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SEROTONERGIC POTENTIATION OF PHOTIC PHASE SHIFTS: EXAMINATION OF RECEPTOR CONTRIBUTIONS AND EARLY BIOCHEMICAL/MOLECULAR EVENTS

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Abstract—The 5-HT mixed agonist/antagonist 1-(2-methoxyphenyl)4-[4-(phthalimido)butyl]-piperazine hydrobromide (NAN-190) has been shown to greatly potentiate photic phase shifts in hamsters. The mechanism of this potentiation has yet to be determined. NAN-190 is believed to act primarily through the 5-HT_{1A} receptor, but also binds to several other receptors, making it uncertain as to which receptor underlies its potentiation of photic phase shifts. Also uncertain are the intracellular changes in the suprachiasmatic nucleus (SCN) which are associated with such enhanced phase shifting. Here we examine the role of the 5-HT_{1A} receptor as well as the physiological underpinnings, in terms of both gene expression and biochemical activation, in the behavioral responses to photic stimuli following pretreatment with NAN-190. Administration of NAN-190 to wildtype mice significantly potentiated late subjective night photic phase shifts, while mice lacking the 5-HT_{1A} receptor (knockouts) exhibited an attenuated behavioral response to light when pretreated with NAN-190. In wildtype mice, the protein product of the immediate-early gene c-fos, induced following photic stimulation, was found to be significantly decreased with NAN-190 pretreatment. Similarly, the levels of phosphorylated CREB protein, involved in a biochemical pathway leading to gene transcription, were also attenuated by NAN-190 in the wildtype mice. However, activation of the extracellular signal-regulated kinase I/II (ERK) pathway in wildtype mice, following the light pulse, was not affected by NAN-190 pretreatment, nor was the expression of the circadian clock components *Period1* and *Period2*. These findings suggest that the 5-HT_{1A} receptor plays a critical role in the potentiation effect observed with NAN-190, and that NAN-190 may potentiate photic phase shifts at least partly by down-regulating the activity of some (but not all) genes and biochemical pathways involved in coupling the light signal to the output of the circadian clock.

Key words: suprachiasmatic nucleus, 5-HT, fos, *period* genes, P-ERK, P-CREB.

Circadian rhythms are regulated by a neural clock located in the suprachiasmatic nucleus (SCN; Moore and Eichler, 1972; Stephan and Zucker, 1972). This clock is normally synchronized to our day/night cycle through exposure to light. Desynchrony between our internal clock and our activity cycle caused by travel or shift work can lead to adverse symptoms that result in higher incidence of disease and accidents (Knutsson, 2003; van Mark et al., 2006). Finding ways to rapidly realign our circadian clock with our life schedule could ameliorate some of these symptoms. Some studies have suggested that certain serotonergic drugs may be able to enhance normal clock resetting to light (Rea et al., 1995; Gannon, 2003).

The SCN receives dense serotonergic innervation from the median raphe nucleus, which plays a role in modulating the circadian system (Meyer-Bernstein and Morin, 1996). Serotonin (5-HT) has been shown to modulate the effects of light by dampening the excitatory signals sent to the cells of the SCN as a result of the light exposure (Meyer-Bernstein and Morin, 1996; Mistlberger, 2000). A specific class of serotonergic compounds, termed mixed agonists/antagonists due to their actions as agonists at somatodendritic autoreceptors in the raphe nuclei as well as antagonists at postsynaptic receptors on non-serotonergic cells, has been shown to enhance the effects of light on the circadian system (Gannon, 2003). Included in this class of 5-HT mixed agonist/antagonist is 1-(2-methoxyphenyl)4-[4-(phthalimido)butyl]-piperazine hydrobromide (NAN-190). When administered during late subjective night, NAN-190 (Rea et al., 1995) or related drugs (Gannon, 2003) are known to significantly potentiate photic phase advances in the hamster, inducing shifts in the activity rhythm of up to 8 h in magnitude. NAN-190 can also significantly potentiate phase delays to light in the early night, although the magnitude of this effect is much more modest (Rea et al., 1995). These drugs are believed to counteract 5-HT's inhibitory activity at the SCN through two complementary and parallel mechanisms: by activating somatodendritic autoreceptors in the median raphe thereby decreasing 5-HT output, while simultaneously blocking 5-HT_{1A} receptors in the SCN (Gannon, 2003; Sterniczuk et al., 2008). This decreased inhibition to the SCN is suspected to lead to increased exci-

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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine monophospate; CRE, cAMP response element; CREB, cAMP response element binding protein; CT, circadian time; DAB, diaminobenzidine; DD, constant darkness; DIG, digoxigenin; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GRP, gastrin-releasing peptide; ICC, immunocytochemistry; IEGs, immediate-early genes; −IR, −immunoreactive; KO, knockout; *mPer1*, mouse *Period1* gene; *mPer2*, mouse *Period2* gene; NAN-190, 1-(2-methoxyphenyl)4-[4-(phthalimido)butyl]-piperazine hydrobromide; NMDA, *N*-methyl-D-aspartic acid; PBS, phosphate-buffered saline; PBSx, phosphate-buffered saline with 0.1% Triton X-100; ROD, relative optical density; SCN, suprachiasmatic nucleus; VP, vasopressin; WT, wildtype.

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tation following photic input, thus allowing an overall potentiated phase shifting effect.

Although NAN-190 is able to greatly potentiate photic phase shifts (Rea et al., 1995; Kessler et al., 2008), the receptor activation leading to this effect is still not well understood. Originally, NAN-190 was thought to be strictly a 5-HT_{1A} antagonist, but it has now been shown to have high affinity for the α_1-adrenergic receptor, modest affinity for 5-HT_{1B}, 5-HT_7 and D_2 receptors, as well as measurable, but low, affinity for 5-HT_2, D_1 and β-adrenergic receptors (Glennon et al., 1988; Lovenberg et al., 1993). Other pharmacological studies also support the role of the 5-HT_{1A} receptor in serotonergic potentiation of photic phase shifts (Moriya et al., 1998), however some of the drugs used in that study have recently been shown to be less selective than previously thought (Chemel et al., 2006; Marona-Lewicka and Nichols, 2009). Given the lack of receptor specificity often observed with a pharmacological approach, complimentary techniques are required to confirm that the 5-HT_{1A} receptor underlies serotonergic potentiation of photic phase shifts. One method by which to determine the location of action of non-selective drugs, such as NAN-190, is through the use of mice genetically modified to lack a specific receptor subtype (Sprouse et al., 2005). Transgenic mice missing the 5-HT_{1A} receptor may be useful in determining the role of the 5-HT_{1A} receptor subtype in phenomena such as the photic phase shift potentiation observed with NAN-190.

In addition to the uncertainty of the precise receptor that mediates the enhancement of photic phase shifting, it is equally uncertain as to what the cellular and molecular underpinnings are of the photic phase shift potentiation observed with serotonergic mixed agonists/antagonists such as NAN-190. It was originally proposed that mixed agonists/antagonists induce their potentiation of photic phase shifts by blocking the inhibitory effects of serotonin, mainly at the retinal terminals (Gannon, 2003). More recently though, it has been shown that NAN-190 directly affects both the retinorecipient cells responsive to the glutamate agonist *N*-methyl-D-aspartic acid (NMDA), as well as those cells in the dorsolateral SCN that are responsive to gastrin-releasing peptide (GRP; Sterniczuk et al., 2008). Yet, it is still unclear exactly what NAN-190 is doing at the molecular level within those cell sub-populations in order to enhance photic phase shifts to such a large degree.

Behavioral phase shifts to light exposure in the subjective night are accompanied by the induced expression of numerous genes in the cells located in the ventrolateral core of the SCN, including immediate-early genes (IEGs) such as *c-fos*, along with the clock genes *Period 1* (*Per1*) and *Period 2* (*Per2*; Guido et al., 1999a,b; Hamada et al., 2004). Second-messenger signaling pathways, including the pathway leading to the phosphorylation of the extracellular signal-regulated kinase I/II (ERK), serve to couple this incoming photic input to the transcription of IEGs and *Period* genes (Butcher et al., 2003). Similarly, the phosphorylated form of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), which is also induced following light exposure, subsequently binds to the cAMP response element (CRE) of the promoter region of several genes, including *c-fos*, *Per1*, and *Per2*, inducing transcription (Antle and Silver, 2005; Antle et al., 2009). Thus, the phase shifting effects of light consistently activate the second-messenger pathways of both ERK and CREB (von Gall et al., 1998; Dziema et al., 2003).

The serotonergic system has also been shown to modulate the aforementioned effects of photic input on the light-induced gene expression in the cells of the SCN. Both agonists and antagonists to the serotonin system that are known to alter circadian activity rhythms have also been shown to modulate the light-induced gene expression in the SCN (Glass et al., 1994; Amir et al., 1998; Takahashi et al., 2002). Serotonergic agonists that attenuate light-induced phase shifts can also attenuate the subsequent Fos protein expression observed in the SCN (Amir et al., 1998). Conversely, the selective 5-HT_{1A} receptor agonist MKC-242, which has the ability to potentiate photic phase advances and delays, has been found to concomitantly prolong the light-induced expression of *mPer1* and *mPer2* in the mouse SCN during the late subjective night (Moriya et al., 1998; Takahashi et al., 2002). Paradoxically, while NAN-190 potentiates photic phase shifts, it partially blocks Fos expression in the SCN as a whole following a light pulse (Recio et al., 1996).

The present study first examined if NAN-190 could also potentiate phase shifts in mice. Next, this study attempted to determine the contribution of the 5-HT_{1A} receptor to the phase shift potentiation observed with NAN-190, through the use of mice genetically engineered to lack the 5-HT_{1A} receptor. We also sought to explore the molecular underpinnings of the photic phase shift potentiation observed with NAN-190 in mice, by examining the regionally-specific expression of several proteins and genes involved in the transduction of the incoming light signal into phase shifts of the behavioral output of the circadian clock.

EXPERIMENTAL PROCEDURES

Animals and housing

A total of 117 male mice were used, consisting of 21 5-HT_{1A} receptor knockout (KO) mice and 23 5-HT_{1A} receptor wildtype (WT) mice, as well as 73 C57BL/6J mice obtained from the University of Calgary Life and Environmental Science Animal Resource Centre. These experiments were the only studies in which these mice were used, with the exception of three KO and three WT mice used to quantify the effect and duration of NAN-190 on behavioral state (these mice had previously been used for behavioral studies, and were pharmacologically naive). The knockout and wildtype mice were generated from a breeding colony established in our laboratory from mice originally developed and generously donated by Dr. Thomas Shenk (Princeton University, Princeton, NJ, USA), provided by Dr. Miklos Toth (Cornell University Medical College, New York, NY, USA) and bred on the C57BL/6J background. For information regarding the generation of the 5-HT_{1A} receptor KO mice see Parks et al. (1998). Animals from our breeding colony were genotyped using a previously described protocol (see Smith et al., 2008). Briefly, a pair of KO primers (amplifying a 400 base-pair product that included a portion of the neomycin cassette that replaced the start codon of the 5-HT_{1A} receptor gene) as well as a pair of WT primers (amplifying a 238 base-pair product that included a portion