offen, D. *et al.* Vasoactive intestinal peptide (VIP) prevents neurotoxicity in neuronal cultures: relevance to neuroprotection in Parkinson's disease. *Brain research* **854**, 257–262 (2000).
37. Steingart, R. A., Solomon, B., Brenneman, D. E., Fridkin, M. & Gozes, I. VIP and peptides related to activity-dependent neurotrophic factor protect PC12 cells against oxidative stress. *Journal of molecular neuroscience: MN* **15**, 137–145, doi: 10.1385/JMN:15:3:137 (2000).
38. Gozes, I. & Brenneman, D. E. Activity-dependent neurotrophic factor (ADNF). An extracellular neuroprotective chaperonin? *Journal of molecular neuroscience: MN* **7**, 235–244, doi: 10.1007/BF02737061 (1996).
39. Brenneman, D. E. & Gozes, I. A femtomolar-acting neuroprotective peptide. *The Journal of clinical investigation* **97**, 2299–2307, doi: 10.1172/JCI118672 (1996).
40. Brenneman, D. E. *et al.* Activity-dependent neurotrophic factor: structure-activity relationships of femtomolar-acting peptides. *The Journal of pharmacology and experimental therapeutics* **285**, 619–627 (1998).
41. Roberson, R., Kuddo, T., Benassou, I., Abebe, D. & Spong, C. Y. Neuroprotective peptides influence cytokine and chemokine alterations in a model of fetal alcohol syndrome. *American journal of obstetrics and gynecology* **207**, 499 e491–495, doi: 10.1016/j.ajog.2012.10.005 (2012).
42. Matsuoka, Y. *et al.* Intranasal NAP administration reduces accumulation of amyloid peptide and tau hyperphosphorylation in a transgenic mouse model of Alzheimer's disease at early pathological stage. *Journal of molecular neuroscience: MN* **31**, 165–170 (2007).
43. Matsuoka, Y. *et al.* A neuronal microtubule-interacting agent, NAPVSIQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease. *The Journal of pharmacology and experimental therapeutics* **325**, 146–153, doi: 10.1124/jpet.107.130526 (2008).
44. Idan-Feldman, A. *et al.* Davunetide (NAP) as a preventative treatment for central nervous system complications in a diabetes rat model. *Neurobiology of disease* **44**, 327–339, doi: 10.1016/j.nbd.2011.06.020 (2011).
45. Fernandez-Montesinos, R. *et al.* Activity-dependent neuroprotective protein (ADNP) expression in the amyloid precursor protein/presenilin 1 mouse model of Alzheimer's disease. *Journal of molecular neuroscience: MN* **41**, 114–120, doi: 10.1007/s12031-009-9300-x (2010).
46. Gozes, I. & Divinski, I. NAP, a neuroprotective drug candidate in clinical trials, stimulates microtubule assembly in the living cell. *Current Alzheimer research* **4**, 507–509 (2007).
47. Holtser-Cochav, M., Divinski, I. & Gozes, I. Tubulin is the target binding site for NAP-related peptides: ADNF-9, D-NAP, and D-SAL. *Journal of molecular neuroscience: MN* **28**, 303–307, doi: 10.1385/JMN:28:3:303 (2006).
48. Smith-Swintosky, V. L., Gozes, I., Brenneman, D. E., D'Andrea, M. R. & Plata-Salaman, C. R. Activity-dependent neurotrophic factor-9 and NAP promote neurite outgrowth in rat hippocampal and cortical cultures. *Journal of molecular neuroscience: MN* **25**, 225–238, doi: 10.1385/JMN:25:3:225 (2005).
49. Sanyal, S. Genomic mapping and expression patterns of C380, OK6 and D42 enhancer trap lines in the larval nervous system of *Drosophila*. *Gene expression patterns: GEP* **9**, 371–380, doi: 10.1016/j.gep.2009.01.002 (2009).
50. Berger, C., Renner, S., Luer, K. & Technau, G. M. The commonly used marker ELAV is transiently expressed in neuroblasts and glial cells in the *Drosophila* embryonic CNS. *Dev Dyn* **236**, 3562–3568, doi: 10.1002/dvdy.21372 (2007).
51. Cowan, C. M. & Mudher, A. Are tau aggregates toxic or protective in tauopathies? *Frontiers in neurology* **4**, 114, doi: 10.3389/fneur.2013.00114 (2013).
52. Johnson, G. V. & Stoothoff, W. H. Tau phosphorylation in neuronal cell function and dysfunction. *Journal of cell science* **117**, 5721–5729, doi: 10.1242/jcs.01558 (2004).
53. Noble, W. *et al.* Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 6990–6995, doi: 10.1073/pnas.0500466102 (2005).
54. Brunden, K. R. *et al.* The characterization of microtubule-stabilizing drugs as possible therapeutic agents for Alzheimer's disease and related tauopathies. *Pharmacological research* **63**, 341–351, doi: 10.1016/j.phrs.2010.12.002 (2011).
55. Yanamandra, K. *et al.* Anti-tau antibodies that block tau aggregate seeding *in vitro* markedly decrease pathology and improve cognition *in vivo*. *Neuron* **80**, 402–414, doi: 10.1016/j.neuron.2013.07.046 (2013).
56. Cowan, C. M. *et al.* Rescue from tau-induced neuronal dysfunction produces insoluble tau oligomers. *Scientific reports* **5**, 17191, doi: 10.1038/srep17191 (2015).

57. Sudo, H. & Baas, P. W. Strategies for diminishing katanin-based loss of microtubules in tauopathic neurodegenerative diseases. *Human molecular genetics* **20**, 763–778, doi: 10.1093/hmg/ddq521 (2011).
58. Incerti, M. *et al.* Prevention of learning deficit in a Down syndrome model. *Obstetrics and gynecology* **117**, 354–361, doi: 10.1097/AOG.0b013e3182051ca5 (2011).
59. Brenneman, D. E. *et al.* Protective peptides that are orally active and mechanistically nonchiral. *The Journal of pharmacology and experimental therapeutics* **309**, 1190–1197, doi: 10.1124/jpet.103.063891 (2004).
60. Spong, C. Y., Abebe, D. T., Gozes, I., Brenneman, D. E. & Hill, J. M. Prevention of fetal demise and growth restriction in a mouse model of fetal alcohol syndrome. *The Journal of pharmacology and experimental therapeutics* **297**, 774–779 (2001).
61. Toso, L. *et al.* Learning enhancement with neuropeptides. *American journal of obstetrics and gynecology* **194**, 1153–1158, discussion 1158–1159, doi: 10.1016/j.ajog.2005.12.023 (2006).
62. Lu, W., Lakonishok, M. & Gelfand, V. I. Kinesin-1-powered microtubule sliding initiates axonal regeneration in Drosophila cultured neurons. *Molecular biology of the cell* **26**, 1296–1307, doi: 10.1091/mbc.E14-10-1423 (2015).
63. Gozes, I. *et al.* A novel signaling molecule for neuropeptide action: activity-dependent neuroprotective protein. *Annals of the New York Academy of Sciences* **897**, 125–135 (1999).
64. Wilkemeyer, M. F. *et al.* Differential effects of ethanol antagonism and neuroprotection in peptide fragment NAPVSIQ prevention of ethanol-induced developmental toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 8543–8548, doi: 10.1073/pnas.1331636100 (2003).
65. Sinadinos, C. *et al.* Live axonal transport disruption by mutant huntingtin fragments in Drosophila motor neuron axons. *Neurobiology of disease* **34**, 389–395, doi: 10.1016/j.nbd.2009.02.012 (2009).

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Author Contributions

S.Q. carried out experiments; S.Q. and A.M. contributed to scientific design and wrote the manuscript; M.S. contributed to western blotting experiments and L.C. contributed to larval locomotion assays. All authors reviewed the manuscript.

Additional Information

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