CHAPTER 4

Postsynaptic TrkB-Signaling Has Distinct Roles in Spine Maintenance in Adult Visual Cortex and Hippocampus


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Abstract
In adult primary visual cortex (V1), dendritic spines are more persistent than during development. Brain-derived neurotrophic factor (BDNF) increases synaptic strength and its levels rise during cortical development. We therefore asked whether postsynaptic BDNF-signaling through its receptor TrkB regulates spine persistence in adult V1. This question has been difficult to address as most methods utilized to alter TrkB-signaling in vivo affect cortical development or cannot distinguish between pre- and postsynaptic mechanisms. We circumvented these problems by employing transgenic mice expressing a dominant negative TrkB-EGFP fusion protein in sparse pyramidal neurons of the adult neocortex and hippocampus producing a Golgi-staining like pattern. In adult V1 this resulted in reduced mushroom spine maintenance and synaptic efficacy accompanied by an increase in long and thin spines and filopodia. In contrast, mushroom spine maintenance was unaffected in CA1, indicating that TrkB plays fundamentally different roles in structural plasticity in these brain areas.

Introduction
During development, synapse formation and elimination are regulated by molecular cues, spontaneous activity and experience (Hubel et al., 1977; Shatz and Stryker, 1978). Most glutamatergic synapses on excitatory neurons are situated on dendritic spines. Live imaging of neurons expressing GFP has provided important information on the dynamics of spine formation and maintenance (Fischer et al., 1998; Grutzendler et al., 2002; Holtmaat et al., 2005; Wu et al., 1995; Yuste and Bonhoeffer, 2004; Zuo et
Filopodia are short-lived finger-shaped protrusions and believed to be precursors of dendritic spines (Portera-Cailliau et al., 2003; Ziv and Smith, 1996). Newly formed spines are often thin or long and appear and disappear within days. Some mature into mushroom or stubby spines which are more stable and often persist for months (Holtmaat et al., 2005; Zuo et al., 2005a). There are strong correlations between spine size, spine persistence, synaptic efficacy and the number of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) at the postsynaptic density (PSD) (Matsuzaki et al., 2001; Nusser et al., 1998; Zuo et al., 2005a). With development and ageing of the cortex there is a shift towards larger and more persistent spine types (Grutzendler et al., 2002; Holtmaat et al., 2005; Trachtenberg et al., 2002).

Spine dynamics are influenced by plasticity. Long term potentiation in hippocampus is associated with an increase in spine size (Matsuzaki et al., 2004) and spine formation (Yuste and Bonhoeffer, 2001), while long term depression (LTD) is associated with spine elimination (Nagerl et al., 2004). Interestingly, reducing synaptic input results in an increase in spine numbers, probably due to homeostatic mechanisms (Kirov and Harris, 1999; Lohmann et al., 2005; Petrak et al., 2005).

Ocular dominance plasticity in V1 is associated with initial pruning and later formation and stabilization of spines (Mataga et al., 2004; Oray et al., 2004) and occurs predominantly during a critical period of development. Maturation of the extracellular matrix (ECM) is a major factor in ending the critical period, probably by increasing spine and axon stability (Mataga et al., 2004; Oray et al., 2004; Pizzorusso et al., 2002).
BDNF signaling through TrkB receptors is a key player in visual plasticity (Cabelli et al., 1995; Galuske et al., 1996). It drives the development of inhibitory innervation, an important factor in ocular dominance plasticity (Hanover et al., 1999; Huang et al., 1999). BDNF is also implicated in directly effecting structural (Genoud et al., 2004; Gorski et al., 2003; Horch et al., 1999) and functional changes (Akaneya et al., 1996; Castren et al., 1993; Kang and Schuman, 1995; Korte et al., 1995; Kovalchuk et al., 2002) in excitatory neurons. Several studies indicate that postsynaptic TrkB signaling stimulates the formation and maturation of spines (Murphy et al., 1998; Shimada et al., 1998). As BDNF expression rises in V1 upon eye opening and reaches maximal levels at early adulthood (Castren et al., 1992; Huang et al., 1999), increased TrkB-signaling may determine the increased spine persistence observed in adult V1. However, a recent study indicated that while postsynaptic TrkB signaling is essential for synapse formation in developing hippocampal neurons, it is dispensable for spine maintenance in adult CA1 (Luikart et al., 2005). Whether the same holds true for adult V1 is currently unknown.

To resolve this issue, we set out to analyze the roles of postsynaptic TrkB signaling in spine maintenance in adult V1 and CA1. This problem has been difficult to address as the various methods used for altering TrkB signaling in vivo also affect cortical development or do not allow dissection of pre- and postsynaptic mechanisms. We bypassed these problems by employing transgenic mice expressing an EGFP fusion protein of the truncated TrkB receptor (TrkB.T1-EGFP) which acts as a dominant negative receptor (Klein et al., 1990a) or a membrane-associated EGFP (EGFP-F) in sparse pyramidal neurons in the adult neocortex and hippocampus starting 6 weeks
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after birth. This Golgi-staining like expression pattern permitted us to study structural modifications in pyramidal neurons mediated by postsynaptic inhibition of TrkB signaling, without disrupting development of the cortical circuitry or ECM. We provide evidence that postsynaptic TrkB signaling is a key determinant of spine maintenance in adult V1, but has much less influence on spines in CA1.

Results

Expression of EGFP-F or TrkB.T1-EGFP in individual neurons of the adult brain

In order to express EGFP-F or TrkB.T1-EGFP in comparable, individual pyramidal neurons in the adult cortex and hippocampus, 3 transgenic mouse lines were created. The first line, Cre-3487, carried a Cre transgene under the control of the Calcium/Calmodulin dependent kinase IIα (CaMKIIα) promoter and defined the Golgi-staining like expression pattern. The other lines, TLG 498 and TLT 817, defined what protein was expressed in a Cre-dependent fashion, i.e. EGFP-F or TrkB.T1-EGFP respectively. In mice double transgenic for CaMKIIα-Cre and TrkB.T1-EGFP or EGFP-F, EGFP fluorescence was detected in isolated pyramidal neurons in layer II/III and V of the neocortex and pyramidal and granule cells of the hippocampus, starting around 6 weeks after birth and accumulating during the following weeks (Fig. 1A and B). Comparable expression patterns were observed in mice double transgenic for CaMKIIα-Cre and TrkB.T1-EGFP or EGFP-F although recombination was less efficient in TrkB.T1-EGFP mice. Representative sections of TrkB.T1-EGFP
expressing neurons in V1 and CA1 are shown in Figures 1C and D. To ensure that the observed mosaicism was mediated by Cre-3487 and was not inherent to the EGFP-F or TrkB.T1-EGFP transgenes we confirmed that when crossed to broad Cre expressing lines, TLG 498 and TLT 817 showed transgene expression in most pyramidal neurons (not shown).

Both TrkB.T1-EGFP and EGFP-F were detected in all compartments of the cell including the spines (Fig. 2A-D) and axons. Biocytin injections showed that all dendritic protrusions were labeled with EGFP-F (Fig 2E-G) or TrkB.T1-EGFP (Fig 2H-J). This excludes the possibility that spines of EGFP-F or TrkB.T1-EGFP expressing neurons appeared different due to variation in EGFP localization. Altogether, these transgenic mice were well suited for studying the different spine types in adult V1 and CA1 neurons.

**TrkB.T1-EGFP expression reduces mushroom spines and increases long and thin spines and filopodia in V1 but not in CA1**

To test if expression of TrkB.T1-EGFP had any effects on spine morphology in adult V1, pyramidal neurons in layer II/III of 8 week old transgenic mice were analyzed by confocal microscopy. At this age, the cells had transgene expression for up to 2 weeks. Dendritic protrusions on basal and proximal and distal apical dendrites were classified in different spine categories (mushroom, long, thin or stubby) or as filopodia and counted (Fig 7B). Compared to EGFP-F expressing neurons, mushroom and stubby spines of TrkB.T1-EGFP neurons were reduced by 60% and 85% respectively (p<0.0001; Fig. 3A). On the other hand, there were 2-3 times more long and thin spines (p<0.0001) and 22 times more filopodia (p<0.0001). The total density of protrusions was reduced by 24%
Protrusion changes in apical and basal dendritic segments were similar (data not shown).

These results suggested that from the onset of TrkB.T1-EGFP expression, the density of mushroom and stubby spines declined, while thin and long spines and filopodia increased. To verify this, we assessed the densities of different spine types before the onset of TrkB.T1-EGFP expression (6 weeks of age). Neurons in V1 from 5 week old wild type mice were labeled with DiI using diolistics. At this age, the critical period is just closing and spine morphologies are becoming similar to the adult situation. We detected a slightly higher density of mushroom spines than in adult EGFP-F expressing neurons (7.8%, p<0.05, Fig. 3A). In addition, we detected higher densities of long spines (2.4-fold, p<0.005) and filopodia (20-fold, P<0.0001) but a 40% lower density of stubby spines (P<0.005) than in adult EGFP-F expressing neurons. In most respects TrkB.T1-EGFP expressing neurons in adult V1 resembled pyramidal neurons in 5 week old V1 more than those in adult V1. However, even compared to neurons in younger V1, TrkB.T1-EGFP expressing cells showed a 65% reduction of mushroom and stubby spines (P<0.0001) and an increase in thin spines (2.3-fold, P<0.0001) and filopodia (1.7-fold, P<0.0001). The fact that the density of long spines on TrkB.T1-EGFP expressing neurons was similar to their density in pyramidal neurons in 5 week old V1 may indicate that reduced TrkB signaling inhibits their maturation into larger and shorter mushroom spines, but only dynamic studies can confirm this.
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Fig 1. Expression of TrkB.T1-EGFP in adult visual cortex and hippocampus (A) TrkB.T1-EGFP expression in primary visual cortex (V1) is mosaic and restricted to pyramidal neurons of layer II/III and V. (B) In hippocampus, TrkB.T1-EGFP expression occurs in pyramidal cells and granule cells. Inverted grayscale images of Cy3-fluorescence are shown. A higher magnification of TrkB.T1-EGFP pyramidal neurons in V1 (C) and CA1 (D) show expression in all cellular compartments. Scale bars – 100 \( \mu \)m (A-B) and 50 \( \mu \)m (C-D).

Fig 2. EGFP-F and TrkB.T1-EGFP label all dendritic protrusions. High magnification projections of confocal images of dendritic protrusions from V1 neurons expressing EGFP-F (A) and TrkB.T1-EGFP (B) show that distinct protrusions are labeled. Similar labeling was observed in EGFP-F (C) and TrkB.T1-EGFP (D) expressing neurons of CA1. Confocal sections of biocytin-filled EGFP-F expressing neurons stained for \( \alpha \)GFP (E) and \( \alpha \)Avidin-Cy3 (F) show that all protrusions are labeled with both GFP and Biocytin (G). Comparable images for TrkB.T1-EGFP are represented (H-J). Scale bar – 5 \( \mu \)m.
Fig 3. TrkB.T1-EGFP expressing neurons in V1, but not in CA1, have less mushroom and stubby spines and more filopodia. (A) TrkB.T1-EGFP expressing pyramidal cells in V1 show a 60-85% reduction in mushroom and stubby spines, a 2- to 3-fold increase in thin and long spines, and a 22-fold increase in filopodia. In comparison with 5-week old wildtype neurons labeled with DiI, TrkB.T1-EGFP expressing neurons have 65% less mushroom and stubby spines and more thin spines (2.3x) and filopodia (1.7x). (B) In CA1 pyramidal cells, stubby spines are reduced by 35%. No significant differences were found in other protrusions. Error bars represent SEM. *** (p<0.0001), ** (p<0.005), * (p<0.05). n=3417 spines for V1; 989 spines for CA1; 328 spines for 5-week old V1.

In CA1 pyramidal cells expressing TrkB.T1-EGFP or EGFP-F, no significant differences were seen in the densities of long (p=0.558), thin (p=0.57) or mushroom (P=0.065) spines or filopodia (p=0.6) (Fig. 3B). There was a moderate decrease of 35% in stubby spines (p<0.01) in
TrkB.T1-EGFP neurons. Together these results show that postsynaptic TrkB signaling is an important regulator of adult spine morphology in adult V1 but much less so in CA1.

**Reduced mushroom spine maintenance of TrkB.T1-EGFP expressing pyramidal cells in V1 but not in CA1**

Over 70% of all spines in adult V1 are persistent for periods of months (Holtmaat et al., 2005; Zuo et al., 2005a). Our observation that 60% of mushroom spines disappear within 2 weeks of TrkB.T1-EGFP expression therefore suggests that spine maintenance is affected rather than the development of new mushroom spines. As large mushroom spines are the most persistent (Trachtenberg et al., 2002), it is expected that spine loss due to natural turnover affects this population the least. However, the spine head diameter/length correlation plot (Fig 4A) shows that mushroom spine heads of TrkB.T1-EGFP expressing neurons are smaller than those of EGFP-F expressing cells with an almost complete loss of mushroom spines with head diameters over 0.8 μm. Mushroom spines of TrkB.T1-EGFP expressing neurons average a mean diameter of 0.49 μm compared to 0.78 μm of EGFP-F expressing neurons (p<0.0001, Fig. 4B). TrkB.T1-EGFP expressing spines are also 30% longer than EGFP-F expressing mushroom spines (p<0.0001) with an average length of 1.5 μm. In CA1, mushroom spines of TrkB.T1-EGFP expressing cells have an average head diameter of 0.7 μm and are similar to those of EGFP-F expressing cells (p=0.69, Fig. 4D). However, spine length increased by 32% resulting in a mean length of 1.32 μm (p<0.0001). This difference was mainly caused by increased spine...
length within the population of spines with heads <0.8 μm (38% increase, p<0.0001 compared to 14% increase in larger spines, p=0.068). Spine length distribution within this population was shifted upwards and spines longer than 1.5 μm increased from 2.5% in EGFP-F neurons to 35% in TrkB.T1-EGFP neurons (P<0.0001, KS-Z value=2.1) (Fig. 4C). Together, these data indicate that postsynaptic TrkB signaling in adult V1 pyramidal neurons is essential for the maintenance of mushroom spines whereas this does not seem to be true for CA1 neurons.

**Decreased mEPSC frequency and amplitude in TrkB.T1-EGFP expressing pyramidal cells**

Under normal circumstances there is a good correlation between spine head size and synaptic efficacy. To test if TrkB.T1-EGFP induced reductions in mushroom spine density and head size had a functional correlate, we measured mEPSCs in neurons expressing EGFP-F (n=4) and TrkB.T1-EGFP (n=4). Amplitude and frequency of mEPSCs were markedly different in EGFP-F and TrkB.T1-EGFP expressing neurons (Fig. 5). The average distribution of time intervals between events was shifted towards larger intervals in TrkB.T1-EGFP expressing neurons (Fig. 5C, P< 0.0001, KS-Z value=11.28), resulting in a median interval of 0.62 ± 0.11 s and 0.19 ± 0.09 s in TrkB.T1-EGFP and EGFP-F expressing cells respectively. The average distribution of amplitudes was shifted towards smaller amplitudes in the TrkB.T1-EGFP expressing cells (Fig. 5D, P<0.0001, KS-Z=7.44) resulting in a strong decrease in the number of events with amplitudes over
25 pA. Median amplitudes of mEPSCs in TrkB.T1-EGFP and EGFP-F expressing cells were 8.9 ± 0.03 pA and 10.95 ± 0.07 pA respectively. To exclude the possibility that the reduction of mEPSCs in TrkB.T1-EGFP expressing cells was due to an acute effect of reduced TrkB signaling, mEPSCs were measured in slices of wildtype C57BL/6 mice in the absence or presence of K252a, a cell permeable inhibitor of Trk phosphorylation (n=7). The Friedman test showed that application of K252a in wildtype mice had no significant effect on either frequency (P=0.565) or amplitude (P=0.779) (data not shown).

**Discussion**

We examined the role of postsynaptic TrkB signaling in spine maintenance by expressing a dominant negative TrkB.T1-EGFP fusion protein in sparse pyramidal neurons of the adult neocortex and hippocampus of transgenic mice. This approach had several advantages over previously used models for studying TrkB’s function in cortical pyramidal cells. First, it permitted us to study morphological changes caused by interfering with TrkB-signaling in individual pyramidal neurons within an unaffected environment. Second, since synaptic partners of transgene expressing neurons were genetically unaffected, pre- and post-synaptic influences of interfering with TrkB-signaling could be discerned. Third, as expression of TrkB.T1-EGFP was confined to the adult brain (>6 wks), neuronal developmental was unaffected.
Fig 4. TrkB.T1-EGFP expression influences spine length and head size in V1 (A) Correlation plot of spine head diameter and spine length shows that in V1, TrkB.T1-EGFP expression results in the loss of the largest mushroom spines and a shift towards longer spines with smaller heads. (B) The mean head diameter of mushroom spines on TrkB.T1-EGFP expressing neurons in V1 is reduced by 40% while their length is increased by 30% (C) In CA1, TrkB.T1-EGFP expression does not affect spine head size, but does result in increased numbers of spine length among spines with small heads (D) In CA1, mushroom spines of TrkB.T1-EGFP expressing neurons do not show a difference in the mean spine head diameter but are 32% longer. Error bars represent SEM. *** (p<0.0001).
We found that after 2 weeks of TrkB.T1-EGFP expression, spine morphology of pyramidal neurons in V1 had become reminiscent of neurons in the much younger visual cortex. Compared to EGFP-F expressing neurons in adult V1, mushroom and stubby spines were reduced by 60-85% while the numbers of filopodia and long and thin spines had increased strongly. Even compared to pyramidal neurons in V1 of younger mice (5 wks), filopodia and thin spine types were more abundant and mature spine types reduced in TrkB.T1-EGFP expressing neurons. These structural changes were accompanied by a reduction in both the amplitude and frequency of mEPSCs. The amplitude of mEPSCs is mainly a reflection of the number of AMPARs at the PSD, which in turn correlates with spine head size (Nusser et al., 1998). Large mushroom spines show strong AMPA currents, while thinner spines may carry “silent synapses” containing N-methyl-D-aspartate receptors but little or no AMPAR (Matsuzaki et al., 2001). In TrkB.T1-EGFP expressing neurons, mEPSCs with large amplitudes disappeared almost entirely and mEPSCs were smaller on the whole. This agreed well with the observed changes in mushroom head sizes (Fig. 4). The frequency of mEPSCs is influenced by the number of AMPAR-containing synapses and the efficiency of presynaptic vesicle release. In TrkB.T1-EGFP expressing cells, the decrease in mEPSC frequency was twice that of the total spine density change but correlated with the reduction in mushroom and stubby spines. This could imply that TrkB.T1-EGFP expressing neurons had more silent synapses, which would fit the observed increase in immature appearing spine types. However, we can not rule out that presynaptic vesicle release was indirectly affected by postsynaptic changes caused by TrkB.T1-EGFP expression.
Fig 5. Reduced mEPSC amplitude and frequency in V1 neurons of TrkB.T1-EGFP (A)
Two sets of typical recordings from EGFP-F and TrkB.T1-EGFP expressing neurons. (B)
Distribution of mEPSC amplitudes in two typical recordings in EGFP-F (left top) and TrkB.T1-
EGFP (right top) expressing neurons and their corresponding mEPSC frequencies (lower panels). (C)
Cumulative probability distribution of inter-event (mEPSC) interval in EGFP-F and TrkB.T1-
EGFP expressing neurons. The frequency in TrkB.T1-EGFP expressing neurons was significantly reduced (P<0.0001,
K-S Z=11.28). Median interval was 0.19 ± 0.09 s in EGFP-F and 0.62 ± 0.11 s in TrkB.T1-EGFP expressing neurons. (D) Cumulative probability distribution of mEPSC amplitude in EGFP-F and TrkB.T1-EGFP expressing neurons. The amplitude was significantly reduced in TrkB.T1-EGFP positive neurons (P<0.0001, K-S Z=7.44). Median amplitude was 10.95 ± 0.07 pA and 8.9 ± 0.3 pA in EGFP-F and TrkB.T1-EGFP positive neurons, respectively. Error bars represent SEM.
Previous experiments have shown that during barrel cortex development, BDNF signaling through postsynaptic TrkB receptors is essential for the insertion of AMPARs at the PSD (Itami et al., 2003) and the development of mature synapses. As new spines also form and mature in adult V1, it is possible that this process is affected by postsynaptic expression of TrkB.T1-EGFP. What would the morphological consequences be of such a defect? Studies employing in vivo 2 photon imaging have shown that in adult V1, over 70% of all spines are persistent (Grutzendler et al., 2002; Holtmaat et al., 2005). Large mushroom spines are the most stable population (Trachtenberg et al., 2002). If the transition of long or thin spines into mushroom spines were to be inhibited for a 2 week time period in adult mice, we expect to see a small reduction in the number of mushroom spines caused by natural turnover, with the population of large mushroom spines being least affected. In contrast, we found a strong decrease in the number of mushroom spines with the largest mushroom spines disappearing altogether in TrkB.T1-EGFP expressing neurons (Fig. 4A). Taken together, these findings indicate that reduced postsynaptic TrkB signaling results in reduced maintenance of large spines and a concomitant reduction in synaptic efficacy.

Recently it was shown that TrkB deficiency in pyramidal neurons of adult CA1 had little effect on their total protrusion density (Luikart et al., 2005). As this contrasted with our observations in V1, we also analyzed spine morphologies of TrkB.T1-EGFP expressing neurons in CA1. In agreement with the previous study, we observed no significant differences in spine densities of EGFP-F or TrkB.T1-EGFP expressing pyramidal cells in CA1. Also, most spine subtypes were unaffected except for a 35% decrease in
stubby spines (Fig. 3B). Head diameters of mushroom spines did not change suggesting that postsynaptic TrkB is not involved in their maintenance. We did observe that small mushroom spines were longer, which may be an indication of reduced transition of long spines into mushroom spines due to TrkB.T1-EGFP expression (Fig. 4C, D), but only dynamic imaging can confirm this. The different roles postsynaptic TrkB plays in the maintenance of spines in V1 and CA1 may well explain why long term TrkB deficiency results in the retraction of neurites and possibly degeneration of neurons in neocortex but not in hippocampus (Xu et al., 2000b).

We observed that TrkB.T1-EGFP expressing neurons in V1 had more long and thin spines and filopodia. The most intuitive explanation is that homeostatic mechanisms induced the formation of novel spines in an attempt to keep total synaptic input constant. A reduction in Ca$^{2+}$ signaling due to the loss of synaptic input could lie at the basis of such a homeostatic response (Lohmann et al., 2005). In addition, some filopodia and thin and long spines may be retracting mushroom spines (Zuo et al., 2005a). Last, an increase in filopodia formation by truncated TrkB expression has also been observed in cultured hippocampal neurons. This effect was independent of BDNF, but involved p75 neurotrophin receptor (p75NTR) (Hartmann et al., 2004). We can not exclude that the same process takes place in adult V1 in which case spine loss and the filopodia formation in TrkB.T1-EGFP expressing neurons would be unrelated events. However, the low levels of p75NTR expression in cortical pyramidal cells and the lack of filopodia formation in the TrkB.T1-EGFP expressing hippocampal neurons in which spine loss is much less prominent make this explanation less likely.
The results presented here define postsynaptic TrkB signals as an important determinant of synapse maintenance in V1. Therefore, the rise of BDNF levels in the developing visual cortex is likely to result in enhanced rigidity of its connections. The fact that TrkB.T1-EGFP expressing neurons showed extensive changes in spine morphology in adult V1 indicates that the mature ECM does not inhibit structural plasticity altogether but supports the view that the ECM and TrkB signaling have synergistic roles in synaptic stability and maintenance (Tropea et al., 2003).

Our observations also imply that in V1, BDNF is capable of bidirectionally regulating synapse strength with increased TrkB signaling resulting in synapse strengthening and decreased signaling resulting in synapse weakening. In this respect it is remarkable that BDNF expression is reduced by stimuli associated with dendritic pruning such as monocular deprivation (Lein and Shatz, 2000) and that its release is reduced by stimuli inducing LTD (Aicardi et al., 2004). Our finding that signals through TrkB are directly or indirectly involved at maintaining AMPARs at the PSD may explain the observation that BDNF interferes with the induction of LTD in the visual cortex (Akaneya et al., 1996) and that monocular deprivation results in reduced surface expression of AMPARs (Heynen et al., 2003). It is striking that the same mechanism is not functional at the Schaffer collateral synapse where mushroom spine maintenance is not affected by postsynaptic TrkB.T1-EGFP expression. A possible mechanistic explanation for this difference is that AMPARs are expressed at much higher levels in hippocampus than in neocortex (Petralia and Wenthold, 1992). This may make hippocampal neurons less sensitive to TrkB mediated differences in AMPAR expression (Brene et al., 2000) or trafficking (Jourdi
et al., 2003) and consequently, less prone to undergo structural changes. This difference seems compatible with the functions of synaptic plasticity in these areas. In V1, plasticity is mostly aimed at setting up and maintaining an efficient circuitry for the processing of visual input which may be achieved effectively through structural changes that are less rapid and more permanent. Faster and less permanent forms of plasticity seem more appropriate for the temporary storage and transfer of information as occurs in hippocampus.

In order to comprehend the underlying biological mechanisms, it is imperative to determine if TrkB signaling regulates synaptic stability at individual synapses or at the cellular level. It is also important to identify the site of BDNF release and to discern its autocrine and paracrine roles. Inactivation of the \textit{bdnf} gene in isolated neurons and analyzing their spine and bouton morphology seems to be an appropriate approach for answering these questions.

\textbf{Materials and Methods}

\textbf{DNA constructs and production of transgenic mice.} Constructs for Cre-dependent expression of TrkB.T1-EGFP or EGFP-F were created as follows: the lox-Stop-lox (LSL) cassette from PBS302 (Gibco-BRL, Bethesda, MD) was cloned into a Thy-1 promoter containing expression vector, rendering pThy-LSL. cDNA encoding AAs 1-477 of TrkB and encompassing a 75 nt fragment of the 5’ untranslated region was cloned into
the polylinker of EGFP-N3 (BD Biosciences, Mountain View, CA). The fragment encoding TrkB.T1-EGFP was cloned into pThy-LSL rendering pThyLSL-TrkB.T1-EGFP. pThyLSL-EGFP-F was created by cloning the fragment encoding EGFP fused to the Ha-ras farnesylation site from pEGFP-F (BD Biosciences) into pThy-LSL. For production of transgenic mice expressing Cre under the control of the CaMKIIα promoter, pJTCre (Tsien et al., 1996) was employed (Fig 6).

Transgenic mice were created by pronuclear injections of linearized DNA into fertilized C57BL/6 oocytes. One of the 7 ThyLSL-TrkB.T1-EGFP founders (TLT-817) showed sufficiently high EGFP levels for spine analysis. Nine ThyLSL-EGFP-F founders were obtained of which 3 showed sufficient EGFP expression. Line TLG-498 was used in this study. Sparse labeling of pyramidal neurons in adult cortex was achieved by generating novel CaMKIIα-Cre transgenic mice. Four CaMKIIα-Cre founders were produced of which 2 showed Cre recombination in a Golgi-staining like pattern. Line Cre-3487 is described here. All experiments involving mice were approved by the institutional animal care and use committee of the Royal Netherlands Academy of Arts and Sciences.
Mice transgenic for Cre under the control of the calcium/calmodulin-dependent kinase IIα (CaMKIIα) promoter are crossed with mice that carry a transgene in which a STOP cassette flanked by loxP sites is inserted between the Thy-1 promoter and the coding sequence for TrkB.T1-EGFP. In double transgenic offspring, mosaic Cre recombination results in the excision of the STOP cassette, resulting in Thy-1-driven TrkB.T1-EGFP expression. Mice expressing membrane-associated EGFP (EGFP-F) were similarly generated and used as controls in the study.

Fig 6. Schematic for mediating Cre-dependent TrkB.T1-EGFP expression in transgenic mice. Mice transgenic for Cre under the control of the calcium/calmodulin-dependent kinase IIα (CaMKIIα) promoter are crossed with mice that carry a transgene in which a STOP cassette flanked by loxP sites is inserted between the Thy-1 promoter and the coding sequence for TrkB.T1-EGFP. In double transgenic offspring, mosaic Cre recombination results in the excision of the STOP cassette, resulting in Thy-1-driven TrkB.T1-EGFP expression. Mice expressing membrane-associated EGFP (EGFP-F) were similarly generated and used as controls in the study.

Fig 7. Classification of dendritic protrusions and schematic of spine counts. (A) Layer II/III pyramidal neurons from V1 were used to count dendritic protrusions in 15-μm segments after the first branch points of distal apical (area 1), proximal apical (area 2), and basal (area 3) dendrites. An autofluorescence image of a TrkB.T1-EGFP-expressing pyramidal neuron in layer II/III of V1 is shown here. (B) Dendritic protrusions were classified as spines and filopodia. Spines were further categorized as mushroom, long, stubby, and thin, depending on the presence of a distinct head and spine length.
Histology and immunohistochemistry. Eight week old mice double transgenic for CaMKIIα-Cre and TrkB.T1-EGFP or EGFP-F were anaesthetized with 0.1 ml/gr bodyweight Nembutal (Janssen Laboratories, Beerse, Belgium) and perfused with 4% paraformaldehyde (PFA) in PBS and postfixed for 2 hrs. Coronal sections of 50 μm were made using a vibratome (Leica VT1000S, Leica, Rijswijk, Netherlands). To allow long-term storage and reduce bleaching, free-floating sections were stained using mouse anti-GFP antibodies (1:500, Chemicon, Temecula, CA) followed by Alexa568 conjugated goat anti-mouse antibodies (1:500, Invitrogen, Breda, Netherlands). Biocytin injected slices (300 μm) were postfixed in 4% PFA and stained with chicken anti-GFP antibodies (1:1000, Chemicon) followed by Alexa488 conjugated anti-chicken antibodies (1:500, Invitrogen) and Cy3 conjugated streptavidin (1:500, Vector Laboratories) for the detection of biocytin.

Diolistics. Diolistics were essentially performed as described previously (Benediktsson et al., 2005). Briefly, 0.15 mg DiI (Invitrogen) was mixed with 50μl methylene chloride and vortexed until completely dissolved. The dissolved dye was added to 12 mg of 1.1μm tungsten particles (Bio-Rad, Veenendaal, Netherlands) on a glass slide. This mixture was spread across the slide and the solvent allowed to evaporate for 2 min. The coated particles were transferred to a 1.5 ml tube, resuspended in 1ml distilled water and sonicated. The suspension was sucked into Tefzel tubing (Bio-Rad) with a syringe and the particles allowed to settle for 2 minutes. The
water was then withdrawn and the tube dried with a flow of nitrogen gas and cut into 13mm pieces.

50μm coronal sections were shot using the Helios Gene Gun (Bio-Rad) at 80 psi through a membrane filter with a 3μm pore size and 8 x 10^5 pores/cm^2 (Corning, Acton, MA). Sections were left for at least 12 hours to ensure good filling of the labeled neurons.

**Confocal microscopy.** EGFP expressing, or DiI or biocytin labeled neurons from layer II/III of V1 or from CA1 were imaged using a Carl Zeiss CLSM 510 Meta confocal microscope (Zeiss, Goettingen, Germany) with Argon (488 nm) and HeNe (543 nm) lasers. The first branch points of basal, proximal apical and distal apical dendrites (Fig. 7A) were imaged at a scaling of 60 nm (63X objective and an optical zoom of 2.5X) with 200 nm steps in the z-plane. The back-projected pinhole was 190 nm. For each image acquisition, the laser intensity and detector gain were adjusted so that the entire detector range was used for the spines. The image stacks were subjected to 3D reconstruction using Zeiss CLSM 510 Meta. At these settings, spine morphology was not different from those that were subjected to blind deconvolution (Huygens Essential, Hilversum, Netherlands).

**Spine classification and image analysis.** Dendritic protrusions were classified as spines (mushroom, long, stubby or thin) or filopodia and quantified as number of protrusions per 15 μm segment (Fig. 7B). Spine analysis was performed on V1 neurons expressing EGFP-F (5 mice, 19 neurons, 80 segments, 1447 spines) and TrkB.T1-EGFP (5 mice, 25
neurons, 141 segments, 1970 spines) and on DiI labeled neurons (2 mice, 10 neurons, 20 distal apical segments, 328 spines). For CA1 neurons, protrusions from basal and distal apical dendrites of neurons expressing EGFP-F (3 mice, 11 neurons, 22 segments, 454 spines) and TrkB.T1-EGFP (4 mice, 8 neurons, 29 segments, 535 spines) were quantified likewise.

Size determination of mushroom spines was carried out using Zeiss CLSM Image browser 5 overlay tools. The longest straight line in the spine head was counted as the head diameter. The length of the entire spine (including head and stalk) was measured using a bent-line tool. Spine size/length correlation plots were performed on mushroom spines from distal apical dendrites of neurons expressing EGFP-F (V1: 116 spines, CA1: 107) and TrkB.T1-EGFP-F (V1: 195 spines, CA1: 47). Statistical significance of differences in spine numbers per segment, spine length or head size was determined by standard student’s t-test. Significance of differences in spine length distributions of CA1 mushroom spines was determined by the Kolmogorov-Smirnov (KS) test.

**Electrophysiology.** Coronal slices (300 μm) of V1 were prepared from 8-10 week old mice. Animals were killed by decapitation and brains chilled in ice-cold carbogenated (95% O2/5% CO2) sucrose-based artificial cerebrospinal fluid (ACSF), containing (in mM): 3.5 KCl, 2.4 CaCl2, 1.3 MgSO4, 1.2 KH2PO4, 10 Glucose, 26 NaHCO3 and 212.5 Sucrose. Slices were stored in carbogenated normal ACSF, comprising (in mM): 126 NaCl, 3 KCl, 2 MgSO4, 2 CaCl2, 10 Glucose, 1.20 NaH2PO4 and 26 NaHCO3, 305 mOsm and pH 7.3.
Slices were transferred to a submerged recording chamber with constant perfusion of carbogenated ACSF. EGFP expressing neurons in layer II/III of V1 were patched under an Axioskop FS upright microscope equipped with infrared differential interference contrast optics (Zeiss). Borosilicate glass patch-pipettes (4–6 MΩ) were filled with K-gluconate internal solution containing (in mM): 154 K-gluconate, 1 KCl, 0.5 EGTA, 10 Heps, 4 Mg-ATP, 4 K₂Phosphocreatine, 0.4 GTP (PH=7.3 and 0.290 mosm) and 3mg/ml biocytin (Invitrogen) for intracellular labeling. In whole-cell configuration, miniature excitatory postsynaptic currents (mEPSCs) were recorded at 18–21°C using a patch-clamp amplifier (EPC8, HEKA Electronics, Lambrecht, Germany) in the presence of 1 µM tetrodotoxin (Alomone Labs, Jerusalem, Israel) while holding the membrane potential at -70mV.

To study the role of acute lack of TrkB signaling, K252a, a cell permeable inhibitor of Trk phosphorylation (Calbiochem, San Diego, CA) was applied (200nM). After stabilization, mEPSCs were recorded for 9 minutes as baseline, 9 minutes in the presence of K252a and 9 minutes after washing it out. Signals were low-pass filtered at 3.0 KHz and digitized at 10 KHz with an ITC-16 computer interface (InstruTECH Corporation, Elmont, NY). Care was taken that series resistance remained below 20 MΩ.

**Data analysis.** Mini Analysis (Synaptosoft Inc., Decatur, GA) was used for analyzing mEPSCs. The amplitude threshold was set at 7 pA, which was more than 3× RMS noise in all recordings. After automatic detection of mEPSCs by the software, each mEPSC was visually inspected. Recordings with a systematic drift in average mEPSC rise time of more than 10% were excluded. mEPSCs with rise times above 3 ms were omitted. Frequencies
and amplitude distributions of mEPSCs in EGFP-F versus TrkB.T1-EGFP
neurons were compared for statistical significance using the KS test.
Statistical analysis of the effect of K252a was done using the Friedman test.

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