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By: Katherine V. Fite, Peter S. Wu, and **Andrew Bellemer**

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Theme: Sensory systems
Topic: Subcortical visual pathways

Keywords: Immediate early gene expression; Rodent visual system; Lateral geniculate nucleus; Intergeniculate leaflet; Ventral lateral geniculate

1. Introduction

The mammalian dorsal raphe nucleus (DRN) contains the majority of serotonergic neurons that innervate widespread areas of the forebrain via several ascending pathways [20,21,51]. Functionally, the DRN is involved in the modulation of a broad range of complex physiological, behavioral, and cognitive systems and has been implicated in a variety of neuropsychiatric disorders [6,20]. Neuronal activity in the DRN is regulated both intrinsically and extrinsically by afferents originating from the medial prefrontal cortex [6,49], lateral habenula, and several hypothalamic nuclei [29,38]. In addition, GABAergic innervation of the DRN arises from multiple external sources as well as from intrinsic interneurons [15].

Optic afferents to the DRN have been described in a number of mammalian species [11–14,26,41,43] and have been most extensively investigated in rodents. In Mongolian gerbils, rats, and Chilean degus, retinal afferents terminate most extensively in the rostral and lateral subdivisions of the DRN [11,12]. In turn, neurons in these subdivisions of the DRN send efferents to the superior colliculus and lateral geniculate complex [23,24,52]. A small subset of retinal ganglion cells that innervate the DRN also innervates the lateral geniculate complex in gerbils [13]. At present, little information is available about how variations in environmental light and photic stimulation may influence neuronal activity in the DRN. Previous neurophysiological studies

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have reported changes in DRN neuronal activity following visual stimulation, but none have provided stimulus-response functions [35,40,44,46]. Trulson and Trulson [46] reported increased responsiveness of serotonergic neurons to visual and auditory stimuli, while Shima et al. [44] described both inhibitory and excitatory responses in the DRN to light flashes.

Quantitative analysis of the immediate-early gene product c-Fos has been used extensively in functional neuroanatomical investigations to identify the location of neuronal populations activated by sensory stimulation, as well as to assess the relative strength or effectiveness of stimulation. Recently, Correa-Lacarcel et al. [8] have reported that low-frequency pulsed photostimulation is particularly effective in activating c-Fos expression in several visual structures, including the lateral geniculate, superior colliculus, and visual cortex. Janusonis and Fite [22] have shown that c-Fos expression varies over the course of the diurnal cycle in the gerbil DRN and is generally lowest in the middle of the light phase and highest in the early portion of the dark phase. To determine whether low-frequency photostimulation might be an effective stimulus for altering c-Fos expression in the DRN, the present study has investigated the effects of pulsed photostimulation at selected times over the course of a 12:12 h light/dark cycle. For comparison, c-Fos expression also was analyzed in several diencephalic retinorecipient nuclei, including the subnuclei of the lateral geniculate complex and in the suprachiasmatic nucleus.

2. Material and methods

2.1. Animals and procedures

Adult male Mongolian gerbils (*Meriones unguiculatus*) were maintained on a 12:12 h light/dark cycle (7 a.m., lights on; 7 p.m., lights off) and were housed two to three animals/cage. All procedures were approved by the University Institutional Animal Care and Use Committee and were in accordance with NIH and USDA guidelines. Ambient room illuminance at cage level was 70–80 lx during the light phase of the diurnal cycle. Two groups of animals, control and photostimulated, were analyzed at four different time points: 8 a.m., 1 p.m., 8 p.m., 1 a.m. (DRN—six animals/group, diencephalic nuclei—four animals/group). Unrestrained experimental animals were exposed to a low-frequency (2 Hz) stroboscopic white light (Xenon source) located immediately adjacent to the animal's cage for 60 min. Illuminance of the pulsed light was ~600 lx, with a square-wave 5-ms pulse duration. Immediately following stimulation, animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg) and were perfused transcardially with saline followed by 400 ml of 4% paraformaldehyde in phosphate buffer (PB; pH 7.2). Control animals were anesthetized and perfused at the same times as experimental animals. Brains were removed and immersed overnight in 30% sucrose in PB at 4 °C. Serial coronal sections were cut on a freezing microtome at 40 μm thickness, saved throughout the mesencephalon and diencephalon, stored in cryoprotectant at −10 °C, and were subsequently processed for c-Fos immunoreactivity.

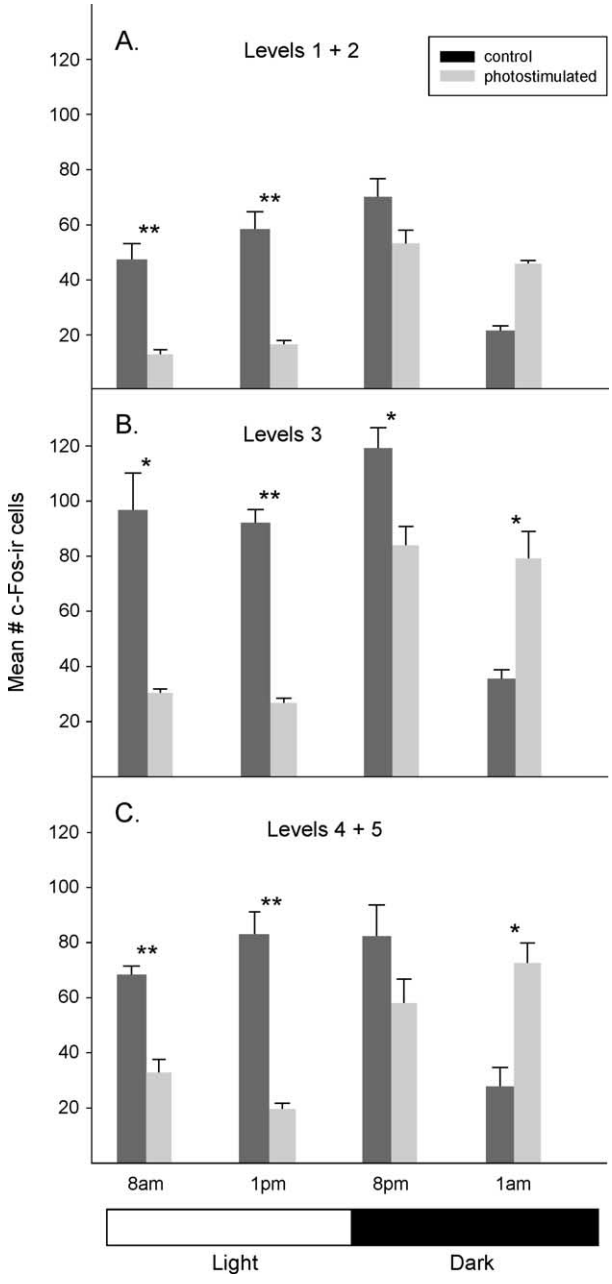

Fig. 1. Mean number of c-Fos-immunoreactive (c-Fos-ir) neurons at different rostro-caudal levels of the dorsal raphe nucleus (DRN) in photostimulated (light bars) and control (dark bars) conditions for four time points over the 12:12 h light/dark cycle. (A) Combined cell counts for the anterior DRN levels 1 and 2. (B) Cell counts for the DRN at level 3. (C) Combined cell counts for the caudal DRN at levels 4 and 5. Statistically significant differences in means for photostimulated vs. control conditions are shown (*$p < 0.005$, **$p < 0.001$).

2.2. Immunocytochemistry

Sections selected from each target structure were rinsed three times (5 min each) in 0.1 M phosphate-buffered saline (PBS; pH 7.4), incubated in 0.3% H_20_2 in PBS (30 min) rinsed two times (5 min each) in PBS, and followed by incubation in 3% normal goat serum (NGS; Vector Laboratories) and 0.25% Triton-X 100 (TX) in PBS for 2 h. Sections were then incubated in rabbit anti-c-Fos IgG solution (Oncogene Research Products, diluted 1:10,000) containing 3% NGS and 0.25% TX in PBS for 3 days at 4 °C. Sections were then rinsed six times (10 min each) in PBS, incubated in goat antirabbit IgG (Vector Laboratories, 5 μg/ml) with 3% NGS and 0.25% TX in PBS for 2 h, rinsed three times (10 min each) in PBS, incubated in a 1:100 avidin–biotin–peroxidase (ABC Elite, Vector Laboratories) solution containing 0.5% TX in PBS, and then rinsed two times (10 min each) in PBS. Sections were preincubated in a 0.05% 3, 3′-diaminobenzidine (DAB) solution in PBS for 1 min then reacted by adding 0.01% H_20_2 to the solution. Following incubation for 8 min, sections were rinsed three times (5 min each) in PBS, mounted on gelatin-subbed slides and allowed to air dry at room temperature, then cleared with Hemo-De and coverslipped with Permount.

2.3. Tissue analysis

For the DRN, c-Fos-immunoreactive (c-Fos-ir) neurons were counted in two alternate sections (six animals/condition for each time point) selected from each of five successive rostro-caudal levels, as previously defined by Janusonis et al. [22,23], based upon the distribution pattern of serotonin-immunostained neurons in this species. Photographic images were obtained of the entire right lateral and medial subdivisions of the DRN at levels 1, 2, and 3. The entire areal profile of the DRN was analyzed at levels 4 and 5. Graphic templates were created to consistently delineate the regions of analysis at each rostro-caudal level, and these were superimposed on gray-scale images of each section to be analyzed. Digital gray-scale images were obtained using a Kodak DC290 Zoom digital camera mounted on a Leica DMR light microscope and were displayed using Adobe Photoshop 6.0. The overall background brightness level (overall digital, gray-scale value) was analyzed for each selected area of the DRN analyzed, and 50% of this value was defined as a criterion value. c-Fos-positive neurons darker than this value were counted (see also Ref. [22]). A criterion background brightness threshold level of 50% was selected, and c-Fos-ir nuclei darker than this value were counted (see also Refs. [5,22,34]). A mean number of c-

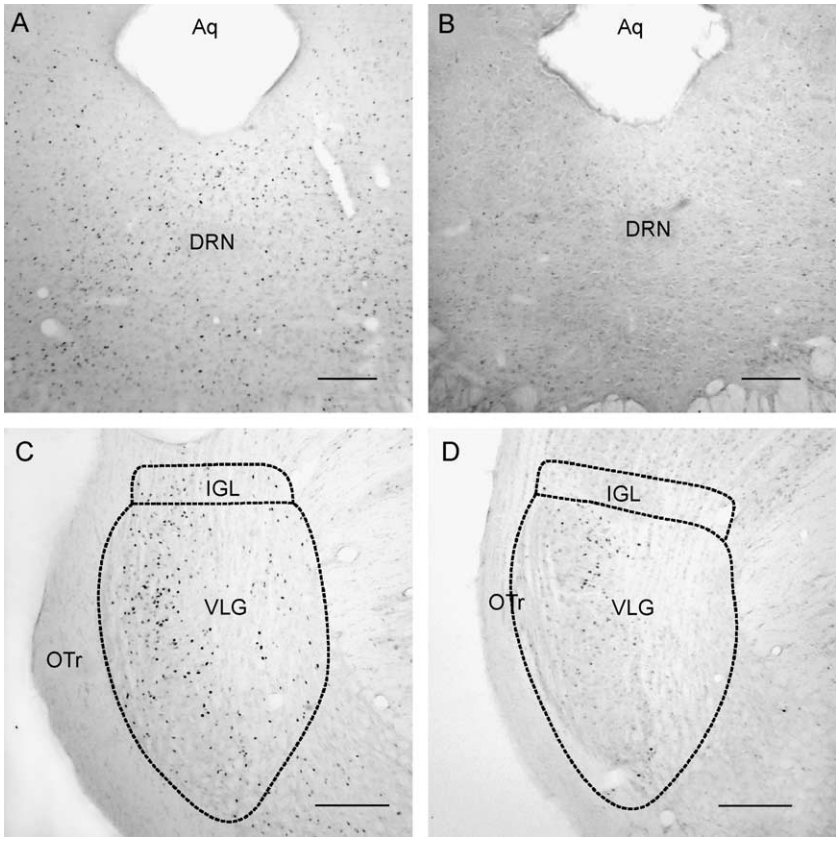

Fig. 2. Pattern of c-Fos-immunoreactive (c-Fos-ir) neurons at 1 p.m. in controls (A, C) and following low-frequency photostimulation (B, D) in the dorsal raphe nucleus (DRN) (level 3) and in the intergeniculate leaflet (IGL) and ventral geniculate nucleus (VLG). Both regions show substantial reduction in c-Fos-ir cell numbers following photostimulation. Aq—aqueduct, OTr—optic tract; scale bar = 200 μm.

Fos-ir nuclei was calculated for each DRN level, for each condition (photostimulated vs. control), and for each selected time point. To obtain a c-Fos expression profile representative of the entire DRN, these values were combined across all five levels for each condition and time point. Statistical analyses included a two-way analysis of variance (ANOVA), with pair-wise post hoc comparisons between experimental and control groups at each time point.

For diencephalic retinorecipient nuclei, digital images were obtained and analyzed using methods similar to those described above. Nuclear boundaries for the right dorsal lateral geniculate (DLG), ventral lateral geniculate (VLG), and intergeniculate leaflet (IGL) were initially determined for each section in dark-field illumination (which revealed the areal extent of each subnucleus) and also with reference to an existing set of coronal sections containing anterogradely labeled optic axons in this species [12,31]. For each selected time point, digital gray-scale images were obtained from two adjacent alternate sections (four animals/condition/time) containing well-defined profiles of the VLG and IGL. c-Fos positive nuclei were counted in the entire areal profile of these two nuclei. Due to the greater areal extent of the DLG, a 300-μ^2 template counting region was used (approximately half the DLG profile), located equidistant from the medial and lateral edges of the DLG. For the suprachiasmatic nucleus (SCN), boundaries of the nucleus were determined with reference to the location and extent of anterogradely labeled optic axons in an existing series of coronal sections [12,31]. Two alternate sections containing the largest and most distinct profile of the SCN were scanned and analyzed for c-Fos-ir neurons.

3. Results

3.1. Dorsal raphe nucleus

Although the actual number of c-Fos-positive neurons varied at different rostro-caudal levels of the DRN, the profile of c-Fos expression over the diurnal cycle was quite similar at each level (Fig. 1). Photostimulation was consistently associated with a lower number of c-Fos-ir neurons at each level for three of four selected time points when compared with control animals, and a substantially lower number of c-Fos-ir neurons occurred in animals photostimulated during the normal light phase (8 a.m., 1 p.m.) compared with those stimulated during the dark phase of the light/dark cycle (8 p.m., 1 a.m.) (Figs. 1 and 2A). c-Fos-ir cell counts combined across all five rostro-caudal levels (Fig. 3A) showed that, overall, 65–70% fewer c-Fos-ir neurons occurred in photostimulated vs. control animals in the DRN. In animals photostimulated during the normal dark period (8 p.m., 1 a.m.), the mean number of c-Fos-positive neurons was greater than in the light period (8 a.m., 1 p.m.) and early in the dark period (8 p.m.), the number of

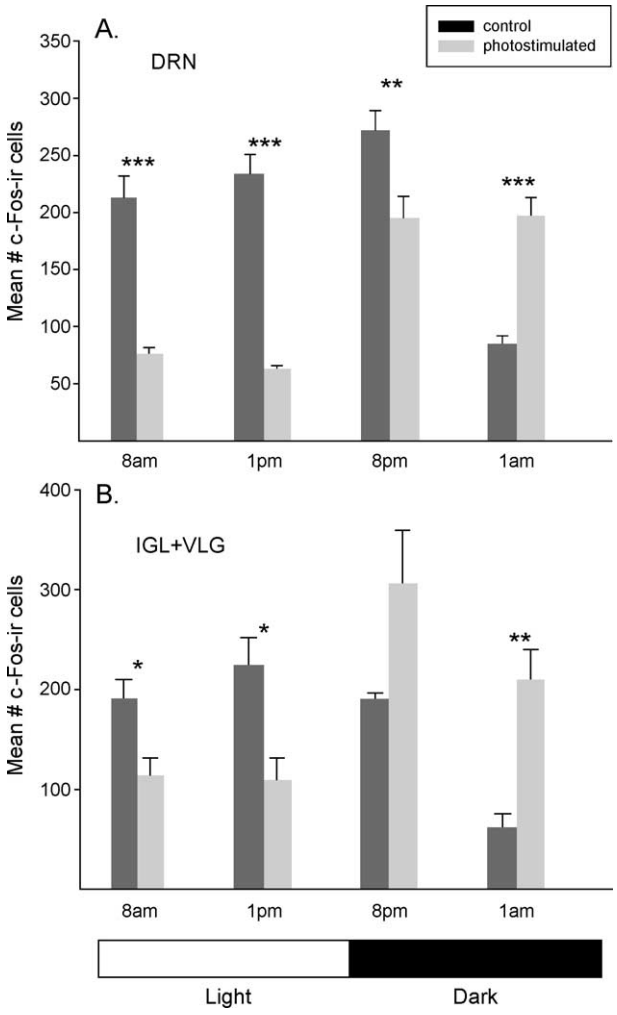

Fig. 3. (A) Mean number of c-Fos immunoreactive (c-Fos-ir) neurons in photostimulated (light bars) vs. control (dark bars) conditions at each of four selected times over the light/dark cycle. (A) c-Fos-ir neuron counts combined over all DRN rostro-caudal levels. (B) c-Fos-ir neuron counts combined for the intergeniculate leaflet (IGL) and ventral lateral geniculate (VLG). Statistically significant differences in means for photostimulated vs. control conditions at each time point are shown (*$p < 0.025$, **$p < 0.01$, ***$p < 0.001$).

c-Fos-ir neurons in photostimulated animals remained lower than in control animals. By the middle of the dark period (1 a.m.), the number of c-Fos-ir neurons in controls had decreased significantly, while, in photostimulated animals, the number remained similar to the value seen early in the dark period. A two-way ANOVA showed significant main effects of condition and time ($p < 0.001$), with a significant condition–time interaction ($p < 0.001$). Pair-wise post hoc comparisons between means for control vs. photostimulated animals revealed significant differences ranging from $p < 0.005$ to $p < 0.001$.

3.2. Lateral geniculate subnuclei

The profiles of neuronal c-Fos expression observed in both the intergeniculate leaflet (IGL) and ventral geniculate

nucleus (VLG) for photostimulated vs. control animals were quite similar; therefore, results from these adjacent subnuclei were combined (Fig. 3B). The overall profile of c-Fos expression in the IGL/VLG for animals photostimulated during the light period resembled that observed in the DRN (i.e., lower numbers of c-Fos-ir neurons compared with controls). As in the DRN, the greatest number of IGL/VLG c-Fos-ir neurons observed in photostimulated animals occurred during the middle of the dark period (1 a.m.). In contrast, the dorsal lateral geniculate subnucleus (DLG) showed no significant differences in the number of c-Fos-ir neurons in controls vs. photostimulated animals for three of the four time points examined (8 a.m., 1 p.m., 8 p.m.) (Fig. 3A). The lowest number of c-Fos-ir neurons occurred in controls at 1 a.m., while the highest number occurred in photostimulated animals at 1 a.m., as in the other nuclei examined.

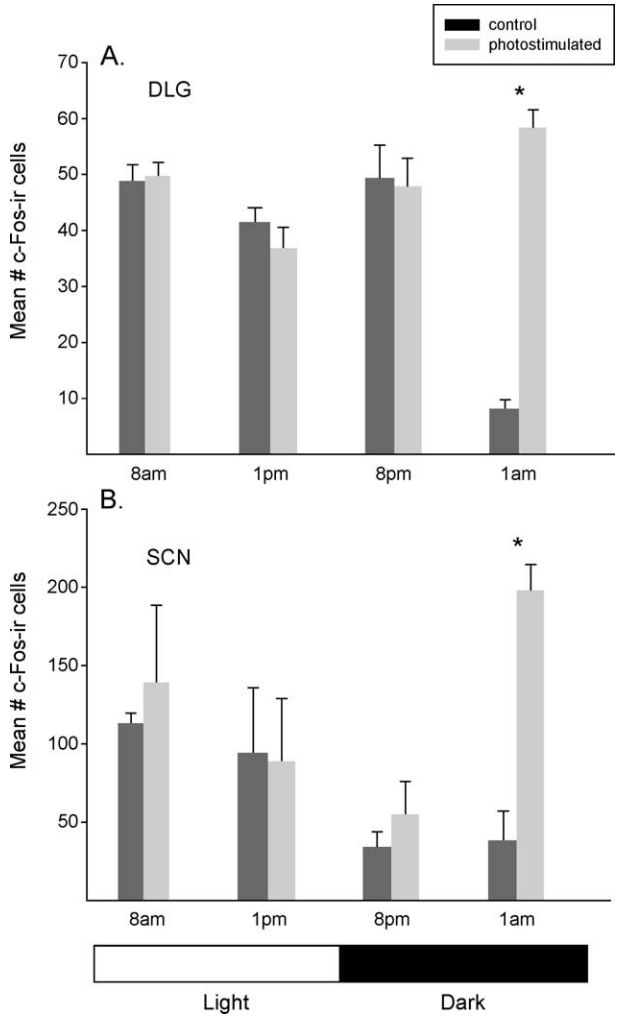

Fig. 4. (A) Mean number of c-Fos immunoreactive (c-Fos-ir) neurons in photostimulated (light bars) vs. control (dark bars) conditions at each of four selected times over the light/dark cycle. (A) c-Fos-ir neuron counts for the dorsal lateral geniculate (DLG). (B) c-Fos-ir counts for the suprachiasmatic nucleus (SCN). Statistically significant differences in means for photostimulated vs. control conditions are shown (* $p < 0.001$).

For the IGL/VGL, a two-way ANOVA showed significant main effects of time ($p < 0.002$), with a significant interaction between time and condition ($p < 0.001$) Pair-wise post hoc comparisons showed significant differences ($p < 0.025$–0.01) between controls and photostimulated animals at three of four time points analyzed. For the DLG, two-way ANOVA indicated a main effect of time ($p < 0.001$) and condition ($p < 0.001$), with an interaction between time and condition ($p < 0.044$). Pair-wise post hoc comparisons showed a significant difference between conditions only at 1 a.m. ($p < 0.001$).

3.3. Suprachiasmatic nucleus

In the SCN, a downward trend in the number of c-Fos-ir neurons occurred in both photostimulated and control animals between 8 a.m. and 8 p.m. At 1 a.m., the number of c-Fos-ir neurons in controls vs. photostimulated animals were significantly different, as also occurred in the DLG and IGL/VGL (Fig. 4B). A two-way ANOVA showed a significant main effect of time ($p < 0.046$), with a significant interaction between time and condition ($p < .044$). Pair-wise post hoc comparisons revealed a significant difference between photostimulated and control conditions only at 1 a.m. ($p < 0.001$).

4. Discussion

The present findings demonstrate that exposure to a low-frequency visual stimulus can substantially alter c-Fos expression in the DRN, and this effect appears to involve a reduction or suppression of c-Fos expression when photostimulation occurs during the normal light and early dark phase of the diurnal cycle. While the number of DRN c-Fos-ir neurons occurring in photostimulated animals showed some increase early in the dark period, it remained significantly lower than in control animals. In contrast, c-Fos expression was significantly higher in photostimulated vs. controls in the middark period, indicating that time of day and/or the state of adaptation of the retina can influence the effects of photostimulation on c-Fos expression.

Since we have previously demonstrated that direct first-order retinal input to the DRN exists in gerbils and several other rodent species and also that some retinal ganglion cells project both to the lateral geniculate and DRN in gerbils [11,13,12], the use of low-frequency pulsed stimulation allowed a direct comparison of c-Fos expression in the DRN with that in the lateral geniculate and suprachiasmatic nucleus using a visual stimulus shown previously to be highly effective in stimulating c-Fos expression in central visual structures [8]. In general, pulsed or “on–off” stimulation constitutes a highly salient visual stimulus (an effect that has been documented extensively at every level in the visual pathway [42]) and also may lead to increased arousal and alertness. Since the

spontaneous firing rate of DRN serotonergic neurons consistently occurs within the range of 1–3 Hz, we hypothesized that a pulsed visual stimulus in this low-frequency range would be effective in influencing c-Fos expression in the DRN. That assumption proved correct, although the observed reduction/suppression effect on c-Fos expression was not anticipated. As we have previously shown in gerbils, although the greatest density of optic afferents occur in the dorsomedial and lateral subdivisions of the DRN, retinal terminals do occur throughout the DRN as a diffuse matrix of extremely fine arborizations [12]. No significant differences were observed in the diurnal profile of c-Fos expression in control vs. photostimulated animals in relation to the five rostro-caudal levels analyzed nor in relation to internal subdivisions of the DRN, indicating that the effects of light stimulation are consistent throughout the entire DRN in this species.

Since no evidence currently exists for second-order optic input to the DRN, the observed effects of photostimulation are most likely mediated via the direct retinal pathway to the DRN in this species [13,12]. Conceivably, activation of retinal afferents could lead to inhibitory effects on c-Fos expression that are mediated via GABAergic neurocircuitry in the DRN involving both intrinsic and extrinsic components [15]. Preliminary findings with confocal imaging also indicate that, following intraocular tracer injections, anterogradely labeled optic axonal puncta in the DRN occur in close association with serotonergic neuronal somas and initial dendritic segments. Puncta also have been observed in contact with small nonserotonergic perikarya, whose size indicates that they are interneurons (Fite, unpublished observations). The neurochemical identity of neurons that shows changes in c-Fos expression following light stimulation in the DRN is as yet unknown. We have previously provided evidence that, while some colocalization of c-Fos and serotonergic neurons occurs in the gerbil DRN [22]; the percentage of colocalization was actually quite low (11%) even at the time of day that c-Fos expression reached at its highest level. This earlier finding has been recently confirmed; furthermore, pulsed light stimulation does not increase the very low percentage of serotonin/c-Fos colocalized neurons in the DRN (A. Seeling and K. Fite, unpublished data).

Relatively few prior studies have reported decreases in c-Fos expression following modality-specific sensory stimulation. However, Beaule et al. [1] found that 30 min of moderate light exposure suppressed c-Fos expression in the SCN at light/dark transition times, specifically, in the dorsomedial "shell" region, which shows rhythmic Fos expression and receives a GABAergic projection from the retinorecipient ventrolateral core region. In the auditory system, Keilmann and Herdegen [27] showed that low-frequency stimulation (8 Hz) reduced the number of c-Fos-ir neurons in the acoustic pathway of rats, and Van Luijtelaar et al. [48], using a sensory-gating stimulus paradigm in rats, observed less activation in the auditory pathways, septum, and hippocampus following a brief double-click stimulus compared with a longer interclick interval.

Although the expression of c-Fos has been used extensively as an index of stimulus-induced neuronal activation, there is evidence that it may not be closely linked to changes in neuronal firing rates since c-Fos is induced by changes in signal-transduction pathways and second-messenger systems and not by depolarization per se [19]. Furthermore, brain regions with high levels of neuronal activity often do not show significant levels of c-Fos expression [28]. Previous studies showing reduction in c-Fos expression following sensory stimulation [27,48] have used repetitive stimulation which may induce changes in transcriptional regulators and, ultimately, limit or block the expression of c-Fos through an intracellular decrease in adenylyl cyclase activity, a reduction in cAMP, and decreased induction of the c-Fos gene, thereby down-regulating c-Fos expression in a negative-feedback manner,as Grahn et al. have proposed [18]. Certain kinds of stimulation may not activate second-messenger systems sufficiently, and some neurons may not use Fos when stimulated transynaptically [19].

The profile of c-Fos expression to photostimulation observed in the DRN during the light phase also was similar to that seen in the IGL/VLG, which is consistent with a variety of evidence showing a functional relationship between these regions. Efferent projections from the DRN to the IGL are well documented [30,33], and a variety of evidence indicates that the DRN can exert strong modulatory influences on the IGL. These include serotonergic inhibitory effects on spontaneous activity and photic responses [53], alterations in rhythmic and neuronal oscillations induced by electrolytic lesions of the DRN, or electrical stimulation [3]. Furthermore, Peters et al. [37] have previously reported that light pulses during the light or dark period elevate c-Fos mRNA levels in the IGL that are substantially higher during the dark phase, which also is consistent with results in the present study.

In the DLG and SCN, no significant differences were observed in controls vs. photostimulated animals except at 1 a.m.; c-Fos-ir cell numbers for control vs. photostimulated animals were indistinguishable at the three other time points examined. Essentially, both normal room illumination (control condition) and exposure to low-frequency photostimulation yielded similar c-Fos-ir cell numbers during the light period. Correa-Lacarcel et al. [8] also have reported lower levels of c-Fos expression in the DLG than in the IGL and VGL following low-frequency visual stimulation, and Prichard et al. [39] showed that acute shifts in illumination induce c-Fos expression in the IGL and VGL but not in the DLG. Thus, despite their close physical proximity, the DLG and the IGL/VGL show quite different profiles of c-Fos expression to low-frequency photostimulation. c-Fos expression in the SCN showed a marked downward trend for both control and photostimulated animals over the course of the light phase and into the early dark period. Previously, Colwell and Foster [7] have shown that c-Fos expression is

highest in the SCN at the onset of the light period then decreases and remains low until onset of the next light period. Light stimulation during the dark period appears to be most effective for inducing SCN c-Fos expression, suggesting that a phase-dependent c-Fos response may be functionally linked to circadian entrainment [10,25]. Although the SCN receives efferents from the IGL, its profile of c-Fos expression is quite different from that of the IGL/VGL [36], as we have confirmed in the present study.

Serotonin has been consistently implicated in modulatory effects of photic stimulation in a number of visual structures, and serotonergic innervation from the DRN is most extensive to the ventral region of the lateral geniculate complex, which includes the IGL and VLG [32,47,50]. Furthermore, the DRN is interconnected with the median raphe (MRN), which provides direct serotonergic innervation to the SCN [33,45]. Electrical stimulation of either the DRN or the adjacent median raphe nucleus reduces light-induced c-Fos in the SCN proportional to the intensity of stimulation [30], leads to the release of 5-HT in the SCN [9,16], and reduces expression of c-Fos in the SCN to light stimulation. Thus, the DRN may indirectly influence the SCN and circadian system via a multisynaptic pathway modulating photic and/or nonphotic phase-shifting effects through the IGL [17].

Finally, it is possible that variations in the light/dark cycle and amount of environmental light may alter serotonin levels in the DRN itself. Cagampang et al. [4] previously have shown that a rapid synthesis of serotonin occurs in the DRN in rats during the early portion of the light phase followed by lower levels over the remaining portion of the light phase. Recent analysis of the immunocytochemical staining density of DRN serotonergic neurons in gerbils over the light/dark cycle [2] has revealed a temporal profile that closely resembles that reported by Cagampang et al. [4]. Such evidence suggests that environmental light stimulation may directly influence both neuronal activation and serotonin levels in the DRN and that these effects depend upon time of day and light/dark exposure condition. Whether the profile of c-Fos expression observed in the DRN is linked to the specific conditions of photostimulation used in the present study or would vary according to other stimulus parameters remains to be determined.

Acknowledgments

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