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AMPAKINES CAUSE SUSTAINED INCREASES IN BRAIN-DERIVED NEUROTROPHIC FACTOR SIGNALING AT EXCITATORY SYNAPSES WITHOUT CHANGES IN AMPA RECEPTOR SUBUNIT EXPRESSION

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Abstract—Recent demonstrations that positive modulators of AMPA-type glutamate receptors (ampakines) increase neuronal brain-derived neurotrophic factor (BDNF) expression have suggested a novel strategy for treating neurodegenerative diseases. However, reports that AMPA and BDNF receptors are down-regulated by prolonged activation raise concerns about the extent to which activity-induced increases in BDNF levels can be sustained without compromising glutamate receptor function. The present study constitutes an initial test of whether ampakines can cause enduring increases in BDNF content and signaling without affecting AMPA receptor (AMPAR) expression. Prolonged (12–24 h) treatment with the ampakine CX614 reduced AMPAR subunit (glutamate receptor subunit (GluR) 1-3) mRNA and protein levels in cultured rat hippocampal slices whereas treatment with AMPAR antagonists had the opposite effects. The cholinergic agonist carbachol also depressed GluR1-3 mRNA levels, suggesting that AMPAR down-regulation is a global response to extended periods of elevated neuronal activity. Analyses of time courses and thresholds indicated that BDNF expression is influenced by lower doses of, and shorter treatments with, the ampakine than is AMPAR expression. Accordingly, daily 3 h infusions of CX614 chronically elevated BDNF content with no effect on GluR1-3 concentrations. Restorative deconvolution microscopy provided the first evidence that chronic up-regulation of BDNF is accompanied by increased activation of the neurotrophin's TrkB-Fc receptor at spine synapses. These results show that changes in BDNF and AMPAR expression are dissociable and that up-regulation of the former leads to enhanced trophic signaling at excitatory synapses. These findings are encouraging with regard to the feasibility of using ampakines to tonically enhance BDNF-dependent functions in adult brain.

Key words: AMPA receptor modulator, synaptic scaling, gene expression, TrkB, histone deacetylase, neurotrophin.

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Abbreviations: AMPAR, AMPA receptor; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; DMSO, dimethyl sulfoxide; GluR, glutamate receptor subunit; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride; ir, immunoreactivity; LTP, long-term potentiation; SNK, Student-Newman-Keuls; str., stratum; TSA, trichostatin A.

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Brain-derived neurotrophic factor (BDNF) promotes synaptic plasticity (Kang and Schuman, 1995; Kramár et al., 2004; Kuipers and Bramham, 2006) and is protective in animal models of brain injury and of neurodegenerative diseases. Accordingly, there is an intense and ongoing effort to find physiologically plausible means for increasing BDNF supply in adult brain, either through delivery of exogenous factor or by amplifying synthesis. The discovery that BDNF expression is positively regulated by neuronal activity (Zafra et al., 1990; Isackson et al., 1991; Gall and Lauterborn, 2000) pointed to the possibility of using positive modulation of excitatory transmission for the latter purpose and tests of this idea were successful. Ampakines, a family of compounds that slow the deactivation and desensitization of AMPA-type glutamate receptors and thereby enhance fast excitatory transmission (Staubli et al., 1994a; Lynch and Gall, 2006; Arai and Kessler, 2007), markedly increase expression of BDNF and nerve growth factor (Lauterborn et al., 2000). Up-regulation is triggered by structurally distinct families of positive modulators (Legutko et al., 2001; Dicou et al., 2003) and occurs *in vivo* after peripheral administration (Lauterborn et al., 2000; Mackowiak et al., 2002; Dicou et al., 2003). There is also evidence that *in vivo* ampakine treatment reduces neuronal death in animal models of Parkinson's disease (O'Neill et al., 2004a) and excitotoxic brain damage (Bahr et al., 2002; Dicou et al., 2003), and can reverse deficits in hippocampal long-term potentiation (LTP) (Rex et al., 2006; Simmons et al., submitted for publication); in the latter instances, improved survival and function were associated with elevated BDNF (Dicou et al., 2003; Rex et al., 2006).

However, other studies have identified counterbalancing, homeostatic changes that return synaptic strength to normal levels in the face of chronic increases or decreases in neuronal activity. Such bidirectional processes of synaptic scaling have been demonstrated for AMPA receptor (AMPAR)–mediated transmission after prolonged exposure to TTX or GABA receptor antagonists, to cite two examples (O'Brien et al., 1998; Turrigiano et al., 1998). Although the observed effects could in principle have a pre- or post-synaptic locus, altered responses to applied glutamate as well as changes in dendritic protein levels (O'Brien et al., 1998; Turrigiano et al., 1998) suggest that synaptic scaling is predominantly postsynaptic (Wierenga et al., 2005). There is evidence that prolonged exposure to AMPAR modulators elicits compensatory changes in the postsynaptic machinery as well. Continuous incubation of cultured hippocampal slices with the ampakine CX614 rapidly increased BDNF mRNA (over 3–12 h) but this was

followed by a gradual decrease to control values over the next 36 h (Lauterborn et al., 2000). A similar time course was described for a structurally distinct AMPAR modulator (LY392098) in studies using dissociated hippocampal neurons (Legutko et al., 2001). Further analysis of slice cultures showed that the fall in BDNF expression was accompanied by decreases in AMPAR (glutamate receptor subunit (GluR) 1, GluR2) mRNA and protein levels (Lauterborn et al., 2003; Jourdi et al., 2005) suggesting that the decline in the ampakine response resulted, in part, from a loss of target receptors.

These findings raise the possibility that negative scaling of AMPAR-mediated transmission constitutes a limit on the use of ampakines for generating chronic increases in neurotrophin levels. Evaluation of the issue requires information on whether and to what degree the drug's effects on BDNF and AMPAR gene expression are dissociable with different treatment regimens. Also of critical importance is the largely unexplored question of whether *BDNF signaling* at synapses is actually enhanced by elevating brain concentrations of the neurotrophin and, if so, does the effect undergo time-dependent compensatory changes. The present studies addressed the first of these points by asking if near-threshold conditions for increasing BDNF expression have detectable effects on AMPAR concentrations and then determining if predicted treatment regimens could sustain BDNF protein levels for days without altering AMPAR protein levels. Restorative deconvolution microscopy and double immunofluorescence labeling were then used to test if the up-regulation of BDNF content increases activation of the neurotrophin's TrkB receptors at excitatory synapses in hippocampal field CA1. Results of these studies indicate that appropriate ampakine treatment regimens produce marked and sustained increases in BDNF protein, and BDNF signaling at glutamatergic synapses, without detectably affecting AMPAR protein levels.

EXPERIMENTAL PROCEDURES

Preparation of cultured hippocampal slices

Cultured hippocampal–entorhinal slices were prepared from Sprague–Dawley rat pups (9 to 11-day postnatal; Simonsen Laboratories, Gilroy, CA, USA) (n=40) as previously described (Lauterborn et al., 2000). The cultured slices included hippocampus, entorhinal cortex and portions of the adjacent neocortex. For each rat pup, slices from both hippocampi were explanted onto four Millicel-CM biomembrane inserts (Millipore, St. Louis, MO, USA) with four slices/insert and maintained with medium containing 20% horse serum (pH 7.2); unless otherwise stated, reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The slices were maintained in an interface configuration in a humidified incubator at 37 °C in 5% CO_2 and medium was changed every other day; experiments used explants after 10–14 days *in vitro* (DIV). All experiments were performed in accordance with NIH guidelines, and protocols were approved by the Institutional Animal Care and Use Committee with care to minimize both distress to the animals and numbers of animals used.

Drug treatments

The positive AMPAR modulator CX614 (a.k.a., LiD37, BDP-37) (Arai et al., 2000; Lauterborn et al., 2000) was generously provided by Cortex Pharmaceuticals, Inc. (Irvine, CA, USA). Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). CX614, GYKI-52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride) (GYKI) (Tocris Bioscience, Ellisville, MO, USA), and trichostatin A (TSA) (Tocris Bioscience) were dissolved in 100% dimethyl sulfoxide (DMSO); carbachol, AMPA (Tocris Bioscience) and the TrkB ligand scavenger TrkB-Fc (R&D Systems, Minneapolis, MN, USA) were dissolved in serum-free medium. All drug stocks were stored at −20 °C. Treatment schedules and drug concentrations are presented in the Results section with each experiment. In all cases, control cultures were treated with equivalent concentrations of vehicle and received the same schedule of medium changes as paired, drug-treated cultures. In preliminary studies, DMSO diluted 1:2000 (the highest concentration used in the present studies) was found to have no effect on mRNA levels relative to naive-control cultures at all time points examined.

Treatments were terminated by tissue fixation (4% paraformaldehyde) for *in situ* hybridization and immunofluorescence analyses or by freezing for Western blots and BDNF immunoassays.

In situ hybridization

Fixed hippocampal slices were cryoprotected, and sectioned (20 μm) parallel to the broad explant surface using a freezing microtome. Sections were mounted onto Superfrost Plus slides (Fisher Scientific, Tustin, CA, USA) and processed for *in situ* hybridization as described (Lauterborn et al., 2000), with the hybridization incubation at 60 °C for 16–20 h and the ^{35}S-labeled cRNA probes at 1×10^7 cpm/ml. The GluR1, GluR2 and GluR3 cRNAs were transcribed from *Bgl*I, *Bam*HI, and *Sal*I digests of p59/2, pRB14, and pRB312, respectively (Gold et al., 1997): the cDNAs are complementary to 895, 900, and 720 bases, respectively, of the 3′-ends including non-coding regions. Previous work showed there is no cross-hybridization between these subunit cRNAs and the heterotypic GluR mRNAs (Gold et al., 1997). After the final post-hybridization wash, the tissue was air-dried and processed for Biomax film (Eastman Kodak, Rochester, NY, USA) autoradiography with exposure times of 1–2 days. *In situ* hybridization labeling densities were measured from film autoradiograms, and calibrated relative to film images of commercial ^{14}C-labeled standards (American Radiochemicals Inc., St. Louis, MO, USA) using the AIS imaging system (Imaging Research Inc., St. Catherines, Ontario) as described (Lauterborn et al., 2000). Hybridization densities were measured for the internal blade of dentate gyrus stratum (str.) granulosum, CA1b-c and CA3a-b str. pyramidale, and, as an estimate of background, the internal blade of the dentate molecular layer.

Western blot analyses

Homogenates were prepared from previously frozen hippocampal slice cultures by tip sonication in 0.32 M sucrose, 1 mM EDTA, 0.1 M Tris (pH 7.4) and protease inhibitor cocktail (PIC Complete™, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Sample protein levels were adjusted to normalize μg/ml protein content. Samples were then diluted 1:1 by volume with 2× sample buffer (10% glycerol, 0.5% sodium dodecylsulfate, 62.5 mM Tris (Fisher Scientific, Pittsburgh, PA, USA) (pH 6.9), 5% 2-mercaptoethanol, and 0.1% Bromophenol Blue), boiled 10 min, separated by 7.5% PAGE (30 μg/lane) and transferred onto polyvinylidene difluoride membranes (Hybond-P™, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked in 5% non-fat dry milk, 1% bovine serum albumin in Tris-buffered saline