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Tau's role in the developing brain: implications for intellectual disability

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Microdeletions encompassing the *MAPT* (Tau) locus resulting in intellectual disability raised the hypothesis that Tau may regulate early functions in the developing brain. Our results indicate that neuronal migration was inhibited in mouse brains following Tau reduction. In addition, the leading edge of radially migrating neurons was aberrant in spite of normal morphology of radial glia. Furthermore, intracellular mitochondrial transport and morphology were affected. In early postnatal brains, a portion of Tau knocked down neurons reached the cortical plate. Nevertheless, they exhibited far less developed dendrites and a striking reduction in connectivity evident by the size of boutons. Our novel results strongly implicate *MAPT* as a dosage-sensitive gene in this locus involved in intellectual disability. Furthermore, our results are likely to impact our understanding of other diseases involving Tau.

INTRODUCTION

Tau is a microtubule-associated protein (MAP), well known for its involvement in a group of neurodegenerative diseases collectively known as tauopathies (reviewed in 1–4). This is probably the reason that most tau-related studies involve overexpression of the protein in relation to the adult brain. The most common tauopathy is Alzheimer's disease where hyperphosphorylated tau accumulates within paired helical filaments. Mutations within the *MAPT* (Tau) locus result in frontotemporal dementia with Parkinsonism (5,6), whereas microdeletions of a region encompassing the *MAPT* gene result in moderate intellectual disability with associated dysmorphic features (7–11). The frequency of the microdeletion syndrome was estimated to be 1:13 000 to 1:20 000, thus suggesting it to be a common underlying cause for intellectual disability. Today, we know that copy number variations are an important component of the molecular mechanism underlying many brain diseases, such as intellectual disability, autism and schizophrenia (12–22). Yet, in only a handful of examples, the identity of the dosage-sensitive gene(s) within the disease locus has been revealed. *MAPT* is one of the few genes within the microdeleted locus, it is strongly expressed in the developing brain (23,24) and it has been suggested to play a role in neuronal migration. There are multiple examples indicating a strong link between intellectual disability and abnormalities in neuronal migration (25,26). Nevertheless, so far the role of Tau in neuronal migration has not been proven unequivocally. Mice deleted for Tau exhibited a reduction in microtubule density in small caliber axons (27), as well as muscle weakness and memory disturbances (28). It has been proposed that developmental functional redundancy by increased expression of other MAPs may explain the relatively mild phenotype (27). This hypothesis was corroborated by the observed neuronal migration phenotype in mice mutated for both tau and *MAP1b*, where the single *MAP1b* mutant mice already demonstrate a neuronal migration phenotype (29). We investigated the role of tau in the developing mouse brain using *in utero* electroporation which has been shown to circumvent gene redundancy as previously demonstrated in case of the *Dcx* family of proteins (30,31) and in case of the *MARK* family of proteins (32). Our results show several novel and important roles for tau in the developing cerebral cortex, which have not been previously demonstrated. We show for the first time that reduction in Tau inhibits neuronal migration in the developing cortex. The leading edge of radially migrating neurons was aberrant in spite of normal morphology of radial glia. Furthermore, we discovered a new role for

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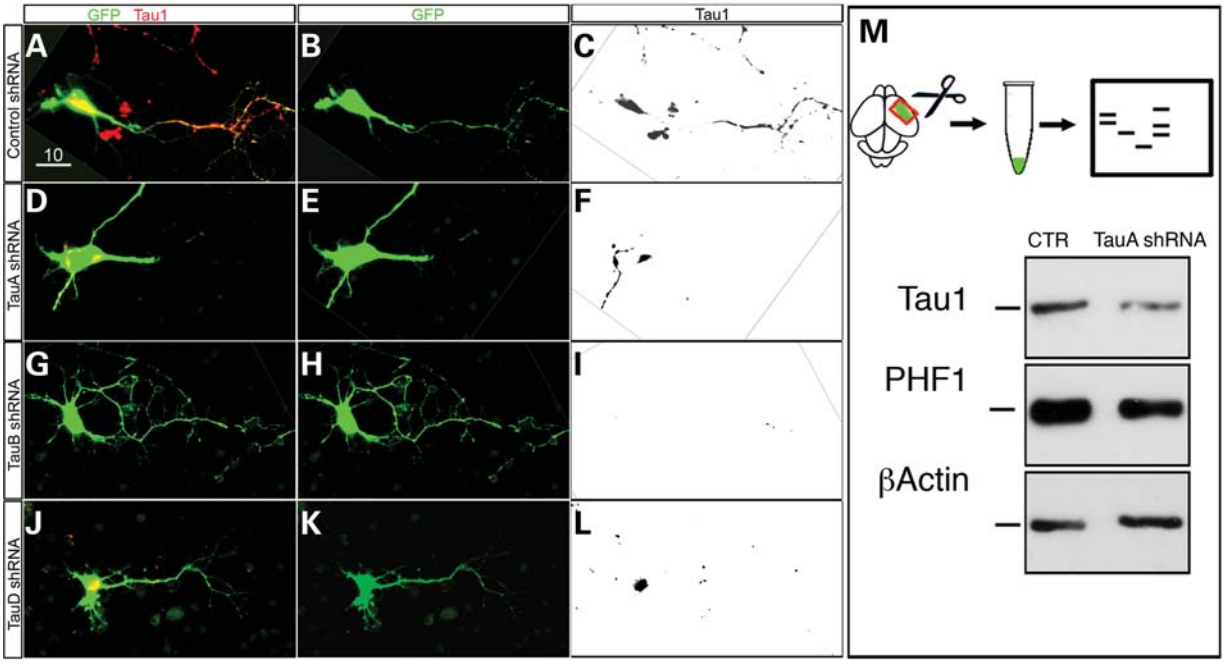

Figure 1. Knock down of Tau in cortical neurons. E14 mouse embryos were co-electroporated *ex utero* with GFP and Tau shRNA plasmids. Cortical neurons were prepared from the electroporated brains and after 2 DIV fixed and stained with Tau1 antibodies. Control shRNA (**A**–**C**), Tau in red merged with the green cell (A, **D**, **G**, **J**), electroporated neurons identified by GFP (B, **E**, **H**, **K**) and immunoreactivity of Tau in black (C, **F**, **I**, **L**). A marked reduction in Tau levels in the somatodendritic and the axonal compartments was noted for all shRNA sequences tested (D–K) in comparison to control shRNA (A–C). (**M**) Tau1 and PHF-1 show reduced reactivity in lysates extracted from Tau shRNA A-electroporated regions of E18 brains, 4 days after electroporation. β-Actin levels indicate similar loading (lower panel).

tau in regulation of mitochondria; both intracellular mitochondrial transport and morphology were severely affected following tau knockdown. In early postnatal brains, a portion of Tau knocked down neurons reached the cortical plate (CP). Nevertheless, they exhibited smaller somas, far less-developed dendrites and a striking reduction in connectivity. Collectively, our results support the previously raised hypothesis that Tau's deletion contributes to the pathophysiology of the 17q21.31 microdeletion syndrome.

RESULTS

Tau levels are effectively reduced *in vivo*

To study the effect of reduced Tau levels on cortical development, we introduced gene-specific shRNA sequences in primary cortical neurons. Brains of E14 mouse embryos were co-electroporated with Tau shRNA and green fluorescent protein (GFP) (3:1 ratio) *ex utero*. Immediately following electroporation, primary cultures were prepared from the treated cortices. The cells were fixed and stained after 2 days growth *in vitro*. Four shRNA sequences were tested for their ability to reduce Tau expression (Fig. 1). Three of the tested sequences (shRNA A, B and D) almost eliminated Tau1 immunoreactivity in the axons of the cultured neurons (Fig. 1A–L). The ability of shRNA A to knockdown Tau levels *in vivo* was further evaluated using an independent method. Two and 4 days post-electroporation, the GFP-positive areas were dissected out from 3 to 5 different electroporated brains. Brain lysates were pooled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blot analysis (Fig. 1M and Supplementary Material, Fig. S1). Tau levels decreased as demonstrated using either Tau1 (recognizing a relatively wide repertoire of Tau species), K9JA (pan-Tau antibody) or PHF-1 (recognizing phosphorylated Tau at Ser396/Ser404) antibodies. Constant actin or emerin levels indicated equal loading. Furthermore, we noted equal amount of LIS1 and DCX, two additional MAPs as well as a slight elevation in the levels of MAP-2. Thus, we concluded that three shRNA sequences successfully reduced endogenous Tau. We then tested whether Tau knockdown affects the dynamics of microtubules in cultured cortical neurons by tracking microtubules using the plus-tip binding protein EB1-mCherry. We noted that microtubules were more dynamic in primary cortical neurons with reduced Tau levels (Supplementary Material, Fig. S2).

Tau reduction impairs radial neuronal migration

Taking into consideration the role of Tau in regulation of microtubule dynamics and regulation of microtubule-associated motors, we questioned whether reduction in the levels of the Tau protein affects radial neuronal migration in the developing cortex. Developing embryonic brains were electroporated at E14.5 by *in utero* electroporation together with a GFP expression plasmid, and the position of the electroporated cells was analyzed 4 days later. Our results indicate that control pyramidal neurons born at E14.5 migrate to the CP and typically occupy layers II/III (Fig. 2A). Notably, all Tau shRNA sequences

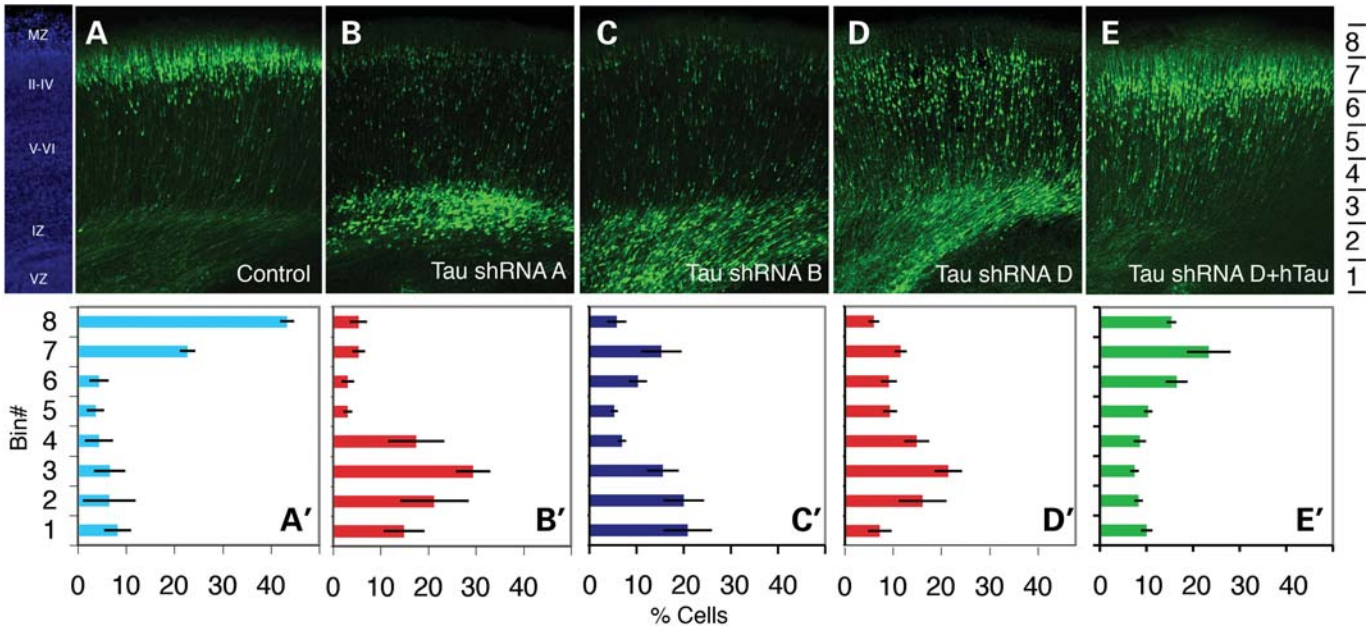

Figure 2. Acute reduction in Tau levels inhibits neuronal migration. E14 mouse embryos were co-electroporated with control (**A**) or Tau shRNAs (**B**–**D**) and GFP plasmids. The location of GFP-positive cells was examined 4 days after electroporation. The proportion of cells located in eight arbitrary bins along the cortex was measured and plotted (A′–D′). (**E** and E′) Partial rescue of the neuronal migration phenotype following Tau knockdown is achieved by introduction of hTau, resistant to Tau shRNA D.

effectively inhibited the ability of the GFP-positive-treated cells to reach the outer layers of the CP (Fig. 2). Two-way analysis of variance (ANOVA) detected significant differences in the proportion of shRNA neurons located in the different bins in comparison with control neurons. The most severe effect was noted for Tau shRNA A (3/8 bins $P < 0.001$, 2/8 bins $P < 0.01$, $n = 8$). To exclude off-target events, human Tau (hTau) was co-introduced with Tau shRNA. Human and mouse Tau are functionally conserved; however. hTau is resistant to Tau shRNA D sequence. For this experiment, we used hTau23, which is the most abundant isoform in the developing brain containing three microtubule-binding repeats (33). Following addition of hTau, some of the observed neuronal migration defects were alleviated (Fig. 2E and E′), and this treatment significantly improved neuronal migration of the Tau shRNA D-treated neurons (1/8 bins $P < 0.01$, 1/8 bins $P < 0.05$, $n = 8$). A closer examination of the stalled neurons revealed a cell autonomous morphological defect (Fig. 3). The leading edge of cells with reduced Tau levels was crooked and thinner than in control cells (Fig. 3A–F). This observation was further strengthened by quantitative analyses of the straightness and thickness of the leading edge. Tau shRNA A and D significantly reduced the straightness of the leading edge, whereas the reduction observed following treatment with Tau shRNA B was not statistically different (one-way ANOVA, $P < 0.01$ considered very significant in case of Tau A and D, $n = 41$, 31, 60, 37 for control, shRNA A, B and D, respectively) (Fig. 3E). To estimate the thickness of the leading edge, we measured a cross-section in a distance of 15 μm from the cell body (Fig. 3F). The cross-sections surfaces of the control neurons were significantly larger (>60%) than the TauA shRNA-treated neurons (two-tailed *t*-test, $P = 0.04$, control 7 ± 1.05 μm^2, TauA shRNA 4.90 ± 0.76 μm^2, $n = 16$). To rule out the possibility that the deformation of the leading edge of migrating neurons is a secondary effect, radial glia fibers were examined. Some radial glia cells were GFP-positive following *in utero* electroporation. These cells were readily identified by the location of their cell bodies and their typical elongated processes that can be followed along the thickness of the cortex (Fig. 3G and H). No morphological differences in GFP-positive radial glia fibers were noted in any of the treatments. Additionally, radial glia in the area of the stalled cells appeared normal as judged by DiO backfills from the cortex outer surface (Fig. 3I). The axons extending from Tau shRNA-treated neurons were somewhat shorter than the control at E16 (Supplementary Material, Fig. S3). However, this phenotype may be secondary due to their inhibited migration. Collectively, our findings indicate that knockdown of Tau impairs neuronal migration and affects the morphology of the leading edge in a cell autonomous manner.

Tau knockdown affects the morphology and the distribution of mitochondria

Increased expression of Tau has been demonstrated to affect the transport of mitochondria in the axon of cultured neurons (34). Therefore, we speculated that mitochondria may be affected following Tau knockdown. To test this hypothesis, we examined the motility, distribution and morphology of mitochondria in migrating neurons where Tau had been knocked down (Fig. 4). The mitochondria were labeled with either mCherry or DsRed fused to mitochondria localization sequence and imaged in live organotypic slices (Fig. 4A–H) as well as in fixed slices (Fig. 4I–L). In control neurons, the mitochondria found in the cell soma and the leading edge (Fig. 4A–C) exhibited high motility as seen in a kymograph presentation (Fig. 4D). The

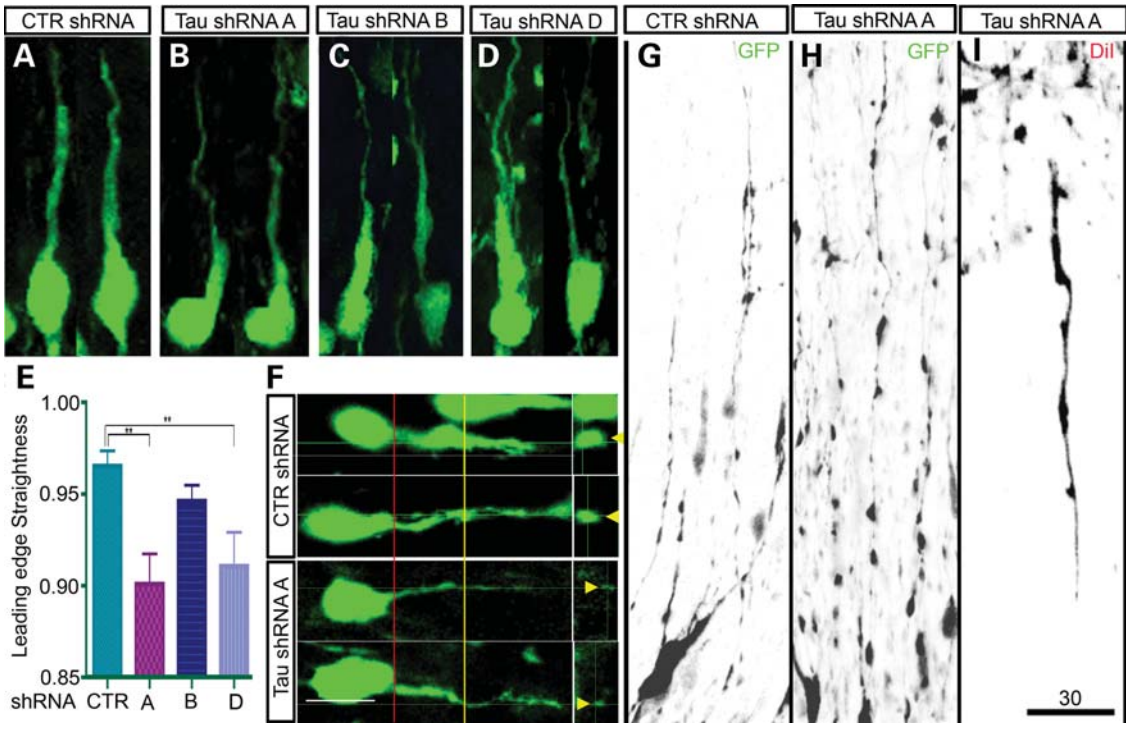

Figure 3. Abnormal morphology of bipolar migrating neurons with reduced Tau levels. (**A**–**D**) GFP-positive cells treated with Tau shRNA (B–D) exhibit a thin and crooked leading edge in comparison to control cells (A). The straightness of the leading edge was measured using Imaris and plotted in (**E**). (**F**) Cross-sections of leading edge of neurons were taken at a distance of 15 μm from the cell body. The position of the end of the cell body is marked with a red line, the position of the cross-section is marked with a yellow line, the z-stack of the position at the yellow line is on the right panel and the sections are labeled with a yellow arrowhead. (**G**–**I**) Radial glia in the vicinity of GFP-positive cells in either control (G) or Tau shRNA-treated brains (H) are indistinguishable. DiO Backfills of radial glia in Tau shRNA-treated brains (I) reveals no obvious morphological abnormalities.

labeled mitochondria in Tau shRNA-treated neurons localized primarily to the cell body and poorly penetrated the abnormal leading edge (Fig. 4E–G). Moreover, the motility of the mitochondria that did penetrate the apical aspect of the leading edge was reduced in comparison with control neurons as demonstrated in time-lapse images (Fig. 4A–C and E–G) and typical kymographs obtained from control and Tau shRNA A-treated cells, respectively (Fig. 4D and H). Examination of fixed neurons substantiated the live imaging observations (Fig. 4I and J compared with K and L). We cannot exclude the possibility that the ability of mitochondria to enter the leading edge was impaired at least in part due to the reduced thickness of the leading edge. Nevertheless, even in control cells with a relatively thin leading edge, the mitochondria were transported to a greater distance than in Tau shRNA-treated neurons, which exhibited rather thick leading processes (Supplementary Material, Fig. S4). These findings prompted us to examine the mitochondria at a higher resolution using electron microscopy (Fig. 4M–R). Immunogold staining for GFP identified the Tau shRNA A or the control shRNA-treated cells. While similar numbers of mitochondria cross-sections were examined in the EM images, Tau shRNA-treated cells contained a considerable proportion of abnormal mitochondria (22% Fig. 4S). Abnormal mitochondria included both enlarged rounded mitochondria that occasionally engulfed cytoplasmic components (Fig. 4M and N) and mitochondria with unusually enlarged vacuoles (Fig. 4O and P). These abnormal mitochondria were not undergoing mitophagy since they were not engulfed in a double membrane. Control neurons contained mitochondria with a normal structure (Fig. 4Q and R). Our results indicate that the knockdown of Tau reduces the mobility of mitochondria into the leading edge and impairs their structure.

Postnatal consequences of reduced Tau levels during embryonic development

To estimate the outcome of the developmental deficits described, we analyzed postnatal (P8) brains of E14 treated embryos. At P8, the control neurons were located in the outer layers of the CP, layers II/III (Fig. 5A), and the majority of the stalled cells observed at E18 had reached a similar position. However, the distribution of Tau shRNA GFP-positive cells included also the less superficial layer IV (Fig. 5B). Additionally, a small number of cells in the Tau shRNA-treated brains remained in an ectopic position deep in the white matter, where usually only axons are located (Fig. 5C and D). We did not observe an increase in apoptotic cells in Tau shRNA-treated brain sections (Supplementary Material, Fig. S5). Interestingly, ectopic cells were able to extend axons across the midline to contralateral targets (Supplementary Material, Fig. S3). Similar results were obtained using two different Tau shRNA sequences (data not shown).

Despite of the finding that many of the Tau shRNA-treated pyramidal neurons were located in the CP at P8, many

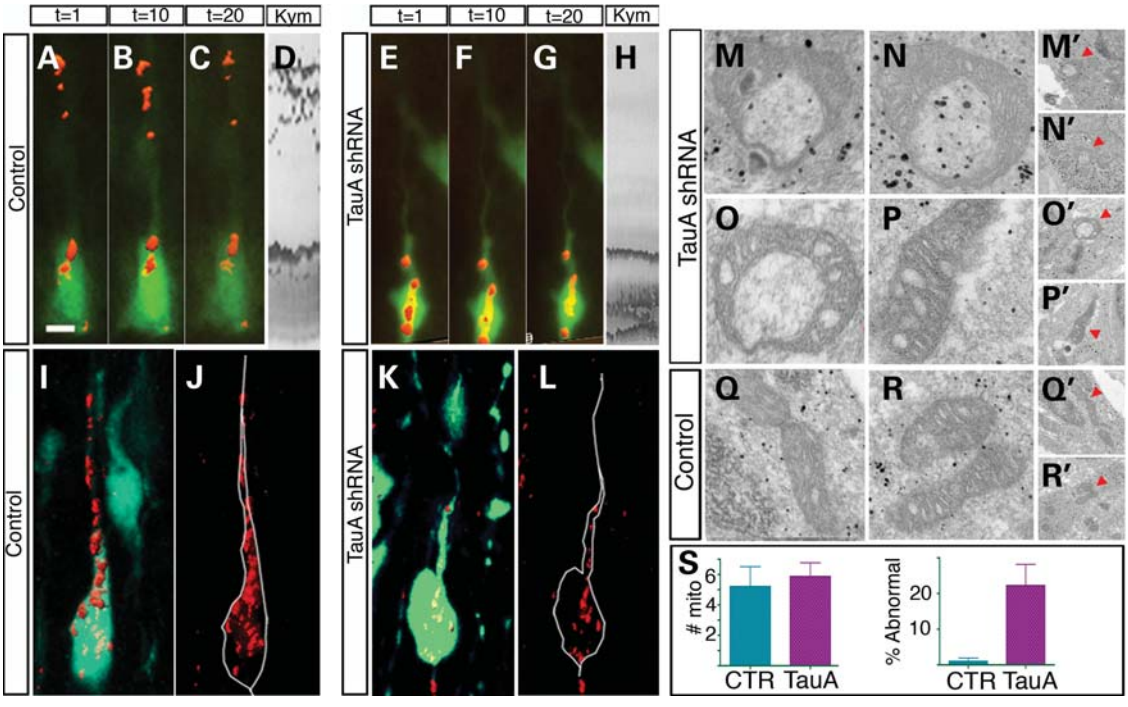

Figure 4. Tau affects mitochondria. Mitochondrial motility and distribution (**A**–**L**). Organotypic slices from control (A–D) and shRNA TauA (E–H) *in utero* electroporated brain sections were imaged. GFP identifies the treated cells and mitochondria were labeled in red using a mito-dsRed plasmid. (A–C) and (E and F) show mitochondria (volumes of dsRed positive puncta) along the shaft of the leading edge at selected times in control (A–C) and Tau shRNA-treated cells (E–G). (D) and (H) show kymographs of control and treated cells, respectively. (I–L) Distribution of mitochondria in control (I and J) and Tau shRNA-treated bipolar cells (K and L). (**M**–**R**) Electron micrographs of mitochondria within control (Q and R) or Tau shRNA migrating neurons (O–R). (M′–R′) Lower magnification images of the cells from which the mitochondrial images M–R are shown, demonstrating that the cells are positive for GFP (black puncta). (**S**) Left panel, the number of mitochondria in the sections were counted and did not differ between treatments. Right panel, the percentage of abnormal mitochondria in normal and Tau shRNA-treated cells in E16 brains is shown.

abnormalities were noted. The cross-sectional areas of the cell soma of the Tau shRNA-treated neurons located in the somatosensory cortex exhibited a significant reduction of 15% ($P = 0.0063$, unpaired t-test, control $385 \pm 12.65\ \mu m^2$, $n = 18$ in comparison to Tau shRNA $325.9 \pm 14.98\ \mu m^2$, $n = 24$) (Fig. 5E and F). A more striking morphological feature was observed when individual cells were traced (representative images are shown in Fig. 6A–C). Tau shRNA-treated cells displayed a much simpler, less branched and shorter apical dendrite in comparison with control cells (Fig. 6A versus B and C). This impression was reflected using the Sholl analysis (Fig. 6D). The shRNA-treated neurons differed very significantly from the controls (two-way ANOVA analysis $P = 0.0068$, $n = 12$). Based on the spectacular differences in neuronal morphology, we postulated that the connectivity might also be impaired in these neurons. Connectivity was evaluated by co-electroporation of a fluorescent synaptic protein expression plasmid (synaptic vesicle protein SV2a) with Tau shRNA A, B or control plasmids. In P8 brains, positive cells were analyzed in the somatosensory cortex. A reduction in the number of SV2a-positive puncta decorating the apical dendrite as well as the basal dendrites was noted in Tau knocked down neurons (Fig. 6E–J). The size and distribution of the SV2a-positive boutons on descending axons was measured and plotted. Small dots were not included in the analysis since they most likely reflect trafficking packets (35,36). The identity of the boutons used for the analysis was validated by immunostaining with additional synaptic markers (Supplementary Material, Fig. S6). All axons displayed similar densities of boutons; the average number of boutons along 10 μm was 3.8, 3.8 and 2.9 in control, Tau shRNA and Tau shRNA B, respectively. However, Tau knocked down axons (using either Tau shRNA A or shRNA B) exhibited an extremely significant smaller size boutons (Fig. 6K–N) (one-way ANOVA $P < 0.0001$, $n = 111$, 103, 106 for control, Tau shRNA A and Tau shRNA B, respectively).

Our data clearly define novel functions for Tau in the developing brain. We demonstrated that reduced Tau levels result in slower radial migration of layer II/III pyramidal neurons to the superficial layers of the cortex, which is accompanied by a defective leading edge in which mitochondria are partially abnormal and less motile. In postnatal brains, some neurons remain in ectopic positions, while others reach their approximate correct position in CP. Nevertheless, these neurons exhibit a smaller cell soma with simpler dendritic trees and less synaptic connections.

DISCUSSION

Tau and neuronal migration

The importance of microtubules in neuronal migration was underscored by the severe neuronal migration abnormalities resulting in lissencephaly (‘smooth brain’) observed in case of mutations of some MAPs such as *LIS1* and *DCX*

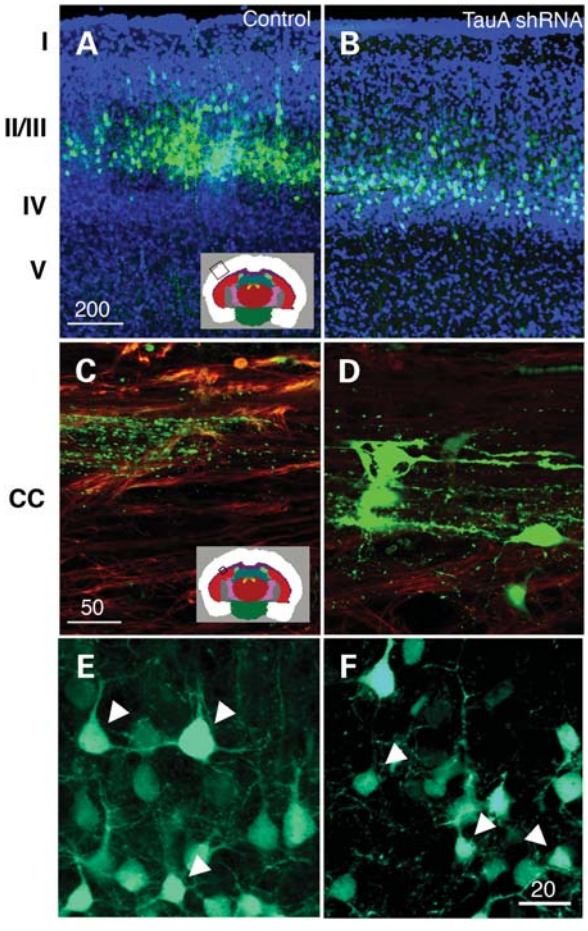

Figure 5. Ectopic location and reduced somal size of pyramidal neurons following reduction in Tau levels in the postnatal brain. (**A** and **B**) Control (A) and Tau shRNA-treated cells (B) show similar positions at postnatal day 8. Tau-treated cells are in layers II/III but are also visible in the deeper layer V. (**C** and **D**) Ectopic cells are often found in the white matter of Tau shRNA-treated brains (D) but not in control brains (C). (**E** and **F**) Larger magnification of GFP-positive cells of control (E) and Tau shRNA-treated brains. Cells with smaller somata are often visible in the treated brains (arrowheads showing typical cells somata).

(37–45), as well as mutations in one of the main tubulin isoforms, alpha-tubulin (TUBA1A) (46–49). The spectrum of diseases associated with abnormal neuronal migration includes schizophrenia (*DISC1*) (50–53), intellectual disability (duplication and triplication in *LIS1*) (54), intellectual disability and/or autism (14-3-3 epsilon/*YWHAE*) (55–59). Furthermore, neuronal motility has been implicated as one of the major pathways underlying autism (15). Most of these diseases will not be diagnosed using current imaging techniques, yet the pathophysiology involves developmental processes occurring in the cerebral cortex. In this respect, our findings that neuronal migration is impaired during development, yet many of the neurons are found in the CP in the postnatal brain, are highly relevant. Such a phenotype has not been reported in Tau knockout mice, yet it is possible that a subtle phenotype exists and has not been detected so far. Alternatively, gene redundancy mechanisms may differ between knockdown and knockout mice. Tau may be inhibiting neuronal migration through its activity as a MAP (60–63). In this context, it is entirely plausible that the knockdown of Tau affects the interaction of other MAPs to the microtubules. For example, Tau and doublecortin (DCX) may compete for binding to microtubules (64) and Tau regulates the access of the microtubule severing protein katanin to microtubules (65). Alternative splicing generates several isoforms of Tau, which differ in their microtubule-binding properties. The most abundant isoform in the developing brain exhibits three microtubule-binding sites, whereas later in life, an isoform containing four microtubule-binding sites is more predominant (33,66). However, it is possible that the effects we observe following Tau reduction in the embryonic brain are not exclusively due to the microtubule-stabilization activity of Tau. Tau may influence neuronal migration by affecting microtubule-associated motors such as kinesin and cytoplasmic dynein (34,67). In this respect, it should be noted that tau23 was a more potent inhibitor for both molecular motors (67). Furthermore, Tau has been shown to interact directly with dynactin, which regulates dynein activity, but can also regulate kinesin (68–71). Tau shRNA-treated migrating neurons exhibit an abnormal leading edge. Such abnormalities have been observed following knockdown of several genes involved in cytoskeletal remodeling. Most pertinent for this study is the knockdown of Mark2/Par-1, one of the main Tau kinases involved in microtubule remodeling (32). In humans, *MARK1*, a *MARK2* paralog, has been suggested as a susceptibility gene for autism (72). Remodeling of microtubules is not the only component required for proper formation of the leading edge; actin is involved as well. Tau activity affects the actin cytoskeleton as well as microtubules (73–75, reviewed in 76). Proper stabilization of actin in the leading edge can be regulated by a variety of factors, for example by phosphorylation of Cofilin mediated by reelin-induced LIM-kinase activation (77). In the absence of reelin, the anchorage of the leading edge to the marginal zone does not take place, and many apical dendrites lose their proper orientation (77). Mutations in reelin severely impair neuronal migration in humans and in mice (reviewed in 78). Reelin has also been considered a susceptibility gene for schizophrenia (reviewed in 79).

Mitochondrial abnormalities in migrating neurons

Tau has been suggested to affect the transport of mitochondria in axons via molecular motors and its effect on microtubules (34,80–83). Our studies demonstrate a novel finding following Tau knockdown. We show that not only the axonal transport in mature neurons is affected but also the mitochondria entering the leading edge of migrating neurons are less motile. In addition, electron microscopy images clearly demonstrate the existence of a population of abnormal mitochondria. Abnormalities in mitochondrial structure and transport are strongly associated with neurodegenerative diseases (reviewed in 84), yet their role in diseases with a known developmental basis has not been strongly established. It has been suggested that some autism patients suffer from mitochondrial malfunctioning (85,86). In addition, a recent study has identified ‘translation in mitochondria’ as one of the biological processes affected in mice displaying neuronal migration abnormalities, mutated in *Lis1*, *Dcx*, *Ywhae* or *Ndel1* (87). These genes, as mentioned above, are associated with human brain diseases,

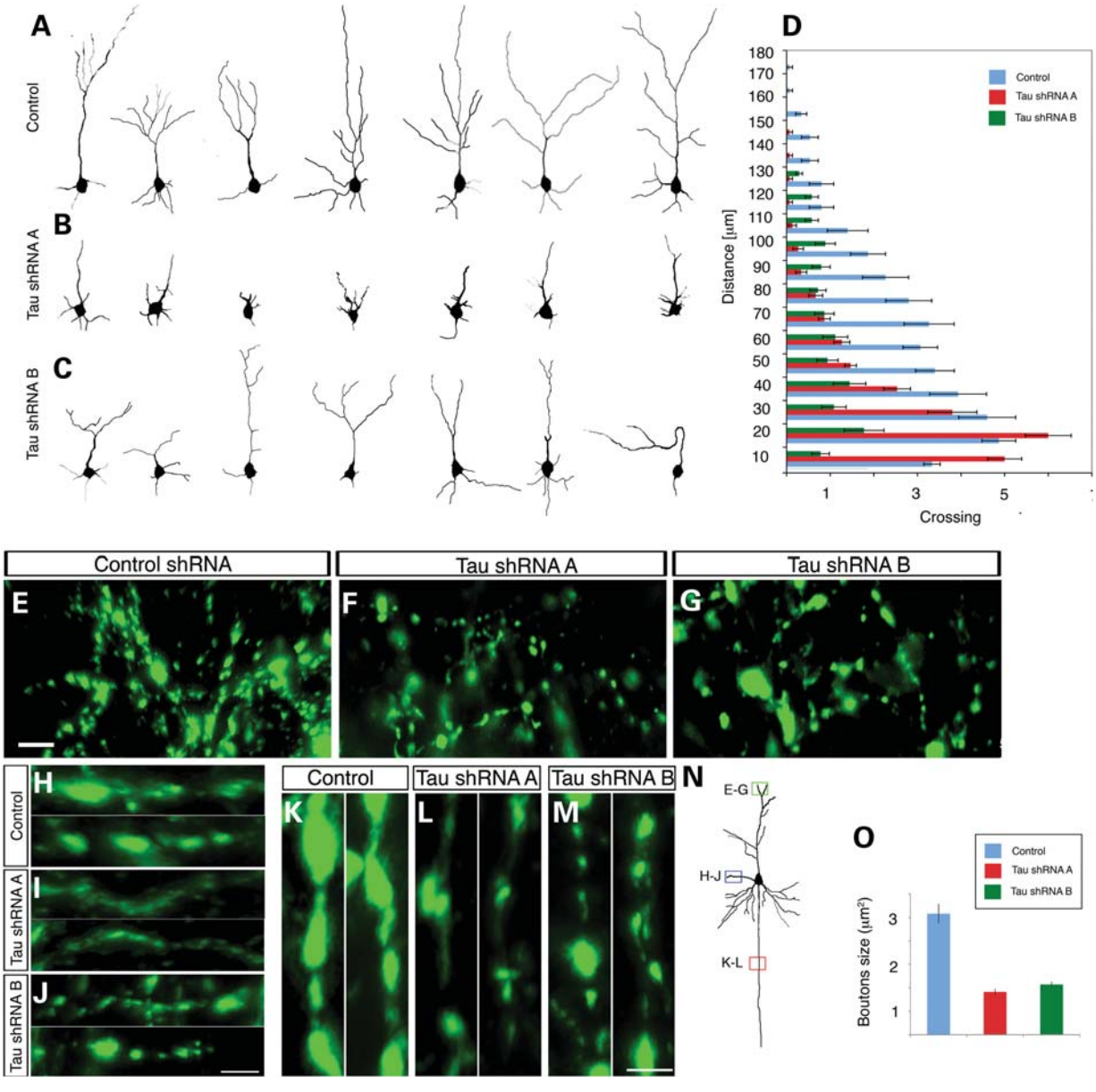

Figure 6. Tau reduction affects connectivity of cortical pyramidal neurons in the somatosensory cortex. (**A**–**D**) Sholl analysis of the complexity of control (A) and Tau shRNA (B and C) treated pyramidal neurons in P8 brains. Tracing of typical pyramidal cells is shown (A–C). The number of crossings in concentric areas around the cells bodies is plotted in (D). (**F**–**N**) Electroporation of SV2a-GFP with control (**E**, H, K) and Tau shRNA-treated cells (F, G, I, J, L, M) allowed the visualization of GFP-positive puncta along various areas of the treated pyramidal neurons at P8. (N) Diagram of apical dendrites (E–G) basal dendrites (H–J) and ascending axons (K–M). (**O**) The size of boutons along the axons of control and Tau shRNA-treated cells was measured and plotted. Size markers are 5 μm.

such as lissencephaly, autism and intellectual disability. Detection of mitochondrial abnormalities further support our notion that there is a tight connection and utilization of similar pathways in neurodegenerative and neuronal migration processes (88).

The role of Tau in the early postnatal brain

Tau has been considered mainly as an axonal protein (89–93); nevertheless, Tau may affect sorting of various proteins to dendrites, and it has been suggested to be involved in postsynaptic targeting of the Src kinase Fyn (94). Other studies have demonstrated an active crosstalk between Tau and Fyn (95,96). We noticed a reduced intensity of Fyn in the dendritic field of postnatal brains treated with Tau shRNA (data not shown). Furthermore, a very significant reduction in the dendritic complexity of neurons was noted, thus our results suggest that Tau participates in shaping the complexity of the dendritic tree, which has not been previously reported.

A smaller cell body and less extensive axodendritic tree is a common feature detected in schizophrenic patients (reviewed in 97), autistic patients with Rett syndrome and in a conditional mouse model for this disease (98,99), as well as in patients

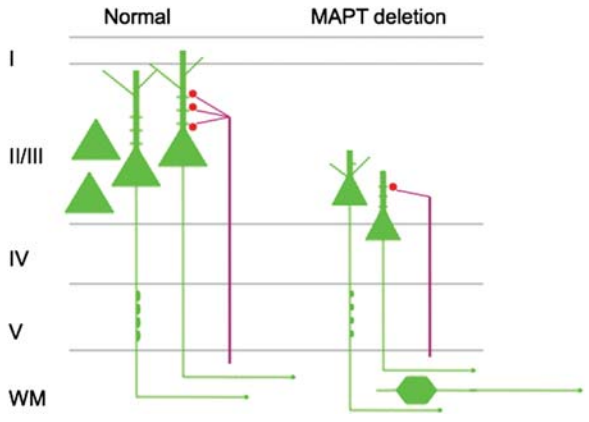

Figure 7. Schematic representation showing the consequences of Tau knockdown in the postnatal day 8 (P8) mouse brain. Cells born at E14 migrate normally to layers II/III, develop a branched apical dendrite and send axons via the (CC) to contralateral targets. These neurons receive input and have visible boutons on their axons. Reduction in Tau levels during the course of development results in a delay of arrival to the CP and therefore cells often occupy the deeper layer V as well as ectopic positions within the white matter. These cells are somewhat smaller with a less-developed dendritic tree. They have fewer synaptic inputs on their dendrites and smaller boutons are visible on their axons.

with mental retardation and their mouse models (reviewed in 100,101). Our experiments revealed that Tau affects the size of the cell body as well as the extent of dendritic arborization (Fig. 7). It has been postulated that the smaller cell bodies correlated to a less extensive or a less active axodendritic tree which the neuron has to support (97). Our findings substantiate this notion because we noticed decreased synapses on dendrites and a reduction in the size of axonal boutons, such effects of the Tau proteins have not been demonstrated to date.

Implications on Tau-related diseases

Tau is one of the six genes within the 17q21.31 microdeletion genomic area. We do not exclude the possibility that other genes also participate in the pathophysiology of the 17q21.31 microdeletion syndrome. Two genes other than Tau may contribute to brain-associated phenotypes. *CRHR1* (corticotropin releasing hormone receptor 1, CRF-R1) is widely expressed in the brain and known to mediate anxiety-related behaviors (102,103). Furthermore, CRF-R1 displays a crosstalk with Tau by mediating stress-induced phosphorylation of Tau (104). An adjacent gene, *IMP5* (intermediate protease 5) is a putative intramembrane protease belonging to the group of GXGD aspartyl proteases (105). Presenilin, one of the known Alzheimer-associated proteins, belongs to this group of proteases.

Humans exhibit two distinct haplotypes in the *MAPT*/tau locus, H1 and H2. The H1 is found in all populations, whereas the H2 haplotype is the minor haplotype in Caucasian populations and is not found in other populations. It has been suggested that the H2 haplotype originated from *Homo neanderthalensis* (106). Interestingly, the architecture of the H2 haplotype contains more extensive sequence homology, perhaps explaining its tendency to undergo microdeletion associated with mental retardation in European populations (107). The *MAPT* haplotypes differ in the level of gene expression; it has been shown that the H1c haplotype is associated with an increased expression of Tau (108). It may be interesting to examine whether the phenotype of microdeletion patients correlate to specific haplotypes. The H1c haplotype is correlated with increased risk for progressive supranuclear palsy, corticobasal degeneration and Alzheimer's disease (108). In regard to Alzheimer's disease, it has been postulated that reduced Tau levels in the diseased brains may have a positive effect, i.e. resulting in reduced toxicity of Abeta (109,110, reviewed in 111).

Collectively, we propose that Tau dosage can explain part of the pathophysiology of 17q21.31 microdeletion patients. Our results suggest that a reduction in Tau for therapeutic purposes in case of neurodegenerative diseases should be carefully evaluated.

MATERIALS AND METHODS

In utero electroporation

In utero electroporation was conducted as previously described (32). Four days after electroporation (unless otherwise stated), E18 embryos were intracardially perfused using 4% paraformaldehyde–phosphate buffered saline (PFA–PBS), the brains were post-fixed overnight and sectioned. Alternatively, pups were anesthetized and perfused postnatally (P8). The location of treated cells was analyzed in sections by measuring the signal detected in eight bins spanning the width of the cortex from at least four different brains per treatment, and the relative percentage of the signal was compared by ANOVA (Prism 4 for Macintosh, GraphPad Software, Inc.). Pictures were taken from 60 μm thick vibrotome sections using Zeiss Confocal or Applied Precision DeltaVision microscopy. To analyze protein content, E18 embryos were removed and electroporated regions were dissected out under a fluorescent dissection microscope. The tissue was flash frozen in liquid N2 prior to possessing. Tissue samples from three to four brains were pooled and lysed in buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich) complemented with phosphatase inhibitors. Brain lysates were separated by SDS–PAGE. shRNA constructs including Tau shRNA A–C are pLKO.1 lentiviral shRNA constructs purchased from Open BioSystems. Control shRNA was previously described (32), and Tau shRNA D (containing mouse Tau sequence 5′–3′) was cloned in pSuper. The shRNA constructs were coelectroporated with either pCAGGS-GFP, SV2A-CFP (35) (gift of Noam Ziv) or mitochondria localization sequence fused to dsRed (gift of Michael Davidson, Florida). Animal protocols were approved by the Weizmann Institute IACUC.

Ex utero electroporation and primary cultures

E14 embryos were removed from pregnant dams. DNA mixtures were injected to the ventricles and electroporation was conducted as described (32). The brains were removed, and the cortices were dissected out, dissociated and plated on PLL/laminin coated glass cover slips. After 2 days in culture, the cells were fixed (PHEM) and stained using the indicated collection of antibodies.

Live imaging

Electroporated brains were harvested 2–3 days after electroporation (E16–E17). Brains were excised in ice-cold L-15 media supplemented with glucose (0.6%), Gentamicin (0.02 mg/ml) and oxygen. Brains were embedded in 3.5% low-melt agarose dissolved in L-15 and cut to 300 μm thick slices by a vibrotome. The explants were placed onto Millicell-CM inserts floating on the neurobasal medium supplemented with B27 and N2, 2 mM GlutaMax, 0.5% glucose and Gentamicin. Images were taken 2 h after plating in Applied Precision DeltaVision microscopy equipped with an environmental chamber. Images were analyzed using Imaris software (Bitplane).

Electron microscopy

Two days after electroporation (E16), the brains were removed and immersed in fixation solution [0.1 M PB; pH 7.4; 4% PFA; 0.1% glutaraldehyde EM grade (Polyscience, Warrington, PA, USA), and 15% saturated picric acid]. The brains were post-fixed for 24 h in glutaraldehyde-free fixative to prevent additional loss of antigenicity. The tissue was thoroughly rinsed in PB and cut on a vibratome to 50 μm thick slices. These vibratome sections were freeze-thawed, blocked (normal goat serum, NGS; 20%) and incubated in an antibody against GFP (Invitrogen); 1:500 in Tris–buffered saline, containing 2% NGS. This was followed by thoroughly washing the sections, which were then incubated in 1.4 nm gold-coupled secondary antibodies (goat anti-rabbit; 1:100; Nanoprobes), followed by silver intensification (HQ-silver; Nanoprobes). Finally, the sections were osmicated and stained with uranyl acetate, dehydrated and flat-embedded in Durcupan (Fluca; Sigma). Thin sections (60 nm) were viewed in an electron microscope (LEO 906E).

Immunohistochemistry

The following antibodies were used to stain primary neuronal cultures and brain sections: Tau1 (Chemicon), anti-Tau PHF-1 (E-M Mandelkow), goat anti-GFP-biotinylated (Vector labs) and rabbit anti-Fyn (FYN3) (Santa Cruz, SC-16). Floating sections or cover slips containing fixed cells were blocked in blocking solution (PBS, 0.1% Triton X-100, 10% HS, 10%FCS) for 30 min. Antibodies were incubated in blocking solution over night at 4°C. After washing, appropriate secondary antibodies (Jackson ImmunoResearch) were diluted in blocking solution, and incubated for 30 min at room temperature. Slices were mounted onto glass slides using Aqua Poly/mount (Polyscience).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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