Transthyretin (TTR) is a protein whose function has been associated with binding and distribution of thyroid hormones in the body and brain. However, little is known regarding the downstream signaling pathways triggered by wild-type TTR in the CNS either in neuroprotection of cerebral ischemia or in physiological conditions. In this study, we investigated how TTR affects hippocampal neurons in physiologic/pathologic conditions. Recombinant TTR significantly boosted neurite outgrowth in mice hippocampal neurons, both in number and length, independently of its ligands. This TTR neuritogenic activity is mediated by the megalin receptor and is lost in megalin-deficient neurons. We also found that TTR activates the mitogen-activated protein kinase (MAPK) pathways (ERK1/2) and Akt through Src, leading to the phosphorylation of transcription factor CREB. In addition, TTR promoted a transient rise in intracellular calcium through NMDA receptors, in a Src/megalin-dependent manner. Moreover, under excitotoxic conditions, TTR stimulation rescued cell death and neurite loss in TTR KO hippocampal neurons, which are more sensitive to excitotoxic degeneration than WT neurons, in a megalin-dependent manner. CREB was also activated by TTR under excitotoxic conditions, contributing to changes in the balance between Bcl2 protein family members, toward anti-apoptotic proteins (Bcl2/BclXL versus Bax). Finally, we clarify that TTR KO mice subjected to pMCAO have larger infarcts than WT mice, because of TTR and megalin neuronal downregulation. Our results indicate that TTR might be regarded as a neurotrophic factor, because it stimulates neurite outgrowth under physiological conditions, and promotes neuroprotection in ischemic conditions.

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Transthyretin provides trophic support via megalin by promoting neurite outgrowth and neuroprotection in cerebral ischemia

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Abbreviations: TTR, transthyretin; pMCAO, permanent middle cerebral artery occlusion; CREB, cAMP response element-binding protein; Bcl2, B-cell lymphoma 2; NMDA, N-methyl-D-aspartate; NR2A, N-methyl-D-aspartate 2A receptor

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conditions in neuronal cultures, and in a mouse model of permanent middle cerebral artery occlusion (pmCAO).

Results

TTR promotes neurite outgrowth in WT and TTR KO
cultured hippocampal neurons through megalin. TTR
was shown to promote neurite outgrowth in the PNS, mainly
in dorsal root ganglia neurons,2 but the molecular signaling
pathways involved are yet to be explored, as well as whether
TTR has a similar role in the CNS. To determine whether TTR
has neuritogenic activity in the CNS, cultured hippocampal
neurons from TTR KO mice were stimulated with recombinant
mouse or human TTR (to address its potential use as a
neuroprotective target in humans) using two concentrations,
55 and 300 μg/ml, that mimic the two pools of TTR in the
body, CSF and sera, respectively.19 These conditions
induced a rise in both neurite number (Figures 1a and b
closed arrow) and neurite length, assessed by MAP2
immunocytochemistry (Figures 1a and b open arrow). The
effect is dose-dependent and identical for human and mouse
TTR. We observed the same neuritogenic activity of TTR in
WT hippocampal neurons incubated with human TTR 300 μg/ ml
(neurite number and length (Figure 1c)). To further confirm
TTR as the protein responsible for these effects, we used an
anti-TTR antibody, which blocked neurite outgrowth
(Figure 1e). Moreover, when TTR KO neurons were
stimulated with recombinant TTR variant (I84S), which has
very low affinity for thyroxine and RBP ligands,20,21 they had
a similar increase in neurite number and outgrowth
(Figure 1d) to WT TTR, proving that TTR neurogenic activity
is not related to its ligands. In the PNS, TTR neuritogenic
activity was shown to be megalin-mediated.2 To investigate
whether this occurred in CNS neurons, we used RAP
(receptor associated protein), an LRP antagonist,22 which
blocks megalin–TTR interaction.2 RAP blocked TTR-induced
increase in neurite number and total length (Figure 1f).
However, because RAP is a broad LRP inhibitor, we prepared
hippocampal neuronal cultures from megalin (+/−) TTR KO
mice embryos, which have significant lower amounts of
megalin than TTR KO cultures (Figure 1j). None of the TTR
concentrations used promoted neurite outgrowth in these
cultures (Figures 1g and h) in opposition with cultures from
the littermates megalin (+/+), TTR KO (Figure 1i),
indicating that TTR neuritogenic activity in the CNS is
megalin-mediated. Both TTR concentrations are over the
Kd determined for the megalin–TTR interaction (500 nM).8
Moreover, expression of LRP1, a megalin-related protein, was
not altered in these megalin (+/−) TTR KO neuronal cultures
(Figure 1i). We analyzed megalin expression in TTR KO
cultured hippocampal neurons and observed that megalin is
expressed in cell body and dendrites (not stained for tau),
and to a lower extent in axons (colocalized with tau)
(Figure 1m).

Signaling pathways activated by TTR are megalin-
dependent in cultured hippocampal neurons. We
addressed the downstream signaling pathways triggered by
WT TTR in the CNS at different levels: (i) phosphorylation
of ERK and Akt (Ser473);23 (ii) intracellular calcium home-
ostasis;23 (iii) Src family kinase activation (Tyr416);24 (iv)
gene transcription activation assessed by CREB phosphor-
ylation (Ser133).25 Stimulation of TTR KO cultured hippo-
campal neurons with recombinant mouse TTR (55 μg/ml),
resulted in a significant rise of the phosphorylated levels of
ERK (35%) and Akt (23%), 30 min after the stimulus
(Figures 2a and c, respectively). ERK phosphorylation levels
were higher in shorter time points (data not shown). TTR
evoked a sustained (30–50%) activation of Src 30 min after
stimulation (Figure 2b) and promoted a significant rise (20%)
of CREB phosphorylation/activation (Figure 2d). As intracel-
lar calcium was shown to induce neurite outgrowth,26
we performed FRET assays using a ultrasensitive calcium
indicator-yellow Cameleon-Nano (YC-Nano15).27 TTR KO
hippocampal neurons stimulated with mouse TTR showed a
significant and transient rise in intracellular calcium concen-
tration, in a dose-dependent manner (Figure 2f), compared
with PBS (Supplementary Figure S1). This effect (calcium
from extracellular source; Supplementary Figure S1) is
observed mainly in neuronal cell body (where the results
were obtained), was also seen in neurites (quantitative data
not shown), demonstrated in the representative image of
FRET assay (Figure 2e). To understand whether megalin

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Figure 1  TTR promotes neurite outgrowth in WT and TTR KO cultured hippocampal neurons through megalin, independent of interaction with its ligands. (a) TTR KO cultured hippocampal neurons were stimulated with recombinant mouse and human TTR (55 and 300 μg/ml), immediately after plating for 24 h, and stained with a MAP2 antibody. Neurite number and neurite sum length were quantified with ImageJ software. The results are the average ± S.E.M. of 3–10 independent experiments. (b) Representative MAP2 staining of TTR KO cultured hippocampal neurons stimulated with TTR. (c) WT cultured hippocampal neurons were also stimulated with recombinant human TTR (300 μg/ml), immediately after plating, for 24 h. Neurite number and neurite sum length were quantified as described in (a and b). The results are the average ± S.E.M. of five independent experiments. (d) Corresponds to the same stimulation of TTR KO hippocampal neurons with a recombinant mutant TTR (TTR I84S 55 μg/ml)). The results are the average ± S.E.M. of three to four independent experiments. (e) The results (neurite sum length) in similar experiments using TTR KO cultured hippocampal neurons treated with mouse TTR with or without an antibody against TTR are shown. The results are the average ± S.E.M. of three to seven independent experiments. (f) TTR KO cultured hippocampal neurons were stimulated with recombinant mouse TTR (300 μg/ml) with or without RAP and stained with MAP2 to quantify neurite number and neurite sum length. The results are the average ± S.E.M. of four to seven independent experiments. Megalin heterozygous TTR KO cultured hippocampal neurons were also stimulated with recombinant mouse TTR (55 and 300 μg/ml) immediately after plating for 24 h. Neurite number and neurite sum length for megalin (+/−) TTR KO (h) and megalin (+/+)) TTR KO littermates (l) were quantified as described in (a and b). The results are the average ± S.E.M. of four to four independent experiments. (g) Representative MAP2 staining of megalin (+/−) TTR KO cultured hippocampal neurons stimulated with TTR. Megalin (j) and LRP1 (I) protein levels in TTR KO versus megalin heterozygous TTR KO hippocampal cultures were determined by western blot (n = 10 and 5, respectively). (m) Representative megalin, tau, MAP2, and Hoechst 33342 staining of TTR KO cultured hippocampal neurons (7 DIV) of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test performed for each condition or Student’s unpaired t-test for two-groups only comparisons. *P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant as compared with the control or as indicated. Scale bar in (b and g) correspond to 20 μm and in (m) to 10 μm.
mediates signaling cascades activated by TTR, we incubated TTR KO neurons with RAP, and observed that it blocked the phosphorylation of ERK (Figure 2g), Akt (Figure 2h) and CREB (Figure 2i). In the same manner, we stimulated megalin +/- TTR KO hippocampal neurons with TTR (55 μg/ml), and observed that TTR stimulation was unable to activate ERK (Figure 2j), Src (Figure 2l), Akt (Figure 2m) and CREB (Figure 2n). The same happened with intracellular
calcium, as detected by FRET (Figure 2o), in opposition to the megalin +/+ TTR KO littermate neurons (Figure 2p). Thus, these signaling pathways activated by TTR through megalin seem to be responsible for TTR neurite outgrowth.

**TTR transiently activates NMDA receptors through a megalin/Src mechanism.** As megalin has a Src homology domain (SH3), and Src constitutes a crucial point of convergence for signaling pathways, we reasoned that Src could be the bridging molecule between megalin and downstream signaling pathways. We performed FRET assay with the calcium probe in TTR KO neurons incubated with TTR in the presence of SKI, a Src inhibitor, and concluded that SKI blocked intracellular calcium rise induced by TTR (Figure 3a). Then, we hypothesized that NMDA could be responsible for this increase in calcium, because Src regulates NMDA receptor activity. Both kynurenic acid (Figure 3b), a broader AMPA/NMDA inhibitor, and MK801 (Figure 3c), a specific NMDA inhibitor, blocked the increase in intracellular calcium promoted by TTR. In addition, 10 μM NMDA stimuli promoted a similar increase in intracellular calcium as mouse TTR (300 μg/ml) stimulus (Figure 3d). To understand whether Src
was also governing TTR signaling pathways, we stimulated TTR KO cultured neurons with mouse TTR (55 μg/ml) and SKI, and observed that it blocked the activation of ERK (Figure 3e), Akt (Figure 3f) and CREB (Figure 3g). Altogether, we concluded that Src is the key regulator of the signaling pathways triggered by TTR/megalin.

**TTR KO hippocampal neurons are more sensitive to an excitotoxic insult than WT counterparts.** To address TTR neuroprotection, we subjected TTR KO and WT cultured hippocampal neurons to excitotoxic conditions, by a transient incubation with high glutamate concentration (125 μM, for 20 min), and further incubating the neurons in culture-conditioned medium for 14 h. These conditions induce 40–50% apoptotic cell death, resembling neuronal death in penumbra of a stroke. TTR KO neurons are more sensitive to this excitotoxic insult than WT neurons, showing less viable dendrites in MAP2 immunocytochemistry assays (Figure 4a) and western blot data (Figure 4b). Axons, observed with Tau staining, are affected to the same extent as dendrites, with TTR KO neuronal cultures showing higher axonal degeneration than WT neuronal cultures (Figures 4c and d). The same was shown in green fluorescent protein (GFP)-transfected WT and TTR KO hippocampal neurons subjected to an excitotoxic insult 48 h after transfection, with transfected TTR KO neurons showing a much lower number of GFP-stained neurites than transfected WT neurons (Figure 4e). To assess the effect of TTR in cell death, we analyzed nuclear morphology by Hoechst 33342, 14 h after the excitotoxic insult. TTR KO neuronal cultures showed significantly more dead neurons than WT counterparts (Figures 4f and g). TTR could prevent neurite degeneration induced by glutamate stimulation, when added (300 μg/ml) after the excitotoxic insult, observed by MAP2 immunostaining (Figure 4h), neurite quantification (Figure 4i) and MAP2 western blot (Figure 4j). Tau protein levels were not rescued (Figure 4j), indicating that TTR is mostly neuroprotective at dendrite level.

**TTR promotes neuronal survival, through megalin.** To investigate the interplay between TTR and megalin in CNS neuroprotection, we subjected megalin (+/−) TTR KO hippocampal neurons to an excitotoxic insult, and added TTR after the insult. We observed that TTR did not prevent dendrite degeneration induced by glutamate, both in dendrite number and total length (Figures 5a and b). To understand whether TTR also rescues neuronal survival with/without megalin, we pre-incubated TTR KO neurons with TTR (300 μg/ml), 6 h before excitotoxic stimulus, and observed a reduced cell death (Figures 5c and d). When added after glutamate, TTR was unable to protect neurons (Figure 5e). To understand whether megalin was involved in TTR neuroprotection at neuronal survival level, we performed the same experimental design using megalin (+/−) TTR KO neurons. We realized that neither TTR pre- or post-incubation, 300 or 55 μg/ml (data not shown), could protect to any extent cell survival (Figures 5f and g). Megalin (+/+ TTR KO littersmates show the same level of neuroprotection observed in TTR KO cultures (data not shown). We found that TTR was capable of activating phospho-CREB (Figure 5i), and probably Akt, with a tendency shown (Figure 5h), in excitotoxic conditions. As megalin was shown to be necessary for the neuroprotective action of TTR, we quantified megalin mRNA levels, after glutamate stimulation (4 h), in both TTR KO and WT cultures. Megalin mRNA was significantly upregulated in WT cultures (Figure 5j), in opposition to TTR KO cultures (Figure 5i). In WT neurons, there is the possibility of endogenous TTR being produced (although not consensual). We did not detect TTR protein by western blot neither in cell extracts nor in culture-conditioned medium (data not shown). However, we found that TTR mRNA is highly upregulated in these cultures after excitotoxic stimulus (4 h) (Figure 5m).

**TTR neuroprotection involves the activation of anti-apoptotic signaling pathways.** As CREB was activated when TTR was added, after excitotoxic conditions (Figure 5i), we investigated whether phospho-CREB activates anti-apoptotic genes, such as members of Bcl2 protein family. We stimulated TTR KO neurons with TTR (human, 55 μg/ml) and observed that Bcl2 (Figure 6a) and BclXL mRNA levels (Figure 6c) were highly upregulated. Bcl2 protein levels were also upregulated 60 min upon TTR stimulation (Figure 6b). Bax was not altered at the mRNA level (Figure 6d) or protein level (Figure 6e). In WT neurons, a similar pattern was seen, that is, upregulation of Bcl2 (Figure 6f) and BclXL mRNA.
levels (Figure 6g), and no change in Bax mRNA (Figure 6h). We expected the balance Bcl2/Bax levels governing cell death to be more relevant in excitotoxic conditions, for cell survival.\textsuperscript{33} Bcl2 mRNA levels were downregulated after glutamate stimulation in both WT (Figure 6i) and TTR KO cultures (Figure 6i). TTR pre-incubation before excitotoxic stimulus resulted in rescue of Bcl2 downregulation, in both TTR KO (Figure 6i) and WT cultures (Figure 6i). Regarding Bax mRNA levels, they did not change significantly after glutamate stimulation in both WT (Figure 6m) and TTR KO cultures (Figure 6j). Yet, when TTR was pre-incubated before the excitotoxic stimulus, Bax levels were significantly downregulated in the WT neuronal cultures (Figure 6m), and with a clear tendency for downregulation in TTR KO cultures (Figure 6j). Taken together, the results clearly indicate that TTR shifts the balance of the Bcl2 protein family members toward anti-apoptotic proteins resulting in less neuronal death.

Figure 3  TTR transiently activates NMDA receptors through a megalin/Src mechanism. (a) Time course of normalized FRET/Donor values in cell body of YC-Nano15-transfected TTR KO cultured hippocampal neurons (7 DIV) stimulated with mouse TTR (55 μg/ml) with or without Src inhibitor Ski (200 nM, 15 min pre-incubation) (for MsTTR, data are from Figure 2f and for MsTTR+SKI, three independent experiments, five to six neurons in each experiment). TTR administration was carried out at the ‘0 s’ line with dashes in the graph), but first time analyzed after stimulation is ‘0.33 s’. (b, c and d) Same experimental design as in (a) but using the NMDA/AMPA receptor inhibit kynurenic acid (b, 2 mM, 15 min pre-incubation) (for MsTTR, data are from Figure 2f and for MsTTR+Kyn acid, three independent experiments, six neurons in each experiment), or the NMDA inhibitor MK801 (c, 50 μM, 15 min pre-incubation) (for MsTTR, data are from Figure 2f and for MsTTR+MK801, three independent experiments, four to six neurons in each experiment) or stimulate neurons with mouse TTR (300 μg/ml) versus an NMDA (10 μM) stimulus (d, for MsTTR, data are from Figure 2f and for NMDA, one independent experiment, five neurons). To address the role of Src in TTR signaling activity, we used the Src inhibitor, SKI, and analyzed ERK (e, n = 3–17), Akt (Ser473) (f, n = 3–6) and CREB phosphorylation levels (Ser133) (g, n = 3–6) following stimulation of TTR KO cultured hippocampal neurons (7 DIV) with recombinant mouse TTR (55 μg/ml) with or without SKI (200 nM, 15 min pre-incubation). The results are the average ± S.E.M. of 3–17 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test performed for each condition as compared with the control or as indicated. ***P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant.
TTR neuroprotection in vivo (pMCAO) is also megalin-dependent. Having identified megalin as the receptor involved in the transduction of TTR neuroprotection in hippocampal neuronal cell culture, we investigated whether megalin was involved and/or affected in a mice stroke model—pMCAO. Wild-type TTR and TTR KO mice (both HSF+/− background) were subjected to ischemia by pMCAO and analyzed 24 h later. Megalin and TTR (only in WT TTR mice, data not shown) immunoreactivity was increased, in the infarct area only, of both WT TTR and TTR KO mice (Figures 7a and b). To clarify megalin upregulation in vivo, we quantified megalin mRNA and protein, in different brain areas of TTR KO and WT pMCAO mice. We found that WT mice upregulated megalin protein and mRNA in the infarct areas (IF P-IF) versus corresponding non-infarct areas (IF-Contra;P-IF Contra) (Figures 7c and d). In opposition, megalin protein and mRNA in TTR KO mice are unchanged between the corresponding brain areas (Figures 7c and d). RAGE and IGF-IR (also bind TTR) did not show any changes between WT and TTR KO mice (data not shown). Through immunohistochemistry, we found that cells that were upregulating megalin in the infarct area of the pMCAO WT mice were neurons, as they fully co-localize with βIII-tubulin (Figure 7e). Moreover, we unraveled, through TUNEL staining, that most of megalin-positive cells in WT TTR mice were alive (80%), whereas in KO TTR mice, only 50% survived (Figures 7f and g). Santos et al.37 also demonstrated that TTR-positive cells were neurons, as we confirmed in this work, but did not clarify whether neurons were alive or dead. Via immunohistochemistry, we observed that all the TTR-positive cells co-localize with megalin (Figure 7h). In addition, by triple staining TTR, TUNEL and Hoechst 33342, we found that most of TTR-positive neurons in WT TTR mice were alive (90%) (Figures 7i and j).

Discussion

In this study, we show that TTR has megalin-dependent neuritogenic activity in cultured hippocampal neurons, either from WT or TTR KO mice under physiologic conditions. More meaningful is the fact that TTR neuronal action is important in pathologic/excitotoxic conditions. TTR is neuroprotective both for neuronal survival and neurite preservation under excitotoxic conditions, through the activation of well-known neuroprotective megalin-dependent signaling pathways and transcription factors. In addition, we demonstrate that TTR neuritogenic activity is a focal cerebral ischemia model is also megalin-dependent.

The neuritogenic activity that we describe for TTR in CNS neurons (Figures 1a and b) has been observed previously in the PNS (dorsal root ganglia neurons),2,34 indicating a widespread action over neuronal populations. Moreover, we describe that TTR function in promoting neurite outgrowth is independent of its ligands, a similar effect observed by Fleming et al.34 in the PNS. Other studies described that TTR KO mice have spatial learning and memory deficits, in Morris water maze testing.35,36 TTR has also been associated with the maintenance of memory capacities during aging, because its hippocampal gene expression is downregulated in aged animals.35 So, this neuritogenic activity of TTR might be correlated with this role of TTR in memory and learning, as seen for other molecules.38 Although it can be due to other TTR pathways, a connection to be explored in the future. In agreement, TTR was found to be highly upregulated in the hippocampus of mice under an enriched environment housing compared with standard housing controls,39 indicating that it might be important to sustain enhanced memory function and recovery from lesion (enriched environment versus standard housing).

Megalin was shown to be important in the neuritogenic activity of other molecules such as metallothioneins40 and α2-macroglobulin (α2-M).41 We now demonstrate that TTR neuritogenic activity in cultured hippocampal neurons (CNS) is mediated by megalin, (Figures 2a-d), whereas LRP1 does not seem to be involved.

We found that TTR interaction with megalin activates the MAP kinase, ERK, Akt and Src, which ultimately lead to the upregulation of the CREB transcription factor (Figures 3a-d), as seen for other LRP ligands. TTR also contributes to a significant rise in intracellular calcium (Figure 5g) that might underlie the neurite outgrowth triggered by TTR. Similarly, α2-M was shown to promote neurite outgrowth in cortical neurons through ERK1/2, CREB and intracellular calcium changes,42 but in this case, LRP1 was involved.43 We show that the rise in intracellular calcium promoted by TTR occurs through NMDA receptors regulated by Src (Figure 5), allowing more calcium influx to neurons, probably through the phosphorylation of Nrf2A/2B subunits, increasing channel gating and the probability of the channel to be in the open state.28 A similar activation of NMDA receptors by other LRP receptors was described by Qiu et al.44 for α2-M. Moreover, the Src kinase family has been described as a crucial point of convergence for signaling pathways that subsequently enhance NMDA receptor activity.28 This can also be a neuroprotective strategy, because it has been already demonstrated in a global ischemia model that NR2A receptor activates ERK1/2-CREB signaling pathways, that ultimately enhance BDNF and Bcl2 expression, decreasing hippocampal CA1 neuronal death.42

Importantly, we demonstrated that TTR KO neurons are more sensitive to excitotoxic insults than WT counterparts, both at neuronal survival and neurite degeneration levels, and that TTR could partially rescue dendrites if added after the insult (Figure 6). This neurite neuroprotection will allow a higher trophic support by the neurotrophic factors (such as BDNF, NGF and TTR) that are exchanged between neuronal processes, allowing the neuronal network to survive and be more resistant to deleterious stimuli.43,44 In a similar manner, TTR was shown to be important in nerve regeneration, as TTR KO mice have dorsal root ganglia neuron neurite outgrowth impairment and decreased regeneration after nerve injury.34 Moreover, local delivery of TTR to the crushed nerves rescues the regeneration phenotype of TTR KO mice.2 Regarding cell survival, TTR was only able to partially protect neurons when added before, but not after, the excitotoxic insult (Figures 7b and c), indicating that the neuroprotective role of TTR in cell survival probably requires de novo protein synthesis. Altogether, neuroprotection by TTR acts in excitotoxic conditions of neuronal cultures and in pMCAO model in a megalin-dependent manner, involving the signaling pathways described above. A recent work also points for megalin as a...
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Materials and Methods

Animals. The number of mice handled for this research was approved by the Institutional and National General Veterinary Board Ethical Committees according to National and European Union rules. Three- to six-month-old TTR wild-type (+/+), TTR KO (−/−) and megalin (+/−) TTR KO mice in a 125/6s/jb background were used for the hippocampal neuronal cultures. Three to six-month-old TTR KO (−/−) and control littersmates under a HSF1 (+/−) background (125/6s/ju) were used for the pmCAO experiments. Megalin heterozygous mice were kindly provided by Dr. Thomas Willnow, Max-Delbrueck Center for Molecular Medicine, Berlin, Germany. The animals were maintained under a 12 h light/dark cycle in type II cages in specific pathogen-free conditions (microbiological health status available). Animals were fed with regular rodent chow and tap water ad libitum. Genotypes were determined from tail-extracted genomic DNA, using primers for the detection of exon 2 of TTR (which is disrupted in TTR −/−) by insertion of a neomycin resistance gene), megalin and neomycin as previously described. ARRIVE guidelines for reporting animal research were taken into consideration in the reporting of the experiments.

Recombinant TTR production and purification. Recombinant mouse and human TTR were produced in a bacterial expression system using Escherichia coli BL21 and purified as previously described. Briefly, after growing the bacteria, the protein was isolated and purified by preparative gel electrophoresis after ion-exchange chromatography. Protein concentration was determined using the Lowry method.

Recombinant GST-RAP production and purification. Recombinant GST-RAP production was previously described. Briefly, expression of the GST-RAP (pGEX-RAP) was induced by treating an Escherichia coli BL21 culture in the exponential phase of growth (A600 nm = 0.4–0.5) with 0.5 mM isopropyl β-D-thiogalactoside for 4–6 h at 30 °C. The protein, with an apparent molecular mass of 65KDa (GST (25 KDa)–RAP (39KDa)) in a SDS-PAGE gel, was extracted and purified from the bacterial pellet through affinity chromatography on glutathione Sepharose 4B (GE Healthcare, Little Chalfont, UK), according to the manufacturer’s recommendations.

Endotoxin removal. To remove endotoxins from recombinant TTR and GST-RAP, a polymer B column (Thermo Scientific, Waltham, MA, USA) was used. Briefly, the column was regenerated with 1% sodium deoxycholate (Sigma, St Louis, MO, USA) and washed with pyrogen-free buffer to remove detergent. Recombinant TTR was applied to the column and incubated during 1 h at room temperature. Aliquots of pyrogen-free buffer were added and the flow-through was collected. Protein concentration was determined using the Bradford method.

Primary hippocampal neuronal cultures. Primary cultures of mouse hippocampal neurons were prepared from the hippocampus of E17-E18 of WT, TTR KO and megalin (+/−) TTR KO mice embryos (125/6s/jb background), as previously described. Neuronal cultures were maintained in serum-free Neurobasal medium (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with B27 (Gibco, Life Technologies), glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml). Cells were kept at 37 °C in a humidified incubator with 5% CO2/95% air, for 24 h, for the neulre outgrowth experiments or 7 days for the western blot experiments, the time required for maturation of hippocampal neurons. Cells were cultured at a density of 90 000 cells/cm² or 80 000 cells/cm² on poly-D-

Figure 4 TTR KO hippocampal neurons are more sensitive to an excitotoxic insult, than WT counterparts. TTR KO and WT cultured hippocampal neurons (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium (14 h). MAP2 (b, n = 6) and Tau (d, n = 5) protein levels were determined by western blot at the indicated time points after excitotoxic stimulation. The results are the average ± S.E.M. of four to five independent experiments. Representative images of MAP2 (a) and Tau (e) are shown. GFP-transfected TTR KO and WT cultured hippocampal neurons (7 DIV) were also subjected to excitotoxic stimulation with glutamate. An immunocytochemistry against GFP was performed; the images are shown in (e), representative of four independent experiments. Cell survival was also assessed 14 h after the excitotoxic insult, using the fluorescent dye Hoechst 33342 (f, the representative image). WT n = 3, TTR KO n = 4). The results are the average ± S.E.M. of three to four independent experiments. (h and i) TTR KO cultured hippocampal neurons (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium with recombinant mouse TTR (300 μg/ml) for 14 h, and stained for MAP2 (h), Neurite number and neurite sum length were quantified using ImageJ software. The results are the average ± S.E.M. of four independent experiments. Using the same experimental design as in (h), MAP2 and Tau protein levels were also assessed by western blot, for the TTR KO cultured hippocampal neurons (j, n = 4). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test performed for each condition or Student’s unpaired t-test for two-groups only analysis. **P < 0.001, *P < 0.01, P < 0.05, n.s., not significant as compared with the control or as indicated. All the scale bars presented correspond to 50 μm.
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Test performed for each condition or Student's unpaired t-test for two-group comparisons. ***P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant as compared with the control or as indicated.

Figure 6  TTR neuroprotection involves the activation of anti-apoptotic signaling pathways. TTR KO cultured hippocampal neurons (7 DIV) were stimulated with recombinant mouse/human TTR (55 μg/ml) in culture-conditioned medium. Anti-apoptotic proteins levels were accessed. Bc2 mRNA levels (a, n = 5) and protein levels (b, n = 6-8) and BclXL mRNA levels (c, n = 4). The results are the average ± S.E.M. of four to eight independent experiments. Pro-apoptotic levels of Bax were also accessed by analysis of mRNA (d, n = 4) and protein levels (e, n = 5-7). The results are the average ± S.E.M. of four to seven in independent experiments. WT cultured hippocampal neurons (7 DIV) were also stimulated with recombinant mouse TTR (55 μg/ml) and the mRNA of Bcl2 protein family members were accessed: Bc2 (f, n = 4), BclXL (g, n = 3) and Bax (h, n = 4). To address TTR neuroprotective activity through the balance of anti-apoptotic signaling pathways, Bc2 and Bax mRNA levels were quantified in excitotoxic conditions with/without human TTR pre-incubation (55 μg/ml) in TTR KO cultured hippocampal neurons (7 DIV) (i, n = 4; j, n = 3) and WT cultured hippocampal neurons (7 DIV) (l, n = 3–5; m, n = 3–5). The results are the average ± S.E.M. of three to six independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test performed for each condition or Student’s unpaired t-test for two-group comparisons. ***P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant as compared with the control or as indicated.

Figure 5  TTR promotes neuronal survival, through a megalin-dependent manner. (a) Megalin (+/–) TTR KO cultured hippocampal neurons (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium with recombinant mouse TTR (300 μg/ml) for 14 h, and stained for MAP2. Representative images are shown (a) and neurite number and neurite sum length were quantified with ImageJ software (b). The results are the average ± S.E.M. of nine independent experiments. (c) TTR KO cultured hippocampal neurons (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min), with a 6 h pre-incubation with recombinant mouse TTR (300 μg/ml) in culture-conditioned medium. Cell survival was accessed 14 h after the excitotoxic insult, using the fluorescent dye Hoechst 33342 (c, representative images; d). The results are the average ± S.E.M. of five independent experiments. In (e), a similar experimental design as in (b) was used, but adding mouse TTR (300 μg/ml) after the excitotoxic stimulus. The results are the average ± S.E.M. of four independent experiments. (g) Using the same approach used as in (d) and (e), but using megalin (+/–) TTR KO cultured hippocampal neurons (7 DIV), mouse TTR (300 μg/ml) was added before and after the excitotoxic stimulus. The results are the average ± S.E.M. of five independent experiments. (f) To address TTR signaling activity after the excitotoxic stimulation, mouse TTR was added to TTR KO hippocampal cultures (7 DIV), 4 h after a glutamate stimulus (125 μM glutamate, 20 min) and Akt (Ser473) (h, n = 3) and CREB phosphorylation (Ser133) (i, n = 3–5) were analyzed by western blot at several time points (30 min, 60 min, 3 h). Megalin mRNA levels were also accessed after an excitotoxic insult (125 μM glutamate, 20 min) in WT cultured hippocampal neurons (7 DIV) (j, n = 5) and in TTR KO cultured hippocampal neurons (7 DIV) (l, n = 9). TTR mRNA levels were also analyzed after an excitotoxic insult (125 μM glutamate, 20 min) in WT cultured hippocampal neurons (7 DIV) (m, n = 4). The results are the average ± S.E.M. of four to five independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test performed for each condition as compared with the control or as indicated or Student’s unpaired t-test for two-groups only analysis. ***P < 0.001, **P < 0.01, *P < 0.05, n.s., not significant. All the scale bars presented correspond to 50 μm.
lysine-coated 6-well microplates (MW6) (for western blot and real-time PCR experiments) or glass coverslips (for immunochemistry studies), respectively. Megalin (+/-) TTR KO mice embryos were all genotyped (5 h express protocol) to separate the megalin (+/-) from the (+/+). Meanwhile, hippocampi were hibernated using Hibernate E medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with diluted 1:100 B27 (Gibco, Life Technologies) and kept at 4 °C. The hippocampal neurons were cultured using a serum-free medium (B27), which does not contain T4 or RBP – TTR ligands. The following pre-established exclusion criteria were used: cell cultures with high % of cell death (> 30%) and/or neurite network undevolved or damaged. For the SKI-I (Src kinase inhibitor I, Abcam (Cambridge, UK), ab120839, 200 nM) experiments, cells were pre-incubated with the inhibitor for 15 min and stimulated with TTR (55 μg/ml) in the presence or absence of the drug, for the time mentioned, at 37 °C. Regarding RAP (receptor-associated protein; LRP inhibitor, custom expressed, 350 μg/ml) experiments, cells were pre-incubated with the inhibitor for 30 min and then stimulated with TTR (55 μg/ml) in the absence or presence of the protein, for the time mentioned, at 37 °C.

Neurite outgrowth analysis. Cultured hippocampal neurons from WT, TTR KO or megalin (+/-) TTR KO mice embryos were isolated under the above-described conditions, plated on poly-D-lysine-coated glass coverslips at a density of 5 x 10^4 cells/cm^2. Recombinant mouse/human TTR (55 μg/ml and 300 μg/ml) was added to cell culture medium immediately after plating. Cells were maintained in culture during 24 h, approximately, in order to allow the precise tracing of all the neurites per neuron. Longer days in vitro do not allow precise neurite quantification per neuron. Neurite outgrowth assay was also performed in 7 DIV TTR KO and megalin (+/-) TTR KO cultures, after an excitotoxic stimulus, which allowed the neurite quantification. Cells were fixed with 4% paraformaldehyde and immunofluorescence was performed against MAP, (1:500, Abcam). The coverslips were mounted in a fluorescent mounting medium (DAKO, Glostrup, Denmark) and imaging was performed on a Zeiss AxioImager Z1 microscope, using a ×20 oil objective. Between 15 and 20 pictures were randomly taken throughout the coverslip, in a blinded manner, for each condition. For the experiments involving the RAP antibody, TTR was pre-incubated 1 h at 37 °C with the antibody, before being added to the cultures. Regarding RAP experiments, RAP (350 μg/ml) was added 30 min before TTR was added to the cultured neurons, after plating. Morphological measurements of neurite outgrowth were performed using the plugin NeuronJ from the ImageJ software. Number and sum length of neurites per cell were the analyzed parameters. At least 60–80 cells were counted for each experimental condition in a blinded manner, and the experiments were repeated in more than three independent preparations. The experimental unit in these assays was each individual culture (always performed with different breeding females in independent neuronal culture isolation procedures).

Cell death assay. Hippocampal neurons were cultured for 7 days on poly-D-lysine-coated glass coverslips as previously described. After the experiments, cells were fixed in 4% sucrose/4% paraformaldehyde (in PBS). The cells were washed twice with PBS and incubated with Hoechst 33342 (0.5 μg/ml) to stain nuclei. Analysis of the nuclear morphology was performed on Zeiss AxioImager Z1 microscope, using a ×20 oil objective. Live and dead cells were counted in a blinded manner, as performed for neurite outgrowth analysis, using ImageJ. The experimental unit in these assays was each individual culture (always performed with different breeding females in independent neuronal culture isolation procedures).

Western blot analysis. Cultured hippocampal neurons and dissected brain areas from pMCAO mice were homogenized in lysis buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100 and 1 x protease inhibitors mixture (GE Healthcare). Total protein concentration was determined using the Bradford method. Fifty micrograms of protein were applied and separated by 4%-10% Tris-Glycine SDS-PAGE (or 3%-7% Tris-Acetate polyacrylamide gels for megalin) and transferred to a nitrocellulose Hybond-C membrane (GE Healthcare), using a wet system, with Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA, USA). Membranes were blocked at least 1 h at room temperature in blocking buffer, 5% bovine serum albumin in phosphate-buffered saline Tween 20 (PBST), and then incubated overnight a 4 °C with primary antibodies diluted in blocking buffer, namely sheep megalin (1:1000; custom made), rabbit megalin (1:750, Abcam, ab129198), rabbit LRP1 (1:5000, Abcam, ab92544), rabbit Pax B (1:1000, Cell Signaling (Danvers, MA, USA, #2772), rabbit BicZ (1:1000, Cell Signaling, #2870), rabbit phospho-Akt (Ser473, 1:1000, Cell Signaling, #4060), rabbit Akt (1:1000, Cell Signaling, #9227), rabbit phospho-p44/p42 MAPK (1:1000, Cell Signaling, #9101), rabbit p44/p42 MAPK (1:1000, Cell Signaling, #9102), rabbit phospho-CREB (Ser133, 1:1000, Cell Signaling, #9198), rabbit CREB (1:1000, Cell Signaling, #9197), rabbit phospho-Src (Tyrl46, 1:1000, Cell Signaling, #6943), mouse Src (1:1000, Cell Signaling, #2110), rabbit anti-MAP2 (1:200; Abcam, ab24040), mouse anti-Tau (1:750, Cell Signaling, #4018), mouse anti-α-tubulin (1:1000 Sigma, T8203). Membranes were then incubated with anti-rabbit IgG-HRP (1:10 000, Binding Site, Birmingham, UK) and anti-mouse IgG-HRP (1:5000, Binding Site), for 1 h at room temperature. Blots were developed using Immun-Star WesternC Chemiluminescent kit (Bio-Rad) and exposed to Bio-Rad ChemiDoc XRS system or ECL Hyperfilm (GE Healthcare), if signal was too low. Quantitative analyses were performed using the Quantity One software or ImageLab from Bio-Rad Laboratories. The experimental unit in the western blot assays was each individual culture (always performed with different breeding females in independent neuronal culture isolation procedures).

mRNA semi-quantification through real-time PCR. Total RNA was extracted from either 7 DIV cultured hippocampal neurons or dissected brain areas from pMCAO mice using TRizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), as previously described.72 RNA quality and integrity was assessed using the Experion automated gel electrophoresis system (Bio-Rad, Hercules, CA, USA), as previously described.73 Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using NanoDrop 1000 (Thermo Scientific). The samples were aliquoted and stored at –80 °C until further use. cDNA synthesis was performed using 1 μg of total RNA and the SuperScript III-tubulin (1:10 000, Sigma, T8203). Samples were stored at –80 °C until further use. Primers used for real-time PCR were designed using 'Beacon Designer' software (Premier Biosoft International, CA).
palo Alto, CA, USA) as described previously. The oligonucleotides used for meganin real-time PCR were forward 5′-GGCTCCTACGAATCCTGCATTCC-3′ and reverse 5′-ACTCAAGGTGGCTCAGATTGC-3′; for Bcl2: forward 5′-TGTGGAG TACGTGACTACTT-3′ and reverse 5′-CAGAGACAGCAGGAGGAGG-3′; for BclXL: forward 5′-GGCCATTCTGATAGTACCAA-3′ and reverse 5′-GTCGAGTGTTAGTGGCAATGG-3′. 18S rRNA was used as reference gene with the following primers: forward 5′-AATCTGTTTGT GTTCCCTTGTTC-3′ and reverse 5′-GCTCTAGAAATTTCCAGATCA-3′. The annealing temperature was 60°C. For gene expression analysis, 1 μl of 1:10 diluted cDNA was added to 10 μl of 2× SYBR Green Master Mix (Bio-Rad) and the final concentration of each primer was 250 nM in 20 μl total volume. The thermocycling reaction was initiated by activation of Taq DNA Polymerase by heating at 95°C for 3 min, followed by 45 cycles of a 15 s denaturation step at 95°C and a 20 s annealing/elongation step at 60°C. The fluorescence was measured after the extension step, using the IQ2 Multicolor Real-Time PCR Detection System (Bio-Rad). The thermocycling reaction, the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence. Data analysis was performed using Pfaff method that adds the efficiency correction (amplification efficiencies of transcripts) to the ΔCP (between target gene and reference gene). Results were normalized with 18S RNA as internal reference gene, because it showed a stable expression in the conditions tested (compared with other reference genes tested). Efficiency correction was carried out for all the independent experiments, in all PCR plates. The experimental unit in pQPCR was each individual culture (always performed with different breeding females in independent neuronal culture isolation procedures).

Transfection. The expression vector containing the GFP gene was the pEGFP-N1 vector (BD Biosciences Clontech, San Jose, CA, USA). The plasmid sequence of pEGFP-N1 was verified by DNA sequencing reactions. Transfection of cultured hippocampal neurons with GFP and yellow cameleon-Nano15 was performed by the calcium phosphate co-precipitation method as previously described, with minor modifications. Briefly, 2 μg of plasmid DNA were diluted in Tri-EDTA (TE) pH 7.3 and mixed with HEPES calcium chloride pH 7.2 (2.5 M CaCl2, 10 mM HEPES). This DNA/TE/Calcium mix was added to 2× HEPES-Tris-buffer saline (TBS), and mixed with HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na2HPO4, 11 mM Dextrose, 42 mM HEPES), pH 7.2. The precipitates were allowed to form for 30 min, with vortex mixing every 5 min, to ensure that the precipitates had similar small sizes. Meanwhile, coverslips with cultured neurons were incubated with cultured conditioned medium with 2 mM of kynurenic acid. The precipitate was added drop wise to each coverslip and incubated at 37°C, 5% CO2, for 3 h. Cells were then washed with acidic (10% CO2) equilibrated culture medium containing 2 mM kynurenic acid and returned to the 37°C/5% CO2 incubator for 15 min. Finally, the medium was replaced with the initial culture-conditioned medium and the cells were incubated in a 37°C/5% CO2 incubator for 46 h to allow protein expression.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. Neurons were then incubated with 5% bovine serum albumin (Sigma) in PBS+0.1% Tween 20, for 1 h at 37°C, to block nonspecific binding, and incubated with primary antibodies, overnight at 4°C. Cells were then washed five times with PBS+0.1% Tween-0.5% bovine serum albumin and incubated with the appropriate secondary antibodies, for 1 h at 37°C. The coverslips were mounted in a fluorescent mounting medium (DAKO) and imaging was performed on a laser scanning Confocal Microscope Leica SP2 or SP5 AOS SE, using the ×40/×63 oil objective. In each set of experiments, the same batch of antibodies (primary and secondary) was used, and images were taken using the same settings, such as camera exposure times. For the t-test survival semi-quantification (Figures 9G and j), cells were counted, that is, either they expressed the indicated epitopes or not. Primary antibodies used were anti-mouse TTR (1:150, Rabbit, custom made), anti-megalin (1:1000, Sheep, custom made), anti-ﬁll-tubulin (1:500, monoclonal mouse, Promega, Madison, WI, USA, G712A), anti-BrdU (TUNEL Assay Kit, Abcam, ab66110); as secondary antibodies Alexa Fluor 488 and 568 (1:750, Life Technologies) were used. The fluorescent dye Hoechst 33342 (0.5 μg/ml–10 min room temperature) was used to stain nuclei.

FRET assay–intracellular calcium concentration. Cells (TRK cultured neurons, 80,000 cells/cm2) were imaged in poly-D-lysine-coated glass-bottom dishes (ibidi GmbH, Martinsried, Germany) 48 h after transfection with yellow cameleon-Nano15 (YC-Nano15), an ultrasensitive Ca2+ FRET probe. This FRET probe has high calcium affinity, enabling the detection of subtle calcium changes associated with intracellular signaling dynamics and neuronal activity, even at the in vitro culture level. Cells were selected for the assay upon two parameters only: transfected with yellow cameleon-Nano15 and the ones that look healthy, with intact neurites. The plasmid was obtained from Addgene (Cambridge, MA, USA) (plasmid #51961). Neuronal cultures were performed using a Neurobasal medium without phenol red (Gibco, Life Technologies) to optimize signal/noise levels. Recombinant TTR stimuli and chemical inhibitors were previously diluted in PBS and applied directly to the conditioned culture medium in the dish. Assays were performed under a humidified and heated chamber, for periods of 20 min. Fluorescence imaging of cells was performed using an epifluorescence inverted...
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microscope (DMI 6000B, Leica Microsystems) with a PlanApo x 63 (N.A. 1.4) glycerol immersion objective. Data acquisition and processing was based on Ferrez-Nogueira et al. Briefly, a mercury lamp coupled to a light attenuator (EL6000, Leica Microsystems) and a bandpass filter was used to excite directly the donor fluorescent protein. The cyan and yellow fluorescence signals were acquired using bandpass filters (480/40 nm and 530/30 nm for cyan and yellow fluorescence, respectively). The setup used a 440/520-nm dichroic mirror (CG1, Leica Microsystems) and the bandpass filters were mounted in external filter wheels (Fast Filter Wheels, Leica Microsystems). The FRET/donor change was calculated using ImageJ software. Customized macros were used to subtract the background from raw images and to create intensity-modulated ratio images. The time axis in the plots was adjusted to consider the TTR administration time as '0 s' (dashed line in the graphs), but first time analyzed after stimulation is '0.33 s'. Drugs and inhibitors used in this FRET assay were always pre-incubated for 15 min before TTR stimulation: SKI (200 mM), kynurenic acid (2 mM, Sigma) and MK801 (50 μM, Calbiochem, San Diego, CA, USA). NM1DA (10 μM, Sigma) was used to stimulate neurons, that is, NM1DA receptors. The experiments performed without calcium or with 60 mM of KCl used a Krebs-Ringer solution. The composition of the solution used in the experiment with KCl consisted of: 119 mM NaCl, 60 mM KCl, 1.0 mM NaH₂PO₄, 2.5 mM CaCl₂. 1.3 mM MgCl₂. 2.0 mM HEPES and 11 mM D-glucose, pH 7.4. The solution used for the experiment without calcium had the same composition with the exception of KCl, which was present at a smaller concentration (2.5 mM), and CaCl₂, which was replaced with 2 mM EGTA. The experimental unit in FRET assays was each individual neuron (four to six neurons were analyzed in each individual culture).

Statistical analysis. Protein values were normalized for α-tubulin or non-phosphorylated forms of the protein in study, whereas mRNA transcripts were normalized for 18 S mRNA. Data are presented as mean ± S.E.M. of at least three different experiments (exact number described in figure legends), performed in independent preparations. A previous power analysis was performed in order to obtain a 25% difference (with 10% S.D.) among two groups, with 90–95% power and were obtained samples sizes of three to six animals or individual cultures. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, when three groups were present:**P < 0.01, *P < 0.05, n.s. (not significant). Unpaired Student’s t-test was used when the comparisons were only between two groups:** *P < 0.01, *P < 0.05, n.s. (not significant).

Conflict of Interest

The authors declare no conflict of interest.

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1. Alshehri B, D’Souza DG, Lee JJ, Petrosas S, Richardson SJ. The diversity of mechanisms influenced by transferrin in neurobiology: development, disease and endocrine disruption. J Neuroendocrinol 2015: 30: 373–303.
2. Fleming CE, Mar FM, Franquinho F, Saraiva MJ, Sousa MM. Transferrin internalization by sensory neurons is megalin mediated and necessary for its neurotrophic activity. J Neurochem 2009; 29: 3220–3223.
3. Santos SD. Lambertsen KL, Claussnitz BH, Akinc A, Alvarez R, Finsen B et al. CSF transferrin neuroprotection in a mouse model of brain ischemia. J Neurochem 2010; 115: 1434–1444.
4. Gao C, Zhang B, Zhang W, Pu S, Yin J, Gao Q. Serum prealbumin (transferrin) predict good outcome in young patients with cerebral infarction. Clin Exp Med 2011; 11: 48–54.
5. Quinta T, Gonçalves I, Baltazar G, Alves CH, Saraiva MJ, Santos CR. 17Beta-oestradiol induces transferrin expression in murine choroid plexus via an oestrogen receptor dependent pathway. Cell Mol Neurobiol 2009; 29: 475–483.
6. Wang X, Liu W, Zhao D, Liu B, Shi Y, Chen B et al. Caenorhabditis elegans transferrin-like protein TTR-mediates recognition of apopotic cells by the CED-1 phagocyte receptor. Nat Cell Biol 2010; 12: 655–664.
7. Sousa MM, Saraiva MJ. Internalization of transferrin. Evidence of a novel yet unidentified receptor-associated protein (RAP)-sensitive receptor. J Biol Chem 2001; 276: 14420–14425.
8. Norden MM, Norden AG, Jacobson C, Willnow TE, Christensen BJ, Thakker RV et al. Evidence for the role of megalin in renal uptake of transferrin. J Biol Chem 2000; 275: 38176–38181.
9. Sousa MM, Yan SD, Stern D, Saraiva MJ. Interaction of the receptor for advanced glycation end products (RAGE) with transferrin triggers nuclear factor kB (NF-kB) activation. Biochem Invest 2000; 80: 1101–1110.
10. Vieira M, Gomes JR, Saraiva MJ. Transferrin induces insulin-like growth factor I nuclear translocation regulating its levels in the hippocampus. Mol Neurobiol 2015; 51: 1468–1479.
11. Alves-Bolero X, Perez-Gonzalez R, Spuch C, Vargas T, Antequera D, Garzon M et al. Megalin interacts with APP and the intracellular adapter protein F656 in neurons. Mol Cell Biol 2010; 30: 325–335.
12. Danto-Moreno, F, Farfan F. New insights into the roles of megalin/LRP2 and the regulation of its functional expression. Biol Rev 2011; 86: 89–105.
13. Cepriano A, Oliveira SM, Guido LF, Magahaesa A, Valenga G, Araque A et al. Transthyretin stabilization by iododiflunisal promotes amyloid-beta peptide clearance, decreases its deposition, and ameliorates cognitive deficits in an Alzheimer’s disease mouse model. J Alzheimers Dis 2014; 39: 357–370.
14. Berri R, Mapiel G, Foli C, Murrell JP, Liesjes JJ, Benson MD. The Ile-84-->Ser amino acid substitution in transthyretin interferes with the interaction with plasma retinol-binding protein. J Biol Chem 1994; 269: 23395–23398.
15. Rebec TF, Deuel FE, Benson MD. Reduced affinity for thyroxine in two of three structural thyroxine-binding prealbumin variants associated with familial amyloidotic polyneuropathy. J Clin Endocrinol Metab 1988; 63: 1432–1437.
16. Hezel J, Goldstein JL, Strickland DK, Ho YK, Brown MS, 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/α2-macroglobulin receptor. J Biol Chem 1991; 266: 21232–21238.
17. Kryukov D, Berezin V, Bock E. Regulators of neurite outgrowth: role of cell adhesion molecules. Ann N Y Acad Sci 2004: 1014–1045.
18. Shin Y, Mantuano E, Iacone G, Campana WM, Gonias SL. Lipid binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. Sci Signal 2009; 2: ra18.
19. Qiu Z, Hyman BT, Rebeck GW. Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. J Biol Chem 2004; 279: 34956–34965.
20. Ronn LC, Dissing S, Holm A, Berezin V, Bock E. Increased intracellular calcium is required for neurite outgrowth induced by a synthetic peptide ligand of NCAM. FEBS Lett 2002; 518: 60–66.
21. Horikawa K, Yamada Y, Matsuda T, Kobayashi K, Hashimoto M, Matsus-uns T et al. Spatiotemporal network activity via ultraviolet-C(2)-indicators, yellow Cameleon-Nano. Nat Methods 2010; 7: 729–732.
22. Saiter MW, Kalia LV. Src kinases: a hub for NFL receptor regulation. Nat Rev Neurol 2004; 1: 317–328.
23. Almeida RD, Marandas BJ, Melo CV, Gomes JR, Mendes CG, Gracie MM et al. Neurprotection by BDNF after glutamate-induced apopotic cell death is mediated by ERK and p38 kinase pathways. Cell Death Differ 2005; 12: 1329–1343.
24. Dwyer TA, Earl DE, Wang L. The utility of a new in vivo model of the stroke penumbra. J Neurosci 2008; 28: 6537–6538.
25. Li X, Masiak E, Reisach N, Busbaum JR. Neuronal production of transferrin in human and murine Alzheimer’s disease: is it protective? J Neurosci 2011; 31: 12483–12490.
26. Wilson BE, Mochon E, Bower LM. Induction of biclx expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 1996; 16: 5546–5556.
27. van der Heide LP, Smith MP. The BCL2 code to dopaminergic development and Parkinson’s disease. Trends Mol Med 2013; 19: 211–216.
28. Fleming CE, Saraiva MJ, Sousa MM. Transthyretin enhances nerve regeneration. J Neurochem 2007; 103: 831–839.
35. Sousa JC, Marques F, Dias-Ferreira E, Cerqueira JJ, Sousa N, Paiva JA. Transthyretin influences spatial reference memory. Neurobiol Learn Mem 2007; 88: 381–385.

36. Buxbaum JN, Roberts AJ, Adame A, Masliah E. Silencing of murine transthyretin and retinol binding protein genes has distinct and shared behavioral and neuropathologic effects. Neuroscience 2014; 275: 352–364.

37. Brouillette J, Quirion R. Transthyretin: a key gene involved in the maintenance of memory capacities during aging. Neurobiol Aging 2006; 28: 1721–1732.

38. He C, Qu X, Cui L, Wang J, Kang JX. Improved spatial learning performance of fat-1 mice is associated with enhanced neurogenesis and neurotogenesis by docosahexaenoic acid. Proc Natl Acad Sci USA 2009; 106: 11370–11375.

39. Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnics Z, Lee VM et al. Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. Cell 2006; 126: 701–713.

40. Fitzgerald M, Naín P, Bartlett CA, Chung RS, West AK, Beazley LD. Metallothionein-IIA promotes neurite growth via the megalin receptor. Exp Brain Res 2007; 183: 171–180.

41. Ou Z, Strickland DK, Hueman BT, Rebeck GW. a2-Macroglobulin exposure reduces pro-survival NMDA 2A receptor signaling mediates postconditioning neuroprotection in the hippocampus. Hippocampus 2015; 25: 286–296.

42. Altar CA, DiStefano PS. Neurotrophin trafficking by anterograde transport. Trends Neurosci 1998; 21: 433–437.

43. Muresanu DF, Buzoianu A, Florian SI, von Wild T. Towards a roadmap in brain protection material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/