TrkB Receptor Ligands Promote Activity-Dependent Inhibitory Synaptogenesis

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Organotypic cerebellar cultures derived from newborn mice were simultaneously exposed to activity-blocking agents and neurotrophins for 2 weeks. Activity-blocked explants treated with the TrkB receptor ligands BDNF and neurotrophin-4 (NT-4) developed a full complement of Purkinje cell inhibitory axosomatic synapses, as defined ultrastructurally, and displayed control cortical discharge rates after recovery from activity blockade. Otherwise untreated activity-blocked cultures and activity-blocked cultures exposed to the TrkB receptor ligand NT-3 had reduced inhibitory synapse development and persistent cortical hyperactivity after recovery. The added TrkB receptor ligands did not induce axonal sprouting to account for increased inhibitory synaptogenesis. Addition of neurotrophins to untreated cerebellar cultures did not increase the complement of Purkinje cell axosomatic synapses. Exposure of cerebellar cultures to a combination of antibodies to BDNF and NT-4 resulted in reduced inhibitory synapse formation, similar to the effects of activity blockade, indicating the necessity for endogenous neurotrophins for development of the full complement of inhibitory synapses in the presence of neuronal activity. Application of antibodies to BDNF and NT-4 to cerebellar explants exposed to picrotoxin to increase neuronal activity prevented the hyperinnervation of Purkinje cell somata by inhibitory terminals characteristic of cultures exposed to picrotoxin alone. These results are consistent with the concept that TrkB receptor ligands promote inhibitory synaptogenesis. The ability of neurotrophins to substitute for neuronal activity in encouraging development of inhibitory synapses may have therapeutic implications.

Key words: neurotrophins; neuronal activity; development; inhibitory synapses; cerebellar cultures; Purkinje cells

Consistent with earlier studies of effects of activity blockade in cultured CNS tissue (Ramakers et al., 1990; Baker and Ruiter, 1991; Furshpan, 1991; Ruiter et al., 1991; Corner and Ramakers, 1992), we reported a sustained increase of cortical spike discharges after transfer of newborn mouse-derived organotypic cerebellar cultures maintained in medium with tetrodotoxin (TTX) and elevated levels of Mg2+ to a physiological recording medium (Seil and Drake-Baumann, 1994). The increased activity correlated with ultrastructural findings of reduced numbers of inhibitory axosomatic and axodendritic synapse profiles on Purkinje cells, whereas numbers of excitatory parallel fiber–Purkinje cell dendritic spine synapse profiles remained at control levels. In contrast, cerebellar cultures continuously exposed to anti-GABA agents [picrotoxin (PTX) and bicuculline] that initially increased neuronal activity had a decreased rate of spontaneous cortical discharge after transfer to a physiological medium 2 weeks later, and the ratio of inhibitory axosomatic synapse profiles to Purkinje cell somatic profiles was twice that of control cultures (Seil et al., 1994a). Excitability of the circuitry in these cultures appeared to be regulated by activity-dependent effects on inhibitory (GABAergic) neurons or synapses.

In a study with postnatal rat dissociated visual cortex cultures containing GABAergic interneurons and target pyramidal cells (Rutherford et al., 1997), it was shown that activity blockade with TTX reduced the percentage of GABA-immunopositive neurons without affecting neuronal survival, whereas relative electrophysiological studies indicated that GABA-mediated inhibition onto pyramidal neurons was decreased, with concomitant increases in pyramidal cell discharge rates. The effects of activity blockade were prevented by simultaneous exposure of the cultures to the TrkB receptor ligand brain-derived neurotrophic factor (BDNF) but not to the TrkA and TrkC receptor ligands nerve growth factor (NGF) and neurotrophin-3 (NT-3), suggesting to the authors that activity regulates cortical inhibition by regulation of BDNF. We subsequently reported in a brief communication (Seil, 1999) that application of BDNF and the other TrkB receptor ligand neurotrophin-4 (NT-4) to cerebellar cultures during activity blockade promoted development of the full complement of inhibitory axosomatic synapses on Purkinje cells, whereas application of NT-3 did not prevent the reduced formation of axosomatic synapses resulting from exposure to activity-blocking agents. Neither Purkinje cell survivability nor size were affected by neurotrophin application in this study, and the results were suggestive of BDNF and NT-4 having a role in the promotion of activity-dependent inhibitory synaptogenesis.

The purpose of the present series of experiments was to extend the study of the results of exogenous application of neurotrophins during activity blockade in cerebellar cultures by examining effects on cortical discharge rates and axonal sprouting, to define the role of endogenous neurotrophins in inhibitory synaptogenesis by application of neurotrophins to cultures in the presence of neuronal activity and by blocking their effect with anti-neurotrophin antibodies, and finally to determine whether increased inhibitory synapse formation consequent to picrotoxin-induced increased activity would be prevented by simultaneous exposure to anti-neurotrophin antibodies.

MATERIALS AND METHODS

Culture procedures. Organotypic cerebellar cultures were prepared from newborn (within 24 hr after birth) Swiss–Webster mice (Harlan Sprague Dawley, Indianapolis, IN and Charles River Laboratories, Hollister, CA) by well established methods (Bornstein and Murray, 1958; Seil, 1979, 1993). The mice were cold-anesthetized and killed by exsanguination after an incision through the heart. After isolation of the cerebellum under aseptic conditions and trimming of their lateral ends, each cerebellum was cross cut with scalp knife blades into seven parasagittal sections 0.5-mm-thick. Each section was placed on a collagen-coated coverslip with a drop of nutrient medium, incorporated into a Maximow assembly, and incubated at 35.5–36°C in the lying-drop position. The nutrient medium was initially changed at 5 d in vitro (DIV) and twice weekly thereafter. Standard nutrient

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medium consisted of two parts 3 1/2U ml low-zinc insulin (Squibb Institute for Medical Research, Princeton, NJ), one part 20% dextrose, eight parts Eagle's minimum essential medium with Hank's base and added l-glutamine, ten parts Simms' X-7 balanced salts solution (full salt solution) with sufficient incorporated HEPES buffer to make its concentration 10^{-2} M in the fully constituted medium, and 12 parts fetal calf serum. Cultures were set up in HEPES buffered organotypic tissue culture dishes (1.5%) in cold cacodylate buffer (0.1M) supplemented with 0.05 M sucrose to 7.4), as described previously (Blank et al., 1982; Seil and Drake-Baumann, 1994). They were post-fixed in cacodylate-buffered 2% osmium tetroxide, dehydrated in a series of cold-graded ethanol and polymerized in LR white resin (London Resin Co., Reading Berkshire, UK). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss (Oberkochen, Germany) EM 10C electron microscope. Single-unit discharge rates were processed for silver or neurofilament protein procedures after electron microscopic procedures. Controls were transferred after 13–16 DIV from the Maximow assemblies to a tissue chamber mounted on the stage of an inverted Zeiss Axiovert microscope. The nutrient medium was replaced with BSS additionally buffered with 1.5 × 10^{-2} M HEPES. Etched tungsten microelectrodes with tip diameters < 1 μm were placed in the absence of neuronal activity. The same neurons were counted at 15 DIV. n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD multiple comparisons test indicates the following: (1) The axosomatic synapse profile to Purkinje cell soma profile ratio in untreated activity-blocked cultures (Mg^{2+}/TTX) is significantly different from the ratios for control and BDNF-treated activity-blocked (Mg^{2+}/TTX/BDNF) cultures (p < 0.001 in both cases), whereas there is no significant difference between control and BDNF-treated activity-blocked explants (p = 0.865). (2) The axosomatic synapse profile to Purkinje cell soma profile ratio in untreated activity-blocked cultures (Mg^{2+}/TTX) is significantly different from the ratios for control and NT-4-treated activity-blocked (Mg^{2+}/TTX/NT-4) cultures (p < 0.001 in both cases), whereas there is no significant difference between untreated activity-blocked and NT-4-treated activity-blocked explants (p = 0.988). (3) The axosomatic synapse profile to Purkinje cell soma profile ratio in untreated activity-blocked cultures (Mg^{2+}/TTX) is significantly different from the ratios for control and NT-4-treated activity-blocked (Mg^{2+}/TTX/NT-4) cultures (p < 0.001 in both cases), whereas there is no significant difference between the ratios for control and NT-4-treated activity-blocked cerebellar explants (p = 0.680).

Electrophysiology. Extracellular electrophysiological recording procedures were as described previously (Leiman and Seil, 1973; Seil and Drake-Baumann, 1994; Seil et al., 1994a). Cultures were transferred after 13–16 DIV from the Maximow assemblies to a tissue chamber mounted on the stage of an inverted Zeiss Axiovert microscope. The nutrient medium was replaced with BSS additionally buffered with 1.5 × 10^{-2} M HEPES. Etched tungsten microelectrodes with tip diameters < 1 μm were placed in the absence of neuronal activity. The same neurons were counted at 15 DIV. n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD test for multiple comparisons.

**Results**

**BDNF and NT-4 promote inhibitory synaptogenesis in the absence of neuronal activity**

Our expanded data on the results of exogenous application of neurotrophins to activity-blocked cerebellar cultures are presented in Table 1, which incorporates the data reported previously in a brief communication (Seil, 1999). Approximately half of the control numbers of Purkinje cell axosomatic synapse profiles were present in cerebellar cultures after 15 d of activity blockade, consistent with our previously reported results (Seil and Drake-Baumann, 1994). Cultures exposed to BDNF or NT-4 during activity blockade had axosomatic synapse profile to Purkinje cell soma profile ratios comparable with those of control cultures, whereas exposure of activity-blocked cultures to NT-3 did not alter the Tukey highly significant difference (HSD) multiple comparisons test using Systat software (SPSS, Chicago IL), p < 0.05 was considered statistically significant.

**Table 1. Ratio of axosomatic synapse profiles to Purkinje cell soma profiles in control, activity-blocked, and neurotrophin-treated activity-blocked organotypic cerebellar cultures**

<table>
<thead>
<tr>
<th>Cultures</th>
<th>No. of control cultures</th>
<th>No. of BDNF-treated cultures</th>
<th>No. of NT-4-treated cultures</th>
<th>Mean ± SEM ratio of synapse profiles to cell profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>103</td>
<td>228</td>
<td></td>
<td>2.21 ± 0.13</td>
</tr>
<tr>
<td>Mg^{2+}/TTX (n = 10)</td>
<td>102</td>
<td>127</td>
<td></td>
<td>1.25 ± 0.10</td>
</tr>
<tr>
<td>Mg^{2+}/TTX/BDNF (n = 11)</td>
<td>102</td>
<td>235</td>
<td></td>
<td>2.30 ± 0.14</td>
</tr>
<tr>
<td>Control (n = 11)</td>
<td>100</td>
<td>200</td>
<td></td>
<td>2.00 ± 0.14</td>
</tr>
<tr>
<td>Mg^{2+}/TTX (n = 11)</td>
<td>104</td>
<td></td>
<td></td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>Mg^{2+}/TTX/NT-3 (n = 11)</td>
<td>103</td>
<td>107</td>
<td></td>
<td>1.04 ± 0.11</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>100</td>
<td>215</td>
<td></td>
<td>2.15 ± 0.13</td>
</tr>
<tr>
<td>Mg^{2+}/TTX (n = 11)</td>
<td>100</td>
<td>121</td>
<td></td>
<td>1.21 ± 0.11</td>
</tr>
<tr>
<td>Mg^{2+}/TTX/NT-4 (n = 11)</td>
<td>104</td>
<td>209</td>
<td></td>
<td>2.01 ± 0.12</td>
</tr>
</tbody>
</table>

Control and untreated activity-blocked cerebellar explants from the same culture well were harvested at 15 DIV. n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD multiple comparisons test indicates the following: (1) The axosomatic synapse profile to Purkinje cell soma profile ratio in untreated activity-blocked cultures (Mg^{2+}/TTX) is significantly different from the ratios for control and BDNF-treated activity-blocked (Mg^{2+}/TTX/BDNF) cultures (p < 0.001 in both cases), whereas there is no significant difference between control and BDNF-treated activity-blocked explants (p = 0.865). (2) The axosomatic synapse profile to Purkinje cell soma profile ratio in untreated activity-blocked cultures (Mg^{2+}/TTX) was processed for silver or neurofilament protein procedures after electron microscopic procedures. Controls were transferred after 13–16 DIV from the Maximow assemblies to a tissue chamber mounted on the stage of an inverted Zeiss Axiovert microscope. The nutrient medium was replaced with BSS additionally buffered with 1.5 × 10^{-2} M HEPES. Etched tungsten microelectrodes with tip diameters < 1 μm were placed in the absence of neuronal activity. The same neurons were counted at 15 DIV. n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD test for multiple comparisons.

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the reduced development of axosomatic synapses that occurred in the absence of neuronal activity. Representative Purkinje cells from control, activity-blocked, and NT-3- and NT-4-treated activity-blocked cultures are shown at low magnification in Figure 1. Presynaptic elements of axosomatic synapse profiles were either basket cell axon terminals or Purkinje cell recurrent axon collateral terminals, as identified by established ultrastructural criteria (Palay and Chan-Palay, 1974; Seil and Drake-Baumann, 1994). There were no evident aberrant axon terminals in any of the culture groups and no differences in Purkinje cell somata among the various groups. Astrocytic ensheathment of Purkinje cells was also unaffected, by either activity blockade, as reported previously (Seil and Drake-Baumann, 1994), or addition of neurotrophins to activity-blocked explants.

**Axonal sprouting is not a factor in neurotrophin-promoted synaptogenesis**

Because neurotrophins have been reported to induce axonal sprouting that could result in formation of increased numbers of synapses (Mansour-Robaey et al., 1994; Cohen-Cory and Fraser, 1995; Rashid et al., 1995), overall neurite density was evaluated in silver stains, and Purkinje cell axonal sprouting was estimated in cultures reacted with antibody to nonphosphorylated neurofilament protein. We had demonstrated previously the sensitivity of the Holmes stain to neurite density in cerebellar cultures exposed to cytosine arabinoside after explantation in which increased neurite density attributable to axonal sprouting was dramatically evident upon qualitative evaluation of silver-stained whole-mount culture preparations (Seil et al., 1980). No increase in neurite density was apparent qualitatively in any of the Holmes-stained preparations in the present study. Purkinje cell recurrent axon collaterals are the source of half of the presynaptic elements of Purkinje cell axosomatic synapses in cerebellar cultures (Seil and Drake-Baumann, 1994; Seil et al., 1994a), and Purkinje cells, their axons, and their target deep nucleus neurons are specifically stained in the reaction with nonphosphorylated neurofilament protein (Seil et al., 1994b). No recurrent axon collateral sprouting attributable to neurotrophin stimulation was evident in neurofilament-stained preparations, as shown by the representative Purkinje cells in Figure 2. Purkinje cells and portions of their axons and axon collaterals from an activity-blocked culture are illustrated in Figure 2A, whereas the activity-blocked Purkinje cells in Figure 2B had been continuously exposed to BDNF. No differences were evident qualitatively in the axonal or axon collateral trees in the two conditions, nor were axons of activity-blocked Purkinje cells distinguishable from axons of Purkinje cells in untreated control cultures.

**Activity-blocked cultures exposed to BDNF and NT-4 have cortical discharge rates equivalent to control cultures**

The results of extracellular recordings of spontaneous cortical activity in cerebellar cultures are presented in Table 2. Although control cultures were immediately active after transfer to the buff-
Figure 2. Whole-mount preparations of cerebellar cultures reacted at 15 DIV with antibody to nonphosphorylated neurofilament protein and processed by the peroxidase-antiperoxidase method (375× magnification). Purkinje cell recurrent axon collaterals are indicated by arrows. A, Purkinje cells from a culture continuously exposed to activity-blocking agents. B, Purkinje cells from an activity-blocked culture treated since explantation with BDNF, 25 ng/ml nutrient medium. Axonal sprouting was not evident in neurotrophin-treated activity-blocked cultures.

Table 2. Spontaneous cortical discharge rates in control, activity-blocked, and neurotrophin-treated activity-blocked organotypic cerebellar cultures

<table>
<thead>
<tr>
<th>Cultures</th>
<th>No. of units recorded</th>
<th>Cortical spikes/sec (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>21</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>Mg2+/TTX (n = 11)</td>
<td>41</td>
<td>1.20 ± 0.11</td>
</tr>
<tr>
<td>Mg2+/TTX/BDNF (n = 12)</td>
<td>41</td>
<td>0.75 ± 0.07</td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>25</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>Mg2+/TTX (n = 11)</td>
<td>46</td>
<td>1.39 ± 0.14</td>
</tr>
<tr>
<td>Mg2+/TTX/NT-3 (n = 10)</td>
<td>34</td>
<td>1.48 ± 0.23</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>36</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>Mg2+/TTX (n = 11)</td>
<td>41</td>
<td>1.65 ± 0.22</td>
</tr>
<tr>
<td>Mg2+/TTX/NT-4 (n = 9)</td>
<td>42</td>
<td>0.91 ± 0.07</td>
</tr>
</tbody>
</table>

Cortical activity was recorded extracellularly from control, activity-blocked, and neurotrophin-treated activity-blocked cultures between 14 and 16 DIV. Activity-blocked and neurotrophin-treated activity-blocked cultures were allowed to recover for at least 45 min before collection of data (see Materials and Methods). n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD multiple comparisons test indicates the following. (1) The untreated activity-blocked cultures in all cases exhibited higher discharge rates than the controls (p = 0.003 and p = 0.001, respectively). (2) The BDNF- or NT-4-treated activity-blocked cultures had cortical activity similar to controls and significantly different from the untreated activity-blocked cultures (p = 0.003 and 0.002, respectively). (3) The NT-3-treated activity-blocked cultures had discharge rates similar to the untreated activity-blocked group.

Figure 3. Extracellular records of spontaneous cortical activity in cerebellar cultures after 2 weeks in vitro. A, Untreated control explant recorded at 14 DIV. B, Increased activity with spike bursts in a cerebellar explant 40 min after recovery from activity blockade induced by continuous exposure to 10⁻⁶ M TTX and 11.1 mM Mg²⁺. The culture had been silent during the first 10 min after transfer to a medium without activity-blocking agents, in contrast to control cultures, which were immediately active after transfer to a physiological recording medium. Recorded at 15 DIV. C, Cortical spikes recorded 45 min after transfer to a recording medium. The culture had been exposed to a combination of activity-blocking agents and BDNF (25 ng/ml medium) for 15 DIV before recording. The spontaneous cortical activity pattern is similar to that of untreated control cultures. Time bar at the bottom, 5 sec.
Neurotrophins have been reported to be synthesized and released in an activity-dependent manner (Zafrá et al., 1991; Lindholm et al., 1994; Bölöni and Thoenen, 1995; Thoenen, 1995). These neurotrophic factors have also been reported to have functional effects at synapses (for review, see Schuman, 1999). BDNF enhanced synaptic currents in hippocampal cultures, an enhancement that was partially prevented by the “universal” Trk receptor inhibitor K252a (Kang and Schuman, 1995). BDNF and NT-4, but not NGF, enhanced synaptic strength at Schaffer collateral–CA1

### Table 3. Ratio of axosomatic synapse profiles to Purkinje cell somatic profiles in organotypic cerebellar cultures treated with neurotrophins, with a combination of antibodies to BDNF and NT-4 (aBDNF/aNT-4), or with PTX with or without antibodies to BDNF and NT-4

<table>
<thead>
<tr>
<th>No. of cell profiles</th>
<th>No. of synapse profiles</th>
<th>Mean ± SEM ratio of synapse to cell profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurotrophins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 9)</td>
<td>81</td>
<td>209</td>
</tr>
<tr>
<td>BDNF (n = 9)</td>
<td>81</td>
<td>212</td>
</tr>
<tr>
<td>NT-3 (n = 9)</td>
<td>81</td>
<td>203</td>
</tr>
<tr>
<td>NT-4 (n = 9)</td>
<td>80</td>
<td>197</td>
</tr>
<tr>
<td>Antibodies to BDNF and NT-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 9)</td>
<td>93</td>
<td>241</td>
</tr>
<tr>
<td>aBDNF/aNT-4 (n = 10)</td>
<td>92</td>
<td>124</td>
</tr>
<tr>
<td>PTX with or without antibodies to BDNF and NT-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 7)</td>
<td>80</td>
<td>183</td>
</tr>
<tr>
<td>PTX (n = 8)</td>
<td>80</td>
<td>277</td>
</tr>
<tr>
<td>PTX/aBDNF/aNT-4 (n = 9)</td>
<td>82</td>
<td>204</td>
</tr>
<tr>
<td>(50 μg/ml each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX/aBDNF/aNT-4 (n = 8)</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Untreated (control) cerebellar explants and neurotrophin-treated, antibody-treated (50 μg/ml medium for each of the antibodies), or picrotoxin- and antibody-treated (at doses of 50 and 100 μg/ml each) cultures were compared at 15 DIV. n is the number of cerebellar cultures from which the data were obtained. One-way ANOVA followed by the Tukey HSD multiple comparisons test indicates that the axosomatic synapse profile to Purkinje cell soma profile ratio in untreated cultures is not significantly different from the ratios for BDNF-, NT-3-, or NT-4-treated explants (p = 0.999, p = 0.989, and p = 0.958, respectively). An independent sample t test indicates that the synapse profile to cell profile ratio in control cultures is significantly different from the ratio for cultures exposed continuously to antibodies to BDNF and NT-4 (aBDNF/aNT-4, p < 0.001). One-way ANOVA followed by the Tukey HSD multiple comparisons test shows that the axosomatic synapse profile to cell soma profile ratio in picrotoxin-treated cultures is significantly different from the ratios for untreated control and PTX-treated cultures continuously exposed to either dose of antibodies to BDNF and NT-4 (PTX/aBDNF/aNT-4, p < 0.001 in all cases), whereas there is no significant difference between the ratios for control and PTX-treated cerebellar explants simultaneously exposed to antibodies to BDNF and NT-4 at a dose of 50 μg/ml each (p = 0.863), but there is a significant difference between ratios for control and PTX-treated cultures simultaneously exposed to antibodies to BDNF and NT-4 at a dose of 100 μg/ml each (p = 0.003).
synapses in hippocampal slices from adult rats, an effect that was blocked by K252a (Levine et al., 1995). Hippocampal long-term potentiation (LTP) was reduced in CA1 of mutant mice that lacked BDNF (Korte et al., 1995), and treatment of hippocampal slices from BDNF knock-out mice with BDNF completely reversed deficits in LTP (Patterson et al., 1996).

Given an association between neuronal activity and the development and maintenance of inhibitory circuitry, activity-dependent effects and neurotrophins, and neurotrophins and regulation of synaptic function, it seemed reasonable to consider the possibility that neurotrophins had a role in the development of inhibitory synapses. That this might, indeed, be the case was supported by the results of our study indicating that BDNF and NT-4 promoted development of inhibitory Purkinje cell axosomatic synapses in cerebellar cultures in the absence of neuronal activity (Seil, 1999).

Support for this concept was strengthened by the results of the present study. Although it was evident from the previous report (Seil, 1999) that Purkinje cell survival and size were not significantly affected by either activity blockade or addition of neurotrophins to activity-blocked cultures, it was shown in the current work that neurotrophin-induced axonal sprouting was also not a significant contributory factor to the BDNF- and NT-4-promoted increase in Purkinje cell axosomatic synapses during activity blockade. Even if axonal sprouting had occurred, the axosomatic synapse regulatory function of the Purkinje cell astrocytic sheath would have had to have been overcome for hyperinnervation of Purkinje cell somata to develop (Seil et al., 1992; Seil, 1996). The only example of hyperinnervation of Purkinje cell somata by inhibitory axon terminals in the presence of intact astrocytic sheaths known to us occurred with continuous exposure of cerebellar cultures to agents that increased neuronal activity (Seil et al., 1994a).

Functional studies indicated a correlation with the morphological results. Activity-blocked Purkinje cells had reduced inhibitory axosomatic synapses and demonstrated persistent cortical hyperactivity after recovery from activity blockade. Purkinje cells treated with the TrkB receptor ligands BDNF or NT-4 during activity blockade developed the full complement of inhibitory axosomatic synapses and displayed cortical activity patterns in the control range after release from blockade. Spontaneous cortical discharge rates appeared to be inversely proportional to the complement of Purkinje cell axosomatic synapses, and activity blockade-induced reduction of Purkinje cell axosomatic synapse development was prevented by exogenous addition of TrkB receptor ligands, thus maintaining the functional integrity of the cortex. These studies also demonstrated the effectiveness of the activity blockade, with or without the presence of neurotrophins, for all cultures exposed to activity-blocking agents were electrically silent for at least 10 min after transfer to a physiological recording medium, even at maximum intervals after the last feeding, which was 4 d.

Collectively, the results of this study indicate that TrkB receptor ligands have a role in the regulation of numbers of synapses, at least with regard to inhibitory synapses. Njä and Purves (1978) had reported previously that NGF increased the number of synapses on superior cervical ganglion neurons. More recently, Vicario-Abejon et al. (1998) described enhancement of the number of functional excitatory glutamatergic synapses by treatment of developing hippocampal cultures with BDNF and NT-3, although BDNF also promoted the formation of inhibitory synaptic connections. Our study was focused on inhibitory synapses because of our earlier finding of development of the full complement of excitatory axosomatic cortical synapses in activity-blocked cerebellar cultures, although numbers of inhibitory synapses were markedly reduced (Seil and Drake-Baumann, 1994). Thus, inhibitory cortical synapses were obvious targets for studies of neurotrophin effects on activity-dependent synaptogenesis, but that is not to say that neurotrophins affect only inhibitory synaptic development, as it appears otherwise from the literature.

The necessity for endogenous neurotrophins for development of the full complement of inhibitory synapses in the presence of neuronal activity was shown by the reduced formation of Purkinje cell axosomatic synapses when the TrkB receptor ligands were functionally blocked with antibodies. The reduction in inhibitory synapse development was similar to that consequent to activity blockade, which presumably reduces neurotrophin release (Blöchl and Thoenen, 1995; Thoenen, 1995) or reduces the responsiveness of neurons to neurotrophins (McAllister et al., 1999). The latter possibility is less likely because added exogenous neurotrophins can promote development of the full complement of inhibitory axosomatic synapses in the absence of neuronal activity (Seil, 1999). There may be a delicate balance, however, between levels of neuronal activity and responsiveness to or release of neurotrophins, because Purkinje cells discharging under control conditions failed to respond with increased axosomatic synapse development upon exogenous addition of the same levels of BDNF and NT-4 that promoted inhibitory synaptogenesis in activity-blocked cultures, an effect that could also be accounted for by a feedback mechanism that inhibited endogenous release in the presence of exogenously supplied neurotrophins. On the other hand, an increase in neuronal activity induced by PTX exposure resulted in hyperinnervation of Purkinje cell somata by inhibitory terminals, an effect that was mitigated by application of antibodies to the TrkB receptor ligands, which is consistent with the concept of accelerated neuronal activity inducing an increased release of neurotrophins, resulting in an increased development of inhibitory synapses. The activity state of the neuron may not only direct the release of neurotrophins but may also define the neurotrophin effect. Quantitative studies with varying concentrations of neurotrophins and levels of neuronal activity may provide some further insight into the nature of the complex interplay between neuronal activity and neurotrophin actions.

The fact that neurotrophins may substitute for neuronal activity in the promotion of synapse development does not necessarily mean that the effects of neuronal activity are mediated by neurotrophins. Regulation of the numbers of synapses may be only one of many aspects of neuronal activity–neurotrophin interaction during synapse development. Recently Cohen-Cory (1999) argued that BDNF modulates, but does not mediate, activity-dependent optic axon arborization because of a difference in mechanisms by which neurotrophins and neuronal activity regulate axonal arborization, with BDNF promoting axonal growth, whereas neuronal activity is involved with stabilization of axonal branching. Opportunities for such differences in mechanisms are probably greatly magnified in the complicated sequence of synaptogenesis, especially when the requirement for a balance between inhibition and excitation for proper circuit function is considered.

The ability of neurotrophins to achieve similar outcomes as neuronal activity may in some circumstances have therapeutic implications. Activity is known to promote functional recovery after CNS insults such as trauma or stroke, both in experimental and clinical situations (Jenkins and Merzenich, 1987; Chollet et al., 1991; Cohen et al., 1991; Elbert et al., 1994; Nudo et al., 1996; Johansson, 2000). Because activity appears to be necessary for the development (and probably restoration) of inhibitory circuitry, thus achieving an appropriate balance between excitation and inhibition and avoiding such negative consequences as seizures, it is conceivable that neurotrophin treatment might serve as an adjunct to such measures as physical therapy and perhaps as a substitute when use of activity-inducing measures is not possible. Such clinical considerations await development of more efficient methods of delivery of neurotrophins to appropriate CNS sites.

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