

# Regulation of NMDA receptor trafficking by amyloid- $\beta$

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**Amyloid- $\beta$  peptide is elevated in the brains of patients with Alzheimer disease and is believed to be causative in the disease process. Amyloid- $\beta$  reduces glutamatergic transmission and inhibits synaptic plasticity, although the underlying mechanisms are unknown. We found that application of amyloid- $\beta$  promoted endocytosis of NMDA receptors in cortical neurons. In addition, neurons from a genetic mouse model of Alzheimer disease expressed reduced amounts of surface NMDA receptors. Reducing amyloid- $\beta$  by treating neurons with a  $\gamma$ -secretase inhibitor restored surface expression of NMDA receptors. Consistent with these data, amyloid- $\beta$  application produced a rapid and persistent depression of NMDA-evoked currents in cortical neurons. Amyloid- $\beta$ -dependent endocytosis of NMDA receptors required the  $\alpha$ -7 nicotinic receptor, protein phosphatase 2B (PP2B) and the tyrosine phosphatase STEP. Dephosphorylation of the NMDA receptor subunit NR2B at Tyr1472 correlated with receptor endocytosis. These data indicate a new mechanism by which amyloid- $\beta$  can cause synaptic dysfunction and contribute to Alzheimer disease pathology.**

Alzheimer disease is a progressive neurodegenerative disease in which patients have declarative memory impairments and increasingly severe dementia. Numerous pathological changes have been described in the postmortem brains of Alzheimer disease patients, including plaques, tangles, inflammation, neuron loss and synapse loss. Cortical and hippocampal synapse density is reduced early in the disease process, and the loss of these synapses correlates strongly with memory impairments<sup>1,2</sup>. The prevention and treatment of Alzheimer disease may therefore require a detailed understanding of how synapses are affected in the disease process.

In patients with Alzheimer disease, a 40- or 42-amino-acid peptide called amyloid- $\beta$  is elevated in the brain. The amount of amyloid- $\beta$  correlates with the onset and severity of memory impairments, consistent with an important role for this peptide in the disease process. This peptide, particularly the 42-amino-acid form (amyloid- $\beta$ <sub>1-42</sub>), is highly hydrophobic and accumulates both extracellularly in plaques and intracellularly in multivesicular bodies<sup>3</sup>. Soluble extracellular amyloid- $\beta$  may initiate memory impairments before the accumulation of plaques<sup>4</sup>.

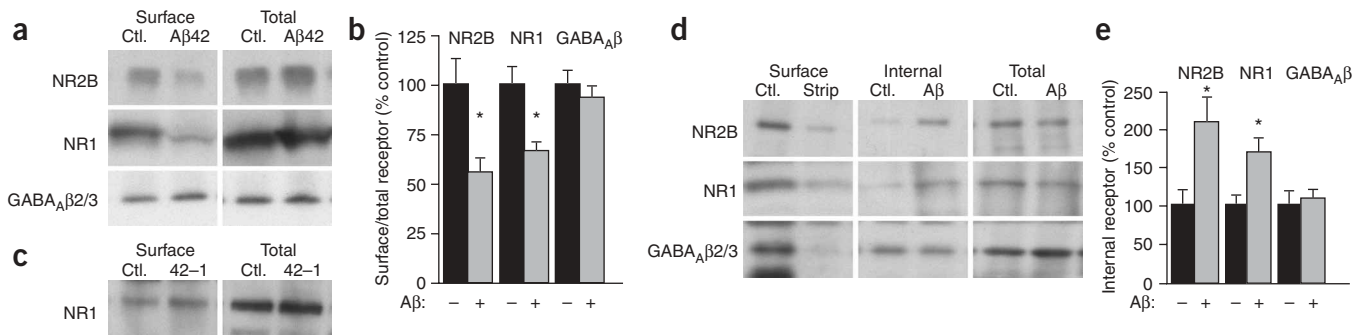
The pathogenic 40- and 42-amino-acid forms of amyloid- $\beta$  are produced by sequential cleavage of the amyloid precursor protein (APP) by enzymatic complexes known as  $\beta$ - and  $\gamma$ -secretases. In all familial forms of Alzheimer disease yet examined, mutations in the APP or in the presenilins, essential components of the  $\gamma$ -secretase, increase production and/or aggregation of amyloid- $\beta$  (ref. 5).

Transgenic mice that overexpress wild-type human APP or APP with a human familial mutation produce increased amyloid- $\beta$  and have alterations in synaptic transmission, plasticity and memory<sup>6,7</sup>. These

memory impairments and synaptic alterations precede neuronal death and plaque formation, indicating that synaptic alterations may be important events that contribute to the memory deficits associated with Alzheimer disease. Amyloid- $\beta$  can reduce long-term potentiation (LTP), a form of synaptic plasticity that is closely associated with learning and memory and can facilitate long-term depression (LTD), an opposing form of synaptic plasticity<sup>4,8</sup>. LTP and LTD involve postsynaptic phosphorylation and glutamate receptor trafficking<sup>9</sup>. The observation that amyloid- $\beta$  reduces LTP and facilitates LTD is suggestive of a role for amyloid- $\beta$  in regulating trafficking of glutamate receptors and postsynaptic phosphorylation, but this has not been previously examined.

In the current study, we investigated the mechanisms by which amyloid- $\beta$  affects signaling through NMDA-type glutamate receptors. Amyloid- $\beta$  deposits first occur in the entorhinal and frontal cortices of Alzheimer disease patients, and the amount of amyloid- $\beta$  in these regions correlates with memory loss<sup>10</sup>. Therefore, we examined the effects of amyloid- $\beta$  on cortical neurons. In cultured cortical neurons, amyloid- $\beta$  promoted endocytosis of NMDA receptors, without affecting trafficking of GABA<sub>A</sub>-type receptors. Application of amyloid- $\beta$  produced a persistent depression of NMDA-evoked currents and reduced signaling to the cAMP response element binding protein (CREB), a transcription factor required for long-term memory and neuronal survival. A reduction in surface-expressed NMDA receptors was also found in neuronal cultures prepared from mice bearing APP with the familial Swedish mutation, with no change in total receptor amounts. Together, these data indicate that amyloid- $\beta$  can regulate glutamatergic receptor endocytosis and that it does so in a genetic

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**Figure 1** Amyloid- $\beta_{1-42}$  promotes endocytosis of glutamate receptor subunits. **(a)** Neurons were treated with control medium (Ctl) or with medium containing amyloid- $\beta_{1-42}$  (A $\beta_{42}$ ) and then surface proteins were measured by biotinylation (see Methods). **(b)** Quantification of biotinylation immunoblots. NR2B:  $P < 0.03$ ,  $n = 4$ ; NR1:  $P < 0.03$ ,  $n = 7$ ; GABA $_A\beta_{2/3}$ :  $P > 0.1$ ,  $n = 5$ . Error bars indicate s.e.m. **(c)** Measurement of surface NR1 after treatment of cultured neurons with the reverse amyloid- $\beta$  peptide 42-1. **(d)** Internalization of surface receptors was measured using a cleavable biotin protocol in cortical cultures. Left panel shows that stripping removes biotin labeling of surface receptors NR2B, NR1 and GABA $_A\beta_{2/3}$ . Center panel shows the amount of internalized surface receptor protein under control conditions, which is measured by biotinylating surface receptors and stripping after 15 min at 37 °C in control medium or in medium containing 1  $\mu$ M amyloid- $\beta$ . Right panel shows a western blot of total receptor protein in control and amyloid- $\beta$ -treated cultures. **(e)** Quantification of immunoblots for receptors labeled with cleavable biotin. NR2B:  $P < 0.03$ ,  $n = 5$ ; NR1:  $P < 0.05$ ,  $n = 5$ .

model of Alzheimer disease. The effect of amyloid- $\beta$  on endocytosis of NMDA receptors is likely to contribute to synaptic dysfunction in Alzheimer disease.

## RESULTS

### Amyloid- $\beta$ decreases surface expression of NMDA receptors

Low amounts of secreted soluble amyloid- $\beta$  regulate glutamatergic currents mediated by both NMDA and AMPA receptors without affecting GABA receptor currents<sup>11</sup>. The effect of amyloid- $\beta$  on NMDA currents is of particular interest as this receptor regulates synapse density and memory formation.

NMDA receptors undergo regulated endocytosis<sup>12-15</sup>. To examine the effect of amyloid- $\beta$  on NMDA receptor endocytosis, cultured cortical neurons were treated with amyloid- $\beta$  (1  $\mu$ M for 1 h; **Supplementary Methods**), and cell surface receptors were quantified using biotinylation<sup>12,16</sup>. Amyloid- $\beta$  reduced the surface expression of the NMDA receptor subunit proteins NR2B and NR1 to  $55 \pm 8\%$  and  $64 \pm 6\%$  of control amounts, respectively; however, no change was observed in the total amount of NR2B or NR1 (**Fig. 1a,b**). As a control for the amyloid- $\beta_{1-42}$  peptide, we treated neurons with the reverse peptide 42-1 (1  $\mu$ M, 1 h) and found that it had no effect on the surface expression of NR1 ( $95 \pm 8\%$  of control; **Fig. 1c**).

NMDA and GABA $_A$  receptors are coexpressed on the cell surface of forebrain neurons but at different synapses<sup>17</sup>. Amyloid- $\beta_{1-42}$  did not affect the surface expression of GABA $_A\beta_{2/3}$  receptor subunits (amyloid- $\beta$ :  $93 \pm 7\%$  of control; **Fig. 1a,b**), indicating that amyloid- $\beta_{1-42}$  affected NMDA receptors but not all other ionotropic receptors.

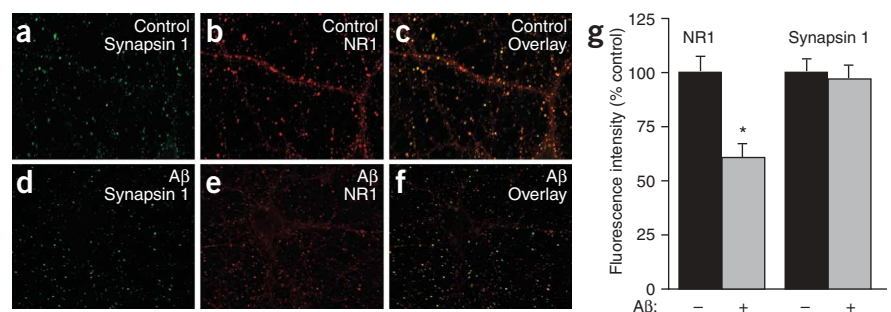
### Amyloid- $\beta_{1-42}$ promotes endocytosis of NMDA receptors

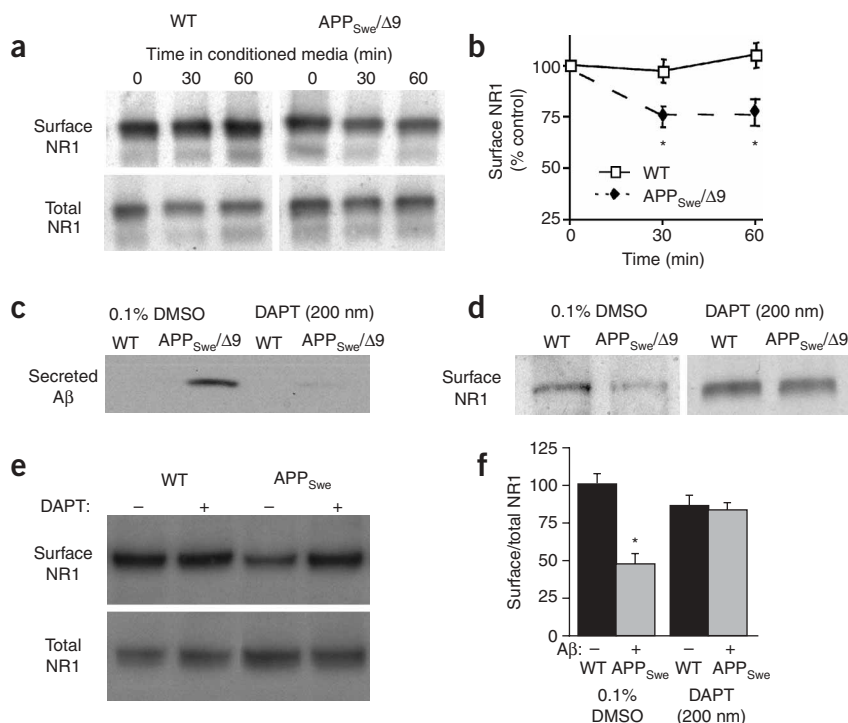
Amyloid- $\beta$  could decrease surface expression of NMDA receptors either by promoting endocytosis of receptor proteins or by preventing surface delivery. Endocytosis of NMDA receptors was examined in cultured cortical neurons using cleavable biotin<sup>18</sup>. Amyloid- $\beta$  promoted the endocytosis of biotin bound to the NMDA receptor subunits NR2B and NR1 (to  $209 \pm 31\%$  and  $171 \pm 16\%$  of control internal biotin amounts, respectively; **Fig. 1d,e**). Amyloid- $\beta$  did not, however, promote endocytosis of GABA $_A\beta_{2/3}$  receptors (**Fig. 1d,e**). These data indicate that amyloid- $\beta$  promotes endocytosis of NMDA receptors, without generally promoting endocytosis of other ionotropic neuronal receptors. These data indicate that APP processing regulates NMDA receptor surface expression.

### Amyloid- $\beta_{1-42}$ reduces synaptic NMDA receptors

We used immunocytochemistry to assay whether amyloid- $\beta$  can reduce the density of NMDA receptors at synapses. Cortical cultures were treated with control medium or with medium containing amyloid- $\beta_{1-42}$  (1  $\mu$ M) for 1 h and were then fixed, permeabilized and stained for the synaptic marker synapsin 1 and the NMDA receptor subunit NR1. NR1 staining showed a punctate distribution that localized with a majority of synapsin 1 in control neurons (**Fig. 2**). Treatment with amyloid- $\beta$  did not affect staining intensity for synapsin 1 but did significantly reduce the presence of NR1 at synaptic sites. In amyloid- $\beta$ -treated cultures, NR1 background staining appeared unchanged, and some puncta were observable; however, localization with synapsin 1 was greatly reduced. Quantification showed that although staining for

**Figure 2** Amyloid- $\beta$  (A $\beta$ ) reduces synaptic NMDA receptors. **(a-f)** Cultured cortical neurons were treated with control medium (**a-c**) or medium containing 1  $\mu$ M amyloid- $\beta$  (**d-f**). Neurons were immunostained for synapsin 1 (**a,d**) and NR1 (**b,e**). Overlays of stained neurons are shown in **c** and **f**. **(g)** Quantification of staining. NR1:  $P < 0.05$ ,  $n = 10$  cells, 150 synapses; synapsin 1:  $P > 0.2$ ,  $n = 150$  synapses in 10 cells. Error bars indicate s.e.m.





**Figure 3** Naturally secreted amyloid- $\beta$  reduces surface NMDA receptors. **(a)** Wild-type medium had no effect on surface NR1 (upper left) or total NR1 (lower left). APP<sub>Swe</sub>/ $\Delta$ 9 medium reduced surface NR1 (upper right) without affecting total NR1 (lower right). **(b)** Quantification shows that APP<sub>Swe</sub>/ $\Delta$ 9 medium reduced NR1 amounts to  $75 \pm 5\%$  within 30 min and  $77 \pm 7\%$  after 60 min. Error bars indicate s.e.m. **(c)** Analysis of amyloid- $\beta$  in conditioned medium from APP<sub>Swe</sub>/ $\Delta$ 9 cells in the presence of the  $\gamma$ -secretase inhibitor DAPT or 0.1% DMSO (vehicle). **(d)** Left: surface NR1 after 60 min in wild-type or APP<sub>Swe</sub>/ $\Delta$ 9 medium from cells grown in 0.1% DMSO. Right: surface NR1 after 60 min in wild-type or APP<sub>Swe</sub>/ $\Delta$ 9 medium from cells grown in the presence of 200 nM DAPT. **(e)** Primary cortical neurons were prepared from APP<sub>Swe</sub> mice or wild-type littermates. Surface expression of NR1 was reduced in neurons from APP<sub>Swe</sub> mice but was restored by DAPT. Total amount of NR1 was not affected by APP<sub>Swe</sub> expression or DAPT treatment (lower panel). **(f)** Quantification of treated cortical neurons.

synapsin remained at  $97 \pm 6\%$  of control amounts, amyloid- $\beta$  reduced staining for NR1 at synaptic sites to  $61 \pm 8\%$  (Fig. 2g). Given that we see no change in total NR1 with western blots, we believe these data reflect a redistribution of NMDA receptors away from synaptic sites and toward nonclustered extrasynaptic sites. Although we did not observe an increase in extrasynaptic staining for NMDA receptors, these observations are consistent with other published accounts of NMDA receptor redistribution<sup>19</sup>.

### Naturally-secreted amyloid- $\beta$ reduces surface NMDA receptors

Because of its hydrophobicity and predilection toward aggregation, synthetic amyloid- $\beta$  may induce effects that are different from those induced by naturally secreted amyloid- $\beta$ . We therefore treated cultured cortical neurons with medium from a neuronal cell line that secretes high amounts of amyloid- $\beta$ : N2A cells that stably express APP<sub>Swe</sub> and presenilin $\Delta$ 9 (APP<sub>Swe</sub>/ $\Delta$ 9; ref. 20). As a control, medium from untransfected wild-type N2A cells, which secrete little endogenous amyloid- $\beta$ , was used. Medium from untransfected N2A cells did not affect surface or total NR1 (Fig. 3a). In contrast, medium from APP<sub>Swe</sub>/ $\Delta$ 9 N2A cells reduced surface NR1 within 30 min but did not change the total amount of NR1 (Fig. 3a). Quantification showed a significant difference ( $P < 0.04$ ) in surface NR1 after a 30-min exposure to the amyloid- $\beta$ -containing cell medium (Fig. 3b). The decrease persisted through 60 min of treatment. Treatment of APP<sub>Swe</sub>/ $\Delta$ 9 N2A cells with the  $\gamma$ -secretase inhibitor DAPT (200 nM for 72 h) substantially reduced the amount of amyloid- $\beta$  (Fig. 3c). DAPT treatment completely blocked the ability of APP<sub>Swe</sub>/ $\Delta$ 9 medium to reduce surface expression of NR1 (APP<sub>Swe</sub>/ $\Delta$ 9 in DAPT:  $97 \pm 5\%$  of wild-type surface NR1; Fig. 3d). These data indicate that processing of APP is necessary for the effects of conditioned medium from APP<sub>Swe</sub>/ $\Delta$ 9-transfected N2A cells on surface expression of NMDA receptors.

### Reduced surface NR1 in neurons from APP<sub>Swe</sub> mice

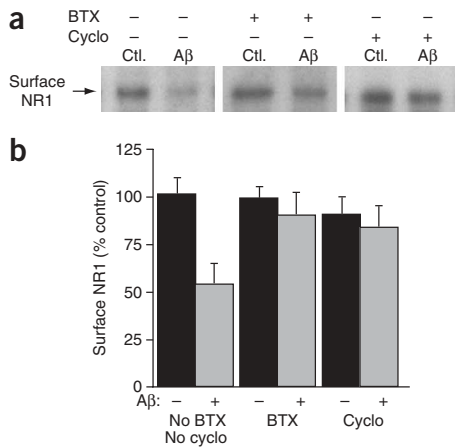
We next investigated whether surface expression of NR1 is reduced in a genetic model of Alzheimer disease. Cortical neurons were cultured

from mice that express human APP with the familial Swedish mutation. These mice have deficiencies in LTP *in vivo*, which correlate with reduced learning and memory<sup>6</sup>. Neurons from these mice survive in culture, extend processes and seem to be generally healthy for up to 3 weeks *in vitro*<sup>3</sup>. Using the biotinylation assay, we found that surface expression of NR1 was much lower in 12-day-old cultured neurons from APP<sub>Swe</sub> mice than in neurons from wild-type littermates, although total receptor protein was not affected (Fig. 3e). Surface expression of NR1 in neurons from APP<sub>Swe</sub> mice was  $46.2 \pm 8.8\%$  of that observed in wild-type littermates ( $n = 10$ ;  $P < 0.01$ ; Fig. 3f). Treatment of wild-type neurons with the  $\gamma$ -secretase inhibitor DAPT for 24 h slightly reduced surface NR1 (control,  $100 \pm 9.8\%$ ; DAPT,  $84.2 \pm 6\%$ ;  $n = 10$ ). Treatment of APP<sub>Swe</sub> neurons with DAPT restored surface NR1 ( $80 \pm 4.2\%$ ;  $n = 7$ ).

### Role of $\alpha$ -7 nicotinic receptor and protein phosphatase 2B

Amyloid- $\beta$  binds to the  $\alpha$ -7 nicotinic receptor with picomolar affinity<sup>21</sup> and promotes Ca<sup>2+</sup> influx in neurons<sup>22</sup>. In cortical neurons,  $\alpha$ -7 nicotinic receptors are expressed postsynaptically at over 70% of synapses, where they may regulate glutamate receptors<sup>23</sup>. Pretreatment with the  $\alpha$ -7 nicotinic receptor antagonist  $\alpha$ -bungarotoxin (BTX; 10  $\mu$ M) for 30 min before treatment with amyloid- $\beta$  partially inhibited the reduction in surface expression of NR1 (BTX,  $98 \pm 5\%$  of control; amyloid- $\beta$  + BTX,  $90 \pm 11\%$  of control; Fig. 4). A structurally distinct antagonist, methyllycaconitine (MLA; 1  $\mu$ M) also substantially inhibited the reduction in surface NR1 (MLA,  $90 \pm 6\%$  of control; amyloid- $\beta$  + MLA,  $87 \pm 8\%$  of control;  $n = 3$ ; see Supplementary Fig. 1 online). These data are consistent with a role for the  $\alpha$ -7 nicotinic receptor in mediating the effects of amyloid- $\beta$  on NMDA receptor endocytosis.

The  $\alpha$ -7 nicotinic receptor can activate PP2B, a Ca<sup>2+</sup>-sensitive enzyme that regulates NMDA receptor transmission and synaptic plasticity<sup>24,25</sup>. Inhibition of PP2B with cyclosporin (20  $\mu$ M) markedly reduced the effects of amyloid- $\beta$ <sub>1-42</sub> on surface expression of NR1 (cyclosporin,  $90 \pm 6\%$  of control; cyclosporin + amyloid- $\beta$ ,  $84 \pm 5\%$  of

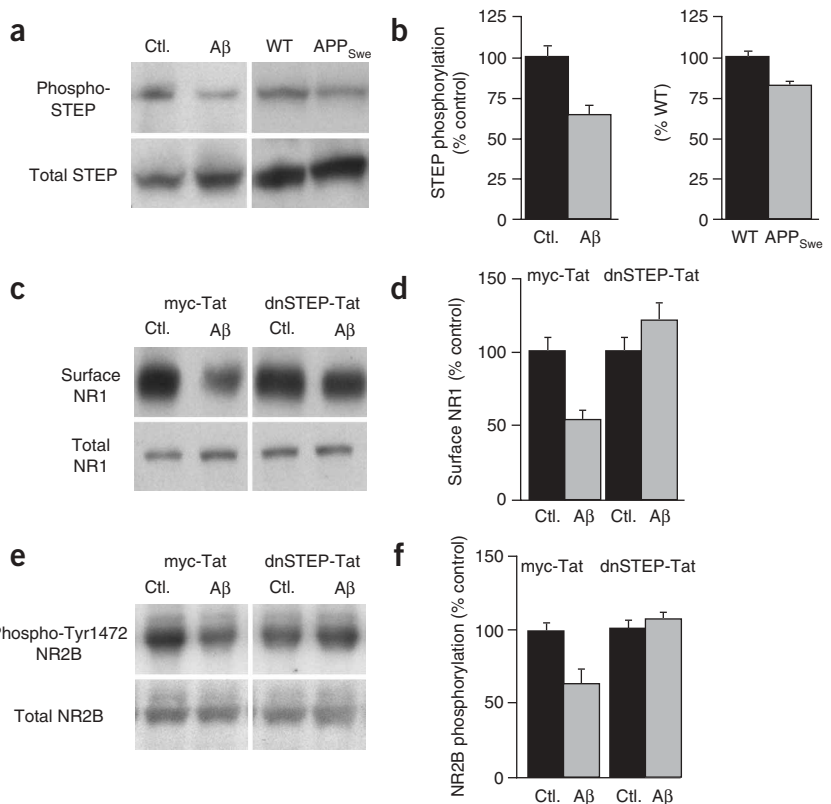


control; **Fig. 4**). Neither BTX or cyclosporin altered the total amount of NR1. These data indicate that PP2B activity is necessary for amyloid- $\beta$ -induced endocytosis of NMDA receptors.

### Amyloid- $\beta$ activates the tyrosine phosphatase STEP

Tyrosine phosphorylation regulates NMDA receptor function<sup>26</sup> and surface expression<sup>27</sup>. One tyrosine phosphatase that has been shown to regulate NMDA receptor function is the striatal-enriched phosphatase (STEP)<sup>28</sup>. STEP is expressed in brain regions that receive dopaminergic input including the striatum, hippocampus and cortex. STEP is alternatively spliced to produce a 61-kDa membrane-associated protein (STEP<sub>61</sub>) and a 46-kDa cytosolic fragment (STEP<sub>46</sub>). STEP<sub>61</sub> is present in postsynaptic densities and coimmunoprecipitates with the NMDA receptor complex<sup>28</sup>, although its specific binding partners within the complex remain unknown. STEP activity reduces NMDA currents and the induction of LTP<sup>28</sup>. Both STEP<sub>61</sub> and STEP<sub>46</sub> are phosphorylated by protein kinase A (PKA) at a common site (STEP<sub>61</sub> at Ser221 and STEP<sub>46</sub> at Ser49) within its substrate-binding domain. Phosphorylation of STEP at this site by PKA reduces the affinity of STEP for its substrates, whereas dephosphorylation by PP2B activates STEP<sup>29</sup>.

**Figure 5** Amyloid- $\beta$  promotes dephosphorylation of STEP, which controls glutamate receptor trafficking. **(a)** Left: neurons were treated with control medium or medium containing amyloid- $\beta$ , and the tyrosine phosphatase STEP was immunoprecipitated. Right: STEP protein was immunoprecipitated from the cortices of 10-month-old APP<sub>Swe</sub> mice or their wild-type littermates. **(b)** Amyloid- $\beta$  treatment reduced phosphorylation of STEP ( $n = 5$ ,  $P < 0.04$ ). In the brains of APP<sub>Swe</sub> mice, STEP phosphorylation was reduced ( $n = 4$ ,  $P < 0.05$ ). Error bars indicate s.e.m. **(c)** dnSTEP-TAT reduces the effect of amyloid- $\beta$  on NR1, whereas myc-Tat has no effect. **(d)** Quantification shows that in myc-Tat, amyloid- $\beta$  reduced surface NR1 to  $54 \pm 8\%$  of myc-Tat alone ( $n = 5$ ;  $P < 0.05$ ). **(e)** dnSTEP-Tat blocks the dephosphorylation of Tyr1472 that occurs in the presence of amyloid- $\beta$ . Total NR2B was not affected. **(f)** Quantification shows that amyloid- $\beta$  reduced phosphorylation of NR2B at Tyr1472 ( $n = 5$ ;  $P < 0.05$ ).

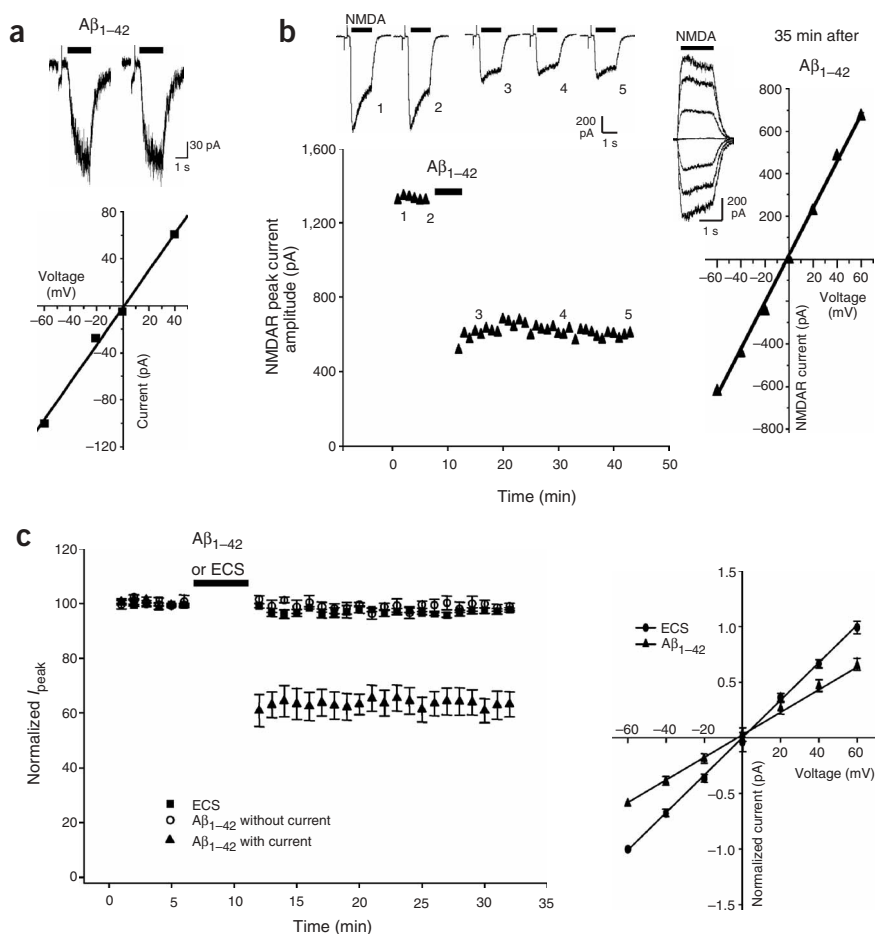


**Figure 4** Amyloid- $\beta$  reduces surface expression of NMDA receptors through  $\alpha$ -7 nicotinic receptors and PP2B. **(a)** Surface NR1 amounts were monitored in neuron cultures that were incubated in parallel in the absence or presence of BTX or cyclosporin for 30 min before amyloid- $\beta$  treatment ( $1 \mu\text{M}$  for 1 h). Inhibitors were included during the 1-h amyloid- $\beta$  treatment. **(b)** Quantification shows that amyloid- $\beta$  reduced surface NR1 to  $54 \pm 10\%$  of control amounts ( $n = 4$ ;  $P < 0.05$ ). BTX with control or amyloid- $\beta$  medium:  $n = 6$ ;  $P > 0.05$ ; cyclosporin with control or amyloid- $\beta$  medium:  $n = 4$ ;  $P > 0.05$ . Error bars indicate s.e.m.

We hypothesized that the amyloid- $\beta$ -induced activation of PP2B might lead to dephosphorylation and activation of STEP. Amyloid- $\beta$  treatment reduced phosphorylation of STEP to  $64 \pm 5\%$  of control amounts without affecting total amounts of STEP<sub>61</sub> (**Fig. 5a,b**). We measured the phosphorylation of STEP *in vivo* in the cerebral cortices of 10-month-old APP<sub>Swe</sub> mice. Phosphorylation of STEP was significantly lower in the brains of the APP<sub>Swe</sub> mice than in their wild-type littermates ( $83 \pm 3\%$  of wild-type amounts; **Fig. 5a,b**).

To study the role of STEP in NMDA receptor trafficking further, we used a dominant-negative fusion protein. We made a recombinant fusion protein of STEP<sub>46</sub> with two mutations: an inactive enzymatic domain (C300S) and a mutation to mimic dephosphorylation (S49A). These two mutations make the fusion protein a dominant negative, as it promotes substrate interaction but not dephosphorylation<sup>29</sup>. The fusion protein was coupled with the HIV-Tat peptide, which efficiently enters neurons (dnSTEP-Tat). We also coupled Tat to myc, to control for any effect of the Tat protein on its own (myc-Tat). A series of control experiments showed that the Tat fusion proteins were efficiently transduced into cells within 30 min when treated at 1–5  $\mu\text{M}$  (data not shown).

Pretreatment with dnSTEP-Tat ( $5 \mu\text{M}$  for 45 min) caused no significant change in surface NR1 ( $99.5 \pm 10\%$  of myc-Tat control)



**Figure 6** Amyloid- $\beta$  depresses NMDA receptor (NMDAR) currents. **(a)** Top: recordings from a cultured cortical neuron showing responses to two applications of amyloid- $\beta$ . Bottom: current-voltage ( $I$ - $V$ ) relationship for currents evoked by amyloid- $\beta$  from another cortical neuron. **(b)** Left: the NMDAR peak current amplitude recorded in a cultured cortical neuron is shown before and after treating the neuron with amyloid- $\beta$ . Traces above the graph illustrate responses to the test applications recorded from this cell; NMDA and glycine were applied during the period indicated by the bar above each trace. The traces were taken before and after treating with amyloid- $\beta$  peptide at the time points indicated by numbers, which correspond to those in the graph. Right: The  $I$ - $V$  relationship of the peak current 35 min after treatment with amyloid- $\beta$ . Traces above the  $I$ - $V$  curve illustrate superimposed responses to the test applications recorded at membrane potentials from  $-60$  mV (bottom) to  $+60$  mV (top) in increments of  $20$  mV. **(c)** Left: normalized NMDAR peak currents (mean  $\pm$  s.e.m.) are plotted before and after treatment with amyloid- $\beta$ . Peak currents were normalized to the average of first six responses. The cells were treated either with regular ECS (filled squares); with ECS plus amyloid- $\beta$  peptide, which did not induce an inward current (open circles), or with ECS plus amyloid- $\beta$  peptide, which did induce an inward current (filled triangles). Right: the normalized  $I$ - $V$  relationship of NMDA-evoked peak currents from cells  $20$  min after treatment with ECS only (filled circle;  $n = 3$  cells) or ECS plus amyloid- $\beta$  (filled triangles).

but blocked amyloid- $\beta$ -induced endocytosis of NR1 (**Fig. 5c,d**). In the presence of dnSTEP-Tat, amyloid- $\beta$  treatment slightly increased surface NR1 to  $121 \pm 12\%$  of control amounts ( $n = 5$ ; **Fig. 5c,d**); however, this increase was not significant as compared with treatment with dnSTEP-Tat alone ( $P > 0.16$ ). The Tat peptides did not alter total amounts of NR1 (**Fig. 5c**).

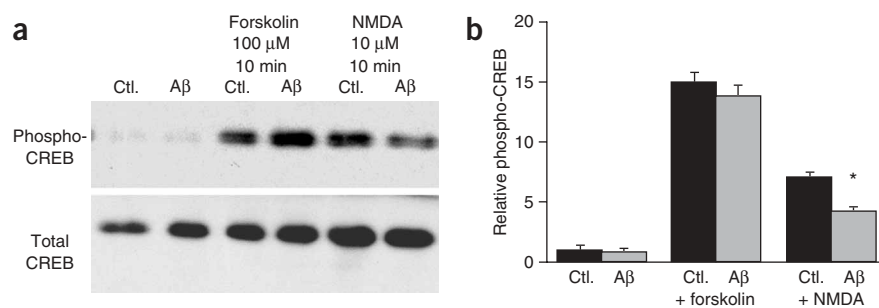
The NR2B subunit regulates NMDA trafficking and is phosphorylated at three tyrosine residues in its C terminus. One phosphotyrosine residue (Tyr1472) is in a region that regulates NMDA receptor endocytosis<sup>13</sup> and binding of the receptor to the synaptic scaffolding protein PSD-95 and the clathrin adaptor protein AP-2 (ref. 30). We hypothesized that the amyloid- $\beta$ -induced activation of STEP would promote dephosphorylation of Tyr1472. Amyloid- $\beta$  ( $1 \mu\text{M}$  for  $1$  h) promoted dephosphorylation of NR2B to  $62 \pm 7\%$  of control amounts (in the presence of myc-Tat; **Fig. 5e,f**). In the presence of dnSTEP-Tat, however, this dephosphorylation was blocked ( $107 \pm 11\%$  of control amounts;  $n = 5$ ). These data are consistent with the hypothesis that amyloid- $\beta$  promotes dephosphorylation and endocytosis of the NMDA receptor by activating STEP.

### Amyloid- $\beta$ rapidly depresses NMDA-evoked currents

Reducing the density of NMDA receptors at the cell surface is expected to reduce NMDA receptor-mediated currents as well. Accordingly, we carried out whole-cell patch-clamp recordings in pyramidal neurons. Amyloid- $\beta$  ( $1 \mu\text{M}$ ), when applied by rapid local perfusion, caused an inward current that developed over the first  $1$  s of the start of the application and ended within  $1$  s after the application ceased

(**Fig. 6a**). The reversal potential for this current was approximately  $0$  mV, indicating that, with the ionic composition of the recording solutions used, it was a nonselective cation current. The amplitude of the current recorded at  $-60$  mV ranged from  $40$  to  $800$  pA. This current was reliably evoked with repeated amyloid- $\beta$  application but was observed in fewer than half the neurons tested. These cells appeared morphologically similar to cells that responded to amyloid- $\beta$  and did not differ in holding current, input resistance or characteristics of NMDA-evoked responses (data not shown). It is not clear if the cells that had no response to amyloid- $\beta$  are indicative of a population of neurons that are resistant to amyloid- $\beta$  under these experimental conditions.

To investigate the effect of amyloid- $\beta$  on NMDA currents, we probed NMDA receptors with regularly timed test applications ( $3$ -s duration) of NMDA ( $50 \mu\text{M}$ ) and glycine ( $1 \mu\text{M}$ ). Under these conditions, currents evoked by NMDA are stable for more than  $45$  min<sup>14</sup>. We recorded test NMDA-evoked currents for at least  $10$  min to establish a stable baseline and then applied amyloid- $\beta$  ( $1 \mu\text{M}$ ) for  $5$  min; the test applications of NMDA were not made during the period when amyloid- $\beta$  was applied. In neurons where amyloid- $\beta$  evoked an inward current (which was sustained for the  $5$ -min application; data not shown), the amplitude of NMDA-evoked currents was decreased and remained decreased for the remainder of the recording period (up to  $30$  min; **Fig. 6b,c**). On average, the amplitude of the NMDA-evoked currents was reduced to  $63.1 \pm 4.6\%$  of the amplitude before amyloid- $\beta$  application ( $n = 8$  cells; **Fig. 6c**). By contrast, in cells where amyloid- $\beta$  did not evoke an inward current, we found that NMDA



**Figure 7** Amyloid- $\beta$  reduces NMDA-induced CREB phosphorylation. **(a)** Cultured neurons were treated with amyloid- $\beta$  for 1 h and then were treated with forskolin (100  $\mu$ M) or NMDA (10  $\mu$ M) for 10 min. Immediately after treatment, western blotting was performed for CREB phosphorylated at Ser133 (top) or for total CREB (bottom). **(b)** Both forskolin and NMDA induced CREB phosphorylation. Amyloid- $\beta$  did not affect basal CREB phosphorylation (which decreased slightly to  $0.94 \pm 0.1$  of control values;  $n = 5$ ;  $P > 0.2$ ). NMDA increased CREB phosphorylation in control cultures, but the phosphorylation was significantly reduced in amyloid- $\beta$ -treated cultures ( $n = 6$ ;  $P < 0.03$ ).

currents were not altered and remained at  $98.4 \pm 1.3\%$  of the baseline ( $n = 5$  cells; **Fig. 6c**). Likewise, after a 5-min control application of extracellular solution (ECS), NMDA-evoked currents remained at  $97.6 \pm 1.1\%$  of the baseline ( $n = 5$  cells; **Fig. 6c**). The depression of NMDA currents by amyloid- $\beta_{1-42}$  was not associated with a change in the reversal potential, which was near 0 mV after applying amyloid- $\beta_{1-42}$  or ECS (**Fig. 6c**). Thus, the whole-cell NMDA-evoked conductance, rather than the driving force, was decreased by amyloid- $\beta$ .

### Amyloid- $\beta$ reduces NMDA signaling to CREB

We examined whether amyloid- $\beta$  could influence NMDA receptor-mediated biochemical signaling. Stimulation of synaptic NMDA receptors promotes transcriptional activation through phosphorylation of CREB at Ser133 (ref. 31). Pharmacological or genetic inhibition of adenylyl cyclase or PKA significantly reduces NMDA-induced phosphorylation of CREB<sup>31</sup>. In addition, NMDA receptor activation can also lead to phosphorylation of CREB through direct activation of the RAS/MAP kinase pathway and/or CaMKIV<sup>31</sup>.

Amyloid- $\beta$  regulates activity-dependent CREB phosphorylation<sup>32</sup>. To test whether this effect is mediated by NMDA receptors, neurons that were cultured for 7–10 d were first treated with tetrodotoxin (1  $\mu$ M) for 2 h to reduce basal CREB phosphorylation and then for an additional 1 h with tetrodotoxin and either amyloid- $\beta_{1-42}$  (1  $\mu$ M) or control medium. To stimulate CREB phosphorylation, neurons were treated with NMDA (10  $\mu$ M) or the adenylyl cyclase activator forskolin (100  $\mu$ M) for 10 min. Treatment with amyloid- $\beta$  did not affect basal phosphorylation of CREB (**Fig. 7a**). Treatment with NMDA robustly increased phosphorylation of CREB, although this effect was reduced by amyloid- $\beta$  treatment (**Fig. 7a**). In control cultures, NMDA treatment increased CREB phosphorylation by a factor of  $7.1 \pm 0.39$  over basal amounts, whereas in amyloid- $\beta$ -treated cultures, NMDA treatment increased CREB phosphorylation by a factor of  $4.20 \pm 0.41$  over basal amounts (**Fig. 7b**). In control studies, forskolin increased CREB phosphorylation by a factor of  $14.9 \pm 1.1$  (**Fig. 7b**). After amyloid- $\beta$  treatment, forskolin increased CREB phosphorylation by a factor of  $13.7 \pm 1.2$  ( $n = 4$ ; **Fig. 7b**).

### DISCUSSION

Despite a wide consensus that amyloid- $\beta$  is a central causative factor in Alzheimer disease, it remains unclear how this peptide causes synaptic

dysfunction, synapse loss and neuronal death. The most notable result from the current study is that amyloid- $\beta$  is capable of regulating the surface expression and endocytosis of NMDA-type glutamate receptors. Our results are consistent with recent findings demonstrating that nontoxic amounts of secreted amyloid- $\beta$  reduce LTP and glutamatergic transmission<sup>11</sup>. Prolonged depression of NMDA receptor-mediated transmission may initiate the pathological changes observed in Alzheimer disease.

Synaptic NMDA receptor activation promotes the phosphorylation of CREB and transcription of target genes. These target genes include those involved in synapse formation, neuronal survival (BDNF<sup>33,34</sup>, BCL-2 (ref. 35)) and long-term memory formation (C/EBP)<sup>36</sup>. Decreased CREB phosphorylation

has been detected in the brains of Alzheimer disease patients<sup>37</sup>, indicating that amyloid- $\beta$  may reduce neuronal growth and survival through this transcription factor.

Amyloid- $\beta$  binds to several cell surface proteins and initiates numerous signaling cascades. We find that amyloid- $\beta$ -induced endocytosis of NMDA receptors involves the activation of the  $\alpha$ -7 nicotinic receptor and PP2B (**Supplementary Fig. 2**). These findings are consistent with previous data demonstrating that amyloid- $\beta$  can bind and activate  $\alpha$ -7 nicotinic receptors and that this class of receptors can reduce NMDA receptor signaling through PP2B<sup>24,25</sup>. Given that we did not see a complete blockade of NMDA receptor endocytosis with the nicotinic receptor antagonist BTX, there may be additional receptor mechanisms involved in this process.

PP2B promotes dephosphorylation of the tyrosine phosphatase STEP. PP2B may act directly on STEP as a substrate or through PP1 and inhibitor-1 or DARPP-32 (ref. 38). Either way, dephosphorylation of STEP via PP2B promotes its activity and interaction with substrates<sup>29</sup>. STEP gates NMDA receptor activation and LTP<sup>28</sup>. The present data suggest a mechanism by which this occurs and indicate that amyloid- $\beta$  promotes dephosphorylation and activation of STEP in cultured neurons and in the brains of 10-month-old APP<sub>Swe</sub> mice. In addition, STEP regulates amyloid- $\beta$ -induced endocytosis of NMDA receptors. These findings are consistent with a role for STEP in regulation of NMDA transmission.

Although it remains unclear which STEP substrates are essential in regulating receptor trafficking, our data indicate that STEP regulates NR2B phosphorylation. Phosphorylation of the NR2B subunit of the NMDA receptor at Tyr1472 is positively correlated with NMDA currents<sup>39</sup>. This tyrosine is of interest as it regulates binding of the NMDA receptor to PSD-95 as well as to the clathrin adaptor protein AP-2, and phosphorylation of this tyrosine may regulate interactions with endocytosis machinery<sup>30</sup>. Dephosphorylation of NR2B may trigger clathrin-coated endocytosis. Future studies should address the role of the clathrin-coated machinery in the pathology of Alzheimer disease.

Alzheimer disease involves multiple neurological changes ranging from plaques and tangles to reduced synapse and neuron density and inflammatory responses. Our data indicate that a critical signaling pathway allows amyloid- $\beta$  to regulate synaptic function and plasticity. Genetic or pharmacological inhibition of NMDA receptors reduces learning and memory in rodents, indicating that amyloid- $\beta$ -induced loss of surface NMDA receptors may markedly affect memory

and behavior. Further studies will be necessary to understand precisely how NMDA receptor endocytosis is regulated by amyloid- $\beta$  and if and how this contributes to dementia. In addition, it will be of great interest to understand how secreted amyloid- $\beta$  interacts with the amyloid- $\beta$  that accumulates intracellularly, proximally to synapses. Both secreted and intracellular amyloid- $\beta$  seem to affect synapses. Further exploration of the interaction of intracellular and extracellular amyloid- $\beta$  at synapses may lead to a better understanding of Alzheimer disease pathology and may promote new therapeutic approaches for the disease.

## METHODS

**Patch-clamp recordings.** Whole-cell recordings were made from cortical neuron cultures using procedures described in detail previously<sup>14</sup>. All experiments were done in accordance with guidelines on animal research of Rockefeller University, University of Toronto or Cornell University. Recordings were made at room temperature (20–22 °C). After attaining the whole-cell configuration, the recorded neurons were voltage clamped using an Axopatch 1-D amplifier to –60 mV, except where indicated otherwise. The neurons were continually perfused with electrophysiological saline (ECS), containing 140 mM NaCl, 1.3 mM CaCl<sub>2</sub>, 5 mM KCl, 25 mM HEPES, 33 mM glucose, 500 nM tetrodotoxin and 10  $\mu$ M bicuculline (pH 7.35, osmolarity 330 mOsm), by a computer-controlled, multibarreled fast perfusion system. NMDA receptor currents were induced by 50  $\mu$ M NMDA and 1  $\mu$ M glycine. Data were recorded, digitized with DigiData1200A, filtered (2 kHz) and acquired by the pClamp8.1 program. Recordings in which the series resistance varied by more than 10% were rejected. The intracellular solution contained 20 mM CsCl, 125 mM CsCH<sub>3</sub>SO<sub>3</sub>, 2 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 10 mM HEPES and 4 mM K<sub>2</sub>ATP, with pH 7.28 and 285 mOsm.

**Surface biotinylation assay.** After treatment, neurons were placed on ice and rinsed in cold PBS. Neurons were then incubated in PBS containing 1.5 mg/ml sulfo-NHS-LC-biotin (Pierce) for 20 min at 4 °C. Neurons were rinsed twice in PBS and then lysed in 200  $\mu$ l PBS with complete protease inhibitor cocktail (Roche) + 0.1% SDS + 1% Triton X-100. To determine the total protein concentration by immunoblotting, 10% of the cell lysate was removed and diluted in sample buffer. To isolate biotinylated proteins, 80% of the cell lysate was incubated with NeutrAvidin agarose (50  $\mu$ l; Pierce). Western blots were carried out and data were quantified by comparing the ratio of biotinylated to total protein for a given culture and normalizing to control untreated cultures, unless stated otherwise.

**Cleavable biotinylation assay.** Neurons were rinsed in PBS and then incubated with cold 1.5 mg/ml cleavable biotin reagent in PBS (EZ-Link Sulfo-NHS-SS biotin; Pierce) at 4 °C for 20 min. Cultures were then rinsed in warm medium and incubated at 37 °C in control medium or in medium containing amyloid- $\beta$  for 15 min. Immediately after treatment, neurons were placed on ice and rinsed in cold stripping buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% FBS, pH 8.5–9.0). Neurons were then lysed in 200  $\mu$ l PBS with complete protease inhibitor cocktail (Roche) + 0.1% SDS + 1% Triton X-100. Lysates were briefly sonicated and centrifuged to remove insoluble material. Biotinylated proteins were isolated by incubation with NeutrAvidin agarose (50  $\mu$ l; Pierce). Isolated proteins were rinsed in buffer three times and then boiled in 20  $\mu$ l sample buffer. Western blots were then carried out. Quantification was done by comparing the densitometric value for internalized biotinylated protein in the presence of amyloid- $\beta$  to the value in control cultures and normalizing to the value in control cultures.

**Generation and purification of myc-Tat and Tat-STEP46 S-A/C-S fusion protein.** STEP46 S-A/C-S was rendered cell permeable by inserting Tat (YGRKKRRQRRR), the cell membrane transduction domain of HIV type 1 (ref. 40), at the amino terminus using PCR. Tat-STEP46 S-A/C-S (dnSTEP-Tat) was then cloned in the bacterial expression vector pTrcHis2-TOPO (Invitrogen) and transformed into *Escherichia coli* BL21 for high expression of the fusion protein. The fusion protein was purified using TALON metal affinity resin (Clontech) according to manufacturer's protocol. The myc-Tat peptide was synthesized by the W.M. Keck Biotechnology Resource Center at Yale University.

Note: Supplementary information is available on the Nature Neuroscience website.

## ACKNOWLEDGMENTS

We thank M. Greenberg for the gift of phospho-NR2B antibody and G. Thinakaran and S. Sisodia for the gift of stably transfected N2A cells. We also thank J. Shepherd and members of the Greengard and Gouras labs for helpful discussions. This work was supported by the Fisher Foundation for Alzheimer's Research, US National Institutes of Health grant AG09464 (P.G.T. and G.K.G.) and National Institute of Mental Health grant 52711 and 01527 (P.J.L.).

## COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 18 April; accepted 22 June 2005

Published online at <http://www.nature.com/natureneuroscience/>

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