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Chronic baclofen desensitizes GABA_B-mediated G-protein activation and stimulates phosphorylation of kinases in mesocorticolimbic rat brain

Bradley M.T. Keegan^{1,2}, Thomas J.R. Beveridge^{1,2,&}, Jeffrey J. Pezor^{2,3,&}, Ruoyu Xiao^{1,2}, Tammy Sexton^{1,2}, Steven R. Childers^{1,2}, and Allyn C. Howlett^{1,2,*}

¹Center for the Neurobiology of Addiction Treatment

²Department of Physiology and Pharmacology, Wake Forest School of Medicine

³Department of Chemistry, Wake Forest University, Winston-Salem, NC 27157 USA

Abstract

The GABA_B receptor is a therapeutic target for CNS and neuropathic disorders; however, few preclinical studies have explored effects of chronic stimulation. This study evaluated acute and chronic baclofen treatments on GABA_B-activated G-proteins and signaling protein phosphorylation as indicators of GABA_B signaling capacity. Brain sections from rats acutely administered baclofen (5 mg/kg, i.p.) showed no significant differences from controls in GABA_B-stimulated GTPγS binding in any brain region, but displayed significantly greater phosphorylation/activation of focal adhesion kinase (pFAK^{Tyr397}) in mesocorticolimbic regions (caudate putamen, cortex, hippocampus, thalamus) and elevated phosphorylated/activated glycogen synthase kinase 3-β (pGSK3β^{Tyr216}) in the prefrontal cortex, cerebral cortex, caudate putamen, nucleus accumbens, thalamus, septum, and globus pallidus. In rats administered chronic baclofen (5 mg/kg, t.i.d. for five days), GABA_B-stimulated GTPγS binding was significantly diminished in the prefrontal cortex, septum, amygdala, and parabrachial nucleus compared to controls. This effect was specific to GABA_B receptors: there was no effect of chronic baclofen treatment on adenosine A₁-stimulated GTPγS binding in any region. Chronically-treated rats also exhibited increases in pFAK^{Tyr397} and pGSK3β^{Tyr216} compared to controls, and displayed widespread elevations in phosphorylated dopamine- and cAMP-regulated phosphoprotein-32 (pDARPP-32^{Thr34}) compared to acutely-treated or control rats. We postulate that those neuroadaptive effects of GABA_B stimulation mediated by G-proteins and their sequelae correlate with tolerance to several of baclofen's effects, whereas sustained signaling via kinase cascades points to cross-talk between GABA_B receptors and alternative mechanisms that are resistant to

*Correspondence: Allyn C. Howlett, Department of Physiology and Pharmacology, Wake Forest University Health Sciences, One Medical Center Blvd., Winston-Salem, NC 27157 USA, ahowlett@wakehealth.edu.

&Present Addresses for TJRB: Clinical Sciences, Ferring Pharmaceuticals Inc., 100 Interpace Parkway, Parsippany, NJ 07054; JJP: Aon Hewitt, 3350 Riverwood Parkway, Suite 80, Atlanta, GA 30339

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desensitization. Both desensitized and sustained signaling pathways should be considered in the development of pharmacotherapies targeting the GABA system.

Graphical abstract

Keywords

baclofen; GABA_B receptor; focal adhesion kinase (FAK)/protein tyrosine kinase 2 (PTK2); glycogen synthase kinase 3 (GSK3); dopamine and cAMP-regulated phosphoprotein-32 (DARPP-32)/protein phosphatase 1 regulatory subunit 1B (PP1RB)

1. Introduction

γ -Aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the mammalian brain, plays an integral role in several neuropsychiatric and neurodegenerative pathologies. Modulators of the GABA system constitute novel treatments for Parkinson's and Alzheimer's diseases, as well as neuropsychiatric disorders such as anxiety, depression, and drug addiction (for review, see (Kumar et al., 2013)). Because GABA receptors are expressed throughout the limbic system (Bischoff et al., 1999), they are thought to mediate many emotional behaviors associated with these disorders through modulation of both dopaminergic and glutamatergic systems. GABA_B receptors are class C G-protein-coupled receptors (GPCRs) that exist as heterodimers of GABA_{B1} and GABA_{B2} subunits (Jones et al., 1998). Notably, GABA_{B1} subunits contain the extracellular ligand-recognition site (Galvez et al., 2000), whereas GABA_{B2} subunits facilitate intracellular receptor interaction with G_{i/o}-type G-proteins (Havlickova et al., 2002). GABA_B receptors function to inhibit adenylyl cyclase activity and thus decrease intracellular levels of cAMP. Yet, pre- and postsynaptic GABA_B receptors achieve neuronal inhibition in markedly different ways. Presynaptic GABA_B receptors occur as either autoreceptors or heteroreceptors to inhibit calcium entry into the presynaptic terminal and prevent vesicular neurotransmitter release (Bowery, 2006; Ladera et al., 2008). In contrast, postsynaptic GABA_B receptors predominantly exert their inhibitory effects through release of G $\beta\gamma$ subunits that open inwardly rectifying K_{ir}3-type potassium channels, producing late inhibitory postsynaptic potentials and hyperpolarization (Filip and Frankowska, 2008).

Baclofen, a full agonist at GABA_B receptors, has been used clinically in the treatment of pain and spasticity, conditions which allow for acute treatment paradigms (Bertman and Advokat, 1995). The drug exhibits a short half-life, ranging between 4.5 h (Anderson et al., 1984) and 6.8 h (Wuis et al., 1989), such that it is typically prescribed three or four times daily to treat these conditions. Baclofen therapies are also being investigated for their potential to counterbalance over-stimulation of the dopamine (DA) system caused by alcohol, cocaine, and other addictive drugs; as presynaptic GABA_B receptors located on mesolimbic neuron terminals in the nucleus accumbens (NAc) can effectively inhibit the release of DA (Brebner et al., 2005; Halbout et al., 2011; Xi and Stein, 1998). With emerging evidence that baclofen has therapeutic benefit in addiction treatment (Addolorato et al., 2006; Agabio et al., 2013; Kahn et al., 2009), there is additional need for preclinical studies to evaluate the effects of chronic administration of the drug. Studies have reported a reduction in efficacy following chronic administration of baclofen and the need to increase the dose to maintain its antispasmodic effect (Heetla et al., 2009; Nielsen et al., 2002). These clinical findings are consistent with the observation that tolerance develops to baclofen's locomotor effects after 5 to 11 days of baclofen in rodents (Beveridge et al., 2013; Gianutsos and K. E. Moore, 1978; Levy and Proudfit, 1977). Interestingly, region-specific reductions in cerebral metabolism were observed in acutely treated rats compared with controls, but not in chronically treated animals (Beveridge et al., 2013). These findings emphasized that region-specific changes in neuronal activity must be investigated in order to properly evaluate baclofen's potential as a treatment for conditions that require use of the drug for extended periods.

Molecular mechanisms underlying the development of tolerance to many of baclofen's effects remain unclear. (Kohout and Lefkowitz, 2003) speculated that chronic administration of baclofen might induce either desensitization or changes in GABA_B receptor density. We therefore examined intracellular signal transduction using [³⁵S]GTPγS binding analysis to identify changes in GABA_B-stimulated G-protein activation. We also examined the effects of acute versus chronic GABA_B receptor activation on phosphorylation patterns of three key signaling pathway regulators which served as indicators of changes in fundamental cellular processes: focal adhesion kinase (FAK), glycogen synthase kinase 3-β (GSK3β), and dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32). FAK (also known as protein tyrosine kinase 2, PTK2) is an ubiquitously expressed non-receptor tyrosine kinase that is recruited by integrins and growth factors of the extracellular matrix for incorporation into focal adhesion complexes under conditions of synaptic plasticity (Leask, 2013; Monje et al., 2012). GSK3β is a highly conserved and constitutively active serine/threonine protein kinase whose dysregulation has been implicated in a wide variety of dopamine-associated psychiatric diseases including substance addiction (Shi et al., 2014), bipolar disorder, and schizophrenia (Jope and Roh, 2006). DARPP-32 (also known as protein phosphatase 1 regulatory subunit 1B, PPR1B) is phosphorylated at Thr34 by the cAMP-activated protein kinase A (PKA). The extent of DARPP-32 phosphorylation is commonly used as a measure of psychostimulant activity, as this protein is abundantly expressed in dopaminergic cells involved in the addiction pathology (for review, see (Le Novère et al., 2008). In our study, rat brain sections were evaluated following acute and chronic baclofen treatments in order to

identify those brain regions exhibiting variations in cellular signaling following extended periods of administration compared to a single treatment.

2. Materials and Methods

2.1 Animal Treatments

Animal procedure protocols were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee (IUCAC) and conformed to the principles set forth in the NIH Guide for the Care and Use of Laboratory Animals. All animal experiments were performed in an effort to minimize the number of animals used and the degree of animal suffering. Male Sprague-Dawley rats (280–300 g) (Harlan Industries, Indianapolis, IN, USA) were housed in a temperature- and humidity-controlled vivarium on a 12-hour light/dark cycle (lights on at 7:00 am) and were given unrestricted access to food and water. Rats were randomly assigned to one of three treatment groups: control (n=8), acute baclofen (n=8), or chronic baclofen (n=8). Animals were administered i.p. injections of either 150 mM NaCl saline/vehicle (control), or (±)-baclofen (Sigma Aldrich, St. Louis, MO) (5 mg/kg at 1 mL/kg) (chronic) t.i.d. at 9:00 am, 12:00 pm, and 3:00 pm for five consecutive days as previously described (Beveridge et al., 2013). On the sixth day, the animals were given a final injection of either vehicle (control) or drug (chronic) 15 min prior to sacrifice. The acute treatment group received saline vehicle i.p., t.i.d. for five days and a single i.p. injection of baclofen (5 mg/kg at 1 mL/kg) on the sixth day 15 min prior to sacrifice. This dose of baclofen had been shown to block the development of cocaine sensitization (Frankowska et al., 2009) and reduce responding for amphetamine on both fixed and progressive ratio schedules (Brebner et al., 2005). Animals were sacrificed via sodium pentobarbital (100 mg/kg, i.v.). Brains were removed, quick-frozen in isopentane at -45 °C, and preserved at -80 °C.

2.2 [³⁵S]GTP γ S Binding Analysis

Frozen brains were sliced as coronal sections (20 μ m) using a cryostat microtome maintained at -22 °C and the sections were thawed onto glass slides for [³⁵S]GTP γ S autoradiography (Sim et al., 1995). Brain sections were washed with TME (50 mM Tris-HCl, pH 7.4; 3 mM MgCl₂; 0.2 mM EGTA; 100 mM NaCl) for 10 min at 25 °C prior to incubation with TME assay buffer containing 2 mM GDP for 15 min at 25 °C. Sections were then incubated for two hours at 25 °C in TME assay buffer containing 2 mM GDP, 100 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, an A₁ adenosine receptor antagonist), and 0.04 nM [³⁵S]GTP γ S in the presence and absence of 300 μ M baclofen (Sim et al., 1996a). In some assays, the specific GABA_B antagonist CGP-54626 ((3-N[[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-P-cyclohexylmethylphosphinic acid; Tocris) was added at 1 μ M to block baclofen stimulation of GABA_B receptors (Brugger et al., 1993). For assay of adenosine A₁-stimulated [³⁵S]GTP γ S binding, sections were incubated with 1 μ M of the adenosine A₁ agonist phenylisopropyladenosine (PIA) in the absence of DPCPX (R. J. Moore et al., 2000). The sections were washed twice with 50 mM Tris-HCl, pH 7.4 at 4 °C, rinsed once with deionized water, and were exposed to phosphor-imaging screens overnight. Screen images were captured with a Sony XC-77 video camera, and quantitative densitometric analysis was performed on regions of interest using NIH

ImageJ software (National Institute of Health, Bethesda, MD, USA). Regions of interest were defined by user-defined settings in NIH Image software that selected areas of highest optical density. Optical densities were quantitated by comparison with [^{14}C] brain paste standards and values corrected to nCi/g [^{35}S]. [^{35}S]GTP γ S binding data were expressed as percent of net agonist-stimulated binding in sections from saline-treated control rats.

2.3 Immunohistochemistry: In-Cell Western™ Analysis

Frozen coronal sections (30 μm) were prepared from the same brains used above for [^{35}S]GTP γ S binding using a cryostat microtome maintained at -22°C , and were then placed flat onto frozen phosphate-buffered formalin (1.5 mM KH_2PO_4 , 2.7 mM KCl, 8 mM Na_2HPO_4 , 150 mM NaCl; 30% sucrose (w/v); 3% paraformaldehyde (v/v), pH 7.4) in a well of a 24-well plate. Thawed sections were stored at 4°C in fixative. Kinase phosphorylation was measured using an In-Cell Western assay as previously described (Blume et al., 2013; Kearns, 2004). To summarize, rat brain sections were rinsed six times with Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.4; 137 mM NaCl), blocked overnight at 4°C in Blocking Buffer (TBS containing 0.1% IGEPAL and 50% Odyssey® Blocking Buffer (LI-COR Biosciences®, Lincoln, NE, USA)), and incubated with affinity-purified primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilutions 1:250 or 1:500) in Blocking Buffer overnight at 4°C . The primary antibodies used were anti-FAK (A-17), anti-pFAK (2D11), anti-GSK3 β (11B9), anti-pGSK3 β (Tyr216), anti-DARPP-32 (N-19), and anti-pDARPP-32 (Thr34). Two primary antibodies were applied together: 1) a “total protein” antibody recognizing a non-modifiable region of the protein, and 2) a phosphoprotein antibody specific for only the phosphorylated species of the protein. Tissue sections were then washed four times with TBS containing 0.1% Tween-20 (TBST) and incubated with secondary antibodies conjugated to one of two IRDye® fluorophores (700 nm and 800 nm) (LI-COR Biosciences®; dilution 1:1500) in Blocking Buffer for two hours at 25°C . Sections were washed four times with TBST, allowed to dry overnight at 4°C , and visualized using LI-COR Odyssey Infrared Imaging System software (LI-COR Biosciences®). Images (TIFF files) and signal intensity values were quantified using NIH ImageJ software. Phosphoprotein fluorescence intensity was normalized to the total protein fluorescence intensity in each of the indicated brain regions and expressed as relative fluorescence units (RFU).

2.4 Data Analysis

Brain regions of interest were selected for analyses from each section according to the stereotaxic atlas of the rat brain (Paxinos and Watson, 2014). Densitometric data from [^{35}S]GTP γ S binding were expressed as net agonist-stimulated binding (nCi/g) in sections. Agonist-stimulated G-protein activation is expressed as mean $\pm$ standard error of the mean (SEM) of triplicate sections from 6-8 animals per group for comparison between treatment groups. The mean $\pm$ SEM of normalized RFU values from immunohistochemical assays were used to compare relative phosphorylation levels of each kinase between treatment groups ($n = 8$). Results were expressed as percent of net intensity ratio (RFU value) of saline-treated rat brain regions. Statistically significant differences in GABA B -stimulated [^{35}S]GTP γ S binding and kinase phosphorylation between groups were determined by one-way ANOVA (between-subjects factor: treatment condition) followed by Tukey's multiple

comparisons tests with single pooled variance. In all cases, the family-wise significance and confidence threshold (α) was 0.05.

3. Results

To compare effects of acute versus chronic treatments of baclofen, brain sections were examined from animals following acute (5 mg/kg, single dose i.p.) and chronic (5 mg/kg, t.i.d. for five days) administration schedules. Effects of baclofen treatment on GABA_B activation of G-proteins in various regions were determined by [³⁵S]GTP γ S autoradiography using baclofen as GABA_B agonist (Sim et al., 1996a). We have used similar methods in the past to detect the development of region-selective and time-dependent desensitization of GPCRs following prolonged treatment with their respective agonists (Maher et al., 2001; 2005; Martin et al., 2007; O'Connor et al., 2005; Selley et al., 1997). The pharmacological specificity of baclofen-stimulated [³⁵S]GTP γ S binding is confirmed in Figure 1, which shows autoradiograms of baclofen-stimulated [³⁵S]GTP γ S binding in rat brain coronal sections assayed in the presence and absence of the GABA_B antagonist CGP-54626 (Brugger et al., 1993). These autoradiograms show that stimulation of [³⁵S]GTP γ S binding by baclofen (300 μ M) is completely blocked by CGP-54626 (1 μ M, a concentration of antagonist that blocked stimulation of [³⁵S]GTP γ S binding by 300 μ M baclofen in rat cerebellar membranes; data not shown). These results show that baclofen-stimulated [³⁵S]GTP γ S binding is a specific measure of GABA_B activation of G-proteins in rat brain sections.

Representative autoradiograms from sections prepared from the three treatment groups of rats (saline, acute baclofen, and chronic baclofen) are shown in Figure 2. These sections reveal high levels of baclofen-stimulated [³⁵S]GTP γ S binding in the prefrontal cortex and cingulate cortex (top row), lateral septum (second row), hippocampus, amygdala, and periaqueductal gray (third row), as well as the cerebellum and parabrachial nucleus (bottom row). As these autoradiograms suggest, there was no evident effect of acute baclofen treatment on GABA_B-stimulated [³⁵S]GTP γ S binding in any region; in contrast, chronic baclofen treatment appeared to decrease agonist-stimulated binding in several regions. To quantify these changes, densitometric data were analyzed by brain region (Table 1), with values expressed as nCi/g of net baclofen-stimulated binding from densitometric analysis. Results showed significant baclofen stimulation of [³⁵S]GTP γ S binding in eight brain regions. Acute treatment with baclofen had no significant effect in any of these regions; however, chronic treatment with baclofen significantly reduced levels of baclofen-stimulated [³⁵S]GTP γ S binding in the prefrontal cortex, lateral septum, amygdala, and parabrachial nucleus ($p < 0.05$ vs. saline). Although some reductions in baclofen-stimulated [³⁵S]GTP γ S binding were also observed in the cingulate cortex and hippocampus of rats chronically treated with baclofen, these effects failed to reach statistical significance ($0.05 < p < 0.10$). Chronic baclofen treatment had no detectable effect on GABA_B-stimulated [³⁵S]GTP γ S binding in the cerebellum or periaqueductal gray.

These results were normalized as percent of the [³⁵S]GTP γ S binding in sections from saline-treated (control) animals (Figure 3). Once again, acute baclofen treatment had no significant effect on baclofen-stimulated [³⁵S]GTP γ S binding in any brain region. However, baclofen-

stimulated binding was significantly decreased in the prefrontal cortex (PFC), lateral septum (Sept), amygdala (Amyg), and parabrachial nucleus (PBN) of rats chronically administered baclofen compared to vehicle-treated controls. The most dramatic decreases were observed in parabrachial nucleus (45% decrease vs. control) and septum (35% decrease).

To determine whether these reductions in baclofen-stimulated [^{35}S]GTP γ S binding were specific to GABA $_B$ receptors, adjacent sections from the same animals in these treatment groups were assayed for adenosine A $_1$ -activated G-proteins by [^{35}S]GTP γ S autoradiography using the A $_1$ agonist PIA. A $_1$ -stimulated [^{35}S]GTP γ S binding was chosen as a control because A $_1$ receptors activate G $_{i/o}$ proteins in many of the same brain regions as GABA $_B$ receptors (R. J. Moore et al., 2000). Figure 4 shows the results of densitometric analysis of A $_1$ -stimulated [^{35}S]GTP γ S binding in sections from these rats, expressed as percent of values in saline control rats. In contrast to the effects on GABA $_B$ -activated G-proteins, chronic treatment with baclofen had no effect on stimulation of [^{35}S]GTP γ S binding by the A $_1$ agonist in any region. Of particular note was that chronic baclofen treatment had no effect on A $_1$ -stimulated [^{35}S]GTP γ S binding in the prefrontal cortex, lateral septum, or amygdala; brain regions that displayed significant reductions in GABA $_B$ -stimulation of G-proteins in the same animals.

We also surveyed for changes in tyrosine phosphorylation of kinases that are pertinent to cell signaling in the brain. Representative images of sections prepared from saline-treated rats for detection of total FAK, p-FAK, total pGSK3 β , and pGSK3 β are shown in Figure 5. Quantitative immunohistochemical analyses were performed to quantitate levels of immunodetectable phosphoproteins, which were normalized to total protein for each brain region of interest (Figures 6-8). First, we were interested in patterns of FAK auto-activation at Tyr397. As shown in Figure 6, acute baclofen treated rats displayed significantly increased pFAK^{Tyr397} in the cortex, caudate putamen (Caudate), thalamus (Thal), and hippocampus (Hippo) compared to vehicle-treated control animals. There was also a tendency for higher levels of pFAK^{Tyr397} to be detected in the globus pallidus (Glob Pal) following acute baclofen treatment, but this effect failed achieve statistical significance ($p=0.089$). Following chronic baclofen treatment, rats displayed significantly increased pFAK^{Tyr397} in the cortex, prefrontal cortex, caudate putamen, thalamus, and hippocampus compared to vehicle-treated control rats ($p<0.05$).

Next, we explored the proposal that GABA $_B$ receptors might regulate GSK3 β activity through phosphorylation of Tyr216, the auto-activation site (Cole et al., 2004; Lu et al., 2012). Figure 7 shows that rats acutely administered baclofen exhibited elevated levels of pGSK3 β ^{Tyr216} in the cortex, prefrontal cortex, caudate putamen, globus pallidus, nucleus accumbens (NAc), thalamus, and lateral septum compared to saline controls. Following chronic baclofen treatment, rats displayed significantly increased pGSK3 β ^{Tyr216} in the cortex, caudate putamen, globus pallidus, thalamus, and lateral septum compared to vehicle-treated controls. In chronically treated rats, there also tended to be higher levels of pGSK3 β ^{Tyr216} in the hippocampus ($p=0.066$) and prefrontal cortex ($p=0.74$), although these effects did not achieve statistical significance. Follow-up statistical analyses further revealed that levels of pGSK3 β ^{Tyr216} in rats acutely administered baclofen were significantly higher than those chronically treated in the cortex ($p=0.024$) and globus pallidus ($p=0.032$).

Lastly, the extent of DARPP-32 Thr34 phosphorylation in each brain region following acute versus chronic administration of baclofen is presented in Figure 8. Higher levels of pDARPP-32^{Thr34} were detected in the substantia nigra (SN) of animals treated acutely with baclofen compared to saline controls. There was a similar but insignificant tendency for increased pDARPP-32^{Thr34} in the cortex ($p=0.053$) and lateral septum ($p=0.052$) of acutely treated animals compared to controls. Following chronic baclofen treatment, rats displayed significantly increased pDARPP-32^{Thr34} in the cortex, nucleus accumbens, thalamus, hippocampus, substantia nigra (SN), amygdala, and cerebellum compared to saline controls. In chronically treated rats, there also tended to be higher levels of pDARPP-32^{Thr34} in the ventral tegmental area (VTA) ($p=0.063$), prefrontal cortex ($p=0.064$), and globus pallidus ($p=0.064$), although these effects failed to achieve significance. Further analysis of acute versus chronic treatments revealed significantly higher levels of pDARPP-32^{Thr34} in the thalamus, amygdala, and cerebellum of rats chronically administered baclofen compared to those that received baclofen acutely. In addition, chronically treated rats also tended to display higher levels of pDARPP-32^{Thr34} in the VTA ($p=0.067$) compared to those treated acutely with baclofen.

4. Discussion

The aim of this study was to determine neurobiological changes that occur in the brain as a result of acute versus chronic administration of the GABA_B receptor agonist baclofen. Few preclinical studies have assessed the consequences of extended-term baclofen treatments; nevertheless, there are reports of a decline in efficacy and the development of tolerance to many of baclofen's effects (Heetla et al., 2009; Lehmann et al., 2003; Soni et al., 2003). In the clinic, tolerance to baclofen's untoward side effects, but not its therapeutic effects, might offer a significant pharmacokinetic advantage. However, the intracellular mechanisms by which GABA_B receptor agonists might exert their acute versus chronic effects are not well understood at this time. This report documents neuropharmacological changes observed in both GABA_B receptor-stimulated G-protein activation and phosphorylation of FAK^{Tyr397}, GSK3 β ^{Tyr216}, and DARPP-32^{Thr34} in various brain regions implicated in psychiatric disorders.

4.1 Chronic baclofen treatment attenuates GABA_B receptor-mediated activation of G-proteins

We recently reported the development of tolerance to baclofen's locomotor effects following chronic treatment (Beveridge et al., 2013). Our current data using the same treatment regimen demonstrate region-specific decreases in GABA_B-stimulated G-protein activation arises following chronic administration of baclofen. Taken together, these findings lead to the conclusion that desensitization to GABA_B-mediated signal transduction might account for the locomotor tolerance. Tolerance to baclofen's sedative and antinociceptive effects may also be attributed to GABA_B receptor desensitization, where baclofen-stimulated [³⁵S]GTP γ S binding was nearly abolished in lumbar spinal tissue of rats chronically administered baclofen for seven days (Sands et al., 2003).

The current study shows that chronic treatment of rats with baclofen decreased GABA_B-activated G-proteins to different degrees in different brain regions; indeed, in cerebellum

and periaqueductal gray, there was no effect of chronic baclofen treatment at all, despite relatively high levels of GABA_B-activated G-proteins in these regions. The mechanism of these regional differences is not known; they likely involve regional differences in receptor kinases, β -arrestins, and other molecules involved in receptor desensitization and/or down-regulation. Regardless of the mechanism, this finding of regional differences in receptor desensitization is found in many other studies. For example, our laboratory has previously reported such differences after chronic treatment with morphine, heroin, ⁹-THC, buspirone, and cocaine analogs (O'Connor et al., 2005; Sim et al., 1996a; 1996b; Sim-Selley et al., 2000).

Our finding that a significant decrease in GABA_B receptor-activated G-proteins occurred in the medial prefrontal cortex (mPFC) is consistent with cocaine self-administration as well as sensitization phenomena. Specifically, the dorsal mPFC is important for cocaine- and cue-primed reinstatement of cocaine self-administration in rats (Fuchs et al., 2005). Baclofen injected into the mPFC prior to cocaine injections blocked the initiation of cocaine-induced motor activity sensitization (Steketee and Beyer, 2005). However, baclofen delivered to the mPFC of cocaine-sensitized rats did not block the locomotor effects of a subsequent dose of cocaine (Jayaram and Steketee, 2004). Using microdialysis, these authors later showed that seven days of repeated cocaine exposure (associated with cocaine sensitization) increased GABA levels in the mPFC, and suggested that this increase in GABA neurotransmission following repeated cocaine might be due to a reduction in GABA_B presynaptic autoreceptor function (Jayaram and Steketee, 2005). In consort with these studies, we have shown that baclofen and cocaine induce changes in PET-imaged metabolic activity in many of the same regions of the non-human primate mPFC (Porrino et al., 2013). Moreover, baclofen's effects on the mPFC are associated with its capacity to attenuate acute cocaine-mediated decline in cognition in a delayed match to sample test (Porrino et al., 2013). Taken together, all of these indicate that GABA_B receptor desensitization in the PFC is a key effect linked to cocaine-invoked neuroadaptations and that this might limit baclofen's efficacy as a therapy for addiction.

We report that significant decreases in GABA_B receptor-activated G-proteins also occurred in the basolateral amygdala and lateral septum. GABA neurotransmission within the lateral amygdala has been linked to foot-shock fear conditioning and expression of anxiety (Lange et al., 2014). The lateral septum plays a critical role in depression as determined in the forced swim test (Singewald et al., 2011). Anti-anxiolytic effects of baclofen have been reported in clinical treatment of people for alcohol use disorders (Addolorato et al., 2006), and reduced the incidence of panic attacks in a clinical trial of continued treatment with baclofen for four weeks for panic disorder (Breslow et al., 1989). The authors did not report tolerance to these affects after long-term treatment (Addolorato et al., 2006; Breslow et al., 1989).

The results of the current study cannot differentiate between true receptor desensitization (i.e., uncoupling between the GABA_B receptor and G-proteins) versus receptor down-regulation (i.e., decrease in the number of GABA_B receptors) since a decrease in agonist-stimulated [³⁵S]GTP γ S binding would be produced by either mechanism. Sands et al. (2003) showed that GABA_B receptor desensitization in response to chronic baclofen was not

associated with changes in the mRNA level of GABA_{B1} or GABA_{B2} subunits. Similarly, Lehmann et al. (2003) reported that tolerance to baclofen's hypothermic effects arose without significant changes in GABA_B receptor density or mRNA levels. Both reports support the notion that GABA_B receptors undergo agonist-dependent modification rather than down-regulation. On the other hand, (Malcangio et al., 1993) reported that 21 days of (-)-baclofen administration led to significantly reduced (71%) density of spinal cord GABA_B receptors in autoradiographs, supporting a model whereby GABA_B receptor desensitization occurs via dynamic changes in surface expression of the receptor. These seemingly contradictory conclusions might suggest a mechanistic progression in GABA_B tolerance beginning with modification of G-protein coupling during "sub-chronic" baclofen treatment (5-7 days) followed by long-term adjustments in receptor surface density after extended administration (21 days). To differentiate between these different mechanisms is beyond the scope of the current study; nevertheless, it is important to point out that either desensitization or down-regulation would attenuate GABA_B receptor-mediated signal transduction.

4.2 Baclofen treatment alters auto-phosphorylation of tyrosine kinases

Many of the GABA_B receptor's cellular effects are neuroprotective under conditions of oxidative stress, injury, and glucose deprivation (Tu et al., 2010). We therefore chose to examine changes in the phosphorylation states of two proteins that mediate cellular responses to these conditions, FAK and GSK3 β . We report significantly elevated levels of auto-phosphorylated pFAK^{Tyr397} in several mesocorticolimbic rat brain regions following both acute and chronic baclofen. Focal adhesion complexes serve as cellular attachments to the extracellular matrix (ECM) by linking the actin cytoskeleton to ECM proteins and can mediate cell survival by allowing cells to rapidly adapt to changes in their microenvironments. Typically, FAK is recruited by integrins and growth factors for incorporation into focal adhesions. Activation of FAK involves auto-phosphorylation at Tyr397 (Parsons, 2003) primarily in response to integrin clustering (Cooper et al., 2003). pFAK^{Tyr397} assumes a conformation with a high affinity binding site for several substrates including Src family kinases (Src), which catalyze the phosphorylation of additional tyrosine residues on FAK for maximal activation (Parsons, 2003; Toutant et al., 2000) and stimulation of its downstream PI3K/Akt, Grb2-SOS/Ras/Raf/MEK/ERK, and Rho/Rac effector pathways (Calalb et al., 1996). GPCRs can elicit rapid increases in phosphorylation of FAK at Tyr397 (Rozengurt, 1995; Salazar and Rozengurt, 2001; Zachary and Rozengurt, 1992). In fact, G_{i/o}-coupled (Dalton et al., 2013; Karanian et al., 2005) and G_{q/11}-coupled receptors (Slack, 1998) are capable of stimulating pFAK^{Tyr397} through mechanisms that require a direct interaction of integrins with G-protein subunits (Gong et al., 2010). Moreover, FAK is thought to play a critical role in GPCR transactivation of receptor tyrosine kinases (RTKs), an effect which can occur in the absence of RTK ligands (Knezevic et al., 2009; Lin et al., 2012; Rozengurt, 2007). Auto-phosphorylation of pFAK^{Tyr397}, which occurs in response to GABA_B receptor activation and cross-talk with integrins, is necessary for the assembly of GABA_B receptor, G-proteins, IGF-1 receptor, Src, and Akt into a single protein complex for the transactivation of IGF-1 receptors (Lin et al., 2012). Notably, GABA_B agonists and positive allosteric modulators both induce transactivation of the IGF-1 receptor in primary neurons *in vitro* through a mechanism

involving $G_{\beta\gamma}$ subunits, phospholipase C, and Ca^{2+} (Baloucouné et al., 2012; Lin et al., 2012; Tu et al., 2010).

GSK3 has been described as a ‘master regulator’ of cellular processes due to its wide range of substrates including metabolic proteins, structural proteins, and transcription factors (for review, see (Silva et al., 2014). GSK3 β is a serine/threonine kinase that has been linked to hyper-DA-associated behaviors and psychiatric disorders such as bipolar disorder, schizophrenia, and attention deficit disorder (for review, see (Li and Gao, 2011). Auto-phosphorylation at Tyr216 is associated with enhanced activation of GSK3 β and permits this kinase to function constitutively (Hughes et al., 1993). Phosphorylated pGSK3 β^{Tyr216} plays a critical role in important processes such as memory formation through regulation of long-term potentiation (LTP) (Peineau et al., 2007), inhibition of cAMP responsive element-binding protein (CREB) (Bullock and Habener, 1998; Hansen et al., 2004), and promotion of actin and tubulin assembly during memory formation (Koivisto et al., 2004).

It is not clear how baclofen is regulating GSK3 β Tyr 216 auto-phosphorylation in the brain regions studied herein. Whereas (Lu et al., 2012) observed phosphorylation of Ser9 in cells following *in vitro* treatment with a GABA $_B$ agonist, they failed to detect significant changes in phosphorylation at Tyr216. In fact, regulation of pGSK3 β^{Tyr216} activation is most closely associated with changes in DA receptor signaling, cocaine and other stimulants readily enhance GSK3 β activity, and pGSK3 β mediates the development of sensitization to many effects of these stimulants (Xu et al., 2009). Because high concentrations of DA activate D $_2$ dopamine receptors (G $_{i/o}$ -coupled), D $_2$ receptors predominantly mediate behavioral and locomotor responses to dopamine release through a cAMP-independent mechanism first described by (Beaulieu et al., 2004). DA stimulation of D $_2$ receptors results in the dephosphorylation of Akt at Thr308, inhibiting its kinase activity (Beaulieu et al., 2005). Reduced Akt activity allows for the disinhibition of pGSK3 β by removal of the phosphate from Ser9, a reaction catalyzed by the DA-activated protein phosphatase 2A (for review, see (Li and Gao, 2011). Cocaine and other DA-releasing stimulants readily enhance GSK3 β activity, and pGSK3 β mediates the development of sensitization to many effects of these stimulants (Xu et al., 2009). It is therefore possible that the GABAergic response may regulate other dopaminergic neurotransmitter systems in these brain regions. We report that rats administered acute and chronic baclofen displayed increased pGSK3 β^{Tyr216} in regions receiving dopaminergic input (prefrontal cortex, cortex, hippocampus, thalamus, caudate putamen). For these reasons, we postulate that chronic baclofen resulted in a loss of the tonic inhibitory GABA $_B$ receptor activity at DA-releasing neuronal terminals. Resultant increases in dopamine D $_2$ receptor activation could have inhibited phosphorylation of Ser9, permitting GSK3 β to undergo greater Tyr216 auto-phosphorylation.

4.3. GABA $_B$ desensitization alters phosphorylation of DARPP-32 Thr34

Region-specific elevations in pDARPP-32 Thr34 observed after chronic, but not acute, baclofen administration are consistent with the suggestion that activation of the GABA system might increase phosphorylation of DARPP-32 Thr34 through inhibition of dephosphorylation (Snyder et al., 1994). However, we attribute these changes in DARPP-32 phosphorylation to be a direct consequence of decreased GABA $_B$ receptor activation of G $_{i/o}$

proteins. The loss of inhibitory GABA_B tone and subsequent disinhibition of adenylyl cyclase would be expected to lead to elevated basal levels of tonic cAMP synthesis and cAMP-stimulated PKA activity. If true, one would expect this effect to be most pronounced in areas with the greatest populations of GABA_B receptors. Indeed, the most significant changes in pDARPP-32^{Thr34} were observed in regions where GABA_B receptors are most densely expressed: thalamic nuclei, cerebellum, amygdala, cortex, hippocampus, habenula, substantia nigra, VTA, nucleus accumbens septi, globus pallidus, and hypothalamus (Bischoff et al., 1999; Bowery et al., 1987). PKA-activated pDARPP-32^{Thr34} affects downstream regulation of GSK3 β , CREB, and c-Fos proteins through inhibition of PP1 and is associated with the effects of many commonly abused drugs including cocaine, amphetamine, nicotine, ethanol, and morphine (Svenningsson et al., 2005). Beyond regulation of DARPP-32, increased PKA activity affects the regulation of a number of other protein phosphatase 1 (PP1) inhibitors and has a plethora of other downstream consequences for the cell.

5. Conclusions

Given that GABA_B receptors are expressed throughout the CNS, regulate neuronal excitability and neurotransmitter release in many different neuronal pathways, and are involved in nearly all brain functions, it is no wonder that dysregulation of GABA_B signaling has been implicated in a number of pathologies such as mood disorders (depression and anxiety), neurodegenerative disorders (Parkinson's and Alzheimer's diseases) and substance use disorders (for review, see (Kumar et al., 2013). Observed differences in GABA_B-stimulated G-protein activation between rats administered vehicle and chronic baclofen are indicative of GABA_B receptor desensitization and/or down-regulation. Consequently, increases in pDARPP-32^{Thr34} imply that GABA_B receptor desensitization (i.e., suppressed G_{i/o} protein activity) permits tonic G_s protein activation of cAMP production and increased PKA activation of DARPP-32. On the other hand, changes in baclofen-stimulated, G-protein-independent tyrosine phosphorylation of FAK^{Tyr397} and GSK3 β ^{Tyr216} did not exhibit desensitization. Hence, future studies should seek to uncover these different molecular mechanisms of GABA_B receptor effectors in order to rationally design medications based on the appropriate signaling mechanism involved in the desired therapeutic responses.

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Abbreviations

CREB	cAMP responsive element-binding protein
DA	dopamine
DARPP-32	dopamine and cAMP-regulated neuronal phosphoprotein-32
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
FAK	focal adhesion kinase
GABA	γ -aminobutyric acid
GSK3β	glycogen synthase kinase 3- β
GPCRs	G-protein-coupled receptors
NAc	nucleus accumbens
PKA	protein kinase A

Highlights

- Chronic baclofen reduced GABA_B-mediated G-protein activation in rat brain sections.
- Acute baclofen increased tyrosine phosphorylation of FAK and GSK3 β .
- FAK and GSK3 β phosphorylation was sustained throughout chronic baclofen.
- Acute and chronic baclofen increased DARPP-32 phosphorylation in rat brain regions.
- Chronic baclofen desensitized GABA_B G-proteins, but left tyrosine kinase signaling.

Figure 1. Autoradiograms of baclofen-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in coronal rat brain sections, showing blockade of stimulated binding by the GABA_B antagonist CGP-54626
Sections were incubated in the absence of agonist alone (BASAL), with 300 μM baclofen (BACLOFEN), or 300 μM baclofen in the presence of 1 μM CGP-54626 (BAC + CGP) as described in the Materials and Methods section. Activation of G-proteins by baclofen was reduced to virtually basal levels in all brain regions examined.

Figure 2. Representative autoradiograms showing the effect of acute and chronic baclofen treatments on GABA_B-stimulated [³⁵S]GTPγS binding in coronal rat brain sections
Densitometric values of specific baclofen-stimulated GTPγ[³⁵S] binding were determined as described in the Materials and Methods section, and are denoted as progressing from low to high (blue, green, yellow red).

Figure 3. Effect of acute and chronic baclofen treatments on GABA_B-stimulated [35S]GTPγS binding in coronal rat brain sections

Slide-mounted tissue sections were assayed for baclofen-stimulated [35S]GTPγS binding as described in the Materials and Methods section, and densities from the autoradiograms for each region of interest are presented as mean values ± SEM (n=6-8 per group); * p < 0.05 significantly different from saline (one-way ANOVA, Tukey's test for multiple comparisons).

Figure 4. Effect of acute and chronic baclofen treatments on adenosine A₁-stimulated [³⁵S]GTPγS binding in coronal rat brain sections

Adjacent sections from the same animals assayed in Table 1 and Figure 3 were assayed for A₁-stimulated [³⁵S]GTPγS binding using 1 μM PIA, as an A₁ agonist as described in the Materials and Methods section. Optical densities were determined for each region of interest, and data are presented as mean values ± SEM of percent values in saline animals (n=6-8 per group); No significant effects of either treatment were observed in any region (one-way ANOVA, Tukey's test for multiple comparisons).

Figure 5. Representative images of coronal rat brain sections from saline-treated rats used in immunohistochemical analysis for detection of changes in phosphorylation of signaling proteins Sections were incubated with two primary antibodies: one antibody recognizing a non-modifiable region of the protein (“Total”) and one phosphoprotein-specific antibody (“p”). Secondary antibodies conjugated to two different IRDye® fluorophores were applied for visualization with LI-COR Odyssey Infrared Imaging System software (LI-COR Biosciences®), as described in the Materials and Methods section. The sections shown were colorized red (total) and green (phosphoprotein) for visualization.

Figure 6. Effect of acute and chronic baclofen treatments on FAK auto-phosphorylation at Tyr397 in select rat brain regions

Fixed coronal sections were immunostained for detection of pFAK^{Tyr216} and total FAK and visualized by Li-Cor infrared imaging system. The integrated intensity of pFAK was normalized to total FAK in each region and data are reported as mean $\pm$ SEM (n=6-8); * $p < 0.05$ significantly different from saline (one-way ANOVA, Tukey's test for multiple comparisons).

Figure 7. Effect of acute and chronic baclofen treatments on GSK3β phosphorylation at Tyr216 in select rat brain regions

Fixed coronal sections were immunostained for detection of pGSK3β^{Tyr216} and total GSK3β and visualized by Li-Cor infrared imaging system. The integrated intensity of pGSK3β was normalized to total GSK3β in each region and data are reported as mean values ± SEM (n=6-8 per group); * $p < 0.05$ significantly different from saline, # $p < 0.05$ significantly different from acute baclofen treatment (one-way ANOVA, Tukey's test for multiple comparisons).

Figure 8. Effect of acute and chronic baclofen treatments on DARPP-32 phosphorylation at Thr34 in select rat brain regions

Fixed coronal sections were immunostained for detection of pDARPP-32^{Thr34} and total DARPP-32 and visualized by Li-Cor infrared imaging system. The integrated intensity of pDARPP-32^{Thr34} was normalized to total DARPP-32 in each region and data are reported as mean values $\pm$ SEM (n=6-8 per group); * $p < 0.05$ significantly different from saline, # $p < 0.05$ significantly different from acute baclofen treatment (one-way ANOVA, Tukey's test for multiple comparisons).

Table 1
Effects of saline, acute baclofen, and chronic baclofen treatment of rats on baclofen-stimulated [35 S]GTP γ S binding in brain sections

Net baclofen-stimulated [35 S]GTP γ S binding, nCi/g:			
Region	Saline	Acute baclofen	Chronic baclofen
Prefrontal Cortex	79.8 $\pm$ 6.8	74.3 $\pm$ 6.7	59.6 $\pm$ 5.8 *
Cingulate Cortex	51.5 $\pm$ 5.2	49.4 $\pm$ 3.3	39.2 $\pm$ 4.5
Septum	128 $\pm$ 11	133 $\pm$ 10	85.7 $\pm$ 7 *
Hippocampus	54.3 $\pm$ 5.7	59.7 $\pm$ 3.5	46.7 $\pm$ 3.9
Amygdala	92.3 $\pm$ 7.2	91.8 $\pm$ 6.2	71.0 $\pm$ 4.9 *
Periaqueductal Gray	46.3 $\pm$ 5.7	42.4 $\pm$ 5.3	44.9 $\pm$ 5.8
Parabrachial Nucleus	47.3 $\pm$ 4.8	43.8 $\pm$ 4.5	27.8 $\pm$ 4.1 *
Cerebellum	127 $\pm$ 6.8	121 $\pm$ 5.6	121 $\pm$ 5.1

Rats were treated with saline, or with baclofen (acute, single injection; chronic, 5 days injections t.i.d.) and brain sections were assayed for GABA B -activated G-proteins by [35 S]GTP γ S autoradiography using 300 μ M baclofen as a full agonist, as described in the Materials and Methods section. Data are expressed as net agonist-stimulated [35 S]GTP γ S binding (nCi/g), determined by densitometric analysis of autoradiograms using ^{14}C standards corrected for ^{35}S ;

* $P < 0.05$ significantly different from saline (one-way ANOVA, Tukey's test for multiple comparisons).