Long-term actions of BDNF on inhibitory synaptic transmission in identified neurons of the rat substantia gelatinosa

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Submitted 19 May 2011; accepted in final form 8 April 2012

Lu VB, Colmers WF, Smith PA. Long-term actions of BDNF on inhibitory synaptic transmission in identified neurons of the rat substantia gelatinosa. J Neurophysiol 108: 441–452, 2012. First published April 11, 2012; doi:10.1152/jn.00457.2011.—Peripheral nerve injury promotes the release of brain-derived neurotrophic factor (BDNF) from spinal microglial cells and primary afferent terminals. This induces an increase in dorsal horn excitability that contributes to “central sensitization” and to the onset of neuropathic pain. Although it is accepted that impairment of GABAergic and/or glycinergic inhibition contributes to this process, certain lines of evidence suggest that BABA release in the dorsal horn may increase after nerve injury. To resolve these contradictory findings, we exposed rat spinal cord neurons in defined-medium organotypic culture to 200 ng/ml BDNF for 6 days to mimic the change in spinal BDNF levels that accompanies peripheral nerve injury. Morphological and electrophysiological criteria and glutamic acid decarboxylase (GAD) immunohistochemistry were used to distinguish putative inhibitory tonic-islet-central neurons from putative excitatory delay-radial neurons. Whole cell recording in the presence of 1 μM tetrodotoxin showed that BDNF increased the amplitude of GABAergic and glycinergic miniature inhibitory postsynaptic currents (mIPSCs) in both cell types. It also increased the amplitude and frequency of spontaneous, action potential-dependent IPSCs (sIPSCs) in putative excitatory neurons. By contrast, BDNF reduced sIPSC amplitude in inhibitory neurons but frequency was unchanged. This increase in inhibitory drive to excitatory neurons and decreased inhibitory drive to inhibitory neurons seems inconsistent with the observation that BDNF increases overall dorsal horn excitability. One of several explanations for this discrepancy is that the action of BDNF in the substantia gelatinosa is dominated by previously documented increases in excitatory synaptic transmission rather than by impendence of inhibitory transmission.

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sensitization, changes such as these would be expected to impede its onset.

Further investigations of nerve injury- or BDNF-induced changes in inhibitory synaptic transmission are clearly warranted, but these are complicated by the heterogeneity of the neuronal population (Graham et al. 2007; Lu et al. 2009a).

Several electrophysiological phenotypes can be identified in substantia gelatinosa neurons both in acute slices and in organotypic culture. In response to depolarizing current commands, neurons evoke a tonic, delay, irregular, phasic, or transient firing pattern (Balasubramanyan et al. 2006; Lu et al. 2006, 2009a). A variety of neuronal morphologies have also been described, including islet, central, radial, and vertical or “stalked” cells (Hantman et al. 2004; Lu and Perl 2003, 2005; Lu et al. 2009a; Todd et al. 2010; Yasaka et al. 2007, 2010). In the present study, we have analyzed data from tonic-islet-central neurons and delay-radial neurons (Lu et al. 2009a). This is because virtually all tonic-firing neurons that exhibit an islet cell morphology are inhibitory GABA-glycinergic, whereas those that exhibit a delayed firing pattern and radial morphology (delay-radial neurons) are excitatory glutamatergic (Cui et al. 2011; Grudt and Perl 2002; Labrakakis et al. 2009; Lu and Perl 2003, 2005; Yasaka et al. 2010; Zhang and Dougherty 2011; Zheng et al. 2010). Analysis of the action of BDNF on these two neuron types allowed us to determine whether inhibitory transmission onto excitatory neurons is modulated in a different way from inhibitory transmission to inhibitory neurons. Experiments were carried out on rat substantia gelatinosa neurons in defined-medium organotypic culture (Biggs et al. 2011b; Lu et al. 2006) so that neurons could be exposed to BDNF for 6 days or more to mimic the effect of CCI in vivo (Lu et al. 2007, 2009a).

**MATERIALS AND METHODS**

All experiments were carried out with ethical approval from the University of Alberta Health Sciences Laboratory Animal Welfare Committee, which operates under the guidelines of the Canadian Council for Animal Care.

Defined-medium organotypic cultures of spinal cord slices. Organotypic culture slices were prepared and maintained in defined medium as described previously (Lu et al. 2006). Thus day 13–14 rat fetuses were delivered by cesarean section from timed-pregnant Sprague-Dawley rats (Charles River, Saint-Constant, PQ, Canada) under 2–5% isoflurane anesthesia. The female rat was then euthanized by intracardiac chloral hydrate (10.5%). Under 2–5% isoflurane anesthesia. The female rat was then euthanized by intracardiac chloral hydrate (10.5%). Sprague-Dawley rats (Charles River, Saint-Constant, PQ, Canada) fetuses were delivered by caesarean section from timed-pregnant females. All experiments were carried out with ethical approval from the Animal Care Committee, which operates under the guidelines of the Canadian Council for Animal Care.

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**ELECTROPHYSIOLOGY**

Whole cell patch-clamp recordings were obtained from neurons in organotypic slice cultures under infrared differential interference contrast optics (Fig. 1A). Neurons selected for recording were located 250–800 μm from the dorsal edge of the cultures in an area presumed to reflect the substantia gelatinosa and up to a depth of 100 μm from the surface. Neurons were categorized according to their firing pattern in response to depolarizing current commands as tonic; delay, phase, irregular, or transient (Lu et al. 2006, 2007).

Recordings were obtained with an NI SEC-05L amplifier (NI electronic, Tann, Germany) in discontinuous current- or voltage-clamp mode. For recording, slices were superfused at room temperature (~22°C) with 95% O2-5% CO2-saturated aCSF that contained (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH2PO4, 26 NaHCO3, 1.3 MgSO4, 2.5 CaCl2, and 25 d-glucose, pH 7.4. Patch pipettes were pulled from thin-walled borosilicate glass (1.5/1.12 mm OD/ID; WPI, Sarasota, FL) to 5- to 10-MΩ resistances when filled with an internal solution containing (in mM) 130 potassium gluconate, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.2, 290–300 mosM.

Spontaneous IPSCs (sIPSCs) were recorded for 3 min with the neuron clamped at 0 mV. At this voltage, Cl−-mediated inhibitory events are outward currents (estimated ECl− = −80 mV) whereas excitatory currents are inward but of small amplitude as they would occur close to their reversal potential (Chen et al. 2009). Although it was possible to pharmacologically isolate sIPSCs by using 10 μM CNQX plus 50 μM AP-5 to block both the AMPA and NMDA receptor components of spontaneous excitatory postsynaptic currents (sEPSCs), this antagonist mixture sometimes attenuated or occasionally completely blocked sIPSCs (Lu et al. 2009b). This presumably reflected impeded transmission of inhibitory synaptic drive to the inhibitory interneurons that were responsible for sIPSC generation. In view of this variable effect of CNQX/AP-5 on sIPSCs, we elected to use a holding potential of 0 mV rather than pharmacological methods to separate sIPSCs from sEPSCs. The validity of this approach is supported by the observation that 10 μM bicuculline plus 1 μM strychnine completely eliminated all spontaneous outward current activity recorded at 0 mV (Fig. 1B).

mIPSCs were recorded in a manner similar to sIPSCs but in the presence of 1 μM tetrodotoxin (TTX; Alomone Laboratories) to block action potential-dependent neurotransmitter release (Edwards et al. 1990) and to thereby reveal any actions of BDNF on the release process per se. Since mIPSCs with decay τ > 20 ms are likely GABAergic whereas those with τ < 20 ms are likely glycinergic (Keller et al. 2001), we were able to distinguish putative GABAergic events from glycinergic events on the basis of their rates of decay. The validity of this approach is supported by the experiments illustrated in Fig. 1, D–I. These show that 1 μM strychnine preferentially affects glycinergic mIPSCs with τ < 20 ms whereas 10 μM bicuculline affects GABAergic mIPSCs with τ > 20 ms.

Glutamic acid decarboxylase staining and morphological analysis. To identify dorsal horn neurons expressing glutamic acid decarboxylase (GAD), a marker of GABAergic neurons, neurons recorded in organotypic culture slices were labeled with biocytin for post hoc identification and probed with immunohistochemical techniques. Pri-
mary anti-GAD antibodies (1:8,000, rabbit; Chemicon, Temecula, CA) in 2% normal goat serum (NGS; Rockland, Gilbertsville, PA) and 0.3% Triton X-100 solution in PBS were incubated with organotypic cultured slices for 48 h at 4°C. Secondary antibodies (1:300, anti-rabbit conjugated to Alexa 488; Molecular Probes, Eugene, OR) in 2% NGS and 0.3% Triton X-100 in PBS were applied for 2.5 h. A Texas red-streptavidin conjugate used to stain biocytin-filled cells was added after 2 h of the start of incubation with the secondary antibody solution. Slices were washed thoroughly with distilled water before mounting on microscope slides. Stained slices were allowed to dry sufficiently before a coverslip was applied with Prolong Gold (Molecular Probes, Eugene, OR). The concentration of antibodies was optimized by using control slices to produce sufficient staining and reduce back-ground. The fluorescent dyes that were used, Texas red and Alexa 488, did not produce spectral crossover in staining controls (data not shown). Staining was visualized with a Zeiss inverted confocal laser scanning microscope (LSM 510; Zeiss, Toronto, ON, Canada) equipped with appropriate lasers (HeNe1, 543 nm; argon, 488 nm) and filters. Fluorescent confocal images and three-dimensional (3D) reconstructions were acquired with Zeiss LSM 510 imaging software (Zeiss LSM image browser, v. 3, 2, 70). Approximately 50% of the neurons recorded and categorized electrophysiologically were filled with biocytin. Successful recovery and visualization of morphology was possible for ~50% of these cells. Of these, only ~50% were unequivocally assignable to the islet central or radial neuron categories. This meant that morphological characterization was only obtained from ~12.5% of the neurons studied electrophysiologically.

Islet-central cells were identified in 3D reconstructions by the predominance of their rostrocaudal projections and radial cells by the presence of processes radiating in all directions from the soma (Grudt and Perl 2002) (see Fig. 2, B and D).

Data analysis and statistical testing. All data were acquired with Axon Instruments pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). Analysis was confined to recordings from tonic-firing neurons that displayed islet or central cell morphology (tonic-islet-central neurons) (Lu et al. 2009a), delayed-firing neurons that exhibited radiacl cell morphology (delay-radial neurons), and neurons exhibiting GAD immunoreactivity (GAD+ neurons).

Synaptic events were analyzed with Mini Analysis software (Synaptosoft, Decatur, GA). Peaks of events were first automatically detected by the software according to a set of threshold criteria. All detected events were then visually reexamined and accepted only if they displayed a monophasic rise time to peak <25 ms, a smooth offset, and an amplitude greater than five times the background noise. Typical recordings of sIPSCs are illustrated in Fig. 1C. We occasionally observed very large (up to 500 pA) sIPSCs that had deflections on their rising phase indicative of addition of multiple single events (Fig. 1C, right). Since these events contributed to the total amount of GABA released, they were not excluded from the analysis and were considered as single events. They are likely the consequences of bursts of action potentials that we previously observed in presynaptic neurons.

To generate cumulative probability plots for both amplitude and interevent time interval, the same number of events (100–200 events...
acquired after an initial 1 min of recording) from each neuron was pooled for each cell type and input into the Mini Analysis program. The Kolmogorov-Smirnov two-sample statistical test (KS test) was used to compare the distribution of events between control and BDNF-treated groups. Data for all mISPCs were initially analyzed to prepare event lists of amplitudes, interevent intervals (IEIs), and time constants ($\tau$) of decay. To obtain cumulative probability plots for pure GABAergic and glycinergic mIPSCs, events with $\tau > 20$ ms were separated from those with $\tau < 20$ ms.

Drugs and chemicals. Unless stated otherwise, all chemicals were from Sigma (St. Louis, MO). TTX (Alomone Laboratories) was dissolved in distilled water as a 1 mM stock solution and stored at $-20^\circ$C until use. TTX was diluted to a final desired concentration of 1 $\mu$M in external recording solution on the day of the experiment.

RESULTS

Tonic-islet-central and delay-radial neurons. Tonic-firing neurons are defined as low-threshold neurons that discharge APs at regular intervals in response to a depolarizing current command (Balasubramanyan et al. 2006). A typical example is illustrated in Fig. 2A. Figure 2B is a two-dimensional (2D) rendering of a 3D reconstruction of a putative islet or central cell. In our hands, rat spinal cord organotypic slices did not form a monolayer of cells as is the case for many neuron types. Culture thickness approximated the original 300 $\mu$m that was cut (Biggs et al. 2011b; but see also Avossa et al. 2006). Nevertheless, the restricted rostrocaudal projection from central neurons that have shorter projections in this direction (Grudt and Perl 2002). We have therefore used the term tonic-islet-central neurons to define this population (see also Lu et al. 2009a). Delayed-firing neurons are defined as those in which a noticeable delay precedes the initiation of AP discharge in response to depolarizing current (Balasubramanyan et al. 2006). A typical example is illustrated in Fig. 2C. A 2D rendering of a 3D reconstruction of a radial neuron is shown in Fig. 2D. Table 1 documents the input resistance and resting membrane potential of delay-radial and tonic-islet-central neurons.

Table 1. Properties of various neuronal types used in this study

<table>
<thead>
<tr>
<th>Neuronal Type</th>
<th>Resting Membrane Potential, mV</th>
<th>Input Resistance, $M\Omega$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonic-islet-central</td>
<td>$-48.5 \pm 2.8$ ($n=18$)</td>
<td>$551 \pm 68$ ($17$)</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonic-islet-central</td>
<td>$-46.6 \pm 3.0$ ($10$)</td>
<td>$436 \pm 63$ ($12$)</td>
</tr>
<tr>
<td>(BDNF treated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAD+ (control)</td>
<td>$-50.6 \pm 3.0$ ($11$)</td>
<td>$294 \pm 57$ ($10$)</td>
</tr>
<tr>
<td>GAD+ (BDNF treated)</td>
<td>$-48.5 \pm 2.0$ ($13$)</td>
<td>$270 \pm 42$ ($13$)</td>
</tr>
<tr>
<td>Delay-radial (control)</td>
<td>$-44.4 \pm 2.0$ ($21$)</td>
<td>$427 \pm 46$ ($22$)</td>
</tr>
<tr>
<td>Delay-radial (BDNF</td>
<td>$-48.4 \pm 2.3$ ($9$)</td>
<td>$274 \pm 74$ ($11$)</td>
</tr>
<tr>
<td>treated)</td>
<td></td>
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</table>

Values are means $\pm$ SE ($n$ in parentheses). BDNF, brain-derived neurotrophic factor; GAD, glutamic acid decarboxylase.
BDNF increases amplitude and frequency of sIPSCs in delay-radial (putative excitatory) neurons. Since delay-radial neurons are excitatory (Yasaka et al. 2010) and BDNF increases overall dorsal horn excitability (Biggs et al. 2010; Lu et al. 2007, 2009a), it might be predicted that inhibitory synaptic drive to this population would be decreased. However, this did not appear to be the case, as exposure of organotypic spinal cord cultures to BDNF for 6 days increased the amplitude of sIPSCs in delay-radial neurons by 45% and doubled their frequency. These data, which are from 8 control neurons and 12 BDNF-treated neurons, are shown as cumulative probability plots of sIPSC amplitude in Fig. 3A and IEI in Fig. 3C (P < 0.0001, KS test for both). The bar graphs in Figs. 3, B and D, illustrate the same data replotted using mean values ± SE. Increases in amplitude and frequency are again significant (P < 0.0001, t-test for both).

BDNF increases amplitude but not frequency of mIPSCs in delay-radial neurons. The sIPSCs analyzed above comprise both action potential-dependent and action potential-independent events (Edwards et al. 1990; Fredj and Burrone 2009; Sara et al. 2005) mediated by both GABA and glycine. It is possible therefore that the BDNF-induced alterations in action potential-dependent events, promoted by altered firing of presynaptic inhibitory neurons, may account for some of the observed changes (Lu et al. 2009b). To gain insight into effects of BDNF on inhibitory transmission that are independent of presynaptic action potentials, mIPSCs were recorded in the presence of 1 μM TTX. By separating slower mIPSCs (τ > 20 ms) from faster ones (τ < 20 ms), we were able to perform independent analysis of changes in GABAergic and glycine responses (see MATERIALS AND METHODS).

BDNF increased the mean amplitude of GABAergic mIPSCs (Fig. 4B; P < 0.0001). This change was also significant (P < 0.001) with the KS test (Fig. 4A). There was, however, little effect of BDNF on the frequency of GABAergic mIPSCs in delay-radial neurons, as there was no significant change in mean IEI (Fig. 4D) and analysis of data by KS statistics fell just short of defining a significant effect (P = 0.054; Fig. 4C). These data were obtained from four control neurons and five neurons treated with BDNF.

Analysis of the glycine mIPSCs generated by this cohort of neurons revealed a similar trend in the action of BDNF. Thus the amplitude of glycine mIPSCs was clearly increased (Fig. 4, E and F; P < 0.001 for both t-test and KS statistics), whereas there was only a weak tendency towards a decrease in frequency (Fig. 4, G and H; P < 0.003 for KS test but P > 0.5 for t-test).

BDNF decreases amplitude of sIPSCs in tonic-islet-central (putative inhibitory) neurons. Since tonic-islet-central neurons are inhibitory and BDNF increases overall dorsal horn excitability (Lu et al. 2007, 2009a), it might be predicted that inhibitory synaptic drive to this population would be increased, thereby promoting disinhibition. However, this did not appear to be the case, as BDNF reduced the amplitude of sIPSCs in tonic-islet-central neurons by 44%. The data, from 15 control neurons and 9 BDNF-treated neurons, are shown as cumulative probability plots of sIPSC amplitude (P < 0.0001, KS test) in Fig. 5A and replotted using mean values ± SE in Fig. 5B (P < 0.0001, t-test). Careful inspection of the data points as plotted in Fig. 5A also shows that there are only four events (marked with arrows) with amplitudes > 300 pA in the BDNF-treated neurons. This may reflect a reduction in the number of summed sIPSCs (see Fig. 1, B and C).

The effects of BDNF on frequency and IEI of sIPSCs in tonic-islet-central neurons were also complex. The cumulative probability plot in Fig. 5C shows a decrease in the number of short IEIs and an increase in the number of long IEIs (P < 0.002, KS test). There was however, no significant decrease in the mean IEI (Fig. 5D; P > 0.85, t-test). This effect of BDNF on sIPSC frequency in tonic-islet-central neurons is modest compared with its other actions in both delay-radial and tonic-islet-central neurons (Fig. 3, Fig. 5, A and B).

BDNF increases amplitude of mIPSCs in tonic-islet-central neurons. By contrast with the observed decrease in amplitude of sIPSCs (Fig. 5A), BDNF increased the amplitude of GABAergic mIPSCs in tonic-islet-central neurons by 22%. The data are shown as cumulative probability plots (P < 0.001, KS test) in Fig. 6A and replotted using mean data ± SE in Fig. 6B (P < 0.001, t-test). The frequency of GABAergic mIPSCs also appeared to increase (IEI decreased, Fig. 6C; P < 0.02, KS test). This difference was not reflected as a significant decrease in mean IEI (Fig. 6D; P = 0.56, t-test). These data
were obtained from six control neurons and five neurons treated with BDNF.

There was similarity between the actions of BDNF on GABAergic and glycinergic mIPSCs in this sampling of neurons. More larger-amplitude glycinergic IPSCs appeared after BDNF treatment (Fig. 6E), but there was no significant change in mean sIPSC amplitude (Fig. 6F). The frequency of glycinergic mIPSCs was unaffected by BDNF (Fig. 6, G and H).

Effects of BDNF on sIPSCs in GAD+ neurons. The observed decrease in amplitude of sIPSCs in tonic-islet-central neurons (Fig. 5, A and B) and the tendency toward longer IEIs (Fig. 5C) suggest that BDNF may decrease inhibitory drive to inhibitory neurons. We therefore examined the effect of BDNF on spontaneous inhibitory activity in inhibitory neurons that were identified by the presence of GAD immunoreactivity. Of these neurons, 11/24 exhibited a tonic firing pattern, 4/24 exhibited delayed firing, 5/24 exhibited transient firing, and 4/24 irregular firing. No neurons in this sample exhibited phasic firing.

BDNF significantly decreased the frequency and amplitude of sIPSCs in GAD+ neurons (Fig. 7, A–C). Data were collected from 11 control neurons and 13 neurons treated with BDNF. Figure 7, D–F, show localization of GAD immunoreactivity in a

Fig. 4. Effects of BDNF on amplitude and IEI of TTX-resistant mIPSCs in delay-radial neurons. A and B: cumulative probability plot (A) and bar graph (B) illustrating the effect of BDNF on mean amplitude of GABAergic mIPSCs. C and D: effect of BDNF on IEI of GABAergic mIPSCs. The events in the 2 min following the 1st minute of recordings from each neuron were pooled. Illustrated data are from 88 events from 4 neurons in control slices and 79 events from 5 neurons in BDNF-treated slices. Data were also collected for 156 glycinergic mIPSCs seen in the same cohort of control neurons and 118 such events seen in BDNF-treated neurons. E and F: cumulative probability plot (E) and bar graph (F) illustrating the effect of BDNF on mean amplitude of glycinergic mIPSCs. G and H: effect of BDNF on IEI of glycinergic mIPSCs. For bar graphs, error bars indicate SE.

Tonic-Islet-Central Neurons sIPSC analysis

Fig. 5. Effects of BDNF on amplitude and IEI of sIPSCs in tonic-islet-central neurons. A and B: cumulative probability plot [A; note that in the BDNF-treated group, only 4 individual events (indicated by arrows) exceed 250 pA in amplitude] and bar graph (B) illustrating the effect of BDNF on mean amplitude of sIPSCs. C and D: effects of BDNF on IEI of sIPSCs. The first 100 events in the 2 min following the 1st minute of recordings from each neuron were pooled. Illustrated data are from 1,736 events from 15 neurons in control slices and 918 events from 9 neurons in BDNF-treated slices. For bar graphs, error bars indicate SE.
GAD+ biocytin-filled neuron. Resting potential and input resistance of GAD+/H11001 neurons were unaffected by BDNF (Table 1).

Loss of inhibitory neurons as a result of apoptosis has been suggested to play a role in the development of neuropathic pain (Moore et al. 2002), and although others have argued against this mechanism (Polgar and Todd 2008), the extent of apoptosis may relate to the duration of the injury. In our experiments, the proportion of GAD+ neurons encountered in control organotypic cultures was not affected by BDNF exposure (11/19 controls were GAD+/H11001, 11/18 BDNF-treated neurons were GAD+/H11001; \( P > 0.8, \chi^2\)-test), suggesting that BDNF did not markedly alter the survival of this population of neurons.
Comparison of excitation and inhibition of substantia gelatinosa neurons. The observation that BDNF tends to increase inhibitory drive to excitatory delay-radial neurons (Figs. 3 and 4) and to decrease inhibitory drive to inhibitory tonic-islet-central neurons (Figs. 5 and 6) seems inconsistent with its ability to increase overall dorsal excitability (Biggs et al. 2011b; Lu et al. 2007, 2009a). We have previously shown that BDNF increases the frequency of action potential discharge in delay neurons and decreases it in tonic cells. Figure 8 shows similar results when more rigorous criteria are used to define neuronal phenotype. Thus Fig. 8A shows that BDNF increases action potential discharge frequency from 14.7 ± 6.0 to 33.0 ± 12.9 spike/min in excitatory delay-radial neurons. Sample recordings of spontaneous activity in delay-radial neurons in the absence and presence of BDNF are shown in the Fig. 8A, middle and bottom, respectively. Figure 8B shows that BDNF decreases the frequency of action potential discharge in tonic-islet-central neurons from 38.6 ± 6.2 to 15.8 ± 6.2 spikes/min. Sample recordings of spontaneous activity in tonic-islet-central neurons in the absence and presence of BDNF are shown in the Fig. 8A, middle and bottom, respectively.

An explanation for the discrepancies between BDNF-induced changes of inhibitory synaptic drive and changes of action potential frequency may lie in the observation that the frequency of sEPSCs in delay-radial neurons is 4.7 times greater than the frequency of sIPSCs (Fig. 8, C and E) and the frequency of sEPSCs in tonic-islet-central cells is 3.3 times greater than the frequency of sIPSCs (Fig. 8, D and F). Thus the excitatory synaptic drive to both neuron types is greater than the inhibitory synaptic drive. Since we have previously shown that BDNF increases excitatory synaptic drive to delay-radial neurons and decreases it to tonic-islet-central neurons (Lu et al. 2009a), it is possible that BDNF-induced alterations in excitatory synaptic transmission overcome the presently documented changes in inhibitory synaptic transmission within the substantia gelatinosa.

DISCUSSION

The main finding of this study is that BDNF increases inhibitory synaptic drive to putative excitatory substantia gelatinosa neurons yet decreases inhibition of putative inhibitory neurons (Table 2). Although these findings are consistent with studies that infer increases in GABAergic function after nerve injury or BDNF treatment (Bardoni et al. 2007; Fenselau et al. 2011; Pezet et al. 2002), this would predict that BDNF would decrease overall dorsal horn excitability. This clearly does not happen, as it is clearly established that BDNF increases overall excitability of the dorsal horn both in vivo (Coull et al. 2005) and in the organotypic cultures used in the present study (Biggs et al. 2010; Lu et al. 2007, 2009a). It is also difficult to rationalize our findings with a large body of behavioral and physiological results that are consistent with attenuation of GABAergic function in the onset of central sensitization (Baba et al. 2003; Coull et al. 2003, 2005; Laird and Bennett 1992; Loomis et al. 2001; Moore et al. 2002). In view of this, we suggest that the increase in GABAergic and glycinergic activity that we observe is insufficient to detract from the overall increase in excitability that BDNF produces as a result of its enhancing actions on excitatory synaptic transmission (Biggs et al. 2010; Lu et al. 2007, 2009a).

BDNF increases inhibitory synaptic drive to excitatory (delay-radial) neurons. The increase in sIPSC amplitude in delay-radial neurons after BDNF is highly significant (Fig. 3, A and...
show that BDNF decreases the frequency of APs in tonic-islet-central neurons that form inhibitory synapses onto excitatory neurons (Lu and Perl 2003). We thus have no satisfactory explanation for the observed increase in sIPSC frequency in delay-radial neurons (Fig. 3, C and D; Table 2).

**BDNF decreases inhibitory synaptic drive to inhibitory (tonic-islet-central) neurons.** The decrease in sIPSC amplitude in tonic-islet-central neurons is also highly significant (Fig. 5, A and B), whereas any decrease in sIPSC frequency may be regarded as modest (mean frequency unchanged, Fig. 5C; more long IEIs, Fig. 5D). Clearer effects are seen in GAD + neurons, where the both the amplitude and the frequency of sIPSCs are reduced (Fig. 7, A–C). The latter is consistent with our previous findings on tonic as opposed to tonic-islet-central neurons (Lu et al. 2009b) (see Table 2).

The lack of effect of BDNF on GABAergic mIPSCs in tonic-islet-central neurons (Fig. 6, C and D) and the small effect on glycinergic mIPSCs (Fig. 6, G and H) likely also correspond to the lack of change in overall mIPSC frequency seen in the more broadly defined tonic neuron population (Lu et al. 2009b).

The lack of correspondence between the effect of BDNF on sIPSC amplitude, which is decreased (Fig. 5A), and GABA and glycinergic mIPSC amplitude, which is increased, in tonic-islet-central neurons (Fig. 6, A and B; Table 2) is difficult to explain. One possibility is that the quantal size of unitary events (mIPSC amplitude) increases whereas the quantal content of sIPSCs (sIPSC amplitude) decreases. Interestingly, similar observations were reported by Bardoni et al. (2007), who observed an increase in mIPSC amplitude but a decrease in the amplitude of stimulus-evoked IPSCs in an unidentified subgroup of lamina II neurons after acute application of BDNF. This difference may also relate to the suggestion that mIPSCs and sIPSCs reflect release from entirely separate pools of vesicles (Fredj and Burrone 2009). Thus BDNF may decrease release from vesicles that are released by presynaptic action potentials while increasing release from a separate “resting” pool that is responsible for the generation of mIPSCs.

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**Fig. 8.** A and B: comparison of BDNF-induced changes in firing rates in delay-radial and tonic-islet-central neurons. A, top: bar graph comparing the mean number of spontaneous action potentials (APs) generated per minute at resting membrane potential in 11 control (open bar) and 10 BDNF-treated (hatched bar) delay-radial neurons. Data were obtained from 3-min recording in each neuron. The rate of AP discharge is significantly higher in the presence of BDNF (P < 0.05, Student’s t-test). Middle: sample 6-s current-clamp recording of a control delay-radial neuron. Bottom: sample 6-s current-clamp recording of a delay-radial neuron from a BDNF-treated culture. B, top: bar graph comparing the mean number of spontaneous APs generated per minute at resting membrane potential in 13 control and 11 BDNF-treated tonic-islet-central neuron. The rate of AP discharge is significantly lower in the presence of BDNF (P < 0.05, Student’s t-test). Middle: sample 6-s current-clamp recording of a control tonic-islet-central neuron. Bottom: sample 6-s current-clamp recording of a tonic-islet-central neuron from a BDNF-treated culture. C and D: cumulative probability plots for IEI of spontaneous synaptic events in delay-radial (C) and tonic-islet-central (D) neurons [spontaneous excitatory postsynaptic currents (sEPSCs) recorded at −70 mV, sIPSCs recorded at 0 mV]. C: sEPSC data are replotted from Figs. 3C and 5C; sEPSC data are replotted from Lu et al. (2009b). D: E and F: bar graphs to illustrate frequencies of sEPSCs and sIPSCs in delay-radial (E) and tonic-islet-central (F) neurons. Values were obtained with reciprocals of the mean IEIs of the data used to make C and D. Significance of differences was determined by Kolmogorov-Smirnov test, and P values are indicated in graphs.
Effects of BDNF on overall dorsal horn excitability. Although there is clear evidence that impairment of inhibitory synaptic transmission within the dorsal horn contributes to onset of central sensitization (Coull et al. 2005; Keller et al. 2007; Knabl et al. 2008; Prescott et al. 2006, 2008; Zeilhofer 2008; Zeilhofer et al. 2009) and administration of bicuculline and/or strychnine is known to produce signs of allodynia (Loomis et al. 2001), our findings within substantia gelatinosa seem inconsistent with this possibility. Despite our observation that inhibitory drive to excitatory neurons is increased (Fig. 3), delay-radial neurons discharge more spontaneous action potentials after BDNF (Fig. 8A). This may be attributed to the dominance of excitatory over inhibitory drive to these cells (Fig. 8, C and E) and its augmentation by BDNF (Lu et al. 2009a). Similarly, despite the decreased inhibitory drive to tonic-islet-central and GAD+ neurons (Figs. 5 and 7), the firing rate of tonic-islet-central neurons is decreased by BDNF (Fig. 8B). This again may reflect the dominance of excitatory drive over inhibitory to these neurons (Fig. 8, D and F) and our previous observation that it is attenuated by BDNF (Lu et al. 2009a). The predominance of excitation over inhibition in substantia gelatinosa (Santos et al. 2007) was underlined in a recent paired neuronal recording study. Of 221 pairs of neurons studied, only 38 exhibited inhibitory connections, whereas 183 excitatory connections were observed (Santos et al. 2009). We have also noted that 5-day exposure of organotypic cultures to BDNF increases their overall excitability even after blockade of GABA_A receptors with SR95531 (Lu et al. 2009a).

The strong and persistent excitatory effect of BDNF in dorsal horn raises the possibility that an increase in inhibitory function represents a homeostatic response. In other words, the observed upregulation of inhibition may be a response to a pathological increase in excitability rather than a direct consequence of BDNF action. One argument against this is the observation that acute application of BDNF acting through a tyrosine kinase receptor rapidly increases mIPSC amplitude in spinal cord slices (Bardoni et al. 2007).

Use of organotypic cultures in chronic pain studies. The use of organotypic cultures as a model for studying neuropathic pain as mediated by BDNF has been extensively justified in other work from our laboratory (Biggs et al. 2010, 2011b; Lu et al. 2006, 2007, 2009a, 2009b). This work also validates the 6-day exposure protocol we used (Biggs et al. 2010) and provides evidence for the equivalence between neurons in our cultures and those in the dorsal horn of young adult rats. The development of inhibitory transmission in the cultures and its parallel to the in vivo situation is discussed in Lu et al. (2006). It may be argued, however, that the use of organotypic cultures prepared from transversely sectioned rat embryos compromises inhibitory circuitry because islet cells project rostrocaudally (Grudt and Perl 2002). However, a recent study of inhibition in acutely isolated parasagittal slices showed that the frequency of sIPSPs in GABAergic neurons was ~0.5 Hz (Labrakakis et al. 2009). This is actually slower than the frequency of events we observe in tonic-islet-central neurons in organotypic culture (~1.1 Hz, calculated from 0.9-s IEI; Fig. 5D). It is likely therefore that inhibition is relatively intact in our cultures. Nevertheless, neither our data nor those of Labrakakis et al. (2009) take into account the descending GABA/glycinergic inhibition that is present in vivo (Antal et al. 1996; Kato et al. 2006). It is possible therefore that all studies on spinal cord slices in vitro or ex vivo actually underestimate the amount of ongoing inhibition that occurs in the substantia gelatinosa in vivo.

Changes in chloride equilibrium potential? Both BDNF and peripheral nerve injury impair GABAergic inhibition in lamina I by attenuating or reversing the Cl^- concentration gradient after downregulation of K^+-Cl^- cotransporter isoform 2 (KCC2) (Coull et al. 2003, 2005; Doyon et al. 2011; Keller et al. 2007; Prescott et al. 2006). It is likely therefore that impairment of inhibition in lamina I is a major contributor to the nerve injury/BDNF-induced increase in dorsal horn excitability. This effect may be less pronounced in lamina II.
neurons, which appear to have higher capacity for Cl− extrusion than those in lamina I (Ferrini and de Koninck 2009). We have also found that BDNF promotes synchronous oscillatory activity in spinal cord organotypic slices (Biggs et al. 2011a) and that these responses are suppressed by superfusion of GABA (Lu VB, Ballanyi K, and Smith PA, unpublished observations). This suggests that GABA is still capable of generating inhibition in substantia gelatinosa after BDNF exposure. Since sIPSPs are less frequent than sEPSCs in both tonic-islet-central and delay-radial neurons in substantia gelatinosa (Fig. 8, C–F), it is unlikely that changes in inhibition would promote much change in the overall activity in this region. This relative paucity of IPSPs is consistent with the observation that only 31% of neurons in this region exhibit GABA-like and/or glycine-like immunoreactivity (Todd and Sullivan 1990).

Conclusions. Since long-term exposure to BDNF increases overall dorsal horn excitability (Lu et al. 2007, 2009a), up-regulation of excitatory processes in the substantia gelatinosa may overwhelm the upregulation of inhibitory processes described here. By contrast, peripheral nerve injury and/or BDNF are known to impair inhibitory processes in lamina I (Coulé et al. 2003, 2005; Keller et al. 2007; Torsney and MacDermott 2006) and in deeper laminae ( Baba et al. 2003; Schoffmegger et al. 2005; Torsney and MacDermott 2006). The attenuation of GABA and glycineergic mechanisms in these regions but not in the substantia gelatinosa likely contributes to “central sensitization.”

ACKNOWLEDGMENTS

We thank Briana Napier for assistance with the GAD immunohistochemistry experiments.

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GRANTS

This work was supported by the Canadian Institutes of Health Research (CIHR; Funding Reference No. 81089). V. B. Lu received studentship awards from CIHR and from the Alberta Heritage Foundation for Medical Research (AHFMR). W. F. Colmers is a Medical Scientist of the AHFMR.

DISCLOSURES

The authors have no financial arrangements or other arrangements that might lead to a conflict of interest regarding this work.

AUTHOR CONTRIBUTIONS

Author contributions: V.B.L., W.F.C., and P.A.S. conception and design of experiments; V.B.L., W.F.C., and P.A.S. performed experiments; V.B.L. and P.A.S. analyzed data; V.B.L. and P.A.S. interpreted results of experiments; V.B.L. and P.A.S. prepared figures; V.B.L. and P.A.S. drafted manuscript; W.F.C. and P.A.S. edited and revised manuscript; P.A.S. approved final version of manuscript.

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J Neurophysiol doi:10.1152/jn.00457.2011 • www.jn.org

Downloaded from http://jn.physiology.org/ on October 8, 2016
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