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Positive allosteric modulators at $GABA_B$ receptors exert intrinsic actions and enhance the influence of baclofen on light-induced phase shifts of hamster circadian activity rhythms

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ABSTRACT

Light-induced phase shifts of hamster circadian activity rhythms are modulated by $GABA_B$ receptors. Recently, positive allosteric modulators (PAM)s at $GABA_B$ receptors were described, but it is not known whether they affect light-induced entrainment of circadian rhythms. Therefore, we studied the effects of two $GABA_B$ PAMs, GS39783 and RacBHFF, upon light-induced phase advances and delays of hamster circadian wheel-running activity rhythms. Wheel running activity was recorded for Syrian hamsters maintained in constant darkness. Drugs administered intraperitoneally were evaluated for their ability to modulate a light-induced shift of the circadian activity rhythm. Baclofen (3.75–15 mg/kg) dose-dependently inhibited both light-induced phase advances and delays of hamster wheel running rhythms, and its actions were blocked by the selective $GABA_B$ antagonist, SCH50911 (5 mg/kg). Neither GS39783 (3–30 mg/kg) nor RacBHFF (0.63–10 mg/kg) affected phase advances when injected alone, but both GS39783 (3 mg/kg) and RacBHFF (10 mg/kg) *augmented* the inhibitory effect of baclofen (5 mg/kg). At doses above 3 mg/kg, GS39783 and RacBHFF significantly inhibited phase *delays* alone, consistent with the notion of "agonist-allosteric" properties. GS39783 (0.5 mg/kg), but not RacBHFF (10 mg/kg), augmented the inhibitory action of baclofen on phase delays. These data are consistent with the possibility that GS39783 and RacBHFF act as PAMs at $GABA_B$ receptors inhibiting light-induced phase advances, yet that they also posses "allosteric agonist" actions at the (presumably separate) population of $GABA_B$ receptors modulating light-induced phase *delays*. $GABA_B$ receptors clearly warrant further investigation as agents for modulation of circadian dysfunction associated with CNS disorders such as depression.

1. Introduction

Circadian rhythms are central to many physiological systems in mammals, including and not limited to the well known association with sleep but also with rhythmic release of hormones related to growth and metabolism (Levi and Schibler, 2007). Dysfunctions of core timing mechanisms are associated with certain pathological forms of major depression and also with Alzheimer's disease (Germain and Kupfer, 2008; Harper et al., 2001; Millan, 2006), and there appears to be a link as well to metabolic disorders such as diabetes (Bass and Takahashi, 2010). Circadian rhythms are known to be autonomous to different organs and tissues (Dibner et al., 2010), and disease pathology may be related to abnormal clock mechanisms in these peripheral sites, or they may be linked to abnormal timekeeping in the master circadian pacemaker within the suprachiasmatic nucleus (SCN) of the hypothalamus that coordinates the timing of the peripheral pacemakers. Irrespective of their biological origins, it is important to explore pharmacological manipulations of circadian timekeeping that may then be translated into useful therapies.

The circadian pacemaker is comprised of a neuronal network of cells in the suprachiasmatic nucleus that receives input from retinal ganglion cells conveying photic information from the retina to the SCN (Johnson et al., 1988). Input to the SCN from the retina is glutamatergic, but GABA is the most common neurotransmitter found within the SCN itself (Moore and Speh, 1993). GABAergic transmission is required to communicate between cells and regions within the SCN (Albus et al., 2005; Buijs et al., 1994; Liu and Reppert, 2000), and this communication underpins a circadian output signal which leaves the SCN *via* direct and indirect pathways to entrain peripheral targets to the timekeeping of the master pacemaker in the SCN (Guo et al., 2006; Wang et al., 2003). Exogenously applied

Abbreviations: CT, circadian time; DD, constant darkness; DMSO, dimethylsulfoxide; GS39783, N,N′-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine; RacBHFF, 5,7-bis-(1,1-dimethylethyl)-3-hydroxy-3(trifluoromethyl)-2(3H)-benzofuranone; PAM, positive allosteric modulator; SCH50911, (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid; SCN, suprachiasmatic.

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GABAergic compounds modulate the ability of light to entrain the SCN pacemaker in rats and hamsters, and these studies are fairly comprehensive with respect to determining the relative contributions of $GABA_A$ vs. $GABA_B$ receptors in the entraining process (Gillespie et al., 1996, 1997; Mintz et al., 2002; Ralph and Menaker, 1989). However, very little is known as regards the roles of isoforms of $GABA_A$ receptors. Further, while the actions of first-generation $GABA_B$ ligands interacting with orthosteric sites have been documented (Gillespie et al., 1997; Mintz et al., 2002; Ralph and Menaker, 1989), the significance of allosteric sites on $GABA_B$ receptors in the control of circadian behaviors has not to date been studied.

Metabotropic $GABA_B$ receptors comprise obligatory heterodimers of $GABA_{B1}$ and $GABA_{B2}$ subunits, encoded by different genes; the first of which bears the binding site for GABA and the latter subunit is responsible for cell surface membrane insertion and G protein coupling. Allosteric modulators bind to the transmembrane domain of $GABA_B$ receptors to alter the potency and efficacy of GABA binding to the orthosteric site, though possibility of additional sites in intracellular regions should not be discounted (Conn et al., 2009; Mannoury la Cour et al., 2008; Monnier et al., 2011; Rondard et al., 2011). Though allosteric modulators typically have no intrinsic function of their own in principle, very recent evidence suggests that some may act as allosteric agonists in their own right in a context-dependent manner (Conn et al., 2009; Gjoni and Urwyler, 2009; Koek et al., 2010; Rondard et al., 2011). This observation is of considerable therapeutic relevance to their potential clinical utility. In the present study, we examined the influence of two previously characterized and in vivo active $GABA_B$ positive allosteric modulators (PAM) GS39783 and RacBHFF both alone and in interaction with baclofen (Cryan et al., 2004; Froestl, 2010a,b; Mannoury la Cour et al., 2008; Urwyler et al., 2003).

2. Material and methods

2.1. Animals

Male Syrian hamsters were purchased from Charles River Laboratories (Kingston, NY) at 71–80 g and housed under a 14 h:10 h light:dark schedule for several weeks before use. The care and use of the hamsters was approved by the Institutional Animal Care and Use Committee of Valdosta State University. Food and water was provided ad libitum. Each hamster was used from 1–3 times for the experiments as described below in the following section. After each experiment, the hamsters were returned to holding room for one month before being used in the next experiment, therefore, single, randomly assigned drug or vehicle injections were administered to hamsters approximately 45 days apart, with no more than 3 injections per hamster. This study used a total of 220 hamsters.

2.2. Circadian wheel running rhythms

After the hamsters had entrained to the appropriate light schedule, they were transferred to individual cages equipped with running wheels and placed in conditions of constant darkness (DD) for the duration of the experiment (about 3 weeks). Ten days after hamsters were placed in DD, they were removed from their home cages under dim red light at injected intraperitoneally with either drug or vehicle and then returned to their home cage. Forty-five minutes later, the hamster were again removed from their home cage and exposed to 10minutes of dim white light (20 lux) and then returned to their home cage. Hamsters were exposed to light at circadian time (CT) 14 to elicit phase delays in wheel running rhythms or at CT 19 to elicit phase advances in wheel running rhythms. CT 12 is defined as the onset of wheel running activity each day. Some hamsters were injected with drugs but not exposed to light to serve as drug-alone controls. Phase advances or delays were determined by comparing the times of activity onset before and after the day of the light pulse by fitting lines through five consecutive onsets before the light pulse and for five days after the light-induced shift had stabilized, and then comparing the intercept of the lines on the day of the light pulse. Hamsters were returned to the light:dark schedule ten days after the light pulse.

2.3. Drugs

The following drugs were purchased from Tocris Bioscience (Ellisville, MO, USA): baclofen (RS)-4-amino-3-(4-chlorphenyl)butanoic acid); GS39783 N,N'-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine; RacBHFF 5,7-bis-(1,1-dimethylethyl)-3-hydroxy-3(trifluoromethyl)-2(3H)-benzofuranone; SCH50911 (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid. GS39783 and RacBHFF were dissolved in DMSO and baclofen and SCH50911 in water. Each solvent was used as the vehicle in single drug experiments and DMSO was used as the vehicle when multi-drug experiments were performed.

2.4. Statistics

Data are expressed as the mean ± SEM. One-way ANOVAs with Student–Newman–Keuls and Dunnett's post-hoc tests or t-tests were used for statistical analysis (SigmaStat, Point Richmond, CA, USA).

3. Results

3.1. Effects of the $GABA_B$ agonist baclofen and $GABA_B$ antagonist SCH50911 on light-induced phase advances and delays

The $GABA_B$ agonist baclofen (Bowery et al., 1980) inhibited light-induced phase advances of hamster wheel running activity in a dose-dependent manner (ANOVA $F(3,18) = 9.56$, $p<0.001$), with about a 75% inhibition achieved with the 15 mg/kg dose (Table 1). Baclofen also inhibited light-induced phase delays of hamsters wheel running rhythms (ANOVA $F(2,19) = 10.96$, $P<0.001$), and 15 mg/kg baclofen

Table 1
Effects of baclofen and SCH50911 on light-induced phase advances and delays of hamster wheel running rhythms.

Phase advance				
Baclofen				
Vehicle	5 mg/kg	10 mg/kg		15 mg/kg
1.07 ± 0.05 h (6)	0.68 ± 0.17 h (5)	0.66 ± 0.14 h (5) *		0.26 ± 0.08 h (6) *
SCH50911				
Vehicle	0.25 mg/kg	1 mg/kg	5 mg/kg	5 mg/kg (w/o light)
1.38 ± 0.14 h (6)	1.47 ± 0.31 h (6)	1.76 ± 0.17 h (6)	1.67 ± 0.09 h (7)	0.08 ± 0.04 h (3)
Phase delay				
Baclofen				
Vehicle	3.75 mg/kg	15 mg/kg		15 mg/kg (w/o light)
0.84 ± 0.05 h (7)	0.76 ± 0.11 h (7)	0.21 ± 0.13 h (8) *		0.07 ± 0.02 h (3)
SCH50911				
Vehicle	0.25 mg/kg	5 mg/kg		5 mg/kg (w/o light)
0.54 ± 0.05 h (5)	0.51 ± 0.09 h (4)	0.65 ± 0.08 h (5)		0.03 ± 0.03 h (3)

*$p<0.05$ from vehicle, Dunnett's Method.

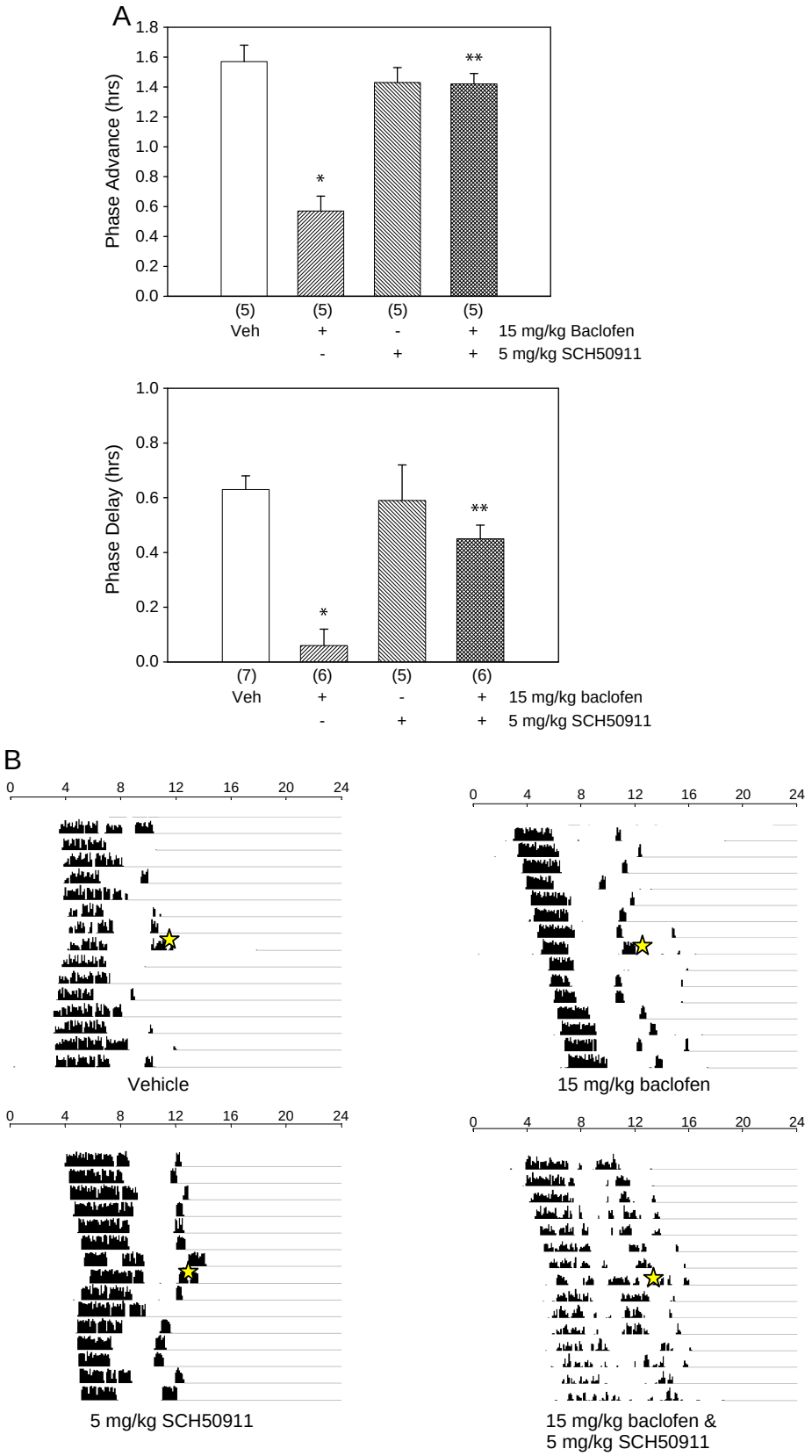

Fig. 1. A. SCH50911 antagonism of baclofen inhibition of light-induced phase advances (top) and phase delays (bottom) of circadian wheel running rhythms. * $p<0.001$ form vehicle, ** $p<0.001$ from baclofen, Student–Newman–Keuls post-hoc test. (n) is indicate din parentheses below the bars. 1B. Representative actograms from the data in Fig. 1A. The number of wheel revolutions are shown as vertical deflections on each line for a twenty-four hr period. A total of fifteen days are shown. Stars indicate the approximate time of the ten-minute, twenty lux light pulse on the day of the experiment.

inhibited phase delays by 75% (Table 1). In contrast, the $GABA_B$ antagonist SCH50911 (Bolser et al., 1995) had no significant effect on light-induced phase advances or delays at doses up to 5 mg/kg SCH50911 (Table 1).

However, 5 mg/kg SCH50911 was able to completely antagonize the inhibitory activity of 15 mg/kg baclofen on both light-induced phase advances and delays (Fig. 1A). For phase advances, light-induced shifts following vehicle injections were 1.6 ± 0.1 h and this was reduced by 64% to 0.6 ± 0.1 h by 15 mg/kg baclofen, but to only 1.4 ± 0.1 h following co-application of 5 mg/kg SCH50911 and 15 mg/kg baclofen (Fig. 1A). For phase delays, light-induced shifts averaged 0.6 ± 0.05 h following vehicle injections, and 15 mg/kg baclofen inhibited these shifts by 90% to 0.06 ± .06 h, but to only 0.5 ± 0.1 h following co-injections of both 15 mg/kg baclofen and 5 mg/kg SCH50911 (Fig. 1A).

3.2. Effects of the $GABA_B$ PAMs GS39783 and RacBHFF on light-induced phase advances and delays

The $GABA_B$ PAM GS39783 (Urwyler et al., 2003) did not significantly modify light-induced phase advances when compared to vehicle (ANOVA $F(3,20) = 2.8$, $P = 0.066$) at doses up to 30 mg/kg GS39783, nor did the $GABA_B$ PAM RacBHFF (Malherbe et al., 2008; ANOVA $F(3,20) = 0.75$, $P = 0.54$) at doses up to 10 mg/kg ; Table 2.

GS39783 had an insignificant (but nevertheless close to 40%) inhibitory effect at a dose 3 mg/kg when compared to vehicle, while 10 mg/kg RacBHFF significantly inhibited light-induced phase delays by nearly 40% as compared to vehicle (Table 2).

3.3. $GABA_B$ PAM modulation of baclofen-induced inhibitions of light-induced phase advances and delays

Both GS39783 and RacBHFF significantly enhanced the inhibitory effect of baclofen on light-induced phase advances of wheel running rhythms. In the first experiment, neither 5 mg/kg baclofen nor 3 mg/kg GS39783 had any effect upon light-induced phase advances when injected by themselves, but when combined they significantly inhibited light-induced phase shifts by more than 40% (veh = 1.2 ± 0.05 and baclofen/GS3978 = 0.7 ± 0.1 h, Fig. 2, top; ANOVA $F(3,22) = 6.495$, $P = 0.003$). Similarly, in a separate experiment neither 5 mg/kg baclofen nor 10 mg/kg RacBHFF had any significant effect on light-induced advances when injected alone, but when injected together they significantly inhibited light-induced phase advances by nearly 40% (veh = 1.3 ± 0.1 h and baclofen/RacBHFF = 0.8 ± 0.1 h, Fig. 2, bottom; ANOVA $F(3,23 = 5.582$, $P = 0.005$).

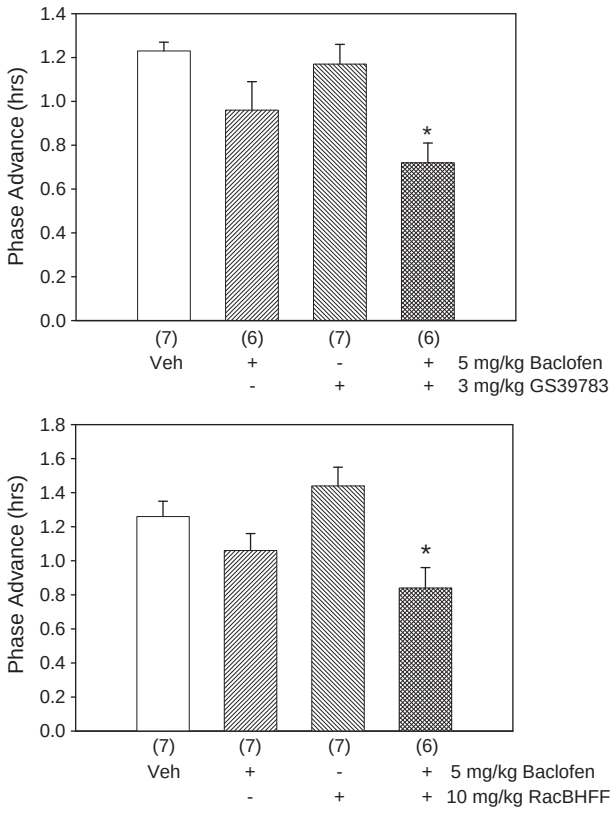

Fig. 2. The $GABA_B$ PAMs GS39783 and RacBHFF enhance baclofen inhibition of light-induced phase advances. * $P<0.05$ from vehicle, Student–Newman–Keuls post-hoc test.

Table 2
Effects of GS39783 and RacBHFF on light-induced phase advances and delays of hamster wheel running rhythms.

Phase advance				
GS39783				
Vehicle	3 mg/kg	10 mg/kg	30 mg/kg	30 mg/kg (w/o light)
1.36 ± 0.07 h (6)	1.46 ± 0.09 h (6)	1.74 ± 0.11 h (6)	1.81 ± 0.20 h (6)	0.04 ± 0.14 h (3)
RacBHFF				
Vehicle	0.63 mg/kg	2.5 mg/kg	10 mg/kg	10 mg/kg (w/o light)
1.36 ± 0.13 h (6)	1.46 ± 0.16 h (6)	1.48 ± 0.11 h (6)	1.62 ± 0.10 h (6)	0.13 ± 0.24 h (4)
Phase delay				
GS39783				
Vehicle	0.5 mg/kg	3 mg/kg		10 mg/kg (w/o light)
0.65 ± 0.08 h (6)	0.52 ± 0.08 h (5)	0.38 ± 0.16 h (5)		0.10 ± 0.13 h (3)
RacBHFF				
Vehicle	10 mg/kg	10 mg/kg (w/o light)		
0.80 ± 0.05 h (7)	0.53 ± 0.09 h (6) *	0.08 ± 0.03 h (3)		

*p<0.05 from vehicle.

For light-induced phase delays, low doses of both baclofen and GS39783 inhibited phase delays by approximately 40% when injected alone; veh = 0.7 ± 0.02 h, 3.75 mg/kg baclofen = 0.4 ± 0.1 h, 0.5 mg/kg GS39783 = 0.4 ± 0.05 h; ANOVA $F(3,27) = 12.328$, $P<0.001$, (Fig. 3, top). When injected together, baclofen and GS39783 inhibited phase delays by nearly twice as much to 0.2 ± 0.1 h, or approximately 75%. However, this effect was not mimicked with RacBHFF. Again, 3.75 mg/kg baclofen and 10 mg/kg RacBHFF inhibited light-induced phase delays by nearly 40% when injected alone; veh = 0.6 ± 0.1 h, 3.75 mg/kg baclofen = 0.4 ± 0.04 h, (*t*-test, $t = 2.477$, $df = 16$); 10 mg/kg RacBHFF = 0.4 ± 0.1 h, (*t*-test, $t = 2.463$, $df = 16$) (Fig. 3, bottom). However, when injected together, baclofen and RacBHFF elicited no further reduction of light-induced phase delays than that seen with either drug alone (Fig. 3, bottom).

4. Discussion

In this study we demonstrate the ability of $GABA_B$ PAMs to enhance the inhibitory effect of baclofen on light-induced phase advances and delays of hamster circadian wheel running activity rhythms. We also pharmacologically confirm that baclofen is acting at $GABA_B$ receptors using the selective $GABA_B$ antagonist SCH50911. Finally, we show that under certain conditions $GABA_B$ PAMs exert

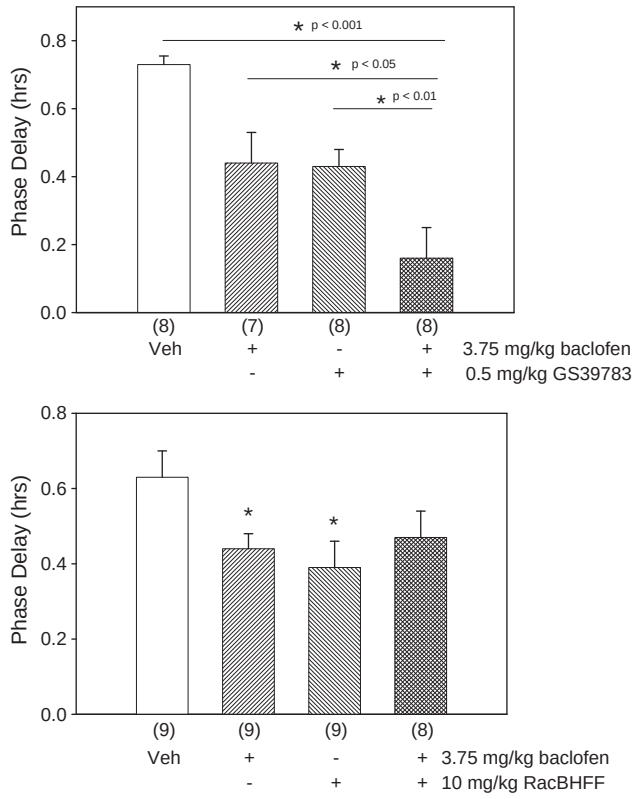

Fig. 3. GS39783, but not RacBHFF enhanced baclofen inhibition of light induced phase delays. Top: * P<as indicated, Student–Newman–Keuls post-hoc. Bottom: * P<0.05 from vehicle (*t*-test).

intrinsic ("allosteric agonist") actions to modulate circadian wheel running activity rhythms.

4.1. Site of baclofen activity in the circadian system

The present characterization of the dose-dependent inhibition of phase shifts by baclofen confirms and extends observations previously reported in the hamster following systemic injection of baclofen (Ralph and Menaker, 1989). Baclofen has a comparable inhibitory effect on light-induced phase shifts in the hamster when injected directly into the SCN (Gillespie et al., 1997), and it also inhibits light-induced induction of c-Fos immunoreactivity in the hamster SCN (Gillespie et al., 1999). There are no anatomical studies describing $GABA_B$ receptors in the hamster to our knowledge, although $GABA_B$ receptor distribution has been described in detail in the rat, where they are found to be both presynaptic, likely on retinohypothalamic projections, and postsynaptic within the SCN (Belenky et al., 2008). Electrophysiological studies also suggest that baclofen inhibits the retinohypothalamic release of glutamate by actions localized within the rat SCN (Gannon et al., 1995; Jiang et al., 1995); in addition, baclofen would appear to recruit inhibitory GABAergic autoreceptors within the rat SCN (Chen and van den Pol, 1998). Finally, with respect to glutamatergic and GABAergic terminals, pharmacological studies suggest further post-synaptic actions of baclofen both in the hamster SCN (Mintz et al., 2002) and in the rat SCN (Jiang et al., 1995). Therefore, there is evidence for both presynaptic and postsynaptic $GABA_B$ receptors in SCN; although inhibition of retinohypothalamic input to the SCN is by far the most likely explanation for the *in vivo* rhythm-modifying actions of baclofen documented herein and elsewhere. Moreover, although systemic injections were used in the present extensive pharmacological study of baclofen, antagonists and PAMs study, the studies cited above clearly define the SCN is the site of baclofen inhibition of light-induced phase shifts; indeed, the effects of baclofen on light induced phase shifts appear to be similar irrespective of whether it is given systemically or directly into the SCN (Gillespie et al., 1999).

4.2. Effects of the $GABA_B$ antagonist SCH50911

Since $GABA_B$ agonists inhibit light-induced phase shifts, it might be postulated that the $GABA_B$ antagonist SCH50911 would act oppositely to enhance light-induced phase shifts of hamster activity rhythms. However, this was not the case. By analogy, it has been reported that $GABA_B$ antagonists do not affect light-induced phase advances when administered either systemically (Ralph and Menaker, 1989) or locally applied into the hamster SCN (Gillespie et al., 1997). It has also been reported that $GABA_B$ antagonists do not affect light-induced c-Fos gene expression in the hamster using times and conditions of administration that reveal a stimulatory influence of baclofen (Gillespie et al., 1999). Clearly, the present results accord with these findings. However, a $GABA_B$ antagonist was reported to enhance light-induced phase delays in hamsters when injected into the SCN (Gillespie et al., 1997) as well as enhancing retinohypothalamic transmission in the rat SCN *in vitro* (Gannon et al., 1995). It is currently unclear why, apart from technical aspects, such differences have been found as regards the actions of $GABA_B$ antagonists but the most likely explanation, underpinned by studies of certain other functions such as cAMP formation in the rat striatum (Gjoni et al., 2006), is that $GABA_B$ receptors in the SCN show only a low and variable degree of spontaneous activity (Froestl, 2010a; Mannoury la Cour et al., 2008) Moreover, it is pertinent to note that a similar lack of antagonist activity is found when examining the influence of diverse classes of serotonergic receptor antagonist on light-induced phase shifts in the hamster despite marked effects of 5-HT itself and certain classes of agonists (Gannon et al., 2009).

4.3. $GABA_B$ PAMs GS39783 and RacBHFF

The $GABA_B$ PAMs GS39783 and RacBHFF both enhanced the inhibitory activity of low doses of baclofen on light-induced phase advances in hamster activity rhythms (Fig. 2), while not having any significant effect themselves (Table 2 and Fig. 2). GS39783 also enhanced the inhibitory effect of low doses of baclofen on light-induced phase delays, though RacBHFF did not (Fig. 3). We currently have not obvious explanation for this discrepancy which requires further study, but in previous work these two agents have likewise not always behaved identically and it is possible that GS39783 possesses somewhat more robust PAM properties (Koek et al., 2010). In any event, these data overall are consistent with PAM actions of both drugs, supporting previous work using other functional models to study their actions at cerebral populations of $GABA_B$ receptor (Cryan et al., 2004; Mombereau et al., 2004; Urwyler et al., 2003). Interestingly, both GS39783 and RacBHFF inhibited light-induced phase delays when given alone in the absence of baclofen, suggesting that they can act as allosteric agonist on the population of $GABA_B$ receptors that mediates phase delays, in contrast to that underlying phase advances. This observation is consistent with the notion that separate populations of $GABA_B$ receptors are involved in phase delays vs. advances (Belenky et al., 2008; Gillespie et al., 1997). The present observations of intrinsic actions of GS39783 and RacBHFF corroborates reports that they act as allosteric agonists using both in vitro and in vivo studies (Froestl, 2010a,b; Gjoni and Urwyler, 2009; Koek et al., 2010; Malherbe et al., 2008). Such functional effects of PAMs are not only interesting from a theoretical viewpoint with regard to molecular models of $GABA_B$ receptors and their activation by orthosteric and allosteric agonists (Mannoury la Cour et al., 2008; Monnier et al., 2011; Rondard et al., 2011), but perhaps more importantly, they underscore present thinking that allosteric ligands can exert

functionally relevant agonist-like actions alone, though interpretation of such data for *in vivo* models are complicated by the endogenous release of GABA itself.

The present observations are of particular interest inasmuch as $GABA_B$ receptors have been implicated in the pathogenesis and control of anxious, depressed and psychotic states (Frankowska et al., 2007; Millan, 2003, 2006; Mombereau et al., 2004) which are associated with circadian rhythm irregularities (Germain and Kupfer, 2008; Kantrowitz et al., 2009; Waddington Lamont et al., 2010). Thus, $GABA_B$ agonists and PAMs may be useful in the control of chronobiological abnormalities in psychiatric disorders in addition to their influence upon mood.

5. Conclusion

In summary, the present data demonstrate that the orthosteric $GABA_B$ agonist baclofen dose-dependently and specifically inhibits light induced phases advances and delays of circadian activity rhythms in hamsters. Its actions were potentiated by $GABA_B$ receptor PAMs which, under certain conditions, behaved as allosteric agonists in exerting actions alone. These observations suggest that $GABA_B$ PAMs justify further investigation for the potential treatment of the circadian rhythm dysfunction that characterizes depression and other CNS disorders.

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