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Promotes the Maturation of GABAergic Synapses

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GABA, the main inhibitory neurotransmitter in the adult brain, has recently emerged as an important signal in network development. Most of the trophic functions of GABA have been attributed to depolarization of the embryonic and neonatal neurons via the activation of ionotropic GABA_A receptors. Here we demonstrate a novel mechanism by which endogenous GABA selectively regulates the development of GABAergic synapses in the developing brain. Using whole-cell patch-clamp recordings on newborn mouse hippocampal neurons lacking functional GABA_B receptors (GABA_B-KO) and time-lapse fluorescence imaging on cultured hippocampal neurons expressing GFP-tagged brain-derived neurotrophic factor (BDNF), we found that activation of metabotropic GABA_B receptors (GABA_B-Rs) triggers secretion of BDNF and promotes the development of perisomatic GABAergic synapses in the newborn mouse hippocampus. Because activation of GABA_B-Rs occurs during the characteristic ongoing physiological network-driven synaptic activity present in the developing hippocampus, our results reveal a new mechanism by which synaptic activity can modulate the development of local GABAergic synaptic connections in the developing brain.

Introduction

The proper development of highly organized structures in the CNS is a complex process that determines appropriate connectivity in the adult. Neurotransmitters released during spontaneous and experience-driven synaptic activity play a crucial role in the formation of neuronal networks (Katz and Shatz, 1996; Zhang and Poo, 2001). The most well documented example is glutamate, the major excitatory transmitter in the vertebrate CNS, which regulates nearly all aspects of neuronal network formation from migration to synaptogenesis (Zhang and Poo, 2001; Manent and Represa, 2007). Recent advances have shown that GABA acts beyond its classical inhibitory role and also functions as an important developmental signal by regulating proliferation, migration, growth, and synapse formation (Ben-Ari et al., 2007). With the observation that the activation of chloride-permeable GABA_A receptors (GABA_A-Rs) depolarizes developing neurons (Cherubini et al., 1991), it was proposed that part of the trophic action of GABA relies on membrane depolarization and subsequent activation of voltage-dependent calcium channels (Ben-Ari et al., 2007). Consistent with this hypothesis, early conversion of GABA-induced depolarization into hyperpolarization impairs synapse formation and dendritic development of the target neurons both in vitro (Chudotvorova et al., 2005) and in vivo (Ge et al., 2006; Cancedda et al., 2007; Wang and Kriegstein, 2008; Reynolds et al., 2008).

In addition to ionotropic GABA_A-Rs, GABA also binds to metabotropic GABA_B-Rs. These receptors are ubiquitously expressed at early stages of development, even before synapses are formed (Frischky et al., 1999; Behar et al., 2001; López-Bendito et al., 2002, 2004), and are activated by endogenous GABA released during early network-driven synaptic activity (McLean et al., 1996; Obrietan and Van den Pol, 1999; Catsicas and Mobbs, 2001; López-Bendito et al., 2003). Although GABA_B-R activity has been reported to modulate cortical neuronal migration (Behar et al., 1996, 2001; López-Bendito et al., 2003), little attention has been paid to the possible contribution of GABA_B-Rs in the functional development of neuronal networks.

In the present study, we tested the ability of CA3 hippocampal pyramidal cells to develop functional synaptic connections in the absence of functional GABA_B-Rs. We found that activation of GABA_B-Rs selectively promotes the development of hippocampal GABAergic synapses via the induction of brain-derived neurotrophic factor (BDNF) secretion. We further show that this process occurs during physiological patterns of synaptic activity. This study reveals a novel role of GABA_B-Rs in regulating the self-refinement of GABAergic synaptic connections in the developing brain.

Materials and Methods

All animal experiments were carried out according to the guidelines laid down by the Inserm animal welfare committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Intact hippocampal formation and slice preparation. Experiments were performed on intact hippocampal formation (IF) and hippocampal slices obtained from newborn [postnatal day 1 (P1) to P24] GABA_A1 receptor subunit knock-out (GABA_A1-KO) and wild-type (GABA_A1-WT) mice (Prosser et al., 2001) and BDNF-KO and BDNF-WT mice
Electrophysiological recordings. Whole-cell patch-clamp recordings of CA3 pyramidal neurons were performed with an Axopatch 200B amplifier (Molecular Devices). To record miniature activity, borosilicate microelectrodes (4–8 MΩ) were filled with the following solution (in mM): 110 NaCl, 30 potassium glutonate, 10 HEPES, 1.1 ES7, 0.1 CaCl₂, 4 MgATP, 0.3 NaGTP, 5-(and)-6-tetramethylrhodamine biocytin (rhodamine, 0.5–1%), pH 7.25, osmolarity = 275 mosmol/L. Criteria for accepting a recording included a resting potential of <-55 mV, an R₅ of >400 MΩ, and an R₆ of <25 MΩ. Capacitance, input, and series resistances were measured online with Ac quis Software (BioLogic).

Miniature GABAₐ receptor-mediated postsynaptic currents (mGABAₐ-PCs) were isolated in the presence of the ionotropic glutamate receptor antagonists [10 μM 2,3-dihydroxy-6-nitro-7-nitrobenzo-2H-quinolinic acid (NBQX), 40 μM D-2-amino-5-phosphovaleric acid (D-APV)] and tetrodotoxin (1 μM TTX) and recorded at a holding potential of –70 mV. Miniature glutamatic post synaptic currents were recorded in the presence of TTX (1 μM) and the GABAₐ receptor antagonist bicuculline (10 μM). Neurons were clamped at –70 mV. The currents were stored on an Axoscope 8.1 (Molecular Devices) and analyzed with the Mini Analysis program (Synaptosoft 5.1). The fact that no false events would be identified was confirmed by visual inspection for each experiment. To generate the average PCs, multiple overlapping events were discarded, and the remaining events were aligned on their rising phase. In the figures, the histograms were constructed using miniature PCs recorded for 10–30 min. To determine the probability of presence of giant depolarizing potentials (GDPs), an average of 13 ± 6 cells (ranging from n = 3 at P24 to n = 23 at P1) were recorded in GABAₐ-WT slices and 14 ± 7 cells (ranging from n = 3 at P24 to n = 27 at P7) in GABAₐ-KO slices.

Peak-scaled analysis of mGABAₐ-PCs was performed as described by Traynelis et al. (1993) using Mini Analysis program (Synaptosoft 5.1). For each recording, we verified the absence of correlation between the decay time course and peak amplitude of mGABAₐ-PCs. For each recording, we used between 50 and 200 events and eliminated all events with a decay time distored by multiple peaks or anomalous noise. Each individual event was scaled to the peak of the mean waveform of the averaged event and subtracted. The mean variance was plotted against mean current. The plot was well fit by a parabolic function that yields the single-channel current iₗ and the number of channels contributing to mGABAₐ-PCs, Nₑ. From iₗ, the single-channel conductance γ can be calculated.

Paired-pulse relationship (PPR) of GABAergic synapses impinging onto CA3 pyramidal neurons was measured using pairs of identical stimuli at a 100 ms interval, delivered at a frequency of 0.01 Hz with a bipolar tungsten electrode placed in the CA3 stratum radiatum. The resulting pairs of postsynaptic GABAₐ currents (GABAₐ-PCs) were isolated in the presence of 10 μM CNQX, 40 μM D-APV, and 5 μM CGP58545. PPR was measured as the following amplitude ratio: second GABAₐ-PCs/first GABAₐ-PCs. Average PPR values were calculated from 20–30 paired stimulations.

Neuron reconstruction and morphometric analysis. Biocytin filling was done using the whole-cell patch-clamp technique. Briefly, after whole-cell access, biocytin (1% in internal pipette solution) filling was done for 15 min. The slices were then fixed in PFA-PBS at 4°C overnight, and the biocytin-filled neurons were visualized using the avidin–biotin method. For 3-D reconstruction, a dendritic tree was digitized directly from the sections by use of a 20× objective on a Nikon microscope, equipped with a motorized stage and coupled to a computer running Neuro lucida software (Micro brightfield), thereby allowing for x–z coordinates of digitized points to be stored and analyzed.

Immunohistochemistry. Brains from P7 mice were fixed in 4% paraformaldehyde (overnight). Cryostat-cut hippocampal sections (30 μm) were preincubated (1 h) in PBS–Triton X-100 (0.3%)–goat serum (3%) and then coincubated overnight at 4°C with antibodies to glutamic acid decarboxylase (Chemicon MA1352) and synaptophysin (Chemicon AB9272). Slices were washed with PBS, and Alexa Fluor 488 donkey antibody to mouse IgG (FluoProbes) and Cy3 donkey antibody to rabbit IgG (Chemicon) were applied (2 h). Sections were visualized with confocal microscopy (LSM 510, Zeiss). Five areas were sampled per animal, in the stratum radiatum and the stratum pyramidale. After the recording sessions, the optical sections were displayed in the form of digital images of 1024 × 1024 pixels and processed using the ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). All the pictures were reviewed to set a threshold to optimize the representation of puncta, and then the same threshold was applied to all images. The GAD65-labeled area fraction, defined as the percentage of GAD65-immunopositive pixels per field (1024 × 1024 pixels), and the size of GAD65 puncta were measured. The proportion of colocalized immunoreactivity (IR) was expressed as the ratio: synaptophysin and GAD65 colocalized area/synaptophysin IR area or GAD65 IR area. For each section, counts were performed blindly in slices taken from three WT and three KO animals.

Cell cultures and transfections. Neurons from postnatal day 0 rat hippocampus were dissociated using trypsin and plated on coverslips coated with polylysyleneimine as previously described (Kuczewski et al., 2008b). Eleven days after plating, neurons were transfected with cDNAs coding for BDNF-GFP (gift from Dr. V. Lessmann, Institute of Physiology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany) according to the OZ Biosciences protocol (Buerli et al., 2007). After transfection, the cultures were incubated at 30°C in 5% CO₂. Immunostaining confirmed that BDNF-GFP is targeted to the dendrite and packed into secretory granules of the regulated pathway of secretion (Kuczewski et al., 2008b).

Time-lapse imaging. Real-time imaging was performed as previously described (Kuczewski et al., 2008b). Fluorescence intensity was measured from dendritic regions containing clusters of BDNF-GFP with ImageJ software after subtraction of background fluorescence. Clusters in which the fluorescence intensity varied during the 5 min control period by >5% were discarded from the analysis. Fluorescence decreases caused by photobleaching and constitutive release were corrected by subtracting the extrapolation of the fluorescence decrease in the first 5 min over the whole recording time. Values are plotted as the percentage of the fluorescence intensity of the last frame before stimulation. Percentage variation in the text and statistical analysis were calculated by comparing the relative fluorescence of the interval –100 to 0 s (control) with that of 400–500 s (after stimuli).

Surface GFP immunofluorescence staining. The procedure for surface BDNF-GFP immunostaining was similar to that previously described (Kuczewski et al., 2008b). Briefly, after being washed in ACSF, the living cultures were incubated at 4°C for 1 h in the presence of an anti-GFP antibody (10 μg/ml Molecular Probes). Cultures were then washed with 0.1 M PBS (4°C, pH 7.4) and fixed for 15 min with 4% paraformaldehyde–4% sucrose. After fixation, the neurons were exposed to a saturating concentration (10 μg/ml) of either anti-rabbit secondary antibody coupled to Cy3 (FluoProbes) for 1.5 h under a nonpermeabilized condition. Quantifications were performed with ImageJ. Surface-bound BDNF-GFP on BDNF-GFP-expressing neurons was expressed as the following ratio: Cy3 and BDNF-GFP colocalized area/BDNF-GFP area.

Phospho-CREB activation and immunocytochemistry. The procedure for phospho-CREB activation and immunocytochemistry was similar to that previously described (Kuczewski et al., 2008b). Briefly, 1 d before stimulation (at 13 DIV) one-half of the culture medium was changed to...
MEM with 2% B27 supplement. To reduce the basal level of CREB phosphorylation, cultures were incubated for 30 min in TTX (1 μM). Five minutes before stimulation, NBQX (10 μM), D-APV (40 μM), and bicuculline (20 μM) were added to medium. The cultures were then stimulated with baclofen (50 μM) for 10 min in the absence or presence of TrkB-IgG (2 μg/ml) or CGP55845 (10 μM). Five to 10 min after stimulation, neurons were fixed for 15 min (4% paraformaldehyde) at 4°C and rinsed several times. Coverslips were then preincubated in PBS–Trition-100 (0.1%)–goat serum (3%) for 1 h at room temperature and incubated overnight with mouse anti-CREB (1:1000) and rabbit anti-phospho-CREB (pCREB: 1:1000) antibodies (Cell Signalling Technology). Immunoreactivities for pCREB and CREB were detected with an Alexa 488-coupled (A488) rabbit secondary antibody (1:500; FluoroProbes) and a Cy3-coupled mouse secondary antibody (1:500; Jackson Immunoresearch Laboratories), respectively. All procedures were performed in phosphate-free solution containing 140 mM NaCl, 5 mM KCl, and 10 mM HEPES-Na, pH 7.4. Images were acquired with an LSM 510 laser scanning confocal microscope (Zeiss). The acquisition of A488 (pCREB) and then Cy3 (CREB) was sequential to avoid overlap of excitation and emission of fluorescence. The optical sections were digitized (1024 × 1024 pixels) and processed using ImageJ software. The pCREB-to-CREB intensity ratio was expressed as means ± SEM. The pCREB:A488 staining intensity versus the CREB:Cy3 staining intensity.

**ELISA.** Brains of wild-type, GABA/B1-KO, and BDNF heterozygote mice were rapidly removed from their skulls at P6. Hippocampi were rapidly dissected out, weighed, and snap frozen in liquid nitrogen and stored at −80°C. BDNF was extracted from hippocampi by mechanical homogenization in a buffer containing 100 mM Tris–HCl, pH 7.0, containing 1 nM CaCl2, 4 μM EDTA, 2% Triton X-100, and the protease inhibitors 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 17 μg/ml PMSF. Homogenates were centrifuged at 14,000 × g for 20 min. Supernatants were collected and analyzed with a commercial two-antibody sandwich ELISA (BDNF Emax immunoassay system; Promega) according to the protocol of the manufacturer. The total protein content of each supernatant was measured with a Bradford protein assay. The BDNF level was expressed as the ratio of BDNF to the total soluble protein concentration. There was no significant difference in the weight of GABA/B1-WT and GABA/B1-KO hippocampi (9.87 ± 0.31 mg/hippocampus vs 9.12 ± 0.56 mg/hippocampus, respectively).

**Results**

**Miniature GABAergic synaptic activity is altered in mice lacking functional GABA/B1 receptors.**

To address the contribution of GABA/B1-Rs to the development of the hippocampal circuit, we recorded miniature GABAergic responses (mGABA/B1-SCs, mGlu-B1-SCs, respectively) from hippocampal CA3 pyramidal cells from P6 to P8 GABA/B1-KO mice, which allowed a complete loss of GABA/B1 receptor function (Prosser et al., 2001). The frequency of mGABA/B1-SCs was significantly reduced in GABA/B1-KO (0.73 ± 0.12 Hz in GABA/B1-KO, n = 13) when compared with their wild-type littermates (1.61 ± 0.26 Hz, n = 10, p = 0.03) (Fig. 1A,B). This decrease in frequency was observed at all developmental stages studied (i.e., from P1 to P10) (Fig. 1C; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The mean amplitude, coefficient of variation of amplitude (CVA), and kinetic properties of mGABA/B1-SCs were unchanged (Fig. 1B; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In contrast to mGABA/B1-SCs, the average frequency of mGlu-B1-SCs was not different between GABA/B1-WT (0.11 ± 0.017 Hz, n = 10) and GABA/B1-KO (0.11 ± 0.016 Hz, n = 12) P6–P8 neurons. The other parameters of mGlu-SCs were also unchanged (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The membrane capacitance of the recorded cells, an indicator of neuronal size (Colin-Le Brun et al., 2004) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), was not different between GABA/B1-WT and GABA/B1-KO (Fig. 1B; supplemental Fig. 1, available at www.jneurosci.org as supplemental material), suggesting that the morphological development of the target CA3 pyramidal cells was not affected. This observation was confirmed by quantitative morphometric analysis of intracellularly biocytin-loaded CA3 pyramidal neurons (Fig. 1D–G). Therefore, the development of GABAergic synapses is selectively impaired in mice lacking functional GABA/B1-Rs.

**Blockade of GABA/B-Rs in vitro mimics the deficit in GABAergic transmission observed in GABA/B1-KO mice.**

In a previous study, we showed that the factors required for the functional maturation of GABAergic synapses in vivo are preserved in the IHFs in vitro (Colin-Le Brun et al., 2004). We therefore used this preparation to overcome the potential pitfalls of genetic manipulations often observed in KO animals. IHFs obtained from P1 GABA/B1-WT mice were incubated at 32°C for 12–16 h in oxygenated control ACSF or with the GABA/B-R antagonist CGP55845 (5 μM). After the incubation period, hippocampal slices were prepared to record mGABA/B-SCs. In slices obtained from CGP55845-treated IHFs, the frequency of mGABA/B-SCs was significantly lower compared with control IHFs [0.107 ± 0.024 Hz (n = 11) vs 0.247 ± 0.049 Hz (n = 10), p = 0.031] (Fig. 1H). The other mGABA/B-SC parameters were unchanged (supplemental Fig. 4a, available at www.jneurosci.org as supplemental material). P1 IHFs obtained from GABA/B1-KO mice were also incubated in control ACSF. The mean frequency of mGABA/B-SCs (0.117 ± 0.026 Hz, n = 8) was significantly lower compared with control GABA/B1-WT IHFs (p = 0.046) but similar to the value obtained in CGP55845-treated GABA/B1-WT IHFs (Fig. 1H). The other mGABA/B-SC parameters were unchanged (supplemental Fig. 4a, available at www.jneurosci.org as supplemental material). Therefore, the pharmacological blockade of the GABA/B-Rs in vitro reproduces the deficit in GABAergic transmission observed in GABA/B1-KO mice, showing that it reflects the absence of GABA/B-R activation by endogenous GABA.

**Reduced perisomatic GABAergic synapses in mice lacking GABA/B receptors**

We next asked whether the deficit in GABAergic synaptic transmission resides in the presynaptic or the postsynaptic site. We first performed a peak-scaled variance analysis of mGABA/B-SCs which allows an estimate of the mean unitary conductance of the GABA/B-Rs (Traynelis et al., 1993) and found no difference between GABA/B1-KO (n = 6) and GABA/B1-WT (n = 11) neurons (Fig. 2A–C). To detect possible changes in the probability of GABA release, we then measured the paired-pulse ratio and CVs of evoked GABA/B-SCs. We found no difference between GABA/B1-WT and GABA/B1-KO mice (n = 5 for both) (Fig. 2D–F). Finally, to investigate possible changes in the density of GABAergic terminals, we performed immunolabeling against GAD65, the synthetic enzyme for GABA. Double immunostaining against GAD65 and synaptophysin confirmed that GAD65 staining corresponds to GABAergic terminals (supplemental Fig. 5a–c, available at www.jneurosci.org as supplemental material). Quantitative analysis shows that, compared with the GABA/B1-WT.
hippocampi, the labeled area fraction was reduced by 20% in the stratum pyramidale of GABAB1-KO (p < 0.001), while the average size of GAD-positive puncta was unchanged (n = 5 pairs of mice, 5 sections per mouse) (Fig. 2). There was, however, no difference in the labeled area fraction and average size of GAD65-positive puncta in the stratum radiatum of GABAB1-KO and GABAB1-WT hippocampi (Fig. 2). Similar results were obtained with synaptophysin, a presynaptic marker (supplemental Fig. 5d,e, available at www.jneurosci.org as supplemental material). Although functional presynaptic modifications cannot be completely excluded, together these data suggest that the deficit in GABAergic synaptic activity observed in GABAB1-KO mice results at least in part from a decrease in the density of proximal GABAergic terminals.

**GABAB-Rs are primarily activated by spontaneous network-driven synaptic activity**

We next asked whether and when GABAB-Rs are activated during ongoing synaptic activity. To address this point, we investigated the effect of the GABAB-R antagonist CGP55845 on GABAB1-WT slices. We found that the duration of GDPs, the characteristic primitive network-driven pattern of synaptic activity (Ben-Ari et al., 1989), was significantly increased in the presence of CGP55845 (5 μM) (from 0.757 ± 0.146 s to 1.738 ± 0.617 s, n = 8, p = 0.0002) (Fig. 3A). However, when applied in the presence of NBQX (10 μM) and D-APV (40 μM), to isolate spontaneous GABA A-PSCs, CGP55845 had no significant effect on the frequency, amplitude and charge transfer of sGABA A-PSCs in GABAB1-WT neurons (n = 9) (Fig. 3B,C). In agreement with previous findings that GABAB-Rs are activated by concomitant release of GABA from several interneurons (Scanziani, 2000), these data show that the activation of GABAB-Rs by endogenous GABA required the presence of spontaneous GDPs.

**The lack of GABAB-R activation is responsible for the deficit in GABAergic transmission**

Network construction is modulated by the level and pattern of spontaneous synaptic activity generated in the developing ner-
GDPs in GABAB1-KO slices are network-driven synaptic events because they were recorded with field electrodes and abolished by TTX (1 μM) (data not shown). As expected, application of CGP55845 (5 μM) had no effect on GDP duration in GABAB1-KO slices (1555 ± 72 ms in CGP55845, n = 4). These data therefore show that GDPs are longer in GABAB1-KO mice and that the lengthening of GDPs results from the lack of functional GABAB-R-mediated inhibition.

We next asked whether the deficit in GABAergic transmission observed in hippocampi deficient in GABAB-Rs is due to the lack of GABAB-R activity per se or is an indirect consequence of the lack of GABAB-R activity, i.e., the lengthening of GDP duration. To address this point, P1 WT IHFs were incubated for 12–16 h in the presence of TTX (1 μM) to block action potential-dependent activity and GDPs. Since GABAB1 receptors are activated during GDPs, we hypothesized that blockade of GDPs with TTX treatment would reduce activation of GABAB-R and lead to a GABAergic deficit similar to that observed in GABAB-R-deficient hippocampi. We found that mGABAA-PSC frequency was indeed significantly reduced in TTX-treated IHFs (0.158 ± 0.037 Hz, n = 15) compared with control IHFs (0.270 ± 0.036 Hz, n = 15, p = 0.045) (Fig. 4F). The other mGABAA-PSC parameters were unchanged (supplemental Fig. 4B, available at www.jneurosci.org as supplemental material). We next attempted to rescue the deficit induced by TTX with the specific GABAB-R agonist baclofen. Baclofen (5 μM for 12–16 h) completely rescued the TTX-induced deficit [from 0.158 ± 0.037 Hz (n = 15) to 0.383 ± 0.076 Hz (n = 11), respectively, p = 0.023] (Fig. 4F; supplemental Fig. 4B, available at www.jneurosci.org as supplemental material). Finally, we investigated the consequences of treatment with baclofen alone, which also blocked GDPs (Tosetti et al., 2004) while activating GABAB-Rs. We found that incubation with baclofen (5 μM) had no significant effect on mGABAA-PSC frequency (0.390 ± 0.082 Hz, n = 11) when compared with control WT IHFs (Fig. 4F; supplemental Fig. 4B, available at www.jneurosci.org as supplemental material). Together, these data show that the deficit in GABAergic synaptic transmission observed in GABAB-R-deficient IHFs is not a consequence of abnormal synaptic activity but rather results from the lack of GABAB-R activation during ongoing synaptic activity.
GABAB<sub>R</sub>-Rs and BDNF signaling interact to regulate the formation of functional GABAergic synapses

We next asked how activity-dependent activation of GABAB<sub>R</sub>-Rs translates into functional development of GABAergic synapses. Considering that BDNF is a powerful modulator of GABAergic synapse development (Lessmann et al., 2003), the recent observation that GABAB<sub>R</sub> activity increases BDNF expression in cultured hippocampal neurons (Ghorbel et al., 2005) prompted us to test the possible contribution of BDNF-tropomyosin-related kinase (TrkB) receptor signaling to this synaptic development. P1 WT IHFs were incubated for 12–16 h with k252a (200 nM), a membrane-permeable inhibitor of protein tyrosine kinase. In k252a-treated IHFs, the frequency of mGABA<sub>A</sub>-PSCs was reduced compared with control IHFs (0.270 ± 0.036 Hz (n = 15) vs 0.105 ± 0.028 Hz (n = 14), p = 0.002) (Fig. 5A). The other mGABA<sub>A</sub>-PSC parameters were unchanged (supplemental Fig. 4c, available at www.jneurosci.org as supplemental material). The deficit induced by k252a was not rescued by baclofen (0.116 ± 0.026 Hz k252a-treated IHFs (n = 10) and 0.105 ± 0.028 Hz (n = 14) in k252a/baclofen-treated IHFs) (Fig. 5A; supplemental Fig. 4c, available at www.jneurosci.org as supplemental material). Moreover, when incubated with k252a, the GABAB<sub>R</sub>-R antagonist had no further effect on mGABA<sub>A</sub>-PSCs [0.088 ± 0.024 Hz (n = 11) in CGP55845/k252a-treated IHFs vs 0.105 ± 0.028 Hz (n = 14) in k252a-treated IHFs] (Fig. 5A; supplemental Fig. 4c, available at www.jneurosci.org as supplemental material). These data show that GABAB<sub>R</sub>-Rs promote the functional maturation of GABAergic synapses by means of a cascade involving tyrosine kinase signaling.

P1 WT IHFs were then incubated with the BDNF scavenger TrkB-IgG (1 μg/ml) or with the NT3 scavenger TrkC-IgG (1 μg/ml) and the BDNF scavenger TrkB-IgG (1 μg/ml) or with the NT3 scavenger TrkC-IgG (1 μg/ml). Figure 3. GABAB<sub>R</sub>-Rs are primarily activated during spontaneous network-driven synaptic activity. A, Representative current traces of spontaneous GDPs recorded in GABAB<sub>1</sub>-WT pyramidal neurons in control conditions and with the GABAB<sub>R</sub>-R antagonist, CGP55845 (5 μM). B, Representative traces of spontaneous GABA<sub>A</sub> receptor-mediated postsynaptic currents (sGABA<sub>A</sub>-PSCs) recorded in the presence of NBOX (10 μM) and D-APV (40 μM) with or without CGP55845 (5 μM). C, Summary plot of the effect of CGP55845 on sGABA<sub>A</sub>-PSC frequency, amplitude, and charge transfer in GABAB<sub>1</sub>-WT neurons expressed as percentages relative to control conditions (ACSF).

Figure 4. Characterization of synaptic activity in the developing mouse hippocampus. A, Representative current traces of spontaneous GDPs recorded in GABAB<sub>1</sub>-WT and KO CA3 pyramidal neurons. B, Plot of the percentage of cells showing GDPs at different postnatal stages of development in GABAB<sub>1</sub>-WT (open symbols) and GABAB<sub>1</sub>-KO (filled symbols). An average of 13 ± 6 cells (ranging from n = 3 at P24 to n = 23 at P1) were recorded in GABAB<sub>1</sub>-WT slices, and 14 ± 7 cells (ranging from n = 3 at P24 to n = 27 at P2) in GABAB<sub>1</sub>-KO slices were also recorded to construct this graph. The Boltzmann fit shows that there is no significant difference in the disappearance of GDPs between GABAB<sub>1</sub>-WT (dashed line) and GABAB<sub>1</sub>-KO (dark line). C–E, Summary plot of GDP frequency (C), amplitude (D), and duration (E) in GABAB<sub>1</sub>-WT (open bars) and GABAB<sub>1</sub>-KO (filled bars). F, Left, Summary plot of the mGABA<sub>A</sub>-PSC frequency in recordings from GABAB<sub>1</sub>-WT intact hippocampi incubated in vitro for 12–16 h in control condition (ACSF), in the presence of TTX (1 μM) alone, TTX and baclofen (5 μM), and baclofen alone. Right, Logarithmic plot of the mGABA<sub>A</sub>-PSC frequency in recordings from GABAB<sub>1</sub>-WT and GABAB<sub>1</sub>-KO intact hippocampi incubated in the corresponding conditions. Each symbol represents the result of one single cell. The dashed line represents the mean value obtained from GABAB<sub>1</sub>-WT incubated in ACSF. baclo, Baclofen.
The frequency of mGABA_A-PSCs was significantly reduced in TrkB-IgG-treated IHFs (0.105 ± 0.033 Hz, n = 8) compared with TrkC-IgG-treated IHFs (0.481 ± 0.087 Hz, n = 14, p = 0.001) (Fig. 5B). The other mGABA_A-PSC parameters were not affected (supplemental Fig. 4d, available at www.jneurosci.org as supplemental material). The GABA_B-R antagonist had no further effect on mGABA_A-PSC frequency when incubated with TrkB-IgG (0.117 ± 0.015 Hz, n = 12) (Fig. 5B; supplemental Fig. 4d, available at www.jneurosci.org as supplemental material) but significantly decreased the frequency of mGABA_A-PSCs when incubated with TrkC-IgG (0.481 ± 0.087 Hz, n = 14, p = 0.006 compared TrkC-IgG-treated IHF) (Fig. 5B; supplemental Fig. 4d, available at www.jneurosci.org as supplemental material). These data show that endogenous GABA and BDNF, acting on GABA_B-Rs and TrkB-Rs, respectively, interact to promote the functional maturation of hippocampal GABAergic synapses.

To confirm the requirement of GABA_B-R and BDNF interaction for the functional maturation of GABAergic synapses, P1 IHFs obtained from BDNF-KO and WT littermates were incubated for 12–16 h in control ACSF or with CGP55845 (5 μM). Incubation with the GABA_B-R antagonist had no effect on the frequency of mGABA_A-PSCs recorded from BDNF-KO IHFs [0.074 ± 0.019 Hz in control IHF (n = 8) vs 0.082 ± 0.019 Hz in CGP55845-treated IHFs (n = 8)] but significantly decreased the frequency of mGABA_A-PSCs recorded from BDNF-WT IHFs [0.248 ± 0.052 Hz in control IHF (n = 12) vs 0.108 ± 0.018 Hz in CGP55845-treated IHFs (n = 9), p = 0.03] (Fig. 5C; supplemental Fig. 4f, available at www.jneurosci.org as supplemental material). Moreover, the frequency of mGABA_A-PSCs from IHFs incubated in control ACSF was significantly reduced in BDNF-KO when compared with their wild-type littermates (p = 0.01) (Fig. 5C), showing that BDNF is required for the functional maturation of hippocampal GABAergic synapses. Altogether, these data strengthen the conclusion that GABA_B-Rs and BDNF interact to promote the maturation of GABAergic synapses in the developing mouse hippocampus.

To better determine the interplay between GABA_B-Rs and BDNF-TrkB signaling, we attempted to rescue the deficit induced by CGP55845 with exogenous BDNF. P1 WT IHFs were incubated with CGP55845 (5 μM) alone or with CGP55845 (5 μM) and BDNF (50 ng/ml) for 12–16 h. BDNF rescued the deficit induced by CGP55845. The mGABA_A-PSC frequency was 0.165 ± 0.042 Hz in CGP55845-treated IHFs (n = 21) and 0.331 ± 0.069 Hz in CGP55845 and BDNF-treated IHFs (n = 17, p = 0.014) (Fig. 5D; supplemental Fig. 4e, available at www.jneurosci.org as supplemen-

Figure 5. GABA_B and BDNF-TrkB signaling interact to promote the formation of functional GABAergic synapses. A, Left, Summary plot of the mGABA_A-PSC frequency in recordings from GABA_B-WT intact hippocampi incubated in vitro for 12–16 h in control conditions (ACSF) or with K252a alone (200 nM), K252a plus CGP55845 (5 μM), or K252a plus baclofen (baco; 5 μM). B, Left, Summary plot of the mGABA_A-PSC frequency in recordings from GABA_B-WT intact hippocampi incubated in vitro for 12–16 h in the presence of TrkC-IgG (1 μg/ml) alone, TrkC-IgG plus GAP5845 (CGP, 3 μM), TrkB-IgG (1 μg/ml) alone, or TrkB-IgG plus GAP5845 (5 μM). C, Left, Summary plot of the mGABA_A-PSC frequency in recordings from BDNF-WT and BDNF-KO intact hippocampi incubated in vitro for 12–16 h in control conditions (ACSF) or with GAP5845 (5 μM). D, Left, Summary plot of the mGABA_A-PSC frequency in recordings from BDNF-WT intact hippocampi incubated in vitro for 12–16 h in control conditions (ACSF) or with BDNF (50 ng/ml), GAP5845 (CGP, 5 μM), or BDNF (50 ng/ml) plus GAP5845 (5 μM). The graphs on the right in A–D represent the logarithmic plot of the mGABA_A-PSC frequency in recordings from the intact hippocampi incubated in the corresponding conditions. Each symbol represents the result of one single cell. The dashed line represents the mean value obtained from GABA_B-WT incubated in ACSF.

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induced by k252a (Fig. 5A), these data show that GABA\textsubscript{A}R-Rs promote the maturation of GABAergic synapses by controlling the amount of available extracellular BDNF.

Activation of GABA\textsubscript{A}R-Rs triggers BDNF release

We next asked how GABA\textsubscript{A}R-Rs control extracellular BDNF availability. GABA\textsubscript{A}R-Rs could modulate the production and/or secretion of BDNF. To determine whether GABA\textsubscript{A}R-Rs modulate BDNF production, we measured the level of BDNF in hippocampi of GABA\textsubscript{A}R-KO and GABA\textsubscript{A}R\textsubscript{WT} by use of an ELISA and found no significant difference (110 ± 7 pg/ml total protein vs 109 ± 10 pg/ml total protein, respectively, n = 5 for both).

In an attempt to determine whether GABA\textsubscript{A}R-Rs could modulate the secretion of BDNF, we sought to measure the amount of BDNF released from mouse hippocampal in vitro upon GABA\textsubscript{A}R activation using ELISA. However, levels of BDNF in these samples were below the threshold for detection of the assay. We therefore examined whether activation of GABA\textsubscript{A}R-Rs triggers BDNF release at the cellular level. With this aim, we transfected cultured hippocampal neurons with GFP-tagged BDNF and monitored dendritic BDNF-GFP secretion with time-lapse fluorescent imaging in living neurons. Using this approach, BDNF-GFP secretion was detectable as a decrease in intracellular GFP fluorescence intensity (Kuczewski et al., 2008b). To exclude indirect effects of synaptic activity, GABAergic and glutamatergic ionotropic receptor antagonists were present. Baclofen (10 \textmu M) led to a significant decrease of dendritic BDNF-GFP fluorescence intensity [−2.6 ± 0.63% change 5 min after baclofen application, n = 62 regions of interest (ROIs) from 14 cells, p = 0.0008 compared with control period, p = 0.01 compared with nonstimulated neurons, n = 107 ROIs from 23 cells] (Fig. 6A–C). The decrease in fluorescence intensity induced by baclofen was prevented by CGP55845 (10 \textmu M, −0.74 ± 0.53% change 5 min after baclofen application, n = 42 ROIs from 11 cells, p = 0.03 compared with baclofen alone) (Fig. 6D) and by nominally Ca\textsuperscript{2+}-free extracellular solution (0.005 ± 0.005% change after 5 min of baclofen application, n = 31 ROIs from 7 cells, p = 0.0006 compared with baclofen alone) (Fig. 6E).

Figure 6. GABA\textsubscript{A}R activation triggers BDNF-GFP secretion. A, Examples of dendritic BDNF-GFP granules are shown before and after baclofen application. Blue and red circles highlight examples of the BDNF-GFP clusters of fluorescence analyzed. The fluorescence was enhanced to near-saturation levels to make fluorescence variation visible. Scale bar, 5 \textmu m. B–E, Average time course of dendritic fluorescence variation in control, nonstimulated neurons (B) (n = 107 ROIs from 23 cells), and in baclofen-stimulated neurons in the absence (C) (n = 62 ROIs from 14 cells) or presence (D) (10 \textmu M, n = 42 ROIs from 11 cells) of CGP55845, or in nominally Ca\textsuperscript{2+}-free external solution (E) (0.5 mM Ca\textsuperscript{2+}, 200 \textmu M Cd\textsuperscript{2+}, n = 31 ROIs from 7 cells). All experiments were performed in the presence of bicuculline (20 \textmu M), NBQX (10 \textmu M), and D-APV (40 \textmu M). F, Surface staining confirmed the BDNF-GFP secretion produced by baclofen. Overlapped images showing intracellular BDNF-GFP fluorescence (green) and secreted BDNF-GFP detected using anti-GFP antibody (red) under nonpermeabilized conditions in control (bicuculline (20 \textmu M), NBQX (10 \textmu M), and D-APV (40 \textmu M)) and baclofen-treated sister cultures. Released BDNF-GFP that bound to the extracellular membrane of BDNF-GFP-expressing neurons appears as a yellow signal. Scale bar, 20 \textmu m. G, Quantitative analysis of surface-bound BDNF-GFP on BDNF-GFP-expressing neurons (yellow signal/green signal) in control (n = 10 cells), baclofen-treated (n = 10 cells), and baclofen (10 \textmu M) and CGP55845 (10 \textmu M)-treated cultures (n = 11 cells) in the presence of bicuculline (20 \textmu M), NBQX (10 \textmu M), and D-APV (40 \textmu M). Surface-bound BDNF-GFP on BDNF-GFP-expressing neurons was also quantified in nontreated (n = 10 cells) and CGP55845 (GFP; 10 \textmu M)-treated cultures (n = 5 cells). Three different sister cultures were used in each condition.
BDNF-GFP on transfected neurons shows that baclofen significantly increased BDNF-GFP release \( (n = 10\) cells for both, 3 cultures, \( p = 0.04 \) compared with control) (Fig. 6A). This effect was prevented by CGP55845 (10 \( \mu M \), \( n = 11\) cells, 3 cultures) (Fig. 6G), which when applied alone had no effect on surface-bound BDNF-GFP in control conditions \( (n = 5\) cells, 3 cultures) (Fig. 6G).

These data show that GABAB-R activation can trigger secretion of BDNF-GFP from hippocampal neurons in culture. However, protein overexpression could have modified the release properties of BDNF. To determine whether GABAB-R activation can trigger secretion of endogenous BDNF, we used the phosphorylated form of the cAMP response element-binding protein CREB (pCREB) as a sensor of endogenous BDNF release (Kuczewski et al., 2008b). Once released, BDNF interacts with TrkB receptors to activate downstream signaling pathways. One of the most common is the ERK (extracellular signal-regulated kinase) pathway, which leads to the phosphorylation of CREB (Ghosh et al., 1994). We therefore tested whether baclofen can induce a BDNF-dependent phosphorylation of CREB. Hippocampal neuronal cultures were stimulated with baclofen (3 and 50 \( \mu M \)) for 10 min in the presence of NQBX (10 \( \mu M \)), D-APV (50 \( \mu M \)), and bicuculline (20 \( \mu M \)). To rule out a possible effect of baclofen treatment on CREB synthesis and to normalize the results obtained from different cultures, the pCREB/CREB ratio was quantified (Fig. 7A, B). At 3 \( \mu M \), baclofen induced a significant increase of the pCREB/CREB ratio \( (30 \pm 11\% \) in control \( (n = 74\) cells, 3 cultures) versus 43 \( \pm 7\% \) in baclofen-treated \( (n = 64\) cells, 3 cultures) cultures, \( p = 0.001 \) ). At 50 \( \mu M \), baclofen induced a threefold increase of the pCREB/CREB ratio \( (from 27 \pm 5\% \) in control \( (n = 42\) cells, 3 cultures) versus 77 \( \pm 13\% \) in baclofen-treated \( (n = 54\) cells, 3 cultures) cultures, \( p = 0.00008 \) ) (Fig. 7B). This increase was prevented by CGP55845 (10 \( \mu M \), \( n = 43\) cells; 3 cultures, \( p = 0.0003 \) compared with baclofen) (Fig. 7B), showing that GABAB-R activation is required. No difference in the pCREB/CREB ratio was observed when the cultures were stimulated by baclofen in the presence of the BDNF scavenger TrkB-IgG (2 \( \mu g/ml\), \( n = 27\) cells, 3 cultures, \( p = 0.0002 \) compared with baclofen) (Fig. 7B). As expected, BDNF (20 \( ng/ml\)) triggered a phosphorylation of CREB and an increase in the pCREB/CREB ratio \( (n = 40\) cells, 3 cultures, \( p = 0.0001 \) compared with control) (Fig. 7B). Thus, GABAB-R activation triggers secretion of endogenous BDNF.

**Discussion**

GABA, the main inhibitory transmitter in the adult vertebrate brain, has recently emerged as an important signal for neuronal development. Besides its classical role in regulating synaptic activity, GABA modulates nearly all key steps of network construction from neuronal migration to experience-dependent refinement of local connections (Ben-Ari et al., 2007). Most of these effects have been attributed to the depolarizing action of GABA, which leads to a postsynaptic rise in intracellular Ca\(^{2+}\) concentration in developing neurons via the activation of chloride-permeable GABA\(_A\)-Rs (Ben-Ari et al., 2007). Here, we reveal a novel mechanism by which endogenous GABA selectively regulates the development of GABAergic synapses. We found that activation of metabotropic GABA\(_B\)-Rs triggers secretion of BDNF and promotes the development of GABAergic synapses in the hippocampus of newborn mice. Moreover, we show that this process occurs during ongoing physiological patterns of activity.

Our study shows that GABAB-R signaling plays a role in self-regulating inhibitory synapse development. We have identified a selective deficit in miniature GABAergic synaptic activity in GABAB1-KO hippocampal pyramidal cells that can be reproduced in vitro in wild-type hippocampi in which GABA\(_B\)-Rs were pharmacologically blocked. Miniature GABAergic activity was also reduced in wild-type hippocampi incubated with TTX, to block action potential-dependent synaptic activity and subsequent activation of GABAB-Rs. The TTX-induced deficit was rescued by the selective GABA\(_B\) receptor agonist, baclofen, showing that GABAB-R activation is required for the functional maturation of GABAergic synapses. Although we cannot completely rule out functional presynaptic modifications, we provide morphological data suggesting that the reduced GABAergic activity in GABAB1-KO mice is at least in part due to a decrease in the number of perisomatic GABAergic terminals. This result is consistent with previous findings that synaptic activity (Chattopadhyaya et al., 2004) and endogenous GABA levels (Chattopadhyaya et al., 2007) regulate cortical basket cell axon branching and perisomatic synapse formation through the activation of GABA\(_A\) and GABAB\(_R\) receptors. Since miniature activity has been proposed to originate from proximal...
GABAergic synapses (Soltész et al., 1995), a decrease in the density of those synapses will significantly affect the frequency of mGABAB-β2-PSCs in GABAβ1-KO pyramidal cells.

The timing of disappearance of GDPs, whose generation critically depends on depolarizing action of GABA (Ben-Ari et al., 2007), was not affected between GABAβ1−KO and WT mice. This result suggests that the depolarizing-to-hyperpolarizing developmental shift in GABA, receptor-mediated responses is not affected in GABAβ1−KO mice. Accordingly, we found that bath-applied isoguvacine, a selective GABAβ, agonist, increased the spiking activity of CA3 pyramidal neurons during the first postnatal week of life in both GABAβ1−KO and WT mice (unpublished observations). Several studies have shown that the depolarizing and excitatory action of GABAβ, receptors is an important signal for neuronal network development (Barbin et al., 1993; Manent et al., 2005; Cancetta et al., 2007; Wang and Kriegstein, 2008); this might explain the lack of structural alterations or modifications in the morphology of CA3 pyramidal neurons in GABAβ1−KO mice.

We show that the mechanism by which GABAβ−Rs promote the formation of GABAergic synapses in vivo likely involves BDNF secretion and subsequent activation of the TrkB signaling pathway. k252a or TrkB-IgG mimicked and occluded the deletion of BDNF secretion and subsequent activation of the TrkB signaling pathway. neuronal cultures (Kuczewski et al., 2009): (1) tetanic stimulation of dendritic BDNF secretion have been directly identified in neuronal cultures (Magby et al., 2006). Our study provides a novel and unexpected mechanism by which synaptic activity can trigger a Ca2+−dependent dendritic release of BDNF.

Important questions remain about the cell type and the location of GABAβ−Rs involved in vivo. Because GABAergic interneurons and glial cells do not produce neurotrophins themselves (Ernfors et al., 1990), BDNF is likely provided by CA3 pyramidal neurons (Brigadski et al., 2005) or the mossy fibers arising from dentate gyrus granular cells (Danzger and McNamara, 2004). GABAβ−R activation can increase the intracellular Ca2+ concentration in neurons (Shen and Slaughter, 1999; Hirono et al., 2001; New et al., 2006). A recent study has shown that the phosphorylation of α-CaMKII, a critical step in BDNF secretion (Kolarow et al., 2007), is enhanced by GABAβ−R activation in the developing rat hippocampus (Xu et al., 2008). Thus, postsynaptic Ca2+ elevation and α-CaMKII phosphorylation might underlie the GABAβ−R-induced secretion of BDNF and development of GABAergic synapses that we found in the present study. Alternatively, GABAβ−Rs can increase Ca2+ concentration in glial cells (Kang et al., 1998; Meier et al., 2008) and indirectly trigger neuronal secretion of BDNF (Elmariah et al., 2005). Further experiments will be required to address this point.

Regulated activity-dependent release of BDNF is crucial for many different aspects of GABAergic and glutamatergic synapse development (Lu et al., 2005). However, because BDNF diffusion (Horch and Katz, 2002) and Ca2+ rise are rather restricted events, spatial proximity is required between the signal that triggers the secretion of BDNF and the target. Synaptic activation of ionotrophic glutamatergic receptors has been previously reported to trigger a localized dendritic release of BDNF (Hartmann et al., 2001) and a potentiation of GABAergic synaptic activity in the developing rat hippocampus (Kuczewski et al., 2008a). However, glutamatergic synapses are restricted to the dendrites. Thus, activation of somatic GABAβ−Rs might trigger the local and specific secretion of BDNF needed for the development of perisomatic GABAergic synapses. Such specificity in the control of BDNF secretion might explain why perisomatic GABAergic synapses, but not glutamatergic or dendritic GABAergic synapses, are impaired in GABAβ1−KO hippocampi. Thus multiple triggers of BDNF secretion might coexist in the hippocampus. Depending on the pattern of activity generated by the neuronal network, BDNF could exert a selective control on the development of different subpopulations of GABAergic synapses. The reason why GABAβ−R-mediated trophic action is selective for perisomatic GABAergic synapses remains to be clarified. One likely possibility is that the effectors linking GABAβ−R activation to BDNF secretion are exclusively localized at the perisomatic level.

GABAβ−Rs are located at perisynaptic or extrasynaptic sites, and thus GABA spillover is needed to activate them. GABA spillover becomes substantial during high-frequency stimulation (Isacson et al., 1993; Xu et al., 2008) or concomitant activation of several interneurons or when the GABA uptake is blocked (Scanziani, 2000). In the developing hippocampus, most of the ongoing synaptic activity is provided by a primitive network-driven pattern, termed GDPs, present both in vitro (Ben-Ari et al., 1989) and in vivo (Leinekugel et al., 2002). GABAergic interneurons fire synchronously during GDPs (Khazipov et al., 1997). GDPs therefore fulfill the criteria for GABAβ−R activation (McLean et al., 1996). Accordingly, we found that the lack of GABAβ−R function in GABAβ1−KO neurons resulted in lengthening of GDPs. In contrast, when GDPs were blocked in GABAβ1−WT neurons, the GABAβ−R antagonist had no significant effect on spontaneous GABAergic activity. Thus, the activation of GABAβ−Rs required the presence of GDPs. We therefore propose that GDPs play an instructive role in the development of the hippocampal GABAergic circuit, providing the critical amount of GABA needed to activate GABAβ−Rs and trigger a subsequent secretion of BDNF. Accordingly, a deficit in GABAergic synaptic activity was observed when GDPs were blocked with TTX, but not when GDPs were blocked with the GABAβ−R agonist baclofen. Thus, baclofen substitutes for GDPs to activate GABAβ−Rs showing that the activation of these receptors is sufficient to support a proper development of at least perisomatic GABAergic synapses. This conclusion is strengthened by the observation that the TTX-induced deficit was rescued by baclofen. Since early spontaneous network-driven activity similar to GDPs and subsequent activation of GABAβ−Rs appear to occur in virtually every developing circuit (Obristian and Van den Pol, 1999; Catsicas and Mobbs, 2001), it is possible that this mechanism is important for synaptic maturation throughout the nervous system.

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