Published in final edited form as:

Eur J Neurosci. 2014 July ; 40(1): 2183–2195. doi:10.1111/ejn.12568.

Reduced levels of brain-derived neurotrophic factor contribute to synaptic imbalance during the critical period of respiratory development in rats

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Abstract

Previously, our electrophysiological studies revealed a transient imbalance between suppressed excitation and enhanced inhibition in hypoglossal motoneurons of rats on postnatal days (P) 12–13, a critical period when abrupt neurochemical, metabolic, ventilatory, and physiological changes occur in the respiratory system. The mechanism underlying the imbalance is poorly understood. We hypothesized that the imbalance was contributed by a reduced expression of brain-derived neurotrophic factor (BDNF), which normally enhances excitation and suppresses inhibition. We also hypothesized that exogenous BDNF would partially reverse this synaptic imbalance. Immunohistochemistry/single neuron optical densitometry, real-time quantitative polymerase chain reaction, and whole-cell patch-clamp recordings were done on hypoglossal motoneurons in brain stem slices of rats during the first three postnatal weeks. Our results indicated that: 1) the levels of BDNF and its high-affinity TrkB receptor mRNAs and proteins were relatively high during the first 1-1½ postnatal weeks, but dropped precipitously at P12–13 before rising again afterwards; 2) exogenous BDNF significantly increased the normally lowered frequency of spontaneous excitatory postsynaptic currents (sEPSCs) but decreased the normally heightened amplitude and frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) during the critical period; 3) exogenous BDNF also decreased the normally heightened frequency of miniature IPSCs (mIPSCs) at P12–13; and 4) the effect of exogenous BDNF was partially blocked by K252a, a TrkB receptor antagonist. Thus, our results are consistent with our hypothesis that BDNF and TrkB play an important role in the synaptic imbalance during the critical period. This may have significant implications for the mechanism underlying Sudden Infant Death Syndrome (SIDS).

Keywords

BDNF/TrkB; critical period; immunohistochemistry; real-time quantitative PCR; whole-cell patch clamp recording

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All authors have no conflict of interest to declare.

Introduction

A delicate balance between excitation and inhibition is crucial to the normal functioning of the CNS (Mody *et al.*, 1994) and is especially important for normal respiratory control. Our previous studies revealed a transient imbalance between suppressed excitation and enhanced inhibition in multiple brain stem respiratory-related nuclei around postnatal days (P) 12–13 in rats (Wong-Riley & Liu, 2005; 2008). There is a sudden decrease in the expression of glutamate and NMDA receptor, and a marked increase in that of GABA, GABA_B receptor, and glycine receptor at P12 (Liu & Wong-Riley, 2002; 2004; 2005; 2006; 2008; 2010a; Gao *et al.*, 2011). The expression of serotonergic neurochemicals also underwent significant reduction at P12 (Liu & Wong-Riley, 2008, 2010a). These changes suggest a transient synaptic imbalance with adverse effects on respiratory control. Indeed, the animals' ventilatory and metabolic responses to hypoxia were also at their weakest (Liu *et al.*, 2006; Liu *et al.*, 2009).

The exact mechanism underlying such an imbalance is poorly understood. We hypothesized that a reduced expression of brain-derived neurotrophic factor (BDNF) and its high-affinity receptor, tyrosine receptor kinase B (TrkB) contributed to the imbalance during the critical period. BDNF plays an important role in neuronal development and synaptic transmission (Huang & Reichardt, 2001; Waterhouse & Xu, 2009). It is critical for respiratory system development (Bouvier *et al.*, 2008) and its dysregulation contributes to Rett syndrome (Ogier *et al.*, 2007). BDNF knockout animals exhibit severe respiratory abnormalities and die within weeks after birth, most likely from respiratory complications (Erickson *et al.*, 1996). BDNF potentiates excitatory and attenuates inhibitory synaptic transmission by acting on presynaptic terminals (Carvalho *et al.*, 2008) and postsynaptic receptors (Tanaka *et al.*, 1997; Brunig *et al.*, 2001). Thus, if its own level is reduced during the critical period, it may contribute to the synaptic imbalance at that time.

Hypoglossal motoneurons (HMs) were chosen because they control tongue extruder muscles that keep upper airway patent during breathing (Lowe, 1980). Their rhythmic bursting activity is synchronous with the respiratory rhythm and is the gold standard for monitoring respiratory activity (Lowe, 1980; Richard & Harper, 1991; Singer & Berger, 1999). Nearly all of inhibitory and excitatory ligand-gated ion channels are present in HMs, and their activation determines the function of the hypoglossal nerve (Greer *et al.*, 1991; Funk *et al.*, 1993). Abnormalities in HMs are implicated in Sudden Infant Death Syndrome (SIDS) (Konrat *et al.*, 1992; Lavezzi *et al.*, 2009). HMs also exhibit all of the neurochemical changes found in other respiratory nuclei during the critical period. Moreover, we have compiled a large database of HM's electrophysiological properties in postnatal development, and they exhibit synaptic imbalance during the critical period (Gao *et al.*, 2011).

To test our hypothesis, immunohistochemistry, real-time quantitative PCR, and whole-cell patch-clamp recordings were done on HMs in rat brain stem slices during the first three postnatal weeks. Our results are consistent with our hypothesis that reduced expressions of BDNF and TrkB contribute to synaptic imbalance during the critical period.

Materials and Methods

All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996), and all protocols were approved by the Medical College of Wisconsin Animal Care and Use Committee (approval can be provided upon request). All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation for immunohistochemistry

A total of 138 Sprague-Dawley rats, both male and female, from 11 litters were used. Rat pups at each postnatal days P0, 2, 3, 4, 5, 7, 10, 11, 12, 13, 14, 17, and 21 were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.; Diamondback Drugs, Scottsdale, AZ) and perfused through the aorta with 4% paraformaldehyde-4% sucrose in 0.1 M sodium phosphate buffered saline (PBS), pH 7.4. Brain stems were then removed, postfixed in the same fixative for 3 h at 4°C, cryoprotected by immersion in increasing concentrations of sucrose (10, 20, and 30%) in 0.1 M PBS at 4°C, then frozen on dry ice and stored at -80°C until use.

Characterization of antibodies

Table 1 includes a brief summary of the antibodies used in the present study. Both antibodies (anti-BDNF and anti-TrkB) have been well characterized and their specificities established by the manufacturer and previous investigators. The anti-BDNF polyclonal antibody (sc-546, Santa Cruz Biotechnology) was a purified immunoglobulin raised against an internal region of human BDNF and did not cross-react with neurotrophic factor 3 (NT-3) or nerve growth factor (NGF). By western blot analysis, this antibody specifically yielded a single band at ~ 14 kDa (Ricci *et al.*, 2004) and did not show any immune signals in brain tissues of BDNF knockout mice (Tongiorgi *et al.*, 2004). The immunoreactivity was abolished with preadsorption of this antibody (Ricci *et al.*, 2004; Tongiorgi *et al.*, 2004). This sc-546 pAb has been used by a number of investigative groups (Ricci *et al.*, 2004; Tongiorgi *et al.*, 2004; Hwang *et al.*, 2005; Tan & Shepherd, 2006; Galvao *et al.*, 2008; Matsumoto *et al.*, 2008). The anti-TrkB rabbit polyclonal antibody (sc-12, Santa Cruz Biotechnology) was a purified immunoglobulin raised against the intracellular C-terminus (amino acid 794–808) of mouse TrkB and did not cross-react with tropomyosin-related kinase A or C (TrkA or TrkC). Its specificity was confirmed by a single band at the expected molecular size (~ 145 KDa) in western blots and in preadsorption tests (Ricci *et al.*, 2004). This antibody has also been used by several groups (Ricci *et al.*, 2004; Hecht *et al.*, 2005; Lee *et al.*, 2007).

Immunohistochemistry

Coronal sections (12-μm thickness) of frozen brain stems were cut with a Leica CM1900 cryostat (Leica Microsystems, Heidelberg, Nussloch, Germany). Ten sets of serial sections were mounted on gelatin-coated slides. In the same litter, sections from 3 (or 4) rats at different ages were mounted on the same slides and processed together. Ages were grouped typically as follows: P2-10-21, P0-3-4-17, P5-7-14, and P11-12-13. All sections from all rats were processed under identical conditions (i.e., time, temperature, and concentration of

reagents) (Liu & Wong-Riley, 2013). Sections were blocked overnight at 4°C with 5% nonfat dry milk-5% normal goat serum-1% Triton X-100 in 0.1 M PBS (pH 7.4). They were then incubated at 4°C for 36 hours in the primary antibodies: 1:100 for rabbit anti-BDNF or 1:1000 for rabbit anti-TrkB receptors, diluted in the same solution as used for blocking. Sections were then rinsed 3 times, 5 minutes each, in PBS, before incubating in the secondary antibodies: 1:100 goat anti-rabbit IgG-HRP (Bio-Rad, Hercules, CA), diluted in the modified blocking solution (without Triton X-100) for 4 hours at room temperature. After rinsing twice with PBS and once with 0.1 M ammonium phosphate buffer (APB), pH 7.0, immunoreactivity was detected with 0.05% DAB-0.004% H₂O₂ in APB for 5 minutes. The reaction was then stopped with APB for 5 minutes, rinsed in PBS three times, dehydrated, and coverslipped. Control sections were processed without primary antibodies or with a non-immune serum in place of the primary antibodies.

The percentage of labeled neurons for BDNF and TrkB in the hypoglossal nucleus (XII) was calculated based on the number of labeled neurons divided by the total number of neurons shown in adjacent Nissl-stained sections through the entire extent of the XII. Approximately 9–12 sections (depending on the age), spanning 1,080–1,440 µm rostrocaudally, and about 800–1,200 neurons in each XII on each side were included in the analysis. Only neurons sectioned through their centers (i.e., with clear nucleus) were counted. The range of percentages during development was based on values obtained at P2, P7, P12, and P21.

Semi-quantitative optical densitometry

Immunoreaction products of BDNF and TrkB receptors in cell bodies of individual neurons in XII were semiquantitatively analyzed by optical densitometry performed with a Zeiss Zonax MPM03 photometer, a 25x objective, and a 2 µm-diameter measuring spot centered on the cytoplasm of individual neurons (Liu & Wong-Riley, 2002). White (tungsten) light was used for illumination, and all lighting conditions were held constant for all of the measurements. As light intensity can directly affect optical densitometric values, a stepped density filter (Edmund Industrial Optics, Barrington, NJ) with 10-step increments of 0.1 and optical density values from 0.1 to 1 was used to precisely adjust the intensity of the light source to a standard value identical for all samples. The white matter was used as an internal standard for measurements because of its very low level of immunoreactivity. Thus, the white matter was set at zero for each section measured.

The optical densitometric value of each labeled neuron in the XII was an average of two to four readings on the cell body (excluding the nucleus). Only those neurons whose nuclei were clearly visible (i.e., sectioned through the middle of the cell body) were measured. To avoid measuring the same neuron more than once, values were taken from cells in sections at least 120 µm apart, as the largest neurons had a maximal diameter of 25–30 µm, with a maximal nuclear diameter of only about 10 µm. About 100 neurons were measured for each marker in each rat, and a total of about 700 neurons from seven rats derived from seven litters (for BDNF) or 1100 neurons from eleven rats derived from eleven litters (for TrkB receptor) at each age for each marker were measured. For statistical analyses, each sample's optical density value for each nucleus of each rat was the average of about 100 labeled neurons. Thus, the sample number of each time point for each marker was seven in Figure

1E and eleven in Figure 1J. A total of 23,400 neurons were measured for the present study. Mean optical density values, standard deviations, and standard errors of the mean for each maker at each age were then obtained.

Photomicrographs were taken with a CCD camera (SPOT RT3) mounted on a light microscope (Nikon E600) using SPOT Advanced 4.6 software (Diagnostic Instruments, Sterling Heights, MI) and processed with Adobe Photoshop 6.0 (San Jose, CA). Contrast and brightness adjustments were identical for all photomicrographs in each plate.

RNA isolation and cDNA synthesis

Another three litters of Sprague-Dawley rats, both male and female were used for real-time quantitative PCR. Rat pups at postnatal days P2, 4, 7, 10, 11, 12, 13, 14, and 21 from each of the three litters were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.; Diamondback Drugs) and decapitated. Hypoglossal nuclei were freshly dissected, and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was treated with DNAase I to remove any trace of contaminating DNA, and the reaction was stopped by heating at 65°C in the presence of EDTA. cDNA was synthesized using iScript cDNA synthesis kit (170-8891, BioRad, Hercules, CA, USA) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RTqPCR)

RTqPCR was carried out in a BioRad iCycler using IQ SYBR Green Supermix (BioRad) and following the manufacturer's instructions. Primer sequences used were as follows: *Actb*, forward: 5' GTGACGTTGACATCCGTAAAGA 3', reverse: 5' GCCGGACTCATCGTACTCC 3'; *BDNF* forward: 5' CCGGTATCCAAAGGCCAACT 3', reverse: 5' CTGCAGCCTTCCTTGGTGTA 3'; and *TrkB* forward: 5' CGGATGTTGCTGACCAAACC 3', reverse: 5' ACCCATCCAGGGGGATCTTA 3'. PCR runs: hot start 3 min at 95°C, denaturation 15 s at 95°C, annealing 30 s according to the T_m of each primer, and extension 35 s at 72°C for 20–40 cycles. Melt curve analyses verified the formation of single desired PCR product. Rat *Actb* (β-actin) was the internal control, and the 2^{-CT} method (Livak & Schmittgen, 2001) was used to calculate the relative amount of transcripts.

Brain stem slice preparations

A total of 128 Sprague-Dawley rats from 35 litters at postnatal days P0 to P16, daily, were used. Rats were anesthetized with isoflurane inhalation and decapitated. The brains were removed quickly and cooled in ice-cold sucrose-cerebrospinal fluid (sucrose-CSF) that contained the following (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgSO₄, 26 NaHCO₃, 25 glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate, pH 7.4. The brain stems were dissected, and horizontal slices (300 μm thick) containing the hypoglossal nucleus were cut using a Vibratome (Microslicer DTK-1000, Ted Pella, Inc., Redding, CA, USA) in ice-cold sucrose-CSF gassed with carbogen (95% O₂-5% CO₂). The slices were transferred to an incubation chamber and maintained for 1 h in artificial CSF (ACSF) that contained the following (in mM): 119 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25

NaH₂PO₄, 26 NaHCO₃, and 10 glucose. The ACSF was saturated with carbogen (95% O₂-5% CO₂) at room temperature (22.5°C).

Electrophysiological Recording

Individual slices were transferred to a recording chamber on the microscope stage equipped with infrared-differential interference contrast microscopy (Olympus BX51W1, Olympus America Inc., PA, USA). Slices were submerged with a constant flow of oxygenated ACSF and stabilized with platinum wire weights. Hypoglossal motoneurons were identified by their location, cell size, and shape (Umekiya & Berger, 1994; Berger *et al.*, 1996; Gao *et al.*, 2011).

For recording sEPSCs, 1 μM strychnine and 10 μM bicuculline were added to ACSF to block glycinergic and GABAergic currents, respectively. For recording sIPSCs, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; an AMPA/kainate receptor antagonist) and 25 μM D-2-amino-5-phosphopentanoic acid (D-APV; a NMDA receptor antagonist) were added to the ACSF to block AMPA and NMDA currents, respectively. For recording the mEPSCs and mIPSCs, 0.5 μM tetrodotoxin (TTX; a voltage-dependent Na⁺ channel blocker) was included in the ACSF. After recording the basic synaptic activity for 10 min, 100 pM BDNF was applied to the bath solution. This concentration of BDNF was chosen because it was found to be effective for neurons at different age groups (Kron *et al.*, 2007). K252a (a TrkB receptor antagonist) was applied subsequently at a concentration of 100 nM.

Patch pipettes were pulled from borosilicate (KG-33) glass capillary tubings (1.5 mm outer diameter; King Precision Glass, CA, USA) with a Narishige PC-10 two-stage electrode puller (Narishige International Inc., NY, USA). When recording sEPSCs or mEPSCs, the pipette solution consisted of (in mM): 125 Cs-gluconate, 10 CsCl, 10 HEPES, 10 EGTA, 1.2 MgCl₂, 2 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine (pH = 7.25). When recording sIPSCs or mIPSCs, the pipette solution consisted of (in mM): 135 CsCl, 10 HEPES, 10 EGTA, 1.2 MgCl₂, 2 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine, pH 7.25 with CsOH. The pipette resistance was 3–5 MΩ when filled with the above saline. Spontaneous and miniature IPSCs and EPSCs were recorded at a V_H of –70 mV in HMs. Whole-cell recordings were made using a patch clamp amplifier (Multiclamp 700B; Molecular Devices, Union City, CA, USA). Data acquisition and analysis were performed using a digitizer (DigiData 1440A) and analysis software pClamp 10 (Molecular Devices) as well as Mini Analysis v. 6.0.3 (Synaptosoft, Decatur, GA, USA). Signals were filtered at 2 kHz and sampled at 10 kHz. Series resistance (10 – 25 MΩ) was monitored throughout the recordings and data were discarded if the resistance changed by > 20%. We performed series resistance compensation (70–80%) for sEPSCs/sIPSCs because their larger amplitude may introduce voltage-clamp errors. However, we did not perform series resistance compensation for miniature PSCs because of the small amplitude of miniature PSCs and the possibility of increasing base line noise (Gao *et al.*, 2011). The basic electrophysiological characteristics of HMs, such as resting membrane potential, membrane capacitance, input resistance, rheobase current and series resistance, were the same as described previously (Gao *et al.*, 2011).

All common chemicals were obtained from Sigma (St. Louis, MO, USA). CNQX and all other drugs (e.g., antagonists) were from Tocris Bioscience (Ellisville, MO, USA).

All recordings were performed at $32 \pm 1^\circ\text{C}$ by using an automatic temperature controller (Warner Instrument, Hamden, CT, USA). All drugs were bath applied in the ACSF and recordings to be analyzed did not commence until the responses were stabilized. In each 10–30 min recording, we analyzed 5 continuous min of stabilized recording.

Data analysis and statistics

The amplitude, frequency, and kinetic properties of spontaneous and miniature EPSC and IPSC were determined by MiniAnalysis software (Mini Analysis software version 6, Synaptosoft). Decay kinetics were measured as the time for the PSC to decay to 37% of its peak amplitude (O'Brien & Berger, 1999). Effects of BDNF on PSC amplitude and frequency were assessed for each cell by comparing control and test plots in cumulative probability (Wang *et al.*, 2006), using the Kolmogorov–Smirnov 2-sample test, which estimates the probability that two distributions are similar. Events that did not return to the baseline or composed by multiple peaks were not included in the kinetic analysis.

Data are presented as the mean $\pm$ SEM. Statistical comparisons were made among the age groups or among the control and BDNF application group by using one-way analysis of variance (ANOVA) (to control for the type I comparisonwise error rate). Among the age groups, when significant differences were found, comparisons were made between successive age groups (e.g., P2 vs. P3, P3 vs. P4, and P5 vs. P6) by using Tukey's Studentized range test (a *post hoc* multiple comparisons, to control for the type I experimentwise error rate). Additional Tukey's tests were conducted between two groups that were not immediately adjacent to each other, and significant differences, if any, were presented in the Results section (but not shown in the graphs to minimize confusion). Values of $p < 0.05$ were considered significant.

Results

BDNF-immunoreactive neurons in the hypoglossal nucleus (XII)

BDNF immunoreactivity (-ir) was visible in cell bodies and proximal dendrites of ~70% – 85% of neurons in the hypoglossal nucleus (XII) (Figs. 1A–D) and in dendrites and axons of the neuropil. The plasma membrane of 25% – 45% of labeled neurons had detectable immunoreaction product (see inset in Fig. 1B). Between P0 and P7, the nuclei of ~50% – 75% of BDNF-ir neurons were also labeled, but the percentage fell to ~25% – 30% in the P10 to P21 age groups (Figs. 1A–D). The nuclear distribution of BDNF is thought to influence transcription (Wetmore *et al.*, 1991; Nitta *et al.*, 1997; Furukawa *et al.*, 1998). Control sections had no specific immunoreactivity above background (data not shown).

BDNF-ir neurons were multipolar, oval, or fusiform in shape and mainly medium or large in size (Figs. 1A–D). Medium-sized neurons ranged from 10.5 to 14.5 μm in diameter at P2 to 14–19.5 μm at P21, and large neurons ranged from 16–19 μm at P2 to 22–27.5 μm at P21. ANOVA indicated significant differences in immunoreactivity among the ages ($P < 0.01$). The intensity of BDNF-ir increased gradually during the first postnatal week with a peak at

P7, followed by a slight decline at P10–11, a significant fall at P12 ($P < 0.01$) that remained low at P13, then a significant rise at P14 ($P < 0.001$) followed by a plateau until P21 (Fig. 1E). Tukey's test revealed that the value at P7 was significantly higher than those of other age groups (except for P5 and P10) ($P < 0.05 - P < 0.001$, significance not shown in Fig. 1E), whereas the values at P12 and P13 were significantly lower than those of other age groups (except for P0 and P21) ($P < 0.05 - P < 0.001$, significance not shown in Fig. 1E).

TrkB-immunoreactive neurons in the hypoglossal nucleus (XII)

TrkB immunoreactivity was clearly visible in ~70% – 90% of neurons in XII (Figs. 1F – I). Labeling was present in cell bodies and proximal dendrites of neurons as well as in dendrites and axons of the neuropil. There was no nuclear labeling. In addition to the cytoplasm, the plasma membrane of 30% – 50% of labeled neurons showed clear immunoreaction product (see inset in Fig. 1G), but the intensity decreased somewhat at P21 (about 15%). From P0 to P10, TrkB-ir was relatively homogeneous in the cytoplasm, but became more punctate and granular afterwards, with ~80% – 90% of neurons at P21 possessing that pattern (arrowheads in Fig. 1I). Some glial cells, much smaller in size than neurons, exhibited TrkB-ir around P11 and later, but they were excluded from our optical densitometric analyses. The sizes, shapes, developmental trends, and prevalence of TrkB-ir neurons were comparable to those of BDNF-ir neurons. Control sections demonstrated no specific immunoreactive product above background (data not shown).

ANOVA indicated significant differences ($P < 0.01$) in the intensity of TrkB-ir among the ages. It was relatively low at P0, increased gradually to peak at P10, then fell significantly at P12 ($P < 0.05$), followed by a gradual rise at P13–14 and a plateau until P21 (Fig. 1J). Tukey's tests revealed that the value at P12 was significantly lower than those at P4, P7, P10, and P14 ($P < 0.01 - P < 0.001$), and that at P10 was significantly higher than those at P0, P13, and P21 ($P < 0.05 - P < 0.01$; significance not shown in Fig. 1J).

BDNF mRNA levels in the hypoglossal nucleus (XII)

The developmental trend of BDNF gene expression in the hypoglossal nucleus was tested with real-time quantitative PCR for rats at P2, 4, 7, 10, 11, 12, 13, 14, and 21 (Fig. 2A). The trend was very similar to that of BDNF protein (compare Fig. 2A with Fig. 1E). The level peaked at P7, which was significantly different from that at P4 ($P < 0.001$). It then fell at P10 ($P < 0.001$), followed by another significant fall at P12 ($P < 0.01$), the lowest level found among all of the time points tested. The level remained low at P13, but rose significantly at P14 ($P < 0.001$), with a further fall at P21 ($P < 0.01$).

TrkB mRNA levels in the hypoglossal nucleus (XII)

The developmental trend of TrkB transcripts was tested in the same samples as those for BDNF. As shown in Figure 2B, the trend was very similar to that of TrkB protein (see Fig. 1F). The level rose significantly at P7 as compared to P4 ($P < 0.001$) and it peaked at P10. The level then fell somewhat at P11 ($P < 0.05$) but had a significant fall at P12 ($P < 0.001$), the lowest point found among all of the ages tested. It then rose at P13 and remained at a moderate level at P14 and at P21.

Electrophysiological recordings of hypoglossal motoneurons (HMs)

Control HMs recorded in the present study were comparable to those in our previous experiments in shape, size and basic electrophysiological characteristics (resting membrane potential, membrane capacitance, input resistance, rheobase current, and series resistance) (Gao *et al.*, 2011) and will not be repeated here. The mean size of hypoglossal motoneurons recorded did not undergo significant change from P0 to P16 ($P > 0.05$). The membrane capacitance also did not change significantly (increased from 51.64 ± 1.44 pF at P0 to 53.02 ± 0.97 at P16; $P > 0.05$). Thus, the amplitude did not undergo major changes due to cell size or to cell membrane capacitance with age from P0 to P16.

Effects of exogenous BDNF on sEPSCs during development

Figure 3A shows representative traces of sEPSCs at P1, P7, P12, and P16 in control, BDNF-treated, and BDNF-plus-K252a-treated HMs. Each neuron recorded was subjected to the same three conditions and the effects appeared after 2 min of BDNF application. As shown in Fig. 3B, the amplitude was relatively low at P3, P12 and P13, but was relatively high at P2, P11 and P16. The significant reductions at P3 ($P < 0.05$, $n = 11$) and P12 ($P < 0.05$, $n = 15$) were comparable to those reported previously (Gao *et al.*, 2011). After BDNF (100 pM) application, the amplitude of sEPSCs did not change significantly (Figs. 3A and 3B), neither did the input resistance of the membrane (data not shown). The same was true after K252a (100 nM) (Fig. 3A and 3B). The cumulative probability plot of sEPSCs' amplitudes at P12 also did not show a significant difference before and after BDNF or BDNF plus K252a application (Fig. 3D). The frequency of sEPSCs was relatively low from P0 to P2, increased gradually at P3, peaked at P7, then plateaued for about 4 days before declining at P12 and P13, followed by an increase at P14 that was sustained until P16 (Figs. 3A and C). When BDNF was applied, the frequency of sEPSCs increased by 6% – 15% before the critical period, by 34% – 41% during the critical period (P12 – P13), and by 10% – 25% after the critical period. The only time points that were significantly different from controls, however, were at P12 ($P < 0.05$, $n = 15$) and P13 ($P < 0.05$, $n = 17$) (Fig. 3C). K252a effectively blocked this increase (Fig. 3A and 3C). The cumulative probability plot of sEPSCs' inter-event interval at P12 was in line with the increase (Fig. 3E). There was a significant shift to the left after BDNF ($P < 0.05$, $n = 15$), indicating an increase in frequency. K252a effectively blocked this shift induced by BDNF. Thus, during development, HMs had a lower frequency at P12 and P13, and exogenous BDNF significantly reversed this trend through TrkB receptors.

Superimposed average trace (averaged from 5 min of recording) of sEPSC before and after BDNF application at P7 and P12 are shown in Fig. 4A and 4B, respectively. The amplitude of sEPSC at P7 was larger than that at P12, but BDNF did not induce a significant change in either one of them. The rise time (Fig. 4C) and decay time (Fig. 4D) of sEPSCs decreased gradually with development, and BDNF did not significantly alter such kinetics, indicating that BDNF did not induce a significant change in channel properties for sEPSCs.

Effects of exogenous BDNF on sIPSCs during development

Figure 5A illustrates original sIPSC traces at P1, P7, P12, and P16 before and after BDNF or with BDNF-plus-K252a. Each neuron recorded was subjected to the same three conditions.

As reported previously (Gao *et al.*, 2011), the amplitude of sIPSCs increased significantly at P12 and P16 and the frequency increased at P3 and P12 (Figs. 5B). BDNF (100 pM) induced a significant decrease in the amplitude of sIPSCs at P12 ($P < 0.05$, $n = 18$) and at P16 ($P < 0.05$, $n = 10$) (Fig. 5B). The frequency of sIPSCs was also significantly decreased by BDNF at P12 ($P < 0.05$, $n = 18$) and at P15 ($P < 0.05$, $n = 13$) (Fig. 5C). The cumulative probability curve of sIPSCs' amplitudes at P12 shifted significantly to the left ($P < 0.05$, $n = 18$) after BDNF application (Fig. 5D), indicating that BDNF reduced the amplitude of sIPSCs at that time, and K252a blocked this effect. The cumulative probability plot of sIPSCs' inter-event interval shifted significantly to the right ($P < 0.05$, $n = 18$) after BDNF application (Fig. 5E), denoting that BDNF decreased the frequency of sIPSCs at P12. K252a blocked this increase effectively (Fig. 5A, 5B and 5C).

Figures 6A and 6B show superimposed average traces (averaged from 5 min of recording) of sIPSCs before and after BDNF application at P7 and P12, respectively. In controls, the amplitude at P12 was larger than that at P7, but BDNF reduced both at both ages, such that the amplitudes became comparable at the two ages. Thus, BDNF eliminated the heightened amplitude at P12. An age-dependent reduction in the 10 – 90% rise time of sIPSCs is evident in Fig. 6C, but BDNF did not induce a significant change with development (Fig. 6C). The decay time to 37% of peak amplitude of sIPSCs also showed a progressive decrease with increasing age, and again, BDNF did not induce a significant change with development (Fig. 6D). These results indicate that the kinetic properties of inhibitory channels were not significantly affected by exogenous BDNF.

A striking finding was that during the first week and half of postnatal development, some HMs showed an increase in the amplitude and/or frequency of sIPSCs with exogenous BDNF. The percent change in amplitude or frequency either in the positive or negative direction after BDNF is shown in Figs. 6E and 6F. In general, responses in the positive direction were more prevalent before P3. However, with development and especially after P8, responses in the negative direction became more dominant. Approximately 50% of HMs at P0–P1 showed either increased or decreased responses to BDNF (percentages of neurons that showed decreased responses are indicated below the downward, black bars in Figs. 6E and 6F). The percentage of HMs that showed increased responses fell progressively with age, such that after P14, virtually all neurons exhibited reduced amplitude and frequency of their sIPSCs with exogenous BDNF. Interestingly, the greatest change in amplitude and frequency occurred at P12.

Effects of exogenous BDNF on mEPSCs and mIPSCs during development

Miniature EPSCs and IPSCs were recorded from P0 to P16 HMs with TTX in the bath solution. Figure 7A illustrates original traces of mEPSCs at P9 in controls (Fig. 7A top row) and after BDNF application (Fig. 7A, 2nd row). BDNF did not significantly alter the amplitude or frequency of mEPSCs. In the presence of BDNF, CNQX (10 μ M) induced nearly 95% elimination of mEPSCs (Fig. 7A, row 3). When both CNQX and D-AP5 were applied, all mEPSCs were virtually eliminated (Fig. 7A, row 4). Figure 7B shows typical mIPSCs during 10 min of stabilized recordings of P9 HMs in control samples (Fig. 7B, top row). Exogenous BDNF induced a 16.77% reduction in the frequency and a 10.83%

reduction in the amplitude of mIPSCs (Fig. 7B, 2nd row). BDNF plus bicuculline (10 μ M) in the bath for 3 min partially inhibited mIPSCs (Fig. 7B, row 3), and the addition of strychnine (1 μ M) virtually eliminated all mIPSCs (Fig. 7B, row 4). These experiments indicate that BDNF did not change significantly the mEPSCs, which were mediated mainly by AMPA receptors (inhibited by CNQX). However, BDNF did decrease (although not reaching significance) the frequency and amplitude of mIPSCs, which were mediated by both glycinergic and GABAergic currents at P9.

The amplitude and frequency of mEPSCs at P10 (before the critical period), P12 (critical period) and P14 (after the critical period) are shown in Fig. 7 C–F. The developmental trend of mEPSC amplitude from P0 to P16 in controls was comparable to that described previously (Gao *et al.*, 2011), with a striking reduction during the critical period (P12–P13). Exogenous BDNF did not change the amplitude of mEPSCs, nor did the BDNF-plus-K252a application (Fig. 7C). BDNF did increase the frequency of mEPSCs by 14.41% at P12, but it did not reach statistical significance ($P > 0.05$, $n = 15$) (Fig. 7D). At the same concentration, BDNF had a weaker effect on mEPSC than on sEPSC (see Fig. 3C). The amplitude of mIPSCs in controls increased dramatically at P12, but BDNF induced a 11.67% reduction (Fig. 7E) that did not reach statistical significance ($P > 0.05$, $n = 17$). Likewise, the frequency of mIPSCs in controls was significantly higher at P12 than at P10 ($P < 0.001$, $n = 11$) (Fig. 7F), but BDNF induced a significant decrease at P12 ($P < 0.05$, $n = 17$) (Fig. 7F). K252a partially reversed the effect of BDNF (Fig. 7F).

Discussion

Our present large-scale, in-depth, developmental studies of the first three postnatal weeks revealed that 1) the levels of BDNF and TrkB protein and mRNA were high during the first 1-1½ postnatal weeks, but dropped precipitously at P12–13 before rising again afterwards; 2) exogenous BDNF significantly increased the normally lowered frequency of sEPSCs but decreased the normally heightened amplitude and frequency of sIPSCs during the critical period; 3) exogenous BDNF also decreased the normally heightened frequency of mIPSCs at P12–13; 4) the effect of exogenous BDNF was partially blocked by K252a, a TrkB antagonist; and 5) the rise time and decay time of spontaneous or miniature IPSCs and EPSCs did not change significantly after BDNF application. Taken together, our results are consistent with our hypothesis that reduced expressions of BDNF and TrkB contribute to the synaptic imbalance during the critical period in HMs, and that exogenous BDNF can partially but significantly reverse such an imbalance.

The role of BDNF outside of the critical period

BDNF, acting via its high-affinity receptor TrkB, has been studied intensively on its growth-promoting and trophic effects during development (Gottmann *et al.*, 2009). It is particularly important for brain stem neurons, including those involved in respiratory control and normal breathing (Del Castillo & Katz, 1954).

BDNF and synaptic transmission are tightly linked in two ways: 1) BDNF is a key modulator of synaptic transmission (Cowansage *et al.*, 2010). It enhances excitatory neurotransmission and suppresses inhibitory ones (Wardle & Poo, 2003). It facilitates

glutamatergic synaptic transmission via both pre- and postsynaptic mechanisms (Schinder *et al.*, 2000), but it attenuates GABAergic and glycinergic inhibitory transmission (Wardle & Poo, 2003; Bardoni *et al.*, 2007). BDNF reduces the frequency of sIPSCs and the amplitude of evoked IPSC (eIPSC) in the Kolliker-Fuse nucleus of postnatal rats (Kron *et al.*, 2007). BDNF also influences neuronal excitability by enhancing membrane conductance and long-term potentiation (Desai *et al.*, 1999; Baker-Herman *et al.*, 2004; Lu *et al.*, 2010); 2) BDNF regulates the release of glutamate and the expressions of NMDA, non-NMDA, GABA receptors, as well as serotonergic receptor 5-HT_{2A} (Tanaka *et al.*, 1997; Narisawa-Saito *et al.*, 1999; Daftary *et al.*, 2012; Kim *et al.*, 2012). However, BDNF itself is regulated by these neurochemicals as well as by 5-HT_{1A} and 5-HT_{2C} receptors (Berninger *et al.*, 1995; Xiong *et al.*, 2002). Thus, a mutual feed-forward and feedback mechanism exists between the activation of BDNF and that of synaptic neurochemicals.

During the first 1½ postnatal weeks, the levels of BDNF and TrkB in HMs are relatively high, and up to 70% – 85% neurons have clear BDNF and TrkB immunoreactivity. This suggests that BDNF and TrkB play an important role in neuronal and synaptic development of HMs. The relatively high BDNF expression during the first 1½ weeks of postnatal life indicates that it was probably operating at or close to a saturated level, so that exogenous BDNF would not have a detectable effect. Significantly, the developmental trend of sEPSC frequency is almost identical to that of BDNF, with a gentle rise from P0 that peaked at P7 and plateaued until P11 (compare Fig. 3C with 1E). As the frequency of postsynaptic currents is driven primarily by presynaptic factors (Bekkers & Stevens, 1990), a positive effect of BDNF on the frequency of sEPSCs (the present study) supports a positive presynaptic influence. This is consistent with BDNF's reported role in enhancing presynaptic glutamate release at excitatory synapses (Poo, 2001; Tyler *et al.*, 2002). Possible insertion of new receptors that may affect the frequency of PSCs (Li & Wolf, 2011) can be ruled out in this case because a) the time frame is relatively short (most of our analyzed data were from 5 to 10 min after BDNF application, during which changes in pTrkB are likely but newly synthesized receptor proteins would take a longer time (Li & Wolf, 2011; Song *et al.*, 2013)); and b) additional receptors would affect both the frequency and amplitude of PSCs (Shinohara, 2012), and we observed a change in only the frequency but not the amplitude of sEPSCs. The amplitude of sEPSCs reflects mainly postsynaptic events (Walmsley *et al.*, 1998). Both NMDA and non-NMDA receptors mediate EPSC currents, with the amplitude of the former being much smaller than that of the latter (Singer *et al.*, 1996; O'Brien & Berger, 1999). Based on the amplitude of sEPSCs recorded in HMs (present study), most of the sEPSCs were mediated by AMPA receptors. These findings are consistent with previous reports that BDNF has relatively little effect on postnatal AMPA receptors (Grosshans *et al.*, 2002). Thus, we suggest that BDNF enhances the development of excitatory synapses in HMs through mainly a presynaptic mechanism during the first one to one-and-half weeks of postnatal life.

The effect of BDNF on the development of sIPSCs is more complex. IPSCs are mediated by GABAergic and/or glycinergic transmission (Wu *et al.*, 1992; O'Brien & Berger, 1999; Gao *et al.*, 2011). In HMs, the frequency of sIPSCs increased progressively during the first two postnatal weeks, peaking at P12 and plateaued thereafter. The amplitude of sIPSC also did

not show a significant increase until P12. Thus, the development of sIPSCs in HMs lags behind that of EPSCs by several days. Previously, we found that there is an apparent switch in the percent contribution to total mIPSCs in HMs, with GABA dominating during the first postnatal week and glycine taking over thereafter (Gao *et al.*, 2011). The expression of Na⁺K⁺Cl⁻-cotransporter 1 (NKCC1; a Cl⁻ importer) is high in HMs (as well as in other respiratory-related nuclei) from P0 to P11, whereas that of K⁺Cl⁻-cotransporter 2 (KCC2; a Cl⁻ exporter) climbs from P0 to P11 (Liu & Wong-Riley, 2012). Although our data do not directly address the issue of depolarization to hyperpolarization shift for Cl⁻-mediated currents, such was reported in the rat HMs postnatally (Singer *et al.*, 1998) and in other regions of the brain (Ben-Ari *et al.*, 2012). Interestingly enough, BDNF induced different sIPSCs responses in different HMs during the first 1½ postnatal weeks (the present study). In one population, exogenous BDNF enhanced the frequency and amplitude of sIPSCs, whereas in the other the effect was suppressive. Such a dichotomy ended around the critical period, after which all HMs recorded had the amplitude and frequency of their sIPSCs suppressed by exogenous BDNF. This implies that not all HMs mature at the same rate, and those that were enhanced by exogenous BDNF represent a more immature group of neurons. More importantly, our data suggest that BDNF has a differential developmental influence on sIPSCs as the Cl⁻-mediated synaptic transmission matures in HMs. Such a transitional role of BDNF has not been reported previously in any respiratory-related nuclei or in other regions of the brain. Interestingly, the effect of BDNF on KCC2 reportedly varies with development, up-regulating it during late embryonic and early postnatal age and down-regulating it at a later stage, especially in the adult (Aguado *et al.*, 2003; Rivera *et al.*, 2004; Ludwig *et al.*, 2011). The exact transitional stage is unknown at present.

After the critical period, the expression of BDNF is maintained at a moderate level. Exogenous BDNF caused a slight increase in the frequency of sEPSC, although it did not reach statistical significance. It is likely that BDNF sustains a positive influence on excitatory synaptic transmission, as it does in other regions of the brain (Martin & Finsterwald, 2011). On the other hand, exogenous BDNF reduced the normally heightened amplitude and frequency of sIPSCs at P15 and at P16, indicating that BDNF's role in the more mature state is to suppress inhibitory transmission.

The role of BDNF during the critical period

The abrupt fall in BDNF and TrkB expressions in HMs during the critical period (P12–13), which exists in all of the normal animals we have examined (the present study), coincides precisely with a significant suppression of excitatory and a significant enhancement of inhibitory synaptic transmission in these neurons at this time (Gao *et al.*, 2011).

Concurrently, the expressions of excitatory neurochemicals (glutamate and NMDA receptor subunit 1) is drastically reduced whereas that of inhibitory neurochemicals (GABA, GABA_B receptors and glycine receptors) is significantly enhanced in HMs and in many other respiratory-related nuclei (Wong-Riley & Liu, 2008). Although the cause and effect are not known at this time, three possibilities can be considered: a) The down-regulation of BDNF/TrkB induces the down-regulation of excitatory neurochemicals and the up-regulation of inhibitory neurochemicals; b) a pre-programmed reduction in the expression of excitatory neurochemicals and a rise in that of inhibitory neurochemicals, in turn, lead to suppressed

expression of BDNF/TrkB; or c) a combination of a and b. Nevertheless, the critical period is a transitional time when the development of excitatory synapses has already peaked (Gao *et al.*, 2011) and refinement of respiratory circuits necessitates inhibitory modulation. We hypothesize that the obligatory down-regulation of BDNF during this time enables inhibitory synapses to flourish, thus leading to a transient imbalance between suppressed excitation and enhanced inhibition within the respiratory network (Wong-Riley & Liu, 2008; Liu & Wong-Riley, 2010c). Such a transition opens a window of vulnerability, during which the animals are less responsive to respiratory stressors such as hypoxia (Liu *et al.*, 2006; Liu *et al.*, 2009). Other changes also contribute to this unstable period, including a switch in subunit composition of GABA_A receptors (from neonatal $\alpha 3$ to mature $\alpha 1$), of glycine receptors (from $\alpha 2$ and $\alpha 3$ to $\alpha 1$), and of NMDA receptors (from 2D to 3B) close to the critical period (Liu & Wong-Riley, 2004; 2006; 2010c). Several serotonergic neurochemicals (including the synthesizing enzyme tryptophan hydroxylase, serotonin transporter SERT, and serotonin receptors 1A, 1B, and 2A) also have a precipitous fall in their expressions during the critical period (Liu & Wong-Riley, 2010a; 2010b). As a reciprocal relationship exists between serotonin and BDNF, such that each affects the synthesis of the other (Daftary *et al.*, 2012; Calabrese *et al.*, 2013), suppressed expression of one will induce likewise in the other, and vice versa. A combination of these factors contribute to a window of susceptibility during the critical period (Liu *et al.*, 2006; Liu *et al.*, 2009), and critical period is a known risk factor for SIDS.

Exogenous BDNF or TrkB agonists reportedly restored wild type breathing frequency in a mouse model of Rett Syndrome, in which BDNF expression and TrkB activation in the brain stem have been reduced (Schmid *et al.*, 2012). Likewise, exogenous BDNF can partially reverse the synaptic imbalance within HMs during the critical period (the present study), when there is a transient down-regulation of BDNF and TrkB in many respiratory-related nuclei (Liu & Wong-riley, 2013; the present study).

Acknowledgments

This study was supported by NIH grant HD048954.

Abbreviations

BDNF	brain-derived neurotrophic factor
HMs	hypoglossal motoneurons
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
SIDS	Sudden Infant Death Syndrome
TrkB	tyrosine receptor kinase B

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Fig. 1.

The expression of BDNF and TrkB during the first three postnatal weeks. (A–E) Brain-derived neurotrophic factor (BDNF) immunoreactive (-ir) neurons and neuropil in the hypoglossal nucleus (XII) at postnatal days P2 (A), P7 (B), P12 (C), and P21 (D). The expression of BDNF was relatively high at P2 and P7, but was markedly reduced at P12, followed by an increase at P21. (E) Optical densitometric measurements of BDNF-ir in individual neurons from P0 to P21. Data points represent mean $\pm$ SEM. BDNF-ir increased gradually during the first postnatal week and peaked at P7, followed by a slight decline at P10–11 and a significant reduction at P12 ($P < 0.01$) that remained low at P13. A significant rise then occurred at P14 ($P < 0.001$) followed by a plateau until P21. Diagrammatic insets in (A) indicate the location of XII in a cross-section of the medulla. Inset in (B) highlights the plasma membrane labeling of BDNF-ir neurons. ANOVA yielded significant differences in the expression of BDNF among the ages in XII ($P < 0.01$). **, $P < 0.01$; ***, $P < 0.001$, revealed by Tukey's Studentized tests between one age group and its immediately adjacent younger age group. Scale bar: 20 μ m (6.66 μ m for insets). (F–J) Tyrosine protein kinase B receptors (TrkB)-ir neurons and neuropil in XII at P2 (F), P7 (G), P12 (H), and P21 (I). TrkB-ir was low at P2, increased at P7, followed by a significant fall at P12 and a higher level at P21. (J) Optical densitometric measurements of TrkB-ir in individual neurons from P0 to P21. There was a gradual rise in the expression during the first 1½ weeks and a peak at P10, then a significant fall at P12 ($P < 0.05$) followed by a gradual rise at P13–14 and a plateau until P21. Diagrammatic insets in (F) indicate the location of XII. Inset in (G)

highlights the plasma membrane labeling of TrkB-ir neurons. Arrowhead in (I) shows the granular appearance of TrkB-ir after P10 as opposed to the more homogeneous appearance before P10. ANOVA yielded significant differences in the expression of TrkB among the ages ($P < 0.01$). *, $P < 0.05$, revealed by Tukey's test between adjacent age groups.

Fig. 2.

mRNA levels of BDNF (A) and TrkB (B) in XII at postnatal days P2, 4, 7, 10, 11, 12, 13, 14 and 21 analyzed by real-time quantitative PCR. (A) Among the ages tested, BDNF level peaked at P7 and was the lowest at P12. It remained low at P13, but rose significantly at P14. (B) TrkB level rose significantly at P7 as compared to P4 and peaked at P10. It then fell at P11 and reached its lowest level at P12. Data points represent mean $\pm$ SEM. $N = 3$ at each time point for each graph. ANOVA indicated significant differences among the groups for BDNF and for TrkB. Tukey's post hoc test revealed significant differences (marked by asterisks) when comparing one age group with its adjacent younger age group. *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$.

Fig. 3.

Amplitudes and frequencies of sEPSCs in HMs changed after BDNF application. Whole cell patch clamp recordings were made at a holding potential of -70 mV in the presence of 10 μ M bicuculline and 1 μ M strychnine. (A) Traces of sEPSCs recorded at different postnatal days (P1, P7, P12, and P16) before and after BDNF or BDNF-plus-K252a application. (B) Mean amplitude (in pA) of sEPSCs from P0 to P16 before and after BDNF or BDNF-plus-K252a application. (C) Mean frequencies (in Hz) of sEPSCs from P0 to P16 before and after BDNF or BDNF-plus-K252a application. BDNF induced a significant rise at P12–13 ($P < 0.05$). (D) Cumulative probability of sEPSCs' amplitudes at P12 before and after BDNF or BDNF-plus-K252a application. (E) Cumulative probability of sEPSCs' inter-event interval at P12 before and after BDNF or BDNF-plus-K252a application. The shift to the left in (E) was significant ($P < 0.05$). Data are presented as mean $\pm$ SEM. * $P < 0.05$ (significance between control and BDNF; i.e., before and after BDNF application), # $P < 0.05$ (significance between adjacent age groups).

Fig. 4.

The rise time and decay time of sEPSCs did not change before and after BDNF application. (A) The average traces overlapped before and after BDNF application at P7. (B) The average traces overlapped before and after BDNF application at P12. (C) The 10–90% rise time of sEPSCs accelerated with development, but did not change after BDNF application. (D) The decay time to 37% of peak amplitude of sEPSCs accelerated with age, but did not change after BDNF application. Data are presented as mean $\pm$ SEM.

Fig. 5.

Amplitudes and frequencies of sIPSCs in HMs changed after BDNF application. Whole cell patch clamp recordings were made at a holding potential of -70 mV in the presence of glutamate antagonists CNQX ($10 \mu\text{M}$) and D-APV ($25 \mu\text{M}$). (A) Traces of sIPSCs recorded at different postnatal days (P1, P7, P12, and P16) before and after the application of BDNF or BDNF-plus-K252a. (B) Mean amplitude (in pA) of sIPSCs from P0 to P16 before and after BDNF or BDNF-plus-K252a application. Control amplitude rose significantly at P12 and P16 ($P < 0.05$). BDNF induced significant decreases at P12 and P16 ($P < 0.05$). K252a effectively blocked the effects of BDNF. (C) Mean frequencies (in Hz) of sIPSCs from P0 to P16 before and after BDNF or BDNF-plus-K252a application. Control frequency rose significantly at P3 and P12 ($P < 0.05$). BDNF induced significant decreases at P12 and P15 ($P < 0.05$). K252a effectively blocked the effects of BDNF. (D) Cumulative probability of sIPSCs' amplitudes at P12 before and after BDNF or BDNF-plus-K252a application. The shift to the left was significant ($P < 0.05$). (E) Cumulative probability of sIPSCs' inter-event interval at P12 before and after BDNF or BDNF-plus-K252a application. The shift to the right was significant ($P < 0.05$). Data are presented as mean $\pm$ SEM. * $P < 0.05$ (significance between control and BDNF), # $P < 0.05$ (significance between adjacent age groups).

Fig. 6.

The rise time and decay time of sIPSCs did not change before and after BDNF application, but two types of cellular responses to BDNF existed in HM during the first 1 ½ weeks. (A) Superimposed average traces of sIPSC before and after BDNF application at P7. (B) Superimposed average traces of sIPSC before and after BDNF application at P12. (C) The 10–90% rise time of sIPSCs accelerated with development, but did not change after BDNF application. (D) The 37% decay time of sIPSCs accelerated with age, but did not change after BDNF application. (E) Percent change in amplitude in either the positive or negative direction after BDNF application. The percent of neurons that exhibited responses to BDNF in the negative direction at different ages are shown below the dark bars. (F) Percent change in frequency in either the positive or negative direction after BDNF application. The percent of neurons that showed a decrease in frequency after exogenous BDNF is indicated below the dark bars. After P14, all neurons showed a reduction in amplitude and frequency of sIPSCs with exogenous BDNF.

Fig. 7.

Amplitudes and frequencies of mEPSCs and mIPSCs changed after BDNF application. Whole cell patch clamp recordings were made at a holding potential of -70 mV in the presence of TTX (0.5 μ M), 10 μ M bicuculline and 1 μ M strychnine for mEPSCs or in the presence of 10 μ M CNQX and 25 μ M D-APV for mIPSCs. (A) Total mEPSCs were recorded in HMs at P9, followed by the application of BDNF, BDNF plus 10 μ M CNQX, and BDNF plus 25 μ M D-APV. (B) Total mIPSCs were recorded in HMs at P9, followed by the application of BDNF, then BDNF plus 10 μ M bicuculline for 3 min, followed by BDNF plus 1 μ M strychnine. (C) Amplitudes of mEPSCs recorded at different postnatal days (P10, P12, and P14) in control, BDNF, and BDNF-plus-K252a. (D) Frequencies of mEPSCs recorded at different postnatal days (P10, P12, and P14) in control, BDNF, and BDNF-plus-K252a. (E) Amplitudes of mIPSCs recorded at different postnatal days (P10, P12, and P14) in control, BDNF, and BDNF-plus-K252a. (F) Frequencies of mIPSCs recorded at different postnatal days (P10, P12, and P14) in control, BDNF, and BDNF-plus-K252a. The frequency of mIPSCs was significantly decreased at P12 after BDNF application ($P < 0.05$). * $P < 0.05$.

Table 1

Primary antibodies used.

Antigen	Immunogen	Manufacturer, species, type	Catalog number	Dilution used
Brain-derived neurotrophic factor (BDNF)	Human BDNF, within an internal region of BDNF	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), rabbit polyclonal IgG, sc-546		1:100
Tropomyosin- related kinase B (TrkB) receptor	Intracellular C-terminus of mouse TrkB (amino acids 794–808)	Santa Cruz Biotechnology, Inc., rabbit polyclonal IgG, sc-12		1:1000