Chronic Elevation of Brain-Derived Neurotrophic Factor by Ampakines

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Received May 1, 2003; accepted June 16, 2003

ABSTRACT

The ampakine CX614 positively modulates \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-gated currents and increases brain-derived neurotrophic factor (BDNF) expression. In rat hippocampal slice cultures, CX614 rapidly increases BDNF gene expression but with time, mRNA levels fall despite the continued presence of active drug. The present study examined this apparent refractory period and the possibility that spaced ampakine treatments could sustain elevated BDNF protein levels. In cultured hippocampal slices, CX614, a second ampakine CX546, and the cholinergic agonist carbachol each increased BDNF mRNA levels with acute (3-h) treatment. After 4-day pre-treatment with CX614, fresh ampakine (CX614 or CX546) did not induce BDNF mRNA, whereas carbachol did. Western blots confirmed that after an extended period of ampakine treatment, AMPA receptor protein levels are indeed reduced, suggesting that with longer treatments receptor down-regulation mediates ampakine insensitivity. Finally, using a “24-h on/24-h off” CX614 treatment protocol, the ampakine refractory state was circumvented, BDNF mRNA was induced with each ampakine application, and elevated BDNF protein levels were maintained through 5 days in vitro. These results suggest that spaced ampakine treatments can be used to sustain elevated neurotrophin levels and to test the utility of this manipulation for neuroprotection by endogenous neurotrophins.

There is considerable evidence that brain-derived neurotrophic factor (BDNF) promotes neuronal survival and differentiation in various brain regions during development. The neurotrophin is expressed at high levels in adult brain and accordingly could play a trophic role throughout life. In accord with this, infusions of BDNF in adult rats increase the survival of cholinergic neurons (for review, see Hefti et al., 1993) and protect hippocampus from ischemic or excitotoxic damage (for review, see Lindvall et al., 1994). These observations raise the possibility that BDNF could have important therapeutic properties with regard to brain injury and age-related pathology. One strategy for exploiting this potential has been to deliver BDNF to brain via infusions or implantation of cells genetically engineered to produce and release the neurotrophin. Alternative approaches would be to pharmacologically elevate the production of endogenous BDNF. Recent studies have shown that marked elevations in BDNF expression can be achieved in vitro and in vivo using ampakines (Lauterborn et al., 2000; Legutko et al., 2001; Mackowiak et al., 2002; Dicou et al., 2003); these compounds belong to several chemical families that marked elevations in BDNF expression can be achieved in vitro and in vivo using ampakines (Lauterborn et al., 2000). Ampakines are of particular interest with regard to potential neurotrophin-based treatments because they 1) readily cross the blood-brain barrier (Staubli et al., 1994a), and experimental studies indicate that this is the route through which they increase BDNF expression (Lauterborn et al., 2000). Ampakines are of particular interest with regard to potential neurotrophin-based treatments because they 1) readily cross the blood-brain barrier (Staubli et al., 1994a); 2) are orally bioactive (Lynch et al., 1997; Goff et al., 2001); 3) have subtle and seemingly positive effects on the forebrain (Staubli et al., 1994a), and experimental studies indicate that this is the route through which they increase BDNF expression (Lauterborn et al., 2000). Ampakines are of particular interest with regard to potential neurotrophin-based treatments because they 1) readily cross the blood-brain barrier (Staubli et al., 1994a); 2) are orally bioactive (Lynch et al., 1997; Goff et al., 2001); 3) have subtle and seemingly positive effects

This study was supported by Cortex Pharmaceuticals awards CP-30783 (to C.M.G.) and CP-28194 (to G.L.) and the University of California Star Biotech Program Grant S99-42 (to C.M.G.).

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; GluR, glutamate receptor; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NMDA, \(N\)-methyl-\(\text{D}\)-aspartate; LY451646, 4-[4-(1-methyl-2-\{[methyl-ethyl]-sulfonyl\}amino)ethyl]phenylbenzenecarbonitrile.
on behavior (for review, see Lynch, 2002); and 4), in preliminary studies, improved cognitive function in humans without evident side effects (Lynch et al., 1997; Lynch 2002). Potential clinical targets for the ampakines include mild cognitive impairment and Alzheimer’s disease, which are characterized by memory deficits that may be ameliorated by increasing neurotrophic support (see Lynch, 2002, for discussion).

The initial studies showing that ampakines increase BDNF production uncovered a potentially important limitation to their use in enhancing neurotrophism. In particular, experiments using prolonged treatments showed that BDNF mRNA levels are maximal by 6 to 12 h and then decline to near control levels by 48 h, despite continued ampakine exposure (Lauterborn et al., 2000; Legutko et al., 2001). The apparent induction of refractoriness with extended ampakine treatments could limit the use of the drugs to periodic increases in neurotrophin content and could interfere with the chronic elevations presumably needed to improve neuronal survival and growth. The present studies were concerned with these issues and had two objectives: 1) identify the causes of the apparent refractoriness of the BDNF response to ampakines, and 2) develop treatment regimens that obviate the refractory state.

Materials and Methods

Preparation of Cultured Hippocampal Slices. Cultured hippocampal slices were prepared from Sprague-Dawley rat pups (9–10 days postnatal; Simonsen Laboratories, Gilroy, CA) (n = 60) as described previously (Lauterborn et al., 2000). In most cases, cultured slices included hippocampus, entorhinal cortex, and portions of the adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate. The adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate. The adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate. The adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate.

Drug Treatments. All experiments were begun on days 11 to 12 in culture. Two positive AMPA receptor modulators (ampakines) were used: CX614 (also known as LiD37 or BDP-37) (Arai et al., 2000; Lauterborn et al., 2000) and CX546 (also known as GR87 or BDP-17) (Lauterborn et al., 2000), generously provided by Cortex Pharmacueticals, Inc. (Irvine, CA). The ampakines were dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and diluted in culture medium as described previously (Lauterborn et al., 2000). In most cultures, slices included hippocampus, entorhinal cortex, and portions of the adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate. The adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate. The adjacent neocortex. Slices were placed onto Millicell-CM biomembrane inserts (Millipore Corporation, Bedford, MA) in a six-well plate.

In Situ Hybridization. For in situ hybridization analysis, hippocampal slices were cryoprotected (20% sucrose in 4% paraformaldehyde/0.1 M phosphate buffer, 1 h) and sectioned (20 µm) parallel to the hippocampal explant surface using a control values. Sections were mounted onto Superfrost/Plus slides (Fisher Scientific Co., Tustin, CA) and then processed for the in situ hybridization localization of BDNF mRNA as described previously (Lauterborn et al., 2000), with hybridization incubation times of 16 to 20 h at 60°C and the 35S-labeled BDNF cRNA probe at a concentration of 1 × 106 cpm/ml. The cRNA to BDNF exon V was generated from Pou1-directed recombinant plasmid pH1112-8, yielding a 540 base-length probe with 384 bases complementary to BDNF exon V-containing mRNA. After a final posthybridization wash in 0.1 × saline sodium citrate buffer (1 × = 0.15 M NaCl/0.015 M Na citrate, pH 7.0) at 60°C, the tissue was air-dried and processed for BioMax film (Eastman Kodak, Rochester, NY) autoradiography with exposure times of 1 day.

Quantification of In Situ Hybridization. Hybridization densities were measured from film autoradiograms, with labeling densities calibrated relative to film images of commercial 14C-labeled standards (American Radiochemicals, St. Louis, MO) using the AID imaging system (Imaging Research, St. Catharines, ON, Canada). For all studies of BDNF mRNA content, analysis focused on the hippocampal granule cell layer because this is one of the more responsive cell types to ampakine treatment, and all of the drugs used had strong effects on BDNF mRNA levels in this cell layer with short treatment intervals (i.e., 3 h) (Lauterborn et al., 2000; infra vide). Hybridization densities were measured for the internal leaf of dentate gyrus stratum granulosum and the adjacent molecular layer. For most in vitro experiments, the significance of effect of treatment was determined by the one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test for individual comparisons. In circumstances where the standard deviations were significantly different between the groups (as determined by Bartlett’s test for homogeneity of variances), significance was determined using the Kruskal-Wallis nonparametric ANOVA followed by the Mann-Whitney U test for individual comparisons. For experiments where two drugs were used together, a two-way ANOVA was performed followed by either Student-Newman-Keuls or Mann-Whitney U tests for planned comparisons. In all instances, statistical analyses were conducted using Prism software (version 3; GraphPad Software, Inc., San Diego, CA), and the 95% confidence level was considered significant. Unless otherwise stated, statistical results presented in the text are for comparison to control values.
body and the avidin-biotin immunohistochemical technique as described previously (Conner et al., 1997) with 0.01 M Tris-buffered saline containing 0.25% Triton X-100 and 2% bovine serum albumin as diluent and diaminobenzidine tetrahydrochloride as chromagen.

**Western Blot Analyses of Membrane Proteins.** Drug effects on AMPA class glutamate receptor protein levels within the synaptic membrane were evaluated using Western blots. Crude synaptic membranes were prepared from hippocampal slice cultures by homogenizing them with sonication in 0.32 M sucrose, 1 mM EGTA, and 100 µM leupeptin. Aliquots of the homogenates were centrifuged at 24,000 g at 4°C for 20 min. The supernatant was discarded, and the pellet was resuspended in distilled water containing 100 µM EGTA. Samples were then centrifuged as described above, and the supernatant was again discarded. The pellet was resuspended in Tris-acetate buffer (100 mM, pH 7.4) containing 100 µM EGTA and centrifuged as described above. This last centrifugation step was repeated, and the final pellet was resuspended in ice-cold Tris-acetate buffer and immediately used for Western blots.

Protein assays were performed using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal volumes of 2× sample buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue) were added to samples that were then boiled for 10 min. Aliquots containing equal amounts of proteins were run on SDS-8% polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were incubated in Tris-buffered saline containing 3% gelatin for 1 h at room temperature and then incubated overnight with primary antibodies in Tris-buffered saline containing 1% gelatin and 0.05% Tween 20. Glutamate receptor 1 (Glur1) and GluR2/3 antibodies were obtained from Chemicon International (Temecula, CA) (1:2,000 dilution); antibodies against actin and synaptophysin were obtained from Sigma-Aldrich and Chemicon International, respectively. Immunostaining was detected by incubation with an alkaline phosphatase-conjugated secondary antibody (Bio-Rad) in Tris-buffered saline containing 1% gelatin and 0.05% Tween 20 for 2 h. Band densities were quantified using ImageQuant software (Amersham Biosciences Inc., Piscataway, NJ).

**Results**

**Ampakines Do Not Lose Potency during Prolonged Incubation.** Perhaps the most obvious explanation for the fall-off in BDNF expression with prolonged ampakine treatment is that the drugs break down after the first few hours in vitro. To test this, three sets of cultured hippocampal slices were treated consecutively for 24 h each with the same ampakine-containing medium (CX614 at 50 µM). Each set of slices was fixed at the end of the 24-h treatment interval and the three sets were processed together, with paired vehicle-treated controls, for in situ hybridization analysis of BDNF mRNA content. As shown in Fig. 1, the ampakine did not lose potency across successive applications. Increases in BDNF cRNA labeling in the dentate gyrus and CA1 were not reliably different for the first, second, and third applications and, in particular, induction during the third application was not smaller than that obtained with the first.

As a further test for loss of potency with prolonged incubation, cultured slices were either treated for 4 days (CX614 at 50 µM) with no further medium or drug change, or given fresh drug/medium every 24 h. Subgroups of slices were harvested at the onset of treatment and every 24 h thereafter up to the 96-h time point. As shown in Fig. 2, the two treatment regimens 1) were equally effective for inducing BDNF expression in stratum granulosum as evaluated at the 24-h time point (p < 0.01 versus control values); and 2) led to a similar decline in BDNF mRNA content with time (p < 0.05, for 48- versus 24-h levels within each regimen). Thus, daily application of fresh CX614-containing medium did not circumvent the decline in BDNF mRNA content that occurs with continuous ampakine exposure.

**BDNF Induction after Prolonged Ampakine Treatment.** A second explanation for the decline of BDNF mRNA levels with extended ampakine treatment is that BDNF gene expression becomes refractory to enhanced excitatory transmission. To test this, cultured hippocampal slices were pretreated for 4 days with 50 µM CX614-containing medium both had markedly elevated BDNF cRNA labeling in strata granulosum (sg) and pyramidale (sp) (calibration bar, 500 µm). D, quantification of BDNF 35S-cRNA labeling in stratum (str.) granulosum and CA1 stratum pyramidale (group means ± S.E.M., 4–5 slices/group) of explants treated for 24 h with vehicle-containing medium (Con) or with successive transfers of the same 50 µM CX614-containing medium: slices in groups 1, 2, and 3 were treated with the CX614-containing medium during the 1st, 2nd, and 3rd days of use, respectively (p = 0.005 and 0.0014 for str. granulosum and CA1 str. pyramidale, respectively; ANOVA; *, p < 0.05; **, p < 0.01; ***, p < 0.001 for comparison with control values; Student-Newman-Keuls).

The rational for selection of test stimuli is as follows: CX546 is an ampakine of the benzamide group that enhances fast, excitatory transmission but differs significantly from CX614 in its binding sites, AMPA receptor subunit preferences, and biophysical effects on receptor kinetics (Arai et al., 2000). Past studies also demonstrated that this compound increases BDNF mRNA expression in explant cultures (Lauterborn et al., 2000). The cholinergic agonist carbachol increases BDNF mRNA levels in dissociated neuronal cultures (Zafra et al., 1990) but has not been previously tested for its effects on hippocampal explants. However, as shown in Fig,
three, preliminary studies confirmed that the cholinergic agonist produces large increases in BDNF expression in slices. Specifically, a 3-h treatment with carbachol alone (50 \(\mu M\)) elevated BDNF mRNA levels in both the dentate gyrus and fields CA3-CA1. This effect was not blocked by cotreatment with 20 \(\mu M\) DNQX or 50 \(\mu M\) dl-2-amino-5-phosphonovaleric acid (Fig. 3D), indicating that the effect of carbachol on BDNF expression is not secondary to an influence on AMPA or NMDA-type glutamate receptors.

After the ampakine pretreatment and 3-h test period, all slices were fixed and processed for in situ hybridization analysis of BDNF mRNA content within stratum granulosum. Control slices (4-day incubation with CX614 followed by 3 h of vehicle treatment) were run along with the three drug-test groups. Results of experiments using CX614 or carbachol as test stimuli are shown in Fig. 4. As can be seen in the two left columns on the left, 3-h treatment of naive slices with 50 \(\mu M\) CX614 induced a more than 4-fold increase in BDNF mRNA content compared with control levels (data not shown). The acute 3-h CX614 test stimulus (second pair of columns), although very effective in naive slices, did not increase BDNF mRNA content in the treated slices (data not shown). The acute 3-h CX614 test stimulus was not statistically different for slices with and without CX614 pretreatment, confirming that the ampakine had little effect on carbachol’s actions. In summary, prolonged treatment with suprathreshold doses of CX614 renders neuronal BDNF expression fully refractory to induction by two structurally distinct ampakines but does not block BDNF induction by cholinergic stimulation. From this, it follows that the refractory period cannot be attributed to a change in the responsiveness of the BDNF gene to induction.
whereas GluR2 levels were reduced only by 24 h of CX614 treatment (\(p < 0.0003\) and \(p < 0.02\), respectively; ANOVA). These effects were not accompanied by significant changes in either synaptophysin or actin levels, indicating that the decrements in AMPA receptor protein levels were not due to a global deterioration of the slices.

**BDNF Can Be Reinduced by CX614 Using an On/Off Treatment Regimen.** Although it is clear that BDNF becomes refractory to ampakines after 12 to 24 h of exposure, it is not known whether and over what period of time this effect dissipates. The question was addressed using an alternating 24-h drug on/24-h drug off treatment regimen; media were changed daily for both ampakine- and vehicle-treated slices. BDNF mRNA levels were assessed with in situ hybridization at time 0 and at 24-h intervals thereafter over 5 days of treatment. As shown in Fig. 6, BDNF mRNA levels were significantly increased above control (time 0) values at the end of every 24-h "drug-on" period and fell to control values by the end of two 24-h "drug-off" periods (\(p > 0.05\) for time 0 values versus 48- and 96-h time points). More importantly, the response to the ampakine after a 24-h off period was as great as that obtained with naive slices; that is, the increases in mRNA levels were as large after the second and third drug applications as they were after the first (Fig. 6). These data demonstrate that BDNF refractory period is relatively brief and that gene expression can be reinduced with spaced ampakine treatments.

Immunoassays were used to assess the effects of CX614 on total BDNF protein levels in cultured slices. The first experiment used a single 24-h treatment with 50 \(\mu\)M CX614 and evaluated separate groups of slices collected at 24, 48, 72, and 120 h after treatment onset to determine the magnitude of the effects of CX614 on levels of the GluR1 and GluR2/3 subunits of the AMPA receptor in cultured slices at 0, 12, and 24 h after adding CX614 (50 \(\mu\)M). As is evident, the ampakine caused a modest but significant decrease in GluR1 at 12 h and in both GluR1 and GluR2/3 at 24 h; statistical analyses confirmed a significant effect of treatment for both GluR1 and GluR2/3 (\(p < 0.0003\) and \(p < 0.02\), respectively; ANOVA). These effects were not accompanied by significant changes in either synaptophysin or actin levels, indicating that the decrements in AMPA receptor protein levels were not due to a global deterioration of the slices.

**Fig. 4.** Prolonged ampakine pretreatment renders BDNF expression refractory to ampakine but not carbachol stimulation. Cultured hippocampal slices were pretreated for 4 days with 50 \(\mu\)M CX614 or vehicle and then tested for effects of CX614 or carbachol (both 50 \(\mu\)M) for 3 h on BDNF mRNA levels in stratum granulosum (columns show densitometric measures of BDNF \(35\)S-cRNA labeling in stratum granulosum; group means \(\pm\) S.E.M., 6–8 slices/group). A shown on left, 3-h treatment with 50 \(\mu\)M CX614 induces a large increase in labeling in previously drug-naïve slices but does not stimulate BDNF expression in slices pretreated with CX614 for 4 days. In contrast, as shown in the graph on the right, carbachol induced BDNF in both drug-naïve slices and slices pretreated for 4 days with CX614 (\(**, p < 0.001\) versus control for each field; Student-Newman-Keuls; ***, \(p = 0.01\), Mann-Whitney U).

**Fig. 5.** Prolonged ampakine treatment down-regulates AMPA receptors. Cultured hippocampal slices were exposed to CX614 (50 \(\mu\)M) for 12 or 24 h. Western blots were used to assess the levels of GluR1, GluR2/3, actin, and synaptophysin in crude synaptic membranes. Results are expressed as percentage of values found in slices exposed to vehicle only and are means \(\pm\) S.E.M. of three to four experiments. As shown, GluR1 levels were reduced by 12 h and further decreased by 24 h of treatment, whereas GluR2 levels were reduced only by 24 h of CX614 treatment (\(*\), \(p < 0.05\); Student-Newman-Keuls).

**Fig. 6.** Spaced ampakine treatments repeatedly induce BDNF expression. Bar graph shows quantification of BDNF \(35\)S-cRNA labeling in stratum granulosum (group means \(\pm\) S.E.M., 6–8 slices/group) in cultured hippocampal slices fixed without treatment (zero time point) or at the end of each 24-h interval in the course of an alternating 24-h drug-on/24-h drug-off treatment regimen (bar underlying graph indicates periods of treatment). As shown, BDNF cRNA labeling was significantly increased at the end of each CX614 treatment period (\(*\), \(p < 0.05\) and \(*\), ***, \(p < 0.001\) versus zero time point; Student-Newman-Keuls) and fell to near control levels at the 48- and 96-h time points when drug was not present.
and duration of increases in BDNF protein content. As shown in Fig. 7 (light columns), a single 24-h CX614 treatment induced a dramatic increase in BDNF protein content (i.e., from 43 ± 18 in control slices to 1,312 ± 118 ng/g at 24 h; mean ± S.E.M.; p < 0.001). After removal of drug, BDNF protein levels were still well elevated at 48 h, declined nearly 40% by 72 h (p < 0.01 for 48 versus 72 h), and were not significantly different from control (time 0) values by the 120-h time point. These results indicate that elevated levels of BDNF protein elicited by a 24-h ampakine treatment last longer than the time interval required for the refractory period to dissipate.

In a second set of experiments, cultures were treated with 50 μM CX614 using the alternating 24-h drug on/24-h drug off regimen; groups of slices were assessed for BDNF protein content before the onset of treatment and at the end of each 24-h interval thereafter. The results of this analysis are plotted in the dark columns of Fig. 7. At 24 h, BDNF protein levels were increased to levels attained in the preceding experiment (1,450 ± 90.6 ng/g; p < 0.001 versus time 0). However, BDNF protein levels were sustained well above control values through 120 h of treatment (p < 0.001 for comparison of 48, 72, 96, and 120 h to time 0 values) with the 24-h on/off treatment regimen.

**CX614 Treatment Increases BDNF Immunostaining.** Activity-induced changes in the levels and distributions of BDNF-immunoreactivity (ir) in vivo have been described previously (for review, see Gall and Lauterborn, 2000). For example, with seizure activity, BDNF-ir is initially reduced but then increases first in neuronal somata and then later in axons and terminal arbors. To determine whether CX614-induced increases in BDNF protein are similarly distributed to neuronal processes in vitro, cultured hippocampal slices were treated with 50 μM CX614 for 24 or 48 h and then processed with paired vehicle-treated control cultures for immunocytochemistry. BDNF-ir in control cultures was most prominent in the mossy fibers (Fig. 8A) and in scattered cells within CA1 stratum pyramidale and neocortical layer 5 (Fig. 8, C and E). The hippocampal distribution corresponds well with BDNF-immunostaining in brain tissue sections, whereas the cortical labeling included relatively greater numbers of immunoreactive perikarya (Conner et al., 1997).

Immunostaining was reduced in the mossy fibers and increased in neuronal perikarya in hippocampal strata pyramidale and granulosum after a 24-h incubation with the ampakine (data not shown). These effects were more pronounced in the 48-h treatment group (Fig. 8, B and D). Changes in BDNF-ir followed a similar pattern in cortex with the exception that perikaryal labeling was virtually absent in the 48-h treatment group. There was a striking increase in immunostained processes throughout the neuropil at 24 and 48 h in both the hippocampus and neocortex (Fig. 8F).
was no indication of glial immunostaining in either control or experimental tissue.

Discussion

The above-mentioned information provides a partial explanation for why BDNF expression becomes refractory to the facilitatory effects of ampakines and, equally important, provides the first evidence that the refractory period can be obviated with appropriate treatment schedules. Based on these findings, it seems that the drugs can be used to produce tonically elevated levels of BDNF, an effect that could be of great value in the treatment of various brain disorders.

Media-transfer experiments spanning several days showed that the decline of BDNF expression with prolonged ampakine exposure was not due to a loss of drug potency, a conclusion that was confirmed in studies using daily applications of fresh drug and media. This led to consideration of the possibility that BDNF gene expression was, for some reason, blocked or actively down-regulated. Effects of this type have been described for nerve growth factor (Elliott and Gall, 2000) and c-fos (Morgan et al., 1987) after seizures. Nerve growth factor gene expression is largely regulated by activating protein-1 binding within its promoter region (D’Mello and Heinrich, 1991) and postseizure increases and decreases in nerve growth factor mRNA content correlate with changes in activating protein-1 composition (Elliott and Gall, 2000). BDNF expression is not regulated by activating protein-1 (Sano et al., 1996) but is suppressed by the neuron-restrictive silencer element (repressor element 1’ (Timmusk et al., 1999). Increased production of the repressor element represents a plausible explanation for the refractory period that emerges with prolonged exposure to ampakines. However, this idea was ruled out by results showing that the efficacy of cholinergic stimulation in inducing BDNF was unaffected by extended ampakine pretreatment. By exclusion, these findings point to AMPA receptors, or their links with the BDNF gene, as the probable sites at which the refractory effect is realized. In accord with this, extended ampakine treatments caused a significant decrease in the concentration of AMPA receptor proteins GluR1 and GluR2/3.

The loss of AMPA receptors with enhanced use as produced by ampakines is not without precedent. Intense activation of the receptors as occurs during seizures down-regulates receptor subunit gene expression (Condorelli et al., 1994; Gold et al., 1996; Grooms et al., 2000). Beyond changes in gene expression, there is an abundance of evidence that surface receptors, including receptor tyrosine kinases, G protein-coupled receptors, and ionotropic neurotransmitter receptors, are endocytosed in response to prolonged agonist exposure or increases in native ligand concentrations (for review, see Carroll et al., 2001). Of particular interest, AMPA receptor surface expression decreases with increases in glutamatergic activity elicited by different experimental manipulations (Lässin et al., 1999), and these effects are blocked by inhibition of clathrin-mediated endocytosis (Lüscher et al., 1999; Wang and Linden, 2000).

The refractory effect would seem to represent a major barrier to the use of ampakines for increasing neurotrophism. However, the present results indicate that hippocampal and cortical neurons quickly regain their responsiveness to ampakines upon washout of the drugs; i.e., a 24-h drug-free period was sufficient to restore the full effect of ampakine treatment on BDNF expression. Previous results demonstrated that BDNF mRNA levels decline (~40%) between 12 and 24 h after the onset of continuous ampakine treatment (Lauterborn et al., 2000), suggesting that the refractory period occurs well in advance of 24 h. Indeed, refractory mechanisms probably emerge hours before the decline in BDNF mRNA is detected but are masked by the half-life of BDNF transcripts (estimated as 0.5 to 2.5 h for longer and shorter transcripts, respectively, Sano et al., 1996; Castren et al., 1998). Thus, it is likely the refractory state develops during the 24-h drug-on intervals and reverses during the 24-h drug-off periods.

In agreement with previous results (Lauterborn et al., 2000), the present enzyme-linked immunosorbent assay measures of BDNF protein content showed that a single 24-h ampakine treatment led to sustained, maximal increases in BDNF protein from 24 to 48 h of treatment onset; this was followed by a slow decline to control levels by the 120-h time point. This prolonged increase in BDNF protein with single treatment suggested that intermittent drug treatments, and associated intermittent increases in BDNF gene expression, would be sufficient to sustain increases in BDNF protein content for several days. This proved to be the case. With alternating 24-h drug-on/drug-off intervals, BDNF protein levels remained elevated and did not drop significantly below the initial 24-h peak, through 5 days of treatment. Although these results provide evidence that elevated BDNF protein levels can be sustained, the ampakine treatment regimen used cannot be considered optimal. As argued above, the time course of changes in BDNF mRNA with continuous ampakine exposure indicates that there is a loss of ampakine sensitivity between 6 and 24 h of treatment onset. Thus, it is likely that shorter drug treatment periods, and comparatively longer interstimulus intervals, could achieve comparable, enduring increases in BDNF protein content while being less likely to elicit significant changes in AMPA receptor responsiveness. Moreover, briefer drug treatments would more closely approximate periods over which these relatively short-lived compounds are at behaviorally effective levels with single doses in vivo (Lynch et al., 1997), and thus would be more informative regarding potential ampakine effects on BDNF expression and AMPA receptor signaling in clinical trials (Lynch et al., 1997; Goff et al., 2001; Lynch, 2002).

BDNF has both autocrine and exocrine trophic functions, and it is generally agreed that the latter requires the neurotrophin to be released from the distal processes of the neuron (for review of in vivo results, see Conner et al., 1998). It is important then that pharmacological strategies for elevating BDNF show that the increased concentrations of the neurotrophin are transported away from the cell body. In the present case, ampakine-driven increases in neuronal activity induced a depletion of BDNF protein from regions of dense axonal immunoreactivity in the control preparation (e.g., the mossy fibers) and both an increase and apparent anterograde axonal transport of BDNF protein in other fields. Depletion of mossy fiber immunostaining is consistent with reports that BDNF release is increased by neuronal activity (Marini et al., 1998; Lever et al., 2001; Balkowiec and Katz, 2002), and by the expectation that depletions would seem to be greatest in sites further from resupply by new synthesis, whereas increases in axonal BDNF-ir in cortical and hip-
Acknowledgments
We thank Yilu Xie, Gus Fowler, Xiaoying Lu, and Jihua Liu for excellent technical assistance.

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