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# Lateral Paracapsular GABAergic Synapses in the Basolateral Amygdala Contribute to the Anxiolytic Effects of $\beta$ 3 Adrenoceptor Activation

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Norepinephrine (NE) is known to play an integral role in the neurobiological response to stress. Exposure to stressful stimuli increases NE levels in brain regions that regulate stress and anxiety, like the basolateral amygdala (BLA). NE is thought to increase excitability in these areas through  $\alpha$ - and  $\beta$ -adrenoceptors (ARs), leading to increased anxiety. Surprisingly, recent studies have shown that systemic  $\beta$ 3-AR agonist administration decreases anxiety-like behaviors, suggesting that  $\beta$ 3-ARs may inhibit excitability in anxiety-related brain regions. Therefore, in this study we integrated electrophysiological and behavioral approaches to test the hypothesis that the anxiolytic effects of  $\beta$ 3-AR agonists may be mediated by an increase in BLA GABAergic inhibition. We examined the effect of a selective  $\beta$ 3-AR agonist, BRL37344 (BRL), on GABAergic synapses arising from local circuit interneurons and inhibitory synapses originating from a recently described population of cells called lateral paracapsular (LPCS) interneurons. Surprisingly, BRL selectively enhanced LPCSevoked inhibitory postsynaptic currents (eIPSCs) with no effect on local GABAergic inhibition. BRL also had no effect on glutamatergic synaptic excitation within the BLA. BRL potentiation of LPCS eIPSCs was blocked by the selective  $\beta$ 3-AR antagonist, SR59230A, or by intracellular dialysis of Rp-CAMPS (cAMP-dependent protein kinase inhibitor), and this enhancement was not associated with any changes in spontaneous IPSCs or LPCS paired-pulse ratio. BRL also increased the amplitude of unitary LPCS IPSCs (uIPSCs) with no effect on uIPSC failure rate. Finally, bilateral BLA microinjection of BRL reduced anxiety-like behaviors in an open-field assay and the elevated plus-maze. Collectively, these data suggest that  $\beta$ 3-AR activation selectively enhances LPCS, but not local, BLA GABAergic synapses, and that increases in LPCS-mediated inhibition may contribute to the anxiolytic profile of  $\beta$ 3-AR agonists. Neuropsychopharmacology (2010) 35, 1886–1896; doi:10.1038/npp.2010.59; published online 21 April 2010

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## INTRODUCTION

The locus coeruleus-norepinephrine system (LC-NE) is a key component of the neurocircuitry involved in the psychologically arousing response to stressful stimuli. At times of acute stress or anxiety, levels of NE are increased throughout the central nervous system (CNS), primarily due to an increase in the firing rate of noradrenergic neurons, the majority of which originate in the LC (Bremner *et al*, 1996). NE, through its actions at postsynaptic  $\alpha$ - and  $\beta$ -adrenergic receptors (ARs), is thought to increase neuronal excitation in downstream target brain regions (Morilak *et al*, 2005). In particular, the LC provides dense NE innervation to the basolateral amygdala (BLA; Fallon *et al*, 1978), a brain region that has been shown to play an integral role in the regulation of anxiety-like behaviors (for review, see LeDoux, 2003). Typically, increased BLA excitation is associated with increased anxiety-like behaviors whereas decreased BLA excitability is associated with anxiolysis (for reviews, see Davis *et al*, 1994; Menard and Treit, 1999). In addition, many studies have shown that intra-BLA infusion of NE or AR agonists can enhance the consolidation of longterm memories of emotionally arousing experiences (Ferry *et al*, 1999; LaLumiere *et al*, 2003; McGaugh, 2004). Therefore, LC-NE neurotransmission in the BLA would be hypothesized to increase BLA excitability and thus contribute to anxiogenic-like effects.

Surprisingly, recent studies have shown that systemic administration of selective agonists of the  $\beta$ 3-AR subtype significantly decrease a broad range of experimental anxiety measures to levels similar to those observed with positive GABA<sub>A</sub> receptor modulators like diazepam (Consoli *et al*, 2007; Stemmelin *et al*, 2008). These findings are particularly unexpected as  $\beta$ 3-ARs are thought to be

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predominantly expressed in the periphery, mainly in the vasculature (Rozec and Gauthier, 2006) and in brown and white adipose tissues (Lowell and Flier, 1997), whereas the localization of functional  $\beta$ 3-ARs within the CNS has not been firmly established. Nevertheless, the anxiolytic profile of  $\beta$ 3-AR agonists suggests that these receptors may be expressed in the CNS and that activation of these receptors may actually inhibit neuronal excitability in brain regions associated with anxiety, like the BLA.

The excitability of BLA pyramidal cells, the principal projection neurons within this nucleus, is tightly regulated by networks of GABAergic interneurons that can profoundly influence the expression of anxiety-like behaviors (Sanders and Shekhar, 1995). Interestingly, recent studies have shown that BLA pyramidal neurons receive inhibitory input from at least two distinct GABAergic pathways. One inhibitory pathway comprises diverse groups of local circuit interneurons within the BLA, which provide a network of predominantly feedback inhibition onto BLA pyramidal neurons (Woodruff and Sah, 2007). The second pathway arises from a distinct class of GABAergic interneurons, termed lateral paracapsular cells (LPCS), which cluster along the border between the BLA and the external capsule and are thought to provide cortical feed-forward inhibition within the BLA (Marowsky et al, 2005; Silberman et al, 2008). Importantly, recent data suggest that  $\beta$ 3 mRNA is expressed in the amygdala (Claustre et al, 2008) and that LPCS-mediated synaptic inhibition may be enhanced by NE (Silberman et al, 2008). Therefore, in this study, we used electrophysiological and behavioral approaches to investigate the hypothesis that activation of LPCS-mediated inhibition in the BLA may contribute to the anxiolytic effects of  $\beta$ 3-AR agonists.

# MATERIALS AND METHODS

# **Slice Preparation**

Transverse amygdala slices (400  $\mu$ m) were prepared from 4- to 6-week-old male Sprague–Dawley rats. In some experiments, amygdala slices were prepared from male Sprague–Dawley rats age-matched to the behavioral experiments described below (approximately 10–12 weeks old). Slices were maintained at ambient temperature for at least 2 h in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3.3 KCl, 2.4 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 10 D-glucose, and 25 NaHCO<sub>3</sub>, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

# **Electrophysiological Recordings**

Slices were transferred to a recording chamber maintained at ambient temperature and superfused with aerated aCSF at 2 ml/min using a calibrated flowmeter (Gilmont Instruments, Racine, WI). Recordings were performed at ambient temperature as previous reports from our laboratory and others have shown that this temperature promotes patch stability without altering the pharmacological properties of GABA<sub>A</sub> IPSCs (Ariwodola and Weiner, 2004; Isoardi *et al*, 2007; Jia *et al*, 2005). Whole-cell patch-clamp recordings of evoked GABA<sub>A</sub> IPSCs (eIPSCs) and AMPA EPSCs were conducted using a filling solution containing 130 mM K-gluconate, 10 mM KCl, 1 mM EGTA, 100 µM CaCl<sub>2</sub>, 2 mM Mg-ATP, 200 µM Tris-guanosine 5'-triphosphate, and 10 mM HEPES, pH adjusted with KOH, 275-280 mOsm. Spontaneous IPSCs (sIPSCs) and unitary IPSCs (uIPSCs) were recorded using a similar filling solution, exchanging equimolar CsCl for K-gluconate and KCl. Typically, 2-3 neurons were recorded from each animal per day. In all experiments, 5 mM N-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium chloride (QX-314) was included in the recording solution to block voltage-gated sodium currents and GABA<sub>B</sub> IPSCs in the BLA neurons being recorded. Whole-cell patch-clamp recordings were conducted from BLA pyramidal neurons voltage-clamped at -30 to -40 mV for eIPSCs and at -60 to -70 mV for EPSCs, sIPSCs, and uIPSCs (not corrected for junction potential). Only cells with a stable access resistance of 5–20 M $\Omega$  were used in these experiments. Whole-cell currents were acquired using an Axoclamp 2B or Axopatch 200B amplifier, digitized (Digidata1200 or Digidata 1321A; Axon Instruments, Union City, CA), and analyzed online and offline using an IBM-compatible personal computer and pClamp 9.0 software (Axon Instruments).

# Pharmacological Isolation of Synaptic Currents

Recording electrodes were prepared from filamented borosilicate glass capillary tubes (inner diameter, 0.86 mm) using a horizontal micropipette puller (P-97; Sutter Instruments, Novato, CA). GABA<sub>A</sub> IPSCs were pharmacologically isolated using a mixture of 50 µM APV and 20 µM DNQX to block NMDA and AMPA/kainate receptors, respectively. AMPA EPSCs were pharmacologically isolated using a mixture of 50 µM APV and 20 µM bicuculline to block NMDA and GABA<sub>A</sub> receptors, respectively. In most experiments, synaptic currents were evoked every 20s by electrical stimulation (0.2 ms duration) using a concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) placed near the recording electrode (within 50-100 µm) to target local interneurons or along the external capsule to target LPCS interneurons (Silberman et al, 2008, 2009). For paired-pulse ratio (PPR) experiments, paired IPSCs with a 50 ms interstimulus interval were evoked every 45 s. In both of these experimental procedures, stimulation intensity was adjusted to evoke responses that were 10-20% of maximal currents (typically 80-120 pA). uIPSCs were recorded in the presence of 50  $\mu$ M APV, 20  $\mu$ M DNQX, and 20  $\mu$ M SCH50911. uIPSCs were evoked by electrical stimulation (0.2 ms duration) with a glass stimulating electrode (septum theta tubing: 1.5 mm outer diameter, 1.2 mm inner diameter, 0.2 mm thick septum; World Precision Instruments, Sarasota, FL) placed along the external capsule to target LPCS interneurons. Minimal stimulation was applied at 0.1 Hz and adjusted to threshold levels to produce both synaptic responses and response failures. Failures were identified by visual inspection methods similar to those reported by others (Braga et al, 2003; Kobayashi et al, 2008; Stevens and Wang, 1994). Increasing the stimulation intensity by 40-60% above threshold for evoking uIPSCs did not change uIPSC amplitude, indicating stimulation of a single presynaptic site. Mean amplitude, decay, and failure rate of uIPSCs were calculated from 25-30 consecutive sweeps before, during, and after drug application. sIPSCs

were digitized at 5–10 kHz in continuous 3 min epochs. No stimulation was used during the recording of sIPSCs. Unless otherwise stated, all drugs used were purchased from Sigma (St Louis, MO). Rp-CAMPS (cAMP-dependent protein kinase inhibitor) and all  $\beta$ -AR modulators were purchased from Tocris (Ellisville, MO). Bath-applied drugs were formed as 100- to 400-fold concentrates suspended in water and applied to slices by calibrated syringe pumps (Razel Scientific Instruments, Stamford, CT).

# **Behavioral Experiments**

Microinjection experiments were conducted on 10 male Sprague–Dawley rats ( $\sim$  300 g) as described previously (Lack *et al*, 2008; McCool and Chappell, 2007). Briefly, subjects were surgically implanted with bilateral guide cannulae (26 gauge) targeted to terminate 1 mm above the BLA injection site (2.8 mm posterior to bregma, 8.2 mm ventral to bregma, and 5.0 mm lateral to the midline; see Figure 1 for approximate location of the guide cannulae) and allowed to recover for 1 week. Subjects were singly housed throughout the duration of this experiment.

Following the 1-week surgical recovery period, each rat was placed in a small holding tub  $(27 \text{ cm} \times 17 \text{ cm} \times 12 \text{ cm})$ for 5 min on 5 consecutive days. During this time, subjects were gently restrained by hand, obturators were removed, and the electronic pump used for microinjections was turned on. Sterile obturators were then inserted back into the guide cannulae, subjects were placed back in their transport cages, and then returned to their home cages. On the sixth day, this same habituation procedure was repeated, the obturators were removed, 33 gauge injector cannulae were inserted into the guide cannulae, and all



**Figure I** Anatomical localization of cannulae placements for BRL microinjection experiments. Figure modified from Paxinos and Watson (1997) illustrating the approximate locations for the guide cannulae tips (correct placements are represented by closed circles, misses by open circles). Numbers on the right indicate the anterior-to-posterior position of each coronal brain section, relative to Bregma. In nine subjects, both cannulae were correctly placed within the BLA. In one subject, one cannula was correctly placed and the other was located medial to the BLA, within the central nucleus of the amygdala. Data from all 10 subjects were included in the statistical analysis.

subjects received an injection of vehicle (0.9% saline) in a volume of  $0.5 \,\mu$ l per side over 60 s. Injection needles were left in place for an additional 30 s after injections and the subjects remained in their holding tubs for an additional 3–4 min. All of these procedures were carried out to habituate the rats to the handling and noises associated with the microinjections and to reduce the novelty and stress associated with this procedure.

Following the habituation period, behavioral testing was then conducted 1 day per week using a randomized, Latinsquare design (0.9% saline, 1µg BRL per side). BRL or saline was injected as described above and the behavioral tests were administered 5 min after injections. Because BRL had not previously been used in microinjection studies, the dose selected was based on an extensive comparison of the effective concentration ranges of NE and other AR agonists used in slice electrophysiological and in vivo microinjection studies (NE: Berlau and McGaugh, 2006; Liu et al, 2006; clonidine ( $\alpha$ 2-AR agonist): Ortiz *et al*, 2007; Stevens *et al*, 2004; isoproterenol ( $\beta$ -AR agonist): Egli *et al*, 2005; Selvage and Rivier, 2003). After completion of the behavioral testing phase of the experiment, all subjects were given a lethal dose of sodium pentobarbital and, once deeply anesthetized, were transcardially perfused with buffered saline, followed by 10% formalin in saline. Brains were removed and stored in 10% formalin for at least 1 week and then brain sections were stained with cresyl violet and examined using light microscopy for confirmation of cannulae placements (Figure 1).

Two tests were used to assess the effect of intra-BLA BRL injections on anxiety-like behaviors. The first test used an open-field exploration assay. In this test, subjects were placed in the center of acrylic plastic chambers  $(42 \text{ cm} \times 42 \text{ cm} \times 30 \text{ cm})$  equipped with infrared photodetectors located in an array of eight photobeams on each wall of the chamber, arranged at regular intervals along the length of the chamber, 2.5 cm above the floor (model-RXYZCM, Digiscan animal activity monitors, Omnitech, Columbus, OH). Exploratory activity was monitored for 60 min, in 5 min time bins, and anxiety-like behavior was assessed as time in the center of the open field  $(15 \times 15 \text{ cm})$ and total distance traveled was used as a measure of locomotor activity (Prut and Belzung, 2003). The second test used a standard plus-maze elevated 72.4 cm from the floor with four radial arms  $(10.2 \times 50.8 \text{ cm})$  of which two opposing arms were enclosed by black polypropylene walls (40.6 cm high; Med Associates, St Albans, VT) and the other two walls were open and illuminated by incandescent light  $(\sim 40 \, \text{lx})$ . Infrared sensors were positioned at the opening to each arm to score an animal's entry or exit and data acquisition was performed using a personal computer interfaced with control units and programmed using MED-PC (Med Associates). Subjects were placed at the central junction, facing an open arm, and activity was recorded for 5 min. Anxiety-like behavior was assessed as the time spent on the open arms and the total number of open arm entries, and total number of entries into the closed arms was used as a measure of locomotor activity. Subjects were randomly assigned to different treatment groups and each subject received only one injection (BRL or saline) followed by an exposure to a single behavioral apparatus (plus-maze or open field) each week. Animals

exposed to one test on any given week were tested on the other apparatus the following week. This experimental design has been shown to reduce the 'one-trial tolerance effect' (File *et al*, 1990; File and Zangrossi, 1993) that can develop when animals are repeatedly exposed to the elevated plus-maze (see McCool and Chappell, 2007).

# Statistics

Drug effects on eIPSCs were quantified as the percentage change in the area under the curve of synaptic currents relative to the mean of control and washout values to assess total charge transfer during synaptic events. Drug effects on EPSCs were quantified as percent change in amplitude and area under the curve of synaptic currents relative to the mean of control and washout values. Effects on PPR were quantified as changes in the ratio of the second peak relative to the first during control and drug conditions. Effects on uIPSCs were quantified as percent change in the amplitude, decay, and failure rate relative to the mean of control and washout values. Effects on sIPSCs were quantified as percent change in the frequency, amplitude, and decay of sIPSC events relative to the mean of control and washout values. Statistical analyses of drug effects were performed using the two-tailed Student's paired or unpaired t-tests, or one-way ANOVA with a minimal level of significance of p < 0.05, followed by the Bonferroni or Kolmogorov-Smirnov *post hoc* tests, where applicable. Behavioral data 1889

were analyzed using paired *t*-tests (elevated plus-maze) or repeated-measures ANOVA followed by Bonferroni *t*-tests for multiple comparisons (open-field activity) with minimal level of significance of p < 0.05.

#### RESULTS

# Effect of the $\beta$ 3 Adrenoceptor Agonist BRL37344 on Local and LPCS GABA<sub>A</sub> eIPSCs

As described above, several recent studies have shown that systemic administration of  $\beta$ 3-AR agonists can reduce a broad range of anxiety-like behaviors to levels similar to those observed with positive GABA<sub>A</sub> receptor modulators like diazepam. Because increased GABAergic inhibition in the BLA is known to produce anxiolysis and because NE enhances LPCS-evoked IPSCs (eIPSCs), we hypothesized that the anxiolytic effect of  $\beta$ 3-AR agonists may be mediated, in part, by a selective increase in LPCS GABAergic synaptic transmission onto BLA pyramidal neurons. To examine this hypothesis, we tested the acute effect of a selective  $\beta$ 3-AR agonist, BRL37344 (BRL), on IPSCs evoked from either local or LPCS GABAergic synapses. Bath application of BRL  $(1-10 \,\mu\text{M})$  significantly potentiated the area of LPCS eIPSCs in a concentrationdependent manner (F = 5.00, p < 0.02; Figure 2c). This enhancement was apparent within 5-10 min, reversed on washout (Figure 2a and b), and was not associated with any



**Figure 2**  $\beta$ 3-Adrenoceptor activation selectively enhances LPCS, but not local, GABA<sub>A</sub> eIPSCs. (a) Representative traces from two individual cells (averages of 10 consecutive sweeps) illustrating the effect of the selective  $\beta$ 3-AR agonist, BRL37344 (BRL; 10  $\mu$ M) on LPCS and local GABA<sub>A</sub> eIPSCs recorded from BLA pyramidal neurons. (b) Representative time courses illustrating the effect of 10  $\mu$ M BRL on local and LPCS eIPSCs recorded from BLA pyramidal neurons. (c) Bar graph summarizing the effect of BRL (1–10  $\mu$ M), on the area of eIPSCs evoked by either local or LPCS stimulation once every 20 s. Solid bars above the points indicate the length of drug application. (c) Bar graph summarizing the effect of BRL (1–10  $\mu$ M), on the area of eIPSCs evoked by either local or LPCS stimulation in the BLA (3-11 cells tested at each concentration). Cells (3–11) were tested at each concentration. BRL potentiation of LPCS synapses was significant at concentrations of 4 and 10  $\mu$ M (p <0.05). (d) Bar graph summarizing the blockade of BRL potentiation of LPCS eIPSCs by pretreatment with the selective  $\beta$ 3-AR antagonist, SR59230A (4  $\mu$ M) but not propranolol (10  $\mu$ M). Numbers in parentheses indicate number of cells used in each experimental group (\*, significant increase of BRL from baseline (represented as % control), p <0.05. <sup>#</sup>, significant differences between groups, p <0.05).

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**Figure 3** BRL37344 does not mimic isoproterenol potentiation of AMPA EPSCs evoked by stimulation of thalamic inputs in the BLA. (a) Traces from representative BLA pyramidal cells showing the average of 10 consecutive AMPA EPSCs recorded before, during, and 10 min after application of 10  $\mu$ M BRL37344 (BRL) or 1  $\mu$ M isoproterenol (ISO). (b) Bar graph summarizing the effect of 10  $\mu$ M BRL, 1  $\mu$ M ISO, and 10  $\mu$ M propranolol + 1  $\mu$ M ISO on the amplitude and area of thalamic AMPA EPSCs recorded from BLA pyramidal neurons. \*, significant increase from baseline (represented as % control, p < 0.01). Numbers in parentheses indicate the number of cells tested under each experimental condition. Note that ISO, but not BRL, significantly potentiates AMPA EPSCs and that this potentiation is completely blocked by pretreatment with propranolol, a  $\beta$ -adrenergic antagonist with some selectivity for  $\beta I/\beta 2$  ARs.

significant changes in holding current or input resistance (data not shown). Significant effects of BRL on LPCS eIPSCs were observed at 4 and 10  $\mu$ M (32.0 ± 10.6 and 68.6 ± 14.6% increases from baseline, respectively, p < 0.05; Figure 2c). In contrast, BRL application had no significant effect on local eIPSCs at any concentration tested (Figure 2a-c). BRL (10  $\mu$ M) also significantly potentiated the area of LPCS eIPSCs in slices prepared from older rats that were age matched to the animals used in the behavioral experiments described below (47.5 ± 15.5%, p < 0.05, n = 10; data not shown) with no significant effect of age (p > 0.05).

BRL has been extensively characterized in receptorbinding studies and functional assays in peripheral tissue where it has been shown to be a potent and selective  $\beta$ 3-AR agonist. For example, BRL has a Ki of 2.51 µM for recombinant rat  $\beta$ 3-ARs expressed in CHOK1 cells (Kullmann *et al*, 2009) and has selectivity ratios of 400 for  $\beta$ 1-ARs and 20 for  $\beta$ 2-ARs in stimulating lipolysis in rat brown adipose tissue (Arch *et al*, 1984). However, BRL potency and selectivity can vary depending on the tissue and functional assay being used (Oriowo *et al*, 1996) and no studies, to date, have characterized this drug in an *in vitro* brain slice electrophysiological preparation. Therefore, we conducted several experiments to address the selectivity of BRL for  $\beta$ 3-ARs under our experimental conditions.

First, we tested the effect of BRL in the presence of a selective  $\beta$ 3-AR antagonist, SR59230A. Pretreatment with 4  $\mu$ M SR59230A, which had no effect on its own (4.3 ± 6.1% decrease from baseline, p > 0.05), completely blocked the effect of 4 and 10  $\mu$ M BRL on LPCS eIPSCs (4.9 ± 6.5 and

3.1  $\pm$  7.1% decreases from baseline, respectively; p > 0.05; Figure 2d). Next, we assessed the effect of BRL in the presence of the  $\beta$ -AR antagonist propranolol, which has higher selectivity for  $\beta$ 1/ $\beta$ 2-ARs relative to  $\beta$ 3-ARs (Baker, 2005). In the presence of propranolol (10  $\mu$ M), BRL (10  $\mu$ M) significantly enhanced the area of LPCS eIPSCs (44.2  $\pm$  6.5% increase from baseline, p<0.05). Moreover, there was no significant difference between the effect of BRL on LPCS eIPSCs in the presence or absence of propranolol (p>0.05).

The BLA receives glutamatergic input through cortical and thalamic pathways (Aggleton et al, 1980; LeDoux et al, 1991) and previous studies have reported that activation of  $\beta$ 1 and/or  $\beta$ 2-AR receptors enhances BLA glutamatergic EPSCs (Abraham et al, 2008; Leibmann et al, 2009). Therefore, to determine if BRL had any activity at  $\beta 1/\beta 2$ receptors in the BLA, we examined the effects of this drug on glutamatergic synaptic transmission onto BLA pyramidal neurons. Bath application of 10 µM BRL, the highest concentration tested in this study, had no effect on the amplitude or area of AMPA EPSCs evoked by stimulation of putative thalamic inputs (5.7  $\pm$  4.1 and 11.3  $\pm$  5.5% increases from baseline, respectively; p > 0.05; Figure 3). In contrast, bath application of 1 µM isoproterenol, a potent nonselective  $\beta$ -AR agonist, significantly potentiated the amplitude and area of AMPA EPSCs ( $68.7 \pm 12.1$  and  $75.8 \pm 11.8\%$ increase from baseline respectively; p < 0.003) and, importantly, this effect was completely blocked by pretreatment with the  $\beta 1/\beta 2$ -AR antagonist propranolol (3.3 ± 10.4 and  $7.8 \pm 14.7\%$  increase from baseline, respectively; p > 0.05; Figure 3). Notably, as mentioned earlier, this same propranolol pretreatment had no effect on BRL potentiation of LPCS IPSCs.

Taken together, these findings strongly suggest that, under our recording conditions, BRL is devoid of  $\beta 1/\beta 2$ -AR activity and selectively potentiates LPCS eIPSCs in the BLA by activation of  $\beta 3$ -ARs.

### Synaptic Locus of $\beta$ 3-AR Potentiation of LPCS eIPSCs

In the next set of experiments, we sought to determine the synaptic locus underlying BRL potentiation of LPCSmediated GABAergic inhibition. We first assessed the effect of 10 µM BRL on sIPSCs. Because sIPSCs primarily reflect the activity of local GABAergic interneurons (Jiang et al, 2009; Kaneko et al, 2008; Silberman et al, 2009), we hypothesized that BRL would have no effect on sIPSC parameters. Consistent with this prediction, bath application of 10 µM BRL, a concentration that significantly enhanced LPCS eIPSCs, had no significant effect on the frequency, amplitude, or decay of sIPSC events  $(2.7 \pm 4.9,$  $-4.9 \pm 3.5$ , and  $6.6 \pm 5.2\%$  increases from baseline, respectively; p > 0.05; Figure 4a). We next examined the effect of 10 µM BRL on the PPR of LPCS GABAergic synapses. Changes in PPR of evoked synaptic responses are typically inversely correlated with changes in neurotransmitter release probability (Siggins et al, 2005). Therefore, if BRL enhanced LPCS eIPSCs through a presynaptic increase in terminal GABA release, this enhancement should be associated with a decrease in PPR. Pairs of LPCS eIPSCs were evoked at an interval-pulse interval of 50 ms and, after an 8–10 min baseline, 10 µM BRL was applied for 8–10 min. BRL significantly increased the peak of both eIPSCs (peak 1,  $55.6 \pm 17.7\%$ ; peak 2,  $38.0 \pm 5.5\%$  increases from control, p < 0.05), however this potentiation was not associated with any significant changes in PPR (control,  $1.4 \pm 0.3$ ; BRL,  $1.3 \pm 0.4$ ; p > 0.05; Figure 4b).

 $\beta$ 3-ARs are Gs-coupled receptors that exert their effects through the initiation of cAMP-PKA signaling cascades in target cells (Vrydag and Michel, 2007). Therefore, to determine if BRL may enhance LPCS GABAergic synapses through a postsynaptic mechanism, we tested the effect of a localized disruption of postsynaptic cAMP signaling on BRL potentiation of eIPSCs. We added 40 µM Rp-CAMPS to the pipette filling solution to disrupt postsynaptic cAMP signaling selectively. After a 10–15 min baseline period to ensure adequate dialysis of Rp-CAMPS, slices were treated with 10 µM BRL (as in Figure 1). Intracellular dialysis of BLA pyramidal neurons with Rp-CAMPS effectively blocked BRL potentiation of LPCS eIPSCs (2.1 ± 8.1% increase from baseline; p < 0.05 compared to effect of BRL alone; Figure 4c).

To further assess the synaptic locus underlying BRL potentiation of LPCS synapses, we also examined the effect of BRL on the failure rate and kinetics of minimally evoked uIPSCs. uIPSCs were blocked by 20  $\mu$ M bicuculline (data not shown), confirming that they are mediated by GABA<sub>A</sub> receptors. Typically, presynaptic alterations in neurotransmitter release probability at a single release site will affect the rate of uIPSCs failures whereas postsynaptic alterations should result in changes in the kinetic properties of successful uIPSCs (Auger and Marty, 2000). Application of 10  $\mu$ M BRL significantly increased the amplitude of uIPSCs



**Figure 4** BRL37344 potentiation of LPCS eIPSCs may be postsynaptic. (a) Application of 10  $\mu$ M BRL has no effect on spontaneous IPSCs. Bar graph summarizing the effect of BRL on frequency, amplitude, and decay of spontaneous IPSCs. Traces above the graph are sample recordings of spontaneous IPSCs from a representative cell under control and BRL conditions. (b) Bar graph summarizing the effect of 10  $\mu$ M BRL on the ratio of paired LPCS eIPSCs (50 ms interstimulus interval). Traces above the graph are averages of five consecutive sweeps from a representative cell illustrating that BRL potentiates LPCS eIPSCs but has no effect on the paired-pulse ratio of these responses. (c) Bar graph summarizing the effect of adding 40  $\mu$ M Rp-CAMPS to the recording pipette solution on BRL potentiation of LPCS eIPSCs. Numbers in parentheses indicate number of eells used in each experimental group (\*, significant increase of BRL from baseline (represented as % control), p < 0.05. #, significant differences between groups, p < 0.05).

 $(57.6 \pm 15.7\%$  increase from control, n = 4, p < 0.05; Figure 5) but had no significant effect on the failure rate or decay of the events  $(13.8 \pm 14.4 \text{ and } 9.1 \pm 4.3\%$  increases from control, respectively, n = 4, p > 0.05 for both; Figure 5).





Figure 5 BRL37344 enhances uIPSC amplitude without altering the failure rate at LPCS synapses. (a) Traces from a representative BLA pyramidal cell showing the average of 25-30 consecutive uIPSCs recorded before, during, and 15 min after 10  $\mu$ M BRL application. Cells were voltage clamped at -60 mV and LPCS GABA<sub>A</sub> uIPSCs were evoked by external capsule stimulation every 10 s. Clearly distinguishable responses and failures can be seen under each condition. ulPSCs were recorded in the presence of 50 µM APV, 20 µM DNQX, and 20 µM SCH50911 to block NMDA, AMPA, and GABA<sub>B</sub> receptors, respectively. (b) Bar graph summarizing the effect of 10  $\mu\text{M}$  BRL on the amplitude, decay, and failure rate of LPCS GABA<sub>A</sub> ulPSCs in a group of four cells. Note that BRL significantly increases uIPSC amplitude but had no effect on either uIPSC decay or failure rate (\*, significant increase by BRL from baseline (represented as % control), p < 0.05).

Taken together, these results support a postsynaptic mechanism underlying BRL potentiation of LPCS GABAergic synapses in the BLA.

#### Intra-BLA Microinjection of BRL37344 Decreases Anxiety-Like Behaviors

Our electrophysiological data suggest that  $\beta$ 3-AR activation selectively enhances LPCS, but not local, GABAergic inhibition in the BLA. To assess the possible behavioral significance of these effects, we examined the effect of intra-BLA infusion of BRL on anxiety-like behaviors using an open-field exploration assay and the elevated plus-maze. Bilateral microinjection of BRL (1 µg per side) had no effect on total distance traveled (Figure 6a) or movement time (data not shown) in the open field. However, a significant main effect of BRL was observed on center exploration time (F = 6.475, p < 0.03; Figure 7b) in this assay. Post hoc analysis revealed that BRL significantly increased center time at the 15, 20, 25, 40, and 50 min time bins. In the elevated plus-maze, intra-BLA injections significantly increased time spent on the open arms (saline,  $7.0 \pm 2.7$  s; BRL, 56.7  $\pm$  7.8 s; t = 6.961; p < 0.0001; Figure 7a) and the number of open arm entries (saline,  $1.0 \pm 0.3$  entries; BRL,  $6.7 \pm 1.0$  entries; t = 4.020; p < 0.003; Figure 7b), consistent with a decrease in anxiety-like behavior (Holmes and Rogers, 1999; Engin and Treit, 2008). In contrast, BRL microinjections had no effect on the number of closed arm entries (saline,  $10.9.2 \pm 1.9$ ; BRL,  $9.0 \pm 1.0$ ; t = -1.41; p > 0.05), a measure of general locomotor activity (Figure 7c). To control for possible 'one-trial tolerance' effects that may have been associated with the withinsubject, Latin-Square design used in this experiment, we compared the magnitude of the BRL-mediated increase in open arm time (relative to saline injection) for the animals



Figure 6 Bilateral intra-BLA microinjection of BRL37344 decreases anxiety-like behavior in an open-field exploration assay. Time courses illustrating that bilateral infusion of BRL (1  $\mu$ g per side) into the BLA of Sprague-Dawley rats has no effect on horizontal locomotor activity (a, counts per 5 min) but significantly increases time spent in the center of the field (b), reflecting a decrease in anxiety-like behavior (N = 10; \*, significant difference relative to saline injection, Bonferroni *t*-tests, p < 0.05).

that received BRL during the first or second exposure to this test. Notably, no order effect was observed as BRL increased open arm time to a similar extent in both groups (BRL on first exposure,  $52.2 \pm 14.3$  s increase in open arm time; BRL on second exposure,  $47.1 \pm 4.6$  s increase in open arm time; p > 0.05, N = 5 per group).

#### DISCUSSION

The results of this study show that the  $\beta$ 3-AR agonist, BRL37344 (BRL), selectively enhances LPCS, but not local, GABAergic synaptic inhibition in the BLA. This effect was concentration dependent and completely blocked by a selective  $\beta$ 3-AR antagonist. BRL potentiation of LPCS eIPSCs was not accompanied by any changes in PPR and BRL had no effect on sIPSC frequency or kinetics. In addition, intracellular dialysis of Rp-CAMPS into BLA pyramidal cells significantly attenuated BRL enhancement of LPCS eIPSCs. BRL also enhanced the amplitude of LPCS uIPSCs with no effect on the failure rate of these events. Finally, bilateral microinjection of BRL into the BLA selectively reduced measures of anxiety-like behavior in the open-field exploration assay and elevated plus-maze. Taken together, these data suggest that  $\beta$ 3-ARs are expressed at a postsynaptic locus at LPCS GABAergic synapses in the BLA and that activation of these receptors significantly enhances LPCS-mediated synaptic inhibition. These findings also provide initial evidence that enhancement of LPCS-mediated BLA inhibition may contribute to the recently described anxiolytic effects of  $\beta$ 3-AR agonists.

Although activation of the AR system is generally associated with increases in neuronal excitability in stress- and

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**Figure 7** Bilateral intra-BLA microinjection of BRL37344 decreases anxiety-like behavior on the elevated plus-maze. Bar graph illustrating that bilateral infusion of BRL (1  $\mu$ g per side) into the BLA of Sprague–Dawley rats selectively increases time spent on the open arms of the maze (a) and the total number of open arm entries (b), reflecting a decrease in anxiety-like behavior, while having no effect on general locomotor activity, assessed by measuring the total number of closed arm entries (c) (N = 10; \*, significant difference relative to saline injection, paired –test, p < 0.001).

anxiety-related brain regions, and thus anxiogenic behaviors (Morilak et al, 2005), recent studies have shown that systemic administration of brain-penetrant  $\beta$ 3-AR selective agonists significantly attenuates a broad range of anxietylike behaviors to levels similar to those observed with classical positive GABA<sub>A</sub> receptor modulators, like benzodiazepines (Consoli et al, 2007; Stemmelin et al, 2008). These findings, combined with recent evidence that the anxiolytic effects of systemically administered  $\beta$ 3-AR agonists likely occur, at least in part, through a centrally mediated mechanism (Claustre et al, 2008), suggest that  $\beta$ 3-AR activation may actually promote inhibitory synaptic activity within some components of the anxiety circuitry. Because the BLA has an integral role in the regulation of anxiety-like behaviors (Engin and Treit, 2008; Menard and Treit, 1999) and  $\beta$ 3-AR mRNA can be detected in the amygdala (Claustre et al, 2008), we first sought to test the hypothesis that stimulation of  $\beta$ 3-ARs may enhance GABAergic inhibition in the BLA.

Interestingly, glutamatergic pyramidal cells in the BLA have been shown to receive input from at least two distinct GABAergic inhibitory pathways, those arising from local and LPCS GABAergic interneurons (Marowsky et al, 2005; Silberman et al, 2008, 2009). Our electrophysiological data show that the selective  $\beta$ 3-AR agonist, BRL, concentration dependently enhanced LPCS GABAergic synaptic transmission in BLA pyramidal neurons, while having no effect at synapses arising from local interneurons. To the best of our knowledge, these data represent the first evidence of functional  $\beta$ 3-ARs in the rat BLA and provide a possible cellular mechanism that may contribute to the anxiolytic profile of  $\beta$ 3-AR agonists. Moreover, the observation that BRL selectively enhanced a discrete subpopulation of GABAergic synapses suggests that  $\beta$ 3-ARs may have a restricted expression profile within the BLA, possibly explaining why previous studies did not detect these receptors in this brain region (Rodriguez et al, 1995; Summers *et al*, 1995). The limited expression of  $\beta$ 3-ARs in the BLA may also suggest that pharmacotherapies targeting this receptor system may have better safety profiles compared with other anxiolytic drugs that target more broadly expressed receptor systems (eg, benzodiazepines).

Although BRL is a potent and selective  $\beta$ 3-AR agonist (Arch *et al*, 1984; Oriowo *et al*, 1996), the pharmacological properties of this drug can vary depending on the tissue and assay used (Oriowo *et al*, 1996; Kullmann *et al*, 2009). As, to the best of our knowledge, BRL had not been characterized in a brain slice electrophysiology preparation before this study, we conducted a series of experiments to determine if this drug was selective for  $\beta$ 3-ARs under our experimental conditions.

We first showed that BRL enhancement of LPCS eIPSCs was blocked by a selective  $\beta$ 3-AR antagonist, SR59230A. Importantly, BRL potentiation was not significantly altered by pretreatment with propranolol, a  $\beta$ -AR antagonist with selectivity for  $\beta 1/\beta 2$ - over  $\beta 3$ -ARs (Baker, 2005). We also showed that, as previously reported (Leibmann et al, 2009), the nonselective  $\beta$ -AR agonist isoproterenol significantly potentiated AMPA EPSCs recorded from BLA pyramidal neurons and this enhancement was completely blocked by propranolol pretreatment. Importantly, 10 µM BRL, the highest concentration used in this study, had no effect on AMPA EPSCs. This latter experiment serves two important purposes. First, it shows that activation of  $\beta$ 3-ARs does not reduce excitatory input onto BLA pyramidal neurons, thus ruling out an alternative synaptic mechanism that could have potentially contributed to the anxiolytic profile of  $\beta$ 3-AR agonists. Second, as isoproterenol potentiation of AMPA EPSCs in the BLA is mediated by  $\beta 1/\beta 2$ -AR activation (Figure 3; Abraham et al, 2008; Leibmann et al, 2009), and BRL does not enhance BLA EPSCs, these data strongly suggest that this drug was selective for  $\beta$ 3-ARs, and was devoid of  $\beta 1/\beta 2$ -AR activity under our experimental conditions.

Although the apparent  $\beta$ 3-AR selectivity of BRL in this study was comparable to that observed in other reports (Oriowo *et al*, 1996; Kullmann *et al*, 2009), the potency of this drug was significantly lower than that reported for  $\beta$ 3-AR mediated effects in peripheral tissue assays (Oriowo *et al*, 1996; Yurtcu *et al*, 2006). Although this difference in potency may reflect differences in the properties of central *vs* peripheral  $\beta$ 3-ARs, it should be noted that the apparent potency of BRL in our studies was similar to, or even higher than, that observed in electrophysiological studies that have characterized BRL modulation of ion channel function in peripheral tissue (Doheny *et al*, 2005; Hristov *et al*, 2008). Therefore, it seems likely that these differences in BRL potency may reflect methodological differences between biochemical and electrophysiological assays rather than actual differences in the pharmacological properties of  $\beta$ 3-ARs in the CNS and periphery.

In this study, BRL had no effect on sIPSC frequency or kinetics. Because sIPSCs most likely originate from local, and not LPCS, interneuronal sources in the BLA (Jiang et al, 2009; Kaneko et al, 2008; Silberman et al, 2009), these findings provide additional support for the hypothesis that  $\beta$ 3-ARs are not located at local interneuronal synapses in the BLA. BRL also had no effect on PPR at LPCS synapses, suggesting that BRL-mediated enhancement of LPCS eIPSCs does not involve an increase in terminal GABA release probability. Furthermore, addition of Rp-CAMPS into the recording solution significantly abated the potentiating effect of BRL on LPCS eIPSCs. These data suggest that  $\beta$ 3-ARs are likely expressed at postsynaptic loci and that  $\beta$ 3-AR enhancement of LPCS GABAergic inhibition may be mediated by a cAMP-dependent mechanism, possibly through enhancement of GABAA receptor efficacy at LPCS synapses (Kapur and MacDonald, 1996). The finding that BRL significantly potentiated the amplitude of LPCS uIPSCs with no effect on the failure rate at these synapses further supports a postsynaptic mechanism of BRL action.

It is also noteworthy that activation of  $\beta 1/\beta 2$ -ARs in the BLA results in profound increases in excitatory synaptic transmission whereas  $\beta$ 3-AR activation selectively increases the function of a discrete subset of GABAergic synapses, particularly as all three  $\beta$ -AR subtypes are positively coupled to Gs (Daly et al, 1981; Ursino et al, 2009). One possible explanation for the distinct pharmacological profile of  $\beta$ -AR subtypes in the BLA may be that  $\beta 1/\beta 2$  ARs are predominantly expressed at excitatory synapses whereas  $\beta$ 3-ARs are segregated to LPCS GABAergic synapses in this brain region. Unfortunately, although a recent study has shown that  $\beta$ 1/2-ARs are expressed on BLA neurons (Qu et al, 2008), the specific synaptic localization of the three  $\beta$ -AR subtypes within this brain region is not known. It will be of interest in future studies to determine if the differential effects of  $\beta$ -AR subtypes on BLA synaptic transmission arise from the differential targeting of  $\beta$ 1/2- and  $\beta$ 3-ARs to excitatory and inhibitory synaptic loci.

Taken together, our electrophysiological findings suggest that BRL selectively enhances LPCS, but not local GABAergic inhibition in the BLA. To begin to address the behavioral significance of these finding, we next assessed the effect of bilateral BLA microinjection of BRL on two measures of anxiety-like behavior. Many studies have shown that intra-BLA microinfusion of drugs that enhance GABAergic inhibition significantly decreases a wide range of anxiety measures (Bueno et al, 2005; Gonzalez et al, 1996; Pesold and Treit, 1995). As predicted from our electrophysiological findings, BRL microinjection into the BLA selectively decreased anxiety-like behaviors in both the open-field exploration assay and elevated plus-maze. Importantly, the anxiolytic effects of BRL microinjections were not associated with any alterations in nonspecific locomotor activity on either test. Collectively, our electrophysiological and behavioral study data provide initial evidence that selective activation of LPCS GABAergic synapses may directly contribute to the recently described anxiolytic effects of systemic  $\beta$ 3 agonist treatment (Consoli *et al*, 2007; Stemmelin *et al*, 2008). In addition, these data provide the first evidence of a behavioral role for LPCS interneurons, as the selective enhancement of LPCS synapses was sufficient to produce robust decreases in anxiety-related behaviors. It should, however, be noted that  $\beta$ 3-AR mRNA has been detected in many other brain regions and  $\beta$ 3-AR activation can increase 5-HT transmission, possibly through a peripheral mechanism, as well as NE neurotransmitter levels through a direct increase in the firing rate of LC neurons (Claustre *et al*, 2008). Therefore, it seems likely that  $\beta$ 3-ARs in multiple brain regions may be important in the regulation of anxiety-like behaviors.

In summary, the electrophysiological and behavioral studies presented here suggest that BRL selectively enhances LPCS GABAergic synapses through a postsynaptic mechanism and that  $\beta$ 3-AR enhancement of LPCS-mediated GABAergic inhibition is sufficient to decrease anxietyrelated measures in the whole animal. Although the behavioral role of LPCS interneurons is not clearly defined, these cells are thought to provide feed-forward cortical inhibition in the BLA (Marowsky et al, 2005) and are thus likely to be activated in response to stressful stimuli. Therefore, these findings suggest that this relatively unstudied cluster of GABAergic interneurons is ideally positioned to modulate sensory input to the BLA and thus influence the expression of anxiety-like behaviors. In addition, although the expression of  $\beta$ 3-ARs may be limited and highly localized within the CNS, our study data reveal that selective activation of  $\beta$ 3-ARs at LPCS synapses in the BLA can significantly modulate behavioral measures of anxiety. Future experiments will be needed to elucidate fully the mechanism underlying  $\beta$ 3-AR-mediated enhancement of LPCS GABAergic synaptic transmission in the BLA and to better define the behavioral role of  $\beta$ 3-ARs and LPCS GABAergic interneurons. However, these findings suggest that LPCS interneurons and  $\beta$ 3-ARs may represent promising targets for the development of novel pharmacotherapies for the treatment of anxiety disorders.

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#### DISCLOSURE

The authors declare no conflict of interests.

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