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Effects of chronic systemic low-impact ampakine treatment on neurotrophin expression in rat brain

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ABSTRACT

Neurotrophin dysregulation has been implicated in a large number of neurodegenerative and neuropsychiatric diseases. Unfortunately, neurotrophins cannot cross the blood brain barrier thus, novel means of up regulating their expression are greatly needed. It has been demonstrated previously that neurotrophins are up regulated in response to increases in brain activity. Therefore, molecules that act as cognitive enhancers may provide a clinical means of up regulating neurotrophin expression. Ampakines are a class of molecules that act as positive allosteric modulators of AMPA-type glutamate receptors. Currently, they are being developed to prevent opioid-induced respiratory depression without sacrificing the analgesic properties of the opioids. In addition, these molecules increase neuronal activity and have been shown to restore age-related deficits in LTP in aged rats. In the current study, we examined whether two different ampakines could increase levels of BDNF and NGF at doses that are active in behavioral measures of cognition. Results demonstrate that ampakines CX516 and CX691 induce differential increases in neurotrophins across several brain regions. Notable increases in NGF were observed in the dentate gyrus and piriform cortex while notable BDNF increases were observed in basolateral and lateral nuclei of the amygdala. Taken together, our data demonstrates that chronic administration of clinically relevant doses of ampakines have the ability to elevate neurotrophin expression in different brain regions, and may have therapeutic benefit in multiple neurodegenerative and/or neuropsychiatric disorders.

1. Introduction

The neurotrophin gene family members (NGF, BDNF, NT3-6) are fundamentally involved in differentiation and survival of neurons during development [1]. Additional studies show a decline in levels of neurotrophins during aging and neurodegenerative disease, suggesting that changes in neurotrophin levels may contribute to age-related behavioral deficits and neurodegenerative mechanisms [2,3]. Furthermore, neurotrophins can reduce age-related, axotomy or neurotoxin-induced neuron loss or diminished function in a variety of brain regions [4]. For example, NGF can restore age-related spatial memory impairments, as well as significantly prevent axotomy-induced cholinergic neuron atrophy. BDNF can also substantially prevent mesencephalic cell death due to axotomy or neurotoxic insult [5]. However, to produce these effects the neurotrophic factors must be directly infused into the brain. Protein/peptide growth factors are not orally bioavailable, and do not cross the blood-brain barrier unaided which severely limits their utility as therapeutics. An alternative approach would be to augment neurotrophin expression selectively in the brain with a peripherally administered compound. It has been known for some time that NGF and BDNF are up-regulated by neuronal activity. Intense neuronal activity, such as during kindled or drug-induced (*i.e.* kainate) seizures greatly enhances BDNF mRNA levels in hippocampus, amygdala, and many cortical areas [6]. More subtle increases in neuronal activity, such as during LTP, produce subtler up regulation of BDNF [7]. Thus, it is possible that compounds known to enhance neuronal activity might also increase neurotrophin levels.

Ampakines are small molecule compounds that specifically act as positive allosteric modulators at the AMPA-type glutamate receptor, thereby enhancing excitatory communication and neuronal activity [8]. Ampakines are subdivided into two distinct classes, known as high and low impact ampakines. High impact ampakines interfere with receptor desensitization and enhance agonist binding [9]. Meanwhile, low impact ampakines primarily accelerate channel opening but do not strongly offset receptor desensitization and do not alter agonist binding affinity [9]. Despite these differences, Greer et al [10,11] have reported *in vivo* target engagement in rats by determining the ability of low impact ampakines to inhibit opioid-induced respiratory depression, an action mediated by brain stem AMPA receptors. They also enhance LTP *in vitro* in hippocampal slices and *in vivo* in freely-moving rats. In

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addition, they enhance performance in several different types of memory tasks to a similar extent as that produced by high impact ampakines [8,10,12,13].

High impact ampakines have previously been shown to up-regulate BDNF mRNA and protein several fold in long-term hippocampal organotypic cultures [14]. We, therefore, sought to determine whether systemic administration of two representative low impact ampakines, CX516 (30 mg/kg) or CX691 (3 mg/kg), for 14 days could induce detectable changes in neurotrophin mRNA levels in rat brain. We chose to specifically examine the effects of ampakines on neurotrophin levels in the hippocampus, amygdala and piriform cortex because these brain regions contain high levels of AMPA glutamate receptors and have been associated with some of the behavioral and cognitive effects produced by ampakines [15–17]. The present results show significant increases in BDNF and NGF mRNA in several brain regions after systemic administration of modest doses of two different low impact ampakines.

2. Materials and methods

All animal procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine (Irvine, CA). Efforts were made to minimize animal suffering and numbers of rats used in the work described.

2.1. Animals

Twenty-four young (3 months), male Sprague-Dawley rats (Jackson Laboratories) were group-housed in a tri-level enriched environment containing a maze of tunnels connecting the three levels. The rats were randomly assigned to three groups and given daily intraperitoneal injections of saline (n = 8), CX516 (30 mg/kg/day; n = 8) or CX691 (3 mg/kg/day; n = 8) for 15 days. Ampakine doses were determined based upon enhancement of performance on an 8 ar m radial maze task as described previously [18]. Doses of CX516 ranged from 6 to 50 mg/kg in the behavioral assay in accordance with doses from previous studies using up to 75 mg/kg CX516 [15]. Doses used for CX691 were chosen in accordance with prior studies [17] although comparatively higher doses of CX691 were used in the clinic [19]. On the day of sacrifice (day 15) rats received the Ampakine or vehicle injection 30 min prior to anesthetization with 100 mg/kg pentobarbital and sacrifice by cardiac perfusion with phosphate-buffered saline. Each brain was fixed *via* cardiac perfusion with cold, freshly-made 4% paraformaldehyde (in 0.1 M sodium phosphate, pH 7.4), carefully removed from the skull and post-fixed overnight (12–18 h). The brains were then cryoprotected in 30% sucrose/1% paraformaldehyde (24–48 h) and coronal slices (40 µm) were prepared with a sliding microtome and stored at 4 °C in 1% paraformaldehyde/0.1 M sodium phosphate, pH 7.4.

2.2. In situ hybridization

BDNF and NGF mRNA were localized in the fixed coronal sections by free-floating *in situ* hybridization using ^{35}S anti-sense c-RNA probes that represented the entire coding region of each mRNA (NT 253–1025 of NGF; NT 78–827 of BDNF; gift of S. Whittemore, Anatomical sciences and neurobiology, University of Louisville School of Medicine). Sense and anti-sense cRNA probes (specific activity: 2×10^9 dpm/µg) were prepared by standard RNA polymerase labeling reactions. Labeled cRNAs were purified by removing unincorporated nucleotides with a Stratagene Push Column, followed by 2 cycles of precipitation from 70% ethanol/0.2 M NaAc in the presence of 250 µg of purified wheat germ tRNA. For *in situ* hybridization, tissue sections were: **1.)** rinsed 3 times in 50 mM TRIS, 5 mM EDTA, pH 8.0 (5X TE); **2.)** digested with 2.5 µg/ml Proteinase K in 5X TE at 25 °C for 10 min and rinsed 3 times in 5X TE; **3.)** pre-hybridized in 50% formamide, 0.5 M NaCl, 50 mM NaPB (pH 7.4), 10 mM EDTA, 50 mM DTT, 0.5% SDS, 5X Denhardts, 200 µg/ml Heparin, 100 µg/ml tRNA at 60 °C for 2 h; **4.)** hybridized in pre-hybridization buffer + 10% dextran sulfate with 8×10^6 cpm/ml (4 ng/ml) at 60 °C for 48 h; 5.) rinsed 2 x 15 min in 0.3 M NaCl, 50 mM NaPB, pH 7.4 at 37 °C; 6.) digested with 20 µg/ml RNAse A + 50 units/ml RNAse T1 in 0.3 M NaCl, 50 mM NaPB, pH 7.4 at 37 °C for 30 min. 7.) washed in 50% formamide, 0.5 M NaCl, 50 mM NaPB pH 7.4, 0.5% SDS, 100 mM 2-mercaptoethanol at 60 °C for 60 min (high criterion wash); 8.) Finally, sections were rinsed in PBS at 25 °C and mounted on Superfrost + microscope slides, air-dried, dehydrated through graded ethanol solutions and exposed to x-ray film. Sense-strand probes of equal specific activity were hybridized to adjacent sections for background assessment.

2.3. Image analysis

X-ray film images were captured with a Dage MTI CCD-300 video camera equipped with a Canon 50 mm macro lens. Images were analyzed with NIH Image 1.62b/Image *J*. Mean signal densities were quantified with Image *J* freeware and then densities of sense strand background were subtracted out. Data were then normalized to appropriate data from vehicle-treated controls and reported in the figures. Rat brain anatomy was determined by comparison of x-ray film images with cognate tissue sections after nissl-staining, with reference to the atlas *Brain Maps: Structure of the Rat Brain* by L.W. Swanson. Results were compared using type 2, 2-tailed students *t*-test compared to appropriate vehicle controls. When standard deviations were significantly different and F-test p value was less than 0.05, type 3, 2-sided *t*-tests were used. Alpha value was set at 0.05, though p-values below 0.07 were indicated as notable trends.

3. Results

In the present study, we investigated whether chronic administration of 2 low-impact ampakines could modulate the levels of neurotrophin expression across several brain regions. Our studies utilized doses of 30 mg/kg CX516 and 3 mg/kg CX691. These doses were selected based upon their ability to significantly increase performance in the 8-arm radial maze (data not shown).

After 15 days of chronic administration of the respective ampakines, rats were sacrificed and neurotrophin mRNA levels were assayed using a radioactive cRNA probe against either BDNF or NGF. In general, neither ampakine significantly increased BDNF levels in the hippocampus (Fig. 1a), other than the statistically significant increases of ~25% in BDNF mRNA in the CA2 and CA3 regions of the hippocampus produced by CX516 (Fig. 1a, $p < 0.05$). In both areas of the lateral amygdala assayed, both ampakines produced increases ranging from ~20-90% (Fig. 1b) in BDNF mRNA levels. In both areas of the basolateral amygdala assayed, ampakines produced increases in BDNF mRNA levels ranging from ~15-90% (Fig. 1b), a much larger effect than that seen in most regions of the hippocampus (compare Figs. 1a and b). In the somatosensory cortex, ampakines produced a disparate effect depending on the area of the somatosensory cortex examined. In the SSp-nII and SSp-nVI regions, ampakines produced increases in BDNF mRNA levels of ~10-80% whereas in the SSp-trII, SSp-trVI and SSs II/III regions, both ampakines failed to produce significant increases in BDNF mRNA levels (Fig. 1c). In the SSs VI region, CX691 produced an approximate 25% increase in BDNF mRNA (Fig. 1c, $p < 0.05$). Fig. 1d shows that BDNF is primarily localized to the CA2 and 3 regions of the hippocampus, the ventral and dorsal blades of the hippocampus and to the amygdala (Fig. 1d). In these regions other than the dentate gyrus, CX516 produces subtle yet significant up regulations in BDNF mRNA (Fig. 1a-b), whereas CX691 is primarily active in the amygdala (Fig. 1b).

We also examined whether ampakines could increase levels of NGF mRNA in the hippocampus. Both ampakines significantly enhanced

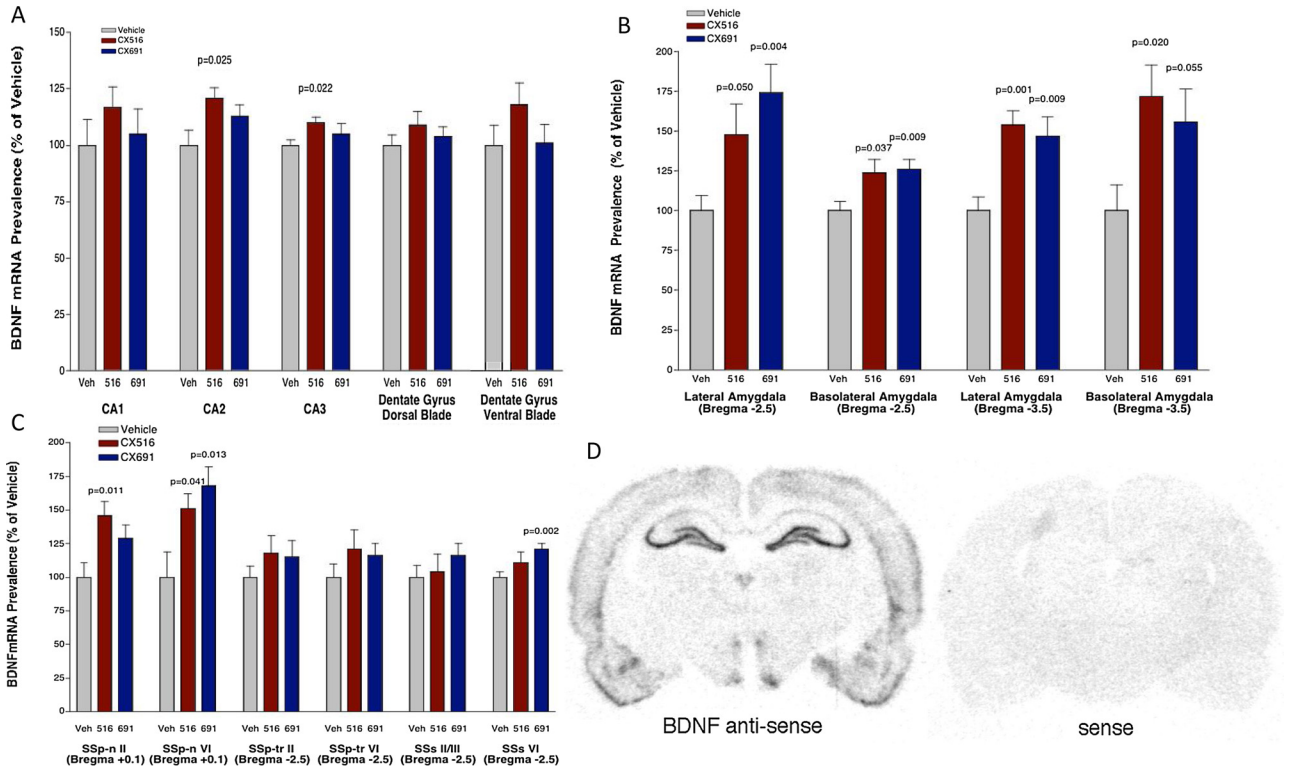

Fig. 1. Effects of Ampakines on BDNF mRNA prevalence in specific brain regions. Mean density levels of signals obtained with BDNF anti-sense cRNA, after correction for sense strand background, are reported as percent of vehicle control rats (n = 8 rats/group for each Ampakine or vehicle). Both sides of the brain were quantified and averaged. Ampakine effect on BDNF levels were examined in (A) the hippocampus, (B) the basolateral and lateral nuclei of the amygdala and (C) the somatosensory cortex. (D) BDNF sense and anti-sense x-ray film images. Significance was judged by 2-tail *t*-test, assuming unequal variance, compared to respective field vehicle-treated control.

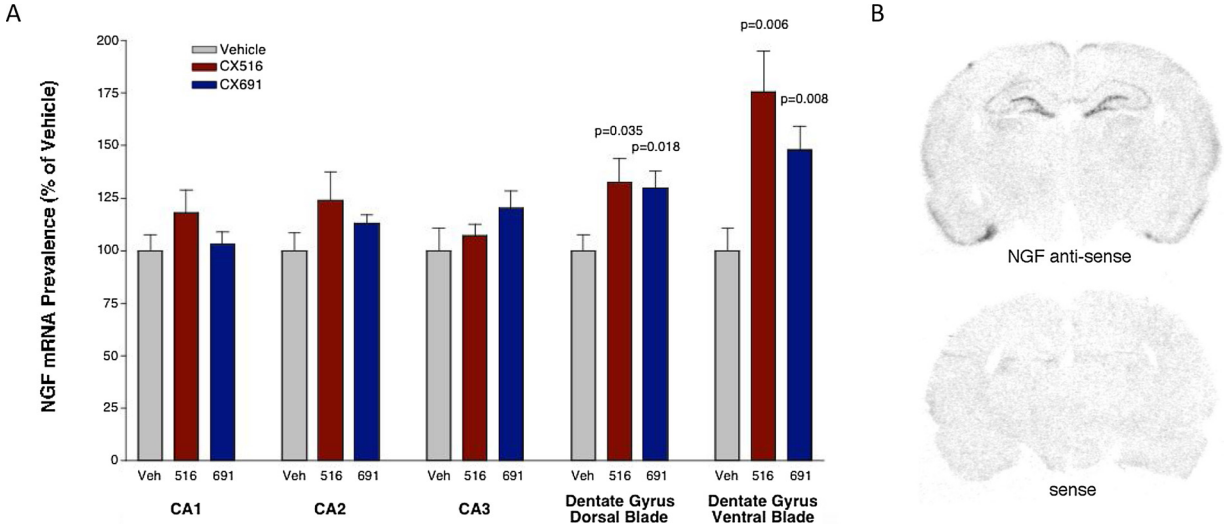

Fig. 2. (A) CX516 and CX691 Enhance NGF mRNA prevalence in dentate gyrus. CX516 and CX691 significantly enhanced NGF message levels in the dentate gyrus granule cell layers, the principal location for NGF in the hippocampus. (B) NGF Sense and anti-sense x-ray film images.

NGF mRNA in the dentate gyrus granule cell layers (Fig. 2), a principal location of NGF in the hippocampus. Both ampakines generally produced a greater increase in NGF mRNA in the ventral blade than in the dorsal blade of the dentate gyrus. In comparison, neither ampakine produced a significant increase in NGF mRNA in CA1, CA2 or CA3 pyramidal fields (Fig. 2). We observe in a representative brain slice image, NGF is primarily localized to the dentate gyrus (Fig. 2b), suggesting low impact ampakines can augment NGF transcription but may not be able to induce de novo NGF transcription in brain regions that do not normally produce it.

Finally, we asked whether chronic ampakine administration could augment levels of both neurotrophins in the piriform cortex. Fig. 3 demonstrates that both ampakines significantly up regulated both BDNF and NGF mRNA levels in 2 distinct layers of the piriform cortex. Both ampakines tended to produce larger increases in NGF mRNA as compared to BDNF mRNA while CX516 produced a notably larger increase in NGF mRNA than in BDNF mRNA in both layers examined (Fig. 3, $p < 0.01$).

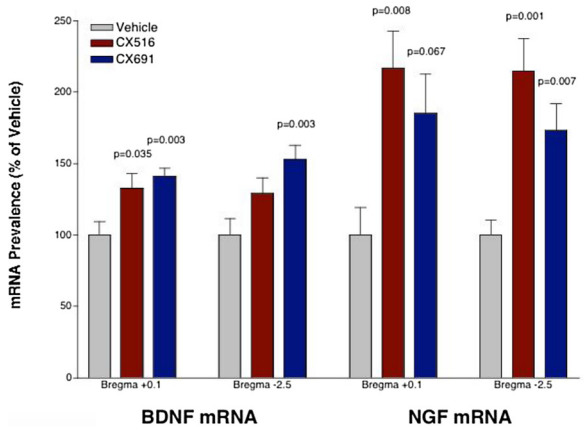

Fig. 3. Ampakines Increase BDNF and NGF mRNAs in Piriform Cortex. Systemic treatment with modest doses of either CX516 (30 mg/kg/day) or CX691 (3 mg/kg/day) significantly up-regulated both BDNF and NGF mRNAs in layer II of piriform cortex, an area involved in processing of olfactory information. The Ampakines enhanced NGF mRNA somewhat more than that of BDNF.

4. Discussion

The present study examined whether 2 low impact or class II ampakines could enhance neurotrophin expression at doses that significantly enhance performance in a behavioral measure of cognition. In general, ampakine administration produced significant increases in BDNF mRNA in basolateral and lateral nuclei of the amygdala and in select cortical areas, including piriform cortex, layer II. Ampakines also produced an increase in NGF mRNA in the dentate gyrus of the hippocampus and in layer II of the piriform cortex.

For nearly two decades, it has been well documented that AMPAR modulation by Class I high impact ampakines greatly increases BDNF levels in organotypic cultures [14]. While preliminary data suggested that low impact ampakines did not increase BDNF levels in hippocampal cultures [20], this lack of effect may have been due to differences between the use of *in vitro* cultures as opposed to *in vivo* administration in the present study. It is also relevant that ampakines increase BDNF mRNA more consistently and to a greater extent in amygdala and somatosensory and piriform cortices.

It is still unknown whether the ampakines used in the present study enhance neurotrophin expression by augmenting ionotropic or metabotropic AMPAR signaling. It has been established that AMPARs, although termed ionotropic, have a Gi-protein linkage and also modulate BDNF expression *via* its interaction with MAP kinase [21]. Our previous work suggests that ampakines augment LTP ~20 min after acute administration [22], a time-scale which generally precludes involvement of de novo gene expression. Furthermore, since enhancing LTP is sufficient to augment neurotrophin levels [7], the ampakines used in the present study may enhance neurotrophin expression by enhancing both AMPAergic currents (thereby increasing LTP) and by increasing metabotropic interactions with effectors like MAP kinase and G-proteins.

Regardless of whether ampakines augment neurotrophin expression by enhancing ionotropic and/or metabotropic AMPAR signaling, the present data give clues as to AMPAR subtypes preferred by the low-impact, class II ampakines. This class of ampakines does not significantly modulate AMPAR agonist binding affinity and does not bind to the well-characterized cyclothiazide binding site [9], which makes their receptor subunit preferences increasingly more difficult to characterize. However, by looking at the neuroanatomical distribution of AMPARs in the rat brain and comparing that data to regions in the brain in which ampakines produced a significant increase in either neurotrophin, we may be able to theorize the subunits for which these ampakines have a higher affinity for. The neuroanatomical distribution of AMPAR subunits in the rat brain as reported by Martin et al [23] demonstrates that in the DG and several areas in the amygdala, where ampakines elicited significant increases in neurotrophin mRNA, Glur1 is expressed at substantially higher levels than other AMPA receptor subunits. This correlation suggests the possibility that the low-impact ampakines used in the study may have relative selectivity for Glur1 than for the 3 other subunits.

Taken together, our data demonstrates that neurotrophin levels can be augmented by chronic administration of pharmacologically relevant doses of low impact ampakines [8]. Importantly, the doses required to increase neurotrophin levels also increase performance in the 8-arm radial maze task, a measure of cognitive ability. This study shows for the first time that low impact ampakines may have the ability to increase NGF levels when administered chronically, and that in some cases, NGF levels are increased to a larger extent than BDNF levels. Furthermore, we demonstrate that it is possible to safely increase BDNF levels with ampakines without the risk of eliciting seizurogenic side effects as observed with cyclothiazide, the prototypical high impact ampakine [24]. However, at this time, these results must be interpreted cautiously, as even significant increases in neurotrophin mRNA may not result in sustained protein levels and adequate secretion into the extracellular space.

Neurotrophin dysregulation has been implicated in the pathophysiology of several neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's diseases and Rett syndrome [25–28]. It has also been implicated in neuropsychiatric disorders such as schizophrenia, depression, generalized anxiety disorder and autism [29–32]. Unfortunately, doses of high impact ampakines required to acutely increase BDNF levels also produce significant epileptogenic effects [24] which would greatly limit their clinical feasibility. On the other hand, because class II or low impact ampakines augment AMPAR currents without interfering with AMPA receptor desensitization, they are not epileptogenic and exhibit high therapeutic ratios, which would make their clinical inception more realistic in the near future. In clinical studies, these ampakines have demonstrated the ability to reverse opioid-induced respiratory depression without nullifying the pain-killing effects of opiates [33], a credible measure of target engagement in humans. The data presented here suggest that these orally bioavailable ampakines may have wider utility in the clinic.

Conflict of interest statement

RespireRx Pharmaceuticals Inc. is developing ampakines to treat opiate-induced respiratory depression in the clinical setting.

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