Presynaptic Plasticity in an Immature Neocortical Network Requires NMDA Receptor Activation and BDNF Release

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INTRODUCTION

During development of the mammalian neocortex, specific patterns of synaptic connectivity are thought to emerge by the long-term stabilization of functionally adequate synapses (Benson et al. 2001; Katz and Shatz 1996). Neocortical synapses have been proposed to initially form as functionally immature contacts that are converted to mature, fully functional synapses by coincident pre- and postsynaptic activity (Isaac et al. 1997; Rumpel et al. 1998). Postsynaptic mechanisms regulating AMPA receptor surface expression are well established to underlie this functional induction of silent synapses (Isaac 2003). However, long-term stabilization of functionally ade-quate synapses requires in addition presynaptic plasticity processes enhancing accumulation and cycling of synaptic vesicles. Unfortunately, because electrophysiological techniques allow only indirect observation of presynaptic changes (Voroshin and Cherubini 2003), the presynaptic expression of long-term plasticity has remained controversial in developing neocortical neurons.

Direct observation of presynaptic vesicle cycling is enabled by the endocytotic uptake of styryl dyes like FM1-43 and their subsequent stimulation-induced release (Betz and Bewick 1992; Ryan et al. 1993). In addition to analyzing basic presynaptic function, presynaptic long-term plasticity has been investigated by performing repeated FM staining and destaining of cycling vesicles in hippocampal neurons. Depending on subtle changes in experimental conditions, an activity-dependent presynaptic potentiation (Ma et al. 1999; Ryan et al. 1996; Micheva and Smith 2005; Zakharenko et al. 2001), no change in vesicle cycling (Micheva and Smith 2005; Ryan et al. 1996), or a presynaptic depression have been reported (Hopf et al. 2002; Stanton et al. 2003). A similar direct observation of long-term plasticity of presynaptic vesicle cycling has not yet been performed in neocortical neurons.

Mechanistically, stabilization of functionally adequate presynaptic release sites has been proposed to be controlled by retrograde signaling from the postsynaptic target in the developing neocortex (Fitzsimonds and Poo 1998; Katz and Shatz 1996). This retrograde signaling is thought to be initiated by activation of postsynaptic N-methyl-d-aspartate (NMDA) receptors and involves release of a retrograde messenger molecule, e.g., the neurotrophin brain-derived neurotrophic factor (BDNF) (Lessmann et al. 2003; Lu 2004; Tao and Poo 2001; Tyler et al. 2002). Again, direct visualization of presynaptic plasticity by repeated FM imaging experiments would allow to study the possible involvement of such a classical retrograde pathway in developing neocortical synapses.

In this paper, we describe pronounced presynaptic long-term plasticity in immature neocortical neurons in culture. Repeated FM staining/destaining experiments revealed an activity-induced potentiation of vesicle cycling that was dependent on NMDA receptor activation. In addition, analysis of BDNF-deficient neurons indicated that BDNF release is necessary for this type of presynaptic plasticity.
METHODOLOGY

Mouse neocortical glial cells were obtained and cultured as microislands as described (Jüngling et al. 2003; Lessmann and Heinmann 1997). Neocortical neurons were taken from E18–19 fetuses of EGFP-expressing mice (Hadjantonakis et al. 1998), were mechanically dissociated after trypsin treatment, were seeded on glial microislands, and were cultured as described (Jüngling et al. 2003; Mohrmann et al. 2003). EGFP-expressing; BDNF+/-; mice were obtained by crossing heterozygous BDNF-knockout mice (Korte et al. 1995) and EGFP-expressing mice (Hadjantonakis et al. 1998). E18–19 fetuses from EGFP-expressing, BDNF+/- females (mated with males of the same genotype) were used to prepare neocortical neurons of different BDNF genotypes for cultivation on glial microislands. Genotyping of fetuses was performed by PCR.

To visualize presynaptic vesicle accumulations on EGFP-labeled dendrites, cycling synaptic vesicles were stained by the uptake of the styryl dye FM4–64 and subsequently destained by stimulation (Betz and Bewick 1992). First, microisland cultures were superfused for 2 min with depolarizing extracellular solution (composition, in mM: 40 KCl, 54 NaCl, 2 CaCl2, 1 MgCl2, and 20 HEPES, pH = 7.3) containing 10 μM FM4–64 (Molecular Probes) to obtain a saturating staining (Mohrmann et al. 2003). After staining, cultures were superfused with a dye-free, low-Ca2+/high-Mg2+ extracellular solution (composition, in mM: 130 NaCl, 5 KCl, 1 CaCl2, 10 MgCl2, and 20 HEPES, pH = 7.3) containing ADVASEP-7 (1 mM, Biotium) to reduce unspecific staining (Kay et al. 1999). Then, digital fluorescence images of FM4–64-stained puncta (excitation: 546 nm; emission: >590 nm) on a EGFP-labeled dendrite were acquired using a ×40 oil-immersion objective (Olympus) in combination with a CCD camera system (CoolSNAPcf., Photometrics; MetaView software, Universal Imaging). For destaining, an extracellular stimulation electrode consisting of a patch pipette (tip diameter: 10 μm, filled with a 1:1 mixture of standard extracellular solution and 1 M NaCl) was located within 50 μm of the FM4–64 puncta studied. A 40-Hz train of electrical stimulations (1 s) was repeated 10 times leading to a complete stimulation-induced destaining. During and after stimulation, digital fluorescence images of the FM4–64 puncta were taken again. After a waiting period of 90 min, the complete staining/destaining procedure was repeated in an identical manner. During the entire experiment, microisland cultures were kept on the stage of an inverted microscope at 34°C and were perfused with ACSF (composition, in mM: 119 NaCl, 2.5 KCl, 1 NaHPO4, 26.2 NaHCO3, 1.5 MgCl2, and 2.5 CaCl2; pH = 7.3 equilibrated with carbogen) with high-Mg2+/low-KCl electrodes (1.3% Ca2+, 86% NaCl, 10% KCl, 1.5 MgCl2, and 2.5 CaCl2) high-Mg2+/low-KCl solution (composition, in mM: 130 NaCl, 5 KCl, 1 CaCl2, 10 MgCl2, and 20 HEPES, pH = 7.3) containing ADVASEP-7 (1 mM, Biotium) to reduce unspecific staining (Kay et al. 1999). Then, digital fluorescence images of FM4–64-stained puncta (excitation: 546 nm; emission: >590 nm) on a EGFP-labeled dendrite were acquired using a ×40 oil-immersion objective (Olympus) in combination with a CCD camera system (CoolSNAPcf., Photometrics; MetaView software, Universal Imaging). For destaining, an extracellular stimulation electrode consisting of a patch pipette (tip diameter: 10 μm, filled with a 1:1 mixture of standard extracellular solution and 1 M NaCl) was located within 50 μm of the FM4–64 puncta studied. A 40-Hz train of electrical stimulations (1 s) was repeated 10 times leading to a complete stimulation-induced destaining. During and after stimulation, digital fluorescence images of the FM4–64 puncta were taken again. After a waiting period of 90 min, the complete staining/destaining procedure was repeated in an identical manner. During the entire experiment, microisland cultures were kept on the stage of an inverted microscope at 34°C and were perfused with ACSF (composition, in mM: 119 NaCl, 2.5 KCl, 1 NaHPO4, 26.2 NaHCO3, 1.5 MgCl2, and 2.5 CaCl2; pH = 7.3 equilibrated with carbogen) with addition of glucose, glutam (Invitrogen), B27-supplement (Invitrogen) and penicillin/streptomycin (Invitrogen). Under these recording conditions the morphology of neurons was stable for 4–5 h as indicated by EGFP fluorescence (data not shown).

For data analysis, a difference image was calculated from the image of the FM4–64-stained puncta taken prior to electrical stimulation and the image taken after destaining at the end of stimulation. To calculate the fluorescence change associated with destaining for individual release sites, the number of actively cycling synaptic vesicles. Destaining of FM puncta was elicited by repetitive (10 times) local extracellular stimulation (40-Hz train for 1 s) until no further destaining could be evoked. Images were taken prior to and during destaining, and a difference image between the image prior to stimulation and the image at the end of stimulation was calculated. To visualize dendrites, neocortical neurons from EGFP-expressing mice (Hadjantonakis et al. 1998) were used and only FM puncta that were located on a dendrite were included in the analysis (Fig. 1A). The mean fluorescence intensity associated with destaining (ΔF1) of individual release sites was calculated from the difference image. Cultures were kept at 34°C at the stage of an inverted microscope throughout the entire experiment. After 90 min a second, identical FM4–64 staining/destaining procedure was performed on the same individual release sites yielding ΔF2.

The vast majority of individual release sites showed an increased mean fluorescence intensity after the second round of FM4–64 staining/destaining (Fig. 1, A and B), suggesting that the number of actively cycling vesicles was increased at most synapses by the activity associated with the first staining/destaining. In addition, the appearance of new functional release sites was frequently observed after the second staining/destaining (Fig. 1, A and B). Quantitatively, 28% of the release sites observed during the second staining/destaining were not detectable during the first round of staining/destaining and thus represent release sites that were either newly formed or were functionally silent at the first staining. In 58% of the release sites, the mean fluorescence intensity increased by >20%, whereas the intensity was constant (±20%) in only 12% of release sites. Release sites that showed a decrease in intensity (1.3% >20% decrease) or that disappeared completely (0.1% >95% decrease) were very rare (Fig. 1D). To further study whether parameters other than the number of cycling vesicles are also changed, we analyzed the FM4–64 destaining kinetics in individual release sites by fitting the stimulation induced fluorescence decay with a single exponential function. However, the mean time constant of fluorescence decay did not significantly differ between the first [6.9 ± 1.4 (SE) s, n = 100] and the second staging/destaining (6.7 ± 1.5 s, n = 100). In addition, also the time constant of destaining in newly appearing release sites was not significantly different (6.6 ± 1.3 s, n = 100).

We next addressed whether the observed presynaptic plasticity depends on the activation of NMDA receptors that occur during the stimulation used for FM4–64 staining and destaining. The preceding experiments were repeated with addition of the NMDA receptor antagonist d-2-amino-5-phosphonopentanoic acid (d-AP5, 25 μM). Strikingly, under these conditions, the mean intensity was constant (±20%) in 83% of individual release sites (Fig. 1, C and D). Release sites that showed a decrease in intensity (8%; >20% decrease) or that disappeared completely (6%; >95% decrease) were now more prominent. The percentage of release sites that showed an increase in the mean fluorescence intensity of >20% dramatically decreased to only 2%. In addition, the appearance of new functional release sites was largely blocked. Thus our results demonstrate that an activity-induced, presynaptic long-term plasticity process occurs in immature neocortical neurons that is strongly dependent on the activation of NMDA receptors.
NMDA receptor-dependent presynaptic plasticity is thought to involve a retrograde messenger molecule, such as BDNF, that mediates signaling from postsynaptic NMDA receptors to presynaptic vesicles. To address this, we studied presynaptic plasticity in cultured neocortical neurons, in which the BDNF gene had been inactivated. To enable fluorescence imaging of dendrites, BDNF+/− mice (Korte et al. 1995) were crossed with EGFP-expressing mice (Hadjantonakis et al. 1998), yielding BDNF+/− EGFP-expressing mice. These mice were bred further to obtain BDNF-wild type EGFP-expressing littermate controls and homozygous BDNF-knockout, EGFP-expressing mice, respectively. In BDNF wild type neurons, an increase in mean fluorescence intensity was observed at 50% of release sites using the same sequential FM4-64 staining/destaining protocol as described in the preceding text (Fig. 2, A–C). Twenty-four percent of the release sites observed during the second staining/destaining were not detectable during the first round of staining/destaining and thus represent newly appearing release sites. In contrast, in homozygous BDNF-knockout neurons, the majority of release sites showed a constant (±20%) mean fluorescence intensity after the sequential FM4-64 staining/destaining, whereas the percentage of release sites that showed an increase in intensity of >20% was strongly decreased (Fig. 2, A–C). Similar to inhibiting NMDA receptors, the appearance of new release sites was blocked. To further confirm that BDNF release is involved in the preceding described presynaptic plasticity, we used trkB receptor bodies (human recombinant trkB/Fc; R&D Systems) as extracellular BDNF scavengers to inhibit the action of BDNF. In wild-type EGFP expressing neurons, predepolarization with an elevated extracellular K+ concentration (40 mM, 3 min) 90 min prior to FM4-64 staining/destaining led to a significantly (KS-test, P < 0.001) increased mean fluorescence intensity of FM 4–64 puncta as compared with nonpredepolarized controls (Fig. 2D). Addition of trkB receptor bodies (1.0 μg/ml) during the entire experiment completely blocked this K+ predepolarization-induced increase in intensity, indicating a crucial role of BDNF release. In summary, the expression and the release of BDNF appeared to be necessary for enabling NMDA receptor-dependent presynaptic long-term plasticity.

**DISCUSSION**

In this paper, repeated FM staining/destaining of individual synaptic release sites in cultured mouse neocortical neurons led to a pronounced increase in the number of actively cycling release sites that showed an intensity decrease of >95% (eliminated), an intensity decrease of >20% (decrease), ≥20% change in intensity (constant), and an intensity increase of >20% (increase). Colored bars: Control conditions. The vast majority of release sites showed an increase in mean fluorescence intensity. Note the frequent appearance of new functional release sites (new sites). Grey bars: experiments in the presence of D-AP5. Note the dramatic reduction in fluorescence increase of individual release sites. In addition, new functional release sites were not observed.
synaptic vesicles. This presynaptic long-term plasticity might be caused by an increased accumulation of synaptic vesicles at individual release sites. Alternatively, the proportion of synaptic vesicles in the cycling pool might increase by recruiting vesicles from the inactive resting pool (Harata et al. 2001; Südhof 2000). Intriguingly, we frequently observed the appearance of new functional release sites within 90 min, which was dependent on NMDA receptor activation. This indicates an activity-dependent, functional induction of presynaptically silent release sites in our neocortical cultures similar to hippocampal neurons (Ma et al. 1999; Voronin and Cherubini 2003). Taken together, our findings indicate a long-term shift in the proportion of actively cycling and inactive vesicles. This presynaptic plasticity was strictly dependent on NMDA receptor activation indicating that the primary induction process might occur postsynaptically and that long-term expression of presynaptic plasticity requires a retrograde messenger also in neocortical neurons (Pratt et al. 2003; Volgushev et al. 2000).

In BDNF-deficient neocortical neurons, the presynaptic plasticity phenomena were strongly reduced. This further supports an important role of BDNF during the developmental maturation of neocortical circuitry (Berardi and Maffei 1999; Cabelli et al. 1995). As proposed previously (Lessmann 1998; Lessmann et al. 2003; Lu 2004; Tao and Poo 2001), BDNF might act as a retrograde messenger that is released in an activity-dependent manner from the postsynaptic neuron (Hartmann et al. 2001) and induces presynaptic long-term plasticity. In this paper, a crucial role of BDNF release was confirmed by the inhibition of presynaptic plasticity in the presence of a BDNF scavenger. In line with our findings, BDNF leads to a long-term enhancement in presynaptic function in cultured hippocampal (Collin et al. 2001; Lessmann and Heumann 1998; Lessmann et al. 1994; Shen et al. 2006; Tyler and Pozzo-Miller 2001; Vicario-Abejon et al. 1998) and immature neocortical neurons (Bradley and Sporns 1999). Alternative to a retrograde mechanism, BDNF expression in the presynaptic cell has been demonstrated to be essential for a presynaptic component of long-term potentiation in hippocampal neurons (Zakharenko et al. 2003). Because recent evidence indicates that presynaptic NMDA receptors might also be involved in long-term synaptic plasticity (Humeau et al. 2003; Sjostrom et al. 2003), a purely presynaptic induction and expression mechanism appears conceivable. However, such a presynaptic mechanism might be less potent in activity-dependent developmental maturation of neocortical circuitry (Pratt et al. 2003). In summary, long-term changes in the functional state of

![FIG. 2. Presynaptic long-term plasticity requires the expression and release of brain-derived neurotrophic factor (BDNF). A–C: repeated FM4-64 staining/destaining was performed in BDNF-deficient neocortical neurons. A: difference images obtained from FM4-64 staining/destaining. Red: FM puncta; white outlines: EGFP-labeled dendrite. Left: 1st FM4-64 staining/destaining. Right: 2nd FM4-64 staining/destaining after 90 min. Typical experiments in BDNF-deficient (BDNF−/−) neurons and in wild-type (BDNF+/+) neurons from littermate mice are shown. Arrow heads indicate release sites with decreasing fluorescence intensity (blue), with constant intensity (green), with increasing intensity (white), and newly appearing release sites (yellow). Scale bars represent 1 μm. B: mean fluorescence intensity (arbitrary units, a.u.) of individual release sites after 2nd FM4-64 staining/destaining (ΔF) in relation to the mean fluorescence intensity after the 1st FM4-64 staining/destaining (ΔF0). N = 448 individual FM puncta from 3 independent experiments for BDNF+/+ neurons (red dots, red line: linear regression) and n = 161 individual FM puncta from 5 independent experiments for BDNF−/− neurons (black dots) were analyzed. Black line represents no change in intensity. Broken line represents detection threshold for the 1st staining. C: percentage of release sites that showed an intensity decrease of >95% (eliminated), an intensity decrease of >20% (decrease), ≥20% change in intensity (constant), and an intensity increase of >20% (increase). Colored bars: Control experiments in BDNF+/+ neurons. Grey bars: experiments in BDNF−/− neurons, in which the majority of release sites showed a constant mean fluorescence intensity. Appearance of new functional release sites (new sites) was not observed in BDNF-deficient neurons. D: extracellular addition of trkB receptor bodies (BDNF scavengers) inhibited the K+/H1001 predepolarization (see text) induced increase in fluorescence intensity in wild type neurons. Cumulative distributions of the mean fluorescence intensities at individual FM puncta are shown.](http://jn.physiology.org/10.1152/jn.01005.2006)
synaptic vesicles, i.e., a shift from the resting pool to the cycling pool, might be an important mechanism in the BDNF-dependent stabilization of the immature neocortical circuitry.

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