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Review

Transactivation of Trk receptors in spinal motor neurons

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Summary. The neurotrophins are a family of trophic factors that have been shown to have neuroprotective effects after traumatic lesions of the nervous system and in animal models of neurodegenerative diseases. They mediate a broad spectrum of biological actions by interacting with tyrosine kinase receptors (Trk). While studies have demonstrated that neurotrophin administration may have beneficial effects, there were difficulties in delivering therapeutic quantities of these factors to spinal motor neurons. We now describe a strategy for applying transactivation of Trk receptors using small molecules, such as adenosine, which can penetrate the blood brain barrier and rescue motor neurons from cell death. Transactivation opens up the possibility of stimulating Trk receptors only in populations of neurons that co-express both Trk and adenosine receptors. We propose in this review to exploit transactivation to improve the survival of motor neurons in a transgenic mouse model of ALS and for other neurodegenerative diseases, such as Alzheimer's and Huntington's disease.

Key words: Trk, G protein-coupled receptor, Transactivation, CGS21680, PACAP

Introduction

The development, maintenance and survival of cells in the vertebrate nervous system are regulated by secreted neurotrophic factor and, indeed, studies have shown that mature neurons depend on trophic support throughout life (Lewin and Barde, 1996; Huang and Reichardt, 2001). The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5) and neurotrophin-3 (NT-3), are among the neurotrophic factors which were demonstrated to exert effects on neuronal biology (Chao et al., 1998). They regulate the development, maintenance and survival of cells in the nervous system by interacting with two types of receptors: the tyrosine kinase (Trk) family and the p75 (Chao, 2003). NGF binds exclusively to Trk-A, NT-4/5 and BDNF to Trk-B and NT-3 to Trk-C, while all neurotrophins bind p75. The p75 receptor has been shown to act as an accessory receptor during Trk-mediated signaling (Hempstead et al., 1991; Bothwell, 1995) and to mediate neuronal death in the absence of Trk receptors (Casaccia-Bonnefil et al., 1996; Terrado et al., 2000).

Activation of the Trk receptors mediates the survival promoting activities of neurotrophins (Chao, 2003). Ligand binding (Fig. 1) initiate the formation of Trk homo-dimers and the auto-phoshorylation of the receptor at several tyrosine residues (Reichardt, 2006). This activation induces the docking of adaptor proteins and activating several downstream signaling cascades, including the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway, the Ras/ERK mitogen-activated protein kinase (MAPK) pathway and the phospholipase C (PLC) α pathway, as well as several small G-proteins, such as the Cdc42-Rac-Rho family and Rap-1, and transcription factors, such as CREB (for a review of Trk signaling see (Reichardt, 2006; Zampieri and Chao, 2006). Notably, the PI3K pathway is the major survival-promoting protein for neurons (Miller and Kaplan, 2001).

Because of potent pro-survival effects elicited by Trk signaling, a number of studies have attempted to use Trk ligands as therapeutic agents in neurodegenerative conditions. Indeed, on the strength of results obtained in animal models, a number of clinical trials have investigated the potential of neurotrophins in Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Parkinson's disease

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(PD), diabetic neuropathy and atrophic macular degeneration. However, these clinical trials have been generally disappointing. Subcutaneous or intrathecal injections of BDNF had minimal beneficial effect in patients with ALS and were associated with strong side effects. Similar studies using NGF for the treatment of Alzheimer's disease and diabetic neuropathy encountered similar outcomes – commonly citing problems with delivery and pharmacokinetics. Neurotrophins are bulky, charged molecules which do not cross the blood-brain barrier easily; therefore, reaching target sites within the central nervous system (CNS) at therapeutically relevant concentrations presents a challenge. Enzymatic degradation poses another problem (Adessi and Soto, 2002) and, although modifications may increase the half-life of the proteins (Frokjaer and Otzen, 2005), they may also decrease treatment potency. Higher dosages increase the risk of side effects as well as initiate a process of receptor down-regulation previously observed in rodents (Knusel et al., 1997; Bibel and Barde, 2000). Lastly, neurotrophins have complex functions in neuronal maintenance and their systemic delivery will undoubtedly incur in side effects due to the indiscriminate activation of multiple signaling pathways.

In addition to the initiation of canonical ligand-receptor events, a novel signal transduction pathway involves receptor transactivation by G protein-coupled receptors (GPCRs). In these events, ligand binding to one receptor indirectly activates a second one via a signaling cascade. Transactivation by GPCRs has been documented for various growth factor receptors, including the epidermal growth factor receptor (EGFR), the platelet-derived growth factor (PDGF) receptor and the Trk receptor (Rajagopal et al., 2004; Arthur et al., 2005; Chen et al., 2006; Mori et al., 2006; Jeanneteau et al., 2008). Many secreted growth factors are initially expressed as membrane-tethered precursors, which undergo regulated proteolysis to release the mature species (Massague and Pandiella, 1993; Chen et al., 1995) and the most commonly reported mechanism for transactivation is based on the shedding of a ligand from the membrane. For example, once activated, GPCRs induce matrix metalloproteases (MMPs) (Fischer et al., 2006) leading to cleavage of pro-EGF (Kue et al., 2002) and the subsequent transactivation of EGFR (Keely et al., 1998). It is important to notice that this mechanism relies on the cognate ligand of a receptor and will result in the activation of the full signaling cascade.

A different, more complex mechanism is observed in the case of transactivation of the Trk receptors by GPCRs (Fig. 1) (Lee and Chao, 2001; Rajagopal et al., 2004). In the absence of NGF and BDNF, adenosine or adenosine agonists, such as CGS21680 which engage the A2A-receptor (Jarvis et al., 1989), and PACAP38 promote the phosphorylation of both the Trk receptors

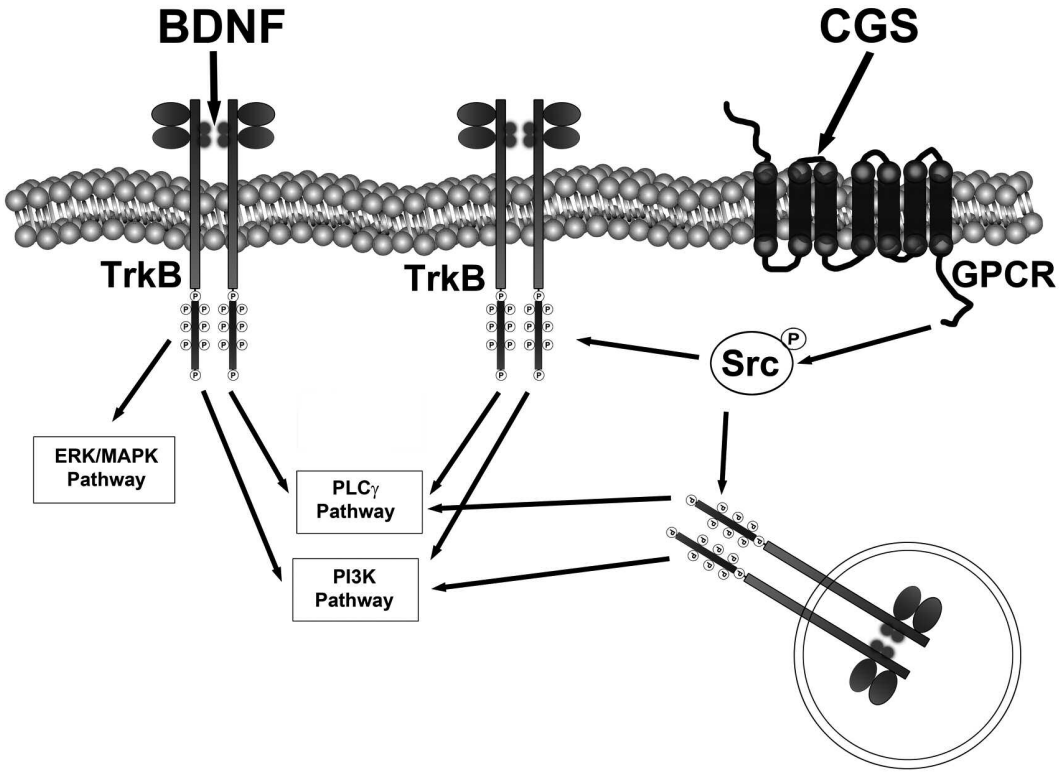

Fig. 1. Mechanisms of Trk signaling. Left panel) Canonical activation of Trk receptors in response to neurotrophin binding. Right panel) Transactivation of Trk receptors by G-Protein coupled receptors in the absence of neurotrophin binding.

and their effectors (Lee et al., 2002). Results have also shown that GPCR stimulation did not promote the synthesis of NGF nor displaced NGF binding (Jarvis et al., 1989; Rajagopal et al., 2004), rather it stimulated intracellular pathways (Rajagopal et al., 2004), such as c-Src and PKC (Arthur et al., 2005).

The mechanism by which GPCRs actually bring about tyrosine phosphorylation of Trk receptors is centered on the activation of the non-receptor tyrosine kinases of the Src family (Luttrell et al., 1999; Lee and Chao, 2001), which are reported to be coupled to most GPCRs that lead to tyrosine receptor transactivation (Thomas and Brugge, 1997). Indeed, when CGS was applied to PC12 cells in conjunction with the Src inhibitor PP1 (Hanke et al., 1996), Rajagopal et al. observed a dose-dependent decrease in the level of phosphorylated Trk receptors (Rajagopal and Chao, 2006). The way in which GPCRs activate Src is not well understood; however, two mechanisms have been proposed. Several GPCRs induce Ca^{2+} flow via activation of phospholipase C. In turn, Ca^{2+} activates the cytoplasmic tyrosine kinase Pyk2 which can associate and activate Src (Dikic et al., 1996; Eguchi et al., 1999). This model is supported by evidence that Trk transactivation by CGS21680 is blocked by pre-incubation with BAPTA/AM, a Ca^{2+} chelating agent (Rajagopal and Chao, 2006). A second mechanism has also received experimental support. The activated ß-adrenergic receptor is phosphorylated by ßark kinases at several sites and associates with the adaptin-type molecule ß-arrestin, which recruits the receptor to coated pits. Evidence shows that Src interacts with the adrenergic receptor–ß-arrestin complex and is activated by this interaction (Luttrell et al., 1999). Regardless of the mechanism and in contrast to neurotrophin-mediated signaling, transactivation of Trks by GPCRs results in the induction of phosphoinositide 3-kinase and Akt signaling cascades but not MAPK (Lee et al., 2002) and, therefore, specifically supplies a survival stimulus in neurons. These findings suggest a possible strategy for selectively activating neurotrophin-mediated survival pathways and bypassing the problems associated with neurotrophin delivery.

The neuroprotective qualities of GPCR ligands have been documented previously. Pituitary adenylate cyclase-activating peptide 38 (PACAP) was shown to have neuroprotective properties after stroke (Chen et al., 2006), retinal degeneration (Atlasz et al., 2009) and oxidative stress (Racz et al., 2007). In a mouse model, chronic treatment with CGS21680 (CGS) attenuated some symptoms of Huntington's disease (Chou et al., 2005). Because BDNF–TrkB signaling has been shown to be required for motor neuron survival (Sendtner et al., 1992), Sendtner and colleagues tested whether CGS transactivation of TrkB *in vivo* could enhance motor neuron survival in a nerve lesion model. Under normal conditions and following facial nerve transection, the motor neurons in the *nucleus facialis* die due to loss of trophic support from their target tissue; however, results demonstrated that application of CGS at the lesion site significantly increases the number of surviving motor neurons (Wiese et al., 2007). Further, analysis of motor neurons from the lesioned side showed that Akt is activated upon treatment with CGS and that the survival response requires the transactivation of TrkB (Wiese et al., 2007).

Results

To evaluate the potential of Trk transactivation as therapeutic tool, CGS or PACAP were delivered by intraperitoneal injections in adult mice. In a pilot study, wild-type mice received CGS (5 μg/g) or saline daily by intraperitoneal injection for 3 days. Following treatment, the spinal cords were isolated, homogenized and subjected to immunoprecipitation with anti-Trk antibodies. The samples were separated by SDS-PAGE and immunostained with specific antibodies against phospho-TrkB or TrkB. Phospho-TrkB immunoreactivity was increased in the spinal cords from CGS treated animals as compared to the ones from animals that received control saline injections (data not shown). Additional spinal cords were subjected to immunofluorescence analysis. As shown in Figure 2, spinal cords sections from CGS treated animals showed a significant increase in phospho-TrkB as compared to control sections. Furthermore, CGS treatment also led to a substantial increase in phospho-Akt reactivity (Fig. 3). These results confirm that GPCR ligands can transactivate TrkB *in vivo* and activate the pro-survival Akt pathway.

Predicting therapeutic outcomes in human patients from any animal model of disease is difficult. However, proof-of-principle studies are important as a first step to the discovery of new therapeutics. Encouraged by our results and the report from Chou et al. that CGS treatment showed efficacy in the animal model of Huntington disease (Chou et al., 2005), we sought to test the same strategy in a model of motor neuron disease.

Motor neuron loss from the lumbar spinal cord is the central pathological finding in the neurodegenerative disease amyotrophic lateral sclerosis (ALS). Approximately 10% of ALS cases are familial (fALS), and of these, 20% have been linked to mutations of the superoxide dismutase (SOD) gene (Rosen, 1993). In the most widely used model of fALS, human mutant cytosolic Cu/Zn SOD1 with a glycine to alanine conversion at the 93rd codon is over-expressed in transgenic mice ($SOD1^{G93A}$ mice) (Gurney et al., 1994). The $SOD1^{G93A}$ mice display an ALS-like pathology with initial symptoms, including fine tremors and weakness, developing in the hind limbs at about 90 days of age, followed by severe hind limb paralysis at 120 days of age (Gurney et al., 1994). Critical motor neuron loss from the lumbar spinal cord occurs coincident with the onset of symptoms (Chiu et al., 1995). In a current study, we delivered CGS (5 μg/g) or PACAP (1.3 nmol/kg) or saline daily by intraperitoneal injection in

pre-symptomatic SOD1^{G93A} mice (age 28 days) for 7 weeks. We hypothesized that the transactivation of TrkB in the lumbar spinal cord would enhance motor neuron survival and result in a delay of disease symptoms. Three parameters were recorded: body weight, grip strength and life span.

Failure to maintain body weight is an indicator of disease onset and progression in the SOD1^{G93A} mouse model of ALS. The average starting body weight (age 28 days) was 16.5±0.47 and 16.8±0.52 g for the saline control- and CGS/PACAP-treated groups, respectively. Although peak body weight occurred at different ages for each animal, when body weight change over time was fitted for each animal, there was a statistically significant difference between groups. The average increase by day 40 from initial body weight was higher in the treated groups (CTRL 0.9±0.2, CGS 2.3±0.2 g, PACAP 2.1±0.1 g) and peak body weight at end of treatment (age 77 days) was higher in treated groups (CTRL 21.1±0.4, CGS 22.6±0.9, PACAP 22.9±1.0 g). The treated animals maintained a higher body weight for 2 week following the end of treatment, at which point body weights were similar in all groups. The data suggested that mice in the treated groups maintained body weight more effectively.

In the SOD1 mouse model, ALS-like pathology is observed first and primarily as a hind limb phenotype; therefore, hind limb grip strength is routinely used to monitor neuromuscular function as a measure of disease progression (Cleveland and Rothstein, 2001). Mice are allowed to grip a triangular bar only with hind limbs, followed by pulling the mice until they released. As early as day 45, an improvement in grip strength was apparent in the CGS- and PACAP-treated mice and, thereafter, these mice performed better than control after end of treatment and into the symptomatic stage (age 100+ days). The average peak strength was 74.3±3.2 g for the control mice, 97.2±4.4 and 95.7±7.1 g for the CGS- and PACAP-treated animals, respectively. There was no statistically significant difference in survival proportions over time when comparing saline control- with CGS- or PACAP-treated SOD1G93A mice. These results indicate that transactivation can overcome several symptoms in the SOD1^{G93A} mice, but does not delay the

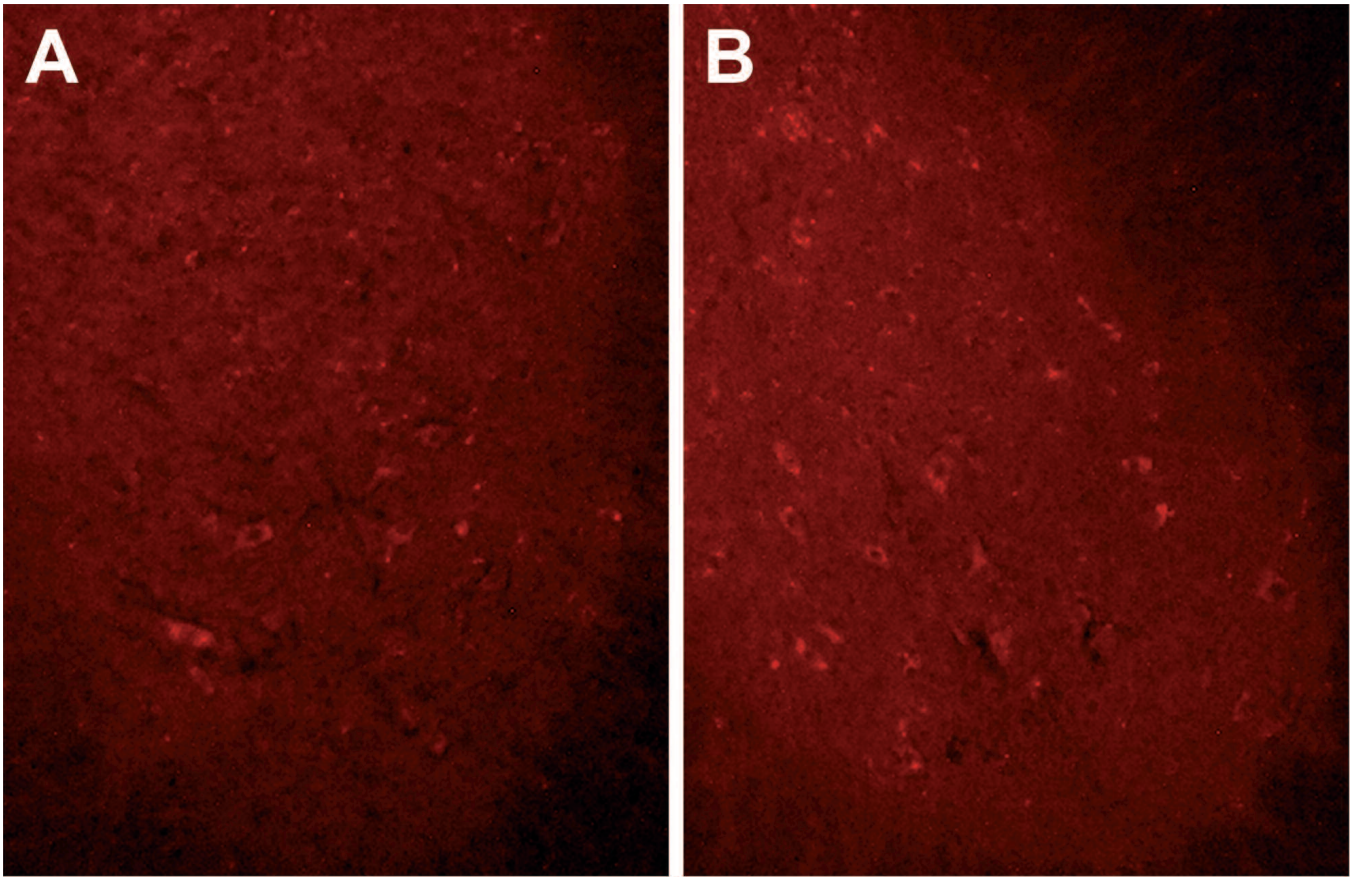

Fig. 2. Transactivation of TrkB in mouse spinal cord. Phospho-TrkB (red) immunostaining of lumbar spinal cord cryosections from wild-type mice treated for 3 days via daily intraperitoneal injections with normal saline **(A)** or CGS21680 **(B)** (5 μg/g).

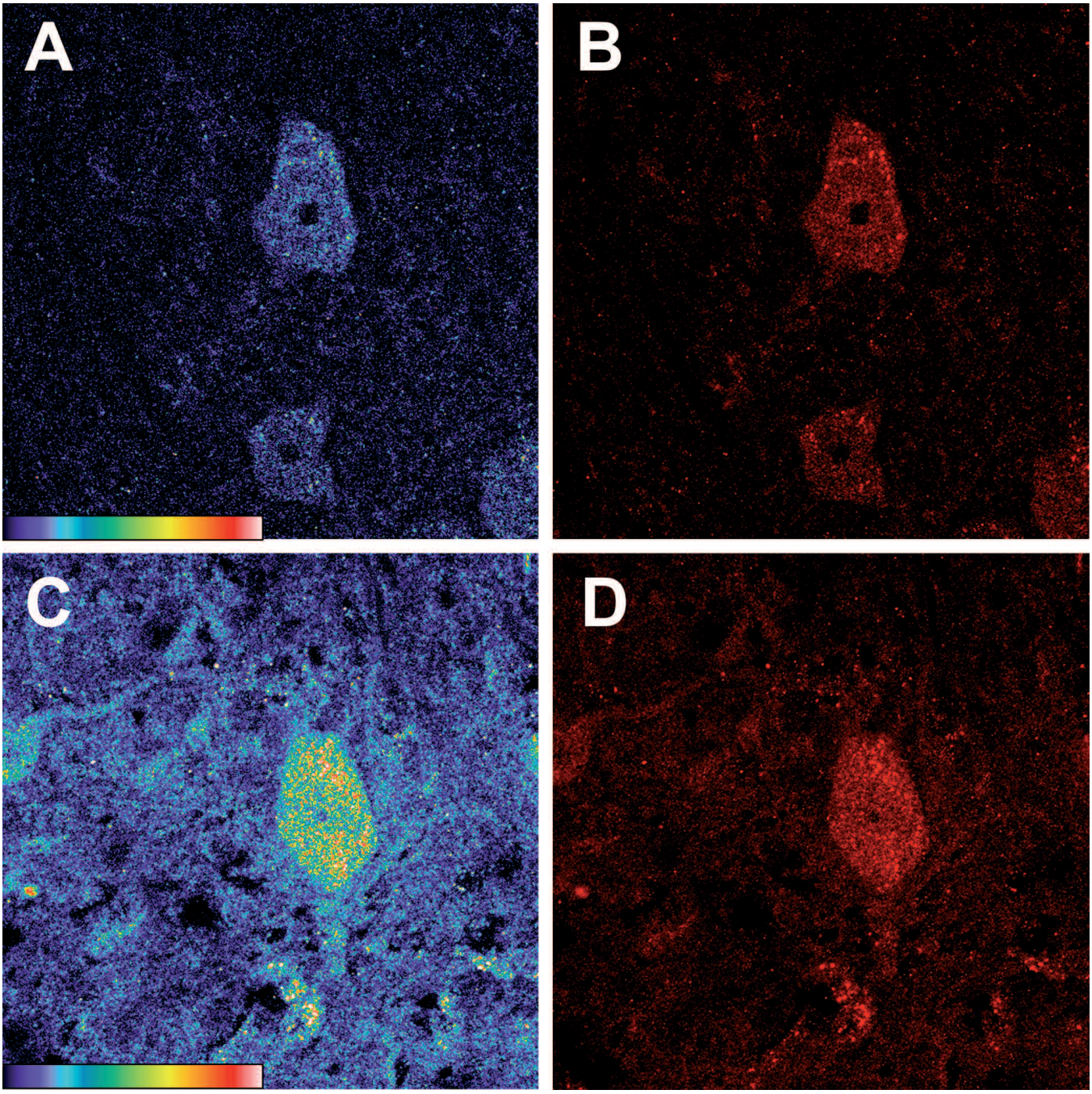

Fig. 3. Induction of Akt signaling by GPCR via TrkB transactivation. Confocal microscopy images of lumbar spinal cord from wild-type mice treated for 3 days via daily intraperitoneal injections with normal saline (**A, B**) or CGS21680 (5 μg/g) (**C, D**). **B, D.** Immunofluorescence labeling of spinal cord sections with phospho-Akt antibodies (red). **A, C.** Quantitative analysis of pixel intensity values within the phospho-Akt images (red is highest).

progression of the disease in this model.

Discussion

Small molecules acting through G protein-coupled receptors can promote trophic activities mediated by receptor tyrosine kinases. Adenosine or PACAP can activate the neurotrophin signaling system in the absence of neurotrophins. This is significant since neurotrophins provide signals to promote neuronal survival, synaptic efficacy and plasticity. Depending upon the circumstances, adenosine and PACAP may be

neuroprotective against injury initiated by ischemia, hypoxia or vascular damage. We have shown that receptor tyrosine kinases represent downstream mediators in G protein-coupled receptor signaling for cell survival in motor neurons. There are hundreds of G protein-coupled receptors and a limited repertoire of receptor tyrosine kinases. Intracellular signaling interactions between individual GPCRs and Trk receptors therefore provide an avenue for developing new approaches to address neurological disorders. Small molecules like adenosine may be used to target populations of neurons that express both adenosine and Trk receptors.

Matching GPCR expression with Trk receptors will identify cell populations that are relevant for a number of nervous system disorders, including cerebral ischemia, amyotrophic lateral sclerosis and Parkinson's diseases and other neurodegenerative conditions. The hypothesis underlying these clinical situations, as well as development of therapeutic strategies using neurotrophic factors assumes that these disease states result in either (1) decreased availability of neurotrophins for the affected neurons; (2) decreased number of neurotrophin receptors on the affected neurons, and/or (3) decreased neuronal survival. These deficits can be ameliorated by the addition of neurotrophic factors.

The strategies to utilize small molecules in place of neurotrophic factors are based on an assumption of symptomatic treatment of impaired neurons. This impairment implies not only cell survival, but also proper functioning of these neurons. With greater understanding of the signal transduction pathways that are activated by GPCR ligands and neurotrophins, alternate strategies can be devised to manipulate these pathways through new drug development. In addition, further understanding of the pathophysiological mechanisms for neurodegenerative disorders will enhance the development of rational therapies that involve the neurotrophin signaling.

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