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The role of the intergeniculate leaflet in the circadian response to light

Kim Edelstein

A Thesis

in

the Department

of

Psychology

Presented in Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

at Concordia University

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ABSTRACT

The role of the intergeniculate leaflet in the circadian reponse to light

Kim Edelstein, Ph.D.

Concordia University, 1998

Mammalian circadian rhythms are generated and regulated by a pacemaker located in the suprachiasmatic nucleus (SCN) in the hypothalamus. Transmission of photic information to the SCN occurs via a direct projection from the retina, the retinohypothalamic tract, and an indirect projection from the retinorecipient intergeniculate leaflet (IGL), the geniculohypothalamic tract. The retinohypothalamic tract is necessary for synchronization of rhythms to environmental light-dark cycles. In contrast, although the IGL is strategically located to convey light information to the SCN, a role for the IGL in photic entrainment has not been established. IGL-lesioned hamsters have been shown to entrain normally to light-dark cycles; effects of the lesion have been manifested in slower rates of re-entrainment to shifts in the light-dark cycle and small changes in the size of light-induced phase shifts.

In the present thesis, the relationship between the IGL and the circadian response to light is explored in two sets of experiments. The first three experiments evaluated activation of IGL neurons by light using immunohistochemical expression of Fos protein as a marker of cellular activation. In rodents, light-induced Fos in SCN neurons is phase dependent and is correlated with the effectiveness of such light to phase shift circadian rhythms. Furthermore, because the IGL has been implicated in the effects of nonphotic stimuli on circadian rhythms, the induction of Fos in the SCN and IGL in response to nonphotic manipulations was assessed. Results demonstrate that, in contrast to light-induced Fos in the SCN, light induces Fos protein in the IGL regardless of circadian time,

and continues to do so as long as the animal is exposed to light. Moreover, nonphotic manipulations induce Fos in both SCN and IGL. However, in the IGL, Fos expression in response to such treatments was enhanced during the light phase of the light-dark cycle. Pharmacological manipulations that attenuate both light-induced phase shift and Fos expression in the SCN are without effect on light-induced Fos expression in the IGL.

The effects of electrolytic lesions of the IGL on circadian temperature rhythms were examined under different lighting schedules in the final two experiments using a telemetry system. Rhythms in these animals were compared to those of intact controls as well as to rhythms of rats with neurotoxic damage to the visual system resulting from neonatal monosodium glutamate (MSG) treatment. IGL-lesioned rats entrained normally to light-dark cycles consisting of 12 hours of light followed by 12 hours of darkness, and showed the disruption of rhythmicity after prolonged constant light housing seen in intact animals. However, in contrast to intact and MSG-treated rats, IGL-lesioned animals exhibited free-running temperature rhythms under a skeleton photoperiod consisting of one-hour of light exposure at times corresponding to dusk and dawn. Because nocturnal animals normally experience light only around dusk and dawn, the inability to entrain to this ecologically relevant lighting schedule suggests that the IGL plays a critical role in photic entrainment.

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LIST OF ABBREVIATIONS

AP-1: activator protein 1

CREB: cyclic AMP response element binding protein

CT: circadian time

DAB: diaminobenzidine

DD: constant dark housing

dLGN: dorsal lateral geniculate nucleus

ENK: enkephalin

GFAP: glial fibrillary acidic protein

GHT: geniculohypothalamic tract

IGL: intergeniculate leaflet

I.P.: intraperitoneal

IR: immunoreactivity

LD12:12: 12-hour light, 12-hour dark cycle

LGN: lateral geniculate nucleus

LL: constant light housing

MSG: monosodium glutamate

NMDA: N-methyl-D-aspartate

NPY: neuropeptide Y

PACAP: pituitary adenylyl cyclase activating peptide

RHT: retinohypothalamic tract

SCN: suprachiasmatic nucleus

SP: substance P

VIP: vasopressin intestinal polypeptide

vLGN: ventral lateral geniculate nucleus

ZT: zeitgeber time

GENERAL INTRODUCTION

Circadian rhythmicity is a ubiquitous feature of physiological and behavioral variables in all organisms, a timekeeping system that has evolved to reflect the solar cycle of the earth's rotation (from Latin: *circa*, around and *dies*, day). In mammals, daily rhythms of sleep and wakefulness, body temperature, feeding, drinking, and hormone secretion have all been well-described (cf. Czeisler, Weitzman, Moore-Ede, Zimmerman, & Kronauer, 1981; Moore-Ede, Sulzman, & Fuller, 1982; Refinetti & Menaker, 1992; Rusak & Zucker, 1979; Zucker, 1971). The study of circadian rhythmicity in non-mammalian species includes work in birds, fish, lizards, fruitflies, molluscs, and plants. Examples of the diversity of rhythms that oscillate with the 24-hour day include photosynthesis and bioluminescence in Gonyaulax, asexual differentiation in Neurospora, optic nerve compound action potentials in the marine molluscs Bulla and Aplysia, and eclosion in Drosophila pupae (cf. Block, Khalsa, McMahon, Michel, & Guesz, 1993; Dunlap, 1990; Hall, 1995; Hastings, 1992; Morse, Fritz, & Hastings, 1990; Rusak & Zucker, 1979).

Circadian rhythms are generated endogenously by one or more pacemakers within the organism and are characterized by their self-sustained oscillation under constant environmental conditions, with a period close to 24 hours (Roenneberg & Foster, 1997; Rusak & Zucker, 1979). Exposure to environmental parameters such as the daily cycles of light and darkness resets the circadian clock resulting in synchronization of period and phase to the 24-hour day. Indeed, environmental light is necessary for stable entrainment of mammalian circadian rhythms (Moore, 1983; Morin, 1994; Pittendrigh & Daan, 1976; Roenneberg & Foster, 1997). Light presented near dusk delays the phase of the clock whereas light presented near dawn advances the phase of the clock (Daan & Pittendrigh, 1976; Roenneberg & Foster, 1997). In addition to light, a variety of nonphotic cues such

as social contact, periodic feeding, exercise, and exposure to novelty have been effective in entraining rhythms (Mistlberger, 1994; Mrosovsky, 1988; Mrosovsky, 1996).

Mammalian circadian rhythms are generated by a dominant circadian pacemaker located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. Evidence that the circadian clock is contained within the SCN is derived from several types of research (for reviews, see Klein, Moore, & Reppert, 1991; Miller, 1993; Rusak & Zucker, 1979). Early studies demonstrated that bilateral ablation of the SCN resulted in loss of circadian rhythms of drinking, locomotor activity, corticosterone secretion, and pineal serotonin N-acetyltransferase activity (Moore & Eichler, 1972; Moore & Klein, 1974; Stephan & Zucker, 1972). Electrical stimulation of the SCN phase shifted circadian activity rhythms (Rusak & Groos, 1982). Transplants of SCN tissue into SCN-lesioned hosts have been shown to restore abolished rhythms, and the period of the recovered rhythm is that of the donor (LeSauter, Lehman, & Silver, 1996; Ralph, Foster, Davis, & Menaker, 1990). SCN cells have been shown to exhibit circadian rhythms in glucose metabolism and electrical activity, both in vivo and in vitro (Green & Gillette, 1982; Inouye & Kawamura, 1979; Newman & Hospod, 1986; Schwartz & Gainer, 1977; see Klein et al., 1991 for review). The metabolic activity rhythm of the SCN is present in utero, and is entrained to the 24-hour day via the maternal SCN (Reppert & Schwartz, 1983; see Reppert, 1995 for review). Moreover, it has been shown that the mean circadian period of the firing rate rhythms in individual SCN neurons in vitro is similar to the circadian period of behavioral rhythms in vivo (Liu, Weaver, Strogatz, & Reppert, 1997; Welsh, Logothetis, Meister, & Reppert, 1995). Taken together, these findings have established the SCN as the site of the central pacemaker regulating circadian rhythmicity in mammals.

Photic information is transmitted to the SCN via two principal pathways; a direct excitatory projection from the retina, the retinohypothalamic tract (RHT) and an indirect projection originating in the retinorecipient intergeniculate leaflet (IGL), part of the

lateral geniculate complex of the thalamus (the geniculohypothalamic tract (GHT); cf. Card & Moore, 1991; Harrington, 1997; Moore & Card, 1994; Morin, 1994). The retinal ganglion cells that project to the SCN have been shown to be unique to the circadian system (Moore, Speh, & Card, 1995), and damage to pathways involved in visual perception alone does not prevent photic entrainment (Dark & Asdourian, 1975; Harrington & Rusak, 1988; Johnson, Moore, & Morin, 1988; Johnson, Moore, & Morin, 1989; Pickard, Ralph, & Menaker, 1987). Visually impaired animals such as the retinally degenerate mouse, the blind mole rat (Spalax ehrenbergi), or rats treated neonatally with the neurotoxin monosodium glutamate (MSG), have been shown to entrain to light-dark (LD) cycles (Edelstein, Pfaus, Rusak, & Amir, 1995; Foster, Provencio, Hudson, Fiske, De Grip, & Menaker 1991; Goldman, Goldman, Riccio, & Terkel, 1997; Pickard, Turek, Lamperti, & Silverman, 1982). In humans, evidence for photic entrainment has been observed in blind individuals who continue to show light-induced melatonin suppression despite the absence of conscious light perception (Czeisler et al., 1995).

Although it is accepted that entrainment of circadian rhythms by light is not a function of the image-forming visual system, the physiological mechanisms underlying photic entrainment are not well understood. Because only disruption of the RHT, and not any other retinofugal projection, has been shown to prevent photic entrainment to normal LD cycles, the RHT is thought to be both necessary and sufficient for photic entrainment (Dark & Asdourian, 1975; Harrington & Rusak, 1988; Johnson et al., 1988; Johnson et al., 1989; Pickard et al., 1987). In contrast, a role for the GHT in the circadian response to light has not been well established. Evidence from IGL lesion studies suggests that the IGL modulates the rate of re-entrainment to shifted LD cycles (Harrington, 1997; Johnson et al., 1989; Pickard, 1989; Zucker, Rusak, & King, 1976). Hamsters with IGL lesions have been shown to exhibit smaller light-induced phase advances (Harrington & Rusak, 1986; Pickard et al., 1987) and larger phase delays (Pickard et al., 1987) compared to intact controls. Longer free running periods have been

observed in IGL-lesioned mice housed in constant darkness (DD; Pickard, 1994), whereas in hamsters conflicting data have been reported (Harrington & Rusak, 1986; Pickard et al., 1987). The IGL is thought to mediate the effects of prolonged constant light (LL) exposure on circadian rhythms in hamsters (Harrington & Rusak, 1986; Harrington & Rusak, 1988; Harrington, 1997; Pickard et al., 1987) but not mice (Pickard, 1994). Hamsters with IGL lesions failed to exhibit the increases in circadian period and splitting of wheel running rhythms into two or more components that have been commonly observed in intact animals under this lighting schedule (Harrington & Rusak, 1986; Harrington & Rusak, 1988; Harrington, 1997; Pickard et al., 1987). Electrophysiological data have indicated that most IGL neurons show sustained activated responses to continuous light (Hale & Sefton, 1978; Harrington & Rusak, 1989; Sumitomo, Sugitani, Fukuda, & Iwama, 1979; Zhang & Rusak, 1989; reviewed in Harrington, 1997), supporting the idea that the IGL is involved in the tonic effects of light on the circadian system. However, the finding that IGL lesion does not prevent entrainment to normal LD cycles (Dark & Asdourian, 1975; Harrington & Rusak, 1988; Johnson et al., 1989; Pickard et al, 1987; Moore and Card, 1994; Harrington, 1997) suggests only a minor contribution of the GHT to the photic entrainment process.

In contrast with the contribution of the IGL to photic entrainment, it is well established that the IGL is necessary for the response of the circadian system to nonphotic cues (see Mrosovsky, 1995; Harrington, 1997 for reviews). Nonphotic stimuli have been shown to phase shift circadian rhythms in a manner that resembles the effects of IGL stimulation or intra-SCN infusion of neuropeptide Y (NPY; one of the transmitters contained in IGL neurons that project to the SCN via the GHT; Albers & Ferris, 1984; Rusak, Meijer, & Harrington, 1989; Shibata & Moore, 1993; reviewed in Mrosovsky, 1995). In contrast with the phase-shifting effects of light during the subjective night, nonphotic phase shifts are characterized by large phase advances during the subjective day (Mrosovsky, 1995). Several experiments have demonstrated that IGL

lesion prevents or attenuates the phase-shifting responses to a variety of nonphotic stimuli including benzodiazepine injection (Biello, Harrington, & Mason, 1991; Marchant, Watson, & Mistlberger, 1997; Maywood, Smith, Hall, & Hastings, 1997; Meyer, Harrington & Rahmani, 1993; Janik & Mrosovsky, 1994), daily saline injection (Maywood et al., 1997) and novel running wheel exposure (Janik & Mrosovsky, 1994; Wickland & Turek, 1994). The results of these lesion studies support a predominant role for the IGL in the response to nonphotic stimuli. However, recent work demonstrating an interaction between the effects of NPY and the circadian response to light suggest that the nature of the relationship between the IGL and photic entrainment has yet to be fully elucidated.

The work described in the present thesis evaluates the role of the IGL in the circadian response to light. Responses of IGL cells under various lighting conditions were studied using the immunohistochemical expression of Fos, protein product of the immediate early gene c-fos, as a marker of neuronal activation. In addition, circadian temperature rhythms were measured using a telemetry system, under the same lighting conditions, to compare intact or sham-operated rats with both IGL-lesioned animals and animals treated neonatally with MSG .

In the following sections, an overview of the role of the IGL in the rat circadian system will be given, with emphasis on the anatomical connections and neurochemical content of the IGL. The effects of environmental light and circadian phase on NPY-IR in the SCN will be described. In addition, the relationship between NPY in the SCN and circadian responses to nonphotic and photic stimuli will be reviewed. Responses of IGL neurons to photic stimulation, and comparisons to SCN neurons, will be discussed in terms of both electrophysiological work and immediate early gene expression. Implications of these studies for the role of the IGL in the circadian response to light will be considered.

Anatomy of the IGL. The IGL was first identified by Hickey and Spear (1976), who recognized the thin layer of neurons that receive bilateral retinal afferents as distinct from the surrounding dorsal lateral geniculate (dLGN) and ventral lateral geniculate (vLGN). Because the IGL cannot be distinguished from the vLGN in Nissl stained material, this region was previously considered to be a part of the vLGN. Recently, the dimensions of the IGL have been redefined using anterograde and retrograde tracers to outline the region that receives retinal input and projects to both the SCN and contralateral IGL, and immunohistochemistry to label neurons distinct to the IGL (Moore & Card, 1994; Morin, Blanchard, & Moore, 1992). Using these techniques the IGL has been shown to lie throughout the rostro-caudal extent of the lateral geniculate nucleus (LGN), approximately 2mm in length (Moore & Card, 1994; Morin, et al., 1992). Golgi impregnations of the LGN indicate that IGL neurons are morphologically distinct from the dLGN and vLGN, and that the dendritic fields of IGL neurons do not project into the neighbouring dLGN or vLGN (Moore & Card, 1994). At its most rostral level, the rat IGL lies ventral to the dLGN and lateral to the rostral vLGN. The middle portion of the IGL is the region initially recognized by Hickey and Spear (1976) as the thin lamina of neurons between the dLGN and vLGN (Moore & Card, 1994). At the most caudal level, the IGL lies ventral to the medial geniculate, dorsal to the lateral terminal nucleus of the accessory optic system and cerebral peduncle, and lateral to the zona incerta (Moore & Card, 1994).

The IGL receive dense, bilateral, overlapping innervation from all parts of the retina, and unlike the retinal afferents projecting to either the dLGN or vLGN, this innervation is not organized in the topographic manner characteristic of the visual system (Hickey & Spear, 1976; Harrington, Nance, & Rusak, 1985; Moore & Card, 1994). Retinal projections to the contralateral IGL are at least twice as dense as those to the ipsilateral IGL (Hickey & Spear, 1976; Moore & Card, 1994). At least some of the retinal ganglion cell axons terminating in the hamster IGL bifurcate, sending collaterals

to the SCN, forming the RHT (Pickard, 1985). Neurons throughout the IGL project to the retinorecipient region of the SCN, forming the GHT (Card & Moore, 1989; Pickard, 1982; Swanson, Cowan, & Jones, 1974). Although the majority of IGL neurons project to the ipsilateral SCN, neurons from each IGL project to both SCNs (Card & Moore, 1989). A commissural projection between the two IGLs exists as well; the neurons forming the geniculogeniculate pathway are distinct from those projecting to the SCN (Card & Moore, 1989; Mikkelsen, 1992; Pickard, 1985). The IGL also projects to the retrochiasmatic area, subparaventricular zone, periventricular nucleus, medial preoptic area, zona incerta, dorsal hypothalamic area, paraventricular thalamic nucleus, periacqueductal gray, accessory optic nuclei, pretectal area, superior colliculus, and deep pineal gland (Horvath, 1998; Mikkelsen & Moller, 1990; Moore, Moga, & Weis, 1996). In addition to retinal and commissural afferents, IGL receives input from the dorsal SCN and adjacent anterior hypothalamic area, the retrochiasmatic area, dorsal raphe, and locus coeruleus (Card & Moore, 1989; Moore & Card, 1994; Watts, Swanson, & Sanchez-Watts, 1984).

Neurochemical content of the IGL. The IGL may be distinguished from other parts of the LGN by the neurochemical content of its neurons (Moore & Card, 1994; Morin et al., 1992). IGL neurons contain GABA, NPY, and Enkephalin (ENK; Mantyh & Kemp, 1983; Moore & Card, 1994; Harrington, 1997; Takatsuji & Tohyama, 1989), and possibly substance P (SP; Takatsuji & Tohyama, 1989; but see Moore & Card, 1994). NPY and ENK are absent from the dLGN. In the rat vLGN, NPY fiber staining is very sparse, and although there are ENK neurons in this region they differ morphologically from those in IGL (Moore & Card, 1994). Glutamic acid decarboxylase (GAD; the GABA-forming enzyme) has been observed in both the dLGN and vLGN, but in the IGL this pattern of staining is very dense, and labels cell bodies and fibers throughout the IGL (Moore & Card, 1994). The IGL may also be distinguished by dense

staining of glial fibrillary acidic protein (GFAP), absent from the surrounding geniculate regions (Botchkina & Morin, 1995; Morin et al., 1989). GFAP-immunoreactivity (IR) is also present in the SCN of rats and hamsters (Morin et al., 1989). Although the presence of astrocytes is a characteristic of the circadian system, the role of glia in circadian rhythmicity is not known.

Retinorecipient NPY neurons are distributed throughout the IGL (Card & Moore, 1988, Card & Moore, 1989; Harrington, Nance, & Rusak, 1985; Harrington, Nance, & Rusak, 1987; Morin et al., 1992; Morin & Blanchard, 1995; Takatsuji, Miguel-Hidalgo, & Tohyama, 1991a). Fibers from NPY neurons in the IGL arborize in the retinorecipient, ventrolateral region of the SCN (Card & Moore, 1988, Card & Moore, 1989; Harrington et al., 1987). Whether RHT and GHT afferents are synapsing on the same population of cells or if they are differentially influencing the activity of separate cell groups is unknown. Approximately 35% of GHT neurons in rat, and 50% of GHT neurons in hamster, are not immunoreactive for NPY and the neurotransmitter content of these neurons is unknown (Card & Moore, 1989; Moore & Card, 1994; Morin et al., 1992). Because most NPY and ENK neurons in the IGL colocalize with GABA, it is unlikely that these neurons are GABAergic either (Moore & Card, 1994).

The most abundant neurotransmitter in the IGL is GABA. GAD-IR has been observed throughout the IGL and dense staining of cell bodies and fibers is evident in IGL of both rat and hamster (Moore & Card, 1994; Morin et al., 1992). GAD-IR colocalizes with either NPY or ENK but not both in rat. There are close to 2000 neurons in the rat IGL, about 1700 of which are GAD-immunopositive and project to either the SCN or contralateral IGL (Moore & Card, 1994). GABAergic cells that colocalize with NPY project to the SCN, and those that colocalize for ENK project to the contralateral IGL.

The commissural projection between IGLs is enkephalinergic; immunohistochemical staining of IGL neurons demonstrates that, in the rat, cells that

contain ENK do not contain NPY, although these cells also receive direct retinal innervation (Card & Moore, 1989; Morin et al., 1992; Takatsuji & Tohyama, 1989; Takatsuji et al., 1991a). In the hamster, some of the NPY-IR neurons that comprise the GHT are also immunoreactive for ENK (Morin et al., 1992; Morin & Blanchard, 1995). However, combined use of immunohistochemistry and retrograde tracing techniques have demonstrated that different neurons comprise the GHT and the geniculogeniculate pathways (Card & Moore, 1989; Morin, Blanchard, & Moore, 1992; Morin & Blanchard, 1995). Fewer than 10% of the cells projecting to the contralateral IGL are NPY-IR in both hamster and rat (Card & Moore, 1989, Morin & Blanchard, 1995).

The IGL contains dense SP-IR fibers (Mantyh & Kemp, 1983; Morin et al., 1992; Moore & Card, 1994; Takatsuji & Tohyama, 1989). The source of the SP fiber plexus is unknown; the finding that enucleation did not alter the density of SP-IR in the IGL, as it did in the SCN, raises the possibility that the SP fibers in the IGL do not originate in the retina (Hartwich, Kalsbeek, Pevet, & Nurnberger, 1994; Morin et al., 1992; Moore & Card, 1994; Miguel-Hidalgo, Senba, Takatsuji, & Tohyama, 1991; Takatsuji et al., 1991b; Takatsuji, Senba, Mantyh, & Tohyama, 1995). Retinal ganglion cells that innervate the IGL do contain pituitary adenylate cyclase-activating peptide (PACAP); in enucleated rats the density of PACAP-IR fibers in both SCN and IGL is greatly reduced (Hannibal et al., 1997). The IGL also receives serotonergic, noradrenergic, and cholinergic inputs from the dorsal raphe, locus coeruleus, and pontine nucleus respectively (Amir, Robinson, Ratovitski, Rea, Stewart, & Simantov, 1998; Mantyh & Kemp, 1983; Morin et al., 1992; Moore & Card, 1994). The neurotransmitter that mediates transmission of photic information from the retina to the IGL is not known. However, transmission of photic information to the SCN as well as to other visual areas such as the dLGN, superior colliculus, and visual cortex occurs via activation of glutamatergic receptors (reviewed in Ebling, 1996; Morin, 1994; Nelson & Sur, 1992). Because in hamsters at least some of the retinal ganglion cells that project to the IGL are

collaterals of those that project to the SCN (Pickard, 1985) and given the prevalence of glutamate neurotransmission in the visual and circadian systems (Ebling, 1996; Nelson & Sur, 1992) a role for glutamate in transmission of photic information to the IGL is plausible.

Functional role of NPY in the circadian system. As discussed above, the source of the NPY fiber plexus in the retinorecipient region of the SCN is the IGL (Card & Moore, 1988, Card & Moore, 1989; Harrington et al., 1987; Moore & Card, 1994). The idea that the IGL may be relevant in entrainment of circadian rhythms arises predominantly from work demonstrating rhythmic expression of NPY-IR in the SCN and effects of NPY on rhythmicity. In this section, the pattern of endogenous expression of NPY in the SCN of animals housed under different lighting schedules will be described and the effects of exogenous application of NPY to the SCN on rhythms will be discussed.

Regulation of NPY levels in the SCN by the LD cycle has been shown (Jhanwar-Uniyal, Beck, Burlet, & Liebowitz, 1990). Greater density of NPY-IR has been observed during the light phase than during the dark phase of the cycle (Calza et al, 1990). A bimodal rhythm of NPY density in the SCN has been reported, with peaks of NPY in the SCN occurring around the transition times (lights on and lights off; Calza, Giardino, Zanni, Velado, Parchi, & Marrama, 1990; Jhanwar-Uniyal et al., 1990; Shinohara, Tominaga, Isobe, & Inouye, 1993).

The fluctuations in NPY in the SCN over the 24-hour day have been shown to be a direct consequence of light-dark transitions at dawn and dusk. In dark-adapted rats, exposure to light for 24 hours starting at circadian time 0 (CT0; by convention CT12 is night onset) resulted in a rapid rise in SCN NPY levels that peaked at CT2 and returned to baseline levels by CT4, after which no further increases occurred. Similarly, light-adapted rats exposed to darkness at CT12 showed an increase in SCN NPY levels in

response to the transition, but no subsequent increase during the next 24 hours. Furthermore, in rats housed in DD for two weeks, decreased density of NPY fiber staining was observed throughout the 24 hour cycle, and no differences were observed between subjective day and subjective night times (Calza et al., 1990). However, the increases in NPY in the SCN have also been shown to be related to circadian phase. Only light pulses starting at CT0 caused an increase in NPY levels (Shinohara, Tominaga, Fukuhara, Otori, & Inouye, 1993). Similarly, light-adapted rats exposed to pulses of darkness at CT 12 showed an increase in NPY that was not observed in animals given dark pulses at any other time point (Shinohara, Tominaga, Fukuhara, et al., 1993).

Neither the intensity of light nor the length of light exposure affected the increase in NPY at dawn. Pretreatment with the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, attenuated the increase in NPY in the SCN after light onset, whereas systemic injection of NMDA at CT0 in DD increased NPY levels in the SCN (Shinohara, Tominaga, Isobe et al., 1993). The results of this work support the idea of a role for glutamate in transmission of photic information to the SCN and an interaction between NPY and light on the circadian system.

Evidence of a role for NPY in nonphotic phase resetting exists both in vitro and in vivo. NPY infusion into the SCN during the subjective day has been shown to phase advance the hamster wheel-running activity rhythm in LL (Albers & Ferris, 1984) or DD (Huhman & Albers, 1994), and infusion of antiserum to NPY into the SCN prevented phase shifts by nonphotic stimuli (Biello, Janik, & Mrosovsky, 1994). Similar patterns have been observed in the SCN cell-firing rhythm in vitro. Application of NPY to the SCN slice during the subjective day resulted in phase advances of the rhythm of SCN cell firing (Biello, Golombek, & Harrington, 1997; Biello, Golombek, Schak, & Harrington, 1997; Golombek, Biello, Rendon, & Harrington, 1996; Medanic & Gillette, 1993; Shibata & Moore, 1993). This effect has been mimicked by application of a Y2 receptor agonist (Golombek et al., 1996; Gribkoff, Pieschl, Wisialowski, van den Pol, & Yocca,

1998), suggesting that Y2 receptors mediate the phase shifting effects of NPY. It is interesting to note that the phase shifting effects of NPY have been blocked by light exposure in vivo (Biello & Mrosovsky, 1995) and glutamate in vitro (Biello, Golombek, & Harrington, 1997).

The responsiveness of NPY in the SCN to changes in circadian phase and lighting cycles suggests that NPY is involved in the circadian response to light in the live animal, and evidence from recent studies supports this idea. It has been shown, for example, that NPY depresses excitatory neurotransmission (van den Pol, Obrietan, Chen, & Belousov, 1996) and blocks glutamate-induced phase shifts in SCN neurons in vitro (Biello Golombek, & Harrington, 1997) and light-induced phase advances in vivo (Weber & Rea, 1997). Intra-SCN infusion of antiserum to NPY potentiated light-induced phase advances at CT18 (Biello, 1995). Furthermore, the finding that NPY-induced phase shifts in SCN neurons occurred via activation of the Y2 receptor, whereas NPY-induced inhibition of cell discharge in the SCN occurred via activation of the Y5 receptor (Gribkoff et al., 1998), raises the possibility that the action of NPY on the responses to light and nonphotic stimuli can occur via different physiological mechanisms.

Electrophysiological responses of IGL neurons to light. Results of electrophysiological work on activity of IGL neurons support a role for the IGL in the response to light. Neurons in the IGL exhibit firing patterns that are similar to those of the SCN in response to retinal illumination. In contrast with visual system cells, neurons in both IGL and SCN have been shown to be more responsive to sustained illumination than to brief light flashes (see Groos & Meijer, 1985; Harrington, 1997 for reviews). Most neurons exhibited sustained increases in firing in response to retinal illumination, although a few cells showed sustained decreases in firing during light exposure. In addition, both IGL and SCN cells that show sustained ON responses to light have also exhibited changes in firing rate in response to changes in light intensity (IGL: Hale &

Sefton, 1978; Harrington & Rusak, 1989; Sumitomo et al., 1979; Zhang & Rusak, 1989; or see Harrington, 1997 for review; SCN: Groos & Mason, 1978; Meijer, Groos, & Rusak, 1986; or see Groos & Meijer 1985, for review). IGL cells identified as projecting to either the SCN or contralateral IGL exhibited these properties as well; most showed sustained activation in response to retinal illumination, a few showed sustained suppression, and photically responsive cells exhibited changes in firing rates with changes in light intensity (Harrington & Rusak, 1989; Zhang & Rusak, 1989). Moreover, electrical stimulation of one IGL suppressed firing rates of cells in the contralateral IGL and inhibited immediate responses to subsequent photic stimulation, possibly reducing the sensitivity of the circadian system to transient changes in illumination (Zhang & Rusak, 1989). The results of these experiments demonstrate that cells in both the SCN and IGL have luminance coding properties enabling the system to detect transitions in environmental illumination at dawn and dusk, providing the cues necessary for photic entrainment.

Immediate early gene expression in the circadian system. Immediate early gene expression is thought to be involved in the signal transduction mechanism coupling cellular activation with long term changes in the cell by regulating transcription of target genes (Morgan & Curran, 1991; Sheng & Greenberg, 1990). Expression of the immediate early gene c-fos has been extensively studied over the last decade. C-fos encodes a nuclear protein Fos, that is rapidly and transiently activated by physiologically relevant stimuli in the nervous system (Morgan & Curran, 1991; Sheng & Greenberg, 1990). Fos protein forms heterodimers with the protein product of one of the immediate early genes of the jun family, c-Jun, JunB or JunD. These heterodimers are components of the mammalian transcription factor activator-protein 1 (AP-1). The investigation of immediate early gene expression in the circadian system has provided insight into the relationship between light and phase shifts in circadian rhythms; indeed induction of c-

fos and junB in the SCN are putative components of the clock resetting mechanism. In the following section, the patterns of induction of Fos and JunB in the retina, SCN, and IGL in response to light pulses as well as levels of expression under constant housing conditions will be described.

In the retina, induction of Fos by light and gating of Fos expression by the circadian clock have been reported (Chambille, Doyle, & Servière, 1993; Earnest, Iadarola, Yeh, & Olschowka, 1990; Nir & Agarwal, 1993). Flashes of light induced c-fos mRNA and Fos protein expression in the ganglion cell layer of the retina of dark-adapted hamsters and mice; greater numbers of Fos-IR cells were observed in animals tested during the subjective night than during the subjective day (Chambille et al., 1993; Nir & Agarwal, 1993; Sagar & Sharp, 1990). However, under LD12:12, LL, or DD housing, greater Fos IR was observed in the retina of animals killed during light exposure regardless of circadian phase (Chambille et al., 1993; Earnest et al., 1990). Increased c-fos mRNA has also been observed around the transition times; transcription of c-fos occurred during the first few hours after dark onset, as well as 30-60 minutes after light onset (Nir & Agarwal, 1993).

In the SCN, the relationship between photic stimuli, Fos induction, and circadian phase has been well-established. Light-induced transcription of c-fos mRNA in the SCN is correlated with the effectiveness of light to phase shift circadian activity rhythms. Light exposure during the subjective night, at times when light is effective at phase shifting circadian rhythms, induces Fos expression in the ventrolateral region of SCN neurons (Aronin, Sagar, Sharp, & Schwartz, 1990; Chambille et al., 1993; Colwell & Foster, 1992; Kornhauser, Nelson, Mayo, & Takahashi, 1990; Rea, 1989; Rusak, McNaughton, Robertson, & Hunt, 1992; Rusak, Robertson, Wisden, & Hunt, 1990; Schwartz, Takeuchi, Shannon, Davis, & Aronin, 1994; Sutin & Kilduff, 1992). Similarly, transcription of junB mRNA and translation of JunB protein in the ventrolateral SCN region has been shown to occur rapidly and transiently following light

pulses given during the subjective night, but not subjective day (Guido, Goguen, Robertson, & Rusak, 1996; Guido, Rusak, & Robertson, 1996; Kornhauser, Nelson, Mayo, & Takahashi, 1992; Rusak et al, 1992; Takeuchi, Shannon, Aronin, & Schwartz, 1993).

Light-induced Fos expression in the SCN is not only gated by circadian phase but also by seasonal daylength. In animals entrained to lighting schedules that simulate the seasonal changes in daylength, the duration of responsiveness of SCN cells to nocturnal light pulses has been shown to be a function of the photoperiod to which the animals were entrained. In the SCN of animals entrained to short days, the phase during which Fos can be induced by night-time light exposure was longer than in animals entrained to long days, indicating that the photoperiodic response to light is gated by the circadian clock (Sumova, Travnickova, Peters, Schwartz, & Illnerova, 1995).

In rats housed under LD12:12, a diurnal rhythm in SCN Fos expression has been reported, with higher levels of Fos-IR during the light phase (Aronin et al., 1990; Earnest et al., 1990). Schwartz et al (1994) showed that Fos expression in the SCN is correlated with zeitgeber time (ZT; by convention ZT0 is onset of the light phase and ZT12 is onset of the dark phase of LD12:12); peaks of c-fos mRNA were observed 30 minutes after light onset, and returned to basal levels by ZT2. Similarly, number of Fos-IR cells in the SCN increased at light onset, reached a maximum at ZT2, and decreased by ZT4, with very few Fos-IR cells in the SCN observed during the dark phase of the cycle (Schwartz et al., 1994). Nocturnal animals also entrain stably to a skeleton photoperiod, an ecologically relevant lighting schedule consisting of two light pulses at times corresponding to dawn and dusk. In rats housed under a skeleton photoperiod and killed after the dawn or dusk light pulse, Fos-IR was observed in the SCN of those animals killed after the dawn pulse only (Schwartz et al., 1994). Because the free running period in rats tends to be greater than 24 hours, rats require the light at dawn (i.e. a daily phase advance) to entrain to the 24 hour day. Thus the Fos expression observed at dawn but not

dusk, supports the idea that Fos expression may be involved in photic entrainment in rats. However, the finding of Fos staining in the SCN of mice in response to light at dawn or dusk regardless of period length (Schwartz, Peters, Aronin, & Bennett, 1996) demonstrates that the relationship between Fos induction in the SCN and photic entrainment is not a simple one.

Rats housed in LL for 4-40 hours exhibited a modest number of Fos-IR cells in ventrolateral SCN during subjective day and subjective night times (Aronin, et al., 1990; Earnest et al., 1990; Earnest, Ouyang, & Olschowka, 1992). In contrast, animals killed at various CTs in DD showed few immunopositive cells in the SCN region, supporting the idea that Fos expression in the SCN is altered by environmental lighting (Aronin, et al., 1990; Earnest et al., 1990; Earnest et al, 1992; Schwartz et al., 1994). However, Chambille et al. (1993) showed rhythms for Fos expression in the SCN of hamsters in DD, with greater numbers of Fos-IR cells in the rostral SCN during the subjective day than during the subjective night. Fos expression in the SCN may be gated by circadian phase under LL conditions as well. Earnest et al (1992) demonstrated a twofold increase in Fos expression in the ventrolateral region of the SCN during the subjective night compared to staining in the subjective day, in rats housed in LL.

SCN Fos expression is coextensive with the distribution of NPY-IR fibers and VIP-IR cells in rats (Earnest et al, 1990; Earnest et al., 1992). Despite the overlap in distribution of VIP cells and light-induced Fos cells, studies examining whether those cells that express light-induced Fos are also immunoreactive for VIP have produced conflicting results (Daikoku, Yokote, Aizawa, & Kawano, 1992; Earnest, DiGiorgio, & Olschowka, 1993). The finding that light exposure decreased VIP-IR and increased GRP-IR in the SCN (Shinohara, Tominaga, Isobè, et al., 1993), further confounds this question. In any case, fewer than 10% of all light-induced Fos cells colocalize with either GRP or VIP in the ventrolateral SCN in rats (Daikoku et al., 1992; Earnest et al, 1993). In addition, Bennett and Schwartz (1994) found that about 10% of the cells that

express light-induced Fos in the SCN are glia. Similarly, in mice, 24% of light-induced Fos-IR cells in the SCN also exhibited immunoreactivity for VIP, 13% for vasopressin, and 7% exhibited GFAP-IR (Castel, Belensky, Cohen, Wagner, & Schwartz, 1997). Thus it appears that Fos expression in the rodent SCN occurs in a heterogeneous population of cells; the common factor amongst these cells if one exists, is unknown.

The threshold level of illumination required for phase shift is the same as that for c-fos induction, and pharmacological manipulations that attenuate light-induced phase shifts, such as treatment with the glutamatergic antagonist dizocilpine maleate (MK-801), also attenuate light-induced Fos expression in the SCN, suggesting that Fos expression in the SCN may play a role in the phase shifting mechanism (Kornhauser et al., 1990). That expression of Fos and JunB are relevant for the phase shifting effects of light has been further supported by the finding that changes in the AP-1 complex in the SCN occurred in response to light pulses given during the subjective night (Kornhauser et al., 1992; Takeuchi et al., 1993). Kornhauser et al. (1992) reported a light-induced increase in AP-1 binding activity in the SCN. In contrast, Takeuchi et al. (1993) found differences not in AP-1 binding activity, but rather in the composition of the AP-1 complex in the SCN of animals killed after light exposure compared to dark controls. Whereas during darkness the AP-1 complex in the SCN consists of cJun and JunD, after light pulses JunB and c-Fos also bind at AP-1 sites (Takeuchi et al., 1993). These findings support the idea that Fos and JunB proteins dimerize in response to phase shifting light pulses, and suggest that changes in the composition of AP-1 leads to transcription of target genes in the SCN. Transcription of c-fos is mediated by phosphorylation of the cAMP response element binding protein (CREB). In the SCN, light-induced CREB phosphorylation is gated by the circadian clock as well; light pulses during the subjective night that induce phase shifts phosphorylate CREB, light pulses during the subjective day are without effect (Ginty et al., 1993). The finding that induction of c-fos, junB, AP-1, and phosphorylation of CREB are regulated by light, and gated by the circadian system, provide the basis for

the hypothesis that these immediate-early genes are a component of the photic entrainment mechanism. This hypothesis is further supported by work demonstrating that intra-SCN infusion of antisense oligonucleotides to c-fos and junB inhibited light-induced phase shifts (Wollnik et al., 1995; Wollnik et al., 1996).

In contrast with the extensive work on Fos in the SCN, few published studies on immediate early gene expression in the circadian system report data for the IGL region. In hamster IGL, light-induced c-fos mRNA or Fos protein has not been consistently observed (Kornhauser et al., 1990; Rusak et al., 1990). In rats, Fos-IR in the IGL region in response to light pulses during the subjective night has been reported in those studies that analyze staining in this region (vLGN: Rea 1989; IGL: Edelstein et al., 1995; Park, Baek, Kim, Kim, & Kim, 1993; Peters, Aronin, & Schwartz, 1996; Rusak, et al. 1990). Park et al. (1993) found that induction of robust Fos expression in the IGL occurred following light pulses of two hours, but not one hour, during the dark phase of LD12:12, although others have observed IGL Fos expression after shorter times of light exposure (60 minutes: Rusak et al., 1990; Edelstein, et al., 1995; 30 minutes: Edelstein & Amir, 1996b; Edelstein & Amir, 1996c; 15 minutes: Amir & Stewart, 1996; 5 minutes: Amir & Stewart, in press). Nevertheless, it is interesting to note that Park et al., (1993) observed similar levels of Fos expression two hours after light onset in rats housed in LD12:12. In the IGL of rats killed over the course of LD12:12, few to moderate numbers of Fos-IR cells have been reported in the light but not the dark phase (Aronin et al., 1990; Earnest et al., 1990). Similarly, in the IGL of animals killed in LL a few Fos-IR cells (<10 cells per IGL section)were observed during subjective day and subjective night times whereas rats killed in DD showed no staining (Earnest et al., 1990; Earnest et al, 1992). Indeed, light-induced c-fos mRNA and Fos protein in the IGL is not phase dependent (see Experiment 2; Edelstein & Amir, 1996a; Peters et al., 1996).

Similar to the results of studies on the identity of light-activated cells in the SCN, little is known about the identity of photically activated IGL cells; using combined

immunofluorescence and Fluorogold retrograde tracing techniques, Peters et al., (1996) demonstrated that 85% of GHT neurons do not exhibit Fos-IR, and 66% of neurons projecting to the contralateral IGL do not exhibit Fos either. Furthermore, novel running wheel exposure induced Fos expression in hamster IGL neurons (Janik, Mikkelsen, & Mrosovsky, 1995; Janik & Mrosovsky, 1992) that also exhibited NPY-IR, whereas only few photically activated cells in the IGL were immunoreactive for NPY (Edelstein & Amir, unpublished observations; Janik et al., 1995).

Night-time light exposure has been shown to induce a normal pattern of Fos expression in the SCN of visually impaired animals, such as retinally degenerate mice, blind mole rats, and MSG-treated rats (Colwell & Foster, 1992; Edelstein et al., 1995; Vuillez, Herbin, Cooper, Nevo, & Pevet, 1994;). Moreover, entrainment to standard laboratory LD cycles has been demonstrated in these animals (Foster et al., 1991; Goldman et al., 1997; Edelstein et al., 1995; Pickard et al., 1982). In contrast, no Fos staining has been found in the IGL of the blind mole rat (Vuillez et al., 1994) or the MSG-treated rat (Edelstein et al., 1995). There is some evidence to suggest that the IGL input to the SCN is not functionally intact in MSG-treated rats (Edelstein et al., 1995) and the extent to which the IGL is involved in the circadian system in Spalax is unknown (Vuillez et al., 1994). These findings underscore the question of whether the IGL is a requisite component of the circadian system under normal lighting conditions.

The Present Thesis. The experiments that comprise this thesis were conducted to explore the role of the IGL in the circadian response to light. Although the IGL is strategically located to convey photic information to the SCN, the role of this structure in the circadian response to light is largely unknown. To begin to study this, the first three experiments examined activation of IGL neurons by light using immunohistochemical expression of Fos protein as a marker of neuronal activation. Moreover, because the IGL has been implicated in the circadian response to nonphotic stimuli, the relationship

between such nonphotic manipulations and Fos expression was examined. In Experiment 1, Fos expression in the IGL and SCN in response to cage change, saline injection, and restraint stress, during the day or night are described. The responses of IGL neurons to light exposure are explored in Experiment 2, by characterizing the profile of Fos protein expression in the IGL of animals housed under LD 12:12, LL, or DD, and killed at different circadian phases under each of these schedules. To further compare light-induced Fos expression in the IGL and SCN, Experiment 3 evaluates the role of glutamate in light-induced Fos in the IGL. Because pretreating animals with glutamatergic antagonists have been shown to attenuate light-induced Fos expression in the SCN, the effects of such treatment on light-induced Fos expression in IGL neurons is explored in this experiment.

The role of the IGL was further evaluated in the final two experiments by comparing circadian rhythms of animals with electrolytic lesions of the IGL to intact controls and to adult rats treated neonatally with MSG. MSG-treated animals housed under LL did not exhibit the disruptions in rhythmicity typically observed in normal rats (Edelstein et al., 1995). That finding, together with the finding in hamsters that the IGL mediated the effects of LL, raised the possibility that neonatal MSG disrupted retinal input to the IGL (discussed in Edelstein et al., 1995). Direct comparisons between the effects of IGL lesions and MSG-induced neurotoxicity on circadian temperature rhythms of adult animals under LD 12:12, LL and DD are made in Experiment 4 using a telemetry system. Finally, findings demonstrating the diurnal rhythmicity of NPY in the SCN, taken together with the effects of NPY on glutamate- or light-induced phase shifts (Biello et al., 1997; Weber & Rea, 1997) raise the possibility of a critical role for the IGL in synchronization of circadian rhythms to discrete pulses of light. Nocturnal animals entrain normally to cycles of brief, discrete pulses of light given at dusk and dawn (DeCoursey, 1986; Pittendrigh and Daan, 1976; Rosenwasser, Boulos, and Terman, 1983; Stephan, 1983). These skeleton photoperiod (SPP) housing conditions provide an

ecologically relevant lighting schedule congruous with light sampling behavior in nocturnal rodents under natural and simulated laboratory conditions (DeCoursey, 1986). Photic entrainment of circadian rhythms in IGL-lesioned animals is evaluated under SPP housing conditions in Experiment 5 using telemetry to measure entrainment of temperature rhythms and Fos expression to measure responsiveness of SCN neurons to light at different times of day.

EXPERIMENT 1

Circadian rhythms can be reset by exposure to photic and nonphotic stimuli. Although light is the most potent zeitgeber for entrainment of circadian rhythms (Pittendrigh & Daan, 1976; Roenneberg & Foster, 1997), a variety of nonphotic cues, such as saline injection and exposure to novel running wheels, are also effective in resetting the circadian clock (Mrosovsky, 1995). The IGL is thought to mediate the effects of such stimuli on circadian rhythms (Biello et al., 1991; Marchant et al., 1997; Maywood et al., 1997; Meyer et al., 1993; Janik & Mrosovsky, 1994; Wickland & Turek, 1994). Associated with light-induced phase shifts is the expression of Fos protein in the SCN and IGL during the night (Chambille et al., 1993; Kornhauser et al., 1990; Rea, 1989; Rusak et al., 1990; Schwartz et al., 1994; Sutin & Kilduff, 1992). Whether Fos is induced by nonphotic stimuli in the SCN and IGL is not clear. Studies in hamsters have shown that subcutaneous saline injection or intraperitoneal (i.p.) triazolam injection do not induce Fos expression in the SCN although these treatments induce phase shifts in circadian activity rhythms (Mead et al., 1992; Zhang, Van Reeth, Zee, Takahashi, & Turek, 1993). Exposure to a novel running wheel induces both phase shifts in activity rhythms and Fos immunoreactive cells in hamster IGL and vLGN regions, but not in the SCN (Janik & Mrosovsky, 1992).

To explore the relationship between nonphotic cues and Fos expression in the rat circadian system during the day or night, the effects of cage change, i.p. saline injection, and restraint stress at zeitgeber time (ZT) 4 (4 hours after lights on; by convention ZT12= dark onset), and ZT16 (four hours after dark onset) on Fos expression in SCN and IGL were evaluated. These results have been published previously (Edelstein & Amir, 1995).

Method

Animals

Male Wistar rats, (300-325g) from Charles River Canada (St. Constant, Quebec) were housed individually in plastic cages under LD12:12 (lights on 08:00-20:00 or 21:00-09:00), with free access to rat chow and water for two weeks prior to the start of the experiment.

Procedure

Three days before testing, rats in their home cages were transferred into two light-tight, ventilated rooms and maintained on the lighting schedules described above. On the day of testing, rats were subjected to one of four treatments, at ZT4 or at ZT16, as described below (n=4, for each treatment, at each time). Rats treated at ZT4 were exposed to ambient light (300 lux) while animals treated at ZT16 were exposed to dim red light during treatment (< 5 lux). Two additional rats housed under each LD schedule, untouched during treatment times, served as controls. All rats received an overdose of sodium pentobarbital (100 mg/kg) following treatment.

Restraint Rats were removed from their home cages and placed into clear, plastic restrainers (length: 17 cm; diameter: 7.5 cm) for 60 minutes.

Intraperitoneal saline injection Rats received an injection of physiological saline (0.9% NaCl; 1ml/kg; i.p.) and were returned to their home cages for 60 minutes.

Cage change Rats were removed from their home cages and placed into clean, empty plastic buckets for 60 minutes.

Preparation of tissue and Fos immunohistochemistry

Anesthetized rats were perfused transcardially with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold, fresh 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Brains were removed and stored overnight in fresh 4% paraformaldehyde at 4°C. Serial coronal brain sections (50μm) through the SCN and

LGN regions (corresponding to plates 22-24 and 36-40, respectively, in the atlas of Paxinos and Watson), were cut from each brain on a vibratome, and processed for Fos immunohistochemistry, as previously described (Edelstein et al., 1995). Tissue sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6, Sigma) 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 Fos protein; NCI/BCB Repository, Quality Biotech, Camden, NJ; lot number 411-081887). This antibody produces one band on Western blots with a molecular weight characteristic of Fos and is therefore believed to recognize Fos protein but not Fos-related antigens (de Togni, Niman, Raymond, Sawchenko, & Verma, 1988). Blocking experiments performed in a subsequent study by exposing tissue sections to the N-terminal Fos peptide (2 μg/ml) in the primary antibody incubation solution prevents Fos-IR staining (Edelstein & Amir, 1996a). The antibody was diluted 1:8000 with a solution of 0.05% Triton X 100 (Sigma) in TBS with 1% normal horse serum (Vector Laboratories, Burlingame, CA). Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for one hour at 4°C with a rat-adsorbed biotinylated anti-mouse IgG made in horse (Vector), diluted 1:33 with 0.05% 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). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris buffer (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB; Sigma) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 (Sigma) and 8% $NiCl_2$ (Sigma). After this final incubation, sections were rinsed with cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and coverslipped with Permount (Fisher).

Data Analysis

Serial brain sections through the SCN and IGL were examined under a microscope and the number of Fos immunopositive cells was recorded for each region, in each animal, using a computerized image analysis system consisting of a Sony XC-77 Video Camera, a Scion LG-3 frame grabber, and NIH Image Software. Mean cell counts of the three sections per region exhibiting maximum Fos expression were obtained for each animal. Counts for the SCN were restricted to the ventrolateral region, and for the IGL separate means were obtained for rostral and caudal parts of this region. Comparisons of group means were conducted using 2x2 (time x treatment) analysis of variance for each region and each manipulation. Pairwise comparisons were conducted using simple main effects tests with a Bonferroni correction of the significance level (alpha/number of comparisons).

Results

In the SCN, a modest number of Fos-IR cells was consistently observed in response to all treatments and tended to be greater during the light phase of the cycle (Figure 1). However, these effects were statistically significant in the i.p. saline-treated condition only (Treatment: $F(1,8)=11.6$, $p<0.01$; Time of day: $F(1,8)=6.06$, $p<0.05$). Untreated control rats showed minimal Fos expression in the SCN at both treatment times.

All treatments induced significant Fos expression in the IGL (Figure 1; Rostral: Cage Change: $F(1,8)=11.7$, $p<0.01$; I.P. Saline: $F(1,8)=11.0$, $p<0.05$; Restraint $F(1,8)=7.3$, $p<0.05$. Caudal: Cage Change: $F(1,8)=48.5$, $p<0.01$; I.P. Saline: $F(1,8)=37.7$, $p<0.01$; Restraint: $F(1,8)=31.9$, $p<0.01$). Furthermore, Fos expression in the IGL was phase dependent; a significantly greater number of Fos-IR cells was observed in the IGL during the light than during the dark phase of the LD cycle regardless of treatment (Figure 1; Rostral: Cage Change: $F(1,8)=72.6$, $p<0.01$; I.P.

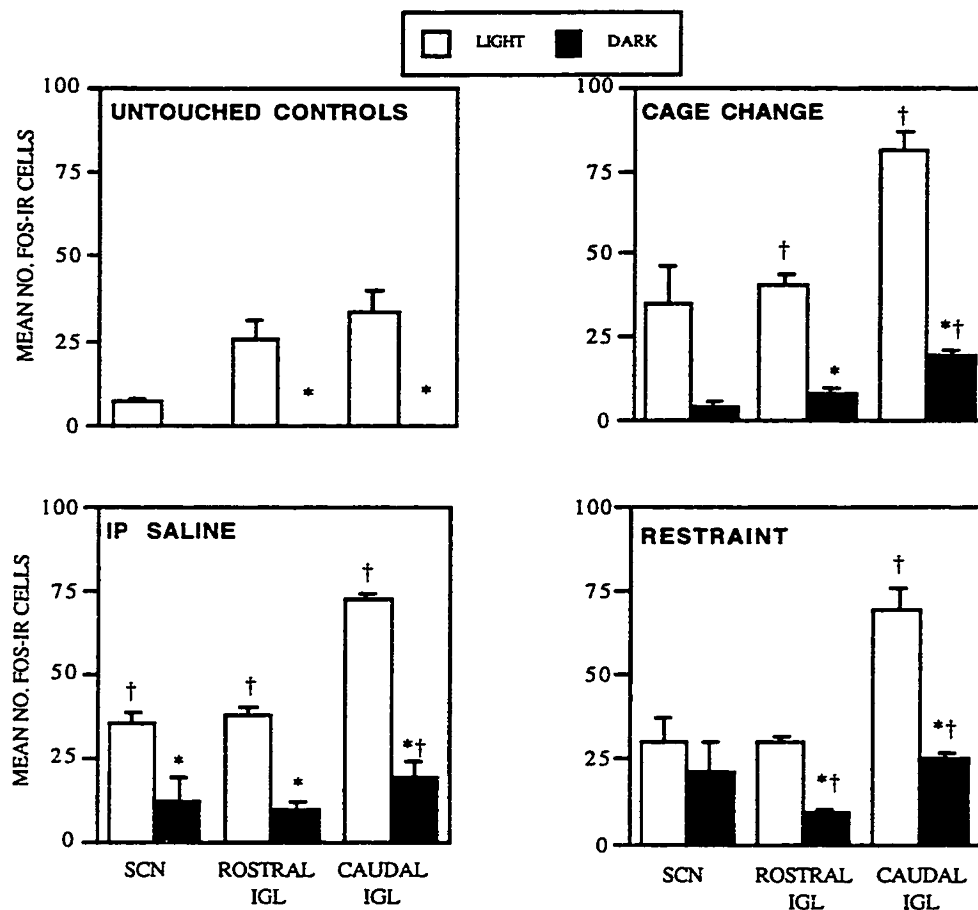

Figure 1. Mean (± sem) numbers of cells showing Fos-like immunoreactivity in the ventrolateral SCN, rostral IGL, and caudal IGL regions of animals treated during the light (ZT4) or dark (ZT16) phase of the cycle. * significant difference between light and dark treatment groups. † significantly different from corresponding control.

Saline: $F(1,8)=68.0$, $p<0.05$; Restraint: $F(1,8)=75.8$, $p<0.01$. Caudal: Cage Change: $F(1,8)=98.3$, $p<0.01$; I.P. Saline: $F(1,8)=89.6$, $p<0.01$; Restraint: $F(1,8)=51.7$, $p<0.01$). Untouched control rats displayed minimal or no Fos-IR cells in the IGL during the dark phase of the cycle. In contrast, in control rats killed during the light phase of the cycle, Fos-IR was observed throughout the IGL. Examples of Fos expression in the SCN and IGL observed in rats exposed to cage change and in untouched controls, during the light and dark phases of the LD cycle, are shown in Figures 2 and 3.

Discussion

The present study demonstrates that nonphotic manipulations induce the expression of Fos protein in rat SCN and IGL. Whether such manipulations induce phase shifts in circadian rhythms in the rat is not known. It is interesting to note that the SCN cells that express Fos in response to these nonphotic manipulations appear to be located in the ventrolateral region of the nucleus, the region containing retinorecipient neurons that is responsive to photic stimulation. However, whereas light-induced Fos expression in the SCN is gated by the circadian clock, there was no significant quantitative difference in the expression of Fos in the SCN in response to nonphotic treatments across time of day. This indicates that the sensitivity of SCN cells to nonphotic manipulations is not circadian in nature. In contrast, in the IGL, a greater number of Fos-IR cells was observed in animals killed during the light phase of the cycle regardless of treatment. Moreover, the IGL of animals exposed to nonphotic manipulations exhibited more robust Fos-IR than untreated control animals, raising the possibility that the response of cells in the IGL to nonphotic stimuli is potentiated during the light phase. Alternatively, activation of the IGL by light may be influenced by the arousal state of the animal. The finding that light-induced cell firing in the IGL and ventral lateral geniculate nucleus is enhanced by electrical stimulation of the tail (Davidowa & Albrecht, 1992) supports this latter idea. Perhaps the nonphotic manipulations used in the present study sensitize light-

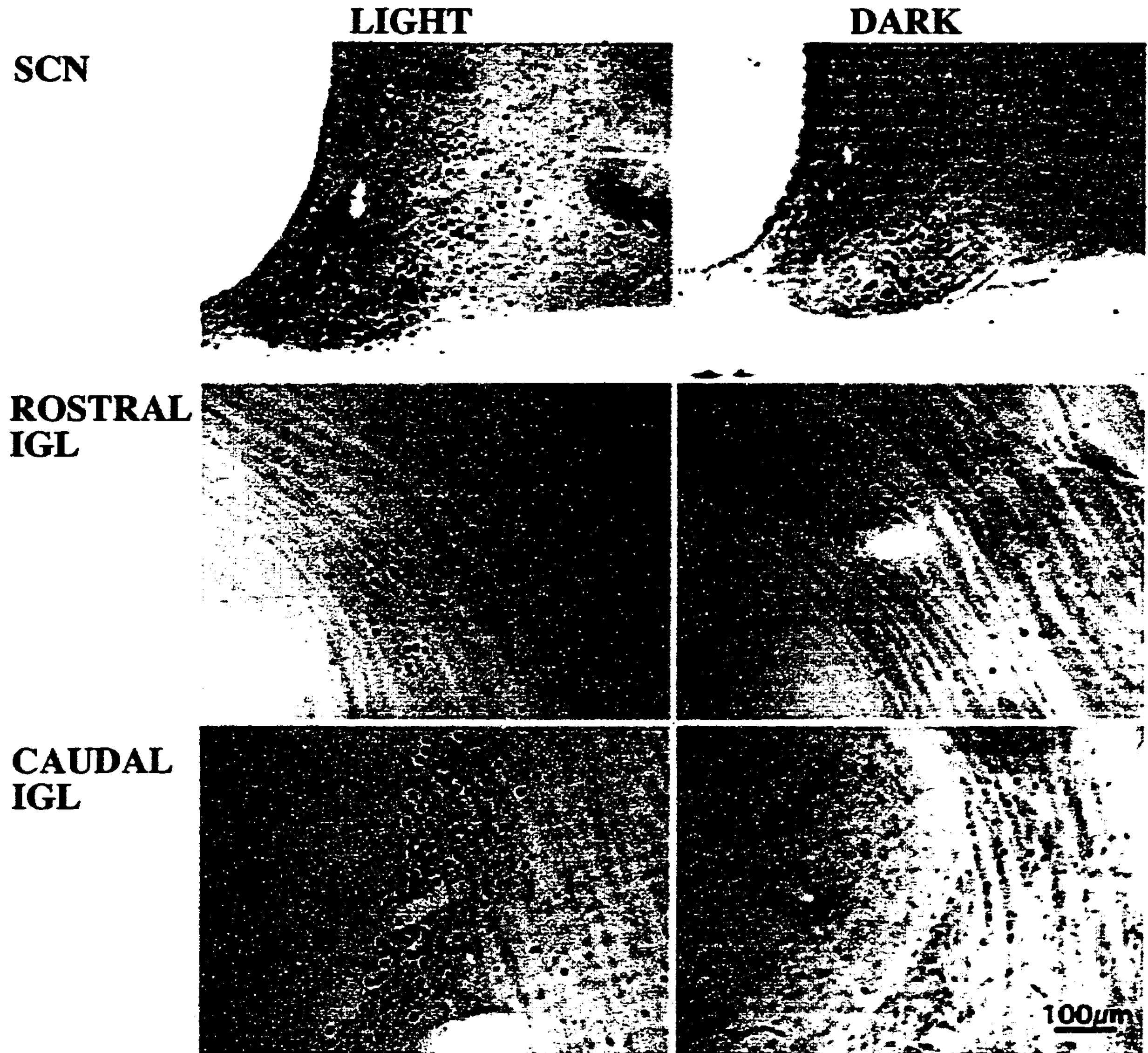

Figure 2. Photomicrographs of Fos-like immunoreactivity in coronal sections through the SCN (top), rostral IGL (middle), and caudal IGL (bottom) of rats removed from their home cages for 60 minutes at ZT4 (left) and ZT16 (right).

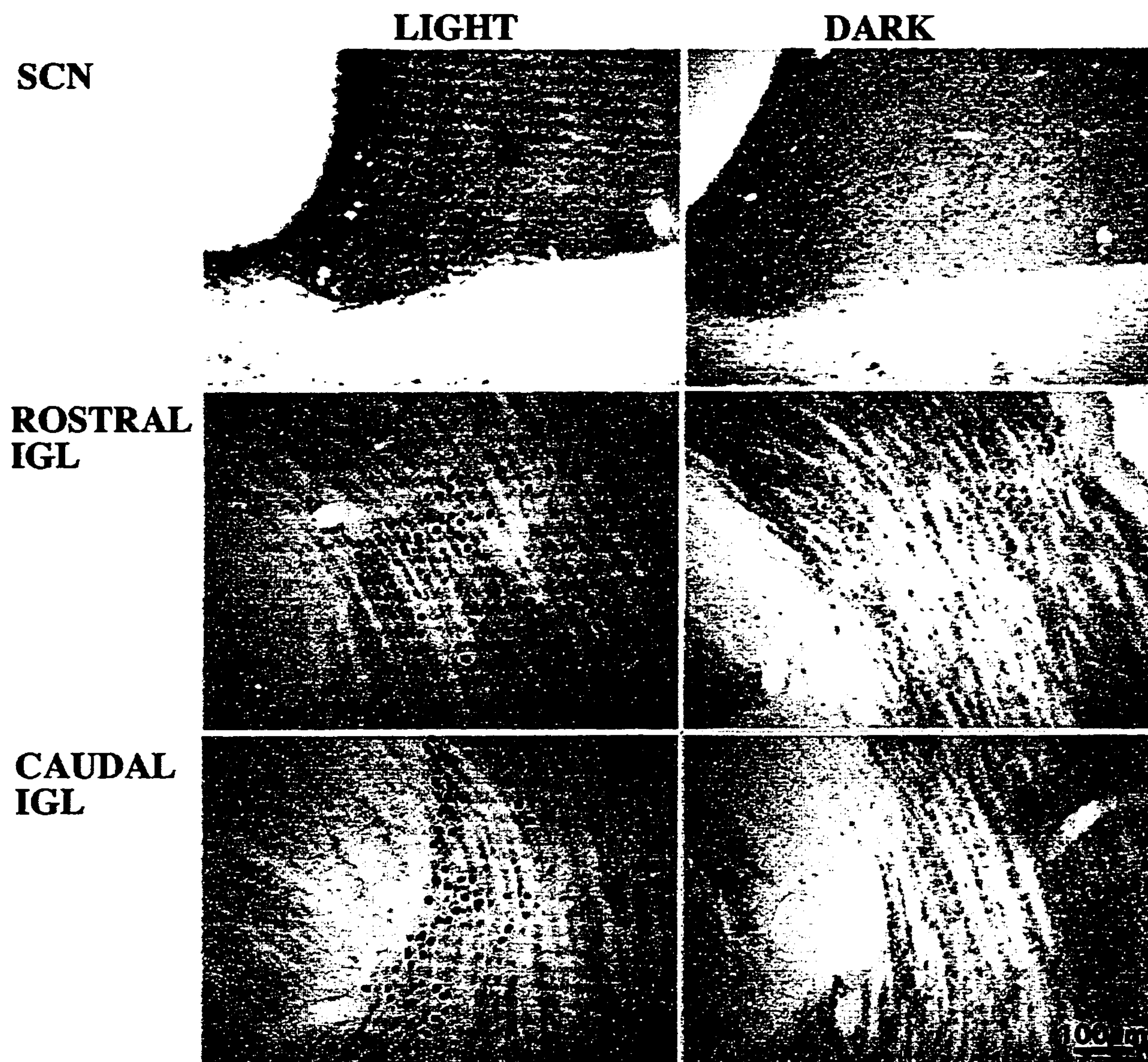

Figure 3. Photomicrographs of Fos-like immunoreactivity in coronal sections through the SCN (top), rostral IGL (middle), and caudal IGL (bottom) of untouched control rats killed at ZT5 (left) and ZT17 (right).

responsive neurons in the IGL to ambient light, resulting in greater Fos protein expression during the light phase of the LD cycle.

Several studies suggest that immediate early genes in general, and c-fos in particular, underlie the molecular basis of photic entrainment of circadian rhythms (Kornhauser et al., 1990; Rusak et al., 1990). Furthermore, it has been suggested that c-fos may be necessary for phase shift of circadian rhythms (Sutin & Kilduff, 1992). Fos induction is correlated with light-pulse induced phase shifts and preventing induction of c-fos and junB with antisense oligonucleotides prevents phase shifts as well (Wollnik et al., 1995; Wollnik et al., 1996). Pharmacological manipulations that block light-induced Fos expression, such as treatments with glutamatergic receptor antagonists (Abe, Rusak, & Robertson, 1991; Ebling et al., 1991; Vindlacheruvu, Ebling, Maywood, & Hastings, 1992), also block light-induced phase shifts. The relationship between immediate early gene expression and phase shifts in response to nonphotic manipulations has not been extensively studied. Janik and Mrosovsky (1992) have reported the expression of Fos in hamster IGL, but not SCN, in response to novelty-induced wheel running, a stimulus that phase shifts circadian activity rhythms. However, other nonphotic stimuli which induce phase shifts in the hamster, such as subcutaneous saline injection (Mead et al., 1992) and i.p. triazolam or saline injection (Zhang et al., 1993) have not been shown to induce Fos expression in hamster SCN, IGL, or vLGN regions. The present study demonstrates that nonphotic stimuli induce robust Fos expression in rat IGL during the day.

These data are consistent with those implicating the IGL in the effects of both photic and nonphotic stimuli on the circadian system. Phase-shifts induced by light pulses or activity-inducing stimuli such as injection of the benzodiazepine triazolam, as well as the circadian response to prolonged constant light exposure, are all altered in the absence of functional connections between the IGL and SCN (Edelstein et al., 1995; Harrington & Rusak, 1986; Harrington & Rusak, 1988; Janik & Mrosovsky, 1994; Johnson et al., 1989; Pickard, 1989; Pickard et al., 1987).

The neural pathways involved in the induction of c-fos in the SCN and IGL in response to the nonphotic manipulations used in this study are not known. In addition to projections from the retina and IGL, the SCN receives dense serotonergic input from the raphe (Azmitia & Segal, 1978; Moga & Moore, 1997; Moore, Halaris, & Jones, 1978), excitatory input from the paraventricular thalamic nucleus (Moga, Weis, & Moore, 1995; Moga & Moore, 1997), noradrenergic input from the locus coeruleus (Legoratti-Sanchez, Guevara-Guzman, & Solano-Flores, 1989; Moga & Moore, 1997), cholinergic input from the basal forebrain and mesopontine tegmentum (Bina, Rusak, & Semba, 1993), as well as widespread input from limbic and hypothalamic areas (Moga & Moore, 1997). The IGL receives input from the dorsal SCN and adjacent anterior hypothalamic area, the retrochiasmatic area, dorsal raphe, and locus coeruleus (Card & Moore, 1989; Moore & Card, 1994; Watts et al., 1984). Manipulations that alter the behavioral state of the animal, such as the ones used in this study, are known to affect these structures (for examples, see Jacobs, 1991; Jones, 1991; Semba, 1991). This, in turn, may result in activation of SCN and IGL cells with subsequent induction of Fos expression.

In summary, nonphotic manipulations induce Fos expression in the rat SCN and IGL, demonstrating that induction of c-fos in these regions is not light specific.

EXPERIMENT 2

Results of Experiment 1 demonstrate that IGL neurons of entrained rats, killed during the light phase of the LD cycle, exhibit Fos-IR (Edelstein & Amir, 1995). Those data suggest that the relationship between Fos expression and circadian phase is different in the IGL than in the SCN. That idea is further supported by results of another study showing Fos expression in the IGL in response to light exposure during the day (Aronin et al., 1990). Light-induced Fos protein expression in the SCN is correlated with the effectiveness of light to induce phase shifts and may participate in the molecular events that reset the circadian clock in response to light (Kornhauser et al., 1990; Rusak et al., 1990; Wollnik et al., 1995; Wollnik et al., 1996). In contrast with the profile of light-induced Fos expression in the SCN, the expression of Fos in the IGL in response to photic stimulation is not well-characterized. Although Fos protein expression has been observed in the IGL in response to light pulses given during the subjective night (Edelstein et al., 1995; Rea, 1989; Rusak et al., 1990) whether such expression is gated by the circadian system is not clear. To further compare induction of Fos in the IGL and SCN by environmental light, this experiment assessed Fos expression in IGL and SCN neurons of rats housed under various lighting conditions (LD12:12, LL, or constant dark; DD) and killed at different circadian phases. Results of this work have been published previously (Edelstein & Amir, 1996).

Method

Animals

Male Wistar rats, (300-325 g; Charles River Canada, St. Constant, Quebec) were housed individually under LD12:12 (~300 lux during lights on) with ad lib food and water in light-tight, ventilated rooms for two weeks before that start of the experiment.

Procedure

Animals were implanted intraperitoneally with precalibrated Mini Mitter transmitters (Mini Mitter, Sunriver, OR) under Metofane anesthesia (methoxyflurane; Janssen Pharmaceuticals, Markham, Ontario) and returned to their home cages. Ten days later they were exposed to either LL or DD for up to 3 weeks. Body temperature was monitored continuously using Dataquest software (Mini Mitter). Circadian time was determined by time of day in animals housed under LD12:12 and by the free running temperature rhythm in animals housed in constant conditions. For rats who were arrhythmic due to prolonged LL housing, projected circadian time was measured from the original LD cycle. Some rats that were housed under LL for 3 weeks were placed into darkness for 6 hours prior to pentobarbital injection. Additional animals housed under LD12:12, whose rhythms were not recorded, were killed during the light or dark phases of the cycle. All animals received an overdose of sodium pentobarbital anesthesia (100 mg/kg i.p; n=2 at each time during LD12:12 and up to 3 days in LL or DD; n=4 at each time during 1-3 weeks LL or DD housing), brains processed for Fos immunohistochemistry, and data analyzed as described in Experiment 1. However, in this and the following experiments, mean numbers of Fos-IR for the entire IGL were calculated from individual means obtained from rostral and caudal cell counts.

Results

Examples of Fos-IR in the IGL of rats killed at different circadian times during LD12:12 or LL housing are shown in Figure 4. Fos expression was observed in the IGL of rats exposed to light during both subjective day and subjective night, suggesting that expression was a result of light exposure and independent of circadian time. As shown in Figure 5, no differences in numbers of Fos-IR cells were seen in the IGL of rats entrained to LD12:12 and killed during the light phase of the cycle at ZT1, ZT5, or ZT10. In the IGL of rats killed during the dark phase of the cycle, Fos-IR was observed at ZT13 but

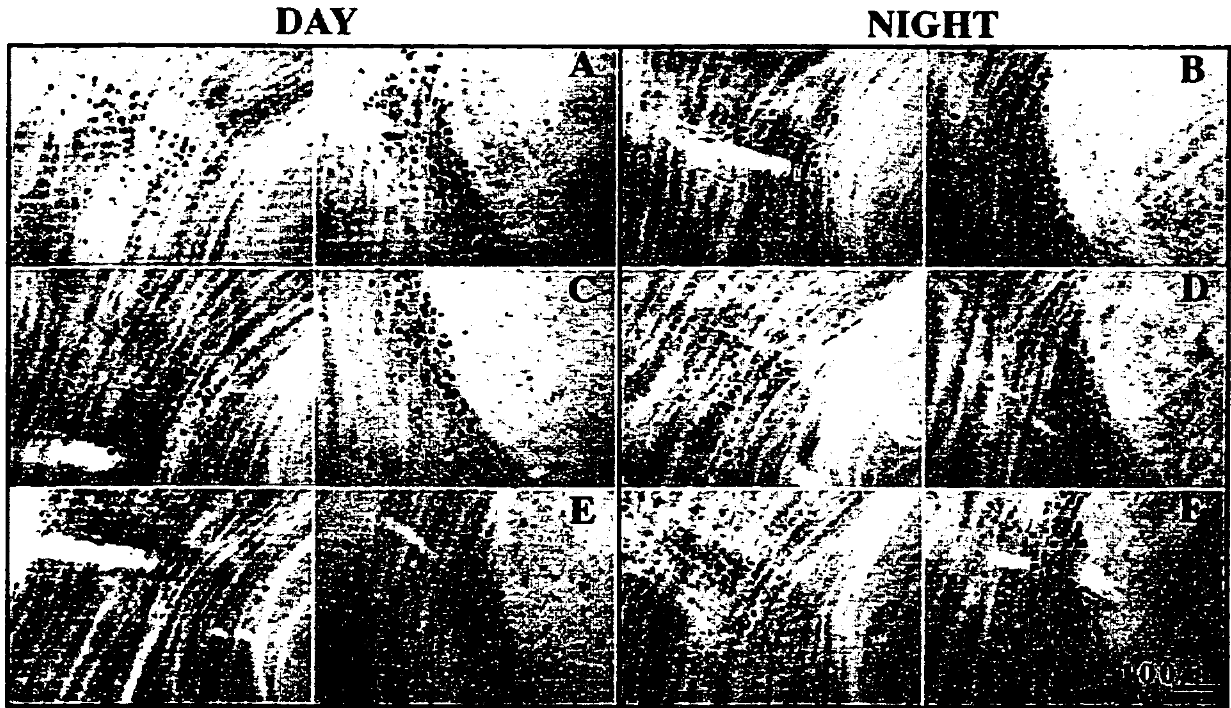

Figure 4. Photomicrographs of Fos immunoreactivity in 50 μm coronal sections through rostral and caudal portions of the IGL of rats entrained to LD12:12 and killed at ZT1 (A); exposed to constant light for 24 hours and killed at a time corresponding to ZT0 (B); after 1 week of LL housing and killed five hours into the subjective day (C) or night (D); after three weeks of exposure to LL and killed during the fifth hour of the projected subjective day (E) or night (F). Solid line = 100 μm.

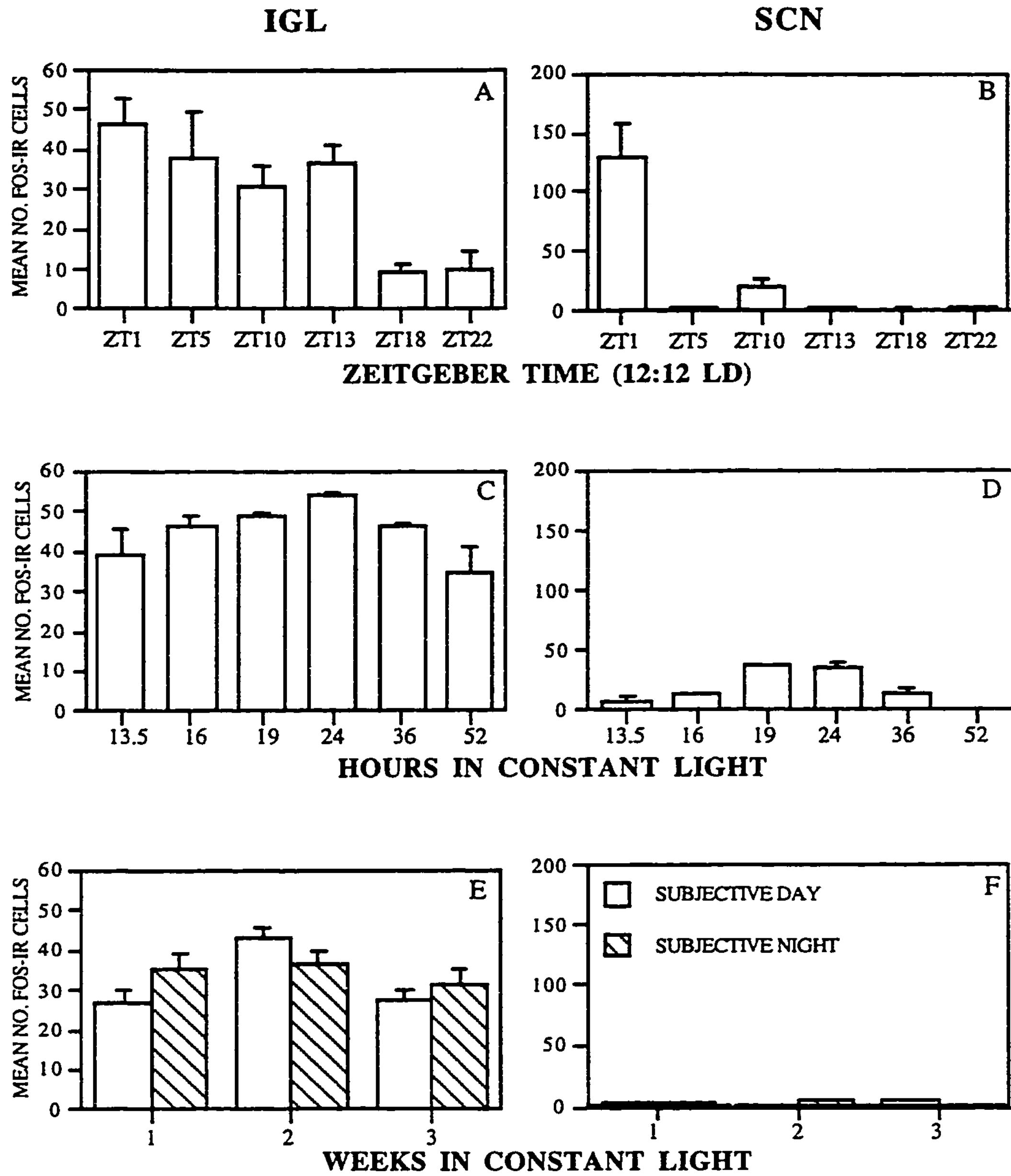

Figure 5. Mean (±sem) numbers of Fos-IR cells per 50 μm tissue section, in unilateral IGL (left) and SCN (right) of rats killed during the normal LD cycle (A,B); 13.5-52 hours (C,D) or 1-3 weeks (E,F) after being exposed to LL (~300 lux).

was reduced at ZT18 or ZT22 (Figure 5; 1 Factor ANOVA; Zeitgeber time: $F(5,6)=5.367$, $p<0.05$). In rats housed in LL, Fos-IR was observed in the IGL of all animals regardless of circadian phase (Figure 5). However, a greater number of Fos-IR cells was observed in IGL of animals housed in LL for 2 weeks compared to 3 weeks (2 Factor ANOVA (Weeks X Phase), significant effect of Weeks: $F(2,18)=4.921$, $p<0.05$). In contrast, few Fos-IR neurons were observed in the IGL of rats killed at similar time-points during DD housing (< 10 Fos-IR cells per IGL; data not shown). Similarly, few Fos-IR neurons were observed in the IGL of rats housed in LL for 3 weeks and then placed in darkness for 6 hours prior to pentobarbital injection (< 10 Fos-IR cells per IGL; data not shown).

In contrast with the effect of light on Fos induction in the IGL, little or no staining was observed in the SCN of animals killed at all times tested during LD12:12, except at ZT1 (1 Factor ANOVA, Zeitgeber time: $F(5,6)=16.628$, $p<0.01$). In those animals, Fos-IR was observed in the ventrolateral region as has been previously reported (Rusak et al., 1990; Schwartz et al., 1994; Figure 5). Furthermore, in animals housed under constant conditions, a few Fos-IR cells were noted in the SCN during the first 24 hours of LL exposure but very little staining was observed thereafter (Figure 5; 1 Factor ANOVA, effect for Hours: $F(5,6)=6.532$, $p<0.05$).

Discussion

The present study demonstrates that light induces expression of Fos protein in cells of the IGL regardless of circadian time, and that such expression persists regardless of length of stimulation. These data are in contrast with the phase dependency of light-induced Fos expression in the SCN. Photic stimulation induces Fos expression in the SCN only at circadian times when such stimulation induces phase shifts (Kornhauser et al., 1990; Rusak et al., 1990; Schwartz et al., 1994). The finding that light-induced Fos expression in the IGL is not phase dependent is supported by another study

demonstrating that two-hour light pulses during either the subjective day or night induce Fos expression in the IGL (Peters et al., 1996). Results of the present work also support electrophysiological studies of light-induced firing rates in IGL neurons. That work showed that most IGL neurons exhibit sustained activation in response to continuous illumination, and that firing rates change in response to changes in light intensity (Harrington & Rusak, 1991). In the present study, IGL neurons appear to show sustained activation, as indicated by the observed Fos-IR at each time tested, in response to constant light.

Although it has previously been shown that Fos protein is rapidly and transiently expressed and that Fos-related antigens (FRAs) accumulate over longer time periods (cf. Sharp, Sagar, Hisanaga, Jasper, & Sharp, 1990), the monoclonal antibody used in the present study does not recognize FRAs (de Togni et al., 1988). Furthermore, blocking studies have demonstrated that exposure to the N-terminal Fos peptide completely abolishes immunostaining (Edelstein & Amir, 1996; Sharp et al., 1990) supporting the idea that this antibody is specific for c-Fos. Thus the observed Fos-IR in IGL neurons of animals tested at any time during the light phase of LD12:12, or during LL, raises the possibility that continuous light exposure induces continuous c-fos transcription and translation in IGL neurons. However, whether the Fos-IR neurons observed at any given time are the same ones displaying Fos-IR at different times of day is not known. Fos protein is thought to regulate its own transcription via a negative feedback loop such that translation of Fos protein shuts off further c-fos mRNA transcription (Morgan & Curran, 1991). Therefore, although it appears that during prolonged light exposure neurons in the IGL express Fos protein continuously, c-fos induction may not persist in individual neurons during such prolonged exposure to light. Rather, it is plausible that as Fos protein expression becomes degraded in one neuron, the neighbouring cell begins transcription of c-fos mRNA, thereby giving the entire IGL region the appearance of Fos-IR.

IGL neurons are innervated by retinal ganglion cell axons, at least some of which bifurcate and project to the ventrolateral portion of the SCN, forming the RHT (Pickard, 1985). The IGL projects to the ventrolateral SCN in a pathway thought to use NPY as a transmitter (Moore & Card, 1994; Harrington, 1997). Although many of the IGL neurons that express Fos in response to light have not been shown to co-express NPY (Janik, et al., 1995) or project to the SCN (Peters et al., 1996), NPY levels in the SCN increased rapidly following light pulses at dawn or dark pulses at dusk (Shinohara, Tominaga, Fukuhara, et al., 1993) suggesting that IGL neurons convey information to the SCN regarding light-dark transitions. The increase in NPY in the SCN in response to light appears to constrain the circadian response to that light; central infusions of antiserum to NPY potentiated light-induced phase advances in hamster wheel-running rhythms (Biello, 1995). These data support the idea that the IGL may be involved in the daily phase advances or delays that synchronize circadian rhythms to the light-dark cycle. However, because light induces Fos protein in IGL neurons at times when it does not do so in SCN neurons, despite transmitting this information via the same retinal ganglion cells, it is likely that different mechanisms regulate Fos induction in these two regions. This is consistent with recent data demonstrating that systemic treatment with the NMDA receptor antagonist MK-801, shown to attenuate light-induced Fos in the SCN (Abe et al., 1991; Amir & Robinson, 1995; Ebling et al, 1991; Rea, Buckley, & Lutton, 1993) had no effect on light-induced Fos in IGL neurons (Edelstein & Amir, 1996b; see Experiment 3).

The IGL is also involved in the effects of LL on circadian rhythmicity. During prolonged LL exposure, hamsters exhibit lengthening of circadian period and splitting of circadian activity rhythms; both of these effects are attenuated in IGL-ablated hamsters (Harrington & Rusak, 1988). Rats housed in LL for several weeks show a progressive loss of circadian rhythms of activity and body temperature, and become arrhythmic after 3-4 weeks of exposure to bright light (Edelstein et al., 1995). This effect of LL is

prevented in animals treated neonatally with monosodium glutamate, a treatment known to damage the visual system (Edelstein et al., 1995). Such treatment also inhibits light-induced Fos expression in the IGL (Edelstein et al., 1995). Taken together, the continuous Fos expression in IGL neurons may provide a clue by which the IGL mediates the response to prolonged LL. That is, IGL neurons may be continuously activated throughout the duration of exposure to LL; this activation, over extended periods of time, may result in disruption of circadian rhythms.

In summary, the results of the present work show that cells in the IGL are continuously activated in response to light, and provide support for existing evidence that the IGL is involved in the circadian response to light, in both entrained and free-running rats.

EXPERIMENT 3

Photic information is transmitted to the SCN along the retinohypothalamic tract (RHT) via activation of NMDA, Kainate, and amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors (Abe et al., 1991; Abe, Rusak, & Robertson, 1992; Amir, 1992; Amir & Robinson, 1995; Colwell, Foster, & Menaker, 1991; Colwell & Menaker, 1992; Colwell, Ralph, & Menaker, 1990; de Vries, Treep, de Pauw, & Meijer, 1994; Ebling et al., 1991; Edelstein & Amir, 1996b; Edelstein & Amir, 1996c; Meijer, van der Zee, & Dietz, 1988; Mikkelsen et al., 1995; Ohi et al., 1991; Rea et al., 1993; Shirakawa & Moore, 1994; Takeuchi et al., 1991; Vindlacheruvu et al., 1992; Zawilska et al., 1997; reviewed in Ebling, 1996). The neurotransmitters mediating the transmission of photic information to the IGL are not known. Because in hamsters at least some of the retinal ganglion cells that project to the IGL are collaterals of those that project to the SCN (Pickard, 1985) and given the prevalence of glutamate neurotransmission in the visual and circadian systems (cf. Ebling, 1996; Morin, 1994; Nelson & Sur, 1992) a role for glutamate in transmission of photic information to the IGL is plausible.

Light-induced activation of glutamatergic receptors in the circadian system is associated with Fos induction in the SCN; treatment with glutamatergic receptor antagonists that blocked light-induced phase shifts attenuated light-induced Fos in SCN neurons (Abe et al., 1991; Abe et al., 1992; Amir & Robinson, 1995; Ebling, 1996; Ebling et al., 1991; Edelstein & Amir, 1996b; Edelstein & Amir, 1996c; Mikkelsen et al., 1995; Rea et al., 1993; Vindlacheruvu et al., 1992). Results from Experiment 2 demonstrate that unlike Fos expression in the SCN, the expression of Fos in IGL neurons is not gated by circadian phase. Light exposure induces Fos in IGL neurons regardless of circadian time (Edelstein & Amir, 1996a; Park et al., 1993; Peters et al., 1996). Furthermore, the findings that neonatal monosodium glutamate treatment prevented light-

induced Fos in the IGL but not SCN of adult rats (Edelstein et al., 1995), and that blockers of nitric oxide synthase attenuated light-induced Fos in rat SCN but not IGL (Amir & Edelstein, 1997), suggest that the physiological mechanism underlying Fos induction in the IGL is different from that of the SCN. To further explore the role of glutamate in light transmission in the circadian system, the present experiment examined the effects of blocking NMDA and non-NMDA receptors on light-induced Fos expression in the rat IGL and SCN. Results of this work have been published in abstract form (Edelstein & Amir, 1996b; Edelstein & Amir, 1996c) and have been submitted for publication.

Method

Animals

Male Wistar rats (300-325 g) were housed individually under LD12:12 with free access to food and water for at least two weeks prior to experimentation. Some animals were implanted with guide cannulae aimed at the right lateral ventricle under sodium pentobarbital anesthesia (65mg/kg i.p.) using standard stereotaxic procedures and the following stereotaxic coordinates: AP: -0.9; ML: -1.7 from bregma; DV: -3.4 from dura, measured at the injector tip. After surgery rats were returned to the animal colony for 7-10 days.

Procedure

Two days before testing, all rats were taken from the animal colony in their home cages, just prior to lights off, and placed in light-tight, ventilated boxes in constant darkness. On test day, animals were injected with either the noncompetitive NMDA receptor antagonist dizocilpine maleate (MK-801; 5mg/kg, i.p. Sigma; n=4), a competitive NMDA receptor antagonist, 3(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 5 nmoles in 5 μl, i.c.v.; RBI; n=4), an AMPA/Kainate antagonist,

6,7 dinitroquinoxaline-2,3-dione (DNQX; 40 nmoles in 5 µl, i.c.v.; RBI; n=4), or both CPP and DNQX (5 nmoles CPP, 40 nmoles DNQX; each in 2.5 µl vehicle; n=4). MK-801 and CPP were dissolved in physiological saline (0.9% NaCl); DNQX was dissolved in 0.25% aqueous polyoxyethelenesorbitan (Tween 80; Sigma). Drug doses used were previously shown to attenuate light-induced Fos in SCN neurons (rats: Edelstein and Amir, unpublished observations; hamsters: Abe et al., 1992). Control animals received either 1ml/kg saline i.p. (n=2), 5 µl saline i.c.v. (n=2), 5 µl 0.25% Tween 80 i.c.v. (n=4), or both saline and 0.25% Tween 80 (2.5 µl each, i.c.v.; n=2). Injections were performed under dim red light (< 5 lux), 15 minutes prior to exposure to a 30-minute light pulse (300 lux at cage level) at projected CT16 (four hours after the onset of the dark phase of the previous LD cycle). Animals were returned to darkness for 30 minutes, given an overdose of sodium pentobarbital (100 mg/kg i.p.), brains processed for Fos immunohistochemistry, and data analyzed as described in Experiment 1.

Results

All drug treatments attenuated light-induced Fos-IR in the SCN compared to vehicle treated controls (Figure 6; MK-801: $t(1,4)=4.030$, $p<0.05$; CPP: $t(1,4)=4.656$, $p<0.01$; DNQX: $t(1,6)=2.630$, $p<0.05$; CPP+DNQX: $t(1,4)=6.650$, $p<0.01$). In contrast, light induced Fos expression in the IGL regardless of drug treatment (Figure 6; MK-801: $t(1,4)=2.337$, $p>0.05$; CPP: $t(1,4)=1.765$, $p>0.05$; DNQX: $t(1,6)=1.635$, $p>0.05$; CPP+DNQX: $t(1,4)=0.314$, $p>0.05$). Few Fos-IR cells were observed in the SCN or IGL of all animals killed without light exposure (<10 cells per section; data not shown), although drug injections induced robust Fos -IR in other brain regions such as the paraventricular thalamic nucleus and piriform cortex, a finding that has been previously described (Gass, Herdegen, Bravo, & Kiessling, 1993). Examples of Fos expression in the SCN and IGL observed in rats pretreated with MK-801 (5mg/kg i.p.) or saline and exposed 15 minutes later to 30 minutes of light at CT16 are shown in Figure 7.

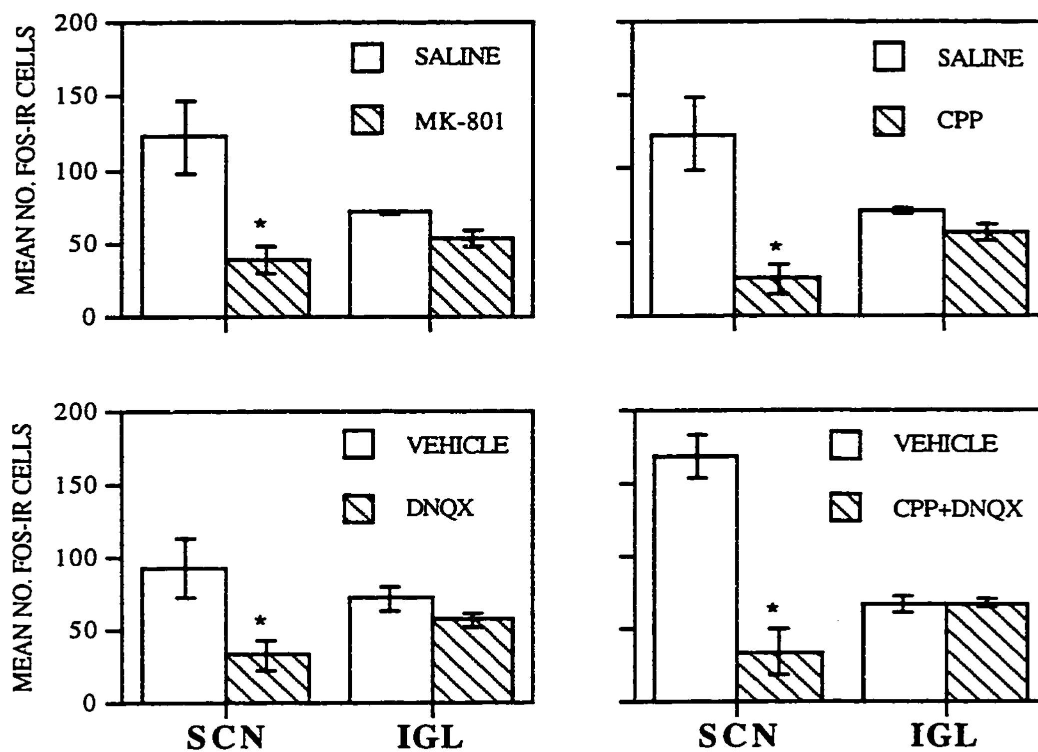

Figure 6. Mean (±sem) numbers of Fos immunoreactive cells per 50 μm tissue section, in unilateral SCN and IGL of rats treated with vehicle or drug 15 minutes prior to a 30-minute light pulse starting at CT16. Top, Left: saline or 5mg/kg MK-801 i.p. Top, Right: saline or 5nmoles CPP, i.c.v. in 5 μl. Bottom, Left: vehicle or 40 nmoles DNQX, i.c.v. in 5 μl. Bottom, Right: vehicle or 5nmoles CPP and 40 nmoles DNQX, i.c.v in 5 μl.

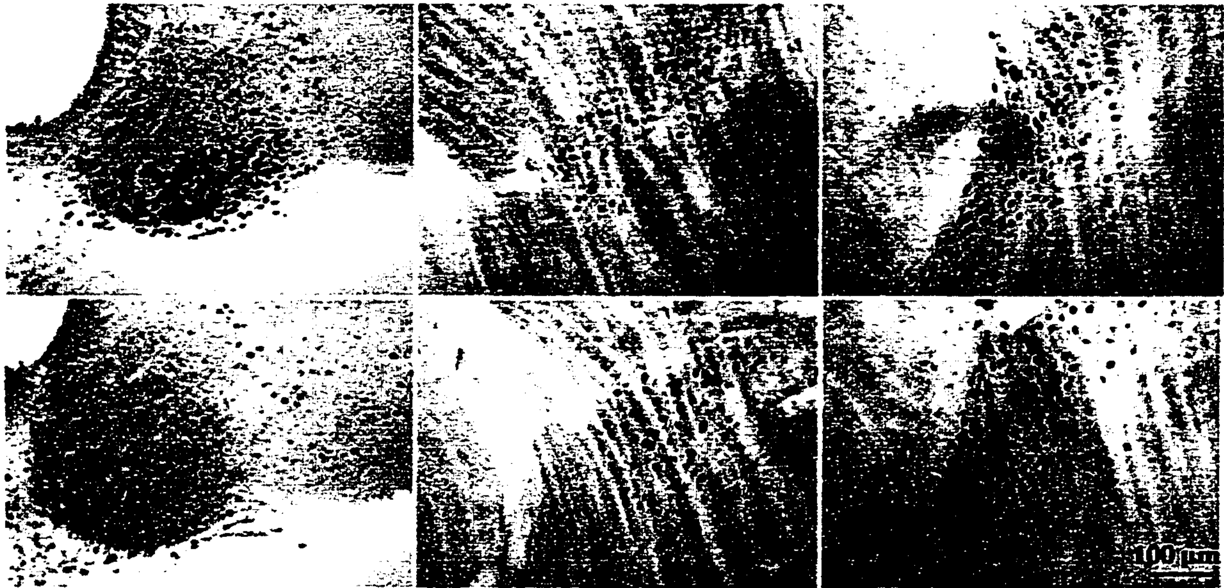

Figure 7. Photomicrographs of Fos immunoreactivity in coronal sections through the SCN (left) and IGL (rostral, middle; caudal, right) of rats treated with saline (top) or MK-801 (bottom) 15 minutes before a 30 minute light pulse at CT16. Scale bar =100 µm.

Because MK-801 has been shown to attenuate the increase in NPY fiber staining observed in the SCN in response to light at dawn (Shinohara, Tominaga, Fukuhara, et al., 1993), and these NPY fibers originate in the IGL (Moore & Card, 1994) the possibility that Fos induction in response to light exposure at dawn occurs via activation of the NMDA receptor whereas light-induced Fos at other circadian times involves different mechanisms was explored. Additional dark-adapted rats were treated with MK-801 (5mg/kg, i.p.) or saline, 15 minutes prior to a 30-minute light pulse at CT1, CT16, or CT19 (n=2 for each treatment, at each time), and brains processed for Fos-IR. Phase of light exposure had no effect on light-induced Fos in the SCN or IGL (SCN: $F(2,6)= 0.393$, $p>0.05$; IGL: $F(2,6)=0.492$, $p>0.05$). MK-801 treatment prior to a 30-minute light pulse at each time point attenuated Fos expression in the SCN and was without effect on Fos in the IGL (Figure 8; SCN: $F(1,6)=19.319$, $p<0.01$; IGL: $F(1,6)=0.603$, $p>0.05$).

Finally, because a previous study reported that two-hour light pulses induce more robust Fos expression in the rat IGL than shorter light pulses, and that MK-801 treatment attenuated this effect of light (Park et al., 1993), additional rats were treated with either MK-801 (5mg/kg, i.p; n=2) or saline (n=1) 15 minutes prior to a 30-minute or two-hour light pulse starting at CT16. Animals exposed to 30 minutes of light were killed at CT17, whereas animals exposed to two hours of light were killed at CT18. MK-801-treated dark-control animals killed two hours after injection were processed for Fos in the same assay for comparison purposes. Two hours of light exposure induced a moderate number of Fos-IR cells in the SCN whereas dense staining was observed in the SCN following a 30-minute light pulse that was attenuated by MK-801 pretreatment (data not shown). In contrast, robust Fos-IR was observed in IGL neurons, regardless of length of light exposure or drug treatment. Furthermore, no differences were observed in numbers of Fos immunoreactive cells in the IGL of animals exposed to 30 minutes or two hours of light (Table 1). Dark control animals exhibited very few Fos-IR cells in SCN and IGL regions (<10 cells per section; data not shown).

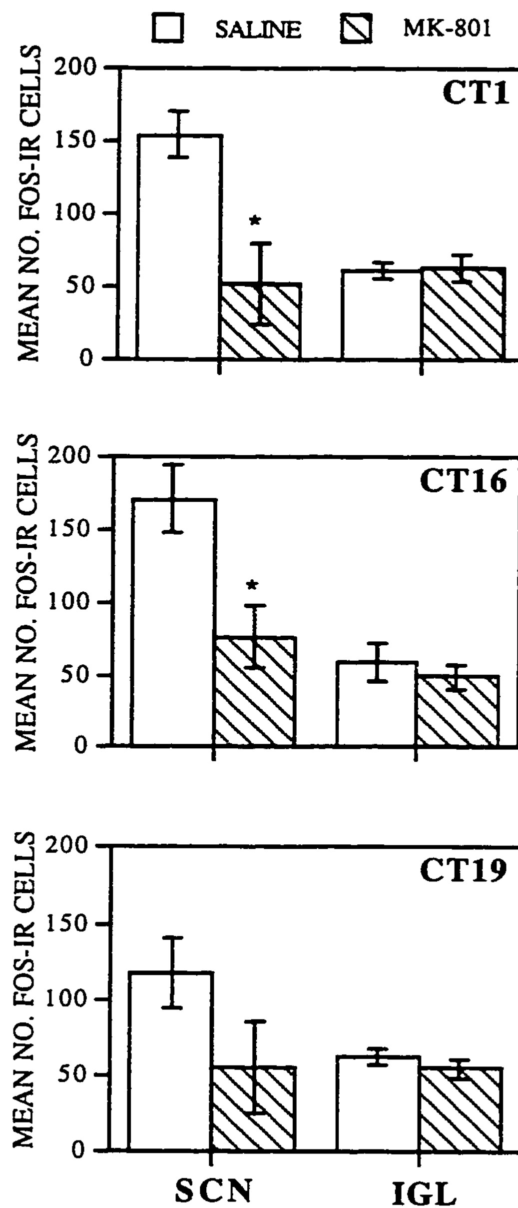

Figure 8. Mean (±sem) numbers of Fos immunoreactive cells per 50 μm tissue section, in unilateral SCN and IGL of rats treated with saline or 5mg/kg MK-801 i.p. 15 minutes prior to a 30-minute light pulse starting at CT1, CT16, or CT19.

Table 1. Mean number of Fos immunoreactive cells per IGL section in animals treated with saline or MK-801 (5mg/kg i.p.) 15 minutes prior to a 30 minute or 2 hour light pulse starting at CT16.

	SALINE	MK-801
30 minutes	46.2	48.6
2 hours	45.6	56.6

Discussion

The results of the present study show that treatments with NMDA and non-NMDA receptor antagonists that attenuate light-induced Fos in the SCN have no effect on light-induced Fos in the IGL. Although similar distributions of NMDA and AMPA/Kainate receptor subtypes have been described in SCN and IGL (Sato et al., 1993; Stamp et al., 1997), it appears that the transmission of light information to the IGL may not be mediated by glutamate receptor activation. These data are in contrast with the established role of glutamate in transmission of photic information in the circadian system.

Glutamatergic drugs have been shown to phase shift circadian rhythms with a phase response curve that resembles the effects of light or optic nerve stimulation on wheel-running activity and SCN cell firing in vitro (Ebling, 1996; Meijer et al., 1988; Mintz & Albers, 1997; Shirakawa & Moore, 1997). Treatment with glutamatergic antagonists prevented these phase shifting effects both in vivo and in vitro (Colwell et al., 1990; Colwell et al., 1991; Colwell & Menaker, 1992; de Vries et al., 1994; Ebling, 1996; Rea et al., 1993; Vindlacheruvu et al., 1992) and attenuated the induction of Fos protein by phase shifting photic stimuli in SCN neurons (Abe et al., 1991; Abe et al., 1992; Amir & Robinson, 1995; Ebling, 1996; Ebling et al., 1991; Edelstein & Amir, 1996b; Edelstein & Amir, 1996c; Mikkelsen et al., 1995; Rea et al., 1993; Vindlacheruvu et al., 1992). Sympathetic system activation in response to light exposure was also prevented by infusion of CPP, the NMDA receptor antagonist, into the SCN (Amir, 1992) The involvement of glutamate in light-induced melatonin suppression has been shown as well; intra-SCN infusion of NMDA or non-NMDA agonists suppressed pineal NAT activity and plasma melatonin levels; light-induced suppression of NAT and melatonin were attenuated by glutamatergic receptor antagonists (Ebling, 1996; Ohi et al., 1991; Takeuchi et al., 1991; but see Rowe & Kennaway, 1996). Taken together, these experiments support a critical role for excitatory amino acid neurotransmission in the

circadian system. However, the results of the present experiment together with the finding that neonatal MSG treatment prevented light-induced Fos expression in the IGL but not SCN (Edelstein et al., 1995) supports the idea that distinct retinal ganglion cells project to these regions. Perhaps the retinal ganglion cells that comprise the RHT are glutamatergic and are not affected by neonatal MSG whereas the retinogeniculate cells, which appear not to be glutamatergic, are sensitive to the neurotoxic effects of neonatal MSG.

The neurotransmitter mechanism responsible for mediating the effects of light on Fos expression in the IGL cannot be specified at this time. One possibility is that substance P (SP) is involved in transmission of photic information to the IGL. Retinal ganglion cells that project to the SCN contain SP (Mikkelsen & Larsen, 1993; Takatsuji et al., 1991) and in hamsters treatment with SP receptor antagonists attenuated light-induced Fos in the dorsal SCN region (Abe, Honma, Shinohara, & Honma, 1996). Intra-SCN infusion of SP during the early subjective night caused small phase delays in wheel running rhythms, although SP infusion was without effect at other circadian times (Piggins & Rusak, 1997). In the rat SCN slice, SP increased firing rates in SCN neurons and induced phase shifts with a phase response curve that resembles the effects of light (Shibata et al., 1992). However, the finding that enucleation does not alter the density of SP-IR in the rat IGL, suggests that the SP fiber plexus in the IGL does not originate in the retina (Hartwich et al., 1994; Miguel-Hidalgo et al., 1991; Mikkelsen & Larsen, 1993; Takatsuji & Tohyama, 1989). Those results, together with preliminary data showing that SP antagonists do not attenuate light-induced Fos in the IGL (Edelstein and Amir, unpublished observations), rule out the possibility that SP mediates light-induced Fos in the rat IGL. Another candidate neurotransmitter is pituitary adenylyl cyclase activating peptide (PACAP). PACAP projections to both SCN and IGL originate in the retina (Hannibal et al., 1997). However, a role for PACAP in the transmission of light information to the IGL seems unlikely given the finding that PACAP application to SCN

in vitro induced phase shifts during the day, in a manner similar to the phase-shifting effects of nonphotic stimuli (Hannibal et al., 1997).

In summary, light-induced Fos expression in the IGL is not attenuated by treatment with NMDA or AMPA/Kainate glutamatergic receptor antagonists. Although there are bifurcating retinal ganglion cells that innervate both the SCN and IGL, it appears that Fos induction by light in the SCN and IGL may be mediated by different mechanisms, if not different retinal ganglion cells. Despite the role of glutamate in transmission of photic and visual information, glutamate may not be involved in light transmission to the IGL.

EXPERIMENT 4

Treatment with MSG early in life leads to multiple behavioral, metabolic, neuroendocrine, and visual abnormalities including acute degeneration of amacrine, bipolar, and ganglion cells in the inner layer of the retina, demyelination of the optic tract, and destruction of neurons in the superficial layers of the superior colliculus (SC; Chambille & Servière, 1993; Cohen, 1967; Kizer, Nemeroff, & Youngblood, 1978; Lucas & Newhouse, 1957; Olney, 1969; Pickard et al., 1982; Seress, Lazar, Kosaras, & Robertson, 1984). Only a fraction of optic nerve fibers have been shown to survive MSG treatment as evidenced by the limited presence of retinal terminals in the SCN, IGL, pretectal olivary nucleus and the lateral third of the superficial layers of the SC (Chambille & Servière, 1993; Kizer et al., 1978; Pickard et al., 1982; Seress et al., 1984). Neonatal MSG treatment also destroys neuronal perikarya in the arcuate nucleus and hippocampal CA1 region, resulting in stunted growth, obesity, and learning impairments in the adult animal (Kizer et al., 1978; Kubo, Kohira, Okano, & Ishikawa, 1993; Nemeroff et al., 1977; Olney, 1969; Olney, 1971; Seress, 1982).

Despite extensive retinal damage, evidence of impairment of the circadian system following MSG treatment is limited. Entrainment of circadian rhythms of food intake, locomotor activity, body temperature, and plasma corticosterone has been demonstrated in MSG-treated animals (Chambille & Servière, 1993; Edelstein et al., 1995; Groos, 1981; Miyabo, Ooya, Yamamura, & Hayashi, 1982; Miyabo, Yamamura, Ooya, Aoyagi, Horikawa, Hayashi, 1985; Pickard et al., 1982). MSG-treated hamsters re-entrained to shifted LD cycles and exhibited light-induced phase shifts in wheel running activity rhythms with a phase response curve similar to controls (Chambille & Servière, 1993; Pickard et al., 1982). Nocturnal light pulses induced Fos expression in the SCN of MSG-treated rats (Edelstein et al., 1995) and hamsters (unpublished data cited in Chambille & Servière, 1993). In addition, light-induced suppression of pineal N-acetyltransferase

(NAT) persisted in these animals (Nemeroff et al., 1977). The absence of overt effects on entrainment, phase shifts, SCN Fos expression, and pineal NAT activity indicates that the RHT remains functionally intact in MSG-treated animals. A recent study showing that the retinal ganglion cells that survive MSG treatment were those that exhibit Fos-IR in response to light flashes suggests that a distinct subset of retinal ganglion cells exists and perhaps those cells comprise the RHT (Chambille, 1998).

In contrast with the lack of effect of MSG on photic entrainment, MSG has been shown to alter the circadian response to prolonged LL housing. Prolonged LL causes disruptions in circadian rhythms, resulting in increases in period, splitting of circadian activity rhythms in hamsters and arrhythmicity in rats (Edelstein et al., 1995; Harrington & Rusak, 1988; Pickard et al., 1987). Because hamsters with IGL lesions are less likely to exhibit LL-induced disruptions in rhythmicity (Harrington & Rusak, 1988; Pickard et al., 1987), it is thought that the IGL mediates the circadian response to prolonged LL. The finding that circadian temperature rhythms of rats treated neonatally with MSG persisted under LL and that phase-shifting light pulses did not induce Fos expression in the IGL in those animals raises the possibility that the IGL mediates the response to LL in rats as it does in hamsters, and that neonatal MSG treatment disrupted the integrity of the retinogeniculate pathway, thereby damaging the IGL, and its input to the SCN.

The role of the IGL in the response to LL in rats is explored more directly in the present study, by comparing circadian temperature rhythms of adult rats treated neonatally with MSG or saline, to rats with bilateral IGL-lesions or sham operations, under LL conditions. In addition, the integrity of the GHT following MSG treatment or IGL lesion is evaluated using immunohistochemical staining of NPY in the SCN (Harrington, 1997; Moore & Card, 1994; Morin, 1994). These data have been previously published in abstract form (Edelstein & Amir, 1997a) and are currently in press.

Method

Animals

Male Wistar rats (Charles River Canada, St. Constant, Quebec) were used in all experiments. Rats given IGL lesions were housed under LD12:12 for at least two weeks prior to surgery. MSG-treated rats were bred at the Concordia University animal facility.

Surgery

Animals (275-300g) were given atropine sulfate (0.06mg/0.1ml, s.c.) 20 minutes prior to sodium pentobarbital anesthesia (65mg/kg i.p.) and implanted intraperitoneally with precalibrated telemetry transmitters (Mini Mitter). Bilateral electrolytic lesions aimed at the IGL were made using a Grass LM4 lesion maker, by passing 2 mA current for 15 seconds through stainless steel electrodes, insulated except for the tip. Electrode placements were at three rostrocaudal positions on each side of the midline: 4.0, 4.7, and 5.4 mm caudal to bregma; 3.8 mm from the midsagittal sinus; and 4.7 mm ventral to dura. Sham operated rats were treated to the same surgical procedures, except that electrodes were lowered to 1mm above the lesion targets and no current was passed.

Monosodium Glutamate Treatment

Wistar rats were bred in the animal facility at Concordia University and housed under LD12:12, with ad libitum access to rat chow and water. At birth, litters were culled to 12 pups with a minimum of six males and three females each, and housed with their dams. Pups from each litter received five subcutaneous injections of either 2 mg/g MSG (Sigma) dissolved in distilled water, or 10% saline (to control for the osmolarity of the MSG) on postnatal days 1,3,5,7, and 9, as previously described (Edelstein, Pfaus, et al., 1995). To prevent leakage of the drug, the injection site was sealed with Collodion (Fisher). Animals were weaned at 21 days of age, and housed with their litter-mates for five days. On day 26, male rats were separated from the females and housed in groups of

two per cage until three months of age. Adult animals were implanted intraperitoneally with precalibrated Mini Mitter transmitters under Metofane (methoxyflurane; Janssen Pharmaceuticals, Markham, Ontario) anesthesia.

Temperature Rhythm Recording and Data Analysis

Animals were housed individually in plastic cages in light-tight, temperature controlled, ventilated rooms. Circadian temperature rhythms were measured under LD12:12 (~300 lux during lights on) for 2 weeks, LL (4 weeks), and constant darkness (DD; 2-4 weeks) using a telemetry system and Dataquest software (Mini Mitter). Body temperature data of individual animals were smoothed with a 90 minute moving average. Fourier analysis was used to assess rhythmicity in the temperature records of each animal under each of the lighting schedules. Period of the circadian temperature rhythm for each animal was calculated during the last 6 days of LL and DD using cosinor analysis. Mean body temperature per day over the final 6 days of each lighting schedule was calculated for each animal. Effects of lighting schedule (LIGHT) and treatment (GROUP) on circadian period and body temperature were evaluated using analysis of variance.

Preparation of Tissue

Animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and brains prepared for immunohistochemistry as described in Experiment 1. Serial coronal brain sections (50 µm) through the SCN were cut from each brain on a vibratome, and processed for NPY immunohistochemistry as described below. The caudal portion of brains of IGL-lesioned and sham operated animals were then cryoprotected in 30% sucrose formalin solution, and frozen coronal sections through the lateral geniculate nucleus were sliced on a cryostat and stained with thionin.

NPY Immunohistochemistry

Tissue sections were washed in cold 50 mM Tris buffered saline (TBS; pH 7.6; Sigma) and incubated in a solution of 30% H_2O_2 (Sigma) in TBS for 30 minutes at room temperature. Tissue sections were then preincubated in a solution of 0.30% Triton X 100 (Sigma) in TBS (0.30 % TTBS) with 4% normal goat serum (NGS; Vector, Burlingame, CA) on an orbital shaker for one hour at room temperature. Tissue sections were then incubated for 48 hours at 4°C with a rabbit polyclonal anti-NPY antibody (Peninsula, Belmont, CA; Lot # 960108-2). The antibody was diluted 1:5000 with a solution of 0.30% TTBS with 2 % NGS. Following incubation in the primary antibody, sections were rinsed in cold TBS and incubated for one hour at 4°C with a biotinylated anti-rabbit IgG made in goat (Vector), diluted 1:66 with 0.30% TTBS with 2% NGS. 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 Standard Elite ABC Kit, Vector). Following incubation with the ABC reagents, sections were rinsed with cold TBS, rinsed again with cold 50 mM Tris buffer (pH 7.6), and again for 10 minutes with 0.05% 3,3'-diaminobenzidine (DAB; Sigma) in 50 mM Tris-HCl. Sections were then incubated on an orbital shaker for 10 minutes in DAB/Tris-HCl with 0.01% H_2O_2 and 8% $NiCl_2$ (Sigma). After this final incubation, sections were rinsed with cold TBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene, and coverslipped with Permount (Fisher).

Histology

Effectiveness of MSG-treatment was confirmed by visual inspection of degeneration of the optic tract, which appears clear and significantly reduced in width following MSG. Completeness of IGL-lesions was verified under a microscope using 2 criteria: absence of NPY fiber staining in the SCN, and inspection of thionin stained sections throughout the LGN.

Results

Histology

Four animals had both complete symmetrical lesions and absence of NPY fiber stain in the SCN (Figure 9). Three of the remaining rats appeared to have either unilateral lesions or asymmetrical lesions as well as some NPY-IR in the SCN. All lesioned animals sustained moderate to extensive damage throughout the dorsal and ventral lateral geniculate regions and the hippocampus at the level of the IGL. One lesioned animal was eliminated from the analysis because of illness during the course of the experiment, and one sham-operated rat was eliminated because of loss of telemetry transmitter signal. Because extent of lesion had no effect on disruption of rhythmicity in LL, data from lesioned animals were analyzed as a group (IGL lesion: n=7; Sham: n=3).

MSG-treated rats all showed clear degeneration of the optic tract, visible to the naked eye. In addition, unlike animals with complete IGL lesions, rats treated neonatally with MSG exhibited a pattern of NPY fiber staining in the SCN similar to saline-treated control rats (MSG: n=8; Saline: n=4; Figure 9).

Circadian Rhythms

All animals entrained to LD12:12 and exhibited free running rhythms under DD housing conditions (Figures 10 and 11). Average period was slightly greater than 24 hours in DD for all treatment groups (Table 2). In contrast, during the last six days of LL housing, IGL-lesioned, sham-operated, and saline-treated rats had no significant rhythm in the circadian range, whereas MSG-treated rats continued to exhibit robust temperature rhythms (Table 2; Figure 12; GROUP: $F(3,18) = 31.460$, $p < 0.001$; LIGHT: $F(1,18)=194.532$, $p < 0.001$; GROUP x LIGHT: $F(3,18)=31.888$, $p<0.001$). Furthermore, the pattern of disruption of rhythms during the LL period appeared to be the same for animals with complete, bilateral electrolytic IGL lesions, animals with incomplete or

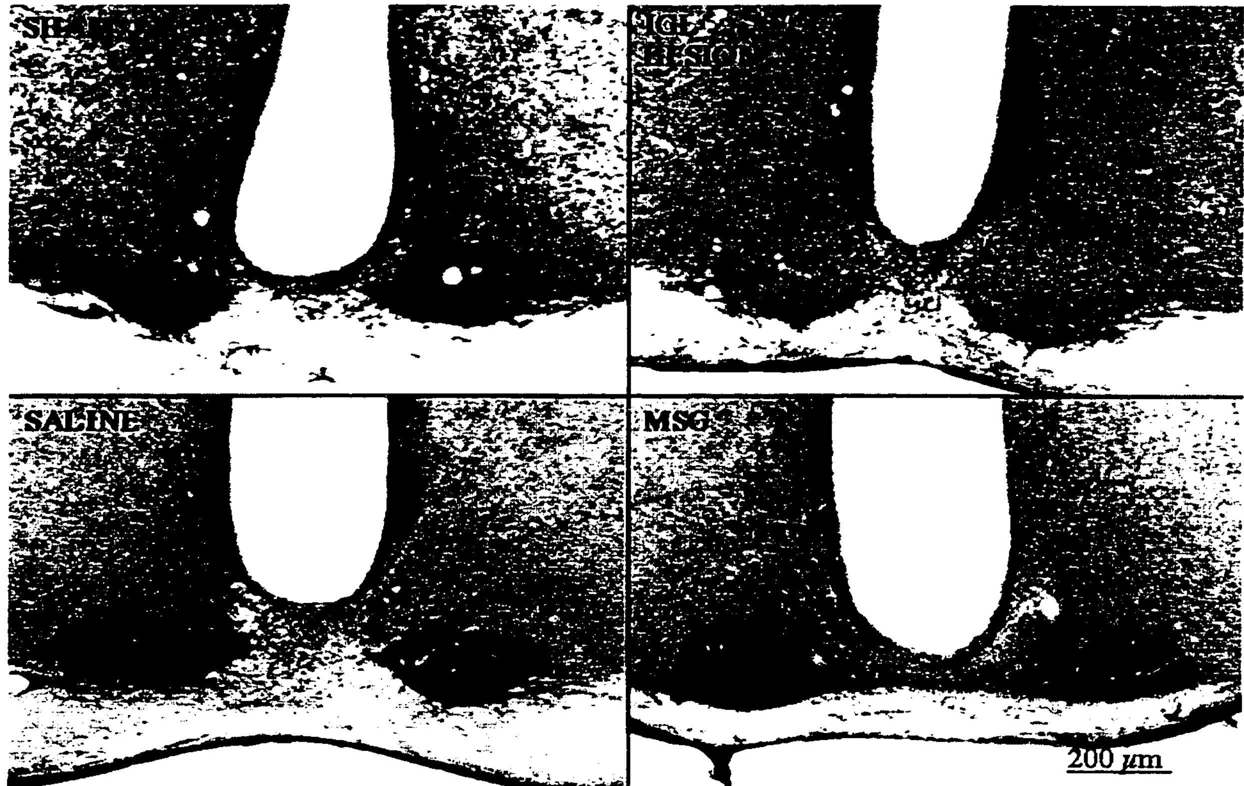

Figure 9. Neuropeptide Y immunoreactivity in coronal sections through the suprachiasmatic nucleus. TOP: sham-operated (left) and intergeniculate leaflet-lesioned (right) rats. Bottom: Adult rats treated neonatally with 10% saline (left) or 2 mg/g monosodium glutamate (right).

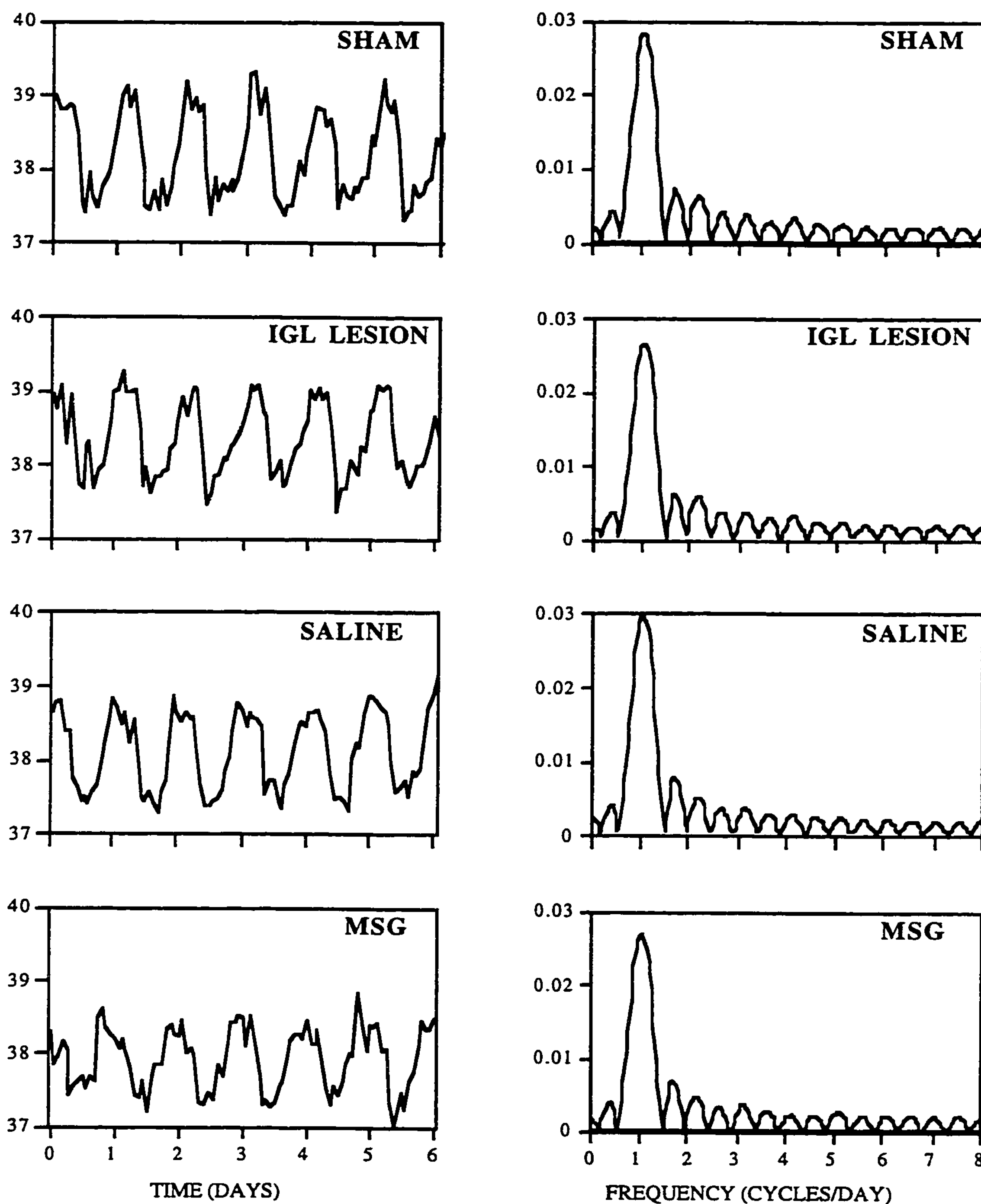

Figure 10. Circadian temperature rhythms (left) and corresponding power spectra based on Fourier analyses (right) of sham-operated or IGL-lesioned rats, and adult rats treated neonatally with 10% saline or 2 mg/g MSG during the last six days of LD 12:12 housing.

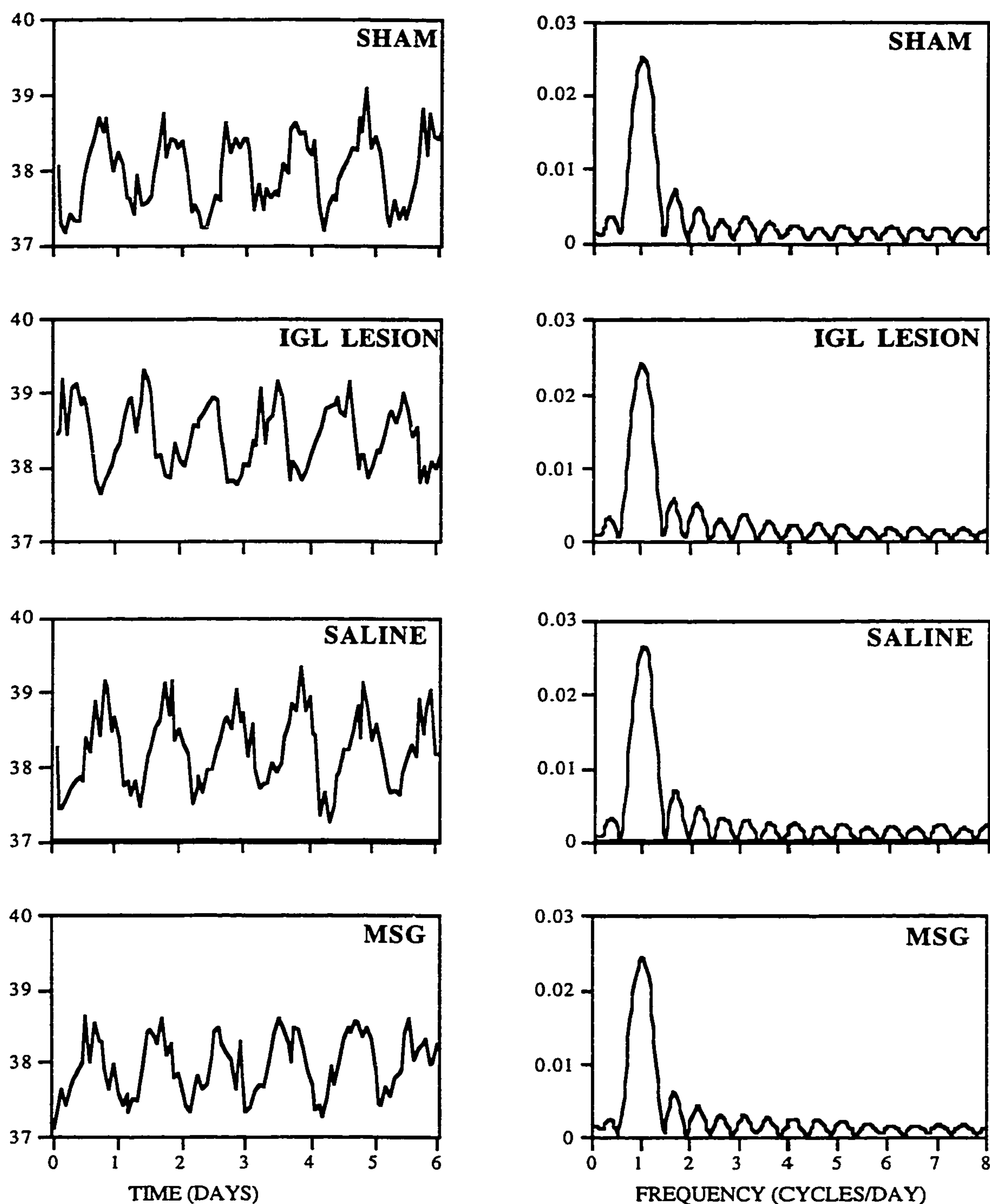

Figure 11. Circadian temperature rhythms (left) and corresponding power spectra based on Fourier analyses (right) of sham-operated or IGL-lesioned rats, and adult rats treated neonatally with 10% saline or 2 mg/g MSG during the last six days of DD housing.

Table 2. Average period of circadian temperature rhythms across the last 6 days of constant light and constant dark housing in adult rats treated neonatally with 10% saline (n=4) or monosodium glutamate (n=8), or in intergeniculate leaflet-lesioned (n=7) and sham-operated (n=3) animals (hours; mean ± sem).

	SHAM	IGL-LESION	SALINE	MSG
LL	N/A	N/A	N/A	24.71 ±0.15
DD	24.28 ±.07	24.33 ±.09	24.31 ±.02	24.23 ±.08

N/A: not applicable

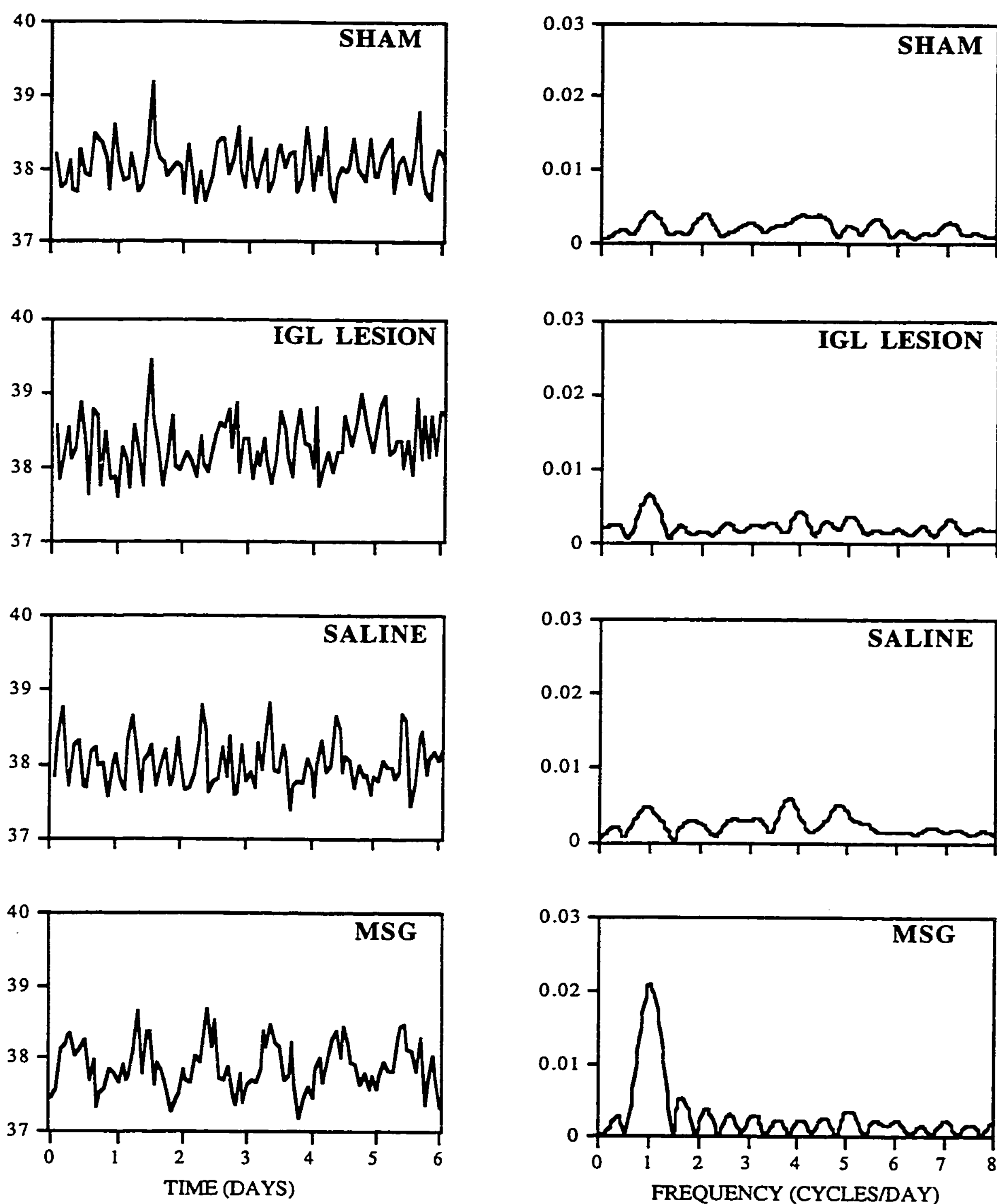

Figure 12. Circadian temperature rhythms (left) and corresponding power spectra based on Fourier analyses (right) of sham-operated or IGL-lesioned rats, and adult rats treated neonatally with 10% saline or 2 mg/g MSG during the last six days of LL housing.

asymmetrical lesions, and sham-operated animals; all animals had no significant period in the circadian range by the third week in LL. MSG-treated animals exhibited a significant increase in free running period during the last six days of LL as compared to the last six days of DD housing ($p < 0.05$; Table 2)

Lighting schedule had no effect on mean body temperature within each treatment group (Table 3; $F(2,36) = 2.703$, $p > 0.05$). However, MSG-treated animals maintained lower average temperatures as compared to the other treatment groups across all lighting schedules (Table 3; $F(3,18)=11.314$; $p < 0.001$).

Discussion

Results of this experiment demonstrate that the IGL does not mediate LL-induced disruption of circadian temperature rhythms in the rat as it does in the hamster, and that the effects of neonatal MSG treatment on the circadian response to LL are not analogous to the effects of IGL ablation. Whereas MSG-treated rats are not sensitive to the effects of LL, IGL-lesioned rats exhibit similar patterns of disruption to temperature and activity rhythms that intact rats do.

The different effects of neonatal MSG and IGL lesion on the circadian system are evident from the pattern of NPY staining in the SCN as well. NPY-IR terminals found in the ventrolateral SCN region originate in the IGL (cf. Harrington, 1997; Moore & Card, 1994); complete bilateral lesions of the IGL eliminate NPY fiber staining in the SCN (Harrington, 1997; Harrington & Rusak, 1988; Moore & Card, 1994; Pickard et al., 1987). In contrast, neonatal MSG treatment has no effect on the pattern of NPY-IR in the SCN (present study). NPY-IR was observed in the ventrolateral region of the SCN in all MSG-treated rats in a pattern similar to that seen in saline-treated animals. This suggests that the GHT is intact in MSG-treated animals. However, whether the diurnal rhythm of NPY-IR in the SCN found in normal rats is preserved in MSG-treated animals is

Table 3. Average daily body temperatures across the last 6 days of 12:12 light-dark cycle, constant light, and constant dark housing in adult rats treated neonatally with 10% saline (n=4) or monosodium glutamate (n=8), or in intergeniculate leaflet-lesioned (n=7) and sham-operated (n=3) animals (°C; mean ± sem).

	SHAM	IGL-LESION	SALINE	MSG
LD	38.27 ± 0.15	38.46 ± 0.09	38.12 ± 0.02	37.85 ± 0.04
LL	38.11 ± 0.19	38.33 ± 0.06	38.12 ± 0.05	37.87 ± 0.04
DD	38.02 ± 0.20	38.39 ± 0.08	38.08 ± 0.06	37.96 ± 0.07

unknown. NPY levels in the SCN exhibited peaks in response to light-dark transitions at dawn and dusk (Shinohara, Tominaga, Fukuhara et al., 1993), suggesting that NPY release is involved in the circadian response to light. Because IGL neurons in MSG-treated animals do not exhibit Fos expression in response to phase shifting light pulses (Edelstein et al., 1995), it is possible that the timing of release of NPY into the SCN is also affected by neonatal MSG treatment. NPY has also been shown to depress excitatory neurotransmission and block glutamate-induced phase shifts in SCN neurons in vitro and light-induced phase advances in vivo (Biello et al., 1997; van den Pol et al., 1997; Weber & Rea, 1997). Perhaps differences in the pattern of release of NPY into the SCN during LL renders MSG-treated rats insensitive to the disruptive effects of this lighting schedule.

Another possibility is that the disruptive effects of LL housing are mediated at the level of the superior colliculus. Neonatal MSG treatment completely destroys neurons of the two superficial layers of the SC (Seress et al., 1984). These layers have reciprocal connections with the IGL and pretectum (Moore et al., 1996; Taylor et al., 1986). Both the IGL and the pretectum receive retinal input and project back to the SCN (Harrington, 1997; Mikkelsen & Vrang, 1994; Moore & Card, 1994; Moore et al., 1996; Scalia, 1972) presumably supplying the circadian pacemaker with photic information that is not provided directly from the RHT. Little is known about the role of the collicular input to these regions. It is possible, however, that these projections allow feedback between the circadian and visual systems to occur. Speculation about the SC as a site of integration of multiple sensory inputs to the circadian clock exists as well (Harrington, 1997; Morin, 1994). The SC regulates saccadic eye movements, and is necessary for orienting visual and motor responses to sensory stimuli (Dean, Redgrave, & Westby, 1989). Animals with collicular lesions exhibited sensory neglect that resulted from both attention and detection deficits, despite normal performance on visual discrimination tasks (Dean & Redgrave, 1984). The absence of input from the SC to the IGL and/or pretectal area in

MSG-treated animals may prevent the disruption of rhythms during prolonged LL, and result in the free running rhythms observed in these animals.

The third possibility is that the LL-induced disruption in rhythms occurs via direct retinal input to the SCN, and that neonatal MSG treatment destroys the retinal projection involved. The RHT is comprised of retinal ganglion cells that are unique in type and morphology (Moore et al., 1995), some of which bifurcate and project to the IGL (Pickard, 1985). Although neonatal MSG treatment destroys 90% of retinal ganglion cells (Chambille & Servière, 1993; Cohen, 1967; Kizer et al., 1978; Lucas & Newhouse, 1957; Olney, 1969), bilateral retinal innervation of the SCN and LGN persists following treatment (Chambille & Servière, 1993; Groos, 1981; Pickard et al., 1982; Seress et al., 1984). Furthermore MSG-treated animals entrain, phase shift, and exhibit light - induced Fos expression in the SCN (Chambille & Servière, 1993; Edelstein et al., 1995; Groos, 1981; Miyabo et al., 1982; Miyabo et al., 1985; Nemeroff, et al., 1977; Pickard et al., 1982). These data support the idea that, despite the reduction in density of retinal terminals in the SCN, the retinal ganglion cells that are necessary for entrainment survive MSG treatment. However, because MSG treatment also affects retinal input to the IGL (as assessed by loss of induction of Fos-IR by light; Edelstein et al., 1995), perhaps the lack of response to LL reflects damage to retinal ganglion cell axons projecting to the IGL in these animals. It is possible that MSG treatment selectively destroys those retinal ganglion cells whose axon collaterals terminate in both SCN and IGL (Pickard, 1985), and that those cells mediate the response to LL. If this is the case, then destruction by MSG would alter the sensitivity of the visual system to the disruptive effects of LL, and prevent induction of Fos expression in the IGL region, an idea supported by our previous findings.

The arrhythmicity that has been observed in normal rats under LL conditions may result from a disruption of circadian pacemaker activity, or may result from the direct suppression of body temperature or general activity levels by light exposure, the masking

effects of light. Although it has been suggested that the IGL is involved in the masking effects of light (Harrington, 1997; Sisk & Stephan, 1982), recent data have shown that rhythms of IGL-lesioned rats (body temperature; Edelstein & Amir, 1997b; see Experiment 5) and hamsters (wheel running activity; Redlin, Vrang, & Mrosovsky; unpublished observations) are at least as sensitive to the masking effects of light as intact animals are. Masking effects are apparent in other species with various types of visual impairments including the retinally degenerate mouse (Mrosovsky, 1994). Although the question of whether MSG-treated animals are sensitive to the masking effects of light has yet to be tested directly, there is no reason to assume that MSG treatment results in a masking-specific impairment of the circadian system. The temperature records of these animals indicate that these rats are sensitive to the masking effects of light; MSG-treated animals exhibit abrupt transitions in body temperature (present study) and general activity (Edelstein & Amir, unpublished observations) at times of light onset and light offset. Such abrupt transitions are thought to occur because of masking effects of light (Mrosovsky, 1994).

It is not yet known whether the disruptive effects of LL on temperature rhythms in rats occur via input to the SCN, or from an independent mechanism that may not involve the SCN. Indeed, neonatal MSG treatment appears to have little effect on visual and circadian behaviors. Despite the severity of the damage to the retina, the only overt manifestation of visual dysfunction in these animals reported is an impaired visual placing response (unpublished data cited in Nemeroff et al., 1977), as well as impaired performance in a visual discrimination task (Kubo et al., 1993). These deficits can be attributed to learning impairments resulting from hippocampal damage (Kubo et al., 1993). Electrophysiological responses of dLGN cells in MSG-treated rats in response to light flashes were not different from saline-treated controls (Groos, 1981), supporting the idea that MSG treated animals should perform normally in visual discrimination tasks. The circadian system in MSG-treated rats is also relatively unimpaired - animals entrain

to LD cycles, phase shift to light pulses, and free run in DD for several weeks (Chambille & Servière, 1993; Edelstein et al., 1995; Miyabo et al., 1982; Miyabo et al., 1985; Nemeroff et al., 1977; Pickard et al., 1982). Thus the insensitivity of MSG treated rats to the disruptive effects of prolonged LL housing is highly unusual, and perhaps reflects damage to multiple brain areas including the visual and circadian systems. It is important to note that LL-induced arrhythmicity in intact animals is thought to be a function of light intensity (Summer, Ferraro, & McCormack, 1984). At dimmer levels of illumination, only a lengthening of period has been observed, whereas brighter light intensities have been shown to produce arrhythmicity (Summer et al., 1984). One possibility is that rhythms of MSG-treated animals would disrupt under brighter light intensities, an idea supported by the finding that the MSG-treated animals in the present experiment exhibited an increased free-running period in LL.

In summary, the disruptive effects of LL exposure on circadian rhythms in the rat are not mediated by a direct IGL input to the SCN. Whether such disruption is due to masking of overt expression of circadian rhythmicity or other mechanisms involving the circadian and/or visual systems is not known. The preservation of circadian rhythmicity in animals treated neonatally with MSG may result from decreased sensitivity to prolonged LL due to retinal or collicular damage.

EXPERIMENT 5

Results of Experiment 4 show that the IGL does not mediate the circadian response to constant light housing in the rat as it does in the hamster. However, the results of the IGL Fos studies (Experiments 1-3) as well as the studies demonstrating an interaction between release of NPY and the circadian response to light raise the possibility of a critical role for the IGL in light-induced resetting of the circadian clock. The diurnal rhythm of NPY in the SCN with peaks occurring in response to light-dark transitions at dawn and dusk (Shinohara, Tominaga, Fukuhara et al., 1993), the inhibitory role of NPY on excitatory neurotransmission (van den Pol et al., 1996) and the blockade of glutamate-induced phase shifts in SCN neurons in vitro (Biello et al., 1997) and light-induced phase advances in vivo (Weber & Rea, 1997) by NPY all support the idea of a role for the IGL in synchronization of circadian rhythms to discrete pulses of light.

Nocturnal animals have been shown to entrain normally to cycles of brief, discrete pulses of light given at dusk and dawn (DeCoursey, 1986; Pittendrigh and Daan, 1976; Rosenwasser, Boulos, and Terman, 1983; Stephan, 1983). Indeed, in the natural environment and in simulated laboratory conditions, nocturnal animals exposed themselves to light only at the transition times (DeCoursey, 1986). In addition, because light exposure suppresses overt expression of rhythmic variables (Mrosovsky, 1994), SPP housing allows for the study of pacemaker entrainment while controlling for the interfering masking effects of light. The involvement of the IGL in entrainment of circadian temperature rhythms to a skeleton photoperiod (SPP) consisting of two one-hour light pulses at times corresponding to dusk and dawn is investigated in this experiment. Results have been published in abstract form (Edelstein & Amir, 1997b) and have been submitted for publication.

Method

Animals

Male Wistar rats (Charles River Canada, St. Constant, Quebec) were used in all experiments and were housed under LD12:12 with free access to food and water for at least two weeks prior to surgery.

Surgery

Animals (275-300g) were implanted intraperitoneally with precalibrated telemetry transmitters (Mini Mitter) and received bilateral electrolytic lesions aimed at the IGL or sham operations as described in Experiment 4.

Temperature Rhythm Recording and Data Analysis

Animals were housed individually in plastic cages in light-tight, temperature controlled, ventilated rooms. Circadian temperature rhythms were measured under LD12:12 (~300 lux during lights on), SPP, and constant darkness (DD) using a telemetry system and Dataquest software (Mini Mitter). The SPP consisted of two one-hour light pulses given at zeitgeber times (ZT) corresponding to lights on and lights off of the previous LD cycle (ZT0-1 and ZT11-12). This lighting schedule maintained the long night of the previous LD cycle (12 hours between light pulses) with a shorter day (10 hours), a paradigm that facilitates stable entrainment in nocturnal animals (Pittendrigh & Daan, 1976; Stephan, 1983). Period of the circadian temperature rhythm for each animal was calculated during the first six days of DD housing using cosinor analysis of body temperature data smoothed with a 90 minute moving average, and group means were compared (t-test). Entrainment to LD cycles was assessed by visual inspection of actograms of body temperature records for each animal, constructed using Dataquest software (Mini Mitter) based on temperature minima and maxima for each animal.

Photic Stimulation

One group of animals was housed in LD12:12 for 3 weeks and killed at ZT1 on days 21-23. A second group of animals was housed in LD12:12 for 7 days, and then released into a SPP. These rats were then killed after either the dawn (ZT0-1) or dusk (ZT11-12) light pulses on days 5 or 6 of the SPP. A third group of animals whose rhythms were not recorded were housed under LD12:12 for 3 weeks after surgery, transferred into DD for 24-36h and then killed after exposure to one hour light pulses in the middle of the projected subjective day (between circadian time (CT) 3-5; where CT0=dawn) or night (between CT15-17). Effects of time of light exposure (PHASE) and treatment (GROUP) on mean number of Fos immunoreactive cells per SCN section per group were evaluated using analyses of variance (ANOVA) where appropriate.

Preparation of Tissue and Histology

Animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and alternate tissue sections from the SCN regions of all brains were processed for either Fos or NPY immunohistochemistry as described in Experiments 1 and 4. The caudal portion of all brains were cryoprotected and sections through the lateral geniculate nucleus were stained with thionin as described in Experiment 4. Completeness of IGL-lesions were verified under a microscope using 2 criteria: absence of NPY fiber staining in the SCN, and inspection of thionin stained sections through the LGN.

Results

All lesioned animals sustained large lesions centered on the IGL, with extensive damage to the lateral geniculate complex, as well as part of the hippocampus and the optic tract. Schematic drawings of the largest and smallest lesion are given in Figure 13. Completeness of the lesion was confirmed by the absence of NPY-IR in the SCN (Figure 14). Two lesioned animals and 1 control animal were eliminated from the analysis

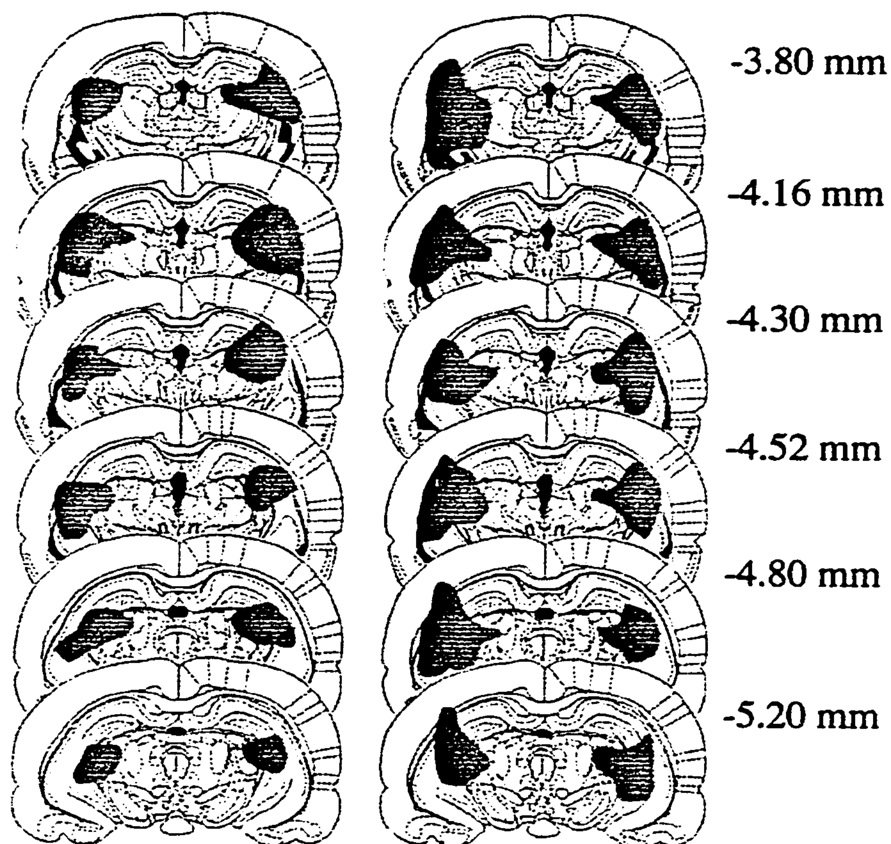

Figure 13. Largest (right) and smallest (left) electrolytic lesions centered on the intergeniculate leaflet in six coronal sections at the levels indicated in millimeters posterior to bregma (from Paxinos and Watson, 1998).

Figure 14. Neuropeptide Y immunoreactivity in coronal sections through the suprachiasmatic nucleus of sham-operated (left) and IGL-lesioned (right) rats.

because they exhibited free running periods of precisely 24 hours, making it impossible to confirm entrainment. Two additional lesioned animals were eliminated from the analysis because of illness during the course of the experiment, and one sham-operated rat was eliminated because of loss of telemetry transmitter signal. Results of the experiments presented below include data for 5 IGL-lesioned and 3 sham-operated rats (Group 1); 7 IGL-lesioned and 3 sham-operated rats (Group 2); and an additional 8 IGL-lesioned and 4 sham-operated rats (Group 3).

Circadian temperature rhythms during LD12:12, SPP and DD

IGL-lesioned and sham-operated animals (Group 1) were housed under LD12:12 for 14 days, followed by 3 weeks of SPP, and were then released into DD for 2 weeks. All animals entrained to LD12:12 and exhibited free running rhythms under DD housing conditions. Average period was slightly greater than 24h in DD for all treatment groups (Table 4), and no differences in free running period were found between groups ($t(8)=0.363$, $p>0.05$). Under the SPP, rhythms of sham-operated rats remained entrained. In contrast, four of the five rats with IGL lesions exhibited free running rhythms. Sample temperature records of an IGL-lesioned and a sham-operated rat during LD12:12, SPP and DD housing are shown in Figure 15.

Circadian temperature rhythms during prolonged SPP housing

The failure to entrain to the SPP in Experiment 1 may reflect a sluggish adjustment of the clock to this novel lighting schedule. If this is so, then following a 180° or 360° shift in the free running rhythm, when the light pulses of the SPP fall at times that light can effectively entrain the rhythm to a 24 hour schedule, perhaps lesioned animals would be able to establish stable entrainment. This hypothesis was evaluated by measuring temperature rhythms of animals with IGL lesions or sham operations (Group 2) housed under LD12:12 and then released into a SPP for 48 days. Sham-operated

Table 4. Period of the body temperature rhythm of IGL-lesioned and sham-operated rats in DD.

	Mean (hours ± sem)	Range (hours; Min - Max)
SHAM (n=3)	24.28 ± 0.07	24.17 - 24.42
IGL-lesion (n=7*)	24.33 ± 0.09	24.08 - 24.75

* includes data from 2 IGL-lesioned animals that exhibited a 24 hour free running period.

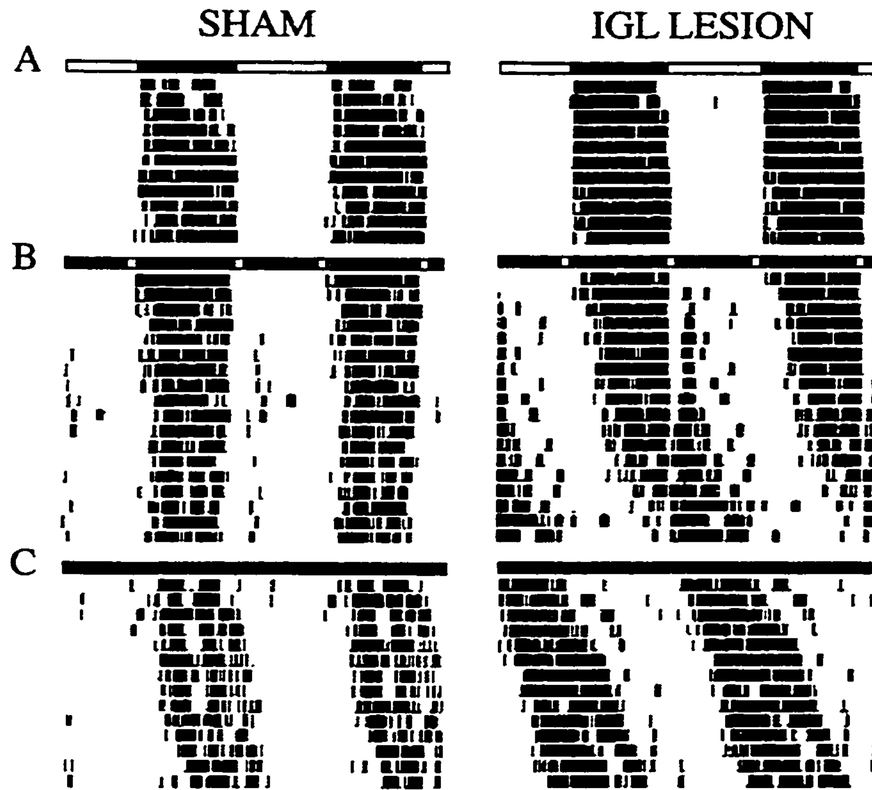

Figure 15. Double-plotted actograms of body temperature records of a sham-operated and an IGL-lesioned rat under LD12:12 (A), SPP (B), and DD (C). Light bars = lights on; dark bars = lights off.

animals maintained steady phase relationships with the SPP throughout this period. One animal entrained throughout the 7 week period; one animal phase jumped after 30 days in the SPP so that the active phase became the shorter period between the two light pulses, and the third animal entrained for 21 days, after which it exhibited rapid delay transients before re-entraining stably to the SPP for the final 15 days of SPP housing. In contrast, four of the seven IGL lesioned rats exhibited free running rhythms throughout the SPP housing period, despite several 360° revolutions through the SPP. The remaining three IGL-lesioned rats free ran for 27-31 days, re-entraining after a 360° revolution in the rhythm. Although IGL-lesioned animals failed to entrain normally to the SPP, relative coordination of the temperature rhythm in response to the light pulses was observed; adjustments in period of the rhythm as a function of phase of the LD cycle occurred. In addition, masking, or acute suppression, of body temperature during times of light exposure was evident in all animals that failed to entrain to the SPP. This suggests that physiological responses to the SPP light pulses are not prevented in IGL-lesioned animals despite extensive damage to the LGN. The relative coordination and masking effects of light observed during the SPP in IGL-lesioned animals is evident in the temperature records of a representative IGL-lesioned animal shown in Figure 16.

Entrainment or masking under LD12:12?

Because IGL-lesioned animals exhibited suppression of body temperature during exposure to the light pulse under the SPP, we considered the possibility that the synchronized temperature rhythm observed under LD12:12 may have been due to masking effects of light on temperature, rather than the entraining effects of light on the clock. To verify whether animals were entrained to LD12:12, free running animals housed in DD (Group 2) were released into LD12:12 for 2 weeks and then released into DD for 10 days. Onset of the free running rhythm was examined relative to the time of lights off of the previous LD cycle, and compared to the predicted onset extrapolated

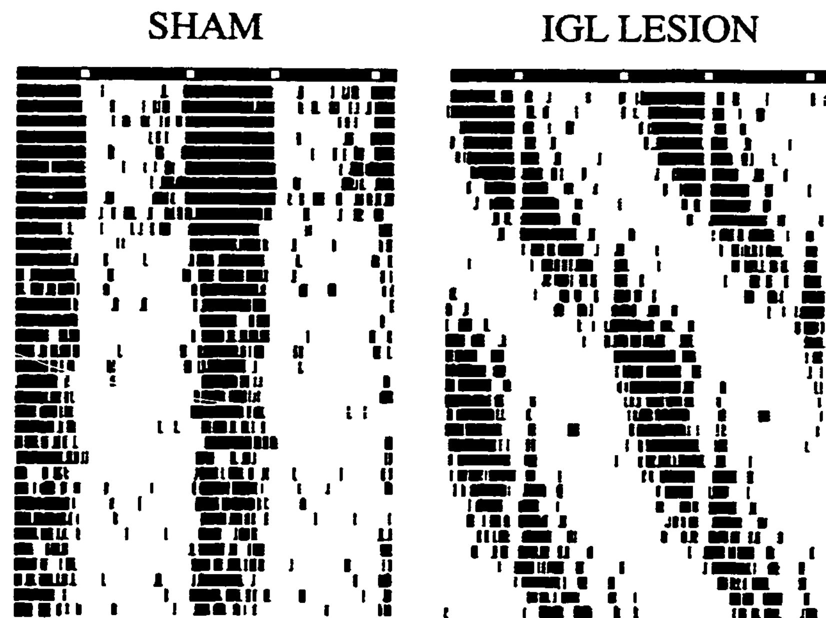

Figure 16. Double-plotted actograms of body temperature records of a sham-operated and an IGL-lesioned rat during prolonged SPP housing. Light bars = lights on; dark bars = lights off.

from the initial free run. No differences between IGL-lesioned and sham-operated animals were observed; in all animals onset of free running occurred at the time that could be predicted from the LD cycle and not from the initial free run, demonstrating that IGL-lesioned rats entrain to full photoperiods (Figure 17).

Light-induced Fos immunoreactivity in the SCN

The failure of IGL-lesioned rats to entrain to the SPP may be due to disruption of light transmission to the LGN as a result of the lesion. To test whether such damage affected either light transmission to the SCN or the response of the SCN to light, we assessed the profile of light-induced Fos protein expression in the SCN. Fos expression in the SCN is induced by light stimuli that phase shift circadian rhythms, and the magnitude of expression correlates with the effectiveness of light as a resetting stimulus (Aronin, et al., 1990; Kornhauser, et al., 1990; Rea, 1989; Rusak, et al., 1990). To study the effect of light exposure at dawn, animals (Group 1) were housed under LD12:12 and killed at ZT1, one hour after light onset. As can be seen in Figure 18A, there was no difference in number of Fos-IR cells in the SCN of animals with IGL-lesions as compared to sham-operated rats ($t(6)=2.308$, $p>0.05$). Thus the SCN of IGL-lesioned animals is as responsive to light at dawn as that of sham operated animals, demonstrating that photic input to the circadian pacemaker is not diminished as a consequence of the lesion.

To confirm that IGL lesions did not alter the response of the SCN to light during SPP housing, animals (Group 2) initially entrained to LD12:12 and subsequently housed under a SPP for four days were killed on the fifth day after either the dawn or dusk light pulse. IGL-lesioned rats exhibited the response to such light pulses previously described in intact rats (Schwartz, et al., 1994) and observed in sham-operated animals in this study; the dawn pulse induced moderate Fos expression in all animals, the dusk pulse induced very few Fos-IR cells in all animals (Figure 18B; Note that only one sham-

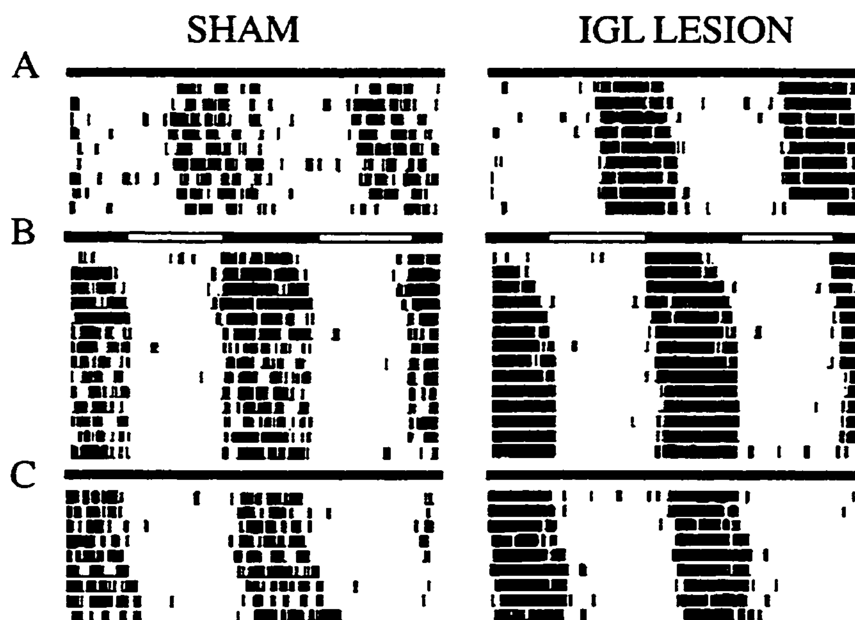

Figure 17. Double-plotted actograms of body temperature records of a sham-operated and an IGL-lesioned rat housed in DD (A), LD12:12 (B), and DD (C). Light bars = lights on; dark bars = lights off.

Figure 18. Mean number of Fos-immunoreactive cells per SCN section in IGL-lesioned and sham-operated rats. A: animals housed under LD 12:12 and killed one hour after light onset (ZT1). B: animals housed under SPP and killed after the dawn (ZT1) or dusk (ZT12) light pulse. C: animals housed in DD and killed after a one hour light pulse during the subjective day (CT3-5) or subjective night (CT15-17).

operated rat was killed after the dawn pulse, therefore an ANOVA was not performed).

Finally, to explore the effect of IGL lesions on the phase response of the SCN to light, additional groups of IGL-lesioned and sham-operated rats (Group 3) were housed in DD for 24-36 hours and then given a one-hour light pulse between CT3-5 or CT15-17. No differences were observed between groups in mean number of SCN Fos immunoreactive cells. Whereas all animals exhibited robust Fos expression in the SCN following light pulses during CT15-17, few Fos-IR neurons were observed in animals exposed to light during the subjective day, between CT3-5 (Figure 18C; $F(1,8)=64.664$, $p<0.001$). These data show that the SCN responds normally to light exposure during the subjective day and night, despite IGL lesion. Because light-induced Fos expression is correlated with photic phase shifting of the rodent circadian system (Kornhauser et al., 1990; Rusak et al., 1990; Wollnik et al., 1995; Wollnik et al., 1996), these data demonstrate that the SCN cells of IGL-lesioned animals are capable of responding to such phase shifting stimuli. Examples of Fos expression observed in the SCN of IGL-lesioned and sham-operated animals killed after light exposure during SPP or DD housing are given in Figure 19.

Discussion

Results of the present experiment demonstrate that the GHT plays a critical role in entrainment of circadian temperature rhythms under SPP housing, a lighting schedule experienced by nocturnal rodents in the natural environment (DeCoursey, 1986). Rats and other nocturnal animals have been shown to entrain normally to this lighting schedule (DeCoursey, 1986; Pittendrigh & Daan, 1976; Rosenwasser et al., 1983; Stephan, 1983). Indeed if housed in simulated den cages, animals emerged from their darkened dens near dawn and dusk, and rhythms were reset according to such light exposures (DeCoursey, 1986). In the present study, IGL-ablated rats exhibited free running rhythms for at least three weeks following release into SPP housing, despite

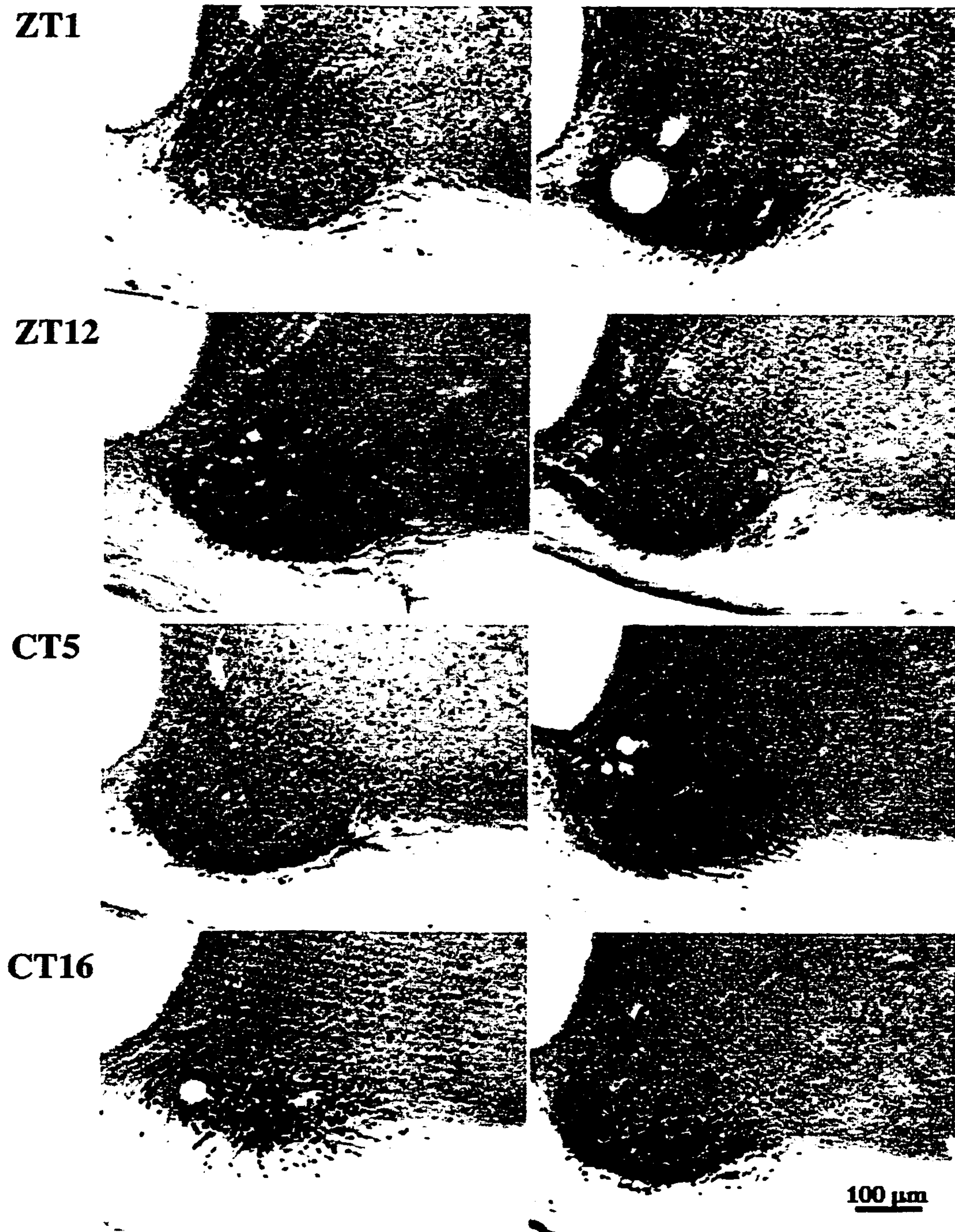

Figure 19. Fos immunoreactivity in the SCN of rats (LEFT: sham; RIGHT: IGL-lesion) following light exposure at ZT0-1 or ZT11-12 of a SPP (TOP) or at CT4-5 or CT15-16 during DD (BOTTOM).

normal entrainment to LD12:12. These animals exhibited normal profiles of light-induced Fos expression under entrained and free running conditions, indicating that the effects of IGL lesion on entrainment to SPP cycles do not result from disruptions in light transmission to the SCN.

Circadian rhythms are synchronized to environmental LD cycles via transmission of photic information from the retina to the SCN (Card & Moore, 1991; Morin, 1994). The RHT is thought to be necessary for photic entrainment to occur; destruction of the RHT results in free running rhythms despite the presence of photic time cues (Johnson et al., 1988). However, because retinal ganglion cell axon collaterals that comprise the RHT send bifurcating axons that terminate in the IGL (Pickard, 1985), destruction of the RHT also damages light transmission to the IGL, and hence transmission of photic information to the SCN along the GHT. It is therefore possible that both the RHT and retinogeniculate pathways are necessary for photic entrainment. Nevertheless, IGL lesion studies have not provided support for a significant contribution of the GHT to photic entrainment (Harrington & Rusak, 1988; Johnson et al., 1989; Pickard et al., 1987; Moore & Card, 1994; Harrington, 1997).

The failure to entrain to SPP housing following IGL lesion cannot be simply explained. One possibility is that IGL lesion alters the phase response curve (PRC) to light such that the phase shift required to entrain to a 24 hour SPP is unattainable. IGL-lesioned hamsters exhibited differences from intact controls in the response to phase shifting light pulses; smaller phase advances (Harrington & Rusak, 1986; Pickard et al., 1987) and an increase in phase delays (Pickard et al, 1987) have been reported, although only at circadian times when light pulses induce maximal advance and delay shifts. Because intact rats have larger phase delay portions than phase advance portions of the PRC to light (Summer & McCormack, 1982), if IGL lesion shifts the PRC further, the 24-hour SPP may be outside the limits of entrainment (Daan & Pittendrigh, 1976;

Pittendrigh & Daan, 1976). However, the effects of IGL lesion on free running period and the PRC to light in rats is unknown. Our finding that IGL-lesioned rats show a normal profile of Fos expression in response to light exposure at different circadian phases, and maintain a normal circadian range of free running periods, suggests that any effects of IGL lesion on PRC and period would be small, an idea supported by previous studies in IGL-lesioned hamsters (Harrington & Rusak, 1986; Pickard et al., 1987).

One question that arises from this work is the difference between discrete exposures to light at dusk and dawn, and continuous light exposure during the light phase of LD12:12. The demonstration that IGL-lesioned animals can entrain to one of these lighting schedules but not the other, raises the possibility that masking of overt rhythmicity contributes to synchronizing the clock to the 24 hour day. It has been suggested that the direct effects of light on rhythmic variables complement pacemaker regulation of those variables (Mrosovsky, 1994). If this is true, then attenuating the masking effects of light on the clock may also eliminate an important element of the entrainment mechanism. Although hamsters with IGL lesions have been shown to be less active than intact animals (Janik & Mrosovsky, 1994; Wickland & Turek, 1994), no differences between IGL-lesioned hamsters or rats have been observed with respect to light-induced suppression of activity and body temperature (rats: present study; hamsters: Redlin, Vrang, & Mrosovsky, unpublished observations) indicating that masking effects of light persist after lesion. Indeed, a recent study in hamsters has shown that IGL-lesioned animals may be more sensitive to the masking effects of light than intact animals (Redlin et al., unpublished observations). Perhaps feedback to the pacemaker in response to light-induced suppressed activity facilitates the phase shift required for entrainment, resulting in a more stably entrained rhythm under full photoperiods.

A related issue is the contribution of nonphotic cues to stable entrainment. Several lines of research support a role for the IGL in nonphotic phase resetting of the circadian clock. Infusion of NPY into the SCN and electrical stimulation of the IGL phase shift

rhythms in a pattern different from that induced by light (Albers & Ferris, 1984; Rusak et al., 1989). Exposure to nonphotic stimuli induces phase shifts characterized by large phase advances during the subjective day and resemble those induced by NPY or IGL stimulation (cf. Mrosovsky, 1995); these phase shifts were prevented in IGL-lesioned animals (Biello et al., 1991; Janik & Mrosovsky, 1994; Marchant, et al., 1997; Maywood et al., 1997; Meyer et al., 1993; Wickland & Turek, 1994; or see Harrington, 1997; Mrosovsky, 1995 for reviews). The IGL has been proposed as the site of integration of photic and nonphotic stimuli that influence the circadian clock (Harrington, 1997; Janik et al., 1995; Moore & Card, 1994; Mrosovsky, 1995; Mrosovsky, 1996). That exposure to nonphotic stimuli can alter circadian responses to photic stimuli or LD cycles is known (Mrosovsky, 1996); the mechanisms underlying such interactions are not. A recent study in hamsters demonstrates the relevance of nonphotic cues on photic entrainment to SPP housing. Under SPP housing, hamsters exposed to a novel running wheel during the subjective day exhibited phase jumps in the entrained activity rhythm, resulting in a switch in subjective day and night times (Sinclair & Mistlberger, 1997). This work underscores the importance of nonphotic zeitgebers on the stability of entrainment. Because IGL-lesioned animals do not respond to activity-mediated nonphotic stimuli, discrete pulses of light may not be sufficient for entrainment, and only under complete LD cycles can entrainment occur.

The absence of NPY turnover in the SCN following complete, bilateral IGL ablation (Harrington, 1997; Moore & Card, 1994) likely contributes to the failure of IGL-lesioned animals to entrain to the SPP. Electrical stimulation of the IGL and NPY infusion into the SCN region phase shift circadian activity rhythms with a PRC that resembles the effects of nonphotic stimuli on the circadian system (Albers & Ferris, 1984; Rusak et al., 1989). Phase shifting effects of NPY are also evident in the SCN in vitro; application of NPY during the mid subjective day resulted in phase advances in the circadian rhythm of cell firing (Gribkoff et al., 1998). Although NPY-induced inhibition

of excitatory neurotransmission has been shown to be independent of circadian time during the subjective day (Gribkoff et al., 1998), the diurnal rhythm of NPY in the SCN and the effects of NPY on glutamate or light-induced phase shifts all suggest that NPY alters the responsiveness of SCN neurons to light as a function of circadian phase (Biello et al., 1997; Shinohara, Tominaga, Fukuhara et al., 1993; van den Pol et al., 1997; Weber & Rea, 1997).

The extensive neural damage to parts of the hippocampus and lateral geniculate nucleus (LGN) sustained by all lesioned animals may contribute to the inability of these animals to entrain to the SPP. The hippocampus has been implicated in the photoperiodic response of hamsters housed under short days; hippocampal damage reduces extent of testicular regression during short photoperiod exposure (Smale & Morin, 1990). In addition, hippocampal damage impairs performance in light-dark discrimination tasks (see Eichenbaum, 1996 for review). Similarly, ventral LGN damage impairs performance on brightness discrimination tasks (cf. Harrington, 1997). The dorsal LGN relays visual information to higher cortical regions and is involved in saccadic suppression, a mechanism underlying visual attention (Burr, Morrone, & Ross, 1994; Sherman & Koch, 1986). In addition the LGN may also integrate photic information from both the circadian and visual systems; perhaps such input to the SCN is necessary for entrainment to discrete pulses of light. However, animals treated neonatally with MSG, who sustain extensive neurotoxic damage to the visual system and the hippocampus (Kizer et al., 1978), exhibited stable entrainment to 24 hour SPP lighting schedules (Edelstein & Amir, unpublished observations). It would be interesting to explore the effects of discrete lesions of these brain areas, or large extra-IGL lesions, in order to more thoroughly evaluate the relative contributions of the IGL and other brain areas to SPP entrainment.

Finally, it is important to consider that IGL ablation results in destruction not only of the GHT but also the hypothalamogeniculate projection originating in the dorsomedial

region of the SCN and terminating in the IGL (Watts et al., 1987). The function of this pathway in the circadian system is not known. It is possible that the results of the present study are due to damage to dorsomedial SCN neurons whose fibers terminate in the IGL.

In summary, the present study demonstrates that the GHT is required for photic entrainment of circadian temperature rhythms to a 24 hour skeleton photoperiod in the rat. It is likely that NPY neurotransmission in the SCN contributes to the response of the circadian clock to light during twilight times, and that the absence of such input in the lesioned animal renders the circadian system incapable of entraining to discrete pulses of light. Whether masking enhances phase resetting of the circadian clock under full photoperiods, and the contribution of nonphotic cues to photic entrainment are important questions for future research.

GENERAL DISCUSSION

The results of the experiments that comprise this thesis support the idea that the IGL plays a critical role in the circadian response to light. The immunohistochemical studies revealed several findings of interest. First, in contrast to the phase dependency of Fos expression in the SCN (Kornhauser et al., 1990; Rusak et al., 1990; Schwartz et al., 1994), it was found that light induced Fos in the IGL independent of circadian phase. This conclusion is supported by another study, showing that two-hour light pulses given at different times of day induced similar levels of Fos expression in IGL neurons (Peters et al., 1996). Second, light-induced Fos was observed in the IGL regardless of duration of light exposure. Third, nonphotic manipulations were found to induce Fos in both the IGL and the SCN; however, unlike in the SCN where the level of Fos expression in response to nonphotic treatment did not vary as a function of circadian phase, in the IGL a greater number of Fos-IR cells was observed during the light phase than during the dark phase of LD12:12. These data show that the profile of light-induced Fos in the IGL is different from that in the SCN. This difference is further emphasized by the finding that glutamatergic antagonists that attenuated light-induced Fos in the SCN had no effect on light-induced Fos in the IGL. Taken together, these findings show that processing of light information in the IGL is distinctly different from that which occurs in the SCN.

Two notable findings concerning the role of the IGL in the regulation of circadian rhythmicity emerge from the second set of experiments. The first, and most important, is that despite the fact that lesions of the IGL permitted normal entrainment to LD12:12, these lesions prevented entrainment to a 24-hour SPP, in which animals were presented with one-hour light pulses at dawn and dusk. This finding shows that the IGL is necessary for entrainment of circadian rhythms to a lighting schedule more like that experienced by nocturnal animals in the natural environment (DeCoursey, 1986).

The second finding is that the IGL does not mediate the disruptive effects of prolonged LL housing on circadian rhythms in rats, as it is thought to do in hamsters (Harrington & Rusak, 1988; Pickard et al., 1987). Body temperature records from animals housed in constant light were found to be similarly arrhythmic in IGL-lesioned rats and in sham-operated rats. This finding brings into question the interpretation of an earlier study by Edelstein et al. (1995) where no disruption of circadian rhythms was found in MSG-treated rats maintained in constant light. Edelstein et al. (1995) attributed that result to an effect of MSG on retinal innervation of the IGL. One important difference between lesioned rats and those treated with MSG is that MSG-treated rats exhibited NPY-IR in the SCN, whereas there was no such staining in IGL-lesioned animals. This would indicate that the GHT is intact following MSG treatment and lends support to the idea that the disruption of circadian rhythms during LL exposure is not mediated directly via the GHT in rats.

Several researchers have proposed that the IGL is involved in the integration of photic and nonphotic information, and that transmission of such information to the SCN provides feedback for the regulation of circadian rhythmicity (Amir & Stewart, 1996; Harrington, 1997; Janik et al., 1995; Moore & Card, 1994; Mrosovsky, 1995; Mrosovsky, 1996). The role of the IGL in the response of the circadian system to nonphotic stimuli has been well-established (for reviews, see Harrington, 1997; Mrosovsky, 1995). Results of the experiments that comprise this thesis now demonstrate that the IGL plays a consequential role in the circadian response to light. Together, these findings lend support to the idea that the IGL may serve to integrate information from photic and nonphotic sources. In addition, however, the findings from this thesis raise several new research questions concerning the induction of immediate early genes in the IGL, and the role of the IGL in photic entrainment. These questions are considered below.

The prolonged expression of Fos in IGL neurons in response to light is unusual, and likely results from activation of individual neurons at different times, so that the

entire IGL region appears to be stained at all times. Sustained immediate early gene expression is usually associated with Fos-related antigens, and not Fos itself (Sharp et al., 1990). Typically, Fos is rapidly and transiently induced in regions of the central nervous system in response to physiologically relevant stimuli (Morgan & Curran, 1991; Sheng & Greenberg, 1990). Fos protein autoregulates transcription of its mRNA via a negative feedback loop; a refractory period of several hours during which further transcription cannot occur is observed in many systems (Morgan & Curran, 1991). In the SCN for example, transcription of c-fos mRNA in response to light pulses occurs within five minutes of stimulation and reaches a maximum about 30 minutes later (Kornhauser et al., 1990). Fos protein levels peak about one hour after onset of the stimulus, and decline to baseline levels within six hours (Colwell & Foster, 1992; Rusak et al., 1990). Although it seems unlikely that individual IGL neurons exhibit sustained Fos expression throughout LL exposure, this possibility should be explored.

To further elucidate the mechanisms underlying light-induced Fos expression in the IGL, several types of experiments need to be performed. In situ hybridization of c-fos mRNA in IGL neurons may provide information about whether prolonged transcription of c-fos mRNA occurs in the IGL of animals exposed to LL, or whether LL interferes with degradation of Fos protein. Colocalization of immunohistochemical expression of Fos protein and in situ hybridization of c-fos mRNA in individual cells (Chaudhuri, Nissanov, Larocque & Rioux, 1997) would provide insight into whether transcription and translation are occurring continuously in individual cells. Perhaps the prolonged Fos expression in LL is not involved in regulating transcription of target genes but rather performs some other function. Characterization of the expression of protein products of c-jun, junB, and junD in the IGL may begin to address this issue. Examination of the changes in the composition of component proteins of the AP-1 site in the IGL, using the methodology of Takeuchi et al. (1993), could provide additional insight into the relationship between light, circadian phase and expression of Fos and

JunB. Preliminary immunohistochemical data demonstrating a pattern of expression of JunB similar to that of Fos in the IGL of animals during the light phase of LD12:12 (Edelstein, d'Abramo, Beaulé, & Amir, 1998), but not during LL, supports the idea that Fos expression in the IGL during the light phase of the cycle may be involved in stimulus-transcription coupling. However, the absence of JunB staining in IGL neurons after 24 hours of LL exposure, during both subjective day and subjective night times (Edelstein et al., 1998) indicates that either Fos is dimerizing with the protein products of other jun genes, or that Fos induction in the IGL represents an as yet unknown function.

The work presented in this thesis also raises new questions about the relationship between light exposure and nonphotic manipulations on Fos expression in the IGL. The finding that IGL Fos expression is greater during the light phase of the cycle, and that nonphotic manipulations enhance that expression, implies that either the response of IGL neurons to nonphotic stimuli is potentiated by light exposure during the day, or alternatively, that nonphotic stimuli enhance the response of IGL neurons to light. Moreover, it is unclear whether the differences in Fos expression during the light and dark phases of the cycle can be attributed to light exposure, circadian phase, or both. Because rats housed in DD and exposed to some of the nonphotic manipulations described in Experiment 1 have been shown to exhibit few Fos-IR cells in either the IGL or the SCN during the subjective day (cage change, saline injection: Edelstein & Amir, unpublished observations), the enhanced response to such stimuli under LD12:12 can be attributed to light exposure. However the finding, from another study, that IGL Fos expression in response to light exposure during the subjective night is not altered when such light was paired with shock (Amir & Stewart, in press), suggests that the potentiation of the response during the day is a function of circadian phase. Another possibility is that exposure to light during the subjective night induces Fos expression in a maximum number of IGL neurons, and, therefore, Fos expression cannot be further enhanced by other stimuli. Exposure to light during the subjective night induces more

robust Fos-IR in both SCN and IGL of dark-adapted animals than in those entrained to LD cycles and exposed to light pulses during the dark phase (Edelstein & Amir, unpublished observations). Comparisons of the responses of IGL neurons to nonphotic manipulations at different circadian phases during DD or LL would provide insight into the relative contributions of light and behavioral state on activation of IGL neurons and may shed light on the nature of the interaction between these two zeitgebers on the circadian system in the live animal.

The finding that ionotropic glutamatergic antagonists do not attenuate Fos expression in the IGL as they do in the SCN, raises the question of whether glutamate or some other neurotransmitter is involved in activation of IGL neurons by light. Although the data indicate that Fos induction in the IGL is not mediated by the same glutamatergic receptors as in the SCN, the effects of metabotropic antagonists on light-induced Fos in the IGL have yet to be evaluated, and therefore glutamate cannot be ruled out as a neurotransmitter. In addition, to better evaluate the importance of glutamate in the transmission of light information to the IGL, the effects of intra-IGL infusion of glutamatergic antagonists on the electrophysiological responses of IGL neurons to light or optic nerve stimulation should be characterized. Similar electrophysiological experiments provided the basis for demonstrating the involvement of glutamate in light transmission to the SCN (Ebling, 1996) and dorsal lateral geniculate nucleus (Nelson & Sur, 1992). Another putative neurotransmitter that could be involved in light transmission to the IGL is SP. Although the retinal projection to the SCN contains SP (Mikkelsen & Larsen, 1993; Takatsuji et al., 1991), it is thought that the SP projection to the IGL may not originate in the retina (Hartwich et al., 1994; Miguel-Hidalgo et al., 1991; Mikkelsen & Larsen, 1993; Takatsuji & Tohyama, 1989). Furthermore, although SP antagonists have been shown to attenuate light-induced Fos in the dorsal SCN region of the hamster (Abe et al., 1996), preliminary data demonstrates no effect of SP antagonists on light-induced Fos in rat IGL (Edelstein & Amir, unpublished

observations). In addition to identification of the neurotransmitters along the retina-IGL pathway, identification of the retinal ganglion cells that project to the IGL might help to clarify these issues. The finding that retinal ganglion cells with bifurcating axons innervate both the SCN and IGL has been demonstrated in the hamster (Pickard, 1985). It is not known, however, whether this is true for other species. Furthermore, the findings that unique retinal ganglion cells innervate the circadian system in the rat (Moore et al., 1995), and that the fraction of retinal ganglion cells that survive the neurotoxic effects of MSG may be those that comprise the RHT in hamsters (Chambille, 1998), suggest that a comparison of the retinal ganglion cells that project to either SCN, IGL, or both, may provide insight into the differences in responsiveness to light in these two regions. It is interesting to note that, in addition to the effects of neonatal MSG on LL-induced disruptions in rhythmicity, this treatment also prevents light-induced Fos in the IGL (Edelstein et al., 1995). This finding, taken together with that of Chambille (1998), raises the possibility that retinal ganglion cells in the rat that innervate both the SCN and IGL are involved in the disruptive effects of LL and are destroyed by neonatal MSG. Other retinal ganglion cells, that survive MSG-induced neurotoxicity, may project only to the SCN; those cells could be involved in photic entrainment to normal light-dark cycles.

It has been shown previously that the RHT is both necessary and sufficient for photic entrainment to complete photoperiods. Damage to all other retinofugal pathways does not prevent entrainment, whereas transection of the RHT renders entrainment impossible (Dark & Asdourian, 1975; Harrington & Rusak, 1988; Johnson et al., 1988; Johnson et al., 1989; Pickard et al., 1987). As noted above, however, transection of the RHT should also damage retinal ganglion cells projecting to the IGL. Therefore, it is possible that both the RHT and the retina-IGL pathway are required for entrainment. Nevertheless, the finding that IGL lesion prevents photic entrainment to skeleton photoperiod housing is startling. This finding raises the possibility that the IGL provides the SCN with information related to daylength; perhaps such information is a requirement

for entrainment to SPP conditions. If this is so, then the absence of light exposure throughout the day prevents entrainment after IGL lesion. Although attempts to address this issue were made in Experiment 5 by analyzing Fos expression in response to the light pulses of the SPP as well as in the middle of the subjective day or night, no effect of IGL lesion was observed in the responsiveness of the SCN to light pulses at those times. However, if such differences do exist, then it is possible that they could not be detected at the few time points studied. For example, in work demonstrating a seasonal response of SCN neurons to daylength, the largest differences observed occurred in response to light pulses in the late subjective night/ early subjective day; animals housed under a short-day photoperiod exhibited peak levels of Fos expression at that time. In contrast, animals housed under a long-day photoperiod exhibited little staining (Sumova et al., 1995). Because Fos expression during the late subjective night is correlated with the effectiveness of light to induce phase advances, perhaps those time points need to be examined more thoroughly, and comparisons between lesioned animals and intact controls made at those times.

The possibility that a 24-hour SPP was outside the limits of entrainment for animals with IGL lesions was considered in the discussion of Experiment 5. The demonstration of enhanced phase delays and smaller phase advances following IGL lesion in the hamster (Harrington & Rusak, 1986; Pickard et al., 1987) suggests that this is possible. To further evaluate this question, changes in the size of phase shifts in response to light pulses given at different times of day (a phase response curve) should be assessed in rats before and after lesion. In addition, the effect of the lesion on free running period in individual animals must be evaluated as well; comparisons of group differences may eliminate any individual variability in such measures. The effect of prior experience with SPP housing on subsequent entrainment to a SPP in IGL-lesioned animals may indicate whether the inability to entrain is related to an inability to adapt to

novel lighting schedules or reflects a deficit in the phase resetting mechanism of the circadian clock under such conditions.

Results of Experiment 5 also demonstrated that intact animals exhibit individual differences to prolonged SPP housing. Although sham-operated animals exhibited stable phase relationships with the SPP, after several weeks one animal phase jumped, and another one exhibited rapid delay transients before re-entraining to the lighting schedule. Such findings suggest that individual differences in stability of entrainment of the circadian system exist. It would be interesting to further evaluate prolonged SPP housing in intact animals to evaluate factors contributing to stability of entrainment, such as differences in free running period, responsiveness to nonphotic stimuli, and previous lighting experience. Such differences may contribute to the ability of an animal to entrain to SPP lighting schedules, and may make it possible to predict whether individual IGL-lesioned animals would eventually entrain to a SPP.

A role for NPY in photic entrainment could be elegantly demonstrated if daily intra-SCN infusion of NPY during light exposure at dawn would restore stable entrainment in IGL-lesioned animals under SPP housing conditions. Similarly, the effects of infusing anti-NPY antibodies or NPY antagonists into the SCN of intact animals and examining the effect on entrainment to SPP housing would provide further information on the contribution of the IGL to photic entrainment under such lighting conditions. Lastly, because infusion of NMDA in to the SCN induces light-like phase shifts, the effects of infusing both NMDA and NPY into the SCN of intact animals under constant darkness may shed light on the relationship between the GHT and photic entrainment to SPP conditions.

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