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Photic Entrainment and Induction of Immediate-Early Genes
Within the Rat Circadian System.

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A Thesis
in
The Department
of
Psychology

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for the Degree of Master of Arts at
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ABSTRACT

Photic Entrainment and Induction of Immediate-Early Genes

Within the Rat Circadian System.

Christian Beaulé

Immediate-early genes (IEG) are transiently expressed within the rodent circadian system in response to nocturnal light. Immediate-early genes are part of the mechanism by which light induces phase shifts of circadian rhythmicity. The two most studied light-induced IEG within the rodent circadian system are Fos and Jun-B. Molecular expression of these two genes within the suprachiasmatic nucleus (SCN) correlates both in magnitude and in the temporal contingency with light-induced behavioral phase shifts. The determination of the role of Fos and Jun-B in circadian clock resetting was previously attempted using light stimuli that induces strong phase shifts. However, the relationship between light-induced IEG in an entrainment context is undocumented in rats. The present study examined the relation between photic induction of Fos and Jun-B in an entrainment context. Male rats, for which the free running period was determined, were entrained for 14 days on a 0.5h:23.5h LD cycle with light onset at dawn. On the fifteenth day, the entraining light pulse was reduced to 10 minutes and brains were processed for IEG immunocytochemistry. Strong Fos induction was observed in the three brain regions investigated: the SCN, intergeniculate leaflet (IGL) and anterior paraventricular thalamus (PVT). Strong Jun-B immunoreactivity was observed only in the SCN. Jun-B labeling was weak in the IGL, and absent in the PVT. Significant correlations were obtained between light-induced IEG in the SCN and the magnitude of the daily phase shift required for entrainment. Further, Fos and Jun-B immunoreactivities correlated in the SCN and in the IGL. These data suggest that the magnitude of Fos and Jun-B induction within the SCN is related to the magnitude of the daily phase shift required for entrainment.

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INTRODUCTION

The living conditions of all organisms are shaped by the daily alternation of day and night. Numerous species ranging from mammals and birds to plants, insects and bacteria respond to this environmental fluctuation by adapting their biological rhythms to the day/night cycle. Such synchronization is called a circadian rhythm and entails both the harmonizing of internal biological rhythms with one another and with the external light/dark (LD) cycle. This process requires a timekeeping mechanism, a clock, capable of such synchronization. The clock must be able to integrate photic information conveyed by the LD cycle and communicate such information to the centers responsible for the generation of the specific components of biological rhythms (i.e. effector centers for endocrine glands, arousal, feeding, sleep etc.).

In mammals, the clock responsible for this synchronization, also called the circadian clock, is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore, 1983; Zucker, Rusak and King, 1976). In the absence of environmental input, the SCN generates rhythms, called free-running rhythms, with periods that approach but typically do not equal 24 hours. To exhibit rhythms with a 24 hour period, the circadian clock has to be entrained by cues from the external world. Although it has been shown that nonphotic cues such as feeding, temperature and social interactions can entrain circadian rhythms (Takahashi and Zats, 1982; Turek, 1985; Zucker *et al.*, 1976), the most effective cue for entrainment is light (Rusak and Zucker, 1979).

The consequences of photic entrainment of circadian rhythms is readily observed as small daily shifts in the phase of biological rhythms such as those of activity and temperature. The molecular mechanism by which light resets the circadian clock and produces such shifts, however, is still unclear although the evidence points to a role for immediate-early genes. Light that affects the circadian clock induces the expression of immediate-early genes in the SCN, leading to late gene transcription that is believed to be

ultimately responsible for behavioral and biological entrainment (Earnest, Iadarola, Yeh, and Olschowka, 1990; Meijer, and Reitveld, 1989; Rusak, Robertson, Wisden, and Hunt, 1990).

The most studied light-induced immediate-early gene present in the SCN is *c-fos* and its protein product Fos; *jun-B* and its product Jun-B have also been studied although to a lesser extent. The expression of both immediate-early genes has been studied primarily in relation to the effects of light pulses given either to free running animals kept in constant dark or during the dark phase of the LD cycle. In these situations light induces large shifts in phase. During entrainment the periodic presentation of light induces much smaller, regular phase shifts. The effect of entraining light on immediate-early gene induction in the circadian system is not well-characterized. The experiment described in the present thesis, therefore, was designed to gain knowledge about the effect of photic entrainment on the induction of the immediate-early genes Fos and Jun-B in the rat circadian system.

The circuitry responsible for the transduction of the photic input to the SCN originates in the retina. Photic information is transmitted to the SCN from ganglion cells of the retina via the retino-hypothalamic tract (RHT) which terminates in the ventral part of the SCN where the retinorecipient cells are located (Johnson, Morin, and Moore, 1988; Moore and Card, 1985; Moore and Lenn, 1972; Pickard, 1985). Fibers of the RHT also convey photic information to the intergeniculate leaflet (IGL) and the ventral lateral geniculate nucleus (vLGN) of the thalamus via a set of collaterals (Pickard, 1985). The RHT conveys excitatory retinal information in the form of a glutamatergic input to the SCN (Mikkelsen et al, 1995; Pickard 1985; Vindlacheruvu, Ebling, Maywood and Hastings, 1992). It has also been assumed that glutamate is involved in the innervation of the IGL and vLGN by the RHT (Pickard, 1985). However, recent evidence showing that ionotropic blockers of glutamate receptors failed to prevent photic induction of Fos in the IGL (Edelstein and Amir, 1998a, submitted) casts doubt on this assumption. A photic feedback fiber pathway, the geniculo-hypothalamic tract (GHT) projects from the IGL/vLGN to the SCN

(Mikkelsen, 1990; Moga and Moore, 1997). Both neuropeptide Y (NPY) and γ -aminobutyric-acid (GABA) are involved in neurotransmission in the GHT (Harrington, Nance and Rusak, 1987; Card and Moore, 1989).

Although the SCN and the IGL are the major components of the circadian clock, other structures projecting to the SCN are believed to be part of a larger circadian timing system. Among these structures are the serotonin (5-HT) containing raphé nuclei that send a dense projection to the ventral SCN (Azmitia and Segal, 1978; van den Pol and Tsujimoto, 1985). Current evidence suggests that the role of the raphé nuclei in circadian functioning is to regulate circadian oscillations by inhibiting the behavioral and molecular responses of the SCN to light (Pickard, Weber, Scott, Riberdy, and Rea, 1996; Selim, Glass, Hauser, and Rea, 1993). Another structure implicated in the larger circadian system is the paraventricular thalamic nucleus (PVT). The PVT receives input from the retina, the SCN and the IGL and sends a diffuse glutamatergic projection back to the SCN (Moga and Moore, 1996; Speh and Moore, 1992). To date, there is one report that hamsters with PVT lesions show normal entrainment to long and short photoperiods as well as normal gonadal regression to the change in lighting schedule (Ebling, Maywood, Humby, Hastings, 1992). Although an intact PVT is not necessary for entrainment to long and short photoperiods, the fact that neuronal activation of this area appears to be dependent on the arousal state of the animal (Peng, Grassi-Zucconi, Bentivoglio, 1995) as well as the retinal and circadian inputs suggests that the PVT may play a role in modulating the information reaching the SCN.

In a recent report, Moga and Moore (1997) investigated the neural inputs to the SCN using both anterograde and retrograde tracers. They report that, in addition to inputs from the retina, IGL, raphé nuclei and PVT, the SCN receives input from the limbic system (infralimbic cortex, lateral septal nucleus, and ventral subiculum) and the hypothalamus (median and medial preoptic nuclei; anteroventral periventricular, ventromedial, dorsomedial, tuberomammillary, and arcuate nuclei; subparaventricular zone; and the

medial preoptic, lateral hypothalamic, and posterior hypothalamic areas). Inputs to the SCN are topographically organized and subdivide the nucleus into two distinct areas: the ventral SCN and the dorsal SCN. The retina, the raphé, and the IGL project predominantly in the ventral SCN. The hypothalamic and limbic inputs, on the other hand, send their projections to the dorsal SCN. Finally, the PVT sends diffuse projections to the entire SCN area. With the exception of the retina and IGL, the precise role of these inputs is not clearly defined. However, it is assumed that inputs terminating in the ventral SCN modulate photic information and that projections ending in the dorsal or entire SCN provide either nonphotic or autonomic/visceral information to the circadian pacemaker.

The SCN projects to a number of thalamic and hypothalamic nuclei including the paraventricular nucleus of the hypothalamus (PVN); the dorsomedial nucleus of hypothalamus (DMH); the medial preoptic nucleus; and the anterior PVT (Buijs, Markmann, Nunes-Cardoso, Hou and Shinn, 1993; Kalsbeek, Teclemariam-Mesbah and Pevet, 1993; Vrang, Larsen, Moller and Mikkelsen, 1995). Most of these nuclei are implicated in autonomic and visceral functions and are assumed to relay the circadian information generated by the SCN to effector centers such as the adrenal or pineal glands.

Typically, two experimental procedures have been used to study the effect of light on rhythms. In the phase shift procedure, the animal is allowed to free run in complete darkness (DD) and is presented with a brief pulse of light at different time points of the sleep-wake cycle. Following the light pulse, the animal is maintained in DD to monitor the effects on the behavioral rhythms. In contrast, when the effect of light is studied under conditions of entrainment, light is presented at the same time each day and thus maintains entrainment by producing a daily phase shift. This latter experimental procedure most closely resembles the effect of the natural environmental light on rhythms. Typically the shifts in rhythm induced by entraining stimuli are smaller than those induced in the phase shift procedure.

It has been demonstrated that light pulses produced behavioral phase shifts in animals kept in DD only when presented during subjective night (the dark phase of the LD cycle, the active phase of the cycle of a nocturnal animal). Light presented during subjective day (the light phase of the LD cycle, the inactive phase of the cycle of a nocturnal animal) does not produce any effect on rhythms (Rusak and Zucker, 1979).

The magnitude and direction of phase shifts is not constant throughout the subjective night. In rodents, light can induce phase shifts ranging from 0 to approximately 120 minutes depending both on its intensity and when in the cycle it is presented. Maximal phase shifts are achieved during the subjective night with bright light around circadian times (CT) 14-16 and 20-22; smaller magnitude phase shifts are obtained close to the transition times corresponding to dawn (CT 24) and dusk (CT 12) (Takahashi and Zats, 1982). Further, light pulses of low intensity produce smaller phase shifts compared to brighter light presented at the same circadian times (Kornhauser, Nelson, Mayo, and Takahashi, 1990).

The direction of the phase shift is also a function of the CT at which light is presented during subjective night. Light falling during early subjective night causes phase delays while light falling towards the end of subjective night causes phase advances (Pittendrigh and Daan, 1976; Rusak and Zucker, 1979). The plot of the circadian time versus the magnitude and direction of phase shifts yields the phase response curve (PRC). A given PRC will describe the relationship between light and phase shifts for a single individual. Once a PRC is determined for an animal, one can predict the direction and magnitude of a phase shift simply by knowing the CT at which the light pulse was administered. Conversely, if one knows the magnitude and direction of the phase shift, the CT at which the stimulus responsible for the shift was applied can be estimated.

The characteristics of the phase response curve obviously have implications for entrainment. The limits of entrainment depend on the period of the clock and on the maximal phase shift that can be obtained for an individual organism. The maximal phase

shift is determined from the phase response curve and will vary across individuals. For example, if the maximal phase shift (either advance or delay) that can be obtained for a given organism is 2 hours, and the clock's period is 24 hours, then the limits of entrainment will be between 22 and 26 hours. Failure to entrain to a stimulus outside the limits of entrainment will lead to free-running or disruption of rhythmicity (Pittendrigh and Daan, 1976; Pittendrigh, 1981).

Rodents entrain to a variety of lighting schedules, however, one artificial manipulation of the LD cycle has dramatic impact on behavioral rhythms: the prolonged exposure to constant light (LL). Long periods of LL will eventually disrupt circadian rhythms, sometimes to the point of loss of rhythmicity (Eastman and Rechtschaffen, 1983). Experimentally, the most frequently used schedule is the 12h:12h LD cycle although it is artificial for nocturnal rodents (rats, hamsters and mice) since it is very unlikely that they will be subjected to such long periods of high illumination. These species can also entrain to more environmentally relevant lighting schedules such as the skeleton photoperiod (SPP) and T-Cycle. In a SPP, two short light pulses (usually 1 hour long, although they can be shorter) are given, separated by long periods of darkness usually ranging from 8-14 hours. These two light pulses represent the transition times corresponding to dawn and dusk. In a T-Cycle, a single light pulse is presented once a day (Pittendrigh and Daan, 1976; Schwartz and Zimmerman, 1990; Stephan, 1983). Although the periods of the SPP and T-Cycle need not add to 24 hours, they must stay within the limits of entrainment (e.g. from 22 to 26 hours) to retain their entraining properties.

The ability of a single light pulse presented at the same time each day to entrain the circadian clock is explained by the PRC. Entrainment to a 24 hour cycle in animals such as rats with long free-running periods (>24 h) should be accomplished by a daily phase advance which would occur when light is presented at dawn (Stephan, 1983); whereas in animals such as mice with short periods (<24 h) entrainment should be accomplished by a daily phase delay when light is presented at dusk (Schwartz and Zimmerman, 1990). In the

event of a light pulse presented only once a day, circadian rhythms will free run until light falls on the region of the PRC leading to the appropriate phase shift required for entrainment. In the SPP the second light pulse appears simply to stabilize the behavioral rhythm by locking it between two light pulses and does not affect entrainment itself (Schwartz, Peters, Aronin, and Bennett, 1996).

Photic phase shifts can only be achieved if there is a transduction process between the photic input on the retina to the clock located in the SCN. This transduction process must then be able to alter SCN function to allow resetting. Although the phenomenon of photic resetting has been known for some time, knowledge about the transduction mechanisms leading to phase shifts and entrainment has emerged fairly recently and is not complete.

Several steps of the signal transduction pathway leading to phase advances and delays in response to light have been identified. Cells of the retinohypothalamic tract terminating in the ventral SCN use glutamate as a neurotransmitter (Mikkelsen *et al.*, 1995; Vindlacheruvu *et al.*, 1992). Glutamate released in response to a light stimulus binds to the N-methyl-D-aspartate (NMDA) receptor (Mikkelsen, Larsen, and Ebling, 1993). Activation of the NMDA receptor stimulates, probably in a phase dependent manner, the enzyme nitric oxide synthase (NOS), producing gaseous nitric oxide (NO) which rapidly diffuses out of SCN cells (Ding *et al.*, 1994). The rise in NO stimulates the phosphorylation of the Ca²⁺/cAMP response element binding protein (CREB), probably through activation of cyclic GMP-dependent protein kinase. The phosphorylation of CREB (P-CREB) appears to be dependent upon glutamate and NO activation of the SCN (Ding *et al.*, 1994; Ding, Fairman, Hurst, Kuriashkina, and Gillette 1997). Furthermore, CREB phosphorylation is achieved in the SCN only by nocturnal light (Ginty *et al.*, 1993). Phosphorylated CREB increases the transcription rate of the genes it regulates by a factor of four (Hunter and Karin, 1992). Although the late response genes regulated by P-CREB are unknown, evidence suggest that immediate-early genes, in particular *c-fos*, a transcription factor

linked to photic resetting, might be specifically induced by CREB activation (Ginty *et al.*, 1993).

A large number of studies have examined the ability of a phase-shifting light stimulus to induce immediate-early genes in the SCN. To date, six immediate-early genes have been shown to be specifically induced by light given during subjective night within the rodent SCN: *c-fos*, *fos-B*, *jun-B*, *zif268*, *nur77* (Rae, 1989; Rusak *et al.*, 1990; Kornhauser *et al.*, 1990; Kornhauser, Nelson, Mayo, and Takahashi, 1992) and recently *egr-3* (Morris, Viswanathan, Kuhlman, Davis, and Weitz, 1998). Other immediate-early genes such as *c-jun* and *jun-D* are also present within the rodent SCN but their induction is relatively insensitive to photic input (Kornhauser *et al.*, 1992; Takeuchi, Shannon, Aronin, and Schwartz, 1993; Rusak, McNaughton, Robertson, and Hunt, 1992). In contrast, only one late response gene has been shown to be light-inducible in the SCN. This gene was found to be the vesicular factor *vgf* the function of this gene is, however, still unknown (Wisor and Takahashi, 1997).

It has been shown that light presented during the dark phase of a nocturnal rodent can induce the expression of the messenger RNA (mRNA) for *c-fos* (Rusak *et al.*, 1990; Kornhauser *et al.*, 1990) as well as the protein product, Fos, in the SCN (Rea, 1989; Earnest *et al.*, 1990; Schwartz, Takeuchi, Shannon, Davis, and Aronin, 1994). One member of the Jun family, *jun-B*, also exhibits strong patterns of light induction, both for the mRNA (Kornhauser *et al.*, 1992) and the protein product (Takeuchi *et al.*, 1993). It is noteworthy that photic stimulation during subjective day that does not induce phase shifts also fails to induce Fos and Jun-B. In addition, the threshold irradiance levels required for both *c-fos* induction and production of behavioral phase shifts by light is the same (Kornhauser *et al.*, 1990). Furthermore, there is evidence for a colocalization of Fos and Jun-B immunoreactive cells within single SCN neurons (Takeuchi *et al.*, 1993). This colocalization is expected since both genes show similar time course of activation and dimerize with one another to activate late response genes through the AP-1 activator protein

complex. The photic induction of Fos and Jun-B in rodent SCN is restricted almost exclusively to the ventral SCN, which is the area that receives RHT innervation (Johnson *et al.*, 1988).

Photic stimulation induces Fos specifically in only one other part of the circadian system, the intergeniculate leaflet (IGL) of the thalamus (Aronin, Sagar, Sharp, and Schwartz, 1990), the other retinorecipient structure involved in entrainment of circadian rhythms. However, Fos induction in the IGL does not appear to be phase dependent since it is always expressed in response to light, irrespective of the CT of light presentation (Edelstein and Amir, 1996). In addition, the duration of the stimulus required for Fos induction in the IGL appears to be considerably longer than in the SCN, although conflicting results have been reported. Robust Fos expression has been observed in the SCN in response to short light pulses (5 minutes) (Kornhauser *et al.*, 1990). In contrast, Fos induction in the IGL has inconsistently been reported in response to 60 or 120 minutes light pulses (Park, Baek, Kim, Kim, and Kim, 1993) while others report strong Fos induction to a 15 minutes light pulse (see Amir and Stewart, 1998a). Interestingly, Fos levels remain elevated in the IGL even after prolonged exposure to light for up to a few days (Edelstein and Amir, 1996). The IGL also expresses Jun-B although its induction is different from Fos. Jun-B is present in rat IGL whenever light is presented. The only exception is when the animal is kept in constant illumination where no expression is observed (unpublished observations). Although the IGL receives retinal input and is assumed to process photic information, there is also evidence that the IGL may process nonphotic events that can influence circadian rhythms (Janik and Mrosovsky, 1992)

Although there is ample evidence to suggest that immediate-early gene induction is a correlate of photic resetting, the question of whether Fos in the SCN is necessary and/or sufficient to produce behavioral phase shifts remains. Various approaches have been used to address this issue. One group reported that microinjection of excitatory amino acid antagonists which block the retinal glutamatergic input to the SCN attenuated both light-

induced phase advances and light-induced Fos in the SCN in response to a phase shifting stimulus (Rea, Michel and Lutton, 1993). However, this manipulation is similar to a lesion of the optic tract that simply “blinds” the SCN to light. In another experiment NMDA injected at CT 18 induced Fos in the retinorecipient ventral SCN but failed to produce phase advances in behavioral rhythms (Rea *et al.*, 1993). These data suggest that the induction of Fos artificially by NMDA injections is not sufficient to induce phase advances.

Recent advances in genetics have yielded a line of gene-targeted mice carrying a null mutation for the *c-fos* gene (Johnson, Spiegelman, and Papaioannou, 1992). The investigation of the circadian system of this mutant mouse revealed that the period of locomotor rhythms was similar to that in wild-type mice. Mutant mice, however, took significantly longer to entrain to a 12:12 LD cycle. In addition, light-induced phase shifts were attenuated in the mutant mice, although the PRC of advances and delays was not affected (Honrado *et al.*, 1996). This suggests that a homozygous null mutation of the *c-fos* gene is not sufficient to completely prevent entrainment and phase shifts. Another mechanism (perhaps a compensatory one), although not as efficient as Fos, must be present in these mice to account for entrainment and phase shifts.

The most convincing evidence for a causal role for Fos in phase shifts comes from a study in which microinjections of antisense oligonucleotides were made into the SCN to block the expression of both *c-fos* and *jun-B*. Application of the antisense was found to block light-induction of Fos and Jun-B mRNAs specifically and to completely prevent phase shifts by light presented at CT 15. Control animals not treated with antisense showed the normal response (both behavioral and molecular) to the light pulse (Wollnick *et al.*, 1995). This is the most convincing report of a role for *c-fos* and *jun-B* in clock resetting since the manipulation adopted did not prevent light from reaching the SCN.

The description of the cascade of events following light stimulation of the SCN described above has emerged from experiments using a phase shifting procedure. Light

stimuli that entrain daily rhythms, however, have somewhat different characteristics and the cascade of events induced by such stimuli in the SCN is less well understood.

Entraining light differs from the phase shifting light pulse used to determine the PRC in that it is presented at the same time each day and presumably induces the same phase shift each day. In addition the two stimuli differ in their ecological validity. A very bright light pulse given during the subjective night is a highly artificial situation that is very unlikely to occur for a nocturnal animal (or a diurnal animal) whereas an entraining light is more natural and behaviorally relevant since it corresponds to the daily transitions of dawn and dusk. Moreover, two species (rats and mice) requiring light at different times for entrainment exhibit an increase in Fos expression occurring within two hours of light onset at dawn when held in a 12:12 LD cycle (Aronin *et al.*, 1990). Mice with short endogenous periods require light at dusk to induce a phase delay to achieve entrainment, whereas rats with long periods require light at dawn to induce a phase advance to achieve entrainment (Schwartz *et al.*, 1996). The latter evidence cast additional doubt on the role of Fos in entrainment. It is of interest, therefore, to know whether the changes in immediate-early gene expression seen following light stimulation in the phase shifting procedure will also occur following the presentation of an entraining light stimulus. In the current study, therefore, the effect of an entraining light stimulus on the expression of both Fos and Jun-B in the SCN, IGL and anterior PVT was determined. The goal was to determine whether the amount of expression of these immediate-early genes would correlate with the size of the phase shift required for entrainment. In addition, the extent to which the induction of Fos and Jun-B was correlated within animals was also investigated.

Materials and Methods

Subjects. A total of 32 male Wistar rats (Charles River Breeding Farms, St-Constant, Québec) weighing between 225-300 g at the beginning of the experiment were used. Each animal had *ad libitum* access to food and water throughout the experiment. Animals water supply was replenished every 5 days and the food supply was replenished every 10 days.

Housing. Animals were housed in clear plastic cages (50 cm X 26.8 cm X 36.4 cm) equipped with a running wheel (34.5 cm in diameter) (Nalgene). A small fan was installed on the top of each plastic cage. Each cage was placed in a sound and light proof chamber (66 cm X 66 cm X 44 cm) equipped with a 15 watt fluorescent light and a ventilation system. The boxes lighting system and the fan on the top of the cage were controlled by their own set of 24 hour outdoor timers (Noma).

Apparatus. Activity data was transmitted from the running wheel to the computer via a magnetic microswitch. Activity rhythms were recorded and analyzed with a microcomputer using the Dataquest III software package (Mini Mitter Co. Inc., Sunriver, OR).

Procedures. Animals were first entrained to a 12h:12h LD cycle for 10-15 days. Then, they were allowed to free-run in complete darkness for 10-15 days to measure the period of their circadian rhythms. Next, animals were switched back to 12h:12h LD cycle for 5-15 days to allow re-entrainment to the original LD cycle. Following the LD cycle animals were presented with a T-Cycle schedule consisting of 0.5h of light and 23.5h of dark. In addition to the present experiment, these animals were part of a conditioning experiment (see Appendix A). Entrainment to the 0.5h:23.5h LD cycle lasted 14 days.

Testing. On the fifteenth day, all animals received light at the usual time (dawn) but its duration was reduced to 10 minutes. Animals were deeply anesthetized with sodium pentobarbital (130 mg/kg I.P. approx.) one hour after stimulus onset.

Tissue preparation. Deeply anesthetized animals were slowly perfused intracardiacally with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). The descending aorta was clamped to force the perfusate into the upper part of the body. Following perfusion, brains were removed and postfixed in 4% paraformaldehyde and stored at 4° C overnight. Free floating coronal brain sections (50 µm in thickness) through the SCN, anterior PVT and IGL were obtained on a vibratome. A total of 40 sections were taken for each animals corresponding to plates 22-24 and 34-37, respectively, in the atlas of Paxinos and Watson (1986). Alternate brain sections were collected to allow immunocytochemistry for both Fos and Jun-B which yielded 20 coronal section per animal per immediate-early gene.

Fos immunocytochemistry. Free floating sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6) and incubated for 48 hours at 4°C with a mouse monoclonal antibody raised against the N-terminal sequence of Fos (corresponding to N-terminal residues 4-17 of human *c-fos*; NCI/BCB Repository, Quality Biotech, Camden NJ). The antibody was diluted 1:8000 with a solution of 0.3% Triton X 100 in TBS with 1% normal horse serum. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a rat-absorbed biotinylated anti-mouse IgG made in horse (Vector Labs), diluted 1:33 with 0.3% triton X 100 in TBS with 1% normal horse serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for two hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris (pH 7.6), and again for 10

minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris with 0.01% H₂O₂ and 8% NiCl₂. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and coverslipped with Permount (Fisher).

Jun-B immunocytochemistry. Free floating sections were washed in cold TBS and incubated at room temperature for 30 minutes in a quenching solution consisting TBS and 30% w/w H₂O₂. Following the quenching phase, sections were rinsed in cold TBS and incubated for 1 hour at room temperature in a pre-blocking solution made of 0.3% Triton X 100 and 3% normal goat serum. Following the pre-blocking phase, sections were transferred directly into a rabbit polyclonal antibody for jun-B (Graciously donated by Dr. Rodrigo Bravo). The antibody was diluted 1:60000 with a solution of 0.3% Triton X 100 in TBS with 0.3% normal goat serum. Sections were incubated for 48 hours at 4°C. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for 1 hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector Labs), diluted 1:200 with 0.3% Triton X 100 in TBS with 2% normal goat serum. Following incubation with secondary antibody, sections were rinsed in cold TBS and incubated for two hours at 4°C with an avidin-biotin-peroxidase complex (Vectastain *Elite* ABC Kit, Vector Labs). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H₂O₂ and 8% NiCl₂. After this final incubation, sections were rinsed in cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and coverslipped with Permount (Fisher).

Immunocytochemical data analysis. Evidence of Fos and Jun-B immunoreactivity was examined under a light microscope (Leitz Laborlux S) and recorded for each region, in each animal. Brain sections through the SCN, anterior PVT and IGL were digitized using a Sony XC-77 Video Camera connected to a Scion LG-3 frame grabber using the NIH Image Software package (version 1.60). The mean number of Fos and Jun-B positive cells for the 5 sections expressing the greatest number of labeled nuclei was computed for the SCN and IGL and the mean number of Fos and Jun-B positive cells for the 3 sections with the most labeled nuclei was computed for the PVT. Correlations between the magnitude of the daily phase shift required for entrainment (see below) and the corresponding mean number of immunoreactive cells for each brain region were computed using Statsview (version 4.1, Abacus Concepts Inc.). Correlations between Fos and Jun-B immunoreactivities in the SCN and IGL were also computed. In addition to the correlation coefficients, regression lines and equations were generated using the same software. Significance of correlation was tested with alpha set at $\alpha = 0.05$.

Behavioral data analysis. Activity data for individual animals was recorded continuously and displayed in ten minutes intervals using the Dataquest III software package (Mini Mitter Co. Inc., Sunriver, OR). Double-plotted behavioral actograms were used to monitor running-wheel activity rhythms at each stage of the experiments. The free running period of the circadian clock was calculated, to the closest minute, from a stable portion of the free-running activity rhythms of the animal (usually 10 days). The absolute number of minutes displayed by the free-running rhythm apart from 24 hours was used in the correlational analyzes and is referred to the magnitude of the daily phase shift required for entrainment.

Results

Behavioral data. All animals displayed the appropriate adjustments to the manipulations made in the LD cycle. Stable entrainment and gradual stabilization of the running wheel activity rhythm was achieved during the initial 10-15 days of the 12h:12h LD cycle.

Rhythms in all animals began to free-run immediately following the removal of light and the period of the cycle was constant for at least 10 days. Free-running periods in individual animals ranged from 24 hours to 24 hours and 34 minutes, resulting in a range of daily phase shifts required for entrainment from 0 minutes to 34 minutes. None of the animals of the present study displayed free-running rhythms shorter than 24 hours, although Wistar rats are known to sometimes have rhythms shorter than 24 hours. Re-entrainment to the original 12:12 LD cycle took from 5-15 days. The transition to the 0.5h:23.5h LD cycle occurred with minimal impact on behavioral rhythms. Representative actograms for 2 animals with different periods are shown in Figures 1 and 2.

Immediate-early genes immunoreactivity. Differential induction of immediate-early genes was observed in the three brain regions investigated. Robust Fos induction in response to the 10-minute morning light pulse was observed in all brain regions observed. In the SCN, Fos immunoreactivity was mostly located in the ventral part with very few nuclei stained outside this region (Figure 3). Dense Fos immunoreactive cells were present throughout the IGL region (Figure 4). The anterior PVT showed robust Fos throughout the region observed (Figure 5). The magnitude of Jun-B immunoreactivity was different in the three regions. The SCN displayed robust Jun-B expression similar in intensity to Fos immunoreactivity, again predominantly located in the ventral part of the nuclei (Figure 6). Localization of Jun-B immunoreactive nuclei was even more restricted to the ventral SCN than Fos (see Figure 3 for comparison). The IGL showed very weak Jun-B immunoreactivity compared to the SCN and compared to Fos in the same region (Figure 7). Refer to Figure 8 for a comparison of the intensities of Fos and Jun-B

immunoreactivities in the IGL. No Jun-B immunoreactivity was induced in the PVT by the present manipulations (Figure 9).

Correlation between immediate-early genes. Significant correlations were obtained between Fos and Jun-B immunoreactivities in two brain regions, the SCN ($r = 0.847$, $p < 0.001$, Figure 10) and the IGL ($r = 0.561$, $p < 0.001$, Figure 11). In addition to significant correlations, the strength of the prediction is much higher compared to the relationship between immediate-early gene and clock resetting ($r^2 = 0.717$ and $r^2 = 0.315$ respectively). A good portion of the variance from the regression line is explained by the correlation between Fos and Jun-B and better predictions can be made on the degree of induction of one immediate-early gene from the other.

Correlation with resetting. Significant correlations (see Appendix B) were found between the expression of both Fos ($r = 0.353$, $p < 0.05$ Figure 12) and Jun-B ($r = 0.430$, $p < 0.05$ Figure 13) in the SCN and the magnitude of the daily phase shift required for entrainment. However, although the correlations are significant, the variance explained by the relationship between the expression of either Fos or Jun-B and the daily phase shift is low ($r^2 = 0.125$ and $r^2 = 0.185$ respectively for Fos and Jun-B), reducing the strength of the predictions of immediate-early gene expression from the daily phase shift. Thus, less than 20 percent of the variability from the regression line can be explained by the correlations. For the other two brain regions investigated (IGL and PVT), none of the correlations between light-induced Fos and Jun-B and the magnitude of the daily phase shift required for entrainment reached significance.

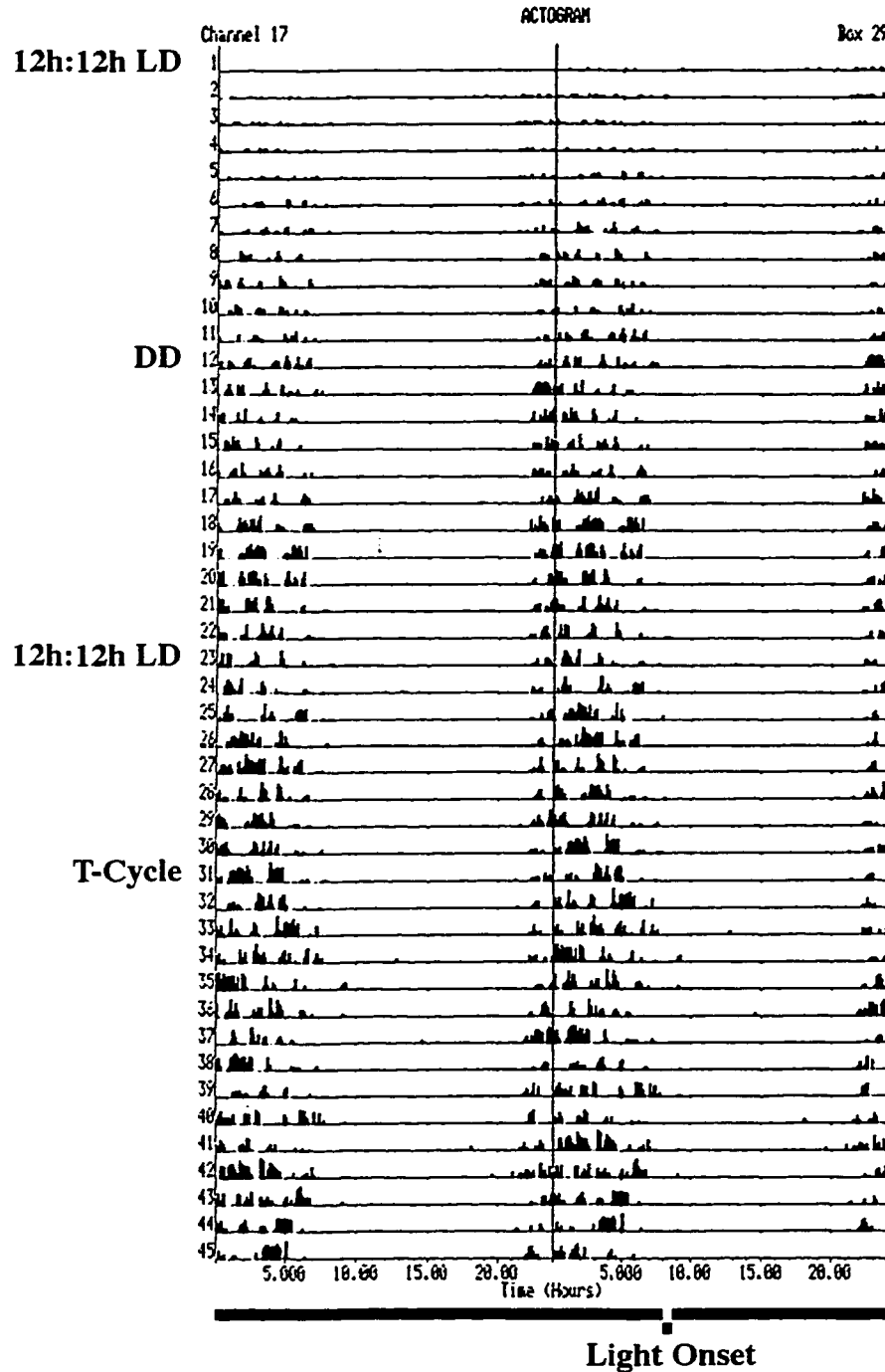


Figure 1. Double plotted behavioral actogram for the running-wheel activity rhythm (summed into 10 minutes bins) throughout the manipulation procedures for one animal with a magnitude of daily resetting required for entrainment of 0 minutes.

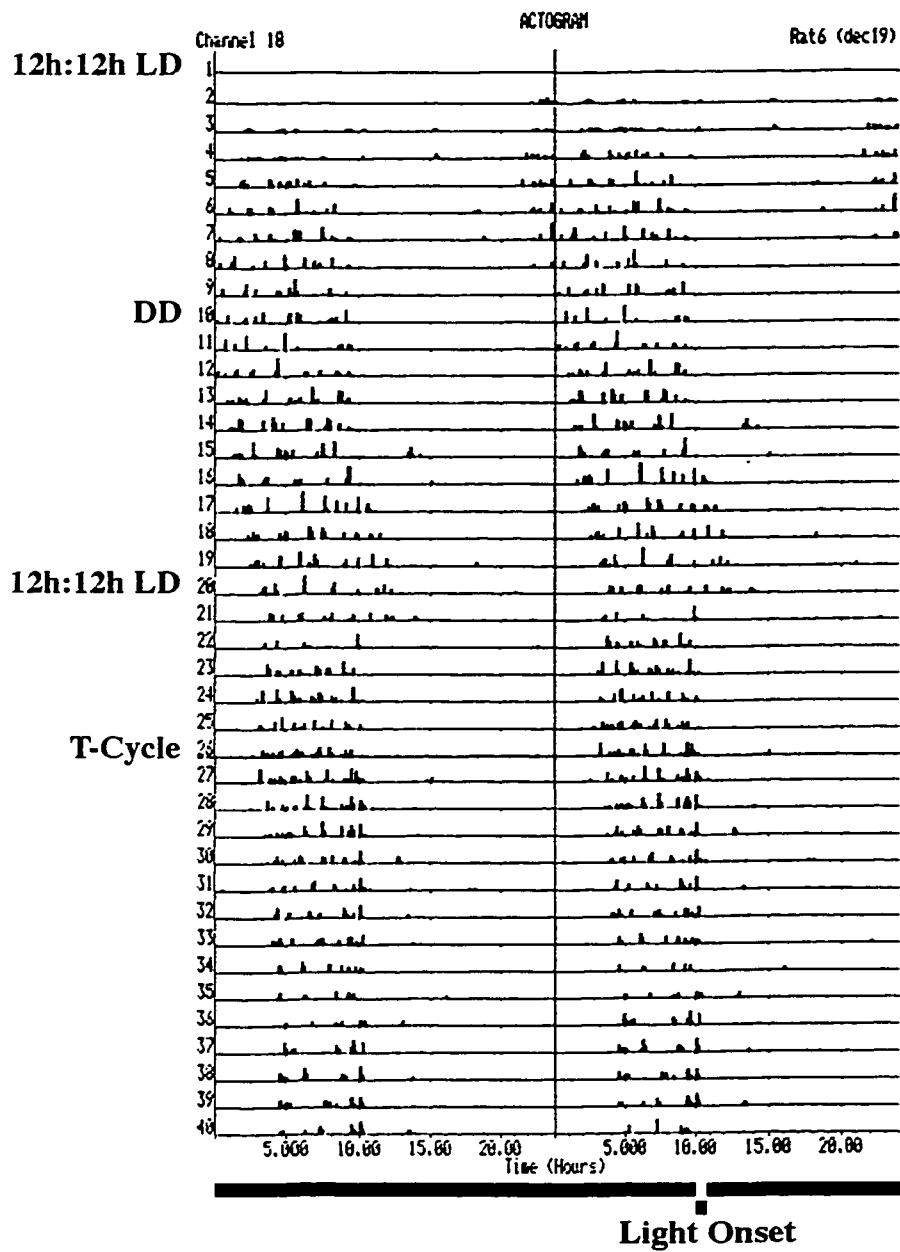


Figure 2. Double plotted behavioral actogram for the running-wheel activity rhythm (summed into 10 minutes bins) throughout the manipulation procedures for one animal with a magnitude of daily resetting required for entrainment of 32 minutes.

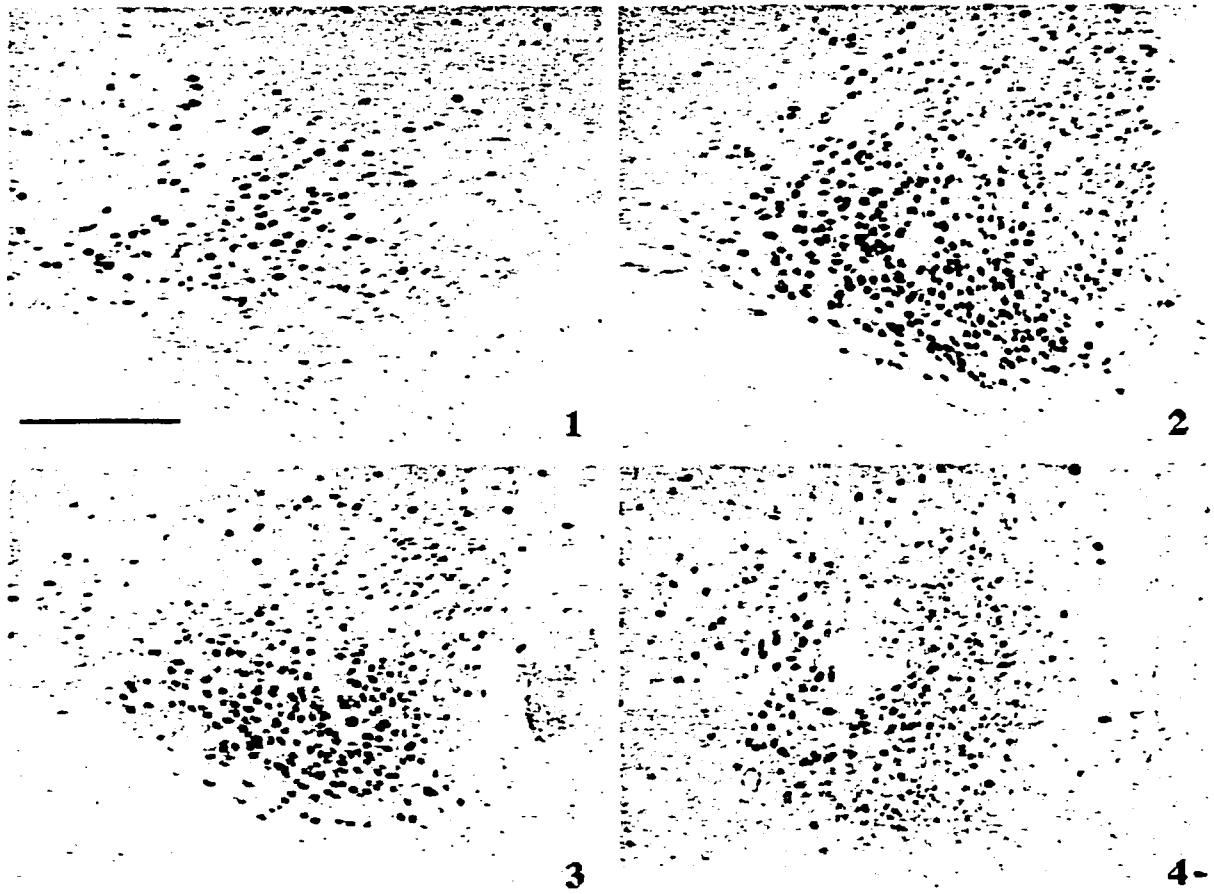
Fos Immunoreactivity

Figure 3. Example of representative Fos immunoreactivity in the SCN from anterior (#1) to posterior (#4) (scale bar 100 μ m).

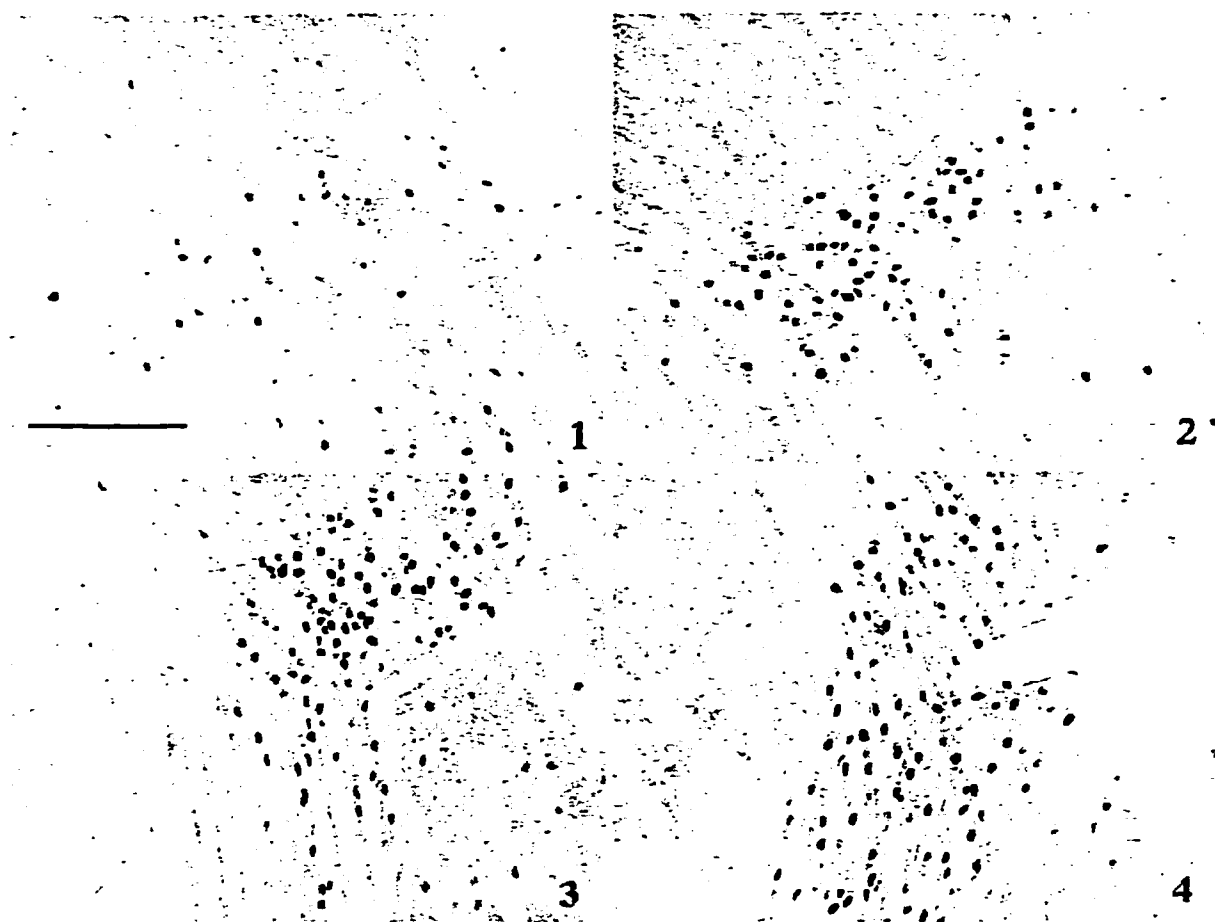
Fos Immunoreactivity

Figure 4. Example of representative Fos immunoreactivity in the IGL from anterior (#1) to posterior (#4) (scale bar 100 μ m).

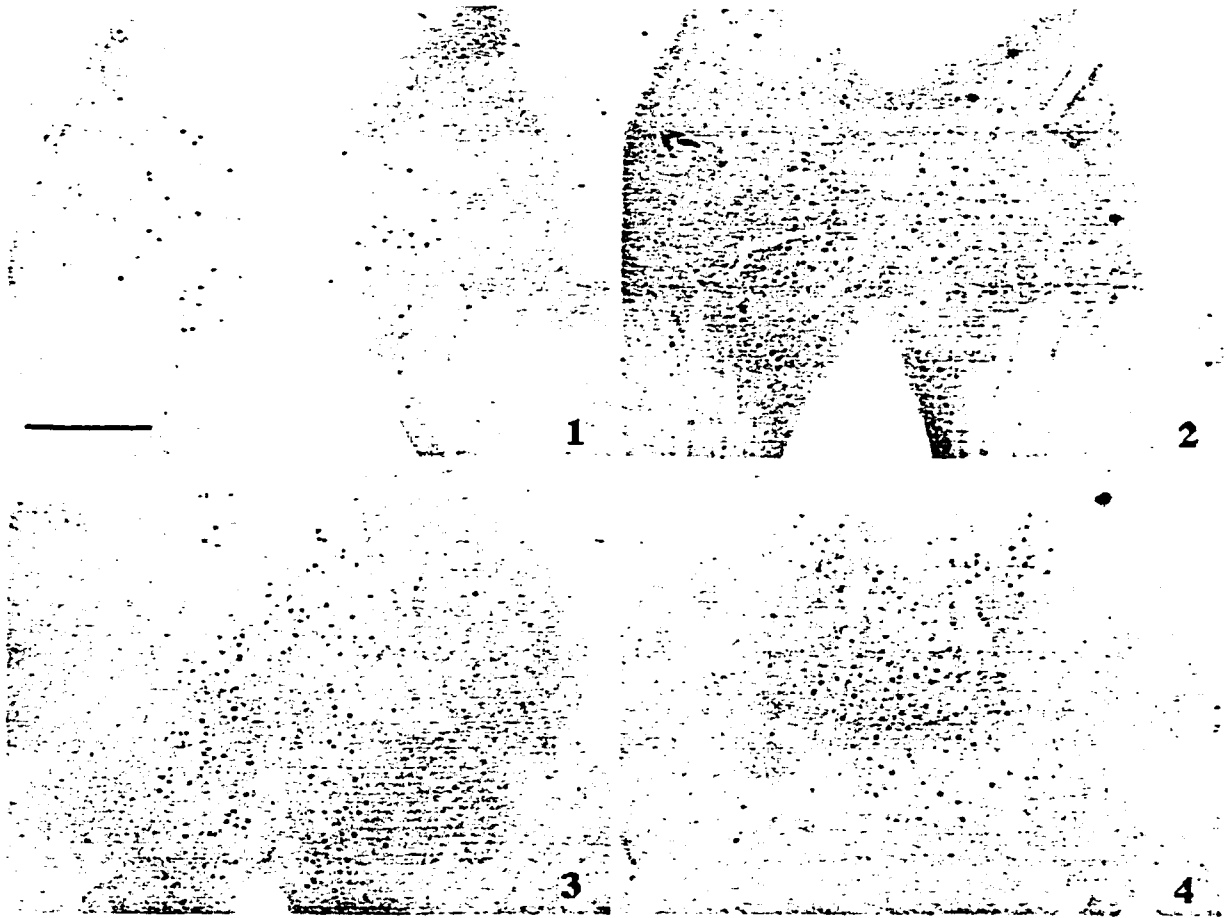
Fos Immunoreactivity

Figure 5. Example of representative Fos immunoreactivity in the PVT from anterior (#1) to medial (#4) (scale bar 200 μ m).

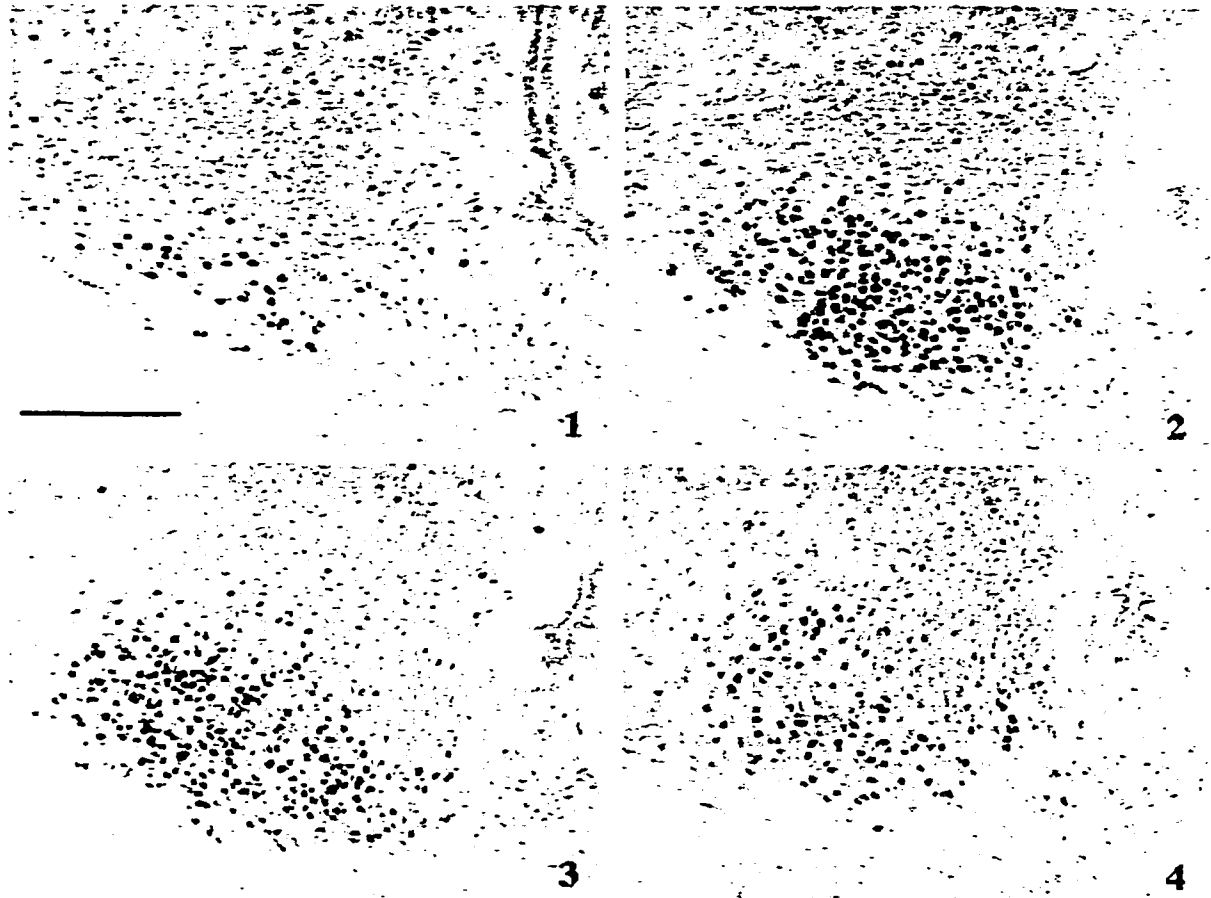
Jun-B Immunoreactivity

Figure 6. Example of representative Jun-B immunoreactivity in the SCN from anterior (#1) to posterior (#4) (scale bar 100 μm)

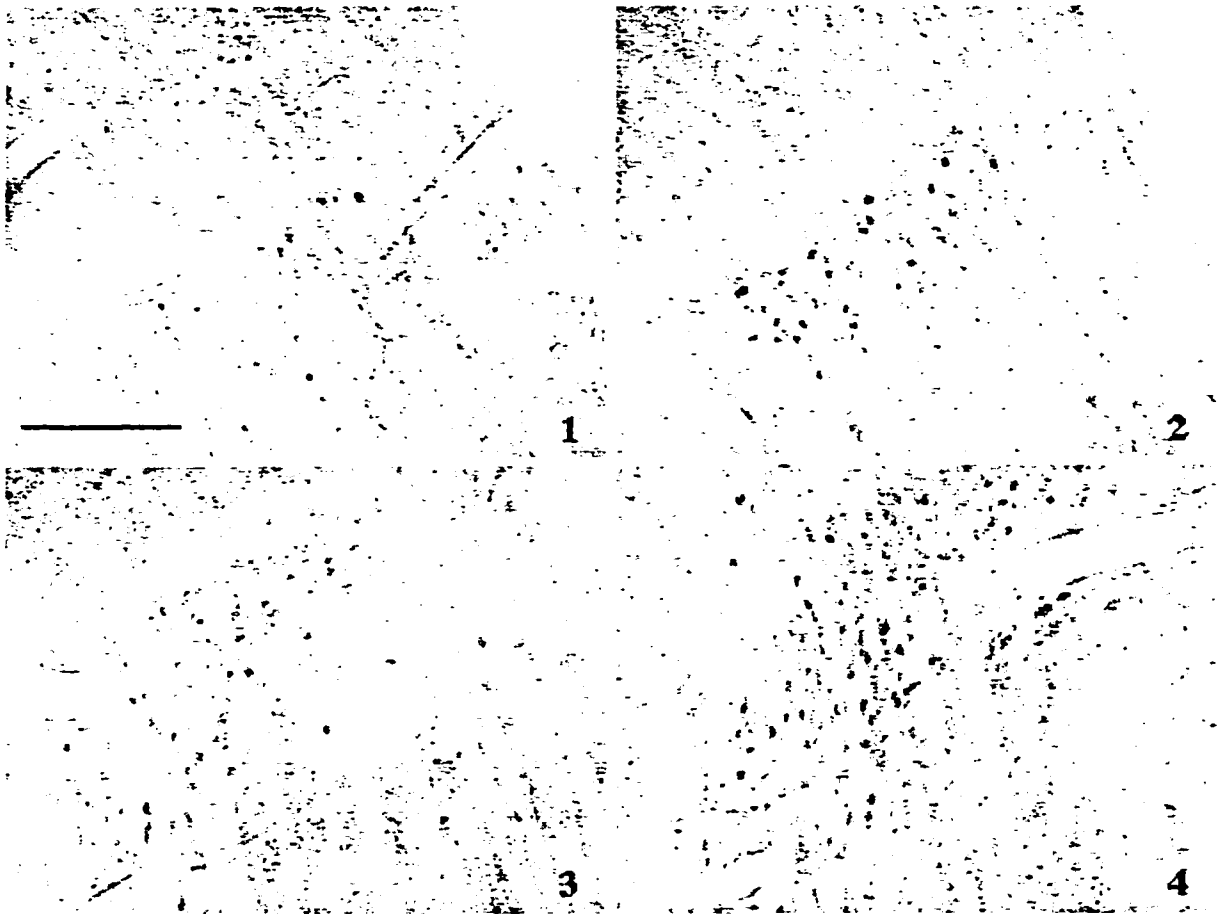
Jun-B Immunoreactivity

Figure 7. Example of representative Jun-B immunoreactivity in the IGL from anterior (#1) to posterior (#4) (scale bar 100 μ m).

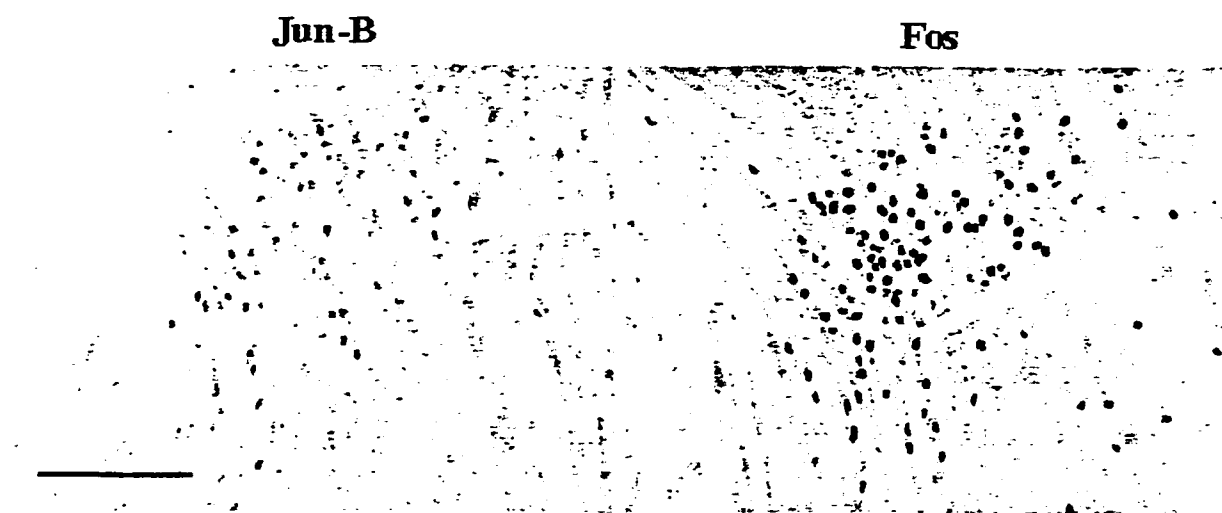


Figure 8. Comparison between the intensity of Jun-B and Fos immunoreactivities within the IGL for the same animal (scale bar 100 μm).

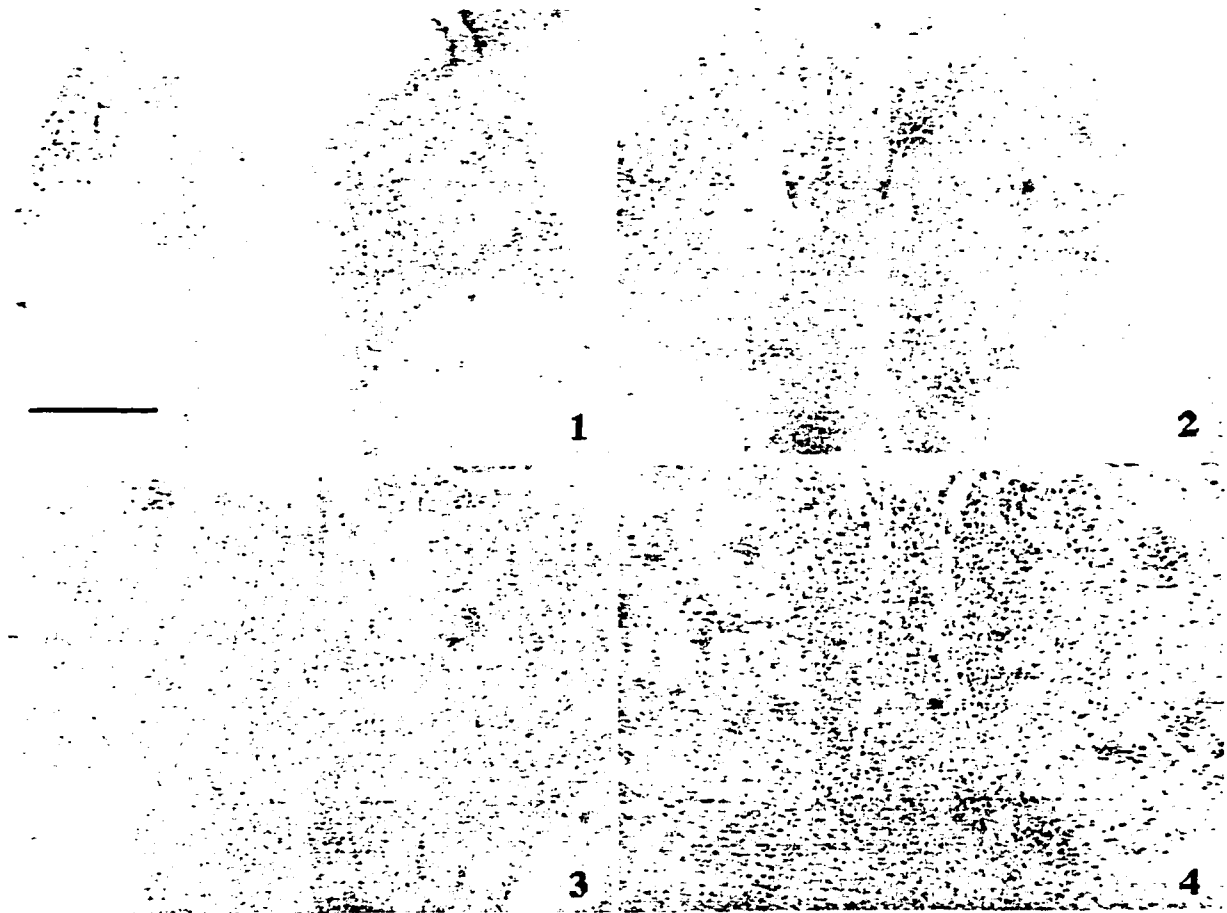


Figure 9. Example of absence of Jun-B immunoreactivity in a representative PVT section from anterior (#1) to medial (#4) (scale bar 200 μm).

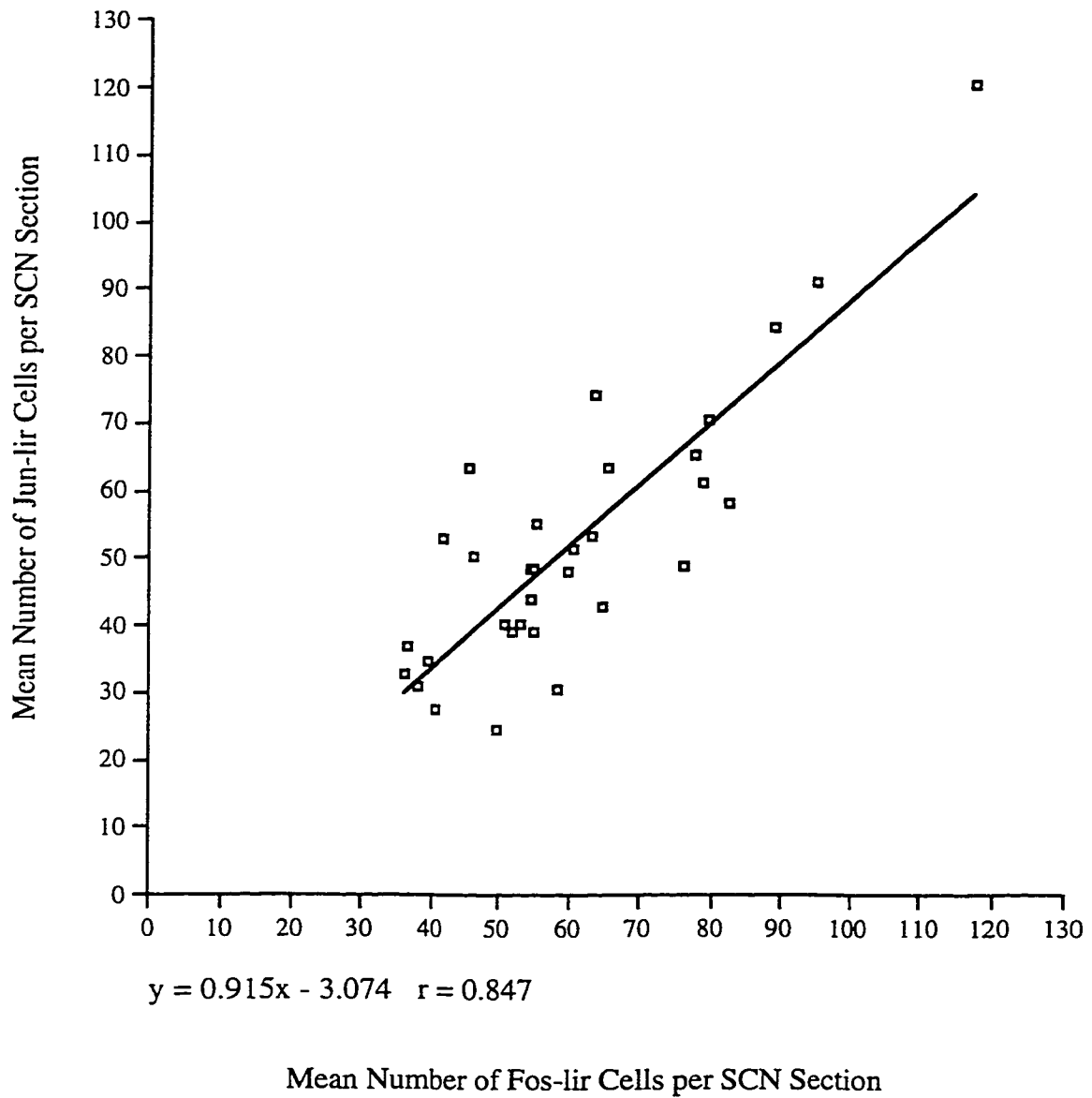


Figure 10. Scatterplot with the regression line and equation representing the relationship between Fos and Jun-B immunoreactivities in the SCN.

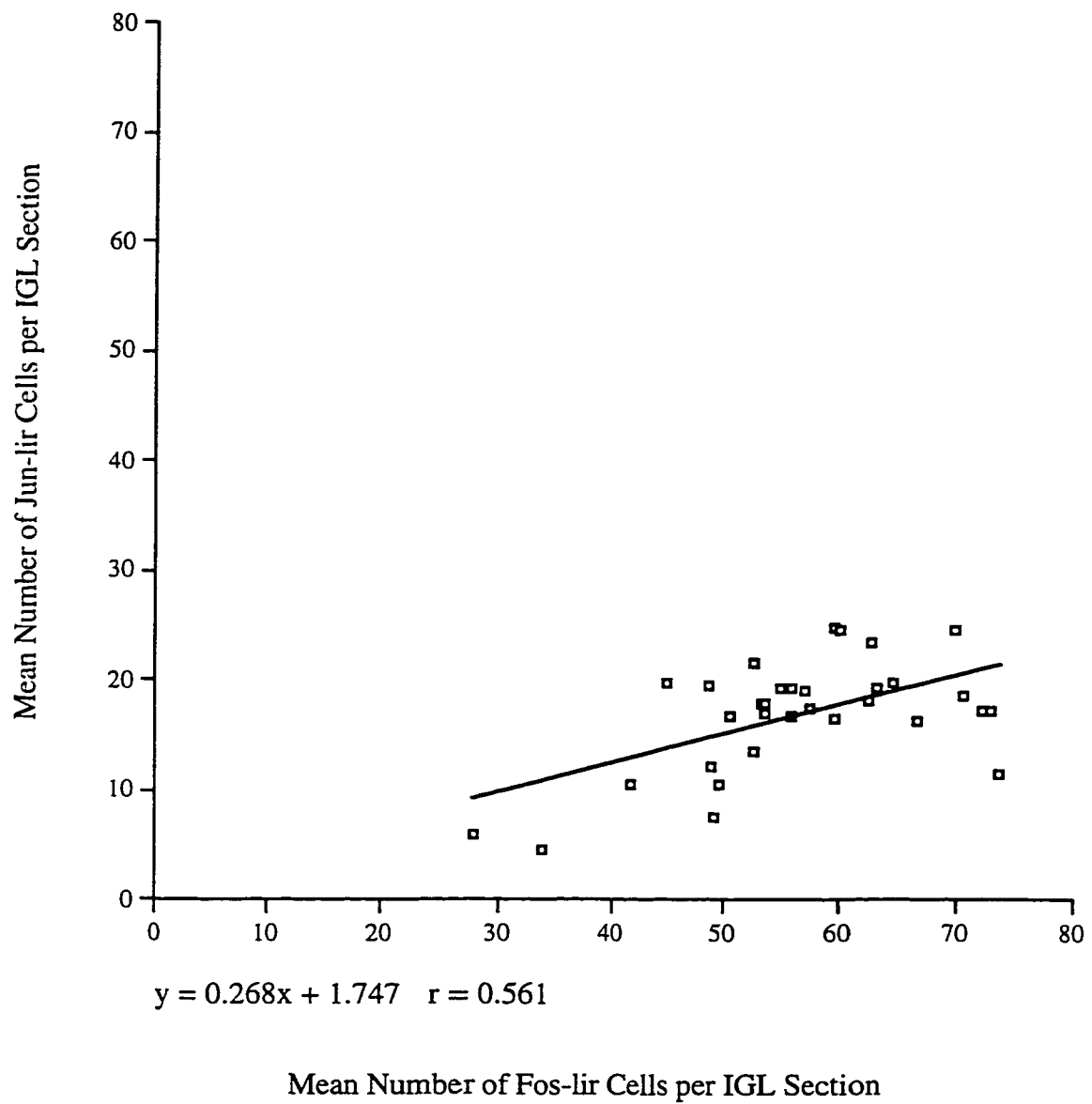


Figure 11. Scatterplot with the regression line and equation representing the relationship between Fos and Jun-B immunoreactivities in the IGL.

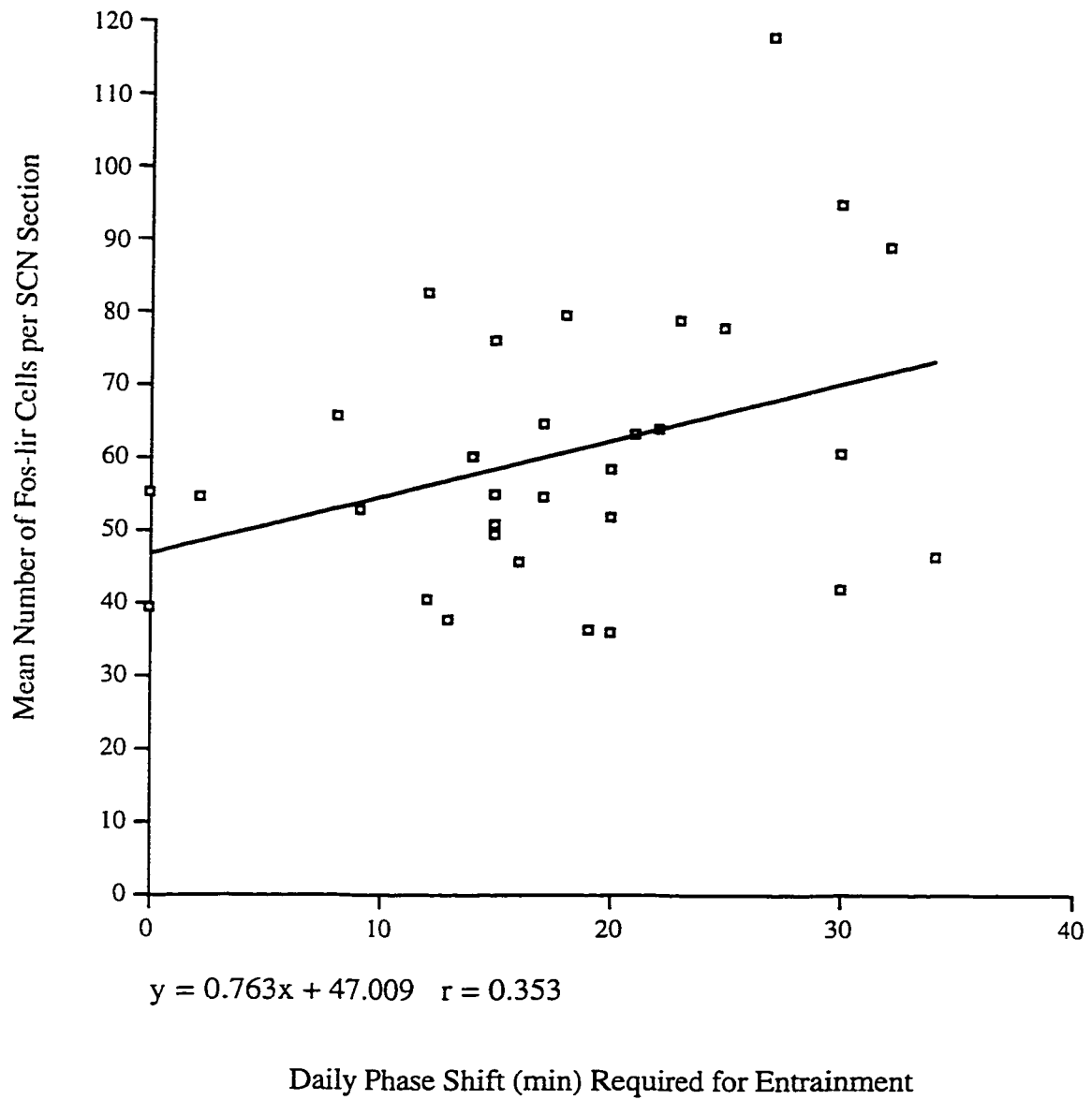


Figure 12. Scatterplot with the regression line and equation representing the relationship between Fos immunoreactivity in the SCN and the phase shift required for entrainment.

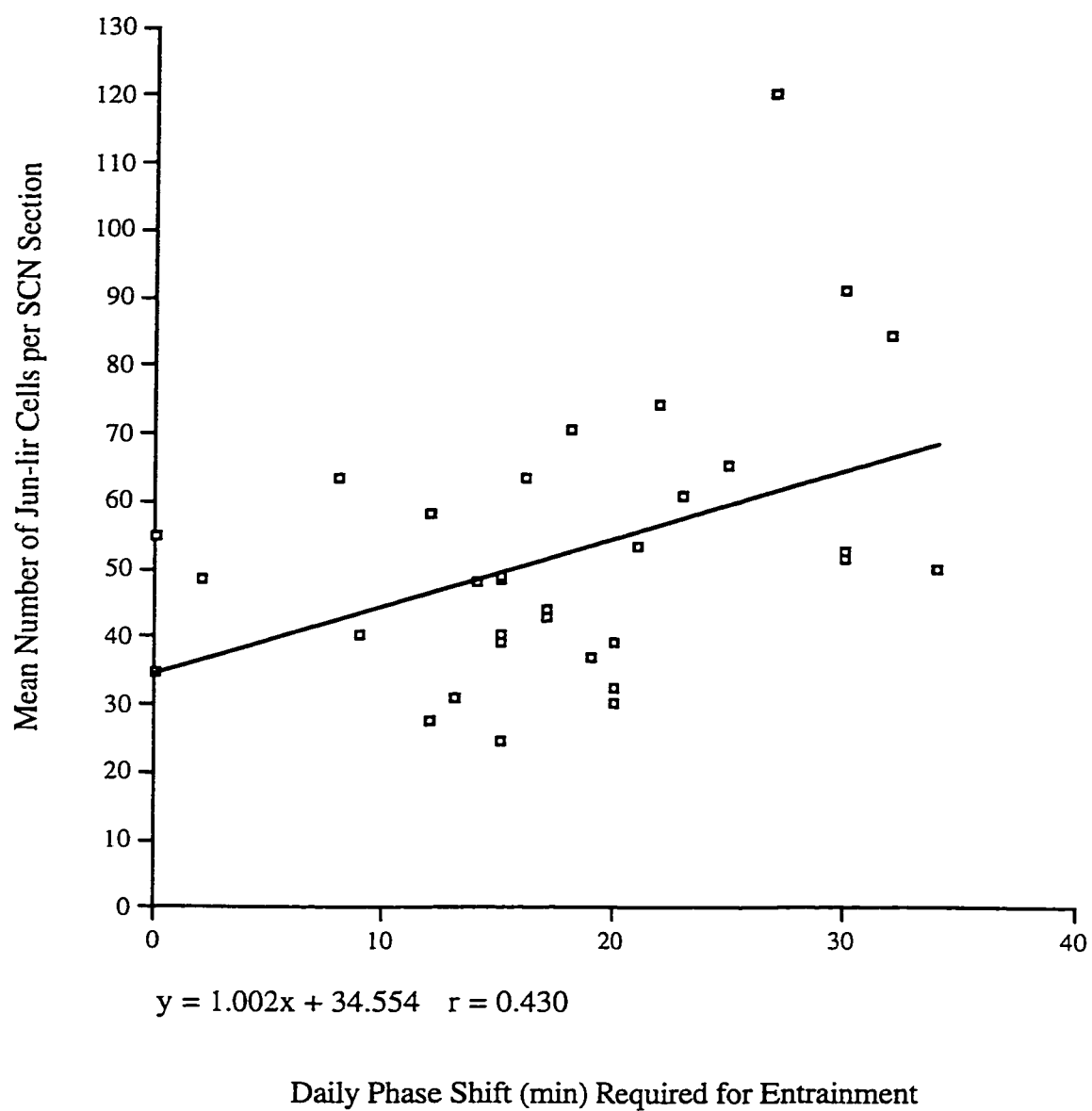


Figure 13. Scatterplot with the regression line and equation representing the relationship between Jun-B immunoreactivity and the phase shift required for entrainment.

Discussion

The results of the present study demonstrate a positive correlation between the number of Fos and the number of Jun-B immunoreactive cells within the SCN and the IGL. Further, a positive correlation between the number of both Fos and Jun-B immunoreactive cells and the magnitude (in minutes) of the daily phase shift required for entrainment was demonstrated in the SCN, but not in the IGL. Furthermore, the study demonstrates different patterns of expression of Fos and Jun-B within the three regions investigated in response to the present experimental conditions. Fos was strongly induced in the SCN, IGL and PVT whereas Jun-B was strongly induced in the SCN, weakly induced in the IGL and not induced in the PVT. Taken together, the data suggest that, within the context of entrainment, the amount of Fos and Jun-B expression can be predicted from the amount of daily phase shift required for entrainment.

The quantification of Fos and Jun-B in alternate brain sections within the same animal allowed for a more meaningful comparison between the magnitude of the expression of the two genes that could not be made when labeling is performed on separate animals. The strong positive relationship (almost a one to one) between Fos and Jun-B immunoreactivities within the SCN adds support to the idea that these two genes act in concert within the same cell to initiate clock resetting. It has been reported that Fos and Jun-B colocalize in SCN neurons (Takeuchi *et al.*, 1993), but the extent of this colocalization is undocumented. The only way to ascertain that all the cells expressing Fos in response to entraining light also express Jun-B would be to perform and quantify a double immunofluorescent staining within the same brain section.

The positive correlation between Fos and Jun-B immunoreactivities in the IGL, although significant, was not as high as in the SCN. Caution is needed however when interpreting Jun-B immunoreactivity in the IGL in the present experiment since the intensity of labeling (the darkness of the stain) was greatly inferior to that of Fos (see below). The

absence of significant correlation between the magnitude of the daily phase shift and the amount of Fos and Jun-B expression in the IGL suggests that the role of these genes in clock resetting is not the same in the IGL as it is in the SCN. Indeed the role of the IGL in daily phase resetting is unresolved. It has been suggested that the IGL is not critical for daily entrainment but serves only to modulate SCN activity by providing additional information about the external environment (Harrington and Rusak, 1986; Pickard, Ralph and Menaker, 1987). Recent data however suggests that the IGL is critical for entrainment to a skeleton photoperiod (Edelstein and Amir, 1998b, submitted).

It has been reported repeatedly that the magnitude (in minutes) of a phase shift induced within the context of the determination of a phase response curve positively correlates with the amount of immediate-early gene expressed within the SCN (Aronin *et al.*, 1990; Earnest *et al.*, 1990). The induction of immediate-early genes associated with this daily entrainment conveys a behavioral significance that does not exist in a context of phase response curve determination. Thus the entrainment context is qualitatively different from the phase shifting context. It is gratifying, therefore, to observe a significant relationship between the magnitude (in minutes) of the daily phase shift required for entrainment and the number of immunoreactive cells (both Fos and Jun-B) within the SCN in the entrainment context.

Although the range of daily phase shifts in the present study is quite narrow, the data argue in favor of a linear relationship between the task involved in clock entrainment (phase resetting) and the mechanism by which it is believed to be initiated in the SCN (immediate-early gene induction). However, the error associated with the prediction of the number of cells expressing immediate-early genes from the length of the daily phase shift necessitates some reservation about the accuracy of such predictions. In the present experiment, the lack of strength of the relationship can be attributed, in part, to the narrow range of daily phase shifts required for entrainment. Further, all animals in the present study exhibited long periods which resulted in entrainment at dawn. Replicating this

experiment using T-Cycles inducing a range of phase shifts from a 90 minutes phase advance to a 90 minutes phase delay would facilitate the determination and quantification of the relationship between the amount and direction of clock resetting and immediate-early gene expression. Another way to approach this problem would be to use a species for which mutants that display exaggerated long or short periods exist (such as the Tau mutant hamsters; Ralph and Menaker, 1988).

The immunocytochemical data from the present study provide information on the transcription factors activated by entraining light. Two findings are of special interest, first, the strong induction of Fos within the IGL in response to a 10 minutes light pulse; and second, the differential magnitude of expression of Jun-B in the three brain regions investigated. The robust induction of Jun-B within the IGL in response to 10 minutes of light is consistent with data from the Amir laboratory that show robust Fos in the IGL with short light pulses (10 and 15 minutes). It contrasts, however, with a report that robust light-induction of Fos within the IGL requires a minimal stimulus duration of more than 60 minutes (Park *et al.*, 1993). Differences in the antibody used might explain these discrepant results. In the present study an antibody which recognizes residue 4-17 of the *c-fos* gene and is not cross-reactive with other members of the Fos family nor with Fos-related antigens was used. Given that Fos-related antigens are induced more slowly and persist longer than Fos (Dragunow, Leah, and Faull, 1991), the pattern of results obtained by Park *et al.* could be explained only if the antibody used in that study was sensitive specifically to Fos-related antigens.

As noted earlier, the photic induction of Jun-B within the three brain regions under investigation varied greatly in intensity of staining observed by the darkness of the stain. It is surprising that in the IGL, the intensity of Jun-B immunoreactivity was much less than that seen in the SCN where the intensity of Jun-B was similar to Fos. The low intensity of the immunoreactivity likely underestimate the number of cells expressing Jun-B in response to light. The latter could explain the 2:1 ratio for the number of Fos:Jun-B cells obtained for

the IGL. If Fos must dimerize with Jun-B to form the AP-1 activator complex in the IGL, such low levels of staining for Jun-B might imply that dimerization is limited by the amount of Jun-B or, that Fos dimerizes with another member of the Jun family such as Jun-D or c-Jun, both of which are present in the IGL (Herdegen *et al.*, 1995). Thus, Jun-B might not be linked to the transduction of photic input to the same extent in the IGL as it is within the SCN. However, caution is needed when assuming quantity of Jun-B protein within a single neuron from the intensity of immunocytochemical staining since it is impossible to presume protein quantity from darkness of the stain.

Another possible explanation for the differential induction of Jun-B within the IGL is that photic activation of Jun-B transcription within neurons of the IGL follows a different time course than it does in the SCN. Animals were perfused one hour after light onset; it might be that Jun-B expression did not reach its full magnitude within this time frame. Performing the perfusion two hours after stimulus onset could easily answer the question. A third explanation relates to the duration of the stimulus required for strong induction of Jun-B in the IGL. The length of the stimulus used (10 minutes) might not be sufficient to induce dark Jun-B staining. Such explanation is similar to the inconsistent reports of Fos induction in the IGL (Park *et al.*, 1993).

In the present experiment, the PVT was examined in an attempt to gain knowledge about its possible role within the circadian system. The induction of Fos observed in the PVT cannot be said to be light-induced due to a lack of dark control animals. In addition, the fact that wakefulness appears to be the key factor in stimulating Fos within PVT neurons (Peng *et al.*, 1995) suggest that light might not be responsible for the Fos observed in the present study. Interestingly, an absence of Jun-B immunoreactivity in the present experiment suggests that, in the PVT, Fos does not dimerize with Jun-B to form the AP-1 activator complex. Reports show that PVT neurons express, in addition to Fos and Jun-B, c-Jun, Jun-D that could act with Fos to form the AP-1 activator (Herdegen *et al.*, 1995). Further, the PVT expresses another transcription factor, Krox-24 (known as

egr-1), which could induce late gene transcription within PVT neurons (Herdegen *et al.*, 1995). Again, from the present results, it is not possible to point to a role for the PVT in circadian functioning.

In the present study, molecular information was obtained for only two of the light inducible immediate-early genes (Fos and Jun-B). An investigation of the remaining four (*fos-B*, *zif268*, *nur77*, and *egr-3*) light -induced transcription factors within a similar entrainment context would contribute to a better understanding of their relationship, if there is one, with daily resetting. In addition, the PVT expresses neither Fos nor Jun-B in response to light. Rather, the Fos expression reported in the present experiment is likely associated with wakefulness of the animal. However, other immediate-early genes are present within this structure and specific investigation of those genes could reveal a connection with photic transmission unappreciated when using Fos and Jun-B.

In summary, the present experiment provides evidence for a relationship between the induction of Fos and Jun-B within SCN neurons and the magnitude of clock resetting under conditions of entrainment. In addition, the quantification of Fos and Jun-B within the same animal yielded information about the relation between the magnitude of induction of Fos and Jun-B previously unappreciated when labeling was performed in different animals. The data suggest that, in areas other than the SCN, Jun-B is not always associated with Fos to mediate the effects of light on the circadian system.

References

- Amir, S., and Stewart, J. (1996). Resetting the clock by a conditioned stimulus. Nature, 379, 542-545
- Amir, S., and Stewart, J. (1998a). Induction of fos expression in the circadian system by unsignaled light is attenuated as a result of previous experience with signaled light: a role for pavlovian conditioning. Neuroscience, 83 (3), 657-661.
- Amir, S., and Stewart, J. (1998b). Conditioning in the circadian system. Chronobiology International, in press.
- Azmitia, E. C., and Segal, M. (1978). An autoradiographic analysis of the differential ascending projections of the dorsal and medial raphé nuclei in the rat. Journal of Comparative Neurology, 179, 641-668.
- Aronin, N. Sagar, S. M., Sharp, F. R., & Schwartz, W. j. (1990). Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei. Proceedings of the National Academy of Sciences, 87, 5959-5962.
- Beaulé, C. and Amir, S. (1997). Experience with signaled light alters the response of the suprachiasmatic nucleus to unsignaled light. Society for Neuroscience Abstract, 18, 789.
- Buijs, R. M., Markmann, M., Nunes-Cardoso, B., Hou, Y. X., & Shinn, S. (1993). Projections of the suprachiasmatic nucleus to stress-related areas in the rat

hypothalamus: a light and electron microscopic study. Journal of Comparative Neurology, 335 (1), 42-54.

Card, J. P. and Moore, R. Y. (1989). Organization of lateral geniculate-hypothalamic connections in the rat. Journal of Comparative Neurology, 284, 135-147.

Ding, J. M., Fairman, L. E., Hurst, W. J., Kuriashkina, L. R., and Gillette, M. U. (1997). Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide. Journal of Neuroscience, 17, 667-675.

Ding, J. M., Shen, D., Weber, T. T., Fairman, L. E., Rea, M. A., and Gillette, M. U. (1994). Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. Science, 266, 1713-1717.

Dragunow, M., Leah, J. D., and Faull, R. L. M. (1991). Prolonged and selective induction of fos-related antigen(s) in striatal neurons after 6-hydroxydopamine lesions of the rat substantia nigra pars compacta. Molecular Brain Research, 10, 355-358.

Earnest, D. J., Iadarola, M., Yeh, H. H., and Olschowka, J. A. (1990). Photic regulation of *c-fos* expression in neural components governing the entrainment of circadian rhythms. Experimental Neurology, 109, 353-361.

Eastman, C. and Rechtschaffen, A. (1983). Circadian temperature and wake rhythms of rats exposed to continuous illumination. Physiology and Behavior, 31, 417-427.

- Ebling, F. J., Maywood E. S., Humby T., Hastings M. H. (1992). Circadian and photoperiodic time measurement in male Syrian hamsters following lesions of the melatonin-binding sites of the paraventricular thalamus. Journal of Biological Rhythms, 7(3), 241-254
- Edelstein, K. & Amir, S. (1996). Constant light induces persistent Fos expression in rat intergeniculate leaflet. Brain Research, 731, 221-225.
- Edelstein, K., and Amir, S. (1998a). Glutamatergic antagonists do not attenuate light-induced fos protein in rat intergeniculate leaflet. Manuscript submitted for publication to Brain Research.
- Edelstein, K., and Amir, S. (1998b). The intergeniculate leaflet is necessary for entrainment of circadian rhythms to a skeleton photoperiod. Manuscript submitted for publication to the Journal of Neuroscience.
- Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., and Greenberg, M. E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science, 260, 238-241.
- Harrington, M. E., and Rusak, B. (1986). Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. Journal of Biological Rhythms, 1, 309-325.
- Harrington, M. E., Nance, D. M. and Rusak, B. (1987). Double-labeling of neuropeptide Y-immunoreactive neurons which project from the geniculate to the suprachiasmatic nuclei. Brain Research, 410, 275-282.

- Herdegen, T., Kovary, K., Buhl, A., Bravo, R., Zimmermann, M., and Gass, P. (1995). Basal expression of the inducible transcription factors c-Jun, JunB, JunD, c-Fos, FosB, and Krox-24 in the adult rat brain. The Journal of Comparative Neurology, 354, 39-56.
- Honrado, G. I., Johnson, R. S., Golombek, D. A., Spiegelman, B. M., Papaioannou, V. E., and Ralph, M. R. (1996). The circadian system of *c-fos* deficient mice. Journal of Comparative Physiology, 178, 563-570.
- Hunter, T., and Karin, M. (1992). The regulation of transcription by phosphorylation. Cell, 70, 375-387.
- Johnson, R. F., Morin, L. P., and Moore, R. Y. (1988). Retinohypothalamic projections in the hamster and rat demonstrated using cholera toxin. Brain Research, 462, 301-312.
- Johnson, R. S., Spiegelman, B. M., and Papaioannou, V. (1992). Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene. Cell, 71, 577-586.
- Janik, D., and Mrosovsky, N. (1992). Gene expression in the geniculate induced by a nonphotic circadian phase shifting stimulus. Neuroreport, 3, 575-578.
- Kalsbeek, A., Teclemariam-Mesbah, R., & Pevet, P. (1993). Efferent projections of the suprachiasmatic nucleus in the golden hamster (*Mesocricetus auratus*). Journal of Comparative Neurology, 332 (3), 293-314.

- Kornhauser, J. M., Nelson, D. E., Mayo, K. E., & Takahashi, J. S. (1990). Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. Neuron, 5, 127-134.
- Kornhauser, J. M., Nelson, D. E., Mayo, K. E., & Takahashi, J. S. (1992). Regulation of jun-B messenger RNA and AP-1 activity by light and a circadian clock. Science, 255, 1581-1584.
- Meijer, J. H. & Rietveld, W. J. (1989). Neurophysiology of the suprachiasmatic circadian pacemaker in rodents. Physiological Reviews, 69, 671-702.
- Mikkelsen, J. D. (1990). Projections from the lateral geniculate nucleus to the hypothalamus of the Mongolian gerbil (*Meriones unguiculatus*): an anterograde and retrograde tracing study. Journal of Comparative Neurology, 299, 493-508.
- Mikkelsen, J. D., Larsen, P. J., and Ebling F. J. (1993). Distribution of N-methyl-D-aspartate (NMDA) receptor mRNAs in the rat suprachiasmatic nucleus. Brain Research, 632, 329-333.
- Mikkelsen, J. D., Larsen, P. J., Mick, G., Vrang, N., Ebling, F. J. P., Maywood, E. S., Hastings, M. H. and Moller, M. (1995). gating of retinal inputs through the suprachiasmatic nucleus: role of excitatory neurotransmission. Neurochemistry International, 27 (3), 263-272.
- Moga, M. M. and Moore, R. Y. (1996). Putative excitatory amino acid projections to the suprachiasmatic nucleus in the rat. Brain Research, 743, 171-177.

- Moga, M. M. and Moore, R. Y. (1997). Organization of the neural inputs to the suprachiasmatic nucleus in the rat. Journal of Comparative Neurology, 389, 508-534.
- Moore, R. Y. (1983). Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. Federation Proceedings, 42, 2783-2789.
- Moore, R. Y. & Card, J. P. (1985). Visual pathways and the entrainment of circadian rhythms. Annals of the New York Academy of Sciences, 453, 123-133.
- Moore, R. Y. and Lenn, N. J. (1972). A retinohypothalamic projection in the rat. Journal of Comparative Neurology, 146, 1-14.
- Morris, M. E., Viswanathan, N., Kuhlman, S., Davis, F. C., and Weitz, C. J. (1998). A screen for genes induced in the suprachiasmatic nucleus by light. Science, 279, 1544-1547.
- Park, H. T., Baek, S. Y., Kim, B. S., Kim, J. B. & Kim, J. J. (1993). Profile of Fos-like immunoreactivity induction by light stimuli in the intergeniculate leaflet is different from that of the suprachiasmatic nucleus. Brain Research, 610, 334-339.
- Paxinos, G., and Watson, C. (1986). The Rat Brain in Stereotaxic Coordinates, 2nd Ed., San Diego: Academic Press.
- Peng, Z. C., Grassi-Zucconi, G., and Bentivoglio, M. (1995). Fos-related protein expression in the midline paraventricular nucleus of the rat thalamus: basal

oscillations and relationship with limbic efferents. Experimental Brain Research, 104, 21-29.

Pickard, G. E. (1985). Bifurcating axons of retinal ganglion cells terminate in the hypothalamic suprachiasmatic nucleus and the intergeniculate leaflet of the thalamus. Neuroscience Letters, 55, 211-217.

Pickard, G. E., Ralph, M. R., and Menaker, M. (1987). The intergeniculate leaflet partially mediates effects of light on circadian rhythms. Journal of Biological Rhythms, 2, 35-56.

Pickard, G. E., Weber, E. T., Scott, P. A, Riberdy, A. F., and Rea, M. A. (1996). 5HT_{1B} receptor agonists inhibit light-induced phase shifts of behavioral circadian rhythms and expression of the immediate early gene *c-fos* in the suprachiasmatic nucleus. The Journal of Neuroscience, 16 (24), 8208-8220.

Pittendrigh, C. S. (1981). Circadian system: entrainment. In J. Aschoff (Ed.), Handbook of Behavioral Neurobiology: Vol. 4. Biological Rhythms (95-124). New-York: Plenum Press

Pittendrigh, C. S. and Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as a clock. Journal of Comparative Physiology, 106, 291-331.

Ralph, M. R., and Menaker, M. (1988). A mutation of the circadian system in the golden hamsters. Science, 241, 1225-1227.

- Rea, M. A. (1989). Light increases fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. Brain Research Bulletin, *23*, 577-581.
- Rea, M. A., Michel, A. M., and Lutton, L. M. (1993). Is fos expression necessary and sufficient to mediate light-induced phase advances of the suprachiasmatic circadian oscillator? Journal of Biological Rhythms, *8*, supp. S59-S64.
- Rusak, B., McNaughton, L., Robertson, H. A., and Hunt, S. P. (1992). Circadian variation in photic regulation of immediate-early gene mRNA in rat suprachiasmatic nucleus cells. Molecular Brain Research, *14*, 124-130.
- Rusak, B., Robertson, H. A., Wisden, W., and Hunt, S. P. (1990). Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. Science, *248*, 1237-1240.
- Rusak, B. and Zucker, I. (1979). Neural regulation of circadian rhythms. Physiological Reviews, *59*, 449-526.
- Schwartz, W. J., Peters, R. V., Aronin, N., and Bennett, M. R. (1996). Unexpected *c-fos* gene expression in the suprachiasmatic nucleus of mice entrained to a skeleton photoperiod. Journal of Biological Rhythms, *11* (1), 35-44.
- Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M., and Aronin, N. (1994). Temporal regulation of light -induced fos and fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. Neuroscience, *58*, 573-583.

- Schwartz, W. J., and Zimmerman, P. (1990). Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. Journal of Neuroscience, *10*, 3685-3694.
- Selim, M., Glass, J. D., Hauser, U. E., and Rea, M. A. (1993). Serotonergic inhibition of light-induced fos protein expression and extracellular glutamate in the suprachiasmatic nuclei. Brain Research, *621*, 181-188.
- Speh, J. C. and Moore, R. Y. (1992). Retinal afferents as demonstrated with unconjugated cholera toxin. Society for Neuroscience Abstract, *18*, 876.
- Stephan, F. K. (1983). Circadian rhythms in the rat: constant darkness, entrainment to T cycles and skeleton photoperiods. Physiology and Behavior, *30*, 451-462.
- Takahashi, J. S., & Zatz, M. (1982). Regulation of circadian rhythmicity. Science, *217*, 1104-1111.
- Takeuchi, J., Shannon, W., Aronin, N., and Schwartz, W. J. (1993). Compositional changes of AP-1 DNA binding proteins are regulated by light in a mammalian circadian clock. Neuron, *11*, 825-836.
- Turek, F. W. (1985). Circadian neural rhythms in mammals. Annual Reviews in Physiology, *47*, 49-64.
- van den Pol, A. N., and Tsujimoto, K. L. (1985). Neurotransmitters of the hypothalamic suprachiasmatic nucleus: Immunocytochemical analysis of 25 neuronal antigens. Neuroscience, *15*, 1049-1086.

- Vindlacheruvu, R. R., Ebling, F. J. P., Maywood, E. S., & Hastings, M. H. (1992). Blockade of glutamatergic neurotransmission in the suprachiasmatic nucleus prevents cellular and behavioral responses of the circadian system to light. European Journal of Neuroscience, *4*, 673-679.
- Vrang, N., Larsen, P. J., Moller, M., & Mikkelsen, J. D. (1995). Topographical organization of the rat suprachiasmatic-paraventricular projection. Journal of Comparative Neurology, *353* (4), 585-603.
- Wisor, J. P., and Takahashi, J. S. (1997). Regulation of the *vgf* gene in the golden hamster suprachiasmatic nucleus by light and by the circadian clock. Journal of Comparative Neurology, *378*, 229-238.
- Wollnick, F., Brysch, W., Uhlmann, E., Gillardon, F., Bravo, R., Zimmerman, M., Schlingensiepen, K. H., and Herdegen, T. (1995). Block of c-Fos and JunB expression by antisense oligonucleotides inhibits light-induced phase shifts of the mammalian circadian clock. European Journal of Neuroscience, *7*, 388-393.
- Zucker, I., Rusak, B., & King, R. G., Jr. (1976). Neural bases for circadian rhythms in rodent behavior. In A. H. Riesen & R. F. Thompson (Eds.), Advances in Psychobiology, Vol. 3 (pp. 35-74). New-York: Wiley.

Appendices

Appendix A

Conditioning protocol. These animals were also used in a conditioning experiment aimed at examining the modulation of immediate-early gene expression in response to light paired with a neutral stimulus. It was previously shown that light previously paired with a neutral stimulus in a pavlovian conditioning protocol induced less Fos in the circadian system when, on test day, the CS was removed (Amir and Stewart, 1996, 1998a, 1998b in press; Beaulé and Amir, 1997). In the previous studies (except Beaulé and Amir, 1997), the conditioning protocol was performed on a light pulse presented during the dark phase of the activity cycle of rats entrained to a 12:12 LD cycle or a skeleton photoperiod. Animals in the present study were entrained to a 0.5h:23.5h LD cycle. The pairings of the neutral stimulus with light was performed on the daily entraining light pulse in an attempt to find a similar modulation of the response to a light but this time in an entrainment context.

As described in the present method section, these rats were first entrained to a 12:12 LD cycle, allowed to free-run for 10-15 days, put back on their original 12:12 LD cycle for 5-10 days and transferred to a 0.5h:23.5h LD cycle. For 18 animals, the entraining light pulse was always preceded by a 20 minute air flow that overlapped with light for 5 minutes. Fourteen animals received explicitly unpaired presentation of the 20 minutes air flow and entraining light. In this group, the airflow was presented once a day, at a different time each day and never within 2 hours of the entraining light pulse. The pairings lasted 14 days.

On the fifteenth day, animals received either light alone or light and fan but this time, light was only 10 minutes. Four groups were formed: conditioning during entrainment, light only on test day (CS-US/US, n = 10); conditioning during entrainment, light and fan on test day (CS-US/CS-US, n = 8); no conditioning during entrainment, light

only on test day (CS≠US/US, n = 8) and no conditioning during entrainment, light and fan on test day (CS≠US/CS-US, n = 6).

Appendix B

Correlation Matrix.

	Fos SCN	Fos IGL	Fos PVT	Jun SCN	Jun IGL	Resetting
Fos SCN	1.000	0.303	0.208	0.847*	0.258	0.353*
Fos IGL		1.000	0.446*	0.044	0.561*	-0.159
Fos PVT			1.000	0.086	0.497*	0.129
Jun SCN				1.000	0.102	0.430*
Jun IGL					1.000	-0.170
Resetting						1.000

* = significant correlations.