P60TRP interferes with the GPCR/secretase pathway to mediate neuronal survival and synaptogenesis

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Abstract

In the present study, we show that overexpression of the G-protein-coupled receptor (GPCR)-associated sorting protein p60TRP (transcription regulator protein) in neural stem cells (NSCs) and in a transgenic mouse model modulates the phosphorylation and proteolytic processing of amyloid precursor protein (App), N-cadherin (Cdh2), presenilin (Psen) and τ protein (Mapt). Our results suggest that p60TRP is an inhibitor of Bace1 (β-site App cleaving enzyme) and Psen. We performed several apoptosis assays [Annexin-V, TdT-mediated dUTP Nick-End Labeling (TUNEL), caspase-3/7] using NSCs and PC12 cells (overexpressing p60TRP and knockdown of p60TRP) to substantiate the neuroprotective role of p60TRP. Functional analyses, both *in vitro* and *in vivo*, revealed that p60TRP promotes neurosynaptogenesis. Characterization of the cognitive function of p60TRP transgenic mice using the radial arm water maze test demonstrated that p60TRP improved memory and learning abilities. The improved cognitive functions could be attributed to increased synaptic connections and plasticity, which was confirmed by the modulation of the γ-aminobutyric acid receptor system and the elevated expression of microtubule-associated protein 2, synaptophysin and Slc17a7 (vesicle glutamate transporter, Vglut1), as well as by the inhibition of Cdh2 cleavage. In conclusion, interference with the p60TRP/ GPCR/secretase signalling pathway might be a new therapeutic target for the treatment of Alzheimer's disease (AD).

Keywords: Alzheimer's disease ● APP ● presenilin ● G-protein

Introduction

G-protein-coupled receptor (GPCR)-associated sorting protein (GPRASP) family proteins are generally involved in the modulation of GPCRs [1–5]. Several proteins, including the sorting nexins and GPRASPs, have been described as regulating the post-endocytic sorting of GPCRs to the degradative pathway [1, 6]. Currently, only one member of the GPRASP protein family has been characterized, GPRASP1, which is an intriguing sorting protein that demonstrates selectivity for specific GPCR family members. GPRASP1 interacts selectively with the δ opioid peptide receptor 1 (OPRD1) and the D2 dopamine receptor (DRD2) but not with the μ OPR1 (OPRM1) or the DRD1 [1, 2, 7]. GPRASP1 binding contributes to the functional down-regulation of OPRD1 [1], DRD2

[2], cannabinoid receptor-1 [8, 9], bradykinin receptors [10] and viral chemokine receptors [11].

Recently another member of the GPRASP protein family, GPRASP2 has been demonstrated to interact with huntingtin protein, HTT, which suggests that members of this protein family may participate in additional activities besides their important role in controlling the function of GPCRs [12]. For example, GPRASP1 was discovered as the Per1 (period homolog 1)-interacting protein in the suprachiasmatic nucleus (also known as Pips) because it interacts with the clock-related protein Per1 and is involved in nerve growth factor (Ngf)-mediated neuronal survival *via* the neurotrophic tyrosine kinase receptor type 1 (Ntrk1), phosphatidylinositol 3-kinase and growth factor receptor bound protein 2-associated protein 1 signalling pathways [13, 14]. Interestingly, Per2 also interacts with GPRASP1 and links the circadian system to oestrogen receptor activities, which suggests that this family may be involved in the regulation of the endocrine system [15]. The expression pattern of Gprasp1 is particularly high in the hypothalamus and the suprachiasmatic nuclei [13].

Recently, we identified the novel protein p60TRP (also known as BHLHB9 or GPRASP3), which contains a basic helix-loop-helix

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(bHLH) motif that distinguishes it from other members of the GPRASP protein family. P60TRP regulates Ngf-dependent neuronal survival and differentiation and is down-regulated in the brains of patients with Alzheimer's disease (AD) [16].

To further explore the potential neurophysiological function of p60TRP with a special focus on AD-related signalling, we generated p60TRP transgenic mice, neural stem cells (NSCs) and PC12 cells overexpressing p60TRP. Our *in vitro* results show that p60TRP mediates neurogenesis through its influence on the expression and signalling of pivotal proteins such as the leukaemia inhibitory factor receptor (Lifr), Notch1, N-cadherin (Cdh2) and the β-amyloid precursor protein (App) [17, 18]. This effect could be the consequence of the direct influence of p60TRP on the secretases Bace1 (β-site App cleaving enzyme) and presenilin-1/2 (Psen1/2). Our data were validated in transgenic mice overexpressing neuronal p60TRP in the brain that exhibited a significantly denser microtubule-associated protein 2 (Mtap2)[+], GABA-B (γ-aminobutyric acid)[+] and Slc17a7 (vesicle glutamate transporter Vglut1)[+] neurite network compared with wild-type littermates. Our data also indicate that p60TRP precludes the amyloidogenic App-cleavage pathway and enhanced cognitive functions in the transgenic mice – thus, providing further evidence that the p60TRP/GPCR/secretase signalling pathway might be a new therapeutic target for the treatment of AD [19–21].

Materials and methods

Reagents

Unless indicated, all reagents used for the biochemical methods were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies

The antibodies used included polyclonal anti-mouse-p60TRP antibody (dilution: 1:5000, rabbit polyclonal, raised against a 'mouse' peptide: aa 38–51: C-GKSRDKGKGKAGSK (9871) and aa 199–212: C-QPVDEINEK-DRPKD (9874); BioGenes GmbH, Berlin, Germany).

Growth factors

Recombinant epidermal growth factor (Egf) and recombinant basic fibroblast growth factor were purchased from Peprotech (Rocky Hill, NJ, USA). Ngf was purchased from Invitrogen (Carlsbad, CA, USA).

Animal experiments

Experimental methods, including the killing of animals, were performed in accordance with the International Guiding Principles for Animal Research (WHO) and were approved by the local Institutional Animal Care & Use Committee (NTU-IACUC). Mouse tissues were isolated from C57BL/6J mice after the humane killing of the animals using approved anaesthetic methods to isolate NSCs and to analyse tissue-specific p60TRP expression. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Establishment of the transgene and the transgenic p60TRP mouse lines

The p60TRP mouse lines were generated analogously to a previously described transgenic 'synapsin-I-Ras' mouse [22].

Mouse brain perfusion, immunohistochemistry (IHC) and immunocytochemistry (ICC)

Mouse brain perfusion, IHC, ICC and NSC cultures (proliferation and differentiation) were performed according to previous reports [18, 23–26].

P60TRP-containing lentivirus stock solution

HEK293FT (American Type Culture Collection [ATCC], Manassas, VA, USA) cells were cultured with high-glucose DMEM supplemented with 10% FBS and antibiotics (complete medium). After being cultured for 12 to 24 hrs, the cells were trypsinized (Trypsin-ethylenediaminetetraacetic acid 0.05%) and transfected with the pLenti expression vector containing the gene of interest (p60TRP in EF.CMV.Gfp-Lenti-vector [elongation factor 1α, cytomegalovirus promoters, green fluorescent protein; JHU-55 (ATCC)]; p60TRP and Gfp were co-expressed from the same vector but from separate promoters *via* transfection using the protocol for ViraPower™ Lentiviral Expression Systems (Invitrogen). The following day, the medium containing Lipofectamine™ 2000 (Invitrogen) was removed, and the cells were washed with Dulbecco's phosphate-buffered saline prior to the addition of serum-free DMEM to harvest the virus-containing supernatant. The supernatant was collected between 24 and 48 hrs in 500 μl aliquots and stored at −80°C [25].

Generation of stable p60TRP-PC12 cells and p60TRP-expressing NSCs

Target PC12 cells were infected with the lentivirus supernatant within 12 to 24 hrs after the cells were split into 6-well plates. The culture medium was removed, and virus-containing supernatant was added to the wells along with Polybrene® (Sigma-Aldrich) at a final concentration of 6 μg/ml, according to the protocol provided by Invitrogen. Green fluorescence (generated by Gfp co-expressed with p60TRP) was observed 48 hrs after infection. Virus-containing-medium was removed after 24 to 48 hrs and replaced with fresh complete medium. The cells were subsequently sub-cultured in 10 cm culture dishes before FACS was performed (fluorescence-activated cell sorting; BD FACSAria flow cytometer, BD Biosciences, Singapore) to obtain a pure population of p60TRP^{+} cells. Similarly, NSCs were infected with the p60TRP lentivirus-containing supernatant, which had been previously collected and cultured in four-well plates for neurosphere formation

before transfer to a flask for culture and eventual FACS purification. As a control, mock (Gfp)-transfected cells were analysed for both PC12 cells and NSCs. The cell lysates were collected to confirm the overexpression of p60TRP by Western blot analysis.

Generation of p60TRP knockdown PC12 cells using the RNA interference technology

PC12 cells were co-transfected with p60TRP small interfering RNA (siRNA) using three different constructs (pSIH1-H1-shRNA-copGfp vector, p60TRP mRNA target sequences: (*i*) 5′-ggagaagagcttagtaataattctgt-3′, (*ii*) 5′-ctggtccgaggtaactatatggccca-3′, (*iii*) 5′-gcagccagagatatgataaatatgaa-3′; System Biosciences, Mountain View, CA, USA) and Gfp using a lentivirus expression system as described above for p60TRP overexpression. As a control, a random shRNA Gfp-vector was used. Single cells were isolated by FACS. Specific silencing of the target p60TRP gene was confirmed by Western blot using a p60TRP-specific antibody [27].

Cell culture

PC12 cells (ATCC) were cultured according to standard procedures [16, 27]. PC12 cells and NSCs were transfected using a lentivirus expression system following established protocols (Invitrogen) [23, 24].

Radial arm water maze (RAWM) test to analyse cognitive functions in p60TRP transgenic mice

The recently described RAWM test was used because it is the most reliable method for detecting memory deficits in transgenic mice; it robustly discriminates the learning abilities between mice that learn well and those that learn poorly [28]. The RAWM is a hybrid of the Morris water maze and the radial arm maze, and it takes advantage of the motivation provided by immersion in water together with the benefit of scoring errors (rather than time or proximity to platform location) associated with the radial arm maze.

Results

Effect of p60TRP on NSC differentiation and survival

We applied the lentivirus-based transfection method to generate p60TRP overexpressing NSCs (NSC-p60) to analyse the effect of p60TRP on neuronal survival and differentiation (Fig. 1). Subsequently, we investigated the effect of p60TRP overexpression in NSCs, in both proliferating and differentiating conditions, by characterizing the expression of various signalling molecules responsible for NSC survival, self-renewal, proliferation and differentiation (Fig. 1B–E). Under proliferating conditions the common stem cell marker nestin (Nes) was down-regulated slightly in NSC-p60 whereas

the higher expression of neuronal markers, such as the Ngf receptor Ntrk1 (also known as TrkA), Tubb3 and Mtap2, revealed that p60TRP induced the differentiation of NSCs even under proliferating conditions in the presence of Egf. Additionally, the glia/progenitor marker, glial fibrillary acidic protein (Gfap), was significantly up-regulated, but the oligodendrocyte markers myelin basic protein (Mbp) and 2′,3′-cyclic nucleotide 3′ phosphodiesterase were moderately down-regulated. The reduced phosphorylation of the signal transducer and activator of transcription 3 (Stat3) also supported higher levels of differentiation in NSC-p60 (Fig. 1D and E). Consistent with this observation, NSCs grown under differentiating conditions also showed that NSC-p60 possessed significantly higher numbers of Tubb3$^+$ and Mtap2$^+$ cells, substantiating the finding that p60TRP enhanced neurogenesis (Fig. 2A–C). Thus, our data demonstrate that p60TRP drives NSCs to differentiate particularly into neuronal progenitors.

Additionally, we studied survival-related signalling proteins in NSCs and observed enhanced phosphorylation of Akt1/2/3, mitogen-activated protein kinases-1/3 (Mapk1/3, also known as Erk1/2), and Bcl2 (survival promoting proteins) and reduced phosphorylation of Mapk8/9 (also known as Jnk1/2) and Gsk3b in NSC-p60. Therefore, we concluded that p60TRP enhanced neuronal survival (Fig. 1D and E). Because p60TRP was originally identified in an AD-related study [16] and interacts with the protein phosphatase 2A (PP2A) [16], which is related to many pivotal signalling pathways, including those that are crucially involved in the pathogenesis of AD [29, 30] and the differentiation of NSCs [31], we performed a PP2A activity assay. We observed considerably higher PP2A activity in NSC-p60. Enhanced PP2A activity further confirmed that p60TRP is a survival and differentiation promoting protein that directly regulates PP2A activity (Fig. 2D) [16].

P60TRP modulates the expression and cleavage of pivotal NSC regulator proteins

We investigated the role of p60TRP in the proteolytic cleavage (regulated intramembrane proteolysis) of proteins that are important for the control of stem cell proliferation, self-renewal and differentiation [33–36]. Although the expression levels of several proteins, such as App and Lifr, showed only a moderate change, their cleavage patterns changed significantly in NSC-p60. The enhanced cleavage of App and Cdh2, the down-regulation and reduced cleavage of Lifr and Notch1, and the unchanged interleukin-6 (IL-6) signal transducer, gp-130, provided additional support for enhanced neurogenesis in response to p60TRP overexpression (Fig. 3A). The differentiation-dependent cleavage of App coincided with reduced App phosphorylation and enhanced expression, cleavage and hyperphosphorylation of the microtubule-associated protein τ (Mapt) (Fig. 3B and D) [37, 38]. Because of the higher levels of App's C-terminal fragment α (CTFα [~11 kD]) in NSC-p60 (Fig. 3B), we examined Bace1 and Psen1/2 expression. Reduced levels of both Bace1 and Psen2 were observed in NSC-p60 (Fig. 3B and C). Interestingly, App phosphorylation remained inhibited despite exposure to Ngf (Fig. 3E). This result was further confounded by
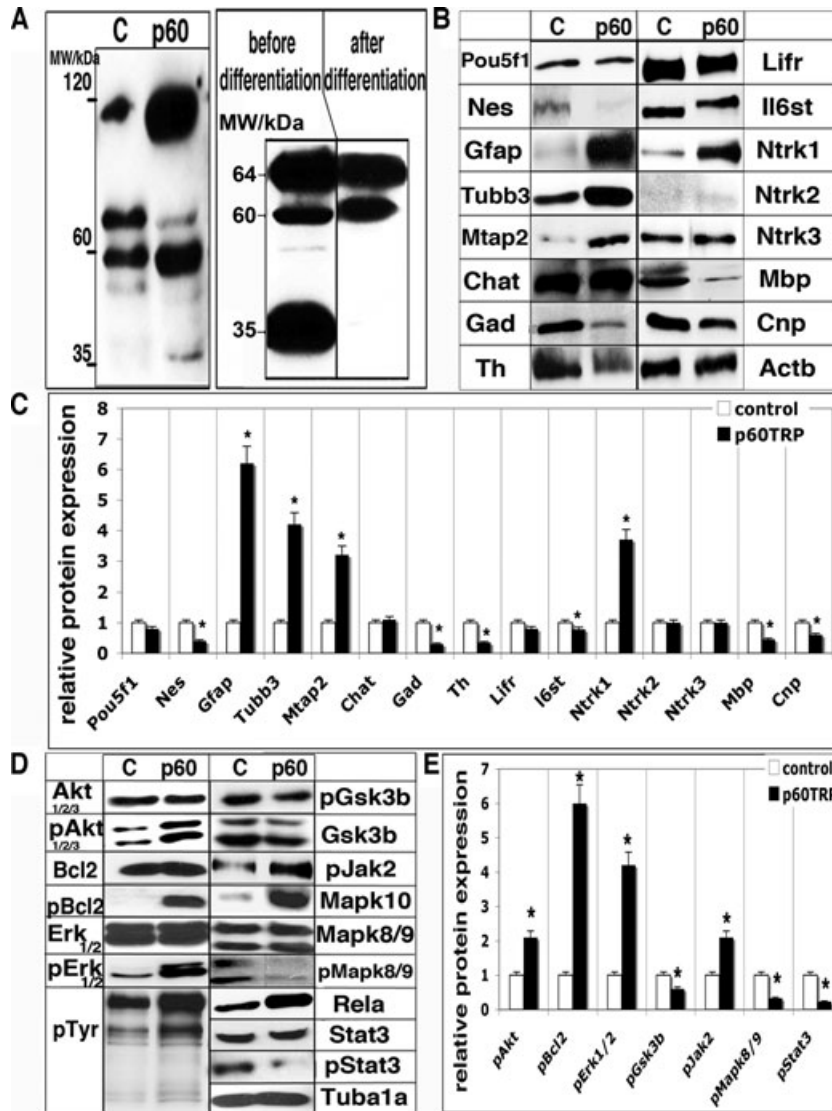
Fig. 1 P60TRP mediates differentiation in proliferating NSCs. Western blot analyses reveal a specific p60TRP expression pattern. Left: Comparing the expression levels of p60TRP in control (C, mock/Gfp-transfected) and p60TRP-transfected NSCs (p60 = p60TRP-transfected) grown under proliferating conditions. The Western blot was performed with a N-terminal-specific p60TRP antibody which revealed a band of p60TRP at ~120 kD (dimer), two bands at ~60 kD (monomer and post-translationally modified monomer), 1–2 bands at ~35 kD (cleaved products of p60TRP (CTF, C-terminal fragment), especially abundant in non-differentiated NSCs). NSCs were grown as described in supplementary 'Materials and methods' before analysis. Right: Comparing the expression pattern of p60TRP in non-transfected NSCs grown under proliferating and differentiating conditions. Western blot was performed with a C-terminal-specific p60TRP antibody. P60TRP undergoes a differentiation-dependent cleavage. Although the 35 kD product is clearly visible in non-differentiated NSCs, it is almost undetectable in differentiated NSCs suggesting that p60TRP and its cleavage product could be used as a potential marker for NSCs (**A**). P60TRP mediates neurogenesis by modulating the expression of several NSC markers, as discussed in the text. NSCs were grown as described in supplementary 'Materials and methods' before Western blot analyses. Of particular interest is the up-regulation of the neuronal markers Ntrk1 and Mtap2 in p60TRP-transfected cells, although Gad and Th expression were inhibited. Actin (Actb) was used as loading control (**B**). Quantitative analyses of the Western blots shown in (B) was performed as described in supplementary 'Materials and methods' (* = $P < 0.05$ compared with controls, $n = 4$) (**C**). P60TRP modulates the expression and phosphorylation of several signalling molecules. NSCs were grown as in (B). P60TRP-transfected NSCs demonstrated a clear increase in the phosphorylation of the anti-apoptotic proteins Bcl2 (Ser70), Akt1/2/3 and Erk1/2, as well as a clear down-regulation of pStat3, which is essential for neural differentiation [18, 32]. Tubulin (Tuba1a) was used as loading control (**D**). Quantitative analyses of the Western blots shown in (D) (*$P < 0.05$ compared with controls, $n = 4$) (**E**).

the observation of increased activation of the Mapk10 (Jnk3) and Cdk5 kinases, which are known to be responsible for the phosphorylation of App [39, 40]. One possible explanation for this observation is that the cells countered the intense dephosphorylation of App mediated by overexpression of p60TRP *via* PP2A activation (Fig. 3F). However, App phosphorylation was not observed in differentiated NSCs (control and NSC-p60), thus indicating that differentiation of NSCs may require the dephosphorylation of App (compare Fig. 3E and G). App dephosphorylation during neuronal differentiation seems to be a general characteristic because we

observed this phenomenon also in other systems, such as Ngf-mediated differentiation of PC12 cells (Fig. 4C) and retinoic-acid-mediated differentiation of neuroblastoma cells (data not shown). However, others have reported increased phosphorylation during neuronal differentiation, it might be possible that the dephosphorylation depends on the system used [41, 42] – in particular, as the specific App-dependent mechanism during NSCs differentiation still needs be investigated [17].

We also found that p60TRP, like GPRASP1 [1], mediated the down-regulation of the δ-opioid receptor, Oprd1 (Fig. S1).

Fig. 2 P60TRP enhances neurogenesis in differentiating NSCs. ICC of differentiated NSCs (control and NSC-p60) reveals that p60TRP enhances neurogenesis, as demonstrated by an increase in Tubb3$^+$ and Mtap2$^+$ (both neuronal markers) cells. NSCs were grown and differentiated as described in supplementary 'Materials and methods' before differentiation was induced for ICC. C: control (mock/Gfp-transfected); p60: p60TRP-transfected NSCs. Scale bar = 100 μm (**A**). Statistical analysis of the data obtained in (**A**). Tubb3$^+$ and Mtap2$^+$ cells were counted manually using a fluorescence microscope (Axiovert 200; Car Zeiss, Göttingen, Germany), and the data are presented as the mean ± SD of three independent experiments performed in duplicate (*$P < 0.05$ compared with controls) (**B**). Western blot analyses of Tubb3 and Gfap in differentiated NSCs indicate that the neuronal marker Tubb3 is expressed at higher levels in differentiated NSC-p60 compared to control NSCs. Gapdh was used as loading control (**C**). Analyses of the PP2A activity assay reveal higher PP2A activity in NSC-p60 compared with control NSCs. The results represent three independent experiments, each performed in triplicate (*$P < 0.05$ compared with controls) (**D**).
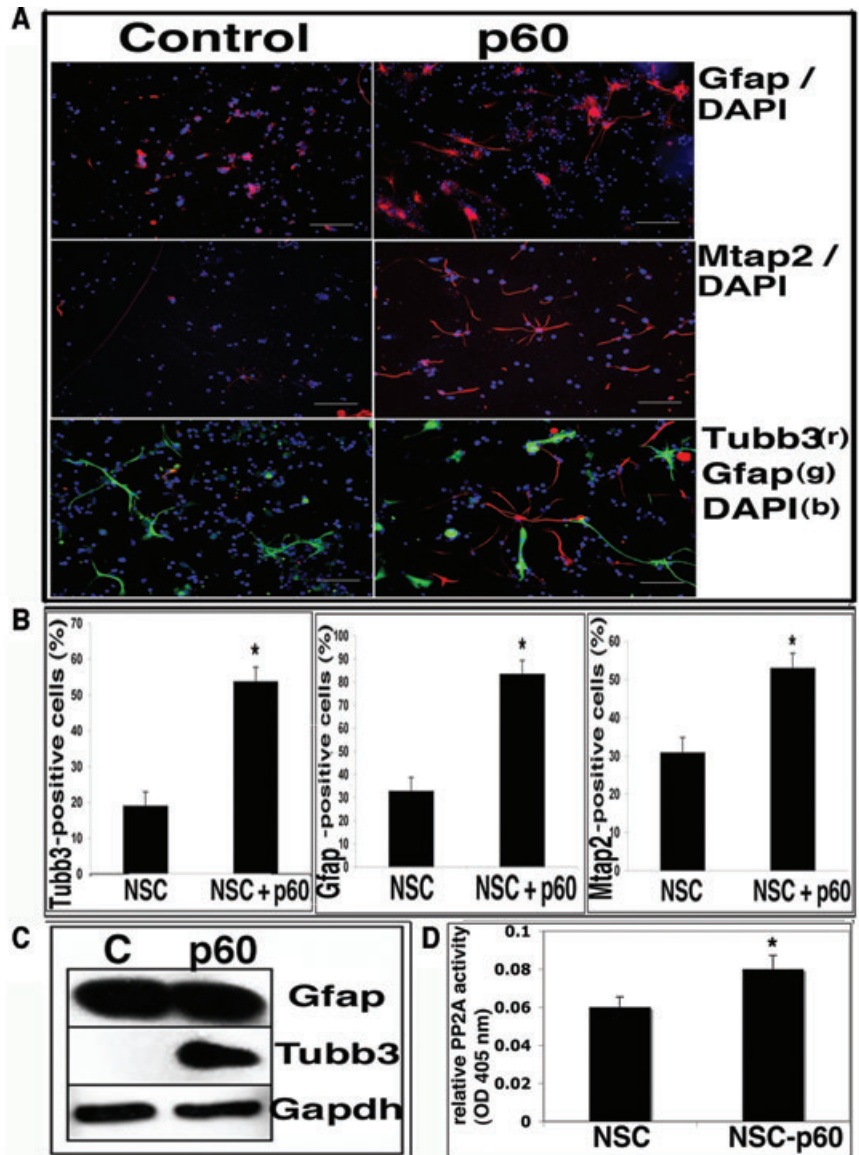
Effect of p60TRP on cell survival and differentiation in PC12 cells

PC12 cells comprise a well-established model system that is frequently used to investigate neuronal survival and differentiation [43]. PC12 cells were utilized in the present study for both p60TRP overexpression and p60TRP mRNA knockdown by specific siRNA to study the effect of p60TRP on survival and Ngf-mediated neuronal differentiation (Fig. S2A).

Investigation of the effects of the characteristic 35 kD band of p60TRP in un-differentiated NSCs (Figs 1A and S2B) revealed a significant increase in cell metabolism. An ATP count-based activity assay with PC12- and NSC-transfected cells revealed significantly

enhanced ATP metabolism in p60TRP-transfected cells (Fig. S2D and E). A higher percentage of p60TRP-transfected NSCs entered the neurogenic lineage, accompanied by higher ATP metabolism. Because knockdown of p60TRP did not affect neurogenesis (data not shown), ATP metabolism remained unchanged.

Next, we investigated the effect of p60TRP on proteins that are important for Ngf-dependent neuronal survival and differentiation in PC12 cells [44]. Of particular interest is the enhanced phosphorylation of Bcl2 at Ser70 (Fig. 4A), which is activated during anti-apoptotic processes [45]. Bcl2 performs its anti-apoptotic activity only when it is phosphorylated at a single site at Ser70, but multi-site phosphorylations have been shown to inactivate the anti-apoptotic function [45–47]. For example, Thr56 phosphorylation inhibits
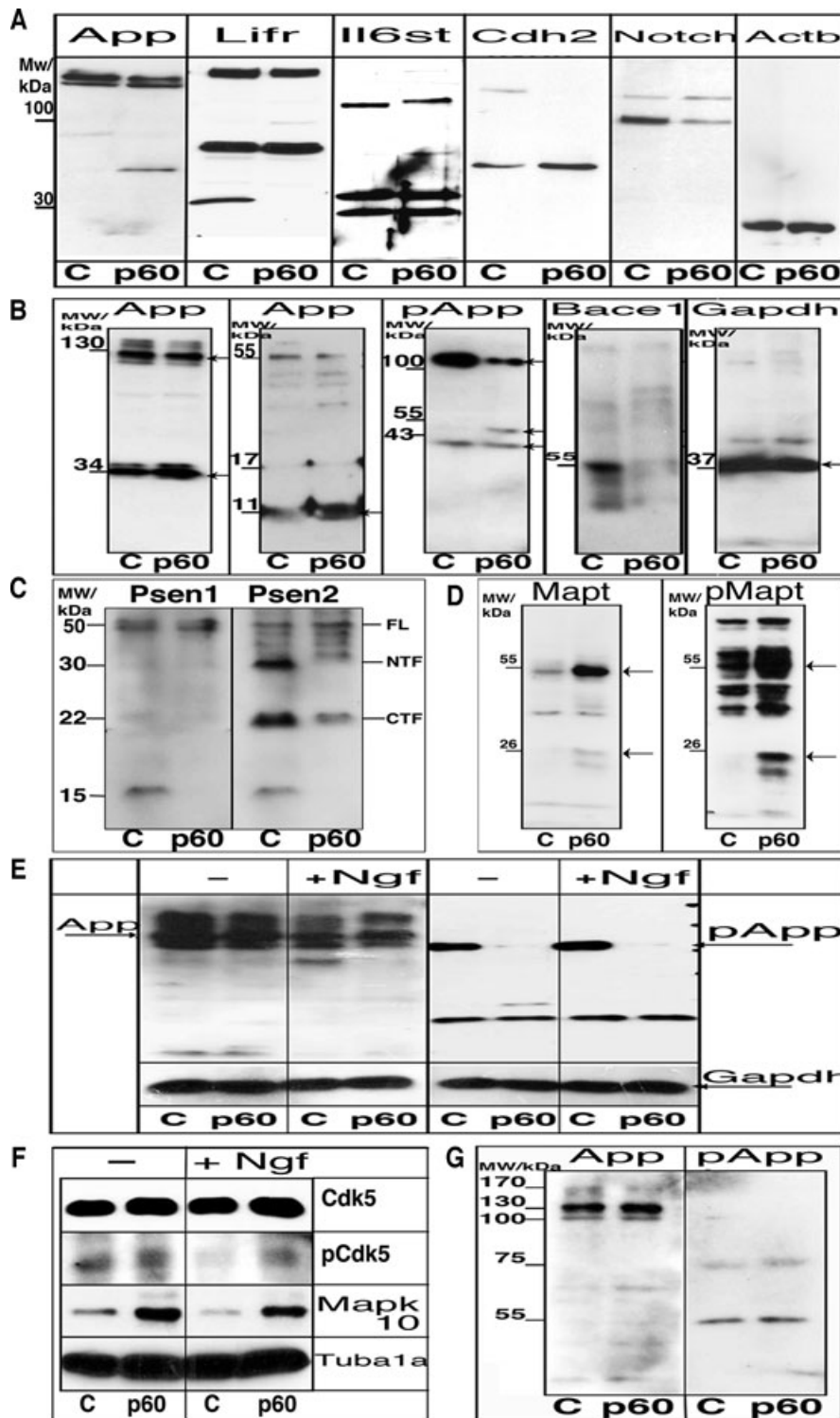
Fig. 3 P60TRP modulates the expression and cleavages of pivotal stem cell proteins. NSCs were grown and stimulated with Ngf (100 ng/ml) as described in supplementary 'Materials and methods' before Western blot analysis. Controls (C) were mock/Gfp-transfected NSCs, p60 = p60TRP-transfected NSCs. p60TRP modulates the expression and cleavage of various stem cell proteins as indicated. Enhanced cleavage of App and Cdh2 but reduced processing of Lifr and Notch are evident. Actin (Actb) was used as loading control (**A**). P60TRP modulates the expression of Bace1 and the cleavage and phosphorylation of App. Reduced phosphorylation of App and inhibited expression of Bace1 in NSC-p60. Left App blot: 8% SDS-PAGE, right App blot: 15% SDS-PAGE was run to see the lower AICD bands of App. An arrow indicates CTFα at ~11 kD. Gapdh was used as loading control (**B**). P60TRP modulates the expression and cleavage of Psen1/2 (NTF/CTF, N-/C-terminal fragment; FL, full-length). P60TRP expressing cells show less functional Psen2 as indicated by reduced levels of NTF and CTF fragments (**C**). P60TRP modulates the expression, cleavage and phosphorylation of Mapt (**D**). P60TRP inhibits App phosphorylation even in the presence of Ngf. Gapdh was used as loading control (**E**). Expression and activation/phosphorylation of Cdk5 and Mapk10 in NSCs by Ngf. P60TRP-transfected NSCs demonstrated higher levels of phosphorylated Mapk10 and Cdk5 compared with control NSCs, although the phosphorylation of App remained inhibited (**E**). Tubulin (Tuba1a) was used as loading control (**F**). NSCs were grown as described in supplementary 'Materials and methods' before differentiation was induced and Western blot analyses were performed. The expression levels and phosphorylation of App were similar in differentiated control (**C**) NSCs and differentiated p60TRP-transfected NSCs. Differentiated NSCs demonstrated diminished phosphorylation of App indicating that p60TRP mediates the differentiation of NSCs *via* the dephosphorylation of App (**G**).

Fig. 4 Overexpression of p60TRP in PC12 cells modulates the expression and activation of proteins critically involved in neuronal survival. Stable PC12 cell lines overexpressing (+p60) and with knockdown of (−p60) p60TRP were established as described in supplementary 'Materials and methods' using a lentivirus-based transfection system. Controls (C) were mock/Gfp-transfected cells. Like in NSCs, p60TRP (+p60) induces the activation/phosphorylation of Bcl2 specifically at Ser70, Akt, and cleaved τ, whereas the phosphorylation of Mapk8/9 (Jnk1/2) is clearly inhibited. Gapdh was used as loading control (**A**). Following a short-term stimulation with Ngf, p60TRP (+p60) enhances the phosphorylation and activation of Ntrk1, whereas the activation/phosphorylation of Mapk8/9 is completely inhibited. Tubulin (Tuba1a) was used as loading control (**B**). As demonstrated, after long-term stimulation with Ngf, p60TRP (+p60) enhances the phosphorylation and activation of Stat3 and Mtap2, whereas the phosphorylation of App is inhibited. Gapdh was used as loading control (**C**). As in NSCs, also in PC12 cells the PP2A activity is enhanced by p60TRP. Values represent the mean (±SD) from three experiments, each performed in triplicate (*$P < 0.05$ compared with controls) (**D**).
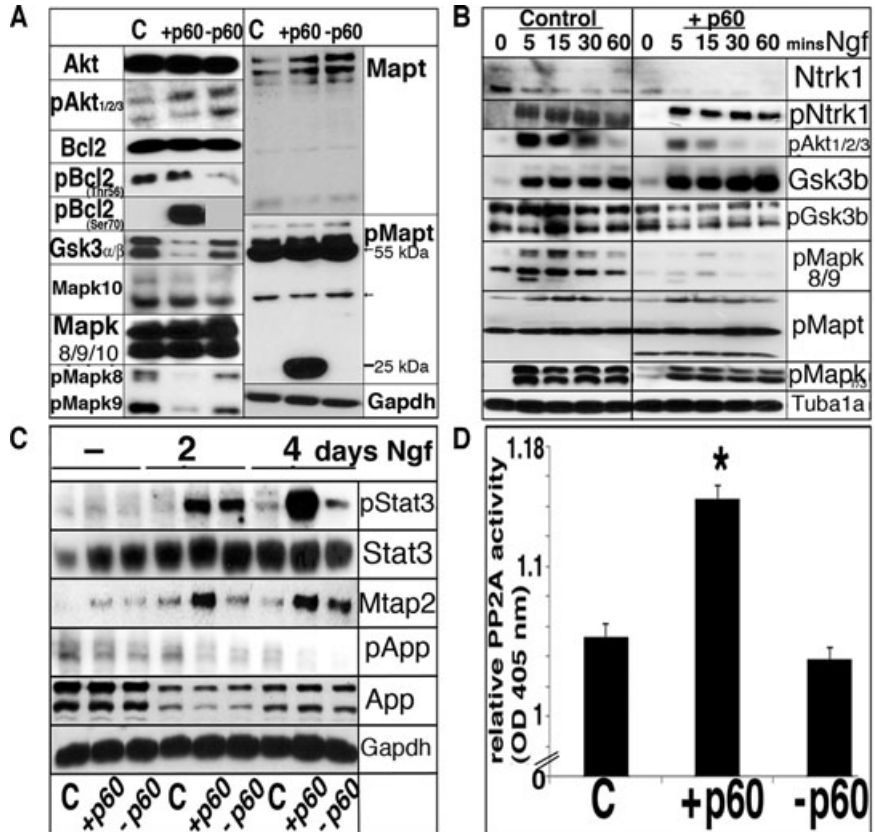
anti-apoptotic Bcl2 activity when other sites are also phosphorylated (Ser70) [48]. Consequently, p60TRP promotes the anti-apoptotic character of Bcl2 by phosphorylating the protein specifically at Ser70. The observation of reduced Mapk8/9 activation in PC12 cells overexpressing p60TRP (Fig. 4A and B) confirms our NSC data and previous results linking the activities of PP2A and Jnk [30]. It is also of interest that a cleaved product of phosphorylated Mapt was detected in NSC-p60 (Fig. 3D), PC12 cells overexpressing p60TRP (Fig. 4A and B) and p60TRP-transgenic mice (Fig. 9B); however, the significance of this cleaved product remains elusive.

To determine the role of p60TRP during neuronal differentiation in PC12 cells, the cells were stimulated with Ngf (Fig. 4B and C). Similar to the results obtained for NSC-p60, PC12-p60 cells showed significantly higher expression of Mtap2 compared to controls (Fig. 4C). Consistent with previous reports, we observed enhanced Stat3 phosphorylation in 2–4 days and reduced activation of Akt within 60 min. in PC12-p60 cells after Ngf-mediated differentiation. We thus concluded that p60TRP enhances Ngf-mediated differentiation in PC12 cells (Fig. 4B and C) [49, 50].

Data obtained from various signalling analyses in NSCs and PC12 cells overexpressing p60TRP strongly indicated it to be a survival promoting protein (Figs 1D and E, 4A and B). Consequently, we performed several apoptosis assays and confirmed the anti-apoptotic effect of p60TRP (Fig. 5).

Because our *in vitro* data demonstrated interesting effects of p60TRP on the expression and processing of App, Psen1/2, Bace1 and Cdh2 and the anti-apoptotic role of p60TRP, we decided to use an *in vivo* system. We developed a novel transgenic mouse model in which the expression of p60TRP was driven by the synapsin-1 promoter in neurons [22] to investigate the significance of p60TRP in neuronal function. Luciferase, a bioluminescence reporter protein, was used to visualize p60TRP *in vivo* in our p60TRP- transgenic mice (Fig. 6).

Neuronal p60TRP expression leads to enhanced neurosynaptogenesis

We analysed transgenic mouse brains by IHC and found that p60TRP induced enhanced neurite formation and dendrite arborization in the hippocampus and cortex, as demonstrated by the significantly higher expression of the neuronal markers Mtap2, neuro-synaptic vesicle marker solute carrier family 17 (Slc17a7, a brain-specific sodium-dependent inorganic phosphate cotransporter, member-7, and a synaptic vesicle marker for glutamatergic neurons (Vglut1)) and synaptophysin (Syp) compared to wild-type littermates (Figs 7, 8A and B). Additionally, rapid Golgi staining demonstrated higher primary and secondary
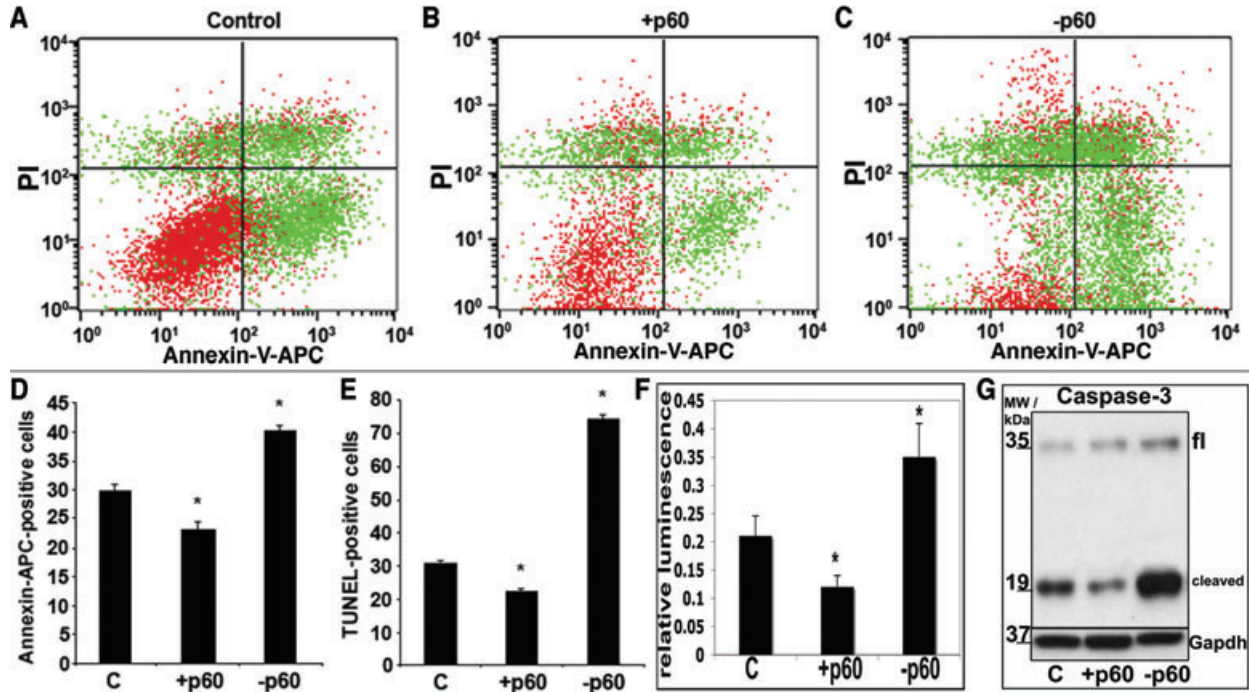
Fig. 5 Overexpression (+p60) and knockdown (−p60) of p60TRP in PC12 cells (C: control, mock-transfected) affects cell survival. Reduced apoptotic cells with lower caspase-3/7 activity are observed among p60TRP overexpressing PC12 cells. Cell survival assays [Annexin-V-APC (APC: allophycocyanin) (**A**)–(**D**), TUNEL- (**E**), and caspase-3/7 (**F**) assays, (**G**) caspase-3 (casp3) Western blot] were analysed as described in supplemental 'Materials and methods'. Data are presented as the mean ± SD of four independent experiments, each performed in duplicate (*$P < 0.05$ compared with controls). P60TRP-overexpressing cells displayed significantly fewer Annexin-V$^+$ (green) (single-positive, early apoptotic cells) and Annexin-V$^+$ (green)-PI$^+$ (red) (double-positive, late apoptotic or dead cells), whereas −p60 cells demonstrated many more Annexin-V$^+$ and Annexin-V$^+$-PI$^+$ cells compared with control cells (**A**)–(**D**). Few TUNEL$^+$ +p60 cells were observed, whereas −p60 cells demonstrated enhanced TUNEL staining (**E**). Reduced caspase-3/7 activity was observed in PC12 cells overexpressing p60TRP (**F**). p60TRP-overexpressing PC12 cells (induced to undergo apoptosis by staurosporine (1 μM) treatment for 5 hrs) showed significant lower expression levels of cleaved (activated) caspase-3; fl: full-length caspase-3; cleaved: cleaved caspase-3. Gapdh was used as loading control (**G**).

apical spines and intense cortical layer-I in p60TRP transgenic mice (Fig. 8C and D).

We then corroborated the effect of neuronal p60TRP overexpression on various crucial signalling proteins by Western blot and verified that p60TRP transgenic mice displayed higher expression of Mtap2 and Slc17a7 in the cortex and hippocampus. The expression of Gabbr1 (GABA-B receptor 1) was reduced in the hippocampus, but higher levels were observed in cortical areas (Fig. 9A, B and I).

Similar to the data obtained for NSCs, p60TRP transgenic mice demonstrated reduced protein levels of the App-cleaving enzyme Bace1 (Fig. 9B). Interestingly, the intensity of the down-regulation of Bace1 was higher in the hippocampus compared with the cortex. Similarly, reduced phosphorylation of App was observed in p60TRP transgenic mice, with an enhanced effect detected in the hippocampus compared with the cortex (Fig. 9D and E). Whereas no obvious differences in expression were observed for Psen1, the expression levels of Psen2 and its cleaved product N-terminal fragment (NTF) were reduced in p60TRP transgenic mice compared

with wild-type littermates (Fig. 9G and H). Cdh2 cleavage was almost abolished in the cortex and hippocampus in our transgenic mice (Fig. 9F). In addition, as detected in NSC-p60 (Fig. 3F), higher levels of activated Cdk5 and Cdk5r1 (a neuron-specific activator of Cdk5) (Fig. 9B) and enhanced activity of PP2A (Fig. 9C) were observed in p60TRP transgenic mice. This result supports the contention that the cells compensate for the enhanced p60TRP-mediated PP2A activity (compare with NSCs and PC12 cells, Figs 2D, 4D and 9C), resulting in the dephosphorylation of App (Fig. 9E) combined with reduced Bace1-mediated App processing [51].

The IHC, rapid Golgi and Western blot data show significantly elevated Mtap2 and Slc17a7 levels and reduced cleavage of Cdh2 and higher number of synaptic connections (Figs 8A–D, and 9A, B and F) in p60TRP transgenic mice compared to wild-type littermates, indicating a greater number of synaptic connections. Therefore, we presumed that p60TRP enhances cognitive functions, and tested this hypothesis using the RAWM. The RAWM demonstrated significantly improved cognitive functions in p60TRP transgenic mice (Fig. 10).

Fig. 6 Overexpression of p60TRP in transgenic mice using a bioluminescence reporter gene. Transgene-vector: Map of the vector used for transgenic expression of p60TRP in mice. Schematic representation of plasmid pTGV.60TRP (TGV: transgenic vector), harbouring the synapsin 1 promoter (Syn1P), p60TRP cDNA, an IRES domain, the luciferase cDNA as a reporter gene to identify p60TRP expression using *in vivo* live bioluminescence imaging, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and the β-globin 3′ untranslated region containing its own polyadenylation signal (β-glob, 3′UTR and pA, respectively) (**A**). Bioluminescence luciferase activity imaging in live mice showing p60TRP expression in the brains of transgenic (tg) mice compared with their wild-type corresponding littermates (left: females, 3 months old, strain 31; right: females, 1 month old, strain 38) (**B**). Bioluminescence luciferase activity imaging of brain slices. Upper panel: mouse strain 38 (female, 1 month old), coronal brain section; lower panel: mouse strain 38 (male, 1 month old), coronal brain section (**C**). Brain imaging of mouse strain 38, sagittal brain section (left: female, 1 month old, right: wild-type female littermate) (**D**). Western blot analysis showing p60TRP overexpression in the cortex (Cx) and hippocampus (Hp) of transgenic mice (strain 38, female, 1 month old) compared to wild-type littermates (C) (**E**). Analysis of luciferase activity assay was performed as described in supplemental 'Materials and methods'. Only p60TRP transgenic mice revealed luciferase activity. The analysis was performed with total brain tissue lysate (strain 31, male, 1 month old) (**F**).
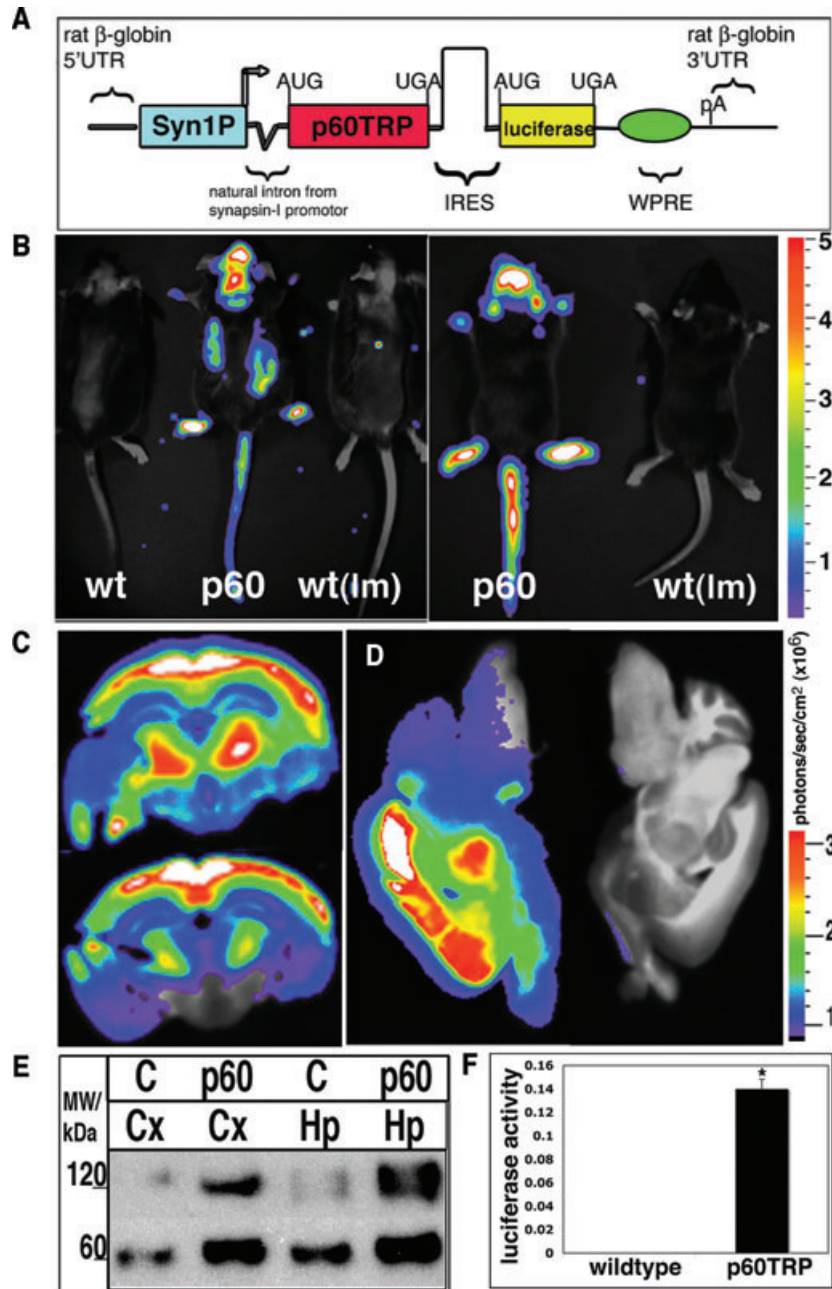
Discussion

P60TRP promotes neurogenesis

In the present study we sought to obtain insight regarding the functional significance of p60TRP. The overexpression and knockdown of p60TRP in PC12 cells revealed its potential anti-apoptotic properties through the activation of Ntrk1 and Bcl2 and the inhibition of the c-Jun N-terminal kinase (Jnk, Mapk8–10) and caspase-3/7 signalling pathways.

We also found that p60TRP induced neurogenesis in NSCs by modulating the expression and signalling pattern of pivotal proteins such App, Lifr, Cdh2 and Notch1. Increased cleavage of Cdh2 and reduced Notch expression, as shown in p60TRP-overexpressing NSCs, indicated an enhanced differentiation potential of NSC-p60 [52, 53]. Accumulating data indicate that in addition to Notch1, the Il6r family signalling pathways appear to play a decisive role
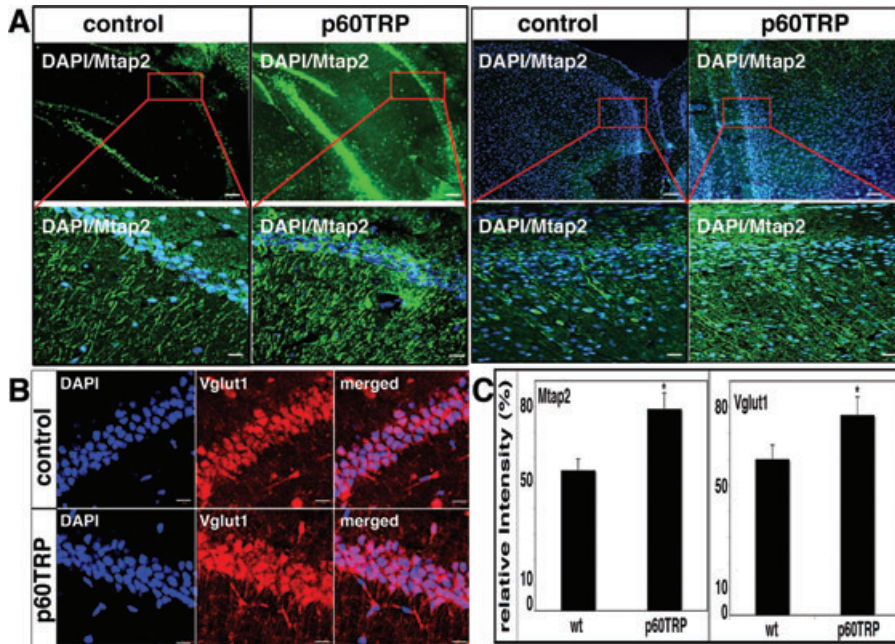
Fig. 7 Overexpression of p60TRP in transgenic mice results in enhanced neurosynaptogenesis in the hippocampus and cortex. IHC was performed as described in supplemental 'Materials and methods'. Brains were isolated from 8-week-old mice (strain 38, female, wild-type (wt) littermates as a control). The brains were sectioned and then stained with an anti-Mtap2 and anti-Vglut1 (Slc17a7) antibody. Representative images are shown in (**A**) and (**B**). Mtap2 IHC of the hippocampus (left) and cortex (right). Red squares indicate enlarged CA1 areas. DAPI (4′,6-diamidino-2-phenylindole; nuclei) was used to stain all cells. Scale bars = 500 μm (upper panels) and = 50 μm (lower panels) (**A**). Vglut1 staining of the enlarged hippocampal CA1 region. Scale bar = 20 μm (**B**). Statistical evaluation of the IHC results shown in (**A**) and (**B**). Analyses of three independent IHC experiments from three different mice (and their respective littermates) and relative intensity (normalized to DAPI staining) measured over the entire CA1-CA3 and DG areas (*$P < 0.05$ compared with wt littermates) (**C**).

during neurogenesis [54–58]. The Il6r family, which includes Lifr, interferes directly with Notch signalling [57]. Members of the Il6 family bind to a receptor complex of the common Il6st (gp130) component and a ligand-specific co-receptor to activate the gp130/Jak/Stat signalling pathway. This pathway regulates target gene transcription [59]. Stat3 is a major effector of the IL-6 family of growth factors, and its phosphorylation is essential for NSC maintenance and gliogenesis [55, 60–64]. In the present study, the NSCs overexpressing p60TRP demonstrated reduced phosphorylation of Stat3, which is a prerequisite to initiate the differentiation leading to the commitment of NSCs to a neuronal fate [18, 32]. In our study, the decreased expression of Lifr-gp45 and an additional cleavage product of Lifr-gp60, or a new cleavage product derived from the cleavage of a separate site in Lifr-gp190, was observed in NSC-p60. Egf-mediated Erk1/2 activation could induce such shedding by phosphorylating Lifr-gp190 [65]. Because Erk1/2 is also one of the main signalling components activated by Ntrk1, it is possible that p60TRP signalling and the modulated shedding of various receptors are linked *via* the activation of Erk1/2. The alteration of Lifr, but not of gp130, protein levels by p60TRP implies a decreased signalling potential through Lifr by Lif, cardiotrophin-1, ciliary neurotrophic factor, oncostatin-M or cardiotrophin-like cytokine factor 1, but not through gp130 by other family members such as IL-6 or IL-11. Thus, the reduced Lifr and Stat3 signalling, together with the enhanced Ntrk1 and Mtap2 expression and Erk1/2 activation mediated by p60TRP in NSCs, leads ultimately to neurogenesis [23, 32, 64].

P60TRP regulates App metabolism by inhibiting the activity of Bace1 and Psen

The processing of Notch and App by the regulated intramembrane proteolysis mechanism is remarkably similar [66]. Notch signalling in NSCs is initiated by the sequential cleavage by Adam10 (a disintegrin metalloprotease) in the extracellular domain and γ-secretase in the transmembrane domain to produce the Notch intracellular domain (NICD). NICD forms a protein complex with various bHLH transcription factors and translocates into the nucleus to regulate gene transcription [54, 55, 57, 58]. Our data revealed a decrease in NICD (120 kD) in NSCs overexpressing p60TRP. This finding could be explained by the inhibition of γ-secretase activity due to the reduced cleavage of Psen1/2 into NTFs and CTFs, respectively [67, 68].

Conversely, App is a conserved and ubiquitous transmembrane glycoprotein that is strongly implicated in the pathogenesis of AD; however, the physiological functions of App are still being investigated intensely [17, 69, 70]. During differentiation, the phosphorylation of the cytoplasmic domain of App at threonine 668 (Thr668) is regulated by Jnk3 and appears to be crucial for intracellular domain (AICD)-mediated signalling [39, 41, 71]. Recent findings have shown that phospho-App-bound Fe65 acts as a downstream element in the App signalling pathway, which negatively regulates neurogenesis [17, 72]. Increased expression of p60TRP induces the dephosphorylation of App by activating PP2A, which inhibits Bace1 [51] activity and causes reduced AICD signalling in p60TRP-overexpressing cells. P60TRP may modulate

Fig. 8 Overexpression of p60TRP in transgenic mice results in enhanced neurosynaptogenesis in the cortex and hippocampus. IHC was performed as described in supplemental 'Materials and methods'. Brains were isolated from 8-week-old mice (strain 38, female, wild-type littermates as control) and sectioned followed by staining with an anti-Syp and anti-Gabbr1 antibody as indicated. DAPI was used for nuclei staining. Top: dentate gyrus; bottom: hippocampal CA3 region. Scale bar = 20 μm. Representative stainings are shown (**A**). Analyses of three independent anti-Syp IHC experiments of three different mice and respective wild-type littermates and relative Syp-intensity (normalized to the DAPI and Gababr1 staining) was measured over the entire CA1-CA4/dentate gyrus area (*$P < 0.05$, compared with wild-type littermates) (**B**). Golgi staining of p60TRP transgenic (line 38, female, 3 months old) and control (wild-type littermates indicated as wild-type (wt)) mice brain. Representative stainings are shown. Scale bar = 500 μm (**C**). Quantitative spine-counting analyses. Number (shown as # on the *Y*-axis) of spines on the primary and secondary apical dendrites were counted per 1000 μm under a 100× objective to measure the spines density (*$P < 0.05$, compared with wild-type littermates) (**D**).
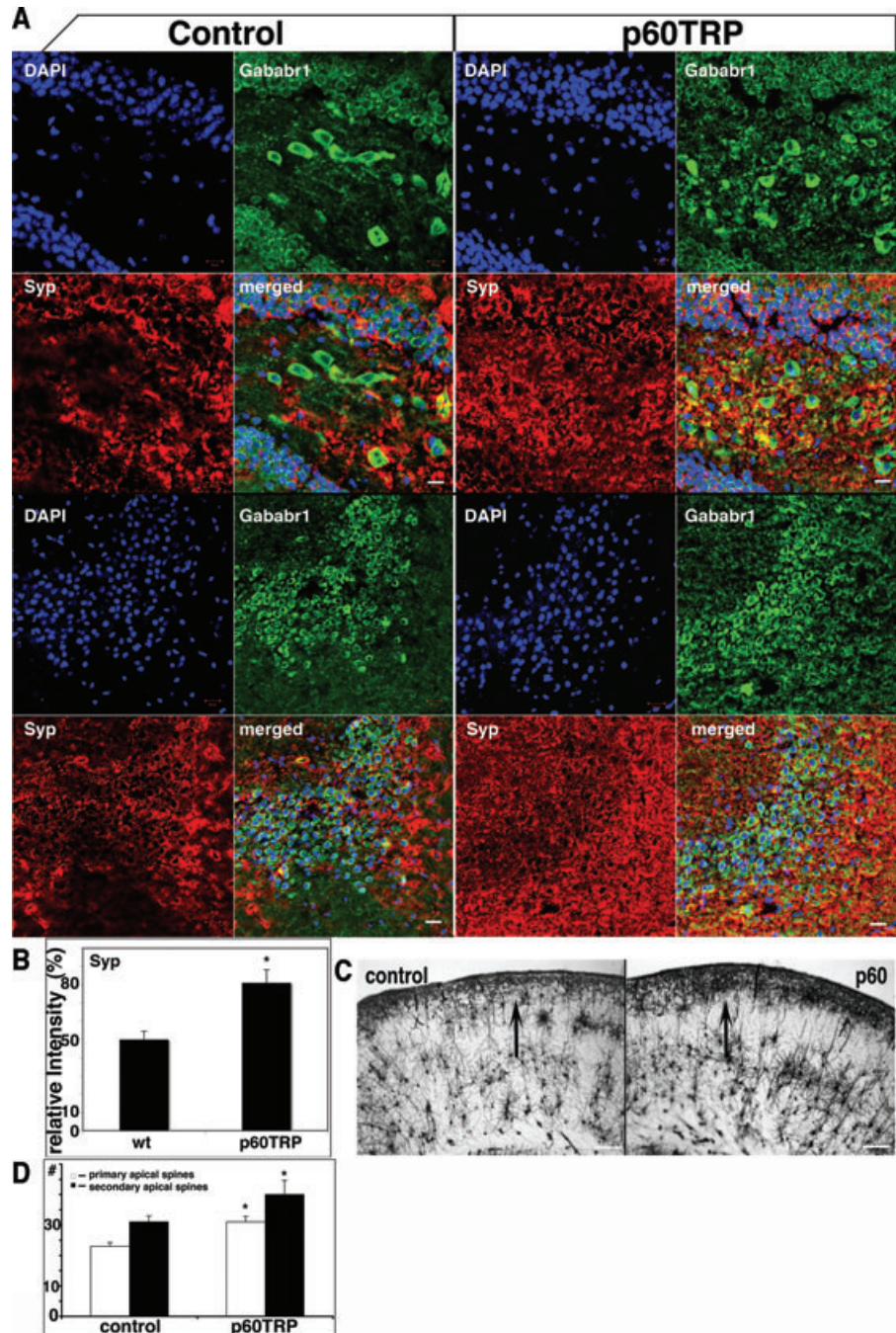
receptor shedding *via* both substrate desensitization/sensitization and specificity: a shift of the secretase cleavage products from Notch1 and Lifr (reduced or no Adam10 and Psen activity) to Cdh2 and App (enhanced Adam10 activity *via* Erk1/2 but reduced Bace1- and Psen-mediated cleavage). All of these effects of p60TRP in NSCs would enhance neurogenesis probably mediated by the inhibition of AICD signalling and enhanced sAppα release (Fig. 11)

[17, 56, 58, 72–78]. This shift is also a non-amyloidogenic pathway because the α-secretase-cleavage precludes the formation of the neurotoxic β-amyloid peptide Aβ. Here, we also show that p60TRP, which interacts with PP2A [16], induces enhanced cleavage of the phosphorylated Mapt protein and increases PP2A activity, consistent with previous reports demonstrating an association between PP2A and Jnk activity in tauopathy [29, 30, 37].
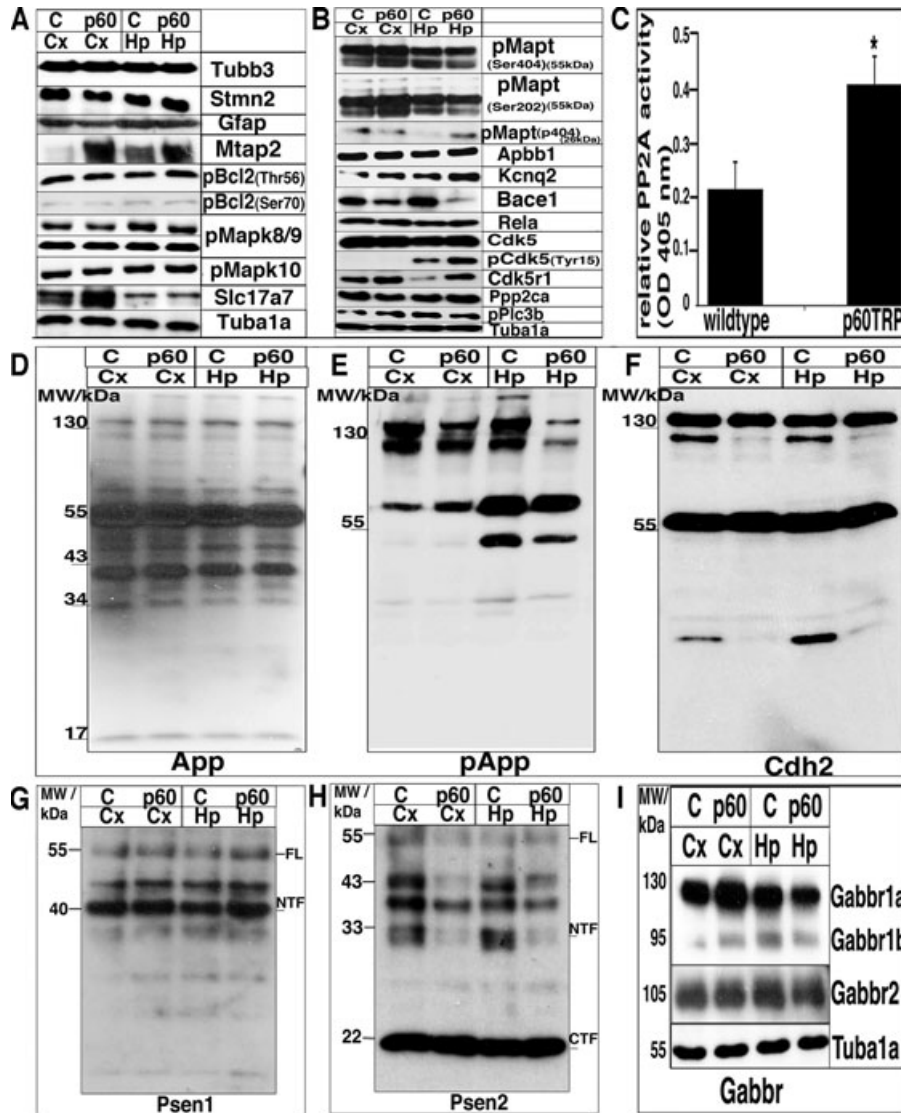
Fig. 9 Overexpression of p60TRP in transgenic mice enhances the expression of neural markers and modulates App-cleavage by reduced Bace1/Psen expression. Western blot analyses of mouse brain cortex (Cx) and hippocampus (Hp) pivotal neural regulator proteins. Control mice were wild-type littermates. Representative data are shown (line 38, female, 2 months old). P60TRP transgenic mice show significantly higher expression of the neuronal markers Mtap2 (in cortex and hippocampus) and Slc17a7 (in cortex; marker for glutamatergic neurons) and Kcnq2 (in cortex and hippocampus; potassium voltage-gated channel) compared with wild-type littermates. Tubulin (Tuba1a) was used as loading control (**A**). Similar to data obtained in NSCs, p60TRP transgenic mice show significantly reduced protein levels of the App-cleaving enzyme Bace1 (β-secretase) whereas higher levels of activated Cdk5 and its regulator Cdk5r1 were observed compared with wild-type littermates. Tubulin (Tuba1a) was used as loading control (**B**). As in NSCs and PC12 cells, p60TRP transgenic mice show higher PP2A activity. The results represent three independent experiments, each performed in triplicate (*$P < 0.05$ compared with controls) (**C**). No obvious App expression difference in transgenic mice compared with wild-type littermates (**D**). As in NSCs, p60TRP transgenic mice show reduced phosphorylated App (full length at 130 kD) compared with wild-type littermates (**E**). N-cadherin (Cdh2) cleavage is reduced in p60TRP mice. Full-length Cdh2 at ~135 kD, NTF ~100 kD, CTF1 ~40–50 kD, CTF2 ~35 kD (the band at 50 kD is unknown or unspecific) (**F**). No obvious Psen1 expression difference in transgenic mice compared with wild-type littermates (**G**). As in NSCs, reduced processing of Psen2 shows lower levels of NTFs in p60TRP transgenic mice compared with wild-type littermates (NTF/CTF, N-/C-terminal fragment; FL: full-length), indicating less active Psen2 (**H**). Higher expression of Gabbr1a in the cortex of the transgenic mice but a slight decrease in the Gabbr1a/b expression in the hippocampus whereas no obvious change in the Gabbr2 expression could be detected compared with wild-type littermates. Tubulin (Tuba1a) was used as loading control (**I**).

P60TRP enhances synaptogenesis

Neural development and the organization of complex neuronal circuits involve numerous processes in which axons select specific partners for synapse formation. Members of the cadherin superfamily are suggested to direct individual axons to their appropriate post-synaptic partners [79, 80]. In neurons, the cadherin–catenin cell-adhesion complex regulates multiple aspects of synaptogenesis and plasticity. Cdh2 contributes to the structural and functional organization of the synaptic complex by ensuring the adhesion between synaptic membranes, organizing the underlying actin cytoskeleton and stabilizing neurotransmitter receptors [79, 81–83]. Analogous to Notch1 and App, the cleavage of Cdh2 results in the shedding of extracellular NTF and the generation of CTF1/2 [84]. Our p60TRP transgenic mice exhibited improved cognitive functions due to increased synaptic plasticity caused by the expression of full-length Cdh2 and increased dendritic arborization as demonstrated by enhanced Mtap2, Syp and Vglut1 expression.

Fig. 10 P60TRP improves cognitive functions in mice. Results obtained from two-day RAWM testing in p60TRP transgenic mice as described in supplemental 'Materials and methods'. Data were collected from three-months-old p60TRP transgenic mice and wild-type littermates. Each block consists of four trials. Average error committed by p60TRP transgenic mice for each trial block. The data shown were obtained from female p60TRP transgenic mouse line-1 (S1 = 38) (diamond, $n = 8$) and wild-type littermates (squares, $n = 8$) (**A**). Average error of male mice for each trial block. The data shown were obtained from p60TRP transgenic mouse line-1 (S1) (diamond, $n = 10$) and wild-type littermates (squares, $n = 10$) (**B**). Average error of male and female mice for each trial block. The data shown were obtained from p60TRP transgenic mouse line-1 (S1) (diamond, $n = 12$) and wild-

type littermates (squares, $n = 12$) (**C**). Average error of male and female mice for each trial block. The data shown were obtained from the p60TRP transgenic mouse line-2 (S2 = 31) (diamond, $n = 12$) and wild-type littermates (squares, $n = 12$). The data shown in A-F were collected independently on separate days with new mice each time. Data are presented as the mean $\pm$ SD (by ANOVA) (*$P < 0.05$ compared with controls) (**D**)–(**F**).
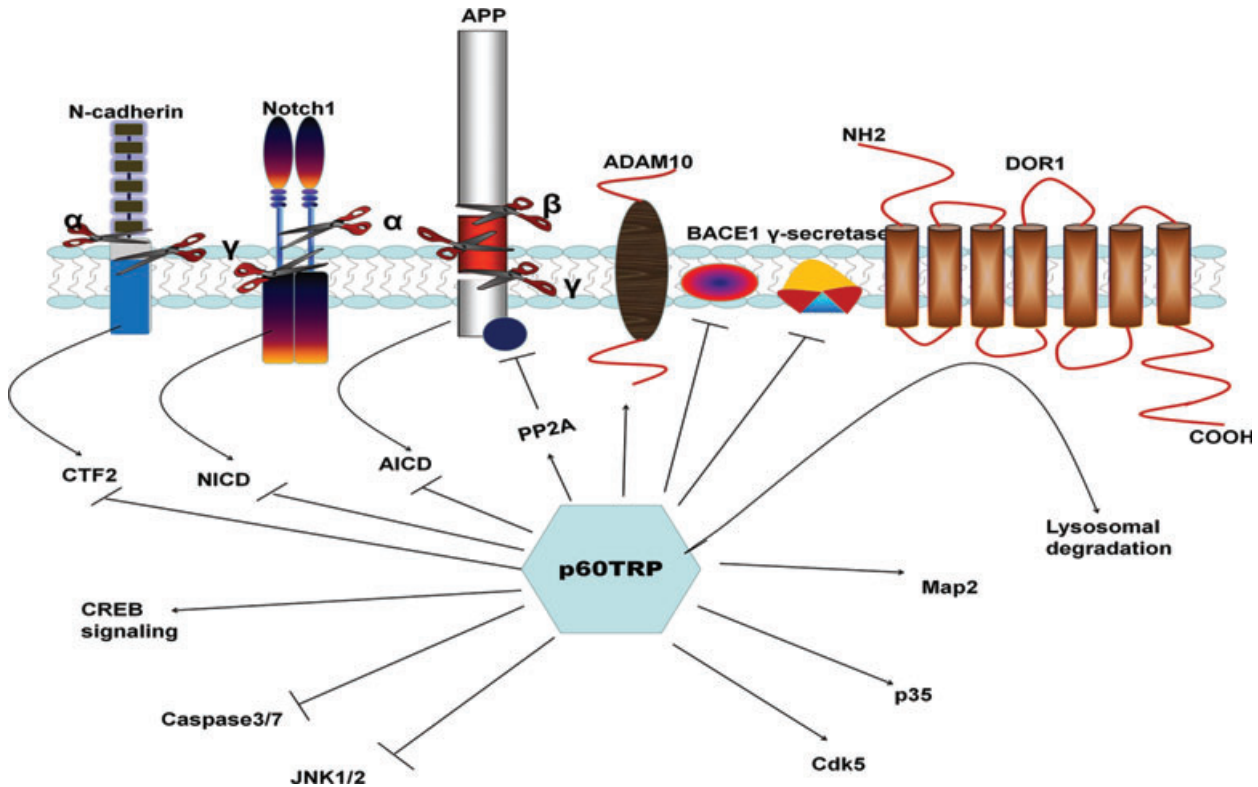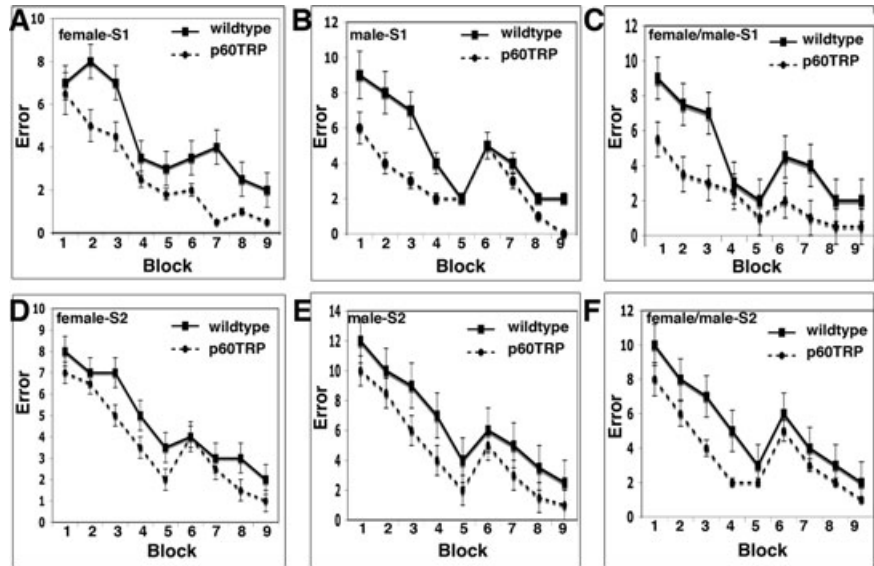

Fig. 11 P60TRP signalling based on the results obtained from different systems including NSCs, PC12 cells and p60TRP transgenic mice. P60TRP mediates neurosynaptogenesis by inducing PP2A activity which results in the dephosphorylation of App and the inhibition of Cdh2 cleavage through the inhibition of Psen. P60TRP also inhibits Bace1 (β-secretase)-mediated processing of App and promotes Adam10-mediated App cleavage resulting in enhanced production of sAppα and hence modulates synaptogenesis. Inhibition of Psen leads to reduced AICD signalling and neurogenesis as well as enhanced Mtap2 expression.

Table 1 p60TRP rescues AD-related deficits

In AD brain	p60TRP-NSCs and neurons of p60TRP transgenic mice
Increased phosphorylation of APP	Reduced phosphorylation of App
Increased activity of β/γ-secretase (Bace1/Psen2)	Reduced activity of β/γ-secretase (Bace1/Psen2)
Reduced activity of PP2A	Increased activity of PP2A
Reduced synaptic connections	Enhanced synaptic connections
Reduced cognitive abilities	Enhanced cognitive abilities

Down-regulation of p60TRP could be a crucial event in the pathogenesis of AD. Interfering with the GPCR signalling pathways may open new potential therapeutic targets for the treatment of AD.

Collectively, our data show that the overexpression of p60TRP repairs the causative molecular events of AD and that the down-regulation of p60TRP may be one of the triggering events leading to AD pathogenesis (Table 1).

As shown previously, reduced PP2A activity, Ntrk1 and p60TRP expression levels may be a common observation in the early AD brain that requires further studies to elaborate the potential significance of p60TRP to become a suitable target for the treatment of AD patients [16, 29, 38, 85–87]. Further investigations also need to be addressed to identify what triggers the down-regulation of p60TRP and under what circumstances this protein gets activated to interfere with the GPCR/secretase complex and to develop better therapeutics for the treatment of AD [20].

Acknowledgement

Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 p60TRP regulates endocytic recycling of the δ-opioid receptor (Oprd1). NSCs were grown as described in 'Materials and Methods' for 4P before stimulated for nine days with the specific Oprd1 agonist SNC80 (10 nM). p60TRP-transfected NSCs show clearly less Oprd1 expression before and upon stimulation with SNC80. Quantitative analysis of representative Western-blots is shown (* = $P < 0.05$, compared with control (Mock/Gfp-transfected)).

Fig. S2 Over-expression of p60TRP in NSCs and PC12 cells enhances the ATP metabolism. Stable PC12 cell lines over-expressing (+p60) and knock-down (-p60) p60TRP were established as described in 'Materials and Methods' using a lentivirus-based transfection system. Controls (C) were mock/Gfp-transfected cells. Tubulin (Tuba1a) was used as loading control (**A**). Comparison of the expression and cleavage pattern of p60TRP in NSCs and PC12 cells. NSCs were grown as described in supplemental 'Materials and Methods' before subjected to cell lyses along with PC12 cells and Western blot analyses were performed. Interestingly, only the proliferating NSCs show the specific p60TRP-cleaved 35 kDa band (as in Fig. 1A) (**B**). p60TRP exists as homo-dimer. p60TRP-expressing NSCs were lysed and treated with 1% DTT (dithiothreitol), 0.05% Nonidet P40 (NP40) and 1% 3-{(3-Cholamidopropyl)dimethylammonio}-1-propanesulfonate (CHAPS) to completely denature the nonionic interactions and disulfide bridges in p60TRP-dimers. 1 = control, 2 = DTT-and other non-ionic detergent-treated lysate (**C**). P60TRP-over-expressing PC12 cells show a higher ATP metabolism ratio than control cells while p60TRP-knock-down remained unchanged (**D**). p60TRP-transfected NSCs show a higher ATP metabolism ratio than control cells. ATP metabolism assays were performed as described in supplemental 'Materials and Methods', four times in duplicates. Quantification in (**D**) and (**E**) is represented as the mean ($\pm$ SD) of four independent determinations, each performed in triplicate (*$P < 0.05$, compared with control cells) (**E**).

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