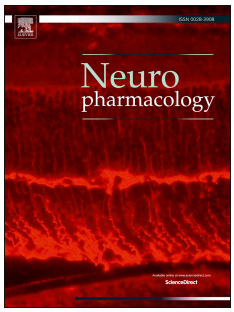
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The $GABA_B$ receptor positive modulator BHF177 attenuated anxiety, but not conditioned fear, in rats

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Running title: BHF177 attenuated unconditioned anxiety in individuals that exhibit a high anxiety state.

Conflict of interest:

AM has received contract research support from Bristol-Myers Squibb Co., Forest Laboratories and Astra-Zeneca, and honoraria/consulting fees from AbbVie, Germany during the past 3 years. AM has a patent on the use of metabotropic glutamate compounds for the treatment of nicotine dependence. VR has received contract research from Johnson and Johnson in the past 3 years. All the other authors report no financial conflicts of interests.

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Abstract

$GABA_B$ (γ-aminobutyric acid B) receptors may be a therapeutic target for anxiety disorders. Here we characterized the effects of the $GABA_B$ receptor positive allosteric modulator (PAM) BHF177 on conditioned and unconditioned physiological responses to threat in the light-enhanced startle (LES), stress-induced hyperthermia, and fear-potentiated startle (FPS) procedures in rats. The effects of BHF177 on LES were compared with those of the $GABA_B$ receptor agonists baclofen and CGP44532, and the positive control buspirone, a 5-HT_{1A} receptor partial agonist with anxiolytic activity in humans. Baclofen (0.4, 0.9 and 1.25 mg/kg) and CGP44532 (0.065, 0.125 and 0.25 mg/kg) administration had significant sedative, but not anxiolytic, activity reflected in overall decrease in the startle response in the LES tests. BHF177 (10, 20 and 40 mg/kg) had no effect on LES, nor did it produce an overall sedative effect. Interesting, however, when rats were grouped by high and low LES responses, BHF177 had anxiolytic-like effects only on LES in high, but not low, LES responding rats. BHF177 also blocked stress-induced hyperthermia, but had no effect on conditioned fear responses in the FPS test. Buspirone (1 and 3 mg/kg) had an anxiolytic-like profile in both LES and FPS tests. These results indicate that BHF177 may specifically attenuate unconditioned anxiety in individuals that exhibit a high anxiety state, and has fewer sedative effects than direct agonists. Thus, BHF177 or other $GABA_B$ receptor PAMs may be promising compounds for alleviating increased anxiety seen in various psychiatric disorders with a superior side-effect profile compared to $GABA_B$ receptor agonists.

1. Introduction

Anxiety disorders are the most prevalent psychiatric disorders across the globe (WHO, 2001). Although the causes of most anxiety disorders are largely unknown, the γ-aminobutyric acid (GABA)-ergic system is one of the most well-known mediators playing a vital role in the pathophysiology of anxiety disorders (Millan, 2003; Mohler, 2012; Pilc and Nowak, 2005). As primary mediators of inhibitory neurotransmission, GABAergic neurons are abundant throughout almost all brain regions (Millan, 2003; Pilc and Nowak, 2005). The ubiquitous nature of the GABA system links its function to that of most other neurotransmitter systems. Specifically, GABAergic pathways exert an inhibitory influence upon the release of many neurotransmitters known to mediate anxiogenic actions, including serotonin, noradrenaline, dopamine and glutamate (Millan, 2003; Mohler, 2012).

GABA signaling is mediated through two receptor classes: the ionotropic $GABA_A$ and $GABA_C$ receptors, and the metabotropic $GABA_B$ receptors (Bormann, 1988; Bowery, 1989). $GABA_A$ receptors are ligand-gated ion channels responsible for the rapid component of inhibitory postsynaptic potentials. $GABA_B$ receptors are G-protein-coupled receptors that inhibit adenylate cyclase activity and mediate the slow and prolonged component of synaptic inhibition (Bormann, 1988; Bowery et al., 2004). For many years, the $GABA_A$ receptor has been a key target for anxiolytic drug development. The benzodiazepines, allosteric modulators of $GABA_A$ receptor that enhance GABAergic neurotransmission, are among the most widely prescribed anxiolytic drugs. However, the repeated treatment with this class of drugs is hampered by dependence liability, sedation, cognitive impairment, ataxia and amnesia (Millan, 2003; Pilc and Nowak, 2005). By contrast, the prototypical $GABA_B$ receptor agonist baclofen, which is used for the treatment of spasticity and skeletal muscle rigidity, has shown to reverse anxiety associated

with alcohol withdrawal, panic disorder, post-traumatic stress and traumatic spinal-cord lesions in clinical studies without abuse potential (Cryan and Kaupmann, 2005). Mice with genetic deletion of the $GABA_{B1}$ receptor subunit show increased anxiety-like behavior in several anxiety-related tests of general avoidance (i.e., light-dark box, elevated zero maze and staircase test) (Mombereau et al., 2004). Similarly, $GABA_{B2}$ receptor subunit deficient mice also exhibit increased anxiety-like behavior in the light-dark box procedure (Mombereau et al., 2005). These genetic deletion studies support the hypothesis that $GABA_B$ receptor is involved in anxiety and increased $GABA_B$ receptor function reduces anxiety-like behavior.

However, $GABA_B$ receptor agonist effects in rodent models of anxiety are inconsistent (Cryan and Kaupmann, 2005; Frankowska et al., 2007; Partyka et al., 2007). This inconsistence is caused, at least partly, by the narrow efficacy dose range of baclofen before side-effects such as sedation, muscle relaxation and hypothermic potential are manifested (Cryan and Kaupmann, 2005; Dalvi and Rodgers, 1996). These side-effects of baclofen limit its use as a tool for behavioral research and as a therapeutic agent, although tolerance develops to some of these undesirable effects (Ong and Kerr, 2005). In contrast, positive allosteric modulators (PAMs) of the $GABA_B$ receptor may be promising for the development of anxiolytic drugs with potentially fewer side effects than agonists at the same receptor (Li et al., 2013). Fully functional $GABA_B$ receptors are heterodimers that consist of $GABA_{B1}$ and $GABA_{B2}$ subunits (Jones et al., 1998; Kaupmann et al., 1998). Endogenous GABA and full receptor agonists bind to the extracellular domain of the $GABA_{B2}$ subunits, whereas PAMs bind to the transmembrane domain of the $GABA_{B2}$ subunits (Guery et al., 2007; Urwyler et al., 2005). Because PAMs increase endogenous, physiological signaling, and cannot activate $GABA_B$ receptors independently of

synaptic activity they may have an improved side-effect profile compared with full receptor agonists (Christopoulos, 2002; Jacobson and Cryan, 2008).

To test our hypothesis that PAMs for $GABA_B$ receptors may have anxiolytic-like properties at non-sedative doses, we examined the efficacy of the novel $GABA_B$ receptor PAM *N*-([1*R*,2*R*,4*S*]-bicyclo[2.2.1]hept-2-yl)-2-methyl-5-(4-[trifluoromethyl]phenyl)-4-pyrimidinamine (BHF177) in rat models of anxiety (Guery et al., 2007). BHF177 is a potent (pEC_{50} = 5.78 ± 0.03; E_{max} (%) = 183 ± 4) and selective $GABA_B$ receptor PAM with good metabolic stability and high bran concentrations after systemic administration (Guery et al., 2007). Early PAMs of the $GABA_B$ receptor, such as CGP7930 and GS39783, are simple symmetric molecules with molecular characteristics that make them likely to exhibit off-target effects. The former contains a strongly electron-rich phenyl ring, and the latter can be expected to be susceptible to nucleophilic attack at the 2- and 5-positions of the pyrimidine ring and oxidation of the ring or the sulfur atom. In studies of combinatorial drug development by synthesis and screening, these characteristics are among those that have been described as leading to pan assay interference, and such compounds are common false positives in such studies (Baell and Holloway, 2010). They represent poor choices for drug development and for in vivo testing, when other alternatives are available. In this case, BHF177 is an excellent alternative, as shows none of these potential deficiencies. Importantly, our previous work and others demonstrated that BHF177 showed more efficacy and/or potency in animal models of drug dependence than other $GABA_B$ receptor PAMs, such as GS39783 and CGP7930 (Maccioni et al., 2009; Paterson et al., 2008; Vlachou et al., 2011a). However, little is known about whether BHF177 has anxiolytic efficacy.

To better understand the potential efficacy of $GABA_B$ receptor activation on anxiety disorders, we used two different startle-related procedures, light-enhanced startle (LES) and fear-

potentiated startle (FPS) test, in the present studies. Both the LES and FPS are anxiety tests that assess passive reflex reactivity and rely on the phenomenon that the acoustic startle response is augmented during threat in mammals (Davis, 1998). It is well documented that startle reactivity is increased during presentation of unconditioned (e.g., foot shock) and conditioned (e.g., shock-paired cue light) aversive stimuli (Davis et al., 1989), or after presentation of more ethologically threatening stimuli (e.g., bright light for rodents) (Walker and Davis, 1997b). These startle-based tests provide translational measures for evaluating the potential efficacy of putative anxiolytic compounds because startle reactivity is a cross-species defensive behavior (Risbrough, 2010). These tests allow for a direct comparison of anxiety-like effects across both unconditioned and conditioned anxiety states. However, $GABA_B$ receptor PAMs have not been tested in these arousal-based models. Thus, findings from the present studies would add a more complete understanding of $GABA_B$ receptor PAM effects on anxiety that complements the literature of these compounds on approach-avoidance based tests (Cryan et al., 2004; Mombereau et al., 2004).

Specifically, we investigated the effects of BHF177 on unconditioned anxiety in the LES and on conditioned fear in the FPS test. Because $GABA_B$ receptor agonists have not yet been tested in the LES model, we also compared BHF177 effects with the effects of the full agonists baclofen and CGP44532. As a positive control we used the $5\text{-}HT_{1A}$ partial agonist buspirone, an effective anxiolytic in generalized anxiety (Ballenger, 1999) that has minimal sedative properties. We did not use a benzodiazepine as we and others have found that the sedation effects of benzodiazepines diminish the utility as positive controls in LES in rats (de Jongh et al., 2002; Walker and Davis, 2002a) and in the equivalent procedure in humans using FPS or darkness (Baas et al., 2002). Finally, we also confirmed anxiolytic activity of BHF177 in a non-startle

based task, the stress-induced hyperthermia (SIH) test which has shown sensitivity to other $GABA_B$ receptor PAMs (Cryan et al., 2004).

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2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River, Raleigh, NC), weighing 300-350 g at the start of the experiments, were housed in groups of two on a 12 h reverse light/dark cycle (lights off from 7 AM to 7 PM) in a colony room with temperature and humidity remaining constant. Food and water were available *ad libitum* in the home cages. On testing days, animals were transferred from the animal facility in transparent individual holding cages and placed in a dark and quiet room for at least 1 h prior to the beginning of testing. Experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and National Research Council's Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Use and Care Committee. An independent group of naive rats was used for each experiment using a between-subjects design for the factor drug dose, except that the same cohort of rats were used for the assessment of baclofen and CGP44532 on LES (see below).

2.2. Drugs

R(+)-baclofen hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally (i.p.) 30 min before testing. CGP44532 was kindly donated by Novartis Pharma AG, and was injected subcutaneously (s.c.), 15 min before testing. Buspirone hydrochloride (Sigma-Aldrich) was injected s.c., 10 min before testing. All of the above compounds were dissolved in sterile 0.9% saline and injected in a volume of 1 ml/kg. BHF177 was synthesized as described previously (Li et al., 2013). BHF177 was suspended in 0.5% methylcellulose and administered orally (p.o.) in a volume of 2 ml/kg, 1 hr before testing. Rats were fasted for

approximately 12 to 16 hrs before p.o. administration of BHF177. The doses of baclofen (0.4, 0.9 and 1.25 mg/kg) were chosen based on previously published studies (Cryan et al., 2004; Frankowska et al., 2007). The doses of CGP44532 (0.065, 0.125 and 0.25 mg/kg) and BHF177 (10, 20, and 40 mg/kg) were chosen based on previous studies in our laboratory and others (Maccioni et al., 2009; Paterson et al., 2008; Vlachou et al., 2011a; Vlachou et al., 2011b). The doses of buspirone (1 and 3 mg/kg) were chosen based on previous publications (Brodkin et al., 2002; Commissaris et al., 2004).

2.3. Acoustic startle apparatus

Eight identical SR Lab ventilated startle chambers with clear Plexiglas cylinders (San Diego Instruments, San Diego, CA) were used for all procedures involving the observation of startle. The Plexiglas cylinders (8.8 cm in diameter and 20.3 cm in length) were mounted on a Plexiglas platform inside a ventilated, sound-attenuated chamber. A high-frequency loudspeaker was located directly above the cylinder housing the rats. For the LES test, a compact fluorescent light bulb (Commercial Electric, Model EDXO-23) on the ceiling was used for the light stimulus (2700-3600 lx). For the FPS test, a 25 W fluorescent bulb (100 µs rise time) was used as the visual conditioned stimulus (CS). In addition, a floor insert made of 10 4-mm diameter stainless steel tubes placed 4 mm apart inside the Plexiglas cylinder was used to deliver foot shocks. Measurement of the startle response is described in detail elsewhere (Mansbach et al., 1988).

2.4. Light-enhanced startle (LES)

The LES procedures were based on those of de Jongh and colleagues (de Jongh et al., 2005) and described in detail elsewhere (Jonkman et al., 2008). At the beginning of the first block, rats

were acclimated in the chamber in the dark for 5 min. Thereafter, the rats were presented with 30 startle stimuli, 10 each at 90, 95, and 105 dB, with an average inter-stimulus interval (ITI) of 30 sec, presented in a pseudorandom order and in dark conditions. The second block of the session was exactly the same as the first block, except that it was presented either in the dark (dark-dark session) or bright light (dark-light session) condition. Thus, one full test of LES consisted of two separate test sessions, one dark-dark to measure habituation of the startle reflex, and one dark-light to measure the startle-enhancing effects of the light. The order of sessions was counterbalanced across subjects within groups. That is, half of the rats started the experiment with a dark-dark session, and the other half began with a dark-light session.

2.5. Fear-potentiated startle (FPS)

The FPS procedures were modified based on those of Walker and colleagues (Walker and Davis, 2002b). Briefly, on days 1 and 2 rats underwent baseline sessions that began with a 5-min acclimation period followed by 30 pulses (100 dB 40-msec) without the presence of the light with an ITI of 30 sec. On days 3 and 4 rats underwent fear conditioning sessions. After 5-min acclimation period, rats received 10 training trials of cue light (CS)–foot shock (unconditioned stimulus; US) pairings with an average ITI of 180 sec (range: 130–230 sec). Each training trial consisted of a 3.7 sec presentation of the light, with a 0.4 mA footshock presented during the last 0.5 sec of the 3.7 sec light. On day 5 rats were given a FPS test session that began with a 5-min acclimation period followed by delivery of a total 60 startle pulses (100 dB 40-msec) with half of the pulses presented during the last 0.5 sec of the CS and the other half without the CS in a pseudorandom order with an average ITI of 60 sec (range: 30–90 sec).

2.6. Stress-induced hyperthermia (SIH)

Immediately after drug administration, rats were returned to their home cage, and the dosing room (room A) was darkened for the remainder of the 1 hr pretreatment period. After the pretreatment period, rats were taken individually to a brightly lit adjacent room (room B) where baseline body temperatures were determined by insertion of a rectal probe lubricated with mineral oil. Core body temperature was assessed using a Physitemp BAT-12 Microprobe Thermometer with a Physitemp RET-2 rat rectal probe (Physitemp Instruments Inc., Clifton, NJ) inserted approximately 2 cm into the rectum; this measure was designated the baseline body temperature, T1, in °C. The rat was then placed back in the home cage and remained in room B. Ten min later, a second body temperature measurement was recorded (T2). The difference between the first and second body temperature measurements (T2 − T1) was used as an index of SIH.

2.7. Experimental design

2.7.1. LES tests:

All naive rats were given one dark-light session first for group assignments (n=15-19/group). Groups were counterbalanced for both average peak startle value in the first block (baseline startle in the dark) and percentage increase of startle value in the second block (light-enhanced startle). A within-subjects design was used for the assessment of BHF177 or buspirone on LES. BHF177 (0, 10, 20 and 40 mg/kg, p.o.) or buspirone (0, 1 and 3 mg/kg, s.c.), were administered before the dark-dark and dark-light sessions using a Latin-square design. Between-subjects design was used for the assessment of baclofen and CGP44532 on LES. Baclofen (0, 0.4, 0.9, 1.25 mg/kg, i.p.) or CGP44532 (0, 0.625, 0.125, 0.25 mg/kg, s.c.) were administered before

the dark-dark and dark-light sessions. There were at least 3 days between dark-dark and dark-light sessions, and 12 days of washout between baclofen tests and CGP44532 tests.

2.7.2. FPS tests:

A between-subjects design was used for the assessment of BHF177 (n=16/group) or buspirone (n=12/group) on FPS. After baseline sessions, rats were assigned to experimental groups to counterbalance for baseline startle reactivity. BHF177 (0, 20 and 40 mg/kg, p.o.) or buspirone (0, 1 and 3 mg/kg, s.c.), were administered before FPS testing on day 5.

2.7.3. SIH tests:

Rats were administered BHF177 (0, 20 and 40 mg/kg, p.o.) using a between-subjects design (n=8/group).

2.8. Statistics

Data are expressed as mean ± SEM. To assess the effect of each compound on LES, a difference score of the peak startle value was calculated for each session by subtracting the startle value of the first block from that of the second block. A composite difference score (difference score in the Dark-Light session – difference score in the Dark-Dark session) was then used to evaluate the effect of each tested compound on LES. The effects of BHF177 or buspirone on baseline startle and LES were analyzed with analyses of variance (ANOVAs) with Drug dose, Session and Block as within-subjects factors. The effects of baclofen or CGP44532 on baseline startle and LES were analyzed with ANOVAs with Drug dose as a between-subjects factor, and Session (Dark-Dark or Dark-Light) and Block (first or second) as within-subject factors. As reported previously (Jonkman et al., 2008), initial LES procedures that included startle intensity consistently showed that significant light potentiation occurred mainly at the 105 dB pulse, not at

90 or 95 dB (see Supplementary Table 1 for example of LES effect across the baclofen study). Hence the data generated were analyzed using the 105 dB pulse only. Because of the variance in LES responses, we also used a median split strategy to examine drug responses in putative "high" and "low" LES responding rats. To validate this procedure we assessed the stability of individual LES responses by comparing LES difference scores in the pre-drug baseline test with vehicle day for each animal, observing a significant correlation ($R=.405$, $p<0.005$, $N=48$). For the FPS tests, cued fear = [(mean "cue" trial startle magnitude – mean "no-cue" trial startle magnitude)/ mean "no-cue" trial startle magnitude] × 100. Context fear = [(mean "no-cue" trial magnitude — mean preshock startle magnitude)/mean preshock startle magnitude] × 100. The effects of BHF177 or buspirone on cue and context fear were analyzed with an ANOVA with Drug dose as a between-subjects factor. The effects of BHF177 on stress-induced hyperthermia were analyzed with an ANOVA. *Post hoc* comparisons were made with Dunnett's test after statistically significant effects in the ANOVAs. The significance level for all analyses was $p<0.05$.

3. Results

3.1. Effect of BHF177 on LES

Significant LES was seen at 105 dB pulse (Table 1; session effect: $F_{1,31} = 14.78$, $P<0.001$; session × block interaction: $F_{1,31} = 25.47$, $P<0.001$). However, there were no significant main effects or interactions with the factor drug, indicating that BHF177 had no effect on startle amplitude or LES at the doses tested. Analysis of the composite difference scores for the 105 dB startle values subsequently confirmed that there was no significant drug effect (Fig. 1). However, when the rats were separated into two groups based on a median split of LES responses

(difference score) after vehicle treatment, BHF177 significantly reduced LES responses in high LES responding rats, but not in low LES responding rats (Fig. 1B; LES trait effect: $F_{1,31}$ = 4.29, P<0.05; session × trait interaction: $F_{1,31}$ = 8.78, P<0.001; drug × trait interaction: $F_{3,90}$ = 3.27, P<0.05). *Post hoc* tests indicated that 40 mg/kg BHF177 significantly decreased LES in high LES responding rats (t=2.91, P<0.05 compared with vehicle treatment; t=2.22, P<0.05 compared with 10 mg/kg BHF177 treatment).

3.2. Effect of buspirone on LES

Analysis of the 105 dB pulse data revealed a significant LES (Table 2; session effect: $F_{1,15}$ = 8.64, P<0.05; session × block interaction: $F_{1,15}$ = 8.46, P<0.05). Buspirone inhibited the enhanced startle responses in the dark-light session (session × drug interaction: $F_{2,30}$ = 4.59, P<0.05), but not in the dark-dark session. Analysis of the difference scores for the 105 dB startle values confirmed that buspirone significantly inhibited anxiolytic-like effects on LES in rats (Fig. 2A). Similar to the effects of BHF177, when the rats were separated into two groups based on a median split of LES responses after vehicle treatment, buspirone significantly decreased the anxiolytic-like effects on LES in high LES responding rats, but not in low LES responding rats (Fig. 2B; LES trait effect: $F_{1,14}$ = 17.27, P<0.001; drug effect: $F_{2,28}$ = 7.73, P<0.01; session × trait interaction: $F_{1,14}$ = 22.08, P<0.001; drug × trait interaction: $F_{3,90}$ = 11.27, P<0.001). *Post hoc* tests indicated that both 1 mg/kg (P<0.001) and 3 mg/kg (P<0.01) buspirone significantly decreased LES in high LES responding rats.

3.3. Effect of baclofen on LES

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Analysis of the 105 dB pulse data (Supplementary Fig. S1A and 1B) revealed a significant light potentiation of startle (session effects: $F_{1,56}$ = 10.57, P<0.001; session × block interaction: $F_{1,56}$ = 11.64, P<0.01) that was unaffected by baclofen treatment. Baclofen did however significantly reduce startle responding independently of light condition (Drug effect: $F_{3,56}$ = 9.22, P<0.001). *Post hoc* tests indicated that the overall startle amplitudes were significantly lowered by 0.9 mg/kg (t=3.40, P<0.01) and 1.25 mg/kg (t=4.87, P<0.001). Analysis of the difference scores for the 105 dB startle values confirmed that there was no significant drug effect (Fig. S1C).

3.4. Effect of CGP44532 on LES

Significant LES was induced at 105 dB pulse (Fig. S2A and S2B; session effect: $F_{1,64}$ = 9.48, P<0.001; session × block interaction: $F_{1,64}$ = 37.99, P<0.001). CGP44532 inhibited startle reactivity in the dark-dark but not dark-light session (session × drug interaction: $F_{3,64}$ = 4.32, P<0.01). *Post-hoc* test indicated that 0.125 mg/kg CGP44532 significantly decreased startle reactivity (t=3.38, P<0.01). Analysis of the difference scores confirmed that CGP44532 had no effect on LES at the doses tested (Fig. S2C).

3.5. Effect of BHF177 on stress-induced hyperthermia

Baseline body temperature (T1) was not significantly influenced by BHF177 (Figure 3A). Stress induced significant increases in body temperature 10 min after the initial recording (T2; time effect: $F_{1,21}$ = 10.5, P<0.01; time × drug interaction: $F_{2,21}$ = 3.05, P<0.05). *Post hoc* tests revealed significant increases of body temperature after vehicle (q = 3.64, p < 0.01) but not after any dose of BHF177. Using a One-Way ANOVA where dose order is not considered there was a

strong trend for BHF177 to inhibit stress-induced hypothermia (Fig 3B; P=0.069), and using linear regression analysis where dose order is considered in the model there was a significant effect of dose ($p < 0.05$, $r^2 = 0.22$). Dunnet's post hoc comparisons supported a significant reduction in the 40 mg/kg group compared to vehicle ($q = 2.48$, $p < 0.05$).

3.6. Effect of BHF177 on FPS

After conditioning, baseline startle amplitudes (i.e., "no cue") during the FPS test were significantly greater than those during the baseline sessions indicating robust context fear (Fig 4A). Pairing of light (CS) and foot shock (US stimuli) resulted in significantly higher startle reactivity during cue trials compared to no-cue trials indicating significant cued fear learning as well (Fig. 4A: trial type: $F_{2,132} = 49.88$, $P<0.001$). BHF177 treatment did not affect startle reactivity under either the context or cued fear conditions (Fig 4B and C respectively).

3.7. Effect of buspirone on FPS

Again, rats showed robust contextual and cued-fear potentiated startle (Figure 5, trial type: $F_{2,129} = 55.59$, $P<0.001$). Buspirone markedly reduced startle responding during cue trials but did not affect startle during no-cue trials (drug effect: $F_{2,129} = 6.21$, $P<0.01$; session × trail type interaction: $F_{4,129} = 3.23$, $P<0.05$). Analysis of the percentage context and cued fear responding confirmed that buspirone selectively reduced cued but not contextual fear (Fig. 5B-C: drug effect of percent cued fear: $F_{2,43} = 9.25$, $P<0.001$). *Post hoc* tests revealed significant decreases in cued fear in the 3 mg/kg ($q = 4.06$, $p < 0.001$) but not the 1 mg/kg treatment group.

4. Discussion

The present study indicated that the $GABA_B$ receptor agonists baclofen and CGP44532 showed sedative, but not anxiolytic, activity, decreasing overall startle responses in the LES test. The $GABA_B$ receptor PAM BHF177 had no effect on LES when all rats were grouped together. However, when rats were grouped based on high and low LES responses, BHF177 administration had anxiolytic-like effects on LES in high, but not low, LES responding rats. These results indicate that BHF177 has relatively low non-specific sedative effects and exert anxiolytic activity in rats that exhibit a high anxiety state. Buspirone, a 5-HT_{1A} receptor partial agonist with anxiolytic activity in humans showed a similar pattern of efficacy, with strongest effects in rats with high LES reactivity. BHF177 also attenuated stress-induced hyperthermia without decreasing baseline body temperature. However, BHF177 did not modify conditioned fear behaviors in the FPS test. Together, these data indicated that BHF177 may have anxiolytic action in tests of non-specific threat or stress response, but not in tests of conditioned fear. Importantly, BHF177 is devoid of classical $GABA_B$ receptor agonist-mediated sedative or hypothermic effects at the doses tested.

Anxiety-related responses and exaggerated fear play an important role in the genesis of anxiety disorders, and share several overlapping neural circuits (Sylvers et al., 2011). It has been shown that both the LES and FPS have predictive validity for standard anxiolytic compounds, including benzodiazepines and 5-HT_{1A} receptor agonists (Davis et al., 1993; Walker and Davis, 2002a). However, the neurobiology that underlies these two procedures is not identical. FPS and LES can be dissociated phenomenologically and anatomically into two different response systems (Walker et al., 2003). The fast onset, short term response elicited by specific threatening stimuli is termed fear, and is critically dependent on the integrity of the central nucleus of the

amygdala (CeA) and can be modeled with the FPS paradigms. The slow onset, long term response elicited by potential and unpredictable threats is termed anxiety, and is critically dependent on the integrity of the bed nucleus of the stria terminalis (BNST) and can be modeled with LES paradigms. In line with previous reports (Commissaris et al., 2004; Mansbach and Geyer, 1988; Walker and Davis, 1997a), the positive control buspirone, a clinically used anxiolytic, inhibited the startle potentiation in the LES procedure and the fear potentiation in the FPS procedure. BHF177 showed anxiolytic effects only in the LES, but not in the FPS, suggesting that this compound is effective in blocking sustained anxiety responses to a potentially threatening context, but not short duration conditioned fear. These findings are consistent with previous reports that the $GABA_B$ receptor PAM GS39783 inhibited anxiety-like behavior in a range of tests of unconditioned anxiety, such as the light/dark box (Mombereau et al., 2004) and the elevated zero maze (Cryan et al., 2004; Mombereau et al., 2004). Furthermore, another $GABA_B$ receptor PAM CGP7930 also exhibited an anxiolytic profile in the elevated zero maze and staircase test in mouse (Jacobson and Cryan, 2008) and in the elevated zero maze in rats (Frankowska et al., 2007). Interestingly, congruent with the lack of effect of BHF177 on FPS in rats in the present study, we previously reported that BHF177 had no specific effect on contextual and cued fear conditioning as assessed by freezing behavior in mice (Li et al., 2013). Recent studies also showed that GS39783 did not modify conditioned fear responses in mice (Sweeney et al., 2013). Taken together, these results indicate that the $GABA_B$ receptor PAMs, such as BHF177 and GS39783, may be effective in attenuating unconditioned anxiety, but not conditioned fear.

An anxiolytic profile of BHF177 was confirmed in the stress-induced hyperthermia test. The SIH paradigm provides an objective assessment of the physiological response to anxiety and has

been validated extensively as a valuable test for detecting conventional and putative anxiolytics (Olivier et al., 2003). Here we found that BHF177 blocked stress-induced hyperthermia without altering baseline body temperature. However, the full agonist baclofen dramatically decreases baseline body temperature (Cryan et al., 2004). In mice, $GABA_B$ receptor PAMs such as GS39783 (Cryan et al., 2004) and CGP7930 (Jacobson and Cryan, 2008) exhibit anxiolytic effects in the stress-induced hyperthermia test without affecting baseline temperature. Taken together, these data suggest that BHF177 has anxiolytic actions in the stress-induced hyperthermia test, but has a better side-effect profile in terms of hypothermia than the $GABA_B$ receptor full agonists.

We have previously reported that BHF177 is ineffective across a wide number of anxiety tests in the mouse, including the light/dark box, the elevated plus maze and the Vogel conflict test (Li et al., 2013). CGP7930 decreased anxiety-like behavior in the elevated zero maze test in mice, but had no effect on the elevated plus maze test in rats (Jacobson and Cryan, 2008). These findings suggest that anxiety-like effects of PAMs may be species dependent. The reason for the species-specific pharmacology of the $GABA_B$ receptor PAMs is unclear. Differences in $GABA_{B1}$ and $GABA_{B2}$ subunit sequence across species may play a role. By using different cell lines expressing either rat or human $GABA_B$ receptors, we observed that $GABA_BR$ receptor PAMs exhibit receptor ortholog selectivity as well as downstream intracellular signal transduction specificity (Sturchler E, Li X, Ladino , Kaczanowska K, Cameron M, Griffin P, Finn M.G., Markou A, and McDonald, unpublished data). These species-dependent effects of $GABA_BR$ receptor PAMs increases the difficulty of identifying potential medications from the drug development perspective, as selection must be based on activity in both the rat and human receptor lines. It also strongly supports a need for confirmation studies in primates before

moving on to human testing. We also cannot rule out variability due to different paradigm constructs and underlying neurochemistry and circuitry across rats and mice (Rodgers, 1997).

The improved side-effect profiles of the $GABA_B$ receptor PAMs compared to agonists for the same receptor are involves less sedation, as supported by the fact that the agonists baclofen and CGP44532 decreased overall startle responses in the LES test, whereas BHF177 did not. The lack of locomotor impairment by BHF177 in the present studies is consistent with reports that BHF177 did not affect food- or sucrose-maintained operant behavior in rats (Maccioni et al., 2009; Paterson et al., 2008), and did not exert an inhibitory effect on any of the locomotion-related parameters in the light/dark box and elevated plus maze in mice (Li et al., 2013). The reduced sedation or muscle relaxation profile of BHF177 compared to full agonists is consistent with that described for other $GABA_B$ receptor PAMs. For example, GS39783 had no effect on locomotor activity, rotarod performance (Cryan et al., 2004), or food- and sucrose-maintained responding (Maccioni et al., 2008; Maccioni et al., 2007; Paterson et al., 2008).

Overall, our data indicated that BHF177 had efficacy in attenuating unconditioned anxiety in the LES and stress-induced hyperthermia tests. Some limitations however are that the BHF177 effect in LES was only apparent when using a median split analysis, which can be confounded by regression to the mean, especially in unstable traits. We found that LES response is moderately stable comparing the relationship between LES difference scores between the initial baseline day used for group matching and the subsequent vehicle day. Additionally, the high dose of 40 mg/kg BHF177 reduced LES compared to not only vehicle but the low dose (10 mg/kg) as well, which suggests that regression to the mean does not fully explain BHF177 effects. Thus, $GABA_B$ PAMs may be more affective medications in disorders characterized by general anxiety (e.g. generalized anxiety) vs. fear based symptoms (e.g. phobias or posttraumatic

stress disorder). The hypothesis that BHF177 may be particularly effective against high anxiety states should also be evaluated in other models, e.g. selectively-bred animal for anxiety, chronic and/or developmental stress paradigms, as these models probe different mechanism of anxiety disorders that may be responsive to $GABA_B$ PAMs.

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Figure Legends

Fig. 1. Effects of BHF177 on light-enhanced startle in rats (n=32). BHF177 had no effect on LES in rats when the data from all rats were analyzed altogether (A). When the rats were subgrouped into two groups based on a median split of LES responses during vehicle treatment, BHF177 significantly decreased the anxiety-like effects on LES in high LES responding rats (B). *$p < 0.05$, compared with vehicle treatment, #$p < 0.05$, compared with 10 mg/kg BHF177.

Figure 2. Effects of buspirone on light-enhanced startle in rats (n=16). Buspirone significantly inhibited overall LES when the data from all rats were analyzed altogether (A). When the rats were subgrouped into two groups based on a median split of LES responses after vehicle treatment, buspirone significantly decreased the anxiety-like effects on LES in high LES responding rats (B). **$p < 0.01$, ***$p < 0.001$, compared with vehicle treatment.

Figure 3. Effects of BHF177 on stress-induced hyperthermia in rats (n=8/group). Significant increases of body temperature induced by stress were observed in vehicle-treated rats, but not in BHF177-treated rats (A). BHF177 significantly reduced stress-induced hyperthermia in rats (B). ##$p < 0.01$, compared with baseline body temperature. ***$p < 0.001$, compared with vehicle group.

Figure 4. Effects of BHF177 on context and cued fear in the fear-potentiated startle test in rats (n=15-16/group). Panel A shows that startle reactivity during baseline sessions, and noise alone (no cue) and noise light (cue) trials during FPS test sessions in different groups. Context fear =

[(no-cue — baseline)/ baseline] × 100. Cued fear = [(cue — no-cue)/ no-cue] × 100. BHF177 had no effect on context fear (A) or cued fear (B) in rats.

Figure 5. Effects of buspirone on context and cued fear in the fear-potentiated startle test in rats (n=15-16/group). Buspirone reduced startle responding during cue trials but did not affect startle during no-cue trials (A). Buspirone significantly blocked cued fear (C), but had no effect on context fear (B) in rats. $^{**}p < 0.01$, compared with vehicle group.

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Table 1 Startle amplitudes in the light-enhanced startle at 105 dB after treatment with BHF177.

BHF177 (mg/kg)	Dark 1.1*	Dark 1.2*	Dark 2.1*	Light 2.2*
0	2452 ± 242	2052 ± 193	2362 ± 209	2619 ± 201
10	2425 ± 203	2045 ± 187	2222 ± 178	2521 ± 204
20	2500 ± 192	1976 ± 167	2359 ± 192	2605 ± 234
40	2293 ± 190	1978 ± 218	2527 ± 272	2530 ± 250

*The LES test consists of one session with two consecutive startle blocks in the dark (Dark 1.1 and Dark 1.2) and a second session with two consecutive startle blocks with a bright light turned on in the second block (Dark 2.1 and Light 2.2). Same conditions for Table 2 and Table S1.

Table 2 Startle amplitudes in the light-enhanced startle at 105 dB after treatment with buspirone.

buspirone (mg/kg)	Dark 1.1	Dark 1.2	Dark 2.1	Light 2.2
0	2566 ± 374	2324 ± 303	2290 ± 411	3139 ± 541
1	2283 ± 384	2199 ± 361	2435 ± 425	2514 ± 428
3	2274 ± 315	2165 ± 248	2143 ± 251	2310 ± 290

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Figure 1:

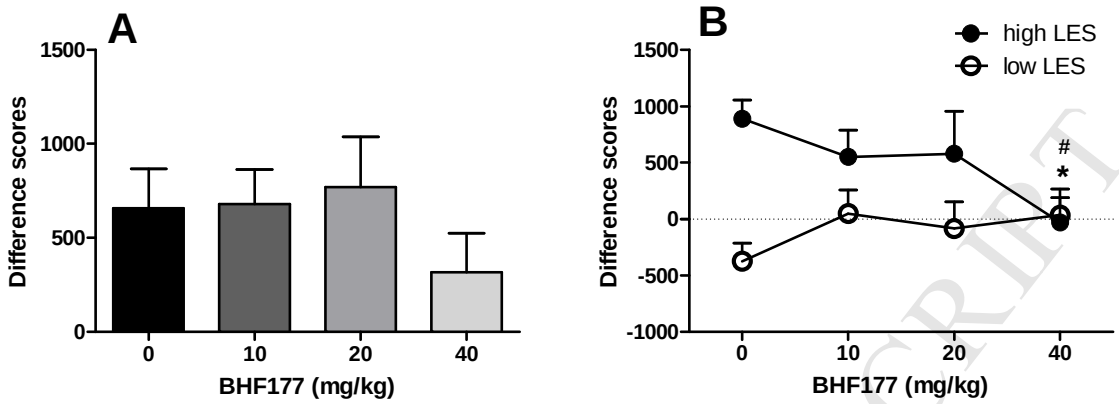
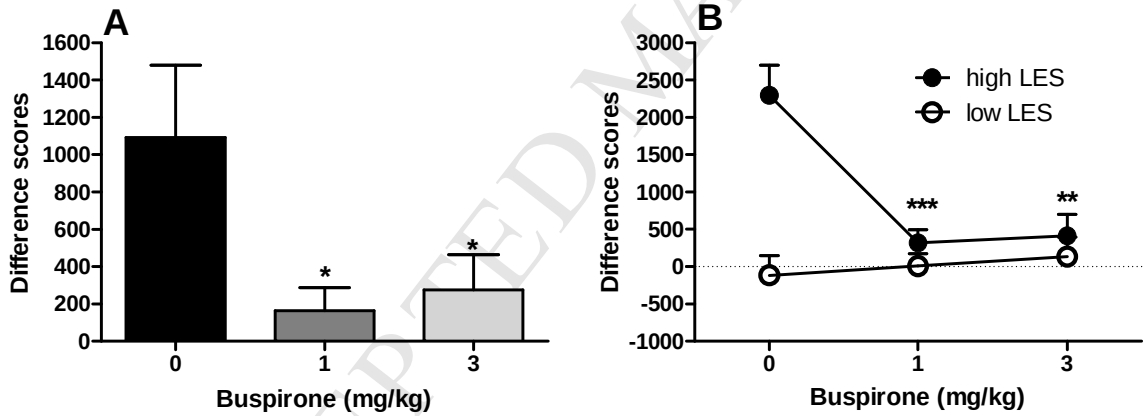


Figure 2:



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Figure 3:

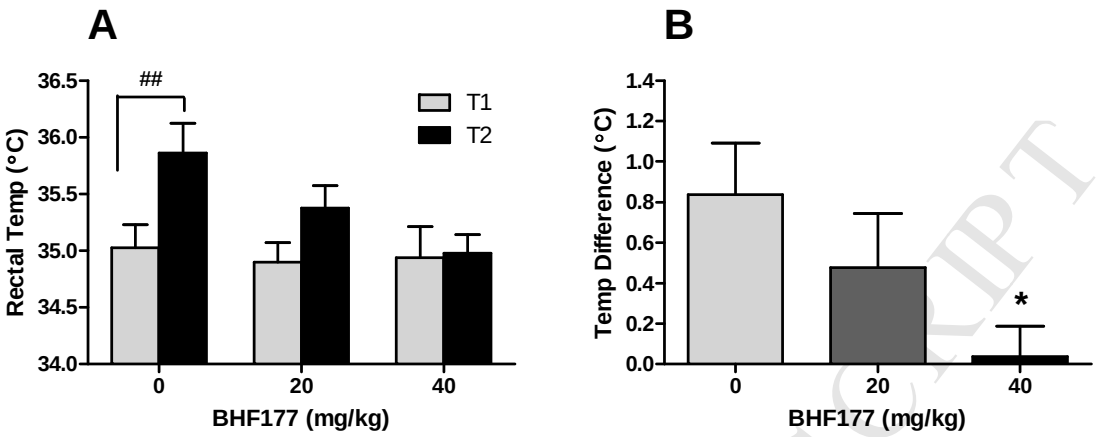


Figure 4:

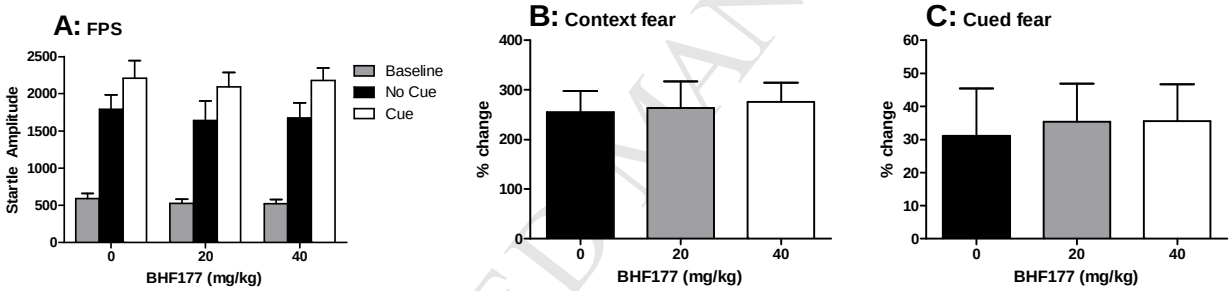
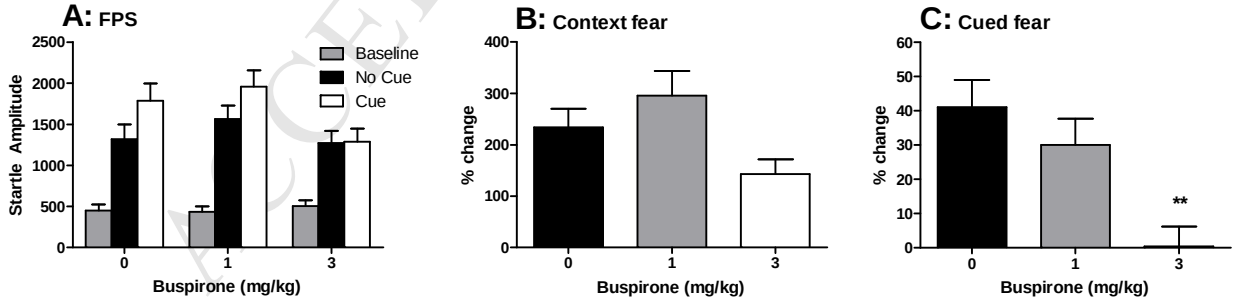


Figure 5:



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- BHF177 has anxiolytic-like effects on light-enhanced startle (LES) in high, but not low, LES responding rats.
- BHF177 has no effect on conditioned fear responses in the fear-potentiated startle test in rats.
- BHF177 blocks stress-induced hyperthermia in rats.

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Table S1 Light-enhanced startle across the three startle pulse intensities after treatment with baclofen.

Pulse (dB)	Baclofen (mg/kg)	Dark 1.1	Dark 1.2	Dark 2.1	Light 2.2
90	0	434	469	449	535
	0.4	397	339	410	546
	0.9	264	256	303	304
	1.25	170	124	146	252
95	0	914	813	937	957
	0.4	792	669	741	1069
	0.9	580	444	600	735
	1.25	389	279	333	458
105	0	2341	2137	2503	2647
	0.4	2173	1845	2057	2485
	0.9	1688	1518	1869	1967
	1.25	1518	1326	1491	1586

Figure S1:

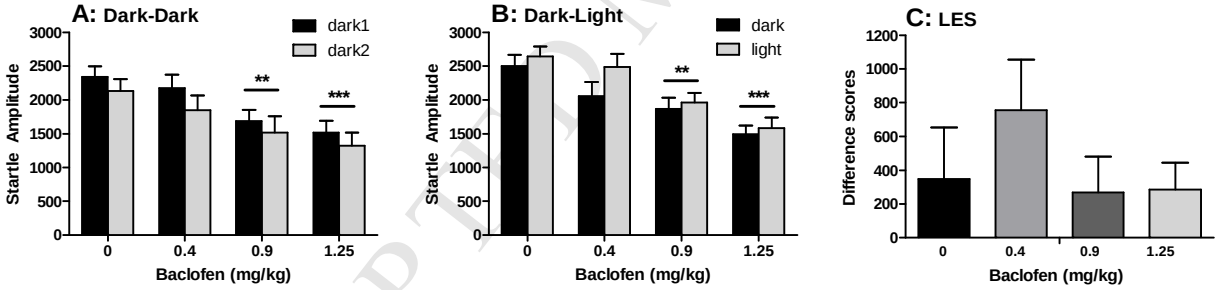


Figure S1. Effects of baclofen on light-enhanced startle in rats (n=15/group). Baclofen decreased overall startle amplitudes in both the dark-dark (A) and dark-light (B) sessions, but had no effect on LES (C) in rats. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with vehicle group.

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Figure S2:

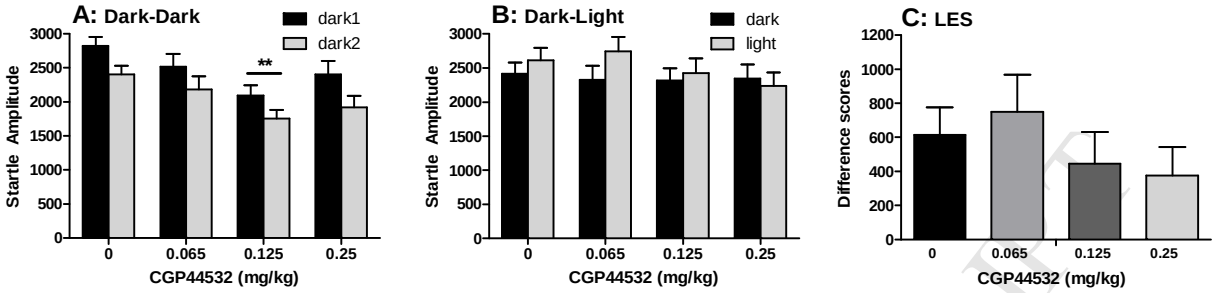


Figure S2. Effects of CGP44532 on light-enhanced startle in rats (n=15-19/group). CGP44532 decreased the startle amplitudes in the dark-dark session (A), but and had no effect on LES (C) in rats. $^{**}p < 0.01$, compared with vehicle group.

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