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Serotonergic enhancement of circadian responses to light: Role of the raphe and intergeniculate leaflet

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Abstract

Light serves as the primary stimulus that synchronizes the circadian clock in the suprachiasmatic nucleus (SCN) to the external day-night cycle. Appropriately timed light exposure can reset the phase of the circadian clock. Some serotonergic drugs that bind to the 5-HT_{1A} receptor can enhance phase shifts to light. The mechanism by which this potentiation occurs is not well understood. In this study we examine where in the hamster brain one of these drugs, BMY7378, might be working. Systemic (5 mg/kg), intra-dorsal raphe and intra-median raphe (both 15.6 nmol in 0.5 µl), but not intra-SCN (7.8 nmol or 15.6 nmol in 0.5 µl) injections of BMY7378 significantly potentiated phase shifts to light. Potentiation of photic shifts persisted when serotonergic innervation of the SCN was lesioned with infusions of the serotonin neurotoxin 5,7-dihydroxytryptamine into the SCN. Light-induced c-Fos expression in the rostral and caudal intergeniculate leaflet (IGL) was attenuated with systemic BMY7378, suggesting that the IGL may be involved in this response. Both complete IGL lesions and depletion of serotonergic innervation of the IGL prevented systemic BMY7378 from potentiating photic phase shifts. Together these findings suggest that the mechanism by which BMY7378 enhances photic responses is by changing the activity of the raphe nuclei to influence how the IGL responds to light, which subsequently influences the SCN as one of its downstream targets. Identification of the network that underlies this potentiation could lead to the development of useful therapeutic interventions for treating sleep and circadian disorders.

Introduction

Daily oscillations in the physiology and behavior of mammals are regulated by an endogenous circadian clock located in the suprachiasmatic nucleus (SCN; Antle & Silver, 2005). These rhythms are synchronized to our day-night cycles primarily by light. When housed in constant conditions, brief light exposure can reset the circadian clock in a phase- and dose-dependent manner (Takahashi et al., 1984), with light late in the subjective night advancing the phase of the clock. Developing approaches to modulate these responses to light could be therapeutically useful in the treatment of jet lag and other circadian disorders.

The SCN receives serotonergic input from the median (Meyer-Bernstein & Morin, 1996) and dorsal raphe (Yamakawa & Antle, 2010). Serotonin opposes the action of light by preventing neurotransmitter release from retinal terminals (Rea & Pickard, 2000) and by attenuating the increased firing rate of SCN neurons that normally results from activation of its retinal inputs (Ying & Rusak, 1994; 1997). Activation of 5-$HT_{1A/7}$ receptors in the SCN attenuates photic shifts (Antle et al., 2003). Circadian responses to light are enhanced when serotonin is depleted (Morin & Blanchard, 1991; Penev *et al.*, 1993).

Drugs that act as 5-HT_{1A} mixed agonists/antagonists show great promise in enhancing circadian responses to light in the late subjective night. Four specific compounds have been identified: NAN-190 (Rea et al., 1995), BMY7378, S15535 (Gannon, 2003) and MKC-242 (Moriya et al., 1998). These drugs bind to the 5-HT_{1A} receptor and are thought to act as agonists at raphe autoreceptors and as antagonists at postsynaptic receptors (Rydelek-Fitzgerald *et al.*, 1990; Claustre *et al.*, 1991; Gannon, 2003). When these drugs are given prior to a light pulse the resulting phase advances can be up to 250% greater than to light alone (Rea *et al.*, 1995; Moriya *et al.*, 1998; Takahashi *et al.*, 2002; Gannon, 2003; Gannon & Millan, 2006; Sterniczuk *et al.*, 2008; Smith *et al.*, 2010; Smith *et al.*, 2015b). Interestingly, both BMY 7378 and NAN-190 have been shown to enhance photic shifts when administered even up to 6 hours after a light pulse (Kessler *et al.*, 2008; Lungwitz & Gannon, 2009). Despite this significant enhancement, relatively little is known about the mechanism by which this potentiation is achieved. The 5-HT_{1A} receptor is required for this potentiation (Smith et al., 2010). In the SCN, light-induced expression of c-Fos, JunB, and PER1 (Recio *et al.*, 1996; Smith *et al.*, 2010; Smith *et al.*, 2015b) and phosphorylation of CREB (Smith et al., 2010) are altered by NAN-190 or BMY7378 pretreatment. It has been suggested that systemic administration of these drugs might enhance circadian responses to light by decreasing serotonin release from the raphe, while simultaneously disinhibiting the retinal terminals in the SCN, leading to greater neurotransmitter release following a light pulse (Gannon, 2003). While this likely accounts for some of the effect, we have previously shown that systemic NAN-190 changes how the SCN responds to intra-SCN injections of agonists for neurotransmitters involved in photic signaling to and within the SCN (Sterniczuk et al., 2008). Specifically, phase advances and delays to NMDA and phase advances to gastrin-releasing peptide (GRP) are enhanced, while phase delays to GRP are attenuated with systemic NAN-190 pretreatment. These results suggest that it is not just modification of retinal

input, but also alterations in how the SCN cells themselves react to their inputs, that underlie how these drugs enhance responses to light. That said, this phenomenon has only been observed with systemic administration of these drugs, greatly limiting our understanding of how they produce the observed potentiation. The present set of experiments examines where in the brain one of these drugs, BMY7378, acts, and the pathways by which it may influence the circadian network.

Material and Methods

Animals

Adult male Syrian hamsters (Crl:LVG(SYR), n=105) were obtained from Charles River (Kingston, NY). Animals were 100-110g upon arrival in the lab. Hamsters were pair-housed in a 14:10LD cycle until the start of the experiments, at which point they were individually housed and placed into constant darkness (DD). Animals had ad libitum access to food (Purina Lab Diet 5001) and water throughout the experiments, and were housed in temperature (21 ± 1ºC) and humidity (50% ± 5% RH) controlled rooms. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

Behavioral analysis

Animals were housed in cages equipped with running wheels (Super Pet Run-Around, 14.6 cm diameter). Rotation of the running wheels was monitored by closure of magnetic switches using Clocklab (Actimetrics, Wilmette, IL). Animals were housed in DD and received manipulations approximately every two weeks. Phase shifts to each manipulation were calculated using the standard Clocklab routine as described previously (Sterniczuk et al., 2008). All manipulations and husbandry were performed using night-vision goggles (General Starlight Company, Richmond Hill, Ontario). All experiments were timed relative to activity onset in constant darkness, which by convention is defined as circadian time (CT) 12 for nocturnal animals. Exposure to experimental light pulses occurred 6 hours after activity onset, or CT18, as this phase elicits maximal phase advances in Syrian hamsters (Daan & Pittendrigh, 1976).

Surgeries

Some animals received cannula implants, radio frequency lesions or neurotoxic lesions (see below). Surgical manipulations of the intergeniculate leaflet (IGL) were bilateral, while all other surgical interventions were unilateral although aimed at midline. All animals undergoing surgery were pretreated with butorphanol (2 mg/kg) for pain management, and were then anesthetized with sodium pentobarbital (~120 mg/kg, CEVA Santé Animale, France). Animals were maintained on oxygen (1 L/min) throughout surgery, and were supplemented with isoflurane (1-3%) as needed throughout surgery.

Cannulation

Some hamsters were surgically implanted with a single guide cannula (C313G, Plastics One, Roanoke, VA) as described previously (Sterniczuk et al., 2008). Guide cannulas were implanted unilaterally and were aimed at either the SCN (n=20, coordinates relative to bregma: +0.3 mm anterior, 0.3 mm lateral and 7.0 mm ventral to skull surface with the incisor bar set to 2 mm below the interaural level), the median raphe nucleus (MRN, n=12; at a 20° lateral angle with the head leveled between bregma and lambda, coordinates relative to bregma: 4.4 mm posterior, 2.3 mm lateral and 5.4 mm ventral to dura) or the dorsal raphe nucleus (DRN, n=5; at a 20° lateral angle, coordinates relative to bregma: 4.7 mm posterior, 1.7 mm lateral and 3.8 mm ventral to dura) such that the tip was 1 mm from the structure. A dummy cannula that extended 1 mm beyond the end of the guide was inserted to maintain patency. Injection cannulas also extended 1 mm beyond the end of the guide cannula.

5,7-DHT lesions

Some hamsters received infusions of 5,7-dihydroxytryptamine (5,7-DHT) as described previously (Yamakawa & Antle, 2010). Animals were pretreated with desipramine (25 mg/kg; Sigma-Aldrich, Oakville, ON, Canada) to protect catecholaminergic terminals. A lower dose of anesthetic was used (~70 mg/kg sodium pentobarbital) as it was observed that desipramine enhanced the potency of the anesthetic. Infusions of 5,7-DHT (0.5 µl; ~24 µg free base/µL in 0.5% ascorbic acid in physiological saline; Sigma-Aldrich) were aimed at either the SCN (n=17, unilateral infusions, at a 10° lateral angle; coordinates relative to bregma: 0.3 mm anterior, 1.25 mm lateral of midline, 7.65 mm ventral to dura; with the incisor bar set to 2 mm below the interaural level) or intergeniculate leaflet (IGL, n=7, bilateral, 0.1 µl at each of 4 locations; 0.6 and 1.6 mm posterior to bregma; ±3.2 and ±3.1 mm lateral to midline; 4.0 and 4.6 mm ventral to dura with the incisor bar set to 2 mm below the interaural level). Infusions were performed over 5 minutes and the injector was left in place after each injection for a further 5 minutes. Control animals (n=4 for SCN, n=5 for IGL) received infusions of 0.5% ascorbic acid in physiological saline.

Radiofrequency lesions

Some hamsters received radiofrequency lesions (n=13) or sham lesions (n=8) aimed at the IGL. Coordinates were the same as for the 5,7-DHT IGL infusions described above. A Teflon-coated stainless steel wire, 200 µm bare diameter, 280 µm coated diameter, with the insulation removed from 0.75 mm of the tip (A-M Systems, Carlsborg WA, USA) was used to create the lesions. Current was then passed through the electrode for 30 seconds using a Grass LM3 radiofrequency lesion maker. Sham operations were performed in a manner similar to the lesions with the exception that the electrode was lowered to coordinates which were 1.0 mm dorsal to the lesion sites listed above and no current was passed through the electrode.

Behavioral Effects of lesions

The activity patterns of animals that received 5,7-DHT lesions of the SCN or IGL, or radiofrequency lesions of the IGL were analyzed in the 7 days prior to their first treatment. The Clocklab Analysis program was used to quantify free running period (tau) and average activity levels. Duration of the active phase (alpha) was quantified by determining the activity onset and offset on each of the 7 days prior to the manipulation, and then calculating the time between each onset and its corresponding offset. The average of these was used as alpha.

Histology/perfusion

To collect brain tissue for histology, all animals were given an overdose of sodium pentobarbital (~840mg/kg). When the animals were deeply anesthetized, they were transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) followed by ~50ml cold 4% paraformaldehyde in PBS. Brains were postfixed overnight at 4°C in paraformaldehyde followed by 24h in 20% sucrose in PBS.

Brains were sliced using a cryostat. Sections (35 µm thick) were collected through the SCN, IGL, MRN and/or DRN as appropriate. To confirm placement of a cannula, brains were stained using a standard cresyl violet nissl stain. Animals were excluded if the tip of the cannula was more that 600 µm from the margin of the SCN, MRN or DRN.

The extent of the IGL radiofrequency lesions were confirmed using immunohistochemistry for neuropeptide Y at the IGL and SCN. Loss of NPY fibers in the ventrolateral SCN was the main indicator of a complete lesion.

Extent of the neurotoxic lesions of serotonergic input to the IGL and SCN were confirmed with immunohistochemistry for the serotonin transporter (SERT) at the SCN or IGL and for serotonin at the level of the median and dorsal raphe nuclei. For 5,7-DHT lesions of serotonin innervation of the IGL, alternate sections were stained for NPY-immunoreactivity.

Immunohistochemistry

All sections were stained for a single antigen using a nickel-enhanced diaminobenzidine protocol described previously (Antle et al., 2008). The following primary antibodies were used: rabbit anti-NPY (1:10,000; (#22940) ImmunoStar, Hudson, WI, USA), rabbit anti-SERT (1:2500; (#24330) ImmunoStar); rabbit anti-serotonin (1: 2500; (#20080) Immunostar), and rabbit anti-c-Fos (1:20,000; (SC- 52) Santa Cruz Biotechnology, Santa Cruz, CA).

Relative optical density (ROD) of NPY at the SCN or SERT at either the SCN or IGL was used to quantify the extent of the respective lesions. Brightfield images were captured using an Olympus BX51 microscope equipped with a QImagine cooled-CCD monochrome camera (QImaging, Burnaby, BC, Canada). Mean grey levels were calculated using ImageJ software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD) for the region of interest (SCN or IGL) and were divided by the mean grey level of adjacent tissue outside the area of normal innervation to obtain the ROD. Calculations were based on bilateral assessments over 2 representative sections. An RGB-LCD adapter was used to capture color images of the 5-HT immunoreactivity in the raphe nuclei to confirm the presence of serotonergic cells

The number of cells immunoreactive for c-Fos was counted bilaterally at 3 specific rostrocaudal levels of the IGL. A region of interest was identified by delineating the IGL based on NPY immunoreactivity on adjacent sections.

Statistical Analyses

Statistical significance was set at $p<0.05$ for all tests, using SigmaPlot (Systat Software, Inc.; San Jose, CA). Factorial ANOVAs, two-tailed paired t-tests or two-tailed independent samples t-tests were used to analyze results, as described below. All significant ANOVAs were followed up by Student-Newman-Keuls post hoc multiple comparisons. Order effects were tested using independent samples t-tests. When an order effect was noted, independent samples comparing only animals receiving treatments in the first round (i.e., treatment naïve animals) were run. Behavioral effects of lesions were quantified with One Way ANOVAs (complete lesion, partial lesion, and intact) for radio frequency lesions of the IGL and 5,7-DHT lesions of the SCN, and using independent samples t-test (lesion vs. sham) for 5,7-DHT lesions of the IGL. All means are reported as ± standard error of the mean (SEM) in figures and ± standard deviation in the text. In all cases, the pairwise planned comparisons were always run to compare BMY7378 to its vehicle control within each condition, and for BMY7378 treatments between lesioned and unlesioned animals.

Experiments

Experiment #1 – Where does BMY7378 act?

We first examined where the 5-HT_{1A} receptor mixed agonist/antagonist 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378, Sigma-Aldrich) acts to enhance circadian responses to light. A number of 5-HT_{1A} mixed agonists/antagonist have been shown to enhance circadian responses to light (Gannon, 2003). Of these, BMY7378 and NAN-190 produce the most robust potentiation. Here we focused on BMY7378 as its solubility in aqueous solutions permitted the use of a less harsh vehicle (i.e., buffered saline) than is needed with other options (e.g., DMSO with NAN-190), thereby facilitating a repeated measures approach where animals received multiple injections. Animals with a cannula aimed at the SCN (n=15), MRN (n=12), DRN (n=5), or unoperated

animals (n=6) were used to answer this question. Animals received BMY7378 or vehicle control (sterile saline) systemically (5 mg/kg i.p.), intra-SCN (7.8 nmol in 0.5 µl), intra-MRN (15.6 nmol in 0.5 µl), or intra-DRN (15.6 nmol in 0.5 µl). An additional set of animals (n=5) was used to examine a higher dose of BMY7378 intra-SCN (15.6 nmol in 0.5 µl). Systemic injections were given 45 minutes prior to a CT18 light pulse, while cannula injections were given 10 minutes prior to the CT18 light pulse. Light pulses (15 minutes) were administered by transferring the animal in their home cage to a light-tight box equipped with a 7.5W white incandescent light bulb (GE S11) partially covered with aluminum foil to achieve 40 lux illuminance at the level of the cage floor. Some animals also received treatments where they received an injection of vehicle or drug without subsequent exposure to light (systemic, n=5; intra-SCN, n=15; intra-MRN, n=4; intra-DRN, n=4). Animals were only used for analysis if they contributed phase shift data to both drug and vehicle conditions. Treatments were given in a counterbalanced fashion. Some SCN cannulated animals (n=4) also contributed data to the systemic administration experiment. The range of days, relative to start of constant darkness, for when each treatment was applied is presented in table 1. Cannula placements were confirmed at the end of the study. A 2 (injection type) x 3 (injection site) factorial ANOVA was used to analyze phase shifts. A paired t-test was used to analyze the effects of the higher dose of intra-SCN BMY7378, and an independent samples t-test was used to compare the two doses of the intra-SCN BMY7378.

Histological examination led to one SCN cannulated animal being excluded due to a missed placement. Another SCN cannulated animal was excluded as its locomotor activity rhythms were not stable enough to provide reliable activity onsets. One of the SCN cannulated animals used for the high dose of BMY7378 was excluded as it did not complete all treatment conditions. One MRN-cannulated animal was excluded due to lost head cap prior to completing all conditions. One unoperated animal was excluded from analysis as its phase shift to the systemic saline and light treatment was more than 6 standard deviations greater than the mean phase shift for this baseline condition. One DRN animal was excluded as its shift to the intra-DRN saline and light treatment was more than 6 standard deviations greater than the mean phase shift for this baseline condition. All other animals were retained for analysis.

Experiment #2 – Is serotonergic innervation of the SCN necessary for BMY7378 potentiation of photic shifts?

We next examined the role of serotonergic innervation of the SCN in enhancements of photic phase shifting by BMY7378. Animals received an infusion of either 5,7-DHT (n=17) or vehicle control (0.5% ascorbic acid in physiological saline, n=4) to the SCN. Upon recovery and establishment of stable freerunning locomotor rhythms in constant darkness, they were treated in counterbalanced fashion with systemic BMY7378 (5 mg/kg i.p. at CT17.25) or saline prior to a 15 minute, 40 lux light pulse at CT18. Due to the potential for serotonergic reinnervation of the SCN starting after about 8 weeks (Morin, 1992), the sham light pulse procedures were not investigated in these animals. Brains were

collected at the end of the study to analyze the extent of the 5-HT lesion. Phase shifts were analyzed using paired t-tests.

Experiment #3 – Does BMY7378 alter light-induced c-Fos in the IGL?

We next examined if BMY7378 would modify light-induced expression of the immediate-early gene c-Fos. Animals were given a systemic BMY7378 (n=4, 5 mg/kg i.p. at CT17.25) or saline (n=4) injection prior to a 15 minute, 40 lux light pulse at CT18. They were then perfused 90 minutes after the start of the light pulse (i.e., at CT19.5). The number of c-Fos-immunoreactive cells was analyzed using a 2 (treatment) x 3 (rostrocaudal level) factorial ANOVA.

Experiment #4 – Is the IGL necessary for BMY7378 potentiation of photic shifts?

We next wanted to determine if the IGL was necessary for the enhancement of photic phase shifts by BMY7378. Animals received bilateral radiofrequency lesions of the IGL (n=13), or sham lesions (n=8). In a counterbalanced fashion separated by two weeks, animals received systemic injections of BMY7378 (5 mg/kg i.p.) or vehicle control (sterile saline) at CT17.25. Forty-five minutes after their injection, at CT18, animals were transferred in their home cages to a light-tight box where they received a 15 minute 40 lux light pulse. Some animals with IGL lesions (n=10) or sham lesions (n=6) also received injections without subsequent light exposure. Brains were collected and processed for NPY immunoreactivity to determine the extent of the IGL lesion. Phase shifts to no-light and light pulses were analyzed separately using 2 (treatment: drug versus saline) x 3 (Lesion extent: sham lesion, partial lesions, complete lesions) factorial ANOVAs.

Experiment #5 – Is serotonergic innervation of the IGL necessary for BMY7378 potentiation of photic shifts?

Our final experiment was to determine if serotonergic innervation of the IGL was necessary for potentiation of photic phase shifts by BMY7378. To test this, animals received bilateral infusions of either the neurotoxin 5,7-DHT (n=7) or vehicle control (0.5% ascorbic acid in sterile saline, n=5) into the IGL. In a counterbalanced fashion separated by two weeks, animals received systemic injections of BMY7378 (5 mg/kg i.p.) or vehicle control (sterile saline) at CT17.25. Forty-five minutes after their injection, at CT18, animals were transferred in their home cages to a light-tight box where they received a 15 minute 40 lux light pulse. Due to the potential for serotonergic reinnervation of the IGL starting after about 8 weeks (Morin, 1992), the sham light pulse procedures were not investigated in these animals. Brains were collected at the end of the study to analyze the extent of the 5-HT lesion. Phase shifts were analyzed using a 2 (treatment) X 2 (lesion) factorial ANOVA.

Results

Experiment #1 – BMY7378 at the MRN or DRN, but not the SCN, is sufficient to potentiate photic shifts

Consistent with previous findings (Gannon, 2003), BMY7378 significantly potentiated phase shifts to light in the late subjective night (main effect of injection type, $F_{(1,34)}$=47.729, p<0.001, Figure 1). However, this was only significant (injection type X injection site interaction, $F_{(3,34)}$=11.873, p<0.001) for BMY7378 administered systemically (p<0.001) and into the MRN (p<0.001) or DRN(p=0.028); intra-SCN injections did not significantly enhance responses to light (p=0.960). The higher dose of intra-SCN BMY7378 (15.6 nmol in 0.5 µl) also did not significantly enhance phase shifts to light (saline + light: 1.66±0.37h; BMY7378 + light 1.48±0.98h; paired $t_{(3)}$=0.390, p=0.723). There was no significant difference between the phase shifts elicited by light following either dose of intra-SCN BMY7378 (independent $t_{(15)}$=0.742, p=0.460). While BMY7378 delivered into the MRN and DRN did significantly enhance photic phase shifts, the resulting phase shifts were significantly smaller than those to light following systemic BMY7378 (MRN, p<0.001; DRN, p=0.002). There were no order effects in response to treatments.

Consistent with previous findings (Gannon, 2003), BMY7378 also produced significant phase shifts on its own (main effect of injection type, $F_{(1,24)}$=23.278, p<0.001), although this was solely due to the phase shifts to systemic BMY7378 (saline alone: -0.06±0.16h; BMY7378 alone: 1.42±0.54h; injection type X injection location interaction $F_{(3,24)}$=21.658, p<0.001), as intra-SCN (saline alone: -0.1±0.2h; BMY7378 alone: 0.004±0.3h), intra-MRN infusions (saline alone: -0.06±0.15h; BMY7378 alone: 0.12±0.24h) and intra-DRN infusions (saline alone: -0.02±0.06h; BMY7378 alone: -0.24±0.11h) elicited no significant phase shifts on their own. There were no order effects in response to treatments.

Experiment #2 – Serotonergic innervation of the SCN is not necessary for BMY7378 potentiation of photic phase shifts

Of the 17 animals given 5,7-DHT infusion to the SCN, 8 were deemed complete or near complete lesions and were used for behavioral analysis. One animal exhibited a 13h advance to the saline + light condition and was excluded from analysis as this response was more than 6 standard deviations from the mean for that control condition. Another 8 animals were excluded from analysis as histological analysis revealed that they had either partial lesions (n=3) or complete misses (n=5). ROD analysis of the complete lesions revealed significantly less optical density in the SCN (1.13±0.06) than sham lesioned animals (1.37±0.13, $t_{(12)}$=4.954, p<0.001). When fibers did remain in the 5,7-DHT-treated animals, they were visible in the ventromedial portion of the SCN (Figure 2). The MRN and DRN were both examined to confirm normal patterns of 5-HT-immunoreactive cells, indicating that the lesions only affected 5-HTergic fibers, and did not cause global 5-HT lesions.

There were no difference in either tau ($F_{(2,13)}$=0.104, p=0.902) or alpha ($F_{(2,13)}$=1.018, p=0.388) between animals with complete lesions, partial lesions and intact 5-HT innervation of the SCN. A significant effect was detected on activity levels ($F_{(2,13)}$=4.575, p=0.031), but post hoc analysis revealed that animals with partial lesions had significantly lower activity levels than those with intact 5-HT innervation. Activity levels for those with complete lesions did not differ from activity levels in either the intact or partial lesion groups.

Systemic administration of BMY7378 significantly increased the magnitude of photic phase shifts induced in the late subjective night, when compared to vehicle control (main effect of treatment: $F_{(1,12)}$ = 46.969, p < 0.001; Figure 2), in both control (p=0.007) and lesioned animals (p=0.005). Overall, animals with 5,7-DHT lesions had smaller phase shifts than did sham lesioned animals in both the saline + light and BMY7378 + light conditions (main effect of lesion: $F_{(1,10)}$=5.223, p=0.045), however there was no significant interaction between treatment and lesion ($F_{(1,12)}$=0.436, p=0.524). While phase shifts to BMY7378 and light appeared somewhat smaller in 5,7-DHT lesioned animals than in sham lesioned animals, the planned comparison revealed that this was not significant (p=0.52). A significant order effect was detected in the response to BMY + light in the animals with complete 5,7-DHT lesions at the SCN. Specifically, animals treated with BMY7378 in the first round had significantly smaller shifts than those in the second round ($t_{(6)}$=3.14, p=0.02), and these shifts were not significantly larger than phase shifts to saline + light ($t_{(6)}$=1.29, p=0.24). Only 3 animals received BMY7378 in the first round, two of whom had phase shifts nearly identical in magnitude as their shifts to their later saline + light treatment. The other hamster from this first round exhibited a much larger shift to the BMY + light treatment than to its later saline + light treatment. This observation contrasts with the second round, where phase shifts following BMY + light were significantly greater than to saline + light ($t_{(6)}$=3.98, p=0.007), and these phase shifts were significantly greater than to their earlier shifts to saline + light ($t_{(4)}$=5.27, p=0.006).

Experiment #3 –BMY7378 alters IGL light-induced c-Fos

Following phase shifting light pulses, prominent c-Fos-immunoreactivity was observed throughout the IGL. Pretreatment with BMY7378 significantly attenuated the number of c-Fos-immunoreactive cells (main effect of treatment, $F_{(1,12)}$=20.505, p<0.01), although this effect was only significant in the rostral and caudal sections (treatment X level interaction, $F_{(2,12)}$=5.601, p<0.05, Figure 3). Although a modest decrease in the number of c-Fos-immunoreactive cells was observed at the mid-IGL level with BMY7378 treatment, this difference was not significant (p<0.09). The number of c-Fos-immunoreactive cells was significantly different between all the rostro-caudal levels of the IGL (main effect of level, $F_{(2,12)}$=43.666, p<0.001), with the highest levels at the caudal portion and lowest levels at the mid-IGL level, thus the lack of significant inhibition at the mid-IGL level may be partially explained by a floor-effect.

Experiment #4 – The IGL is necessary for BMY7378 potentiation of photic phase shifts

Histological analysis revealed three IGL lesion conditions. Normal levels of NPY were observed in SCN sections from sham-lesioned animals (n=8). Three animals exhibited reduced levels of NPY in the SCN and were classified as partial lesions, and 10 animals exhibited no NPY in the SCN and were classified as complete lesions (Figure 4).

Lesioning the IGL did not significantly affect tau ($F_{(2,18)}$=3.039, p=0.07), but did significantly decrease activity levels ($F_{(2,18)}$=18.079, p<0.001) and significantly increase alpha ($F_{(2,18)}$=3.981, p=0.037). With respect to activity levels, both complete and partial lesions yielded significantly lower activity than in intact animals. For alpha, a significant difference was only observed between complete lesions and intact animals.

BMY7378 significantly enhanced responses to light, but only in the sham lesion condition (lesion x treatment interaction, $F_{(2,18)}$=5.892, p=0.011, figure 5). While phase shifts to light in the saline conditions were modestly smaller in the two lesioned groups than in the sham lesion group, this trend was not significant (Sham vs. partial lesion, p=0.651, sham vs. complete lesion, p=0.214). There was no difference between the phase shifts elicited by light with saline or BMY7378 pretreatment in either the partial lesion (p=0.958) or the complete lesion groups (p=0.983). There were no order effects detected in the treatments.

On its own, BMY7378 produced phase advances in the no-light-pulse condition (main effect of treatment $F_{(1,11)}$=6.753, p<0.05). These phase shifts were altered by the extent of the lesion (main effect of lesion, $F_{(2,11)}$=12.406, p=0.002). Specifically, phase shifts to BMY7378 in the sham lesion animals (1.12±0.47h) were significantly larger than shifts to BMY7378 in both the partial lesion animals (0.38±0.45h, p=0.017) and the complete lesion animals (0.21±0.43h, p<0.001). Phase shifts to BMY7378 were only significantly greater than shifts to saline control injections in the sham-lesioned animals (sham lesion saline shift: 0.11±0.27h, p=0.002). Shifts to BMY7378 did not differ significantly from saline controls in either the partial lesion animals (partial lesions saline shift: -0.05±0.17h, p=0.344) or the complete lesion animals (complete lesion saline shift: 0.09±0.18h, p=0.93). There were no order effects detected in the treatments.

Experiment #5 – Serotonergic innervation of the IGL is necessary for BMY7378 potentiation of photic phase shifts

Histological analysis of sequential NPY- and SERT-stained IGL slices following completion of the manipulations revealed normal levels of 5-HTergic innervation of the IGL in sham lesion controls (n=5) and the complete depletion of 5-HT in animals injected with 5,7 DHT (n=7; Figure 6). Lesioning 5-HT

input to the IGL had no effect on alpha ($t_{(10)}$=1.011, p=0.336), tau (($t_{(10)}$=0.96, p=0.36), or activity levels ($t_{(10)}$=0.954, p=0.363).

While BMY7378 enhanced photic phase shifts (main effect of treatment, $F_{(1,10)}$=16.948, p<0.01), this was only observed in the sham treated animals (treatment X lesion interaction, $F_{(1,10)}$=6.578, p<0.05, Figure 7). Phase shifts to saline and light did not differ between sham and lesioned animals (p=0.891). Phase shifts to light following BMY7378 pretreatment were significantly smaller in the lesion animals than in the sham animals (p=0.011). There was no difference between the size of the phase shift to light following either saline or BMY7378 pretreatment in the 5-HT-lesioned animals (p=0.257). There were no order effects detected in the treatments.

Discussion

In this study, we show that the IGL and its 5-HT input are critical for potentiation of photic phase shifts induced by BMY7378. Furthermore, we show that activity of BMY7378 at the raphe nuclei alone is sufficient to yield potentiation of photic phase shifts. Infusions of BMY7378 to just the SCN are not sufficient to enhance photic responses, suggesting that systemic BMY7378 is acting elsewhere to initiate this response. Infusions of BMY7378 to either the DRN or the MRN are sufficient to enhance responses to light, suggesting that systemically administered BMY7378 may modulate raphe output by activating serotonin autoreceptors on raphe neurons. Most animals with lesions of serotonin input to the SCN continued to show enhanced responses to light when pretreated with BMY7378, suggesting that the altered raphe activity does not exclusively use the direct serotonergic connection to the SCN to produce this enhancement. Phase shifts were modestly smaller, albeit not significantly so, in animals with SCN 5,7-DHT lesions treated with BMY7378 and light than in sham lesioned animals, suggesting that the direct serotonergic pathway to the SCN might still contribute to the full response. Systemic BMY7378 attenuated the number of c-Fos-immunoreactive cells in the IGL following a light pulse, consistent with this structure participating in serotonergic enhancement of photic phase shifts. IGL lesions eliminated the enhancement of photic shifts, demonstrating that the IGL is necessary for this phenomenon. The finding that eliminating serotonergic innervation of the IGL also prevented the potentiation suggests that the mechanism underlying BMY7378's potentiation of photic phase shifts requires serotonergic regulation of the IGL's responses to light.

A variety of responses to BMY7378 + light were observed in animals with 5,7-DHT lesions of 5-HT input to the SCN. Of the animals that received BMY7378 in the first round (n=3), two exhibited phase shifts that were not significantly different from their later saline + light treatment, or from animals that received saline + light in the first round. Animals that received BMY7378 + light in the second round, after receiving the saline and light treatment earlier, had significantly larger phase shifts than were observed with saline and light. The reason for this order effect is not clear. Animals from the second round did have complete 5-HT lesions as assayed 2 weeks following their BMY7378 treatment, thus ruling out re-innervation as a possibility for observing potentiation at the later time point. Furthermore,

re-innervation of the SCN following 5,7-DHT lesions does not occur until 8 weeks post-lesion (Morin, 1992). The difference between the animals treated earlier versus those treated later might reflect adaptive changes in 5-HT receptor density resulting from the loss of 5-HT input. However, the BMY7378 manipulations occurred 4 weeks post-lesion in the early group and 6 weeks post-lesion in the later group, and any adaptive changes to lost serotonin input should occur on a much shorter time scale (Prosser *et al.*, 2006). As this difference reflects the responses of just 2 animals that did not exhibit the typical BMY7378 potentiation, these order effects should be interpreted with caution.

It is reasonable to suspect that the IGL would be involved in the potentiation of photic responses. The IGL receives input from both the eye (Harrington, 1997) and the raphe nuclei (Meyer-Bernstein & Morin, 1996; Grossman *et al.*, 2004). 5-HT_{1A} receptors are found in the IGL (Duncan et al., 1999). The IGL contains a number of distinct cell-types that innervate the SCN, including both NPY and non-NPY neurons. NPY and Y1/Y5 receptor agonists attenuate photic phase advances (Weber & Rea, 1997; Lall & Biello, 2002; 2003), and a Y5 receptor antagonist potentiates photic shifts in a manner similar to that observed with both NAN-190 and BMY73787 (Lall & Harrington, 2006). It is possible that BMY7378 exerts its effects by altering raphe activity in such a fashion so as to inhibit NPYergic cells in the IGL (Figure 8). The resulting decrease in NPY release at the SCN would then enhance the circadian response to light. The final mechanism is unlikely to be this simple however, as combining BMY7378 or NAN-190 with a Y5 antagonist elicits greater potentiation than either of the drugs alone. This might suggest separate parallel pathways. Alternatively, the pharmacological effect might be incomplete at the doses used in that study, and therefore intervening at 2 points of a serial pathway might then elicit a greater effect. Finally, it is possible that BMY7378, although requiring the IGL, may also be affecting other components of the circadian system as well. Certainly the role of the IGL in regulating circadian responses to light is complex. In this study, lesion of the IGL modestly, although not significantly, diminished phase shifts to light. Other studies have found that IGL lesions did significantly attenuate phase advances to light (Harrington & Rusak, 1986; Pickard *et al.*, 1987). In no case has significant enhancement of photic phase advances been observed. IGL lesions would certainly lead to a decrease of NPY at the SCN, and thus the effects of BMY7378 cannot be explained by a simple loss of NPY at the SCN alone. It could be that other IGL inputs to the SCN are unchanged or even enhanced following BMY7378 pretreatment (Figure 8), which would not be the case with IGL lesions. This would be consistent with the potentiation observed when just Y5 receptors are blocked (Lall & Harrington, 2006).

Photic phase shifts are associated with induction of *Period* gene expression in the SCN (Hamada et al., 2004). Light-induced *Period* gene expression in the SCN is prolonged by treatments that enhance phase shifts, such as pretreatment with the 5-HT_{1A} partial agonists MKC-242 (Takahashi et al., 2002). Conversely, treatments that oppose photic phase shifts, such as NPY infusions to the SCN (Lall & Biello, 2002), inhibit light-induced *Period* gene expression (Brewer et al., 2002). Given that blocking Y5 receptors enhances responses to light much like NAN-190 and BMY7378, it has been suggested that the

potentiation observed with these 5-HT_{1A} mixed agonists/antagonists might be due to preventing NPY's inhibition on light-induced *Period* gene expression in the SCN (Lall & Harrington, 2006).

Light exposure induces c-Fos expression in the IGL (Park et al., 1993), although the cell phenotype exhibiting this response is unclear, as the majority of c-Fos expressing neurons in the rat IGL following late-night light exposure contain neither NPY nor enkephalin (Juhl et al., 2007). If BMY7378 is indeed acting by decreasing NPY release at the SCN, as suggested above, then the observation that light-induced c-Fos is rarely in NPYergic IGL neurons indicates a multi-cellular pathway. BMY7378 is unlikely to alter the light-responsive IGL neurons directly, otherwise potentiation would still have been observed in the animals with 5-HT lesions at the IGL. Thus it is likely that BMY7378 alters the raphe activity, which then alters the responsiveness of light-activated neurons in the IGL (Figure 8). As these cells likely do not contain NPY (Juhl et al., 2007), then it would be predicted that diminished activity in these light-responsive neurons would lead to diminished activity in downstream NPYergic IGL neurons, that then project to the SCN. This final suggestion is consistent with the observation that few IGL neurons exhibiting c-Fos following a light pulse actually project to the SCN (Muscat & Morin, 2006), and that the IGL cells most strongly activated by light do not contain NPY (Thankachan & Rusak, 2005).

One caveat to the decrease in c-Fos expression in the IGL following light exposure with BMY7378 pretreatment is that this phenomenon may not generalize to all the serotonergic drugs that potentiate photic shifts. One of the serotonergic drugs reported to enhance photic responses (MKC-242) has previously been reported to have no effect on light-induced c-Fos in the IGL (Moriya et al., 1998). The difference between the present result and that study might be due to a difference between mice and hamsters, the specific pharmacological properties of the different drugs, or the rostrocaudal level of the IGL examined. While c-Fos levels were moderately lower at the mid-IGL level, this effect was not significant. Moriya and colleagues (1998) only examined one IGL section per animal, and it is unclear at what rostrocaudal level this assessment was performed. If it was at the mid-caudal level, this may also explain the differences between our studies. The lack of effect that the mid-IGL level may reflect functional heterogeneity of the IGL and its inputs and outputs, or it may reflect a floor-effect for attenuation of light-induced c-Fos at the mid-IGL level where the cross-sectional area is much smaller and there are simply less cells exhibiting light-induced c-Fos than at the more rostral and caudal sections.

While infusions of BMY7378 to the MRN and DRN did significantly potentiate circadian responses to light, these shifts were significantly smaller than those to light following systemic administration. This could be due to a number of interacting factors. First, the final concentration of BMY7378 at the raphe might be different between the two routes of administration. Additionally, as the cannula injection only affects a local area of brain, it is likely that the effect of BMY7378 was not uniform over the whole MRN or DRN in these animals, while all levels of these nuclei would be affected equally with a systemic injection.

The role of the raphe nuclei and serotonin in regulating the circadian system has been extensively studied. The IGL is innervated by the DRN (Meyer-Bernstein & Morin, 1996), while the SCN receives input from both the MRN and DRN (Yamakawa & Antle, 2010). At the SCN, serotonin regulates responses to light (Mistlberger & Antle, 1998; Rea & Pickard, 2000; Antle *et al.*, 2003), in part through the 5-HT_{1A} receptor (Smith *et al.*, 2008; Smith *et al.*, 2015a). Stimulation of the MRN leads to release of 5-HT at the SCN (Dudley *et al.*, 1999) while stimulation of the DRN leads to 5-HT release at both IGL and SCN (Dudley *et al.*, 1999; Glass *et al.*, 2000; Grossman *et al.*, 2004). The DRN regulates the activity of the IGL (Blasiak & Lewandowski, 2003) and serotonin agonist application to the IGL alters neurotransmitter release at the SCN (Glass *et al.*, 2010). The DRN and MRN innervate each other (Sim & Joseph, 1993; Vertes *et al.*, 1999), 1999; Sim and Joseph, 1993), and these interconnections regulate 5-HT release in the SCN (Glass *et al.*, 2003). Global disruption of serotonin leads to changes in properties of entrainment, and enhanced phase delays (Smale *et al.*, 1990; Morin & Blanchard, 1991; Meyer-Bernstein & Morin, 1996; Meyer-Bernstein *et al.*, 1997) and advances (Penev *et al.*, 1993). More targeted lesions of just 5-HT innervation of the SCN, such as those used here, produce more subtle phenotypes with no change in phase shifts to light (Meyer-Bernstein *et al.*, 1997). Together, these data suggest that serotonin modulates circadian responses to light through a variety of locations, including both the IGL and SCN.

Consistent with previous reports, systemic BMY7378 was able to produce phase advances on its own (Gannon, 2003; Lall & Harrington, 2006). The phase shifts to light following BMY7378 treatment are greater than the arithmetic sum of light alone and BMY7378 alone (present results, and Gannon, 2003) consistent with the interpretations that BMY7378 potentiates phase shifts to light. The phase advances to BMY7378 alone were not observed following intra-SCN, intra-MRN, or intra-DRN injections, suggesting that BMY7378 does not cause phase shifts on its own through actions exclusively at the SCN, MRN, or DRN. Additionally, animals with IGL lesions did not phase shift to systemic BMY7378, suggesting that an intact IGL is necessary for this phenomenon. Given that the IGL does have 5-HT_{1A} receptors, it is possible that BMY7378 causes phase shifts by binding to receptors in the IGL. In intact animals, these phase shifts to BMY7378 are small. It is unlikely that the large phase shifts observed with BMY7378 are simply an additive effect of a photic shift and a BMY7378-induced shift, as the phase shifts to the combined treatments are much greater than the arithmetic sum of the two simple shifts (Gannon, 2003; Lall & Harrington, 2006).

In conclusion, the present study has identified the raphe nuclei and the IGL, and the serotonergic pathway connecting them, as being critical components of the circadian network underlying potentiation of photic phase shifts by 5-HT_{1A} mixed agonists/antagonists. Given that this simple treatment can result in phase shifts more than double the size possible with light alone, these treatments might be useful therapeutic interventions to treat sleep and circadian disorders.

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Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HT	Serotonin
ANOVA	Analysis of variance
CT	Circadian time
DD	constant darkness
DRN	Dorsal raphe nucleus
IGL	Intergeniculate leaflet
MRN	Median raphe nucleus
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
PBS	Phosphate buffered saline
ROD	relative optical density
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SERT	Serotonin transporter

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Table 1 Range of days following start of constant darkness that each treatment was administered for each experiment.

Experiment	Saline + LP	BMY + LP	Saline alone	BMY alone
Systemic Injections				
Intact	15 – 65	29 – 57	42 – 94	16 – 80
SCN – 5,7-DHT lesions	17 – 33	18 – 37	–	–
IGL radio frequency lesions	10 – 45	10 – 45	59 – 99	59 – 110
IGL – 5,7-DHT lesions	18 – 29	18 – 42	–	–
Cannula Injections				
Intra-SCN	21 – 52	37 – 52	66 – 80	66 – 80
Intra-MRN	13 – 55	12 – 64	73 – 114	73 – 100
Intra-DRN	18 – 38	18 – 38	54 – 69	55 – 69

Figure Captions

Figure 1 BMY7378 enhances photic phase shifts when delivered systemically or to the MRN or DRN, but not when delivered to the SCN. Top: Actograms from representative animals treated with systemic (I.P.) injections, intra-SCN injections, intra-MRN injections, and intra-DRN injections. Each horizontal line represents a single day's wheel running data, with subsequent days plotted below the previous day. Vertical deflections from the horizontal lines represent 10-minute bins during which wheel running occurred, with the height of the line being proportional to the number of wheel revolutions. The start of the light pulse (40 lux, 15 minutes) is indicated by the diamond (◇), while the time of delivery of the pretreatment is indicated by the circle (○); white for saline and shaded gray for BMY7378. Doses of BMY7378 were 5mg/kg for systemic (I.P.) injections, 7.8nmol in 0.5µl for intra-SCN injections, and 15.6nmol in 0.5µl for intra-MRN and intra-DRN injections. Bottom: Mean phase shifts for each treatment condition. * denotes a significant difference from vehicle control ($p<0.05$).

Figure 2 Elimination of serotonergic innervation of the SCN does not prevent BMY7378 from enhancing the photic phase shifts. The SCN has dense serotonergic innervation, as demonstrated by serotonin transporter (SERT) immunoreactivity (top left, 3V: 3rd ventricle, OX: optic chiasm). Infusion of 5,7-DHT to the SCN region eliminated serotonergic fibers in the SCN (top right, scale bar =100 μm). Dashed boxes in the top row indicate the area depicted in the high magnification images in the next row (scale bar =50 μm). Actograms depicting representative responses from sham-lesioned (left) or 5-HT-lesioned (right) animals. Actograms are from the same animals for which photomicrographs are presented above. Plotting convention is the same as for figure 1. Bottom: mean phase shifts for each treatment condition. * denotes a significant difference from vehicle control ($p<0.05$).

Figure 3 BMY7378 decreases the number of c-Fos-immunoreactive cells in the IGL following a light pulse. Top: representative photomicrographs of the IGL at rostral (first row) mid (middle row) and caudal (last row) from a saline + light pulse-treated animal (2 leftmost columns) and a BMY7378 + light pulse-treated animal (2 rightmost columns). Photo micrographs show the NPY-immunoreactive sections (1st and 3rd columns) used to delineate a region of interest on the adjacent c-Fos-immunoreactive sections (2nd and 4th columns). Scale bar is 100 μm. Bottom: mean number of c-Fos-immunoreactive cells for each treatment condition at the various rostrocaudal levels examined. * denotes a significant difference from vehicle control ($p<0.05$).

Figure 4 Confirmation of completeness of IGL radiofrequency lesions. The completeness of IGL lesions was confirmed by assessing NPY immunoreactivity in the SCN. Sham-lesioned animals (left) exhibit dense NPY immunoreactivity in the ventrolateral SCN. Animals with IGL lesions deemed to be incomplete (middle) still exhibited some NPY immunoreactivity in the ventral SCN. Animals with complete IGL lesions (right) had SCN devoid of NPY immunoreactivity. The NPY immunoreactivity in the PVN arises from the arcuate nucleus, and thus NPY immunoreactivity in the PVN was unaffected by the IGL lesion. 3V: 3rd ventricle, OX: optic chiasm, PVN: paraventricular nucleus, SCN: suprachiasmatic nucleus. Scale bar = 500 μm.

Figure 5 Bilateral radiofrequency lesions of the IGL eliminates potentiation of photic phase shifts by BMY7378. Top: Actograms depicting representative responses from sham-lesioned (top row), partial IGL-lesioned (middle row) and complete IGL-lesioned (bottom row) animals. Plotting convention is the same as for figure 1. Phase shift to light with BMY7378 pretreatment (right column) were compared to phase shifts to light with saline pretreatment (left column), and were only significantly greater in the sham lesion animals (top row). Bottom: mean phase shifts for each treatment condition. * denotes a significant difference from vehicle control ($p<0.05$).

Figure 6 Confirmation of completeness of neurotoxic lesions of 5-HT innervation of the IGL. The IGL was identified using immunoreactivity for NPY (left column). A region of interest was defined based on the NPY-immunoreactivity, and blood vessels (*) were used to align this region of interest on adjacent section stained for serotonin transporter (SERT) immunoreactivity (middle column). This region was examined at high magnification (right column). The approximate locations of the representative high magnification photomicrographs are indicated on the low magnification images by the location of the dashed rectangle. Sham-lesioned animals exhibited prominent fiber immunoreactivity throughout the region (top right). This immunoreactivity was absent in animals receiving infusions of the 5-HT neurotoxin 5,7-DHT to the IGL. DLG: dorsolateral geniculate, IGL: intergeniculate leaflet, VLG: ventrolateral geniculate. Scale bar for the low magnification images (left and middle columns) is 100 μm. Scale bar for the high magnification images (right column) is 50 μm.

Figure 7 Bilateral neurotoxic lesions of 5-HT innervation of the IGL eliminates potentiation of photic phase shifts by BMY7378. Top: Actograms depicting representative responses from a sham-lesioned animal (left), and a complete IGL 5-HT-lesioned animal (right). Plotting convention is the same as for figure 1. Bottom: mean phase shifts for each treatment condition. * denotes a significant difference from vehicle control ($p<0.05$).

Figure 8 Model of how BMY7378 may be acting to enhance circadian responses to light. A) Under normal circumstances, light is detected by the retina and this photic signal is relayed to the SCN and IGL by glutamatergic projections. This results in activation of cells in the SCN and IGL. In the IGL, these retinorecipient, light-activated neurons do not project directly to the SCN (Muscat & Morin, 2006) and do not contain NPY or enkaphalin (Thankachan & Rusak, 2005; Juhl *et al.*, 2007). When BMY7378 is administered prior to a light pulse (B), we hypothesize that it leads to a number of parallel changes. 1) Raphe activity is suppressed through activation of 5-HT_{1A} autoreceptors. 2) Loss of serotonin output at the SCN diminishes retinal inhibition. 3) Activation of retinorecipient, light-activated neurons in the IGL is decreased. 4) Activity of SCN-afferent NPYergic cells in the IGL is prevented. 5) Activity of SCN-afferent IGL cells of an undetermined phenotype is increased. Size of arrows in B relative to A indicates the relative level of activity between the two panels, **with red indicating lower activity than in A and green arrows indicating greater activity than in A**. Direction of arrows indicates if cells are activated (↑) or inhibited (↓). **(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**