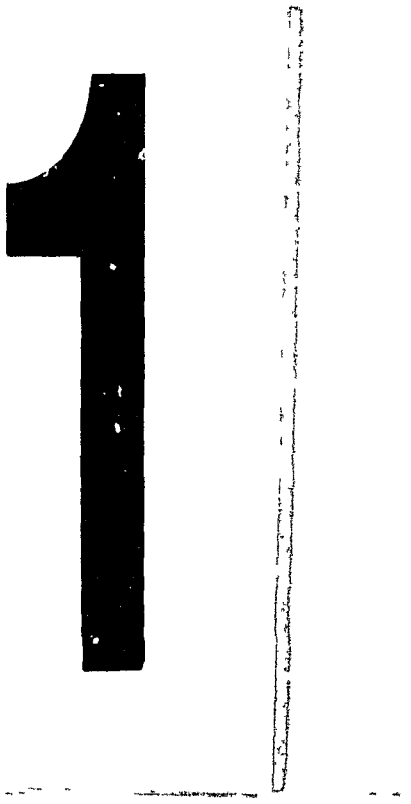

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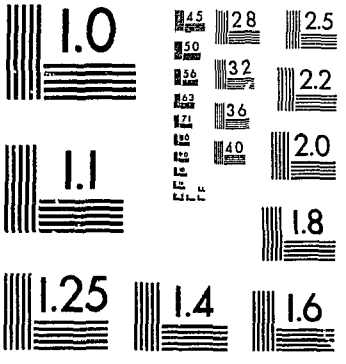

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ANATOMICAL AND BEHAVIOURAL ANALYSIS OF THE ROLE OF ACETYLCHOLINE AND NERVE GROWTH FACTOR IN THE MAMMALIAN CIRCADIAN SYSTEM.

by

K. G. BINA

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
March, 1992

ISBN 0-315-76701-4

Canada

DALHOUSIE UNIVERSITY

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by K. G. Bina

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: 30 March 1992

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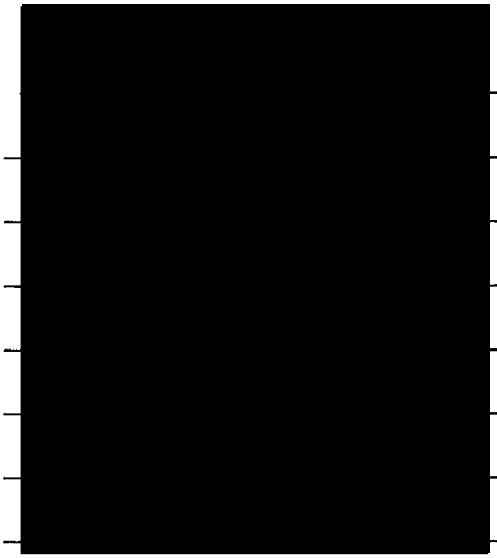
Research Supervisor

Examining Committee



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TITLE: ANATOMICAL AND BEHAVIOURAL ANALYSIS OF THE ROLE OF ACETYLCHOLINE AND NERVE GROWTH FACTOR IN THE MAMMALIAN CIRCADIAN SYSTEM.

DEPARTMENT OR SCHOOL: PSYCHOLOGY/NEUROSCIENCE

DEGREE: Ph.D. CONVOCATION: MAY YEAR: 1992

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I dedicate this thesis to my rodents

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ABSTRACT

In mammals, the generation of circadian rhythms and their synchronization to environmental cues are under the control of the suprachiasmatic nucleus (SCN) of the hypothalamus. Brief light exposure of an animal maintained in otherwise constant darkness is capable of causing a shift in the phase of its circadian rhythms, the direction and amplitude of which depend on the time of administration of the light pulse. A plot of such shifts is called a phase response curve (PRC). Daily repetition of such pulses ultimately entrains the circadian system.

Retinal ganglion cells convey photic information necessary for rhythm entrainment to the SCN, where they release neurotransmitters to initiate the phase shifting process. Various agonists of putative transmitters at these terminals have been injected into the SCN to test for their capacity to mimic the effects of light. A PRC for carbachol, a non-specific cholinergic agonist, partially mimics the PRC for light, implicating the involvement of acetylcholine (ACh) in photic transmission. This study was aimed at investigating the potential roles of ACh and the neurotrophic factor for central cholinergic neurons, nerve growth factor (NGF), in the process of entrainment of activity rhythms in rodents, using several anatomical and behavioural approaches.

Receptor binding studies in hamsters revealed the presence of muscarinic receptors in the SCN, suggesting that cells there are responsive to ACh. Fibers and fiber terminals immunoreactive for choline acetyltransferase (ChAT), the synthetic enzyme for ACh, are found in the rat SCN, suggesting that ACh is released from the terminals of neurons located elsewhere in the brain that project to the SCN. Anterograde tracing studies and a combination of retrograde tracing techniques and immunocytochemistry for ChAT showed that the cholinergic fibers in the SCN arise from cholinergic cells located in the basal forebrain and mesopontine tegmentum.

The ventrolateral SCN shows strong immunoreactivity for receptors for NGF (NGF-R). Retrograde tracing techniques combined with immunocytochemistry for NGF-R and ChAT revealed that some of these receptors are located on the terminals of cholinergic cells in the basal forebrain that project into the SCN. Studies involving optic nerve transection and a combination of retrograde tracing techniques and immunocytochemistry for NGF-R showed that NGF-R are also located on the terminals of retinal ganglion cells in the SCN.

Carbachol, administered through indwelling cannulae in the SCN of hamsters, produces phase advances during late subjective night (circadian time [CT] 22) and subjective day (CT6), whereas it produces phase delays during the early subjective night (CT14). Carbachol-induced phase shifts at all three phases were blocked by atropine, a muscarinic antagonist, and not by mecamylamine, a nicotinic antagonist, suggesting that carbachol mediates its phase shifting effects through muscarinic receptors.

NGF administered in the same way caused phase advances at CT22 and CT6 and phase delays at CT14. NGF-induced phase shifts at CT6 and CT22 were blocked by an antibody to NGF or by the muscarinic antagonist atropine, indicating that its effects are mediated by a cholinergic mechanism. The failure of both anti-NGF and atropine to block such shifts at CT14, and the induction of phase shifts by Cytochrome-c (biochemically similar to NGF without its receptor binding capabilities) at that phase, suggest that phase shifts induced by NGF at this phase are non-specific.

Taken together, these findings suggest that ACh released from the terminals of cholinergic cells that project into the SCN acts on muscarinic receptors, and NGF released from cells in the SCN acts on NGF-R to contribute to phase shifting of the rodent circadian pacemaker.

LIST OF ABBREVIATIONS

^{125}I	-	Iodinated
^{3}H	-	Tritiated
3V	-	Third ventricle
7n	-	Seventh cranial nerve
A	-	Atropine
ac	-	Anterior commissure
AC	-	Atropine + Carbachol
ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase
AH	-	Anterior hypothalamus
AMYG	-	Amygdala
AN	-	Antibody to NGF
ANN	-	Antibody to NGF + NGF
ASD	-	Antisera diluent
BF	-	Basal forebrain
B_{max}	-	Maximal binding
BS	-	Basal ganglia
α-BTX	-	Alpha-bungarotoxin
C	-	Carbachol
cAMP	-	Cyclic adenosine monophosphate
CBC	-	Cholinergic basal nuclear complex
ChAT	-	Choline acetyltransferase
CIN	-	Cingulate cortex
CNN	-	Cranial nerve nuclei
CNS	-	Central nervous system

CPu	-	Caudate-putamen
CT	-	Circadian time
Cyt-c	-	Cytochrome-c
DAB	-	Diaminobenzidine
DD	-	Constant darkness
DR	-	Dorsal raphe nucleus
EAA	-	Excitatory amino acid
EEG	-	Electroencephalogram
ENTO	-	Entorhinal cortex
EPSP	-	Excitatory postsynaptic potential
f	-	Fornix
FG	-	Fluorogold
FITC	-	Fluorescein isothiocyanate
FRON	-	Frontal cortex
GABA	-	Gamma aminobutyric acid
GAD	-	Glutamate decarboxylase
GHT	-	Geniculo-hypothalamic tract
hDBB	-	horizontal limb of the diagonal band of Broca
HIPPO	-	Hippocampus
HRP	-	Horseradish peroxidase
ic	-	Internal capsule
IC	-	Inferior colliculus
IC-50	-	Concentration at which 50% inhibition occurs
IgG	-	Immunoglobulin G
IGL	-	Intergeniculate leaflet
IP	-	Interpeduncular nucleus
kD	-	Kilodalton

Kd	-	Dissociation constant
LC	-	Locus coeruleus
LD	-	Light:dark cycle
LDT	-	Laterodorsal tegmental nucleus
LGN	-	Lateral geniculate nucleus
LL	-	Continuous illumination
LP	-	Light pulse
LS	-	Lateral septum
M	-	Mecamylamine
MC	-	Mecamylamine + Carbachol
MgPA	-	Magnocellular preoptic area
MH	-	Medial habenula
ml	-	Medial lemniscus
MPOA	-	Medial preoptic area
MR	-	Median raphe nucleus
mRNA	-	Messenger ribonucleic acid
MS	-	Medial septum
NAAG	-	N-acetylaspartyl glutamate
n	-	Number
NADPH	-	Dihydro nicotinamide adenine dinucleotide phosphate
NBM	-	Nucleus basalis magnocellularis
N/NGF	-	Nerve growth factor
NGF-R	-	Nerve growth factor receptor
NGS	-	Normal goat serum
NMDA	-	N-methyl D aspartate
NMS	-	N-methyl scopolamine
NPY	-	Neuropeptide-Y

OB	-	Olfactory bulb
on	-	Optic nerve
ox	-	Optic chiasm
PAP	-	Peroxidase-antiperoxidase complex
PAR	-	Parietal cortex
PB	-	Parabrachial nucleus
PBg	-	Parabigeminal nucleus
PBS	-	Phosphate buffered saline
PC-12	-	Pheochromocytoma cell line
PHA-L	-	Phaseolus vulgaris leucoagglutinin
PIR	-	Piriform cortex
PPT	-	Pedunculopontine tegmental nucleus
PRC	-	Phase response curve
PRF/PRT	-	Pontine reticular formation
PVN	-	Paraventricular nucleus
QNB	-	Quinuclidinyl benzilate
RCh	-	Retrochiasmatic area
REM	-	Rapid eye movement
RGC	-	Retinal ganglion cell
RHT	-	Retinohypothalamic tract
RS	-	Rabbit serum
S	-	Saline
SC	-	Superior colliculus
SCG	-	Superior cervical ganglion
SCN	-	Suprachiasmatic nucleus
scp	-	Superior cerebellar peduncle
SEM	-	standard error of the mean

SI	-	Substantia innominata
sm	-	Stria medullaris
SN	-	Substantia nigra
SNAT	-	Serotonin N-acetyl transferase
st	-	Stria terminalis
TEC	-	Tectum
TEMP	-	Temporal cortex
THAL	-	Thalamus
trk	-	Tyrosine kinase
TTX	-	Tetrodotoxin
TBS	-	Tris buffered saline
VD	-	Ventral diencephalon
vDBB	-	Vertical limb of the diagonal band of Broca
VIS	-	Visual cortex
vLGN	-	ventral lateral geniculate nucleus
WGA	-	Wheatgerm agglutinin
ZI	-	Zona incerta

ACKNOWLEDGEMENT

I wish to express my heartfelt thanks to many of my friends and colleagues who were instrumental in the creation and presentation of this thesis in its present format. First and foremost I wish to thank my two mentors, Ben Rusak and Kazue Semba for their advise, patience, encouragement and support during the years of my education at Dalhousie University. Ben, it has been a wonderful learning experience and I am extremely proud to have worked with you and Kazue, thankyou for being such a great friend and mentor.

Autoradiography and receptor binding studies were performed under the excellent supervision of Mike Wilkinson. I thank Pat Jackson for help with surgery, Wolfgang Härtig for help with anterograde tracing technique using Biocytin, Ela Pyza for help in evaluating the data and Hiroshi Abe for help during the last two years. I also wish to express my gratitude to Drs. Ron Lindsay and Patricia Osborne for providing me with the NGF-R and NGF antibodies.

I am indebted to Joan Burns, Christine Burton, Patricia Dickson, Donna Goguen, Heather Grant and Janette Nason for their invaluable technical assistance. I would like to especially thank Ms Goguen for her generous help, patience, abundant love and support. Donna, you were a pleasure to work with.

I wish to thank all the members of my thesis committee. I especially wish to acknowledge the help and support provided by Ian Meinertzhagen during the final days of production of this thesis. I wish to thank all my friends (they are far too many to name) and my everchanging local family for putting up with me. I wish to especially thank Nameera Akhtar, Liz Coscia, Anastasia Droungas, Jo Durup, Tim and Elke Juckes, Chinglu Lee and Ela Pyza.

Last but not the least I wish to thank my family in India for their continual support and encouragement and my dearest friend René Marois for being one of my greatest motivations for the completion of this thesis. I love you for your support.

CHAPTER I

INTRODUCTION

The movement of the Sun, Earth and Moon relative to each other and the Earth's rotation on its own axis expose plants and animals to highly predictable cycling environmental cues, such as those of light, temperature, tides and seasons. Plants and animals in turn demonstrate physiological and behavioural rhythms with periodicities corresponding to these geophysical cycles. Such rhythms are called biological rhythms. These biological rhythms have probably been selected for as a result of the advantages conferred on organisms whose physiology anticipates the consequences of those cycles and whose behaviour allows them to take advantage of the opportunities which arise rhythmically.

Biological rhythms with a 24 hr period are referred to as daily rhythms. The existence of biological rhythms with periods similar to environmental cycles suggests the possibility that they are driven by such cycles. However, when biological rhythms are monitored in plants and animals maintained outside the influence of such environmental cycles and under constant conditions of light, food and temperature, the rhythms persist with a period close to but not equal to 24 hrs. Such endogenously generated, free-running rhythms with periods close to 24 hrs are called circadian (Latin: circa-about; dies-day) rhythms (Halberg, 1959).

Under natural conditions, circadian rhythms are reset every day by daily environmental cycles, particularly by the light-dark cycle. This process by which the rhythm's period and phase are controlled by an exogenous cue (zeitgeber: time giver) is referred to as entrainment. Light is probably one of the strongest zeitgebers to which the mammalian circadian system entrains. A single, brief light pulse, given to an animal expressing a free-

running circadian rhythm as a result of its being maintained in constant darkness, is capable of causing a shift in the phase of this rhythm, the direction and amplitude of which depends on the time of administration of the pulse. Light exposure during the early subjective night (a time in the animal's rhythm during which it behaves as it would during the early hours of the real night; e.g., onset of activity in nocturnal animals) delays the onset of activity and other circadian rhythms while during the late subjective night (a time during which the animal behaves as it would during the late hours of the real night; e.g., end of activity in nocturnal animals) such light exposure advances them (DeCoursey, 1964; Daan and Pittendrigh, 1976; Pittendrigh and Daan, 1976). Light pulses administered during the subjective day have little or no effect on the circadian rhythm. Such a time-dependent responsiveness to an exogenous cue can be represented in the form of a phase response curve (PRC), plotting the direction of the shift against the circadian phase of application of the cue (Figure 1). Exposure of an organism maintained in constant darkness to brief light pulses every 24 hr can entrain its circadian rhythm to a 24 hr period. Under natural conditions, an animal is able to synchronize its endogenous circadian rhythm (either more or less than 24 hr) to the 24 hr day/night cycles, by means of phase delays and phase advances caused by light exposures during dawn and dusk hours.

Exposure of animals, maintained in continuous light (LL), to pulses of darkness produces a mirror image PRC of the light pulse PRC, with phase advances occurring during the mid subjective day and early subjective night and phase delays during the late subjective night and early subjective day (Subbaraj and Chandrashekaran, 1978).

HISTORY OF RESEARCH ON CIRCADIAN RHYTHMS

Although daily rhythms had long been recognized in plants and animals, they were once believed to be passive responses to cycling environmental cues, such as light-dark cycles, resulting from the Earth's rotation on its own axis. In 1729, de Mairan, a French

scientist, studied the daily rhythm of leaf movements in a heliotropic plant (probably *Mimosa pudica* : "touch-me-not") (cited in Moore-Ede et al., 1982). The leaves of this plant normally open during the day and close during the night. De Mairan maintained this plant in a dark room for several days and observed that the plant continued to exhibit daily rhythms of leaf movements, thus demonstrating that these rhythms were not passively driven by light-dark cycles. However, the influence of some unknown 24-hr cue could not be ruled out.

One hundred years passed before these experiments were even replicated. Augustin de Candolle (1832; cited in, Moore-Ede et al., 1982) maintained *Mimosa pudica* in constant darkness and observed that not only did the rhythm in leaf movement persist, but also that its periodicity was not equal to 24 hrs, but was around 22 hrs. This experiment showed for the first time that these rhythms were not produced as a result of the influence of some unobserved cue with 24 hr periodicity, but were instead endogenously generated and would free-run when isolated from 24 hr illumination cycles.

The endogenous nature of circadian rhythms in mammals was not demonstrated until the early 20th century. Simpson and Galbraith (1906; cited in Moore-Ede et al., 1982), Richter (1922) and Johnson (1939) were some of the first to demonstrate the persistence of circadian rhythms under constant conditions in mammals. The existence of endogenous circadian rhythms in humans was not demonstrated until 1962, when Aschoff and Wever showed that individuals isolated in an underground bunker in the absence of environmental cues show free-running activity and rest cycles with a period longer than 24 hrs. Since then circadian rhythms have been described in most eukaryotic phyla. Prokaryotes were believed not to have circadian rhythms, but recent evidence suggests otherwise (Mitsui et al., 1986; Sweeney and Borgese, 1989).

Although the existence of circadian rhythms in mammals was known since the early decades of this century, the region in the mammalian brain responsible for their generation

and entrainment to environmental cues remained an enigma for many more years. The 1960's and 70's marked a period during which neural structures serving these functions were identified and characterized in several phyla, leading to many dramatic developments which established Chronobiology as a new and promising area of science. The past decade has seen the coming together of the fields of molecular biology and chronobiology in an attempt to unravel the cellular substrate(s) of clock function.

In this chapter, I will limit myself to the mammalian circadian system. In the following sections I will describe the brain structure responsible for the generation of circadian rhythms, presenting evidence for its role as a pacemaker. I will also present evidence for the anatomical and neurochemical basis of synchronization (entrainment) of circadian rhythms to exogenous cues, focusing my attention on the neurotransmitter acetylcholine (ACh) and the neurotrophic factor for central cholinergic neurons, nerve growth factor (NGF).

THE SUPRACHIASMATIC NUCLEUS (SCN)

Over the past fifty years several attempts were made to identify the neural centre responsible for generating circadian rhythms in mammals. Richter (1967) made lesions in several regions of the brain, and looked at their effects on the free-running activity rhythms of blinded rats. Only lesions placed in the "ventral median" hypothalamus were able to abolish this rhythm.

Since visual cues are able to entrain circadian rhythms, it is logical to assume that the clock in the mammalian hypothalamus must receive visual input. Thus the localization of the circadian pacemaker was attempted by tracking the photic input pathways into the hypothalamus. By means of autoradiographic and degeneration studies, a retinohypothalamic tract was found to terminate in the SCN, a nucleus in the ventral hypothalamus (Hendrickson et al., 1972; Moore and Lenn, 1972). Loss of adrenal

corticosterone secretion (Moore and Eichler, 1972), drinking and activity rhythms (Stephan and Zucker, 1972b) after selective ablation of the SCN implicated it as the site of the mammalian pacemaker. Neonatal or adult ablation of SCN results in arrhythmicity and an inability to restore rhythmicity even after prolonged survival (Mosko and Moore, 1978). Loss of rhythmicity following ablation of the SCN, however, does not necessarily mean that the SCN has pacemaker properties, since it could simply be acting as a relay station on the output pathway from the pacemaker. Damage to this relay station may result in a break in the circuit, which expresses itself in the form of a disrupted rhythm. Other kinds of evidence are therefore required in order to support the claim for the SCN as a pacemaker.

Partial lesions of the SCN do not result in loss of circadian rhythmicity, suggesting that a few SCN cells are sufficient to maintain circadian rhythmicity (Eastman et al., 1983; Davis and Gorski, 1984; Rietveld, 1984; Pickard and Turek, 1985). These results also suggest that the SCN contains multiple circadian oscillators.

Further evidence for the existence of multiple oscillators in the SCN comes from the observation of splitting of rhythms. Animals maintained in constant conditions for long periods of time under certain lighting conditions may exhibit a dissociation of their activity pattern into two (Hoffmann, 1971; Pittendrigh and Daan, 1976; Pickard et al., 1984) or more components (Boulos and Terman, 1979; Lees et al., 1983). These two components initially run with independent periods, until they obtain a stable phase relationship, and from then on they run with a similar period (Pittendrigh and Daan, 1976). This phenomenon of dissociation of the activity pattern into two components is called splitting. The phenomenon of splitting and other forms of rhythm dissociation indicate that the pacemaker is composed of multiple oscillators, which may reside in the SCN.

FUNCTIONAL SIGNIFICANCE OF THE SCN

Several lines of evidence implicate the SCN as a self-sustained oscillator (Rusak and Zucker, 1979; Meijer and Rietveld, 1989). Inouye and Kawamura (1979) demonstrated that the SCN shows rhythmicity in multiunit activity in freely moving animals and that this rhythmicity persists even after surgical isolation of the SCN. This rhythm follows a circadian pattern with peaks occurring during the subjective day, when the animal is less active, and troughs occurring during subjective night when the animal is highly active. In a diurnal chipmunk, however, the peak in multiunit activity within the SCN also occurs during the subjective day when the activity outside the SCN is high as well, therefore suggesting that the rhythm within the SCN is independent of influences from other brain areas (Sato and Kawamura, 1984). Such a surgical isolation of the SCN, however, abolishes multiunit activity rhythms in other regions of the brain (Inouye and Kawamura, 1982). This evidence suggests that neural afferents to the SCN are not necessary for the generation of circadian oscillations, although, humoral influences on the oscillator cannot be ruled out.

Persistence of a single unit activity rhythm in rats and hamsters has been shown in brain slices containing the SCN, with the highest firing rate seen during the animal's projected lights-on and the lowest seen during the projected lights-off (Kita et al., 1982; Green and Gillette, 1982; Groos and Hendricks, 1982; Shibata et al., 1982; Gillette, 1986; Mason et al., 1987). The circadian variation in single unit activity has been shown to persist for up to three days *in vitro* (Gillette, 1986). A circadian rhythm in vasopressin secretion persists for several cycles in an explanted SCN (Earnest and Sladek, 1986). These results strengthen the evidence that extra-SCN input is not necessary for the maintenance of circadian rhythmicity in electrical or secretory activity within the SCN.

The SCN also shows a circadian rhythm in glucose utilization, similar to the rhythm in electrical activity with peaks during the day and troughs during the night, both *in vivo* and

in a hypothalamic slice (Schwartz and Gainer, 1977; Schwartz et al., 1980; Newman and Hospod, 1986). This rhythm also free-runs in continuous darkness (Schwartz et al., 1980). Developmentally, it is the first rhythm reported to appear (Fuchs and Moore, 1980; Reppert and Schwartz, 1984).

Another strong piece of evidence implicating the SCN as containing the pacemaker comes from restoration of rhythmicity in activity and drinking in SCN lesioned animals after transplantation of fetal SCN tissue into the third ventricle (Drucker-Colin et al., 1984; Sawaki et al., 1984; Lehman et al., 1987; DeCoursey and Buggy, 1989). This restoration of rhythmicity occurs only when the fetal hypothalamic grafts are placed in the third ventricle and when the animals are maintained in light-dark (LD) cycles or constant darkness (DD). Rhythmicity is not restored if the animals receive the grafts into the lateral ventricle (Harrington et al., 1987), or are maintained in continuous light (LL) (Aguilar-Roblero et al., 1986). The implications of the latter finding are unclear, since unablated animals show intact rhythmicity in LL and there is little evidence that photic information can reach an implanted SCN graft. Transplantation of cortical grafts fails to show any rhythmicity under DD, suggesting that hypothalamic tissue containing the SCN is necessary for the restoration of circadian rhythms (García-Hernández et al., 1987).

A more direct and stronger piece of evidence showing that cells in the SCN have pacemaker properties comes from recent work by Ralph et al. (1990). They transplanted fetal tissue containing the SCN from a mutant variety of hamsters, with a very short period activity rhythm (20 hrs in homozygotes), into a wild-type, SCN-lesioned, arrhythmic hamster (which previously had a near 24 hr period) and vice versa. Rhythmicity was restored in the host with the period of the donor, regardless of the genotype of the host, thus suggesting that the SCN contains cells that are responsible for the generation of circadian oscillations of overt behavioural rhythms and that these cells are capable of imposing their oscillations on surrounding brain areas. Transplantation of genetically

engineered cell cultures with selected phenotypes will give a better insight into the pacemaker properties of the SCN.

Electrical stimulation of the SCN in rats and hamsters produces changes in phase and period of free-running circadian rhythms, similar to changes produced by photic stimulation at similar phases (Rusak and Groos, 1982). Stimulation of areas caudal or lateral to the SCN does not produce these effects. Chemical stimulation of the SCN using local applications of glutamate (Meijer et al., 1988), carbachol (Zatz and Brownstein, 1979) and neuropeptide Y (NPY) (Albers and Ferris, 1984) also produces shifts in the phase of the rhythms. These results suggest that neural activity centered in the SCN may be involved in photic entrainment and may function as a pacemaker by influencing other brain areas, by imposing the new phase and period on them after the perturbations. On the other hand, it is likely that various pacemakers exist within and outside the SCN and that they are coupled to each other by means of neurotransmitters such as glutamate, GABA, ACh and/or NPY. Perturbations, either electrical or chemical, may disrupt these couplings and alter the phase relations among these pacemakers.

Whether or not neurotransmission is involved in the generation of rhythms is unclear. Two strong pieces of evidence suggest otherwise. Schwartz et al. (1987) infused tetrodotoxin (TTX), a reversible blocker of voltage dependent sodium channels, into the SCN of freely moving rats for 14 days. During the time of infusion the circadian activity rhythm disappeared. Upon discontinuation of the infusion, the rhythm reappeared, but at a phase at which it would have been if the animal had not been subjected to TTX treatment. This strongly suggests that during the course of the infusion, although generation of sodium dependent action potentials had presumably stopped, the oscillator had continued to run. These results can be explained if one assumes the pacemaker mechanism of the SCN neurons is subcellular and that the electrical signals generated by these cells (and blocked by TTX) are downstream from the clock. Thus the blocking of action potentials may only

amount to the stopping of the 'hands of the clock' and not the 'clock' itself. The second strong piece of evidence is the appearance of a circadian rhythm in glucose utilization in prenatal rats prior to the development of synaptic contacts in the SCN (Lenn et al., 1977; Bernstein and Moore, 1985; Shibata and Moore, 1988). However, the existence of some synaptic contacts cannot be ruled out.

These findings suggest that the generation of circadian rhythms may depend on humoral interactions (Hakim et al., 1991) or on communication among neurons that does not depend on sodium-dependent action potentials, but on another electrochemical mechanism. Evidence for circadian rhythms of electrical activity in the SCN neurons in a slice preparation rules out the possibility of any humoral influence on rhythm generation and any role for non-neuronal cells in rhythm generation or in entrainment remains to be investigated.

ANATOMY OF THE SCN

The SCN are a pair of nuclei in the anterior hypothalamus lying on either side of the third ventricle, bounded ventrally by the optic chiasm. Each of the nuclei consists of up to 10,000 tightly packed cells in the rat (Güldner, 1976; Riley and Moore, 1977; van den Pol, 1980). Each neuron is about 5 to 15 microns in diameter.

Although the SCN in most mammals is located in the same general area, there are variations among species in its position and size. In lower mammals the SCN are found very close to the midline by virtue of the fact that the third ventricle is a narrow vertical slit (Figure 2). By contrast, among primates (including humans) the third ventricle broadens at the base, thereby pushing the SCN laterally (Lydic et al., 1982).

The SCN of rats can be broadly subdivided into two regions - the rostral quarter and the larger caudal area. The rostral subdivision contains small cells which do not receive retinal afferents, either normally or after neonatal ablation of the rest of the SCN (Mosko

and Moore, 1978). The caudal SCN is further subdivided into the dorsomedial and ventrolateral regions. Cells in the dorsomedial part are slightly smaller, and less densely packed than those in the ventrolateral region. These cells are distinct in their elongated shape containing a nucleus with few invaginations (van den Pol, 1980) and fewer cell organelles which are restricted to the poles of the cell. On the other hand, the ventrolateral area contains spherical cells that have highly invaginated nuclei and more cytoplasm with a denser collection of organelles. These cells are often found in close apposition to blood capillaries, suggesting a possible role in neuroendocrine secretion. Dendrites arising from the ventrolateral area show more arborizations but are confined within the SCN, whereas in the dorsomedial area the cells also extend dendrites into the adjacent anterior hypothalamus. Due to the similarity between cells in the dorsomedial area and the adjoining anterior hypothalamus, the boundaries of the SCN in this direction are uncertain, and it is not clear whether this region is functionally similar to the ventrolateral SCN.

AFFERENT CONNECTIONS TO THE SCN

A. Retinal Input

Photic input is received by the SCN mainly by two routes. One is a direct bilateral projection of large retinal ganglion cells to the SCN. This projection is called the retinohypothalamic tract (RHT). The presence of this projection was first demonstrated in rat using autoradiographic methods (Hendrickson et al., 1972; Moore and Lenn, 1972). Further studies, using a variety of tracing techniques, have confirmed this projection in several mammalian species (Moore, 1973; 1978; Pickard and Silverman, 1981; Moore, 1983; Cassone et al., 1988; Johnson et al., 1988).

About 0.1% of all the retinal ganglion cells (RGC) sending their axons through the optic nerve project into the SCN (Mason and Lincoln, 1976). All of the RGCs innervating

the SCN are located in the inner plexiform layer of the retina and have few long dendrites restricted to its proximal half, suggesting that their role lies in detecting fluctuations in illumination (Pickard and Friauf, 1990). This projection is restricted to the ventral and lateral SCN in rat, whereas in hamster the entire SCN except a thin medial crescent seems to receive retinal input (Pickard and Silverman, 1981; Figure 3). In the hamster, the numbers of retinal ganglion cells retrogradely labelled in each eye after unilateral injections of horseradish peroxidase (HRP) in the SCN are identical. Yet, following unilateral injections of HRP into one retina, the label in the contralateral SCN is heavier than in the ipsilateral SCN, suggesting that there is more extensive arborization in the contralateral SCN from equivalent numbers of ganglion cells (Pickard, 1982). In the hamster, on the other hand, the contralateral and ipsilateral RHT terminal densities are approximately equal, although there appears to be a slight contralateral predominance (Johnson et al., 1988) (Figure 3).

The second source of photic input to the SCN is through an indirect retinal projection. Retinal ganglion cells terminate in the dorsal lamina of the internal division of the ventral lateral geniculate nucleus (vLGN) of the thalamus (Höllander and Sanides, 1976). The neurons in this area in turn send bilateral projections via Meynert's commissure to the SCN (Swanson et al., 1974; Ribak and Peters, 1975). The latter projection, called the geniculo-hypothalamic tract (GHT), originates from NPY containing cells located in the intergeniculate leaflet (IGL) (Card and Moore, 1982; Harrington et al., 1987). The ipsilateral projection from the vLGN to the SCN is shown to be twice as heavy as the contralateral projection (Swanson et al., 1974; Ribak and Peters, 1975; Legg, 1979; Pickard, 1982).

Both these optic afferent pathways (RHT and GHT) are composed of unmyelinated fibres and their synapses are restricted to the ventral and lateral regions of the rat SCN (Moore and Lenn, 1972; Moore, 1979). Their terminals are characterized ultrastructurally

by the presence of mitochondria with a swollen electron-lucent matrix. These terminals also have scattered synaptic vesicles and asymmetric densities characteristic of Gray's type I contacts, which are often considered to represent excitatory synapses (Güldner, 1978).

Some of the RHT fibres are collaterals of the optic tract terminating in the vLGN (Mason et al., 1977; Millhouse, 1977; Pickard, 1982) and thus arise from the same ganglion cells. Since the terminal fields of the RHT and GHT overlap within the SCN, a single SCN neuron may be influenced directly and indirectly (via the thalamus) by photic information received by a single ganglion cell.

B. Forebrain Inputs

A substantial input from the paraventricular nucleus of the thalamus reaches the ipsilateral SCN and this projection courses through the periventricular nucleus of the hypothalamus (Pickard, 1982). Cells in both the lateral and medial septal nuclei project, through the diagonal band and caudally through the medial preoptic area, to the ipsilateral SCN (Pickard, 1982). The medial preoptic area and the preoptic PVN innervate the SCN, the latter projection being entirely ipsilateral (Pickard, 1982). The heaviest ipsilateral projection to the SCN arises from the subiculum and the entorhinal cortex of the hippocampal complex (Meibach and Seigal, 1977; Pickard, 1982). The ventromedial hypothalamus and arcuate nucleus provide moderate projections to the SCN. Neurons of the SCN also receive input from the contralateral SCN neurons across the midline (van den Pol, 1980).

C. Brainstem Inputs

The dorsal and median raphe nuclei give rise to serotonergic projections that terminate in the ipsilateral SCN (Fuxe, 1965; Aghajanian et al., 1969; Ajika and Ochi, 1978; Azmitia and Segal 1978; Bobillier et al., 1979). Other minor inputs to the SCN include an ipsi- and

contralateral projection from the dorsal nucleus of the lateral lemniscus and small projections from the dorsal and the ventral tegmental nuclei.

Role of Afferents

Retinohypothalamic tract - Transection of optic nerves, but not the optic tracts, abolishes the entrainment of circadian rhythms to light-dark cycles, suggesting that the RHT is involved in entrainment to photic stimuli (Stephan and Zucker, 1972a; Chase et al., 1969; Rusak, 1977b; Klein and Moore, 1979). Photic stimuli conveyed through the RHT either activate or suppress extracellular single unit activity in SCN neurons (Groos and Meijer, 1985). Most SCN cells respond to prolonged presentations of light stimuli by an increase or decrease in their activity. However, light pulses of very short duration fail to evoke a response (Groos and Mason, 1980; Groos, 1982; Groos and Meijer 1985; Meijer et al., 1986). Within a narrow range of light intensity, the magnitude of excitatory and inhibitory responses of SCN neurons increases as a function of light intensity (Groos and Mason, 1978; 1980; Groos and Meijer, 1985). In nature this range of light intensities is seen during the twilight hours of dawn and dusk, suggesting that SCN neurons are affected differentially at these hours since they change firing rates in opposite directions when light intensity changes in opposite direction. Therefore, SCN neurons are able to discriminate dawn and dusk hours.

Geniculohypothalamic tract - Transection of the optic tracts and bilateral lesions directed at the vLGN in rats have no effect on the responsiveness of SCN neurons to visual stimuli (Sawaki, 1979; Groos and Rusak, 1982) or the behavioural entrainment of locomotor activity (Dark and Asdourian, 1975; Zucker et al., 1976; Groos and Meijer, 1985) suggesting that the RHT is sufficient for conveying photic information to the SCN. However, ablation of the LGN or its retinal innervation in hamsters slows down re-entrainment after a shift in light-dark cycles (Zucker et al., 1976; Rusak, 1977a, 1977b;

Rusak and Boulos, 1981), decreases the period of the free running rhythm in LL only (Harrington and Rusak, 1986; Pickard et al., 1987; Harrington and Rusak, 1988), alters the shape of the light pulse PRC (Harrington and Rusak, 1986; Pickard et al., 1987) and prevents rhythm splitting in continuous bright light (Harrington and Rusak, 1988). Taken together, these results imply that the amount of photic information reaching the SCN is reduced as a result of the ablation. Reduction in the amount of photic information reaching the SCN would result in reduced excitation or inhibition of photically responsive SCN neurons, therefore slowing down the process of re-entrainment. In a nocturnal animal, a decrease in light intensity decreases the free running period (Aschoff, 1965). Therefore the decrease in period could be a result of reduction in photic information reaching the SCN. This hypothesis can also explain why phase advances are reduced in amplitude by intergeniculate leaflet lesions, but not why similar effects are not found for phase delays (Harrington et al., 1986; Pickard, 1987).

EFFERENT PROJECTIONS FROM THE SCN

Using modern anterograde and retrograde tracing methods, autoradiography and degeneration techniques, six major efferent pathways have been identified (Swanson and Cowan, 1975; Berk and Finkelstein, 1981; Stephan et al., 1981; Watts and Swanson, 1987; Watts et al., 1987). These are:

1. Rostrally directed fibres, which arise from cells in the SCN and terminate in the ventral part of the medial preoptic area and the anteroventral periventricular nucleus.
2. Anterodorsally directed fibres, which arise anterodorsally and course through the medial preoptic nucleus and terminate in the ventral part of the intermediate lateral septal nucleus.
3. Fibres arising caudal to the anterodorsally directed fibres, which terminate in the bed nucleus of the stria terminalis and rostral part of paraventricular nucleus of thalamus.

4. Dorsally directed fibres, which constitute the densest projection arising from the SCN cells. Most cells in the dorsal aspect of the SCN project bilaterally to the medial portion of the paraventricular nucleus of the hypothalamus (PVN), with the densest termination in the periventricular area, just ventral to the PVN. These SCN projection neurons are probably the ones that do not receive retinal input. A small projection from the lateral border of the SCN terminates in the anterior hypothalamic area. Few axons continue dorsally to terminate in the paraventricular nucleus of the thalamus after passing through the midline thalamic nuclei. Few other fibres continue caudally to terminate in the dorsomedial nucleus of the hypothalamus, ventromedial nucleus and posterior hypothalamic area.

5. Laterally directed fibres , which leave the SCN laterally, pass along the optic chiasm and terminate in the ventral lateral geniculate nucleus.

6. Posteriorly directed fibres, which pass through anterior hypothalamic and retrochiasmatic areas to terminate in the arcuate nucleus and ventral parts of the ventromedial nucleus and lateral hypothalamic area.

There is little or no direct projection to any brain nuclei that are involved in somatomotor, autonomic or neuroendocrine responses (Watts et al., 1987) thus offering no direct pathway for the SCN's role as a circadian pacemaker for various motor and endocrine functions. The densest projection from the SCN terminates in the sub-paraventricular zone. This region in turn sends much denser projections than the SCN itself, to the other five regions of the brain which the SCN innervates, thus suggesting that this system may act as an amplifier for the information received from the SCN (Watts and Swanson, 1987).

NEUROTRANSMITTERS IN SCN

The presence of various neurotransmitters within the SCN has been implicated using immunocytochemical methods. They can be broadly classified into four groups (van den Pol and Tsujimoto, 1985), as follows:

1. Neurotransmitters of endogenous origin. The perikarya of SCN neurons in the rat show immunoreactivity to antibodies directed to vasopressin (Vandesande et al., 1975; Buijs, 1978), neurophysin (Kucera and Favrod, 1979), somatostatin (Krisch, 1978; Moore et al., 1979), bombesin (Pannula et al., 1982), gastrin releasing peptide (Roth et al., 1982), vasoactive intestinal polypeptide (Fuxe et al., 1977; Loren et al., 1979; Sims et al., 1980; Card et al., 1981), gamma aminobutyric acid (GABA) and glutamate decarboxylase (GAD: a synthetic enzyme for GABA) (van den Pol and Tsujimoto, 1985). Vasopressin-immunoreactive neurons are localized in the rostral and dorsomedial part of the SCN (Vandesande et al., 1975). Vasoactive intestinal polypeptide-containing neurons are found in the ventrolateral part of the SCN (Card et al., 1981). Somatostatin is present in cells throughout the SCN (Moore et al., 1981). Although some of these somatostatin-containing neurons have short axons and project to the PVN, dorsomedial hypothalamus and the vascular organ of lamina terminalis (Watts and Swanson, 1987), most of them are SCN interneurons (Moore et al., 1979).

2. Neurotransmitters of extrinsic origin but found in higher density inside than outside the SCN. Serotonin and NPY fall into this category. Serotonin-containing cells originate in the raphe nucleus and their terminals are restricted to the ventromedial SCN (van den Pol and Tsujimoto, 1985). NPY immunoreactive fibres originate in the LGN (Card and Moore, 1982; Harrington et al., 1987) and innervate the ventral aspects of the SCN in the rostral and caudal levels. More medially they are localized in ventromedial aspects of the SCN. High concentrations of N-acetyl aspartylglutamate (NAAG), a neuron-specific dipeptide which may act as an excitatory neurotransmitter have been localized in the RHT

and the SCN (Moffett et al., 1989; Moffett et al., 1990) implicating glutamate or aspartate as the neurotransmitter candidate at the RHT.

3. Axons of extrinsic origin with similar neurotransmitter density inside and outside the SCN. Immunoreactive fibers for tyrosine hydroxylase and dopamine β-hydroxylase are found throughout the SCN suggesting that axon terminals with dopamine and norepinephrine may be present (van den Pol and Tsujimoto, 1985). Part of the GABA and GAD found in the SCN may also be of extrinsic origin.

4. Axons of extrinsic origin found in low densities within SCN. Prolactin, thyrotropin, neurotensin, adrenocorticotrophic hormone (ACTH) and choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine, are found in very few axons inside the SCN (Brownstein et al., 1975; van den Pol and Tsujimoto, 1985). ACTH is found in higher concentration just bordering SCN, suggesting that dendrites of SCN neurons probably extend into this area, making contact with the ACTH-containing neurons.

Various neurotransmitters have been implicated in transmitting photic information to the SCN and mediating entrainment. Glutamate has been shown to be one of the strongest contenders. NAAG immunoreactivity exists in the RHT and the SCN (Moffett et al., 1989; Moffett et al., 1990). Transection of the optic nerve results in a reduction in immunoreactivity to NAAG in the SCN suggesting the possibility that NAAG or NAAG-derived glutamate may be involved in retino-hypothalamic transmission. Furthermore, glutamate and aspartate have been localized in the ganglion cells of the vertebrate retina (Redburn, 1981) and the enzymes associated with the metabolism of glutamate and aspartate are shown to be present in the optic nerve (Wenthold, 1981). Excitatory amino acids (EAA: such as glutamate or aspartate) may be the neurotransmitter involved in the retinogeniculate pathway in the cat (Kemp & Sillito,1982) and in the retinotectal pathway in goldfish (Langdon & Freeman, 1986).

Pharmacological studies have shown that iontophoretic application of glutamate excites neurons in the SCN both *in vitro* and *in vivo* (Nishino & Koizumi, 1977; Shibata et al., 1983), although some inhibition was also observed. Furthermore, optic nerve stimulation induces the release of tritiated glutamate and aspartate from the SCN in slices of rat hypothalamus (Liou et al., 1986). In addition, antagonists of EAAs, baclofen (an EAA release blocker), and calcium-free medium block the electrical stimulation-induced field potentials. This result is consistent with the work of Cahill and Menaker (1987) which showed that kynurenic acid, a wide spectrum EAA antagonist, blocks the field potentials evoked by optic nerve stimulation. On stimulation of optic nerve and optic chiasm ventral to the SCN, excitatory postsynaptic potentials (EPSPs) are evoked in the SCN neurons (Kim & Dudek, 1989). Twenty to thirty percent of these EPSPs are blocked after bath application of kynurenic acid, whereas, 15-75% of EPSPs are blocked after application of 6-7-dinitroquinoxaline -2,3-dione, a non-NMDA receptor antagonist. These results suggest that EAAs, probably acting on non-NMDA receptors, are involved in generating these EPSPs.

Although glutamate has been shown to be the most likely transmitter candidate at the RHT and it has been shown to be released after optic nerve stimulation, behavioural studies using glutamate have revealed unexpected findings. Glutamate when injected into the SCN produces phase shifts in the wheel-running activity of hamsters (Meijer et al., 1988). Curiously enough, the phase response curve (PRC) for glutamate injections resembles that generated in response to dark pulses, with advances during the mid to late subjective day and phase delays during late subjective night and early subjective day. The reason for this discrepancy between physiological effects and behavioural effects needs to be investigated.

Although EAAs are strong candidates at the RHT, involvement of other putative neurotransmitters cannot be ruled out. In this thesis I will focus my attention on the role of ACh and that of its neurotrophic factor, NGF, in mediating entrainment .

ACETYLCHOLINE

LOCALIZATION OF CENTRAL CHOLINERGIC NEURONS

Cholinergic projection neurons in the central nervous system are located in two distinct groups. One of these groups is located in the forebrain and is sometimes referred to as the cholinergic basal forebrain nuclear complex (CBC) (Figure 4A). The other group is located in the mesopontine tegmentum of the brainstem.

The CBC is a continuum of cholinergic cells located in various classically defined nuclei (Schwaber et al., 1987). Mesulam et al (1983) categorized these nuclei into four groups based on their efferent projections. Ch1 and Ch2 include the medial septum (MS) and the vertical limb of the diagonal band of Broca (vDBB) respectively. These areas of the CBC provide cholinergic projections to the hippocampus through the fornix/fimbria. Ch3 consists of cholinergic cells in the magnocellular preoptic area (MgPA) which project to the olfactory bulb. Cells in the Ch4 group project to the neocortex and this group includes cells from relatively caudal parts of the CBC, including the horizontal limb of the diagonal band of Broca (hDBB), MgPA, the substantia innominata (SI) and the nucleus basalis magnocellularis (NBM) (Bigl et al., 1982; Saper, 1984; Henderson, 1987). The cortical projections from the Ch4 cells are topographically organized in that the more rostral nuclei project to the occipital and cingulate cortices, whereas the caudal nuclei project to the frontal and parietal cortices (Bigl et al., 1982; McKinney et al., 1983).

The mesopontine tegmental cholinergic system is located in the tegmentum of the midbrain and the pons. The cholinergic cells are located in two discrete nuclei, the rostral, pedunculopontine tegmental nucleus (PPT; Ch5 of Mesulam et al., 1983) and the more caudal, laterodorsal tegmental nucleus (LDT; Ch6 of Mesulam et al., 1983) (see Semba and Fibiger, 1989 for review) (Figure 4B). The cholinergic neurons from the PPT and LDT give rise to heavy, ascending projections to various thalamic nuclei (Satoh and Fibiger,

1986; Woolf and Butcher, 1986; Hallanger et al., 1987). The cholinergic neurons in PPT and LDT also innervate the CBC area (Satoh and Fibiger, 1986; Semba et al., 1988; Záborszky et al., 1991). The descending projections from the LDT and PPT terminate in the pontine reticular formation (PRF) (Mitani et al., 1988), the area of the brain involved in the generation of rapid eye movement (REM) sleep.

ROLE OF ACH IN SLEEP AND AROUSAL

PHYSIOLOGICAL EVIDENCE

Activation of the CBC results in cortical arousal or EEG desynchronization (see Semba, 1991 for review). During wakefulness and REM sleep, ACh release in the cortex is about twice as high as that during slow wave sleep (Jasper and Tessier, 1971). Furthermore, lesions made in the NBM slow down the cortical EEG (Buzsáki et al., 1988). On the other hand, electrical stimulation of the NBM results in the activation of cortical EEG and an increase in cortical acetylcholine output (Casamenti et al., 1986). In patients with Alzheimer's disease (characterized by the degeneration of cholinergic cells in the CBC area as well as other cells), there is increased expression of the slow component of the cortical EEG (Coben et al., 1985). Taken together, these findings suggest that during waking, the cholinergic neurons in the CBC are activated, resulting in an increased release of ACh from their terminals in the cortex, which in turn results in cortical arousal.

Stimulation of brainstem reticular formation also results in increased ACh release and EEG activation in the somatosensory and parietal cortices (Kanai and Szerb, 1965). However, there are no direct cholinergic inputs from the brainstem into these cortical areas. Thus, this cortical activation must occur through a projection from the brainstem to the CBC. Several studies using retrograde tracing techniques and immunocytochemistry have shown that monoaminergic neurons located in the dorsal raphe and locus coeruleus project to the CBC (Semba et al., 1988; Jones and Cuello, 1989), making synaptic contact with

the cholinergic neurons (Záborszky, 1989) and cholinergic neurons from the mesopontine tegmentum innervate the CBC area (Semba et al., 1988; Jones and Cuello, 1989) making contact predominantly with non-cholinergic neurons (Bialowas and Frotscher, 1987; Hallanger et al., 1988). This suggests that activation of aminergic neurons in the brainstem activates cholinergic neurons in the CBC, thus resulting in cortical arousal.

The rhythmic oscillations or EEG spindles characteristic of slow wave sleep are produced when thalamocortical neurons switch their activity pattern from the single spike mode to the oscillatory mode in (McCormick, 1989 for review). The thalamic reticular nucleus acts as a pacemaker for the generation of these thalamocortical oscillations or spindles. Transition from one mode to the other is under the control of two mechanisms. In the oscillation mode, inhibitory postsynaptic potentials in cortical neurons which are generated indirectly by thalamic neurons contribute to the conversion of waking EEG desynchronization into spindles, characteristic of slow wave sleep. In the single spike mode, however, the activity of the thalamic reticular nucleus is inhibited by the inputs from the brainstem cholinergic (PPT and LDT) and aminergic nuclei (locus coeruleus and dorsal raphe). Thus, both ACh released from the terminals of PPT and LDT neurons and monoamines released from the locus coeruleus and dorsal raphe inhibit the activity of the thalamic reticular nucleus. This inhibition of the thalamic reticular nucleus results in the disinhibition of cortical neurons and the inhibition of slow wave sleep-associated EEG spindles (Steriade and Deschênes, 1984) and the expression of activated EEG patterns characteristic of both waking and REM sleep. Although the cholinergic inhibition of the thalamus is controversial, two pieces of evidence support this hypothesis. Application of acetylcholine to the thalamic reticular nucleus as well as electrical stimulation of the PPT and LDT results in the inhibition of neurons in the thalamic reticular nucleus.

Cholinergic agonists injected into the PRF induce a REM sleep-like state which includes muscle atonia, rapid eye movement, cortical EEG desynchronization, hippocampal

theta and pontogeniculooccipital waves. These effects of the cholinergic agonists can be blocked by atropine suggesting the involvement of muscarinic receptors. Cholinergic neurons from the LDT and PPT project to the PRF, thus constituting the source of ACh for the induction of REM sleep (Mitani et al., 1988). A subpopulation of cholinergic mesopontine tegmental neurons project to both the thalamus and the PRF (Semba et al., 1990). Thus cholinergic neurons in the mesopontine tegmentum can induce REM sleep through activation of PRF neurons and concurrently suppress thalamic spindles.

Although the cortical EEG patterns during waking and REM sleep look alike, there are physiological differences between the two states, and these may occur as a result of interactions between cholinergic neurons in the mesopontine tegmentum and aminergic neurons located in the locus coeruleus and dorsal raphe (Hobson,1990). According to Hobson (1990) these aminergic neurons enhance stimulus detection and stimulus-dependent learning and memory. During REM sleep (often associated with dreaming), there is a complete suppression of these aminergic neurons resulting in the inattentiveness and amnesia as experienced during dreams. Cholinergic neurons, on the other hand, are normally inhibited during waking (although this is controversial) by the aminergic neurons, except when novel stimuli are encountered, which results in the co-activation of both aminergic and cholinergic neurons (Hobson, 1990).

McCarley and Hobson (1975) proposed a model to explain the phenomenon of brainstem control of REM sleep. They proposed that at the onset of REM sleep cholinergic neurons ("REM-on" neurons) located in the mesopontine tegmentum (PPT and LDT) become active and thus release ACh in the PRF and the thalamus. This results in depolarization of the cells in the PRF and inhibition of cells in the thalamic reticular nucleus. The activation of the PRF manifests itself in the form of the various behavioural markers of REM sleep, and inactivation of the thalamic reticular nucleus results in the suppression of spindles which are typically seen during slow wave sleep. These

mesopontine cholinergic neurons have an excitatory input onto aminergic neurons ("REM-off" neurons) that are located in the dorsal raphe and locus coeruleus. These aminergic neurons, in turn, inhibit the activity of the cholinergic neurons resulting in the termination of REM sleep, and through their activation of cholinergic neurons in the CBC and their facilitation of signal detection capacities the aminergic neurons initiate waking. The aminergic neurons have an inhibitory feedback (autoinhibition), thus slowing down their own activity. The gradual slowing of firing of these REM-off neurons through such feedback eventually disinhibits the REM-on neurons, which gradually increases REM-on activity and suppresses aminergic activity, while releasing ACh to initiate, once again, a REM sleep episode. Although this is an interesting hypothesis, not all the steps presumed in this hypothesis have been proven.

REM/non-REM sleep alternations recur approximately every 90 minutes in adult humans. It is unclear what happens to the REM sleep mechanisms during the waking hours. McCarley and Massaquoi (1986) propose that the REM-off neurons might exhibit circadian variations in their excitability. Thus, during waking hours the REM-off neurons may be very active; as a result the REM-on neurons remain inhibited. There is, however, little physiological evidence to support this hypothesis (Legoratti-Sánchez et al., 1989). Furthermore, there is no anatomical evidence for an efferent projection from the SCN to the dorsal raphe or the locus coeruleus.

ROLE OF ACH IN CIRCADIAN RHYTHMS

Acetylcholine appears to play a major role in mediating entrainment in mammals. The word 'appears' has been used because the available data on the effects of ACh, its agonists and antagonists on circadian rhythms, do not give a clear picture of its exact role (Rusak and Bina, 1990).

One of the first pieces of evidence suggesting a possible role for acetylcholine in mediating the effects of light on circadian rhythms was reported by Zatz and Brownstein (1979). They showed that intraventricular administration of carbachol, an agonist of acetylcholine, mimics the effect of light by either phase shifting or reducing the nocturnal activity of the pineal enzyme, serotonin N-acetyltransferase (SNAT). Since then carbachol administration has been shown to mimic partially the pattern of phase shifting characteristic of light effects on locomotor activity rhythms of mice (Zatz and Herkenham, 1981) and hamsters (Meijer et al., 1988; Wee and Turek, 1989) and the activity rhythms of rats (Mistlberger and Rusak, 1986). The induced phase shifts are permanent, indicating that they are mediated through the central pacemaker and do not reflect an effect on the output mechanism.

Phase response curves for intraventricular injections of carbachol in hamsters maintained in LL and DD show that injections in the early subjective night produce phase delays, while injections in the late subjective night (Earnest and Turek, 1983; 1985) or mid subjective day (Meijer et al., 1988) produce phase advances. Thus, the PRC for carbachol partially mimics the pattern of phase shifting which is characteristic of light effects.

According to Aschoff's rule (Aschoff, 1965), in nocturnal animals, an increase in light intensity results in an increase in the free-running period; i.e. when animals are placed in continuous light (LL), the period of the free-running rhythm increases. As an exception to this rule, however, Murakami et al. (1986) have shown that when the eyelids of rats placed in LL are removed, , thereby increasing the amount of light reaching the SCN, the period of the free-running rhythm of water intake decreases. This shortening is duplicated by bilateral implantation of carbachol pellets near the SCN, thus suggesting that carbachol mimics the effects of light. Similar results were found for the locomotor activity of rats (Furakawa et al., 1987).

ACh content in the rat SCN increases in response to light (Murakami et al., 1984). In sighted animals, the ACh concentrations increase in response to a light pulse administered two hours after the initiation of darkness. This increase in ACh concentration occurs with a delay of 30-60 minutes after the onset of the light pulse, suggesting that the increase may be due to *de novo* synthesis of ACh in the SCN (Murakami et al., 1984). In contrast, in rats tested two weeks after blinding, no rhythm in ACh concentrations was observed, implying that light input is necessary for the increase in levels of ACh. Since the activity of the blinded rats was not monitored, however, it is likely that after two weeks the activities of the blinded rats were desynchronized with respect to each other, and therefore the measurements of ACh concentrations were taken at different circadian times.

Blocking the behavioural effects of carbachol and light using acetylcholine receptor antagonists would suggest that acetylcholine is responsible for mediating the phase shifting effects of light. Alpha-bungarotoxin (α-BTX) and mecamylamine, two acetylcholine receptor antagonists at the nicotinic receptor (however, see chapter 4), have been used to block the phase-shifting effects of light.

Alpha-BTX is a neurotoxin that has the α-subunit of its receptor protein homologous to the skeletal muscle nicotinic cholinergic receptor, suggesting that the toxin probably binds to the nicotinic receptor (Conti-Tranconi et al., 1985). Alpha-BTX, when infused near the suprachiasmatic nucleus, blocked the acute inhibitory effects of nocturnal light on pineal SNAT, without in itself producing any effect on SNAT activity. Like light, it had no effect on daytime levels of SNAT. Mecamylamine has also been shown to block phase advances and phase delays produced by light pulses in hamsters maintained in constant darkness (Keefe et al., 1987).

Unlike nicotinic receptor antagonists, the muscarinic cholinergic receptor antagonists, scopolamine and atropine, are unable to block the phase shifting effect of light (Zatz and Brownstein, 1981), suggesting that the phase shifting effects of carbachol are mediated by

nicotinic receptors. However, contrary evidence also exists. Miller and Billiar (1986) were unable to reproduce the blocking effects of α–BTX on light-induced phase shifts in ovariectomized rats, even though these rats do show reduction in SNAT levels after administration of carbachol, which suggests that they are responsive to carbachol. Furthermore, Pauly and Horseman (1985) showed that hemicholinium-3, a high affinity choline uptake blocker (which by this action blocks *de novo* synthesis of ACh), when administered through indwelling cannulae placed dorsal to the SCN, does not block phase advances or delays caused by light pulses. This study, however, did not present any evidence showing the effectiveness of hemicholinium-3 in completely blocking ACh synthesis and, therefore, the inability of the treatment to block phase shifts to light pulses may have been due to the use of a strong supersaturating light pulse and an incomplete block of ACh synthesis. In contrast, Keefe et al. (1987) used a weak light pulse that was of a strength below the level required to produce maximal phase shifts. They effectively blocked the resulting phase shifts with mecamylamine.

Electrophysiological studies have indicated that cells in the SCN respond to cholinergic agonists, thus implying that these cells are cholinoceptive. Iontophoretic application of ACh to SCN neurons in anesthetized, male rats excited 80% of the neurons tested and inhibited 11% (Nishino and Koizumi, 1977). In addition, 60% of the neurons which were responsive to ACh also responded to optic nerve stimulation (Nishino and Koizumi, 1977) suggesting that these neurons receive photic input. Another study also showed a high degree of correlation between photic and nicotinic responses of SCN neurons (Miller et al., 1987). More than 80% of the photically responsive cells recorded in this study were excited by intravenously administered nicotine. Mecamylamine, had a normalizing effect on cell firing such that the firing rate of photically activated cells was decreased and that of photically suppressed cells was increased. Mecamylamine also blocked responsiveness to photic stimulation, suggesting that cholinergic receptors, located either on the terminals of

retinal ganglion cells that project to the SCN or the retina, are responsible for mediating the effects of light (but see chapter 4).

In contrast to the studies done *in vivo*, ACh perfusions on SCN neurons in brain tissue slices from female rats inhibited neuronal firing (Kow and Pfaff, 1984). These differences in responsiveness reported by Nishino and Koizumi, by Miller et al. and by Kow and Pfaff may be due to a variety of different conditions used in the two sets of studies. The *in vivo* studies used male, anesthetized rats, with the drugs being applied iontophoretically or intravenously. These studies did not control for transneuronal effects of acetylcholine. The *in vitro* study (Kow and Pfaff, 1984) used 500μm thick isolated brain slices of female rats, and drugs were applied through the perfusion medium, with transneuronal effects being blocked through the use of a low calcium medium. Whether the variable response to ACh by SCN neurons in the two sets of studies was because of differences in sex, form of administration of drug or because of transneuronal effects is not known. It is likely that *in vivo* the observed effect is the net effect of ACh on all cells that are responsive to ACh and also have synaptic contact with the single SCN cell that is being recorded from, whereas, *in vitro*, the observed response is just that of the single cell that is being recorded from, because transneuronal effects are either reduced, due to severing of afferents to the SCN, or abolished through the use of a low calcium medium. Shibata et al. (1983) iontophoretically applied ACh to SCN neurons in rat hypothalamic slices and showed that 32% of the cells showed a change in firing rate. The respective percentages of cells that were excited or inhibited by ACh were not reported.

Despite these behavioural and physiological findings indicating cholinergic effects on the SCN, anatomical evidence for the presence of cholinergic receptors in the SCN has been ambiguous. Axon terminals containing ChAT, the enzyme responsible for the synthesis of ACh, have been localized in the SCN of the rat (Brownstein et al., 1975; Block and Billiar, 1981, Ichikawa and Hirata, 1986). Cholinergic receptor localization

studies, on the other hand, have revealed contradictory results. Areas immediately adjacent to the SCN are found to be heavily labelled by ^{125}I-BTX but not by the muscarinic blocker QNB (Segal et al., 1978; Block and Billiar, 1981; Miller et al., 1982; Fuchs and Hoppens, 1987). Although α-BTX binds with very high affinity to nicotinic receptors in peripheral tissues, in the central nervous system there is little overlap between its binding sites and those of ^{3}H-nicotine and ^{3}H-ACh (Clarke et al., 1985; Wonnacott, 1987). The areas around the SCN seem to be heavily labelled by ^{125}I-BTX but show no labelling by either ^{3}H-nicotine or ^{3}H-ACh (Miller et al., 1982; Clarke et al., 1985). The BTX labelling within the SCN is restricted to large neurons in the dorsal and lateral mid-SCN and, dorsally, in mid-caudal SCN. No labelling is found in the caudal most regions of the SCN of rats (Miller and Billiar, 1986). Ovariectomy drastically reduces BTX binding sites around the SCN (Miller et al., 1982) yet it does not prevent phase shifts in response to carbachol infusions into the lateral ventricles (Mistlberger and Rusak, 1986). A recent study using monoclonal antibodies raised against muscarinic and nicotinic receptor proteins has demonstrated the presence of both these receptors in the SCN (van der Zee et al., 1991).

Thus, although both behavioural and neurophysiological results suggest that ACh plays a significant role in mediating entrainment to light, no conclusive statement to this effect can be made at the present time. This thesis will focus on characterizing the cholinergic input to the SCN and reassessing the role of ACh in phase shifting behavioural rhythms.

NERVE GROWTH FACTOR (NGF)

Nerve growth factor (NGF) is a neurotrophic factor that is essential for cell division, cell death, axon outgrowth, synapse formation during foetal development and for the maintenance of adult noradrenergic neurons in the periphery and cholinergic neurons in the CNS, and also for neural regeneration following injury (Levi-Montalcini and Hamburger,

1953; Ross et al., 1991). Large quantities of NGF are found in the submaxillary glands of male mouse (Cohen, 1960). Lower levels are found in other tissues. Low serum levels suggest that it is synthesized at its site of action (Stephani et al., 1987).

Structure of NGF

High molecular weight NGF has a sedimentation coefficient of 7S with a molecular weight of 140,000 (Varon et al., 1968). It consists of three subunits, namely β, γ and α (Server and Shooter, 1977). The stoichiometry of 7S NGF is $\alpha_2\beta\gamma_2$ (for review see Fahnestock, 1991).

The β subunit is responsible for NGF's nerve-growth promoting activity. β-NGF is a non-covalently bound dimer with a molecular weight of 26,000. Strong denaturing agents are capable of separating the dimer into two identical chains of 118 amino acids each (Varon and Shooter, 1970). Both the dimer and the two monomers are biologically active (Frazier et al., 1973; Stach and Shooter, 1974).

The two γ subunits of 7S NGF are responsible for its proteolytic activity (Greene et al., 1969) and have a molecular weight of 26,000 each. This subunit has been implicated in the processing of the β-NGF precursor. It binds to the C-terminal of β-NGF to form the 7S complex.

The α subunit shares significant homology with the γ-subunit, but without any enzymatic activity. Its function is unknown.

NGF Biosynthesis

The β-NGF gene occurs as a single copy on mouse chromosome 3 (Zabel et al., 1985) covering more than 43 kb, and it consists of 5 exons and 4 introns (Selby et al., 1987). The β-NGF protein is synthesized as a series of large precursor molecules (the pre-pro NGF) (Berger and Shooter, 1977) that is processed at N and C terminal ends into the mature 132,00 MW protein. Mature NGF occurs in the 7S form because the binding of the

α and γ subunits to the β-subunit protects it from proteolytic modification and regulates the activity of component subunits. In the 7S form the biological activity is also suppressed due to the interference of α and γ subunits with NGF binding to its receptor (Harris-Warrick et al., 1980; Nicolls and Shooter, 1985). A 2.5S NGF containing one chain of 109 amino acids and another intact chain is biologically active and has been used for evaluating the role of NGF (Fahnestock, 1991).

The Nerve Growth Factor Receptor (NGF-R)

NGF-R is a transmembrane protein with binding sites for NGF lying outside the cell and an effector site mediating the biological signal within the cell. NGF interacts with its receptors with biphasic equilibrium binding kinetics implying the existence of two classes of receptors (Hempstead et al., 1991). NGF binds to 10-15% of its receptors with high affinity (with a dissociation constant: $K_d = 10^{-11}M$) and to the rest with low affinity ($K_d = 10^{-9}M$) (Sutter et al., 1979; Landreth and Shooter, 1980; Schechter and Bothwell, 1981).

Structure of NGF-R

Cloning and sequencing of cDNAs coding for NGF-R predict a polypeptide with a molecular mass of 45kD that increases to about 75-80 kD ($p^{75NGF-R}$) upon glycosylation. This receptor has several distinct domains. The extracellular domain has a signal sequence (N-terminal) of 28 amino acids, followed by four repeats of 40 amino acids with six cysteine residues at conserved positions. The intracellular domain is made up of 21 amino acids in the membrane flanked by 19 amino acids in the extracellular portion and 46 amino acids in the cytoplasmic region. NGF binds to the third and fourth cysteine rich sequences along with part of the second sequence. The transmembrane and

the cytoplasmic domain, although not involved in binding NGF, are necessary for signal transduction (Yan et al., 1991).

The second class of receptor is the protooncogene *trk* ($p^{140prototrk}$), a 140K glycoprotein (Kaplan et al., 1991b; Klein et al., 1991). The protooncogene *trk* belongs to the tyrosine kinase family of membrane receptors with a large extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase catalytic domain. NGF binds to this receptor and induces autophosphorylation by the *trk* encoded tyrosine kinase in Pheochromocytoma (PC12) cells within a minute of binding, reaching a peak in five minutes (Kaplan et al., 1991a; 1991b). The product of *trk* is a transmembrane glycoprotein and is expressed developmentally only in sensory spinal and cranial neurons of neural crest origin (Kaplan et al., 1991a).

Both $p^{140prototrk}$ and $p^{75NGF-R}$ bind NGF with low affinity. In order to form the high affinity NGF binding site, the coexpression of both receptors is required (Hempstead et al., 1991; Loeb et al., 1991).

Localization of NGF in CNS

The highest levels of NGF and NGF mRNA in the CNS are found in the hippocampus and cortex (Goedert et al., 1986; Korsching et al., 1986; Shelton and Reichardt, 1986), regions which are innervated by cholinergic neurons in the basal forebrain (Seiler and Schwab, 1984; Korsching et al., 1985; Korsching, 1986). Radiolabelled NGF injected into the hippocampus is retrogradely transported into the septum and diagonal band of Broca and that injected into the cortex is transported into the nucleus basalis magnocellularis (Schwab et al., 1979; Seiler and Schwab, 1984). In adults, fimbria/fornix transection results in a 50% rise in the NGF content in the hippocampus with no effect on the NGF mRNA content, suggesting that this increase is probably caused by the accumulation of NGF resulting from the breakdown in retrograde transport to the basal

forebrain neurons (Whittemore et al., 1986; Korsching et al., 1986). In neonates, however, similar transections are associated with an elevation in hippocampal mRNA (Whittemore et al., 1986).

Cholinergic neurons in the basal forebrain have also been shown to contain NGF mRNA suggesting that these cells are capable of synthesis of NGF as well as of target-derived neurotrophic support from the hippocampus (Lauterborn et al., 1991)

During development NGF mRNA is transiently expressed in the cerebellum, olfactory bulb (Lu et al., 1989) and in the eye, on retinal ganglion cells (Ayer-Lelievre et al., 1983).

Localization of NGF-R in CNS

Most cholinergic neurons in the basal forebrain contain NGF-R mRNA and protein (Schweitzer, 1987; Dawbarn et al., 1988; Eckenstein, 1988; Kiss et al., 1988; Yan and Johnson, 1988; Batchelor et al., 1989; Koh et al., 1989; Pioro and Cuello, 1990). NGF-R are synthesized in the somata of cholinergic neurons in the basal forebrain and are transported anterogradely to their terminals in the hippocampus and the neocortex (Seiler and Schwab, 1984; Taniuchi and Johnson, 1985; Johnson et al., 1988). High levels of NGF-R are also present in the mesencephalic trigeminal nucleus and vestibulocochlear ganglia. The olfactory bulb, nucleus tractus solitarius, area postrema and the principal and spinal trigeminal nuclei contain high levels of NGF-R protein and low levels of mRNA. The SCN contains high levels of NGF-R protein (Sofroniew et al., 1989) and no detectable amounts of mRNA (Koh et al., 1989).

Mode of action of NGF

NGF is released from the cell bodies of the NGF synthesizing neurons and is taken up at nerve terminals of NGF responsive neurons. At these terminal sites NGF binds to the NGF-R, is internalized and forms a NGF-NGF-R complex which is retrogradely

transported to the somata of the NGF responsive neuron (Hendry et al., 1974; Seiler and Schwab, 1984; Schwab et al., 1979) where it exerts its biological effects. Two views of the intracellular effector mechanisms exist.

One view is that the NGF-NGF-R complex is transported to sites inside the cell and it in itself acts as an intracellular effector. A second view is that the complex itself has no role as an effector and it is internalized, transported to the cell body, degraded in lysosomes or dissociated into its components and recycled. In this view, various second messengers have been implicated in mediating NGF's actions, although no single second messenger has actually been shown to induce all the changes produced by NGF. Furthermore, these second messengers are also induced by other growth factors as well as by other pharmacological agents which lack the action of NGF. These findings suggest that either early events following NGF binding are not important for the transcription of genes specific to differentiation (Levi and Alema, 1991), or that some specific temporal sequence of second messenger activation is needed for differentiation, or that the effects in the target cell are mediated through some hitherto unidentified second messenger(s).

Cyclic adenosine monophosphate (cAMP) has been implicated as the mediator of NGF action, for several reasons: its analogues are able to induce phenotypic changes similar to those induced by NGF; there is a transient rise in cAMP following NGF treatment of PC-12 cells (Schubert and Whitlock, 1977; Schubert et al., 1978): cAMP treatment results in increased ChAT activity (Schubert et al., 1977) and stimulation of neurite outgrowth (Schubert et al., 1978); and lastly NGF activates protein kinase A (PK-A) which in turn increases cAMP. However, contrary evidence exists. Unlike treatment with NGF, cAMP-induced neurite extension does not proceed beyond three days. Furthermore, analogues of cAMP which block cAMP-mediated activation of PK-A do not block NGF induced neurite outgrowth (Richter-Landsberg and Jastroff, 1986).

NGF binding also results in the activation of several protein kinases including PK-A, PK-C, microtubule associated protein (MAP-2) kinase and PK-N (Mutoh and Guroff, 1989). This phosphorylation on serine/threonine residues is maintained as long as NGF is present. Furthermore, the kinase inhibitor K-252a blocks the action of NGF.

Exposure of PC12 cells to NGF also results in the rapid phosphorylation of tyrosine residues (Maher, 1988; 1989). This phosphorylation is unique in that it occurs only transiently and appears to be an important early step in signal transduction following exposure of PC12 cells to NGF (Berg et al., 1991). This phosphorylation is mediated through the $p^{140prototrk}$ receptor. Several proteins such as MAP-2, and phospholipase Cγ1 may be phosphorylated on tyrosine residues in response to the *trk* induced kinase activity (Vetter et al., 1991).

Role of NGF in adult CNS

NGF functions as a trophic factor for the development and maintenance of central cholinergic neurons (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Whittemore and Seiger, 1987). Proper maintenance of these neurons depends on the continued supply of NGF. Deficits in NGF occurring during development lead to developmental neuronal death resulting in decreased neuronal numbers in adults (Cowan et al., 1984), and those occurring in adulthood lead to pathological neuronal death, perhaps resulting in degenerative diseases such as Parkinson's disease or Alzheimer's disease (Appel 1981; Higgins and Mufson, 1989). In aged animals and humans NGF levels in the hippocampus are reduced when compared with young adults (Larkfors et al., 1987), resulting in the lowering of the number of cholinergic neurons in the basal forebrain area (Bartus et al., 1982; Whitehouse et al., 1982). Induced deficits in NGF caused by lack of contact by axotomized neurons with target areas (e.g., following fimbria/fornix transection) also lead to retrograde neuronal degeneration and cell death (Pearson et al., 1983; Hefti, 1986).

NGF infusion following fimbria/fornix transection results in the survival of these cholinergic neurons. NGF infusions combined with a fetal hippocampal graft induces axonal regeneration and extension across the fetal graft (Tuszynski et al., 1990).

There is good evidence that NGF has a trophic role for ganglion cells in the retina. NGF-R is expressed developmentally in the rat visual system; namely, in the retina, optic nerve and superior colliculus (Yan and Johnson, 1988). NGF-R protein and mRNA have also been localized on the retinal ganglion cells and Müller cells of the adult rat retina (Carmignoto et al., 1991). Intraocular injections of NGF into adult rats rescues axotomized retinal ganglion cells from degeneration (Cavicchioli et al., 1989). Following ligation of the optic nerve, NGF-R accumulates on either side of the ligature indicating that NGF-R is transported both retrogradely and anterogradely from the retina (Carmignoto et al., 1991).

The SCN exhibits the most intense neuropil staining of NGF-R in the rat forebrain (Koh et al., 1989; Sofroniew et al., 1989). Recently, using blot hybridization techniques, the presence of NGF mRNA has been demonstrated in the SCN (Ojeda et al., 1991). Furthermore, NGF precursor-like immunoreactivity has also been localized in the SCN, suggesting the possibility that cells in the SCN are capable of synthesizing NGF and/or are involved in the uptake and transport of its precursor molecules (Senut et al., 1990).

It is unclear in what cellular compartment of SCN tissue NGF receptors are found, what the origins of these receptors are and what functions they serve in the SCN. The present study will focus on the localization of NGF-R containing cells that project to the SCN and also evaluate the role of NGF on circadian rhythms and the process of entrainment in the SCN.

In the next chapter, I will describe anatomical experiments performed on rats to identify cholinergic cells in the forebrain and brainstem that project to the SCN using retrograde and anterograde tracing techniques. Localized injections of the retrograde tracer fluorogold

were made into the SCN, and the forebrain and brainstem were processed for the immunocytochemical localization of ChAT. Cells double-labelled with fluorogold and ChAT were identified and mapped. Anterograde tracing was performed to confirm the retrograde labelling of selected areas, by making localized injections of the anterograde tracer, biocytin, into the PPT, LDT and the parabigeminal nucleus in the brainstem, and then localizing labelled fibres in the SCN.

Chapter 3 will report a study which mapped those NGF-R containing cells in the rat forebrain and retina that project into the SCN, using retrograde tracing and immunocytochemistry for NGF-R. Cells triple labelled with the above-mentioned two markers and ChAT were also identified. Projection pathways of NGF-R containing cells to the SCN were traced using lesion and knife cut techniques. In addition, the presence of muscarinic receptors in the SCN was demonstrated using a hydrophilic muscarinic ligand, N-methyl scopolamine.

In Chapter 4, I will describe the effect of microinjections of carbachol into the SCN on the phase of circadian rhythms in hamsters. The cholinergic receptor types mediating this effect were evaluated using specific receptor antagonists of muscarinic and nicotinic receptors.

Chapter 5 will describe the effect of microinjections of NGF into the SCN, on the circadian rhythm phase in hamsters. The role of NGF was further evaluated using antibodies raised against NGF in an attempt to block the effects of NGF.

Figure 1 Panel A shows a schematic representation of the activity rhythm of a nocturnal animal. Each horizontal line represents a single day. Consecutive days are plotted beneath one another. Black areas represent activity and white areas rest. Entrainment to light-dark (LD) cycle occurs with the activity coinciding with the dark phase. Under constant darkness (DD) the animal shows a free-running rhythm, which in this example has a period of less than 24 hr. Light pulses (LP) administered at circadian time (CT) 22 (late subjective night) produce phase advances and at CT14 (early subjective night) produce phase delays. Open squares represent time of administration of the light pulse.

Panel B shows a phase response curve (PRC) for a light pulse in a nocturnal animal. The horizontal axis represents a single day and CT12 is the onset of activity. Phase advances are represented as positive and phase delays as negative. Light pulses administered during the early subjective night produce phase delays and those during the late subjective night produce phase advances. Light pulses administered during the subjective day have no effect on the rhythm.

FIGURE 1

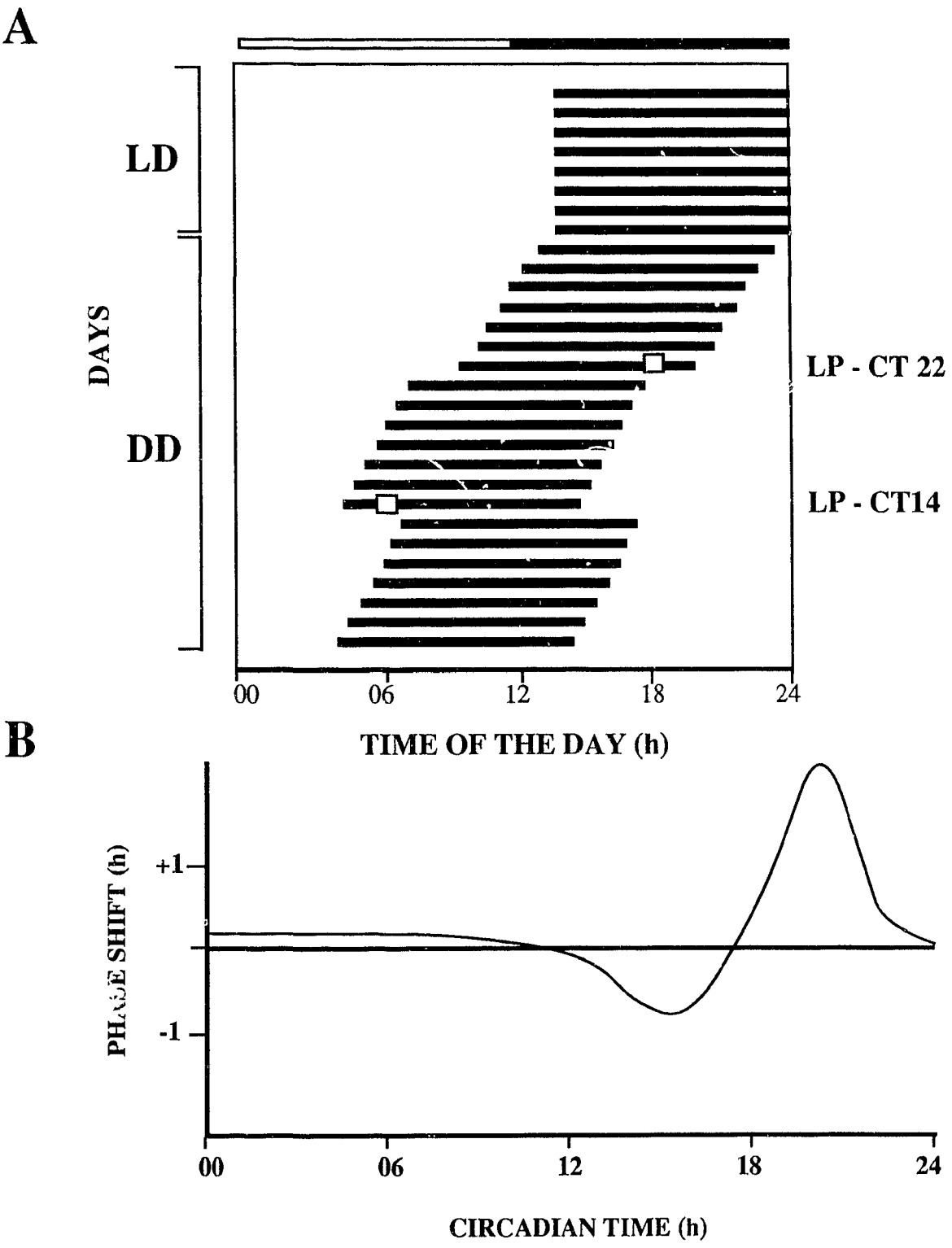

A
LD
DD
DAYS
LP - CT 22
LP - CT14
00
06
12
18
24
TIME OF THE DAY (h)
B
PHASE SHIFT (h)
+1
-1
00
06
12
18
24
CIRCADIAN TIME (h)

Figure 2 Nissl stained sections of the suprachiasmatic nucleus (SCN) of rat (A and B) and hamster (C and D). The top panels represent rostral SCN and the bottom panels represent caudal SCN in rat and hamster. 3V- third ventricle; ox- optic chiasm. Scale bar represents 200 μm.

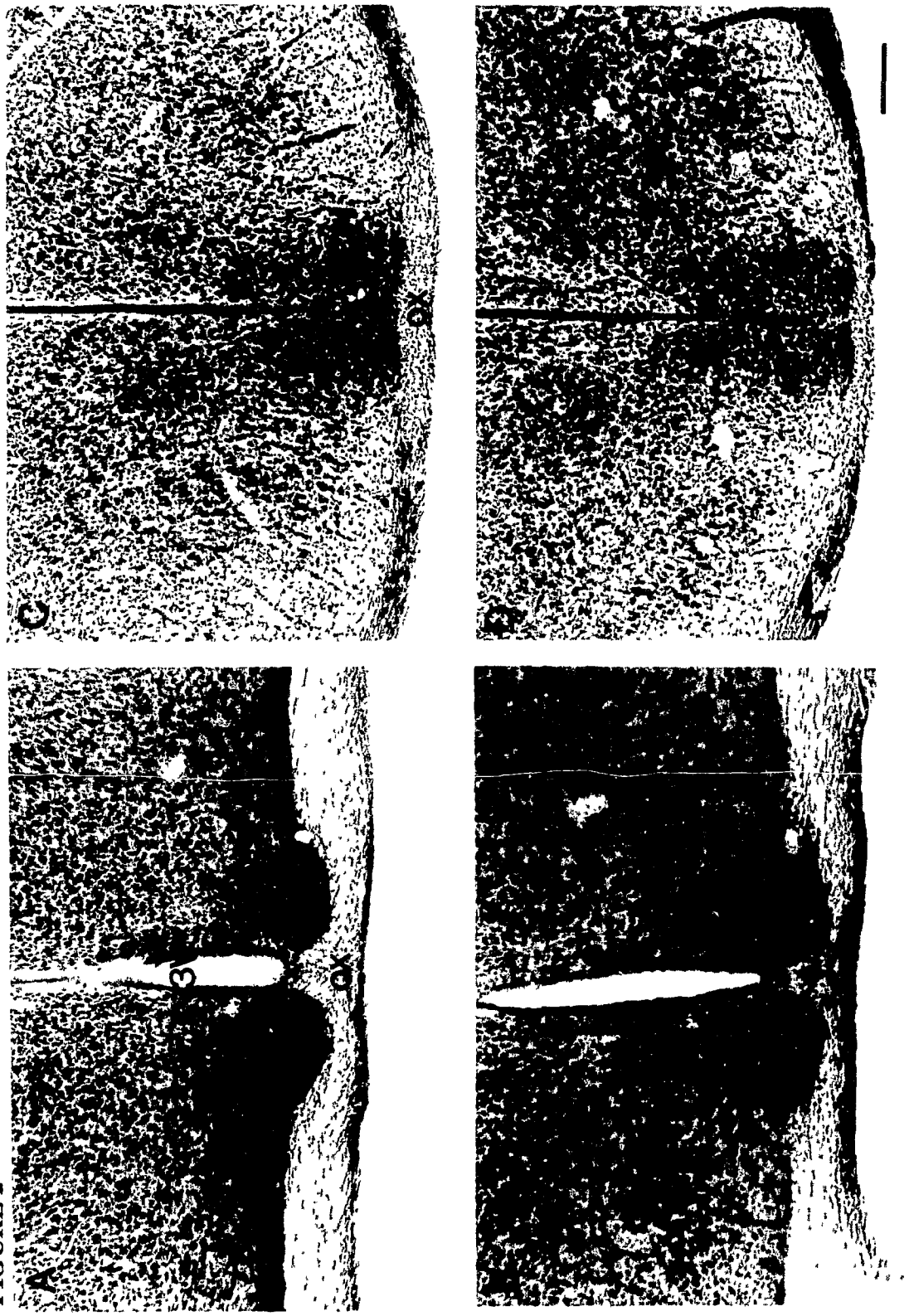


FIGURE 2

Figure 3 Labelling of retinal terminals in the SCN of hamster (A and B) and rat (C and D) following a bilateral injection of the anterograde tracer, choleratoxin, into the eye. Panels A and C represent rostral SCN and B and D represent caudal SCN. Scale bar represents 200 μm.

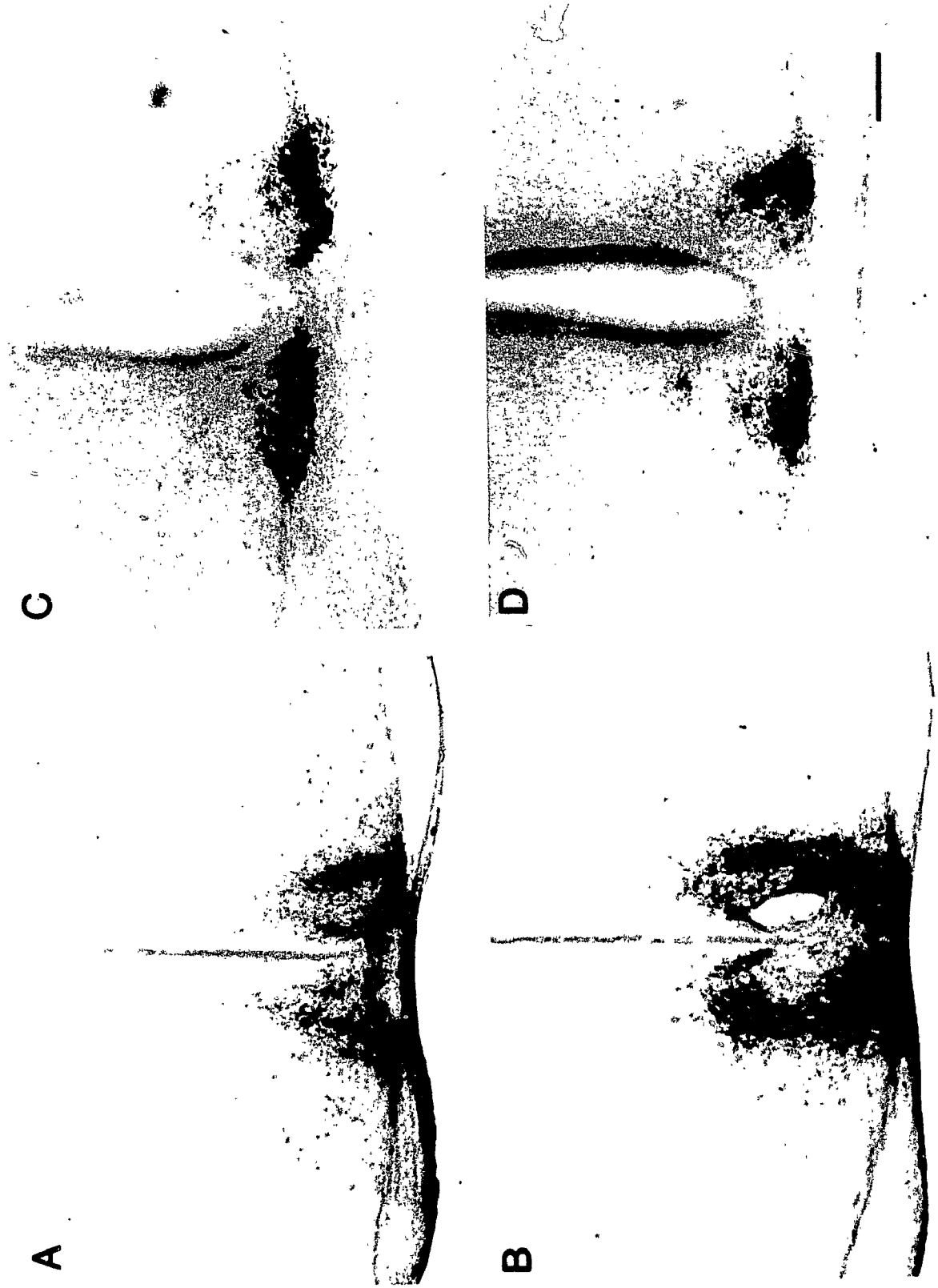
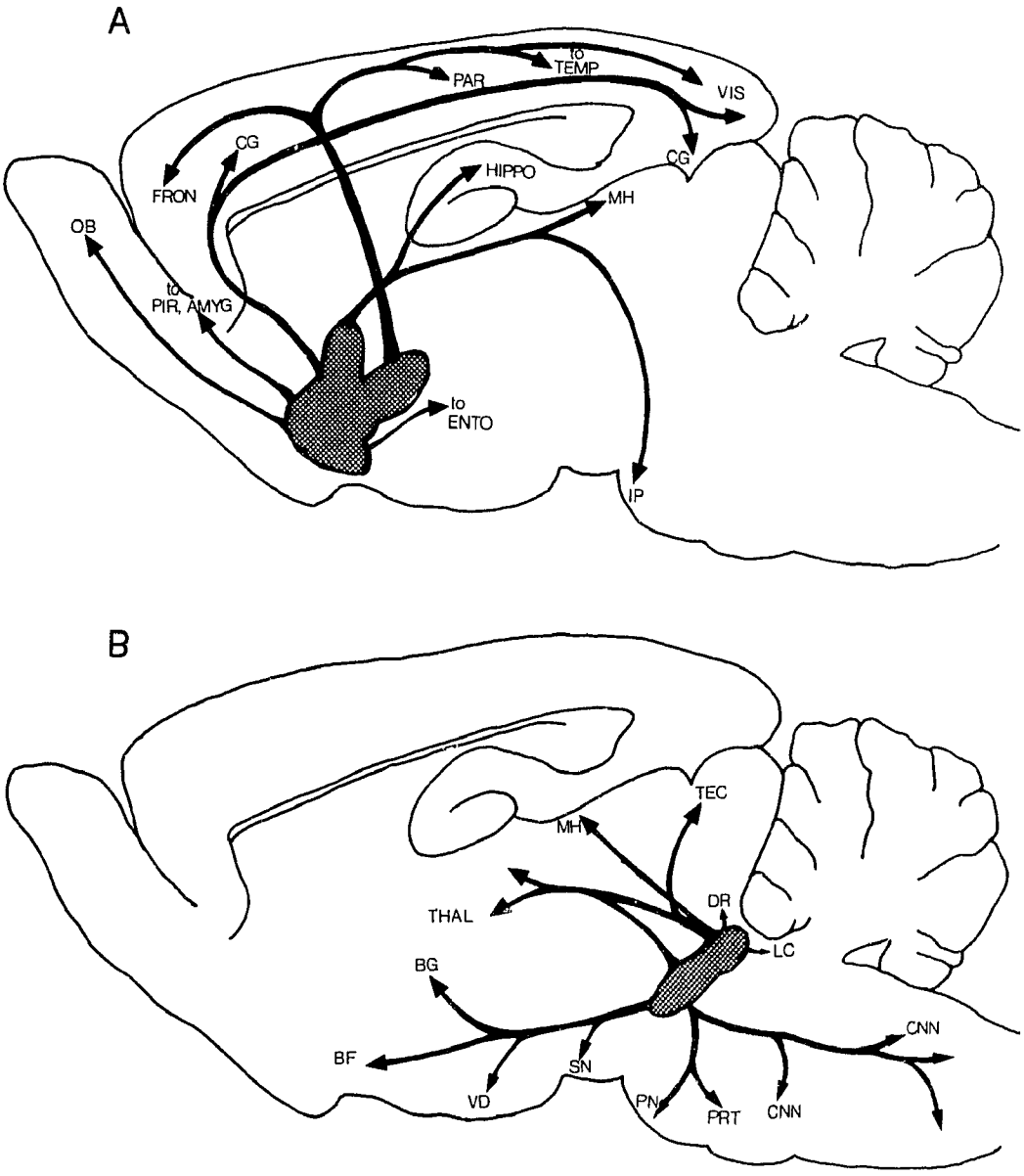


FIGURE 3

Figure 4 Panel A represents efferent pathways of the cholinergic projection neurons in the rat basal forebrain. The shaded area represents the cholinergic neurons located in the medial septum, vertical and horizontal limbs of the diagonal band of Broca, magnocellular preoptic area, substantia innominata and nucleus basalis magnocellularis. Neurons from these areas project to the olfactory bulb (OB), amygdala (AMYG), hippocampus (HIPPO), medial habenula (MH), interpeduncular nucleus (IP), and the cingulate (CG), entorhinal (ENTO), frontal (FRON), parietal (PAR), piriform (PIR), temporal (TEMP), and visual (VIS) cortices.

Panel B represents efferent pathways of the cholinergic projection neurons in the mesopontine tegmentum of the rat. Shaded areas represent cholinergic neurons located in the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT). Neurons from these areas innervate the basal forebrain (BF), basal ganglia (BG), cranial nerve nuclei (CNN), dorsal raphe (DR), locus coeruleus (LC), medial habenula (MH), pontine reticular formation (PRT), substantia nigra (SN), tectum (TEC), thalamus (THAL) and the ventral diencephalon (VD).

FIGURE 4


A
to TEMP
PAR
VIS
CG
CG
FRON
HIPPO
MH
OB
to PIR, AMYG
to ENTO
IP
B
TEC
MH
THAL
DR
LC
BG
CNN
BF
SN
VD
PN
PRT
CNN

CHAPTER II

ANATOMY OF CHOLINERGIC TRANSMISSION IN THE SCN

As described in Chapter I, fibres containing choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine, have been localized in the rat SCN (van den Pol and Tsujimoto, 1985; Ichikawa and Hirata, 1986). In the absence of evidence for ChAT in SCN somata, these fibres can be assumed to derive from afferent projections to the SCN; however, the location of the neurons which contribute these fibres is not known. The SCN receives a projection from cells in the basal forebrain (Pickard, 1982), a region that is known to contain cholinergic neurons. Therefore, this region appeared to be a likely source of the cholinergic projection to the SCN. Consistent with this possibility, Fuller et al (1989) observed that cells in the diagonal band of Broca and in the area immediately lateral to the SCN project to the SCN and also contain acetylcholinesterase (AChE), an enzyme involved in the degradation of ACh.

This chapter will describe two sets of studies carried out in rats and hamsters to clarify the anatomy of cholinergic input to the SCN. The first set of studies carried out in rats involved, first of all, visualization of fibres and fibre terminals immunopositive for ChAT in the SCN using a sensitive technique (Ojima et al., 1988) to confirm the presence of cholinergic fibres in the SCN. Then I used a retrograde tracer, fluorogold, injected into the SCN, in combination with immunohistochemistry for ChAT, to identify cholinergic neurons in the forebrain and brainstem (major brain areas containing cholinergic projection neurons) as afferent sources of these fibres. These projections were further confirmed by injecting an anterograde tracer, biocytin, into the brainstem cholinergic nuclei and visualizing biocytin-labelled fibres and terminals in the SCN.

brainstem cholinergic nuclei. Anterograde studies were not performed in the forebrain because in comparison to the cholinergic neurons in the brainstem, the cholinergic neurons in the forebrain are more diffusely distributed and very large injections spanning, but also restricted to, the entire rostro-caudal extent of the CBC would have been very difficult to produce.

METHODS

Male Sprague Dawley rats weighing 200-250g, obtained from Charles River Canada (St. Constant, Quebec), were housed under a 12:12 light-dark schedule, with free access to food and water.

1. FIBRE LOCALIZATION IN THE SCN

The protocol for perfusion and immunohistochemistry was as described by Ojima et al. (1988). Two rats were deeply anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg) and perfused for a few seconds with 0.1M phosphate buffer (PB; pH 7.4) containing 0.002% calcium chloride followed by an ice cold fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in PB, for three min and then for a further 10 min by 4% paraformaldehyde alone; all perfusions were done at a rate of 10ml/min. The brain was immediately removed, blocked and postfixed in 4% paraformaldehyde at 4°C for 12 hr. The brain was transferred to buffered 30% sucrose at 4°C overnight, rapidly frozen using dry ice and cut on a cryostat into 40μm sections. The sections were collected in 0.1M Tris buffered saline (TBS; pH 7.3), rinsed in three TBS washes of 20 minutes each and incubated in TBS containing 0.1% TritonX-100 for 20 min. The sections were then treated with normal goat serum (NGS) (1:30) in TBS for an hour at room temperature and incubated in a monoclonal antibody against ChAT (1:2.5; Boehringer-Mannheim) with 1:30 NGS and 0.1% TritonX-100, at 37°C on a shaker for 2 days. Subsequently, the sections were rinsed in TBS and incubated for an hour in goat anti-rat IgG (1:50 in TBS containing 1.8% bovine serum albumin [BSA], 1:30 NGS and 0.1% TritonX-100). The

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The second study was carried out in hamsters in order to determine whether cholinergic muscarinic receptors are found in the SCN. Although a number of studies have investigated nicotinic and related receptors in the SCN, few have examined whether muscarinic receptors are present. Given the behavioural results described in chapter IV, it is important to establish whether muscarinic receptors are found in the hamster SCN. This study used a hydrophilic muscarinic ligand N-methyl scopolamine (NMS) and a novel tissue punch technique to permit autoradiographic detection of cell-surface receptors.

The binding studies were conducted in hamsters because all the behavioural studies described in chapters IV and V were performed in hamsters. The tracing and immunohistological studies were conducted using rats because they were done in combination with retrograde labelling of NGF-R containing neurons, in order to study concurrently the source of NGF-R in the SCN. The latter study (chapter III) could not be conducted in hamsters because the monoclonal antibody raised against NGF-R protein in mouse does not recognize the hamster NGF-R protein (unpublished observations). The combined tracing and immunohistological studies could therefore be performed only using rats.

A - LOCALIZATION OF CHOLINERGIC NEURONS PROJECTING TO THE RAT SCN

Three separate sets of experiments were carried out to identify the cholinergic cells which project to the SCN. The first study used a sensitive immunocytochemical method to visualize fibres and terminals expressing ChAT immunoreactivity in the SCN. The second study involved retrograde tracing using fluorogold (Fluorochrome, Englewood, CO) as the tracer, injected into the SCN, and evaluation of both forebrain and brainstem cholinergic regions as potential sources of the cholinergic projection. The third set of studies used biocytin (Sigma Chemical Co., MO) as an anterograde tracer, which was injected into

brainstem cholinergic nuclei. Anterograde studies were not performed in the forebrain because in comparison to the cholinergic neurons in the brainstem, the cholinergic neurons in the forebrain are more diffusely distributed and very large injections spanning, but also restricted to, the entire rostro-caudal extent of the CBC would have been very difficult to produce.

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1. FIBRE LOCALIZATION IN THE SCN

The protocol for perfusion and immunohistochemistry was as described by Ojima et al. (1988). Two rats were deeply anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg) and perfused for a few seconds with 0.1M phosphate buffer (PB; pH 7.4) containing 0.002% calcium chloride followed by an ice cold fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in PB, for three min and then for a further 10 min by 4% paraformaldehyde alone; all perfusions were done at a rate of 10ml/min. The brain was immediately removed, blocked and postfixed in 4% paraformaldehyde at 4°C for 12 hr. The brain was transferred to buffered 30% sucrose at 4°C overnight, rapidly frozen using dry ice and cut on a cryostat into 40µm sections. The sections were collected in 0.1M Tris buffered saline (TBS; pH 7.3), rinsed in three TBS washes of 20 minutes each and incubated in TBS containing 0.1% TritonX-100 for 20 min. The sections were then treated with normal goat serum (NGS) (1:30) in TBS for an hour at room temperature and incubated in a monoclonal antibody against ChAT (1:2.5; Boehringer-Mannheim) with 1:30 NGS and 0.1% TritonX-100, at 37°C on a shaker for 2 days. Subsequently, the sections were rinsed in TBS and incubated for an hour in goat anti-rat IgG (1:50 in TBS containing 1.8% bovine serum albumin [BSA], 1:30 NGS and 0.1% TritonX-100). The

sections were then rinsed, incubated for 1 hr in rat peroxidase-anti-peroxidase complex (at 1:80 in TBS containing 1.8% BSA), rinsed again and incubated in 0.06% diaminobenzidine (DAB) in PBS (0.1M; pH 7.4) for 10 min and subsequently visualized using 0.006% hydrogen peroxide (H_2O_2).

The sections were mounted on gelatinized slides, air-dried, dehydrated through an ascending alcohol series, coverslipped in Permount and viewed through an Olympus microscope.

2. RETROGRADE TRACING

INJECTION AND PERFUSION

Animals were anesthetized with sodium pentobarbital (65 mg/kg; i.p.) and positioned in a Kopf stereotaxic holder with the incisor bar set 5 mm above the interaural line. Unilateral injections of 8% fluorogold (in distilled water) were made into the SCN using a glass microelectrode (outside tip diameter of 25-35 μm) positioned at 1.2 mm anterior and 0.2 mm lateral to bregma and lowered 9.8 mm ventral to the dura. Fluorogold was delivered iontophoretically with a positive current of 2 μA administered over 10 minutes with a duty cycle of 5 seconds on and 5 seconds off. At the end of the injection, the micropipette was kept in place for 5-10 minutes.

After one week survival in their normal lighting cycle, animals were deeply anesthetized with sodium pentobarbital. The descending aorta was clamped and the animal perfused intracardially with 60 ml of 0.9% saline followed by 400 ml of ice cold 4% paraformaldehyde made in 0.1M PB (pH 7.4). Brains were postfixed for 4-5 hrs in 4% paraformaldehyde at 4°C. Coronal sections 50 μm thick were cut from the forebrain and the brainstem on a microslicer. After confirmation of injection sites, every sixth section was processed for immunohistochemistry for ChAT.

IMMUNOHISTOCHEMISTRY

Sections were washed in 0.05 M TBS and then incubated for 48 hours, at 4°C, in a polyclonal antibody raised in rabbit against ChAT (Chemicon International Inc., CA),

diluted to 1:100 in antisera diluent (ASD - 0.3% TritonX-100, 2.0% NGS and 0.01% sodium azide added to 0.05 M TBS). Subsequent to 3 x 20 minute rinses in TBS, the tissue was incubated in 1:50 dilution of Texas red conjugated goat antirabbit IgG (Jackson Immunoresearch Labs. Inc., PA) in TBS and 1% NGS. Sections were mounted on slides, dried overnight, coverslipped with either 1:9 concentration of ethylene diamine (Sigma Chemicals Co., MO) in glycerol or Fluoromount and examined with an Olympus epifluorescence microscope. Fluorogold labelling was examined with an ultraviolet filter cube, and Texas red using the green filter cube. Retrogradely labelled cells which were also labelled with ChAT were mapped and in selected cases photographed.

3. ANTEROGRADE TRACING

Biocytin was used as the anterograde tracer. It is a very effective anterograde tracer which allows the placement of discrete injections as well as visualization of Golgi-like terminal morphology and is transported rapidly without being taken up by fibres of passage (King et al., 1989; Izzo, 1991).

Rats were anesthetized as described above and placed on a Kopf stereotaxic holder with incisor bar 3.3 mm below the interaural line. Injections of 50nl of biocytin (5% in 0.05M Tris buffer, pH 7.6) were made into either the pedunculopontine tegmental nucleus (PPT; coordinates: 7.3 mm posterior to bregma, 1.8 mm lateral to bregma and lowered 5.5 mm ventral to the dura., with the incisor bar placed -3.3 mm below the interaural line), the parabigeminal nucleus (PBg; coordinates: 7.7mm posterior and 2.9mm lateral to bregma and 6.5mm ventral to the skull) or the laterodorsal tegmental nucleus (LDT; coordinates: 8.5 mm posterior and 0.9 lateral to bregma and lowered 6.5 mm ventral to dura) using a glass micropipette (tip diameter 30μm) attached to a 1μl Hamilton syringe. The injections were made over a period of 10 minutes and the pipette was left in place for a further 5 minutes following the injection.

After survival periods ranging from 12 to 20 hours, the animals were given a lethal dose of pentobarbital and perfused transcardially with an initial rinse of 0.9% physiological

saline followed by a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1M PB (pH 7.4). Brains were rapidly removed and postfixed overnight in 4% paraformaldehyde. Coronal sections of the forebrain 50 μm thick containing the SCN region and the brainstem (to localize the injection site) were cut on a microslicer. The sections were thoroughly washed in TBS (pH 7.4) and processed for histochemistry to visualize biocytin.

HISTOCHEMISTRY

Free floating sections were incubated in 0.5% hydrogen peroxide for 30 min in order to decrease the background labelling. Sections were washed in TBS for half an hour and subsequently preincubated in 1% TritonX-100 in TBS for 60 min followed by incubation in Extravidin-peroxidase conjugate (Sigma Chemicals Co., MO) at a concentration of 1:20 for 2 hr. The reaction product was visualized by preincubating the tissue in diaminobenzidine (DAB; 0.5mg/ml) for 5 min followed by incubation in a mixture of DAB and hydrogen peroxide (0.5 ml /ml of a 30% solution) for about 10 min or until the brown reaction product was visible.

Since neurons in the brainstem that are cholinergic have been shown to be dihydro nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase positive (Vincent et al., 1983), the tissue was reacted for NADPH diaphorase histochemistry to determine whether the biocytin injection site in the brainstem overlapped with cholinergic neurons.

NADPH DIAPHORASE HISTOCHEMISTRY

Following biocytin histochemistry the brainstem sections were processed for NADPH diaphorase histochemistry (Scherer-Singler et al., 1983). The sections were incubated in a mixture of β-NADPH (Sigma Chemical Co., MO; 10mg/10ml) and nitroblue tetrazolium (NBT; Sigma Chemical Co., MO; 1mg/10 ml) in TB (pH8.0) at 37°C for 5-10 min. This reaction was performed in the dark, since NBT is light sensitive.

Sections were mounted on gelatinized slides, air dried, dehydrated in an ascending series of alcohols and coverslipped in Permount. The sections were viewed through an Olympus microscope and in selected cases photographed.

RESULTS

ChAT-IMMUNOREACTIVE FIBRES IN THE SCN

Scattered ChAT immunoreactive fibres and terminals were found in the entire rostro-caudal extent of the SCN. Most of the fibre labelling was localized to the ventral SCN, but a few fibres were also found in the dorsal SCN. Two examples of cholinergic fibres in the SCN are shown in Figure 5A and B.

CHOLINERGIC NEURONS RETROGRADELY LABELLED WITH FLUOROGOLD

Injections sites

For the forebrain study, of the 12 animals injected, three had fluorogold injections localized in the SCN. All three injection sites were unilateral with some diffusion into the contralateral SCN. An example of a fluorogold injection site is shown in Figure 6A; the retrogradely labelled cholinergic neurons in this case are mapped in Figure 7. Two other animals used in the brainstem study, had their injection sites localized in the SCN; both of these injections were bilateral. The injection site for the animal whose retrogradely labelled cholinergic neurons are mapped in Figure 9 is shown in Figure 6B.

Forebrain

Retrogradely labelled cells that were also ChAT immunoreactive were observed in the entire rostro-caudal extent of the CBC, but there were regional differences in the density of labelled cells (Figure 7). The largest number of ChAT positive neurons that were retrogradely labelled from the SCN were located in the substantia innominata (SI) and nucleus basalis magnocellularis (NBM) of the CBC (Figure 7, panels F and G). Scattered double-labelled cells were localized in the medial septum and the vertical and horizontal limbs of the diagonal band of Broca. Figure 8 shows an example of a neuron located in the SI region of the CBC that was retrogradely labelled with fluorogold (8A) and immunopositive for ChAT (8B).

Consistent with previous reports (Pickard, 1982) neurons that were not ChAT immunoreactive but labelled with fluorogold were found in the lateral septum, the intergeniculate leaflet (IGL) of the LGN, and the zona incerta.

Brainstem

Figure 9 illustrates the localization of retrogradely labelled neurons in the brainstem, distributed in three distinct brainstem nuclei containing cholinergic neurons, namely the PPT, PBg, and the LDT. Neurons that were retrogradely labelled with fluorogold and were also ChAT positive, were seen in the PPT (Figure 10A and D), LDT (Figure 10B and E) and PBg (Figure 10C and F). Many neurons singly labelled with fluorogold were also present in these nuclei.

Apart from these three nuclei, scattered retrogradely labelled neurons were also found in dorsal and median raphe nuclei, superior colliculus, dorsal tegmental nucleus and parabrachial nucleus. No double-labelled neurons were observed in any of these nuclei.

ANTEROGRADE LABELLING WITH BIOCYTIN

Biocytin injections directed at PPT, PBg and LDT were made in two animals each. Panels A-C of Figure 11 show examples of biocytin injections placed in PPT, PBg and LDT respectively. The location of the injection sites overlapped with NADPH diaphorase positive (thus cholinergic) neurons in the PPT and LDT (not shown). The PBg neurons are not NADPH diaphorase positive, therefore anatomical landmarks were used to determine the location of the injection site.

Scattered anterogradely labelled fibres and terminals were detected in the SCN after injections into PPT, (Figure 11D), PBg (Figure 11E) and LDT (Figure 11F). In each case, these fibres were found throughout the entire rostro-caudal extent of the SCN. Following injections into PBg and PPT, fine fibres could be seen entering the SCN through the optic chiasm. In the case of PPT injections, larger fibres could also be seen coursing through the optic chiasm at the level of caudal SCN. Following both PPT and LDT injections,

extensive fibre labelling was observed in the basal forebrain area. In the case of PPT injections, few scattered fibres were also observed in the lateral hypothalamus.

DISCUSSION

ChAT immunoreactive fibres were detected in the SCN, suggesting the existence of cholinergic input into the SCN. These results are consistent with previous findings (van den Pol and Tsujimoto, 1985; Ichikawa and Hirata, 1986). Ichikawa and Hirata (1986) observed very dense ChAT positive fibres in the ventromedial aspect of the SCN with fine mesh-like ChAT positive fibres distributed elsewhere within the SCN. In the present study, although we observed many fibres in the ventromedial aspects of the SCN we did not find the dense labelling observed by Ichikawa and Hirata (1986). This disparity in the findings may be due to the different sources of antibodies in the two studies.

Cells retrogradely labelled from the SCN were found in both the forebrain and the brainstem. In the forebrain, cells labelled with fluorogold and immunopositive for ChAT were found in the entire rostro-caudal extent of the CBC with highest densities occurring in the caudal nuclei, including the NBM and SI. Non-cholinergic retrogradely labelled cells were observed in the LGN, lateral septum and zona incerta. Although no retrogradely labelled cholinergic cells were found in the lateral septum in the present study, a small cluster of weakly ChAT immunoreactive neurons has been localized in the lateral septum (Kimura et al., 1991).

Retrogradely labelled cells projecting to the SCN have been reported in hamsters using free HRP as a retrograde tracer (Pickard, 1982). That study reported cells labelled with HRP in lateral septum, MS and the vertical limb of DBB. No cells were found in the horizontal limb of DBB, magnocellular preoptic area (MgPA) or any of the caudal nuclei of the CBC. In the present study, however, many cells were found not only in the MS but also throughout the CBC. These differences between the two studies may be due to the type of tracer used. Fluorogold has been shown to be superior to HRP as a retrograde

tracer, and its sensitivity is comparable to that of WGA-HRP (Schmued and Fallon, 1986). It has also been reported that fluorogold is not taken up by undamaged fibres of passage, and that there is little or no transynaptic labelling, since long survival times do not result in increased cell labelling (Schmued and Fallon, 1986). Another possible explanation for the disparity between the two studies is species differences, since the HRP study was done using Syrian hamsters rather than rats.

A cholinergic projection to the rat SCN has been described previously (Fuller and Murakami, 1989). This study used AChE histochemistry as a marker for cholinergic cells and reported double labelled cells in the DBB and an area lateral to the SCN (referred to in the study as "lateral SCN"). In the present study, however, no retrogradely labelled cells or ChAT labelled cells were found in the area lateral to the SCN. These differences may reflect the fact that AChE histochemistry may label a variety of esterases and it is therefore not always a reliable marker for cholinergic neurons. For example, a comparison between AChE histochemistry and ChAT immunocytochemistry has revealed cells in the lateral hypothalamic area labelled by AChE and not by ChAT (Satoh et al., 1983). These cells are non-cholinergic and at least some are believed to be dynorphin-positive cells because they are similar to such cells in their morphology and anatomical distribution patterns (Vincent et al., 1982). In addition, noradrenergic neurons in the locus coeruleus are labelled by AChE but not by ChAT (Satoh et al., 1983).

Another study has reported a cholinergic projection to the hamster SCN using a polyclonal antibody raised against ChAT as the marker for cholinergic neurons and rhodamine-conjugated latex microspheres as the retrograde tracer (Dwyer et al., 1990). That study also reported retrogradely labelled ChAT positive neurons in the area lateral to the SCN (referred to as "lateral SCN" also in this study). We were unable to detect any ChAT positive cells in the area lateral to the SCN. Species differences between our study and theirs may also account for this difference; however, the similarity to the results of Fuller and Murakami (1989) in rats suggests an alternative explanation. It is possible that a

population of cholinergic neurons exists in the area lateral to the SCN, consisting of cells that are weakly ChAT positive and AChE positive, and that these cells were not detectable with the procedures employed in the present study.

In the brainstem, retrogradely labelled cholinergic neurons were localized in three distinct nuclei, namely the PPT, PBg and LDT. As previously reported (Tago et al., 1987) the PPT and LDT contain strongly ChAT positive neurons, whereas neurons in the PBg are only weakly ChAT immunoreactive. Previous studies do not report fibre labelling in the SCN following injections of the anterograde tracers WGA-HRP or PHA-L into the LDT (Satoh and Fibiger, 1986; Cornwall et al., 1990), whereas following the injection of PHA-L into the PPT, labelled fibres are present in the SCN (Hallanger and Wainer, 1988). Although not noted by the authors of the latter study, fibres reaching the SCN are evident in one of their figures (Figure 3D of Hallanger and Wainer, 1988).

Retrogradely labelled neurons in the PBg have been visualized in the cat following HRP injections into the optic chiasm with diffusion of the tracer into the SCN (Baleydier and Magnin, 1979). The PBg is known to innervate the contralateral superior colliculus (Mufson et al., 1986) through a long loop which ascends along the optic tract, crosses the midline in Gudden's supraoptic commissure and returns along the contralateral optic tract (Graybiel, 1978). It is most likely that some of these fibres reach the SCN at the most rostral level of this loop. It is, however, also possible that the labelling in the PBg in the present study is due to a leakage of the tracer into the optic tract. This is suggested by the observation of a few retrogradely labelled cells in the superior colliculus. Since the superior colliculus projects to the lateral posterior nucleus of the thalamus through the supraoptic commissure, the observation of retrogradely labelled cells in the superior colliculus suggests that there was some leakage of the tracer into the supraoptic commissure. Therefore some of the PBg neurons labelled by fluorogold may be the neurons projecting to the superior colliculus via the optic tracts.

Following HRP injections into the SCN of hamsters, brainstem labelling was observed in the ventral and dorsal tegmental area (Pickard, 1982). Dwyer et al. (1990), on the other hand, have reported cholinergic cells labelled with rhodamine microspheres in the PBg, dorsal tegmental nucleus and the parabrachial nucleus in the hamster. They did not observe any double labelling in the LDT. However, it is likely that the retrogradely labelled cholinergic neurons reported to be in the dorsal tegmental nucleus were actually neurons located in the adjoining LDT. Cholinergic neurons have not been described previously in the dorsal tegmental nucleus of rats, but it remains possible that such cells do exist in hamsters.

Anterogradely labelled fibres were observed in the SCN following biocytin injections into PPT, PBg and LDT. These results confirm that the retrogradely labelled neurons observed in the brainstem following fluorogold injections into the SCN were not entirely due to the tracer being taken up by fibres of passage. With all three injections, thin biocytin labelled fibres were observed in the entire rostro-caudal extent of the SCN with the highest density of fibres being found in the ventral SCN. Observation of labelled fibres in the basal forebrain area in the cases of PPT and LDT injections and in the lateral hypothalamus in the case of PPT injections are consistent with previous findings (Satoh and Fibiger, 1986; Hallanger and Wainer, 1988; Cornwall et al., 1990).

As described in chapter I, cholinergic neurons in the CBC and in the brainstem have been implicated in the regulation of sleep and arousal. In addition, carbachol has been shown to produce phase shifts in circadian rhythms in rodents. These findings and the present results taken together suggest that ACh released from the cholinergic neurons in the forebrain and brainstem may produce phase shifts of activity rhythms reflecting the resetting of the clock. The basal forebrain area containing the cholinergic neurons has been shown to receive retinal terminals in both hamsters and rats (Youngstrom et al., 1991; K.G.Bina, unpublished observations). Thus, it is possible that photic input reaching the CBC activates these cholinergic cells resulting in the release of ACh in the SCN. This ACh

could then either act directly on pacemaker cells, or indirectly through interneurons that make synaptic connections with pacemaker cells, to alter the "clock" mechanisms thereby causing a phase shift of rhythms in sleep and wakefulness. The new phase of the clock is transmitted to the forebrain, brainstem and thalamic nuclei involved in the generation and maintenance of sleep and arousal and to autonomic nuclei via efferent connections from the SCN. This model proposes a feedback loop whereby the areas responsible for sleep and wakefulness are linked to the area responsible for timing these events.

One missing link to this hypothesis is that it is not known whether or not the retinal fibres entering the basal forebrain terminate on cholinergic neurons. In addition, there are no direct efferent projections from the SCN to the CBC, to the aminergic brainstem nuclei involved in sleep and arousal, namely the dorsal raphe and the locus coeruleus, or to any of the autonomic nuclei. Indirect and amplified connections to these nuclei made via the paraventricular nucleus of the hypothalamus (PVN), the major target of the SCN efferents, do however, remain a possibility.

B- CHOLINERGIC RECEPTORS IN THE HAMSTER SCN

METHOD

Male Syrian hamsters (*Mesocricetus auratus*), weighing 80-120 g, obtained from Charles River Canada (St. Constant, Quebec) were maintained on a 14:10 light-dark cycle with free access to food and water. N-methyl-^{3}H scopolamine methyl chloride ([^{3}H] NMS; 80-87 Ci/mmol) was obtained from New England Nuclear (NEN, Boston, MA). All other chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO).

Each assay was started five hours after the onset of light. Hamsters were decapitated and their brains rapidly removed and placed in ice-cold Dulbecco's PBS (Gibco, Grand Island, NY). Blocks of hypothalamus (containing the SCN) and occipital cortex (including visual cortex areas 17 and 18), five mm in size were sectioned (400μm) on a tissue

chopper. Using a stainless steel tube, punches 1mm in diameter were made under a dissecting microscope from the SCN area and from the visual cortex. The punches were then placed in separate wells containing 0.5 ml of Dulbeccos PBS for binding assay.

BINDING STUDIES

Time Course: Punches were divided into six groups of 10 each. Four of the punches in each group were treated with 20µl of 10^{-5}M atropine sulphate for determining non-specific binding. Following this, all the ten punches in each group, including the ones already incubating in atropine sulphate, were incubated in 540 µl of 1nM [^{3}H]NMS, a hydrophilic muscarinic receptor ligand, in PBS for 20, 30, 40, 60, 80 or 120 min at 30°C to determine total binding. Following the incubation, 20µl of the buffer was removed for determination of free ligand concentration. The punches were then washed twice in buffer for five minutes each and placed in counting vials containing Formula 963 (NEN) and counted on a LKB 1218 Rackbeta scintillation counter (efficiency: 41.5%). Specific binding was calculated by subtracting non-specific binding from total binding. Brain punches were weighed to determine the mean wet weight. Specific binding was then expressed as femtomoles (fmol) bound/mg protein (Lowry et al., 1951; Wilkinson et al., 1989).

Competition Curve: Carbachol, an agonist of cholinergic receptors, atropine sulphate, an antagonist of muscarinic receptors, pirenzepine, an M1 specific muscarinic antagonist, and methoctramine, an M2 specific muscarinic antagonist were used as displacers. Hypothalamic punches were divided into four groups of 14 each. Each group was subjected to varying concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}M) of one of the displacers or no displacer , just prior to incubation in 1nM [^{3}H]NMS for 2hrs at 30°C. Maximal binding of [^{3}H]NMS in the punches exposed to no displacers was considered as 100% specific binding. The IC_{50} (the concentration of displacer needed to displace 50% of specific binding) for each of the displacers was determined.

Binding Curve: Binding or saturation curves for [^{3}H]NMS in both hypothalamus and visual cortex were determined using varying ligand concentrations (0.5, 1, 2, 4 and 8 nM). Atropine sulphate was used as the displacer and the punches were incubated for 2 hr. Eadie-Hofstee plots of the binding curve were generated to determine the affinity (Kd) of the ligand for the receptor and the receptor density at saturation.

AUTORADIOGRAPHY

Hamsters were rapidly sacrificed by decapitation. The brains were removed and placed in ice-cold Dulbecco's PBS. Brain sections, 30 µm thick, were cut at the level of SCN, on a cryostat and collected on gelatinized glass slides. The glass slides containing the sections were preincubated with 2nM [^{3}H]NMS for 2hr at 30°C. Some of the sections were preincubated in 10^{-5}nM atropine sulphate, pirenzepine or methoctramine before incubation in [^{3}H]NMS. The glass slides were exposed to LKB Ultrofilm for 2 weeks in the dark. The film was developed, viewed under the microscope and in selected cases photographed. Alternate sections were stained for Nissl substance using Cresyl violet to determine the location of SCN.

RESULTS

As shown in Figure 12A, the time course experiment conducted at 30°C showed that [^{3}H]NMS binding initially increased with increasing length of incubation. Binding reached an equilibrium after 2 hr of incubation with no further increase in the number of bound receptors thereafter. Two rinses of five minutes each produced high specific to non-specific binding ratios. Additional washes did not decrease the specific binding significantly. Thus, in all subsequent characterization experiments, the procedure of incubation for 2 hrs at 30°C with two 5 min post-incubation rinses was used.

Competition curves generated using various displacers showed that, of the drugs tested, carbachol is the least potent competitor for muscarinic binding sites, with an IC_{50} of 10^{-4}M (Figure 12B). Pirenzepine and methoctramine, M_1 and M_2 specific antagonists

respectively, have similar IC_{50}s, suggesting that both types of receptor exist in the hypothalamus. Atropine sulphate was the most potent displacer of [^{3}H]NMS with an IC_{50} of 10^{-8}M. In all subsequent experiments 10^{-5}M atropine sulphate was used as a displacer.

Saturation binding performed five hours into the light phase showed that increasing concentrations of ligand (0.5 to 4nM) increased binding saturably, with maximal binding occurring when a concentration of 2nM was used in both hypothalamus and cortex (Figure 13A). Maximal binding (B_{max}) in the hypothalamus was 835 ± 189 fmol/mg protein and that of cortex was 2650 ± 250 fmol/mg wet weight. The K_d values for hypothalamus and cortex were 0.5 and 1.75 nM respectively (Figure 13B).

A comparison between the Nissl stained sections and the autoradiograms revealed that muscarinic receptors are present in low densities in the ventrolateral aspects of SCN (Figure 14A) as well as in the adjoining hypothalamus. On the other hand, very high densities of labelling were seen in the striatum, a finding consistent with earlier published studies (Mash and Potter, 1986; Spencer et al., 1986). Inhibition of NMS binding by both pirenzepine and methoctramine suggests that both M1 and M2 subtypes of muscarinic receptors exist in the SCN (Figure 14B and 14C) and in the adjacent areas of the hypothalamus.

DISCUSSION

These receptor binding data, obtained using a combination of micropunches and a hydrophilic muscarinic ligand, reveal that the hypothalamic area immediately dorsal to the chiasm, including the SCN, contains muscarinic receptors. Previous studies using binding methods to demonstrate muscarinic receptors were performed on homogenized tissue (Por and Bondy, 1981; Kafka et al., 1981; Mash et al., 1985). In such broken cell preparations, not just the surface receptors but internal receptors in the process of being synthesized, as well as unavailable internalized mature receptors are quantified. For the purpose of studying receptor mediated physiological events, however, binding performed

on intact cells, which reveals only cell surface receptors, serves as a useful tool (Wilkinson et al., 1989). In the present study, the use of micropunches retained the cellular integrity, thereby exposing mainly the surface receptors to the hydrophilic ligand [^{3}H]NMS.

A second means of effecting visualization of cell surface receptors is through the use of a ligand that will not cross the cell membrane. Earlier studies used the lipophilic ligand [^{3}H]QNB which crosses cell membranes and thus labels both surface receptors and internalized receptors (Lee et al., 1986; van Huizen et al., 1989). [^{3}H]NMS, on the other hand, is hydrophilic and does not pass through the cell membrane. Thus, a combination of micropunches and the use of a hydrophilic ligand allows the detection of only the cell surface receptors which represent the available receptors for mediating physiological functions.

Binding of [^{3}H]NMS in the hypothalamic tissue of hamster brain was characterized for use in the autoradiographic study with cortical tissue serving as a control. The B_{max} value obtained for cortical tissue is higher than the values obtained by van Huizen et al. (1989) for rat brain slices or by others in homogenized rat tissue (El-Fakahany and Lee, 1986; Lee et al., 1986). This difference may have been due to the species differences or type of assay used. It is likely that intact 'living' tissue kept in physiological conditions will bind more ligand molecules than homogenized tissue lacking dendrites and afferent terminals. There were large differences in the B_{max} and K_d values between the hypothalamic and cortical punches. The high B_{max} in the cortex suggests that there are more than twice as many available muscarinic receptors in the cortex than in the hypothalamus. The K_d value for binding in the hypothalamus is different from that in the cortex, suggesting that different classes of receptors exist in these areas. Higher K_d values in the cortex compared to the hypothalamus suggest that [^{3}H]NMS binds with three times as much affinity in the hypothalamus as in the cortex. Thus the hypothalamus has high-affinity, low-capacity receptors compared with the cortex.

Evidence from the competition curves suggests that both M1 and M2 subtypes of muscarinic receptor exist in the hypothalamus. This conclusion is also consistent with the present autoradiographic findings in the SCN.

Electrophysiological studies both *in vivo* and *in vitro* have demonstrated cholinoceptive neurons in the SCN (Nishino and Koizumi, 1977; Kow and Pfaff, 1984). As described in chapter I, both nicotinic and muscarinic receptors have been localized in the SCN using antibodies raised against their respective receptor proteins (van der Zee et al., 1989; van der Zee et al., 1991). Although reports have described transport of newly synthesized acetylcholine receptors from the retina to the tectum (Henley et al., 1986) and of nicotinic receptors from the retina to all major terminal fields of retinal ganglion cells (Swanson et al., 1987), the only target of the retinal ganglion cells that did not show immunoreactivity to such transported nicotinic receptors was the SCN. This suggests that nicotinic receptors do not exist on retinal terminals in the SCN. In addition, autoradiography for radiolabelled nicotine has revealed an absence of binding in the SCN (Clarke et al., 1985) suggesting that terminals or cells located in the SCN do not contain nicotinic receptors. It has not been determined, however, whether muscarinic receptors are transported from the retina to the SCN, so the possibility remains that the transport of this receptor type takes place. Taken together, the present results and previous findings suggest that the SCN contains cholinoceptive neurons with muscarinic receptors and it is possible, although not proven, that some of these muscarinic receptors are located presynaptically on retinal terminals. However, the existence of a new subtype of nicotinic receptor unrecognized by hitherto available ligands and antibodies cannot be ruled out.

These studies and previously published results indicate that cholinergic cells located in several defined nuclei in the cholinergic basal forebrain and in the PPT, PBg and LDT of the brainstem project into and terminate throughout the SCN. There are populations of both M1 and M2 muscarinic receptors in the SCN on which ACh released from these

terminals may act. The cellular components on which these receptors are localized have yet to be identified.

Figure 5 Photomicrographs of fibres (arrows) in the SCN immunoreactive for choline acetyltransferse (ChAT). Scale bar represents 50 μm.

FIGURE 5

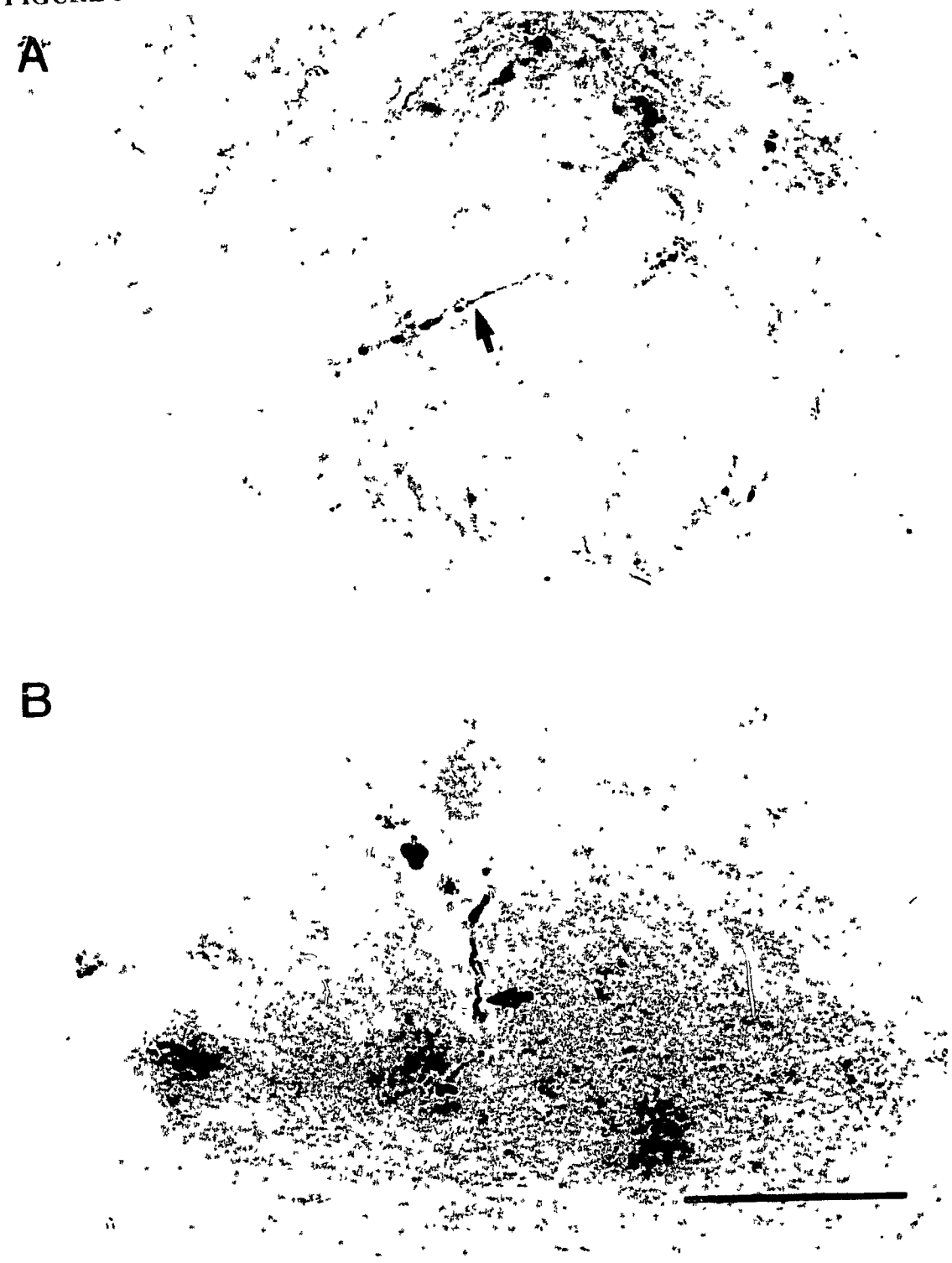


Figure 6 Panels A and B show fluorogold injection sites in rats whose retrograde labelling data are mapped in the forebrain (Figure 7) and the brainstem (Figure 9) respectively. ox- optic chiasm. Scale bar represents 200 microns.

FIGURE 6

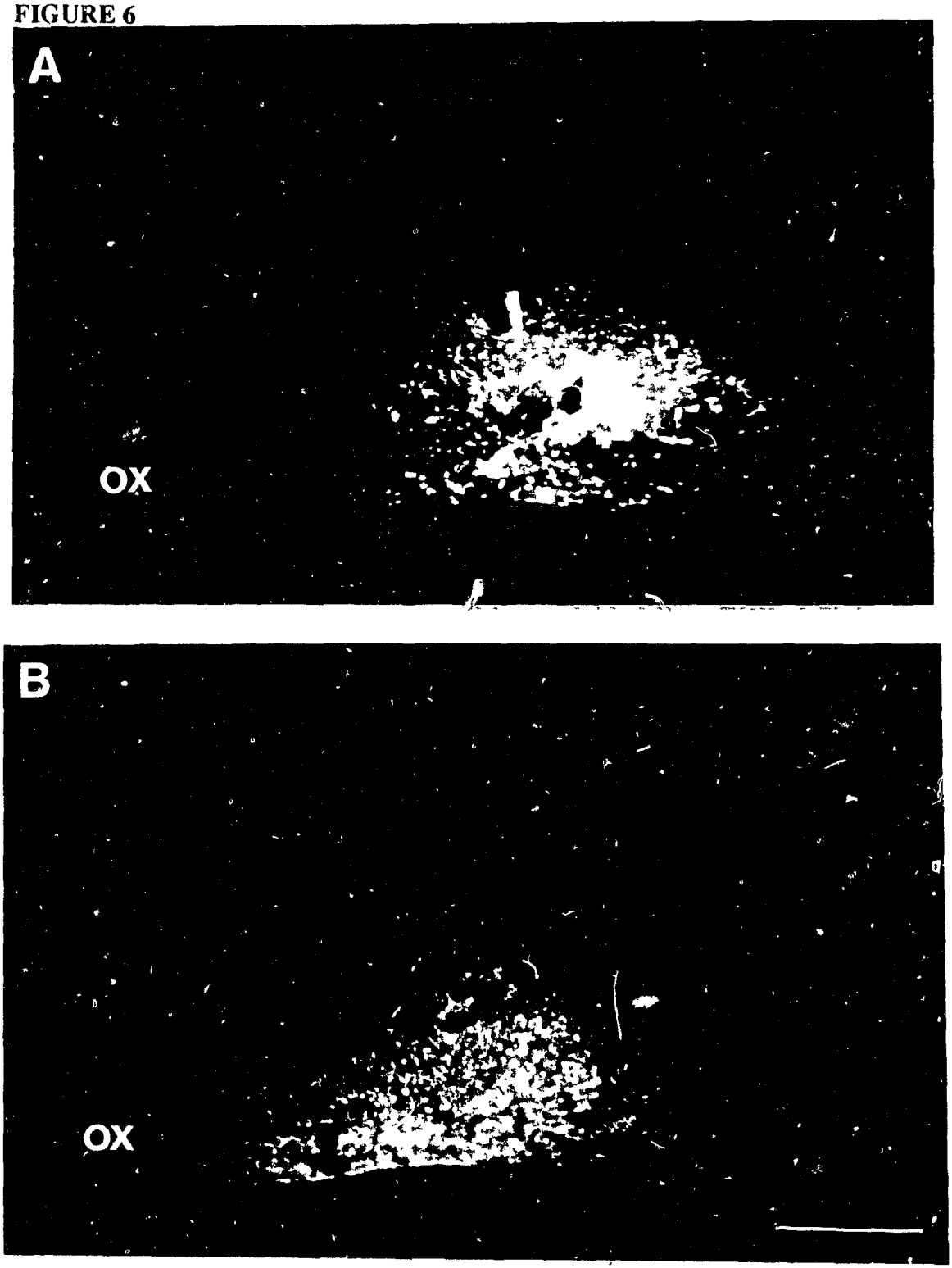
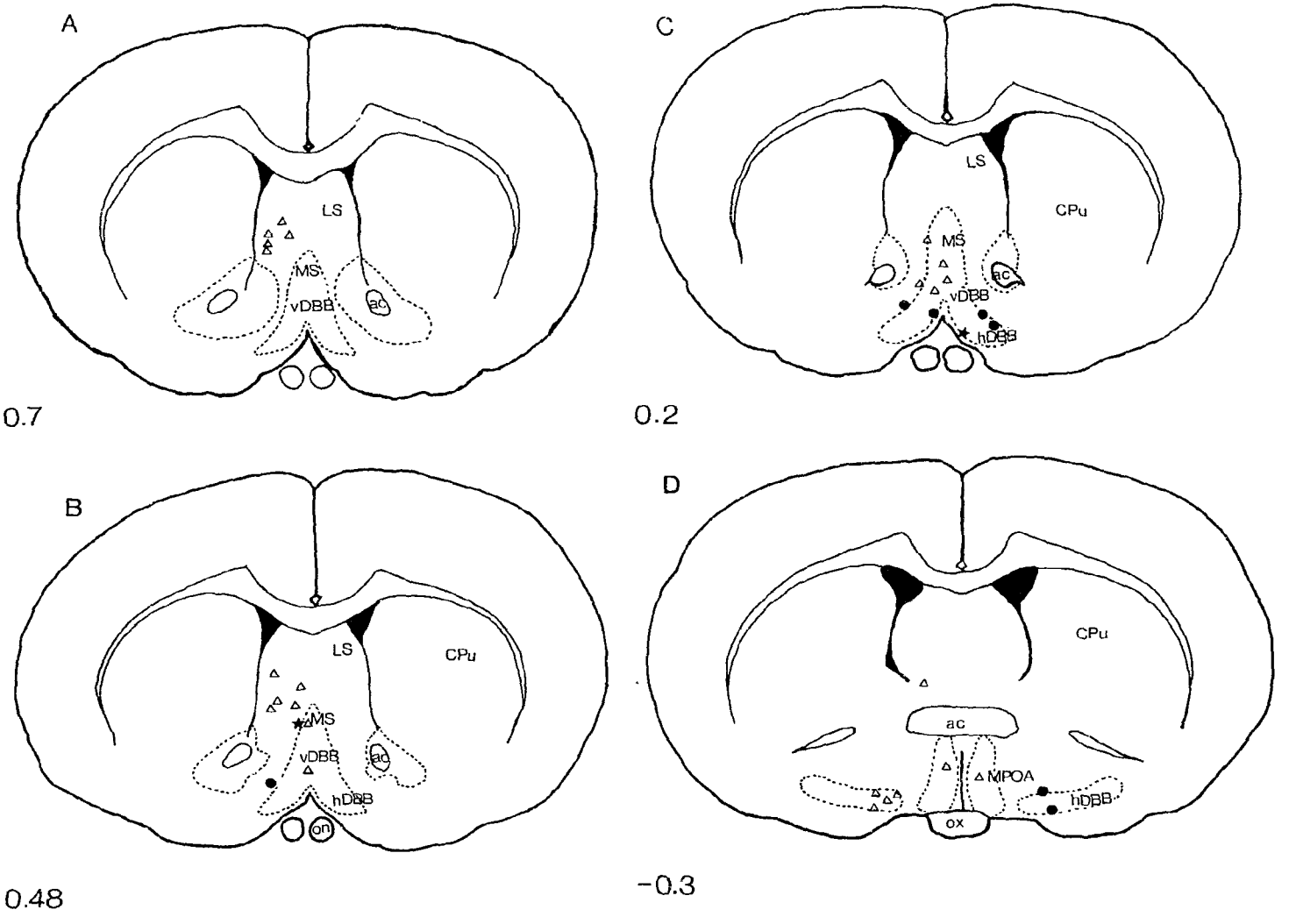


Figure 7 Mapping of cells in the forebrain that are either singly labelled with the retrograde tracer, fluorogold, injected into the SCN, or are labelled with the tracer and are immunoreactive for NGF-R and/or ChAT. Open triangles represent cells that are retrogradely filled with fluorogold. Closed circles represent cells triple labelled with fluorogold, ChAT and NGF-R. Stars represent cells that are labelled with fluorogold and NGF-R alone. Shaded area represents the core of the injection site in the SCN. For full form of abbreviations refer to the list of abbreviations. Numbers on left of individual sections indicate distances in mm from bregma

FIGURE 7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LS
MS
vDBB
ac
0.7
C
LS
CPu
MS
ac
vDBB
hDBB
0.2
B
LS
CPu
MS
vDBB
ac
hDBB
on
0.48
D
CPu
ac
MPOA
hDBB
ox
-0.3

FIGURE 7

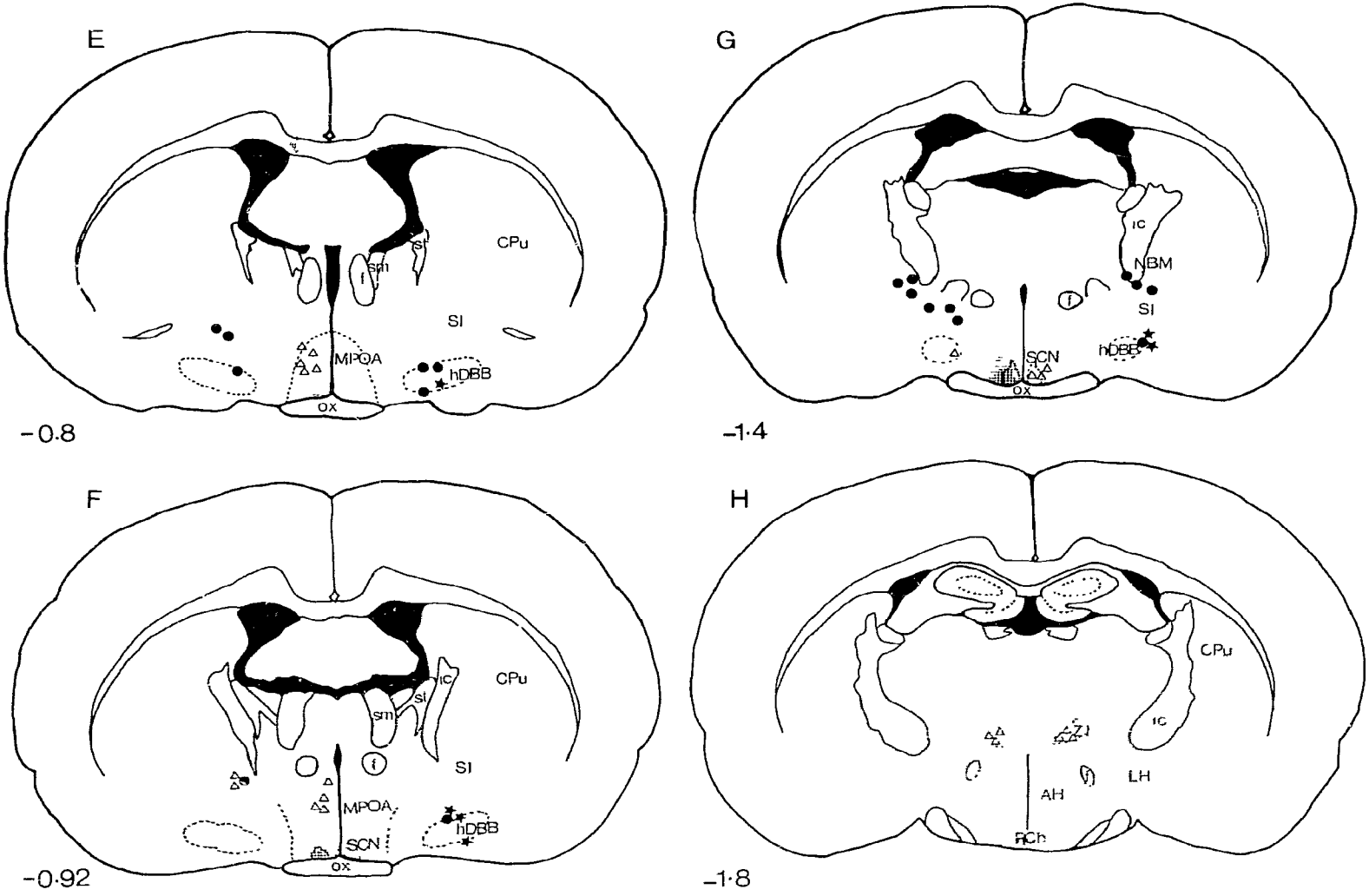

E
CPu
sm
sl
f
SI
MPOA
hDBB
ox
-0.8
G
ic
NBM
SI
f
hDBB
SCN
ox
-1·4
F
CPu
ic
sm
sl
f
SI
MPOA
hDBB
SCN
ox
-0.92
H
CPu
ic
AH
LH
PCh
-1·8

Figure 8 A triple labelled cell in the substantia innominata region of the basal forebrain after injection of the retrograde tracer, fluorogold, into the SCN and immunohistochemistry for NGF-R and ChAT. The cell (arrow) is labelled with fluorogold (A), Texas-red tagged ChAT (B) and FITC tagged NGF-R (C). Most cells in the basal forebrain that are ChAT immunopositive are also NGF-R positive. Scale bar represents 100 μm.

FIGURE 8

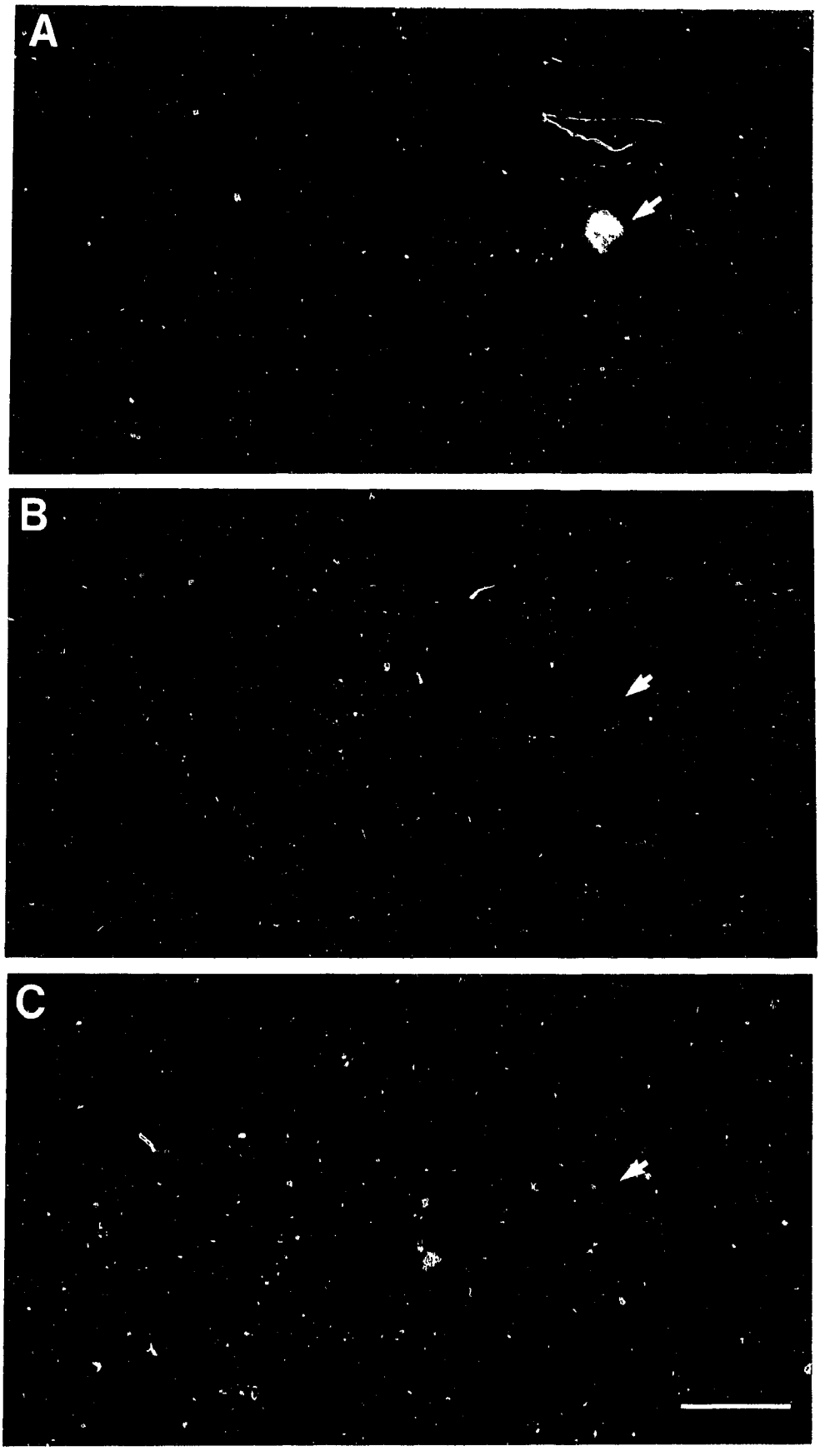


Figure 9 Mapping of neurons in the brainstem retrogradely labelled with fluorogold and immunoreactive for ChAT. Open triangles represent cells retrogradely labelled with fluorogold. Closed triangles represent single cells that are labelled with fluorogold and ChAT. Stars represent group of three cells that are labelled with fluorogold and are immunopositive for ChAT. Full forms of abbreviations are given in the list of abbreviations. Numbers on the left of each section indicate distances from bregma.

FIGURE 9

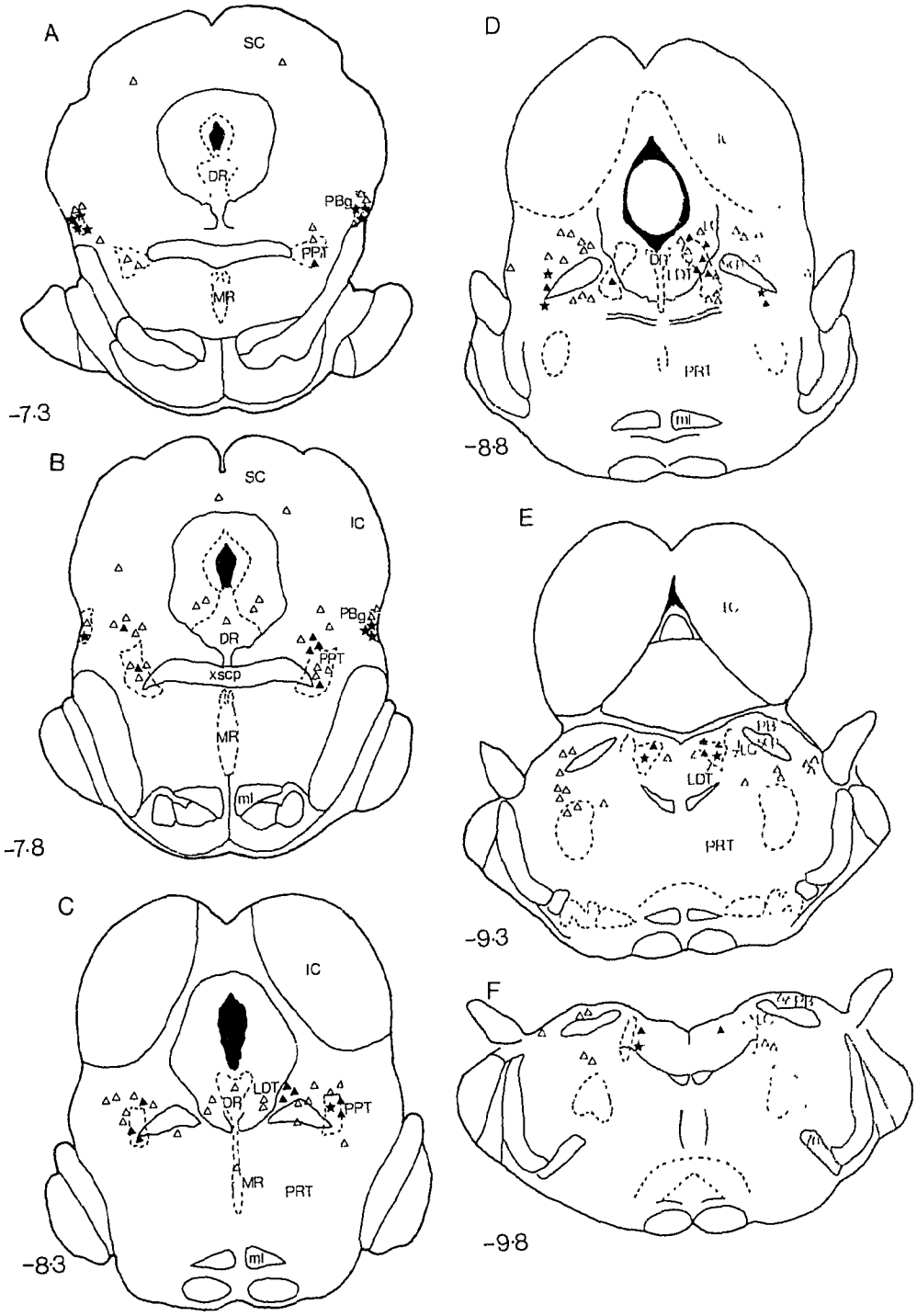

A
SC
DR
PBg
PPT
MR
-7·3
B
SC
IC
DR
PBg
PPT
xscp
MR
ml
-7·8
C
IC
LDT
DR
PPT
MR
PRT
ml
-8·3
D
IC
DR
LDT
PRT
ml
-8·8
E
IC
PB
LC
LDT
PRT
-9·3
F
PB
-9·8

Figure 10 Cells retrogradely labelled with fluorogold and/or ChAT in the brainstem. Panels A, B and C represent cells retrogradely labelled with fluorogold in the PPT, LDT and the PBg respectively. Cells labelled with Texas-red-tagged ChAT in the PPT, LDT and PBg are shown in panels D, E and F. Arrows represent cells that are labelled with fluorogold and ChAT. Arrowheads represent cells labelled with fluorogold but not with ChAT. Scale bar in panels D, E and F represent 100 μm, 50 μm and 200 μm respectively.

FIGURE 10

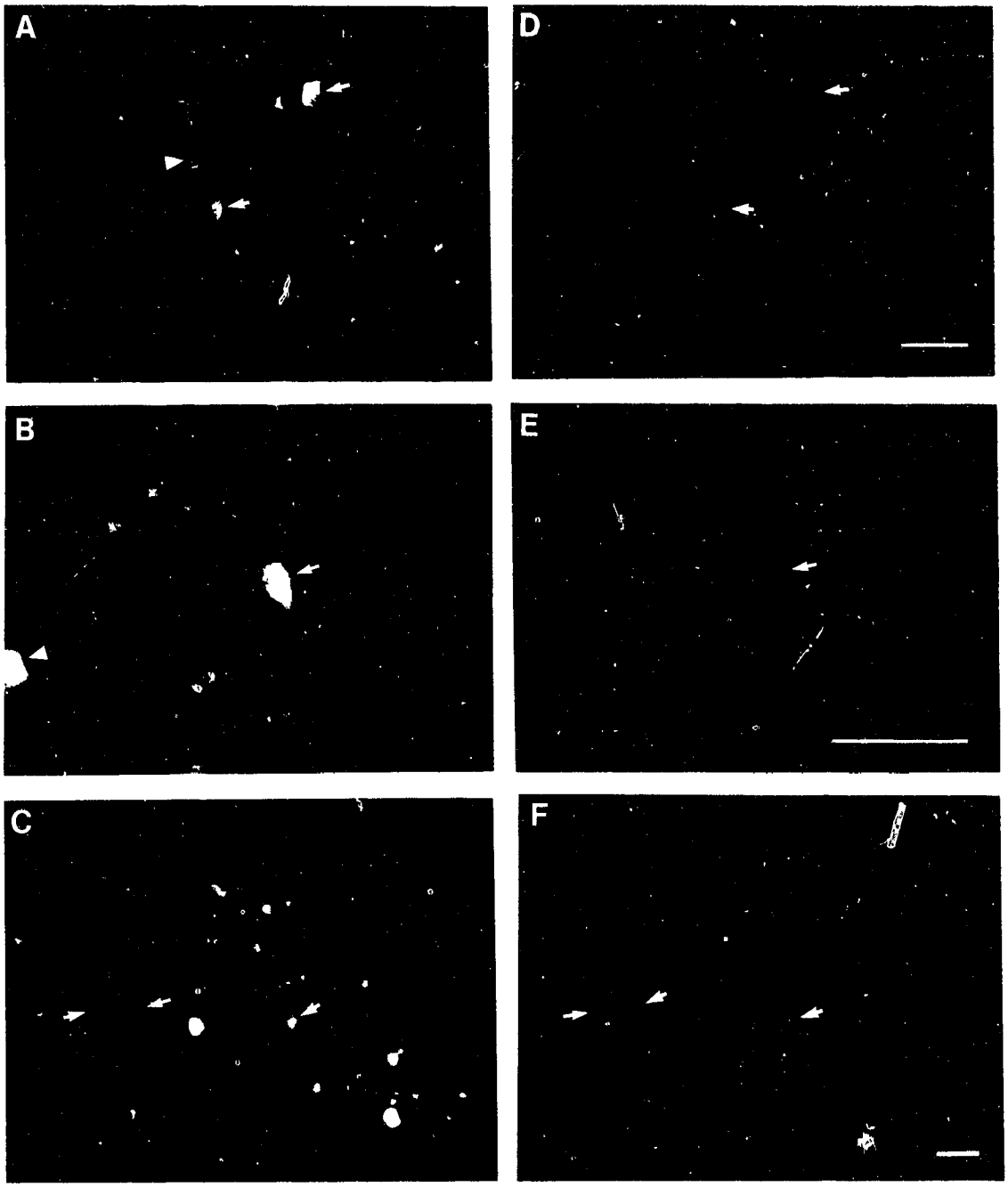
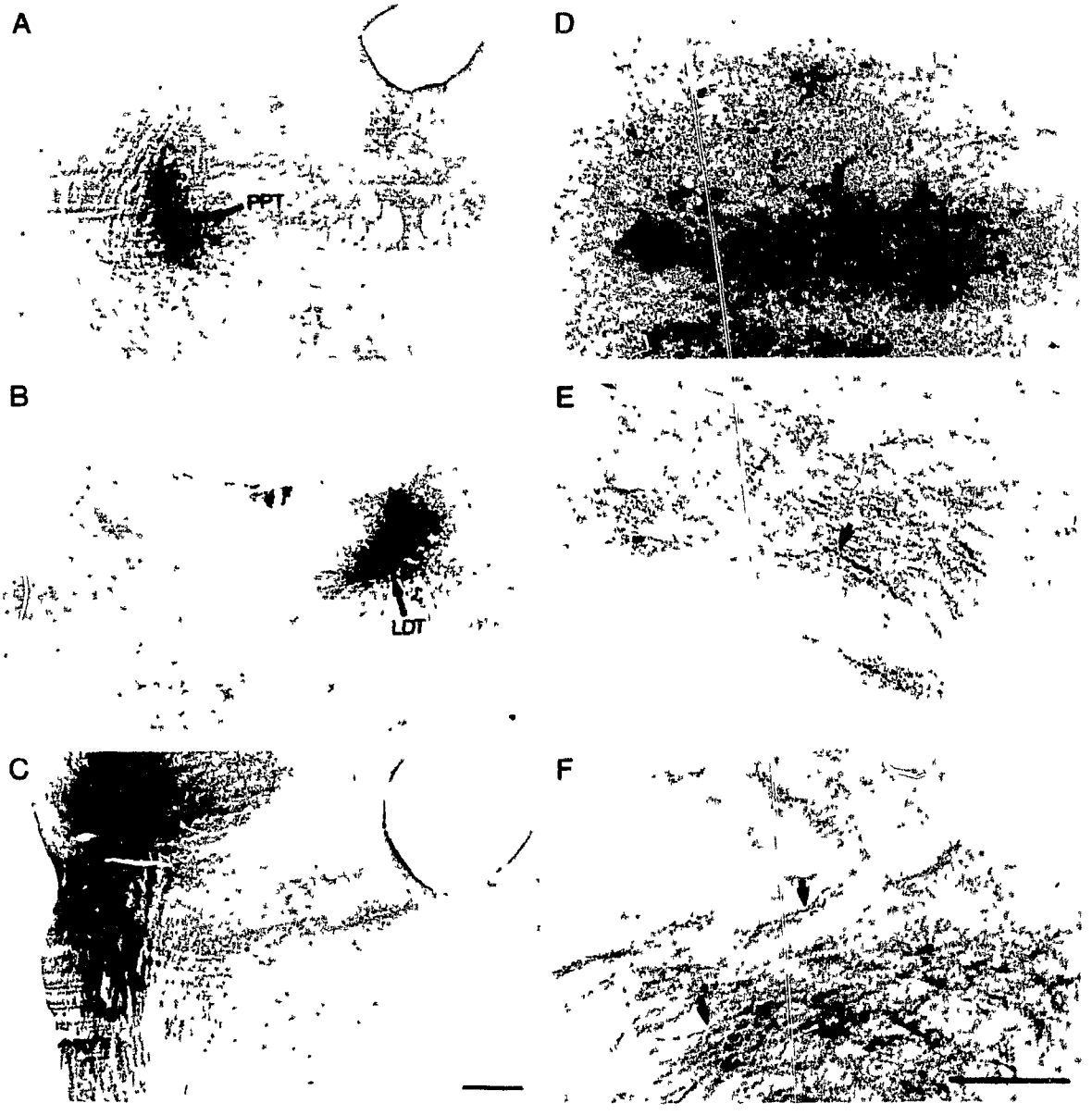
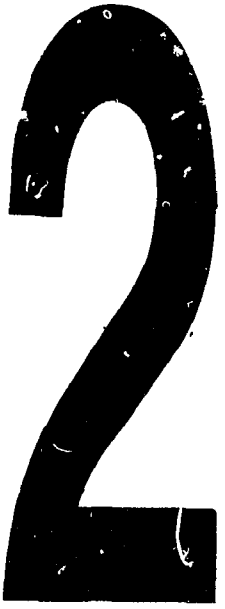


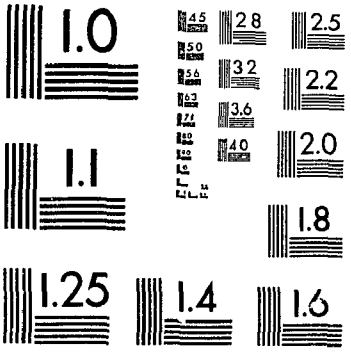
Figure 11 Anterograde labelling of terminals in the SCN is shown in panels D, E and F, following biocytin injections into the PPT (A), LDT (B) and PBg (C) respectively. Scale bar on the left represents 500 μm and that on the right represents 50 μm.

FIGURE 11





PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT



PRECISION[SM] RESOLUTION TARGETS

Figure 12 Panel A represents the time course of binding of 2nM of [^{3}H]NMS to hypothalamic punches. Panel B shows competition curves on blocking the binding of [^{3}H]NMS in hypothalamic tissue for: carbachol, a non-specific cholinergic agonist; pirenzepine, an M1 specific antagonist; methoctramine, an M2 specific antagonist; and atropine, a non-specific muscarinic antagonist.

FIGURE 12

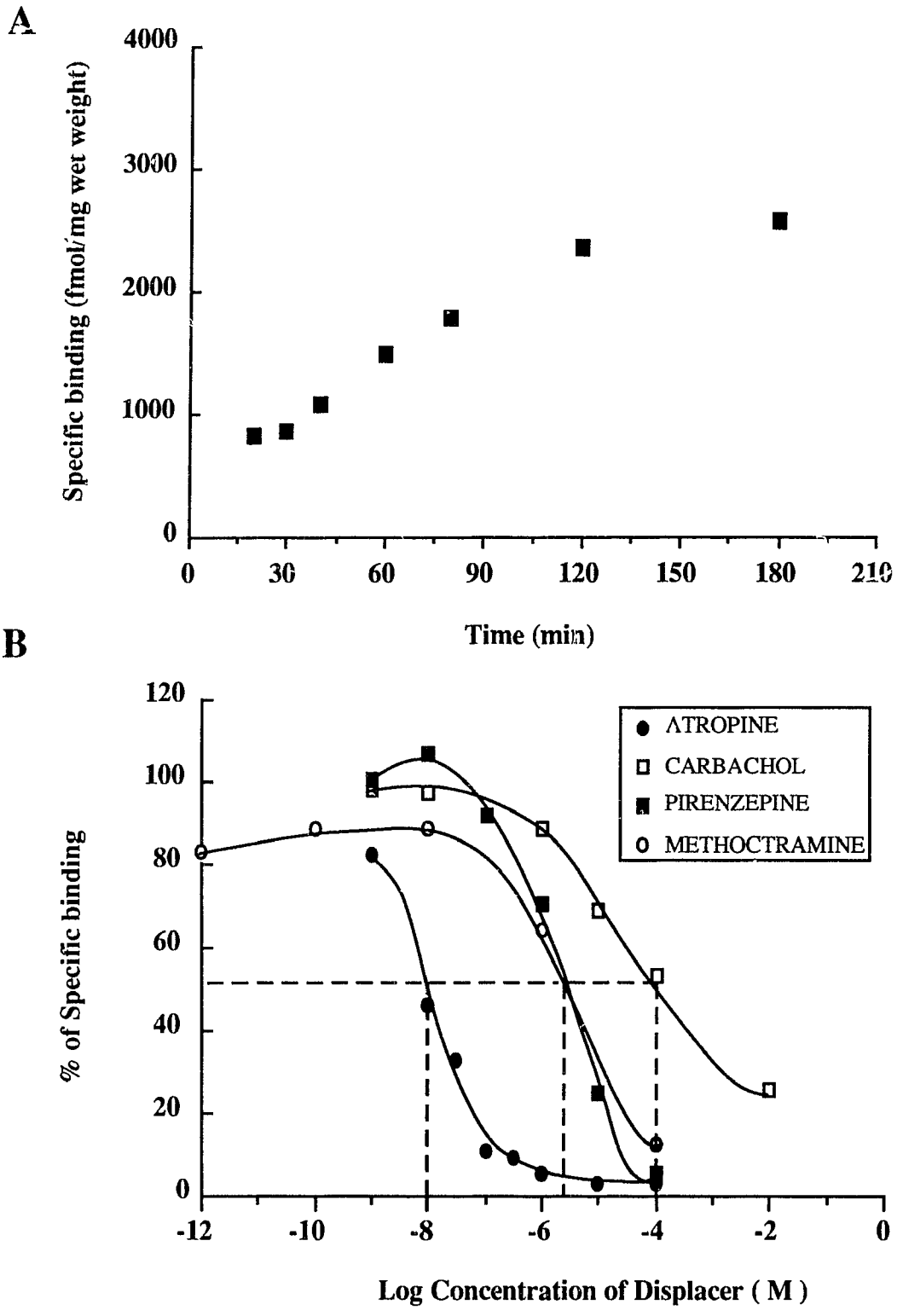

A
Specific binding (fmol/mg wet weight)
4000
3000
2000
1000
0
0
30
60
90
120
150
180
210
Time (min)
B
% of Specific binding
120
100
80
60
40
20
0
-12
-10
-8
-6
-4
-2
0
Log Concentration of Displacer (M)
ATROPINE
CARBACHOL
PIRENZEPINE
METHOCTRAMINE

Figure 13 Panel A shows the saturation curve for binding of [^{3}H]NMS to hypothalamic and cortical punches following incubation in varying concentrations of ligand. Panel B represents the Scatchard plot for hypothalamus and cortex.

FIGURE 13

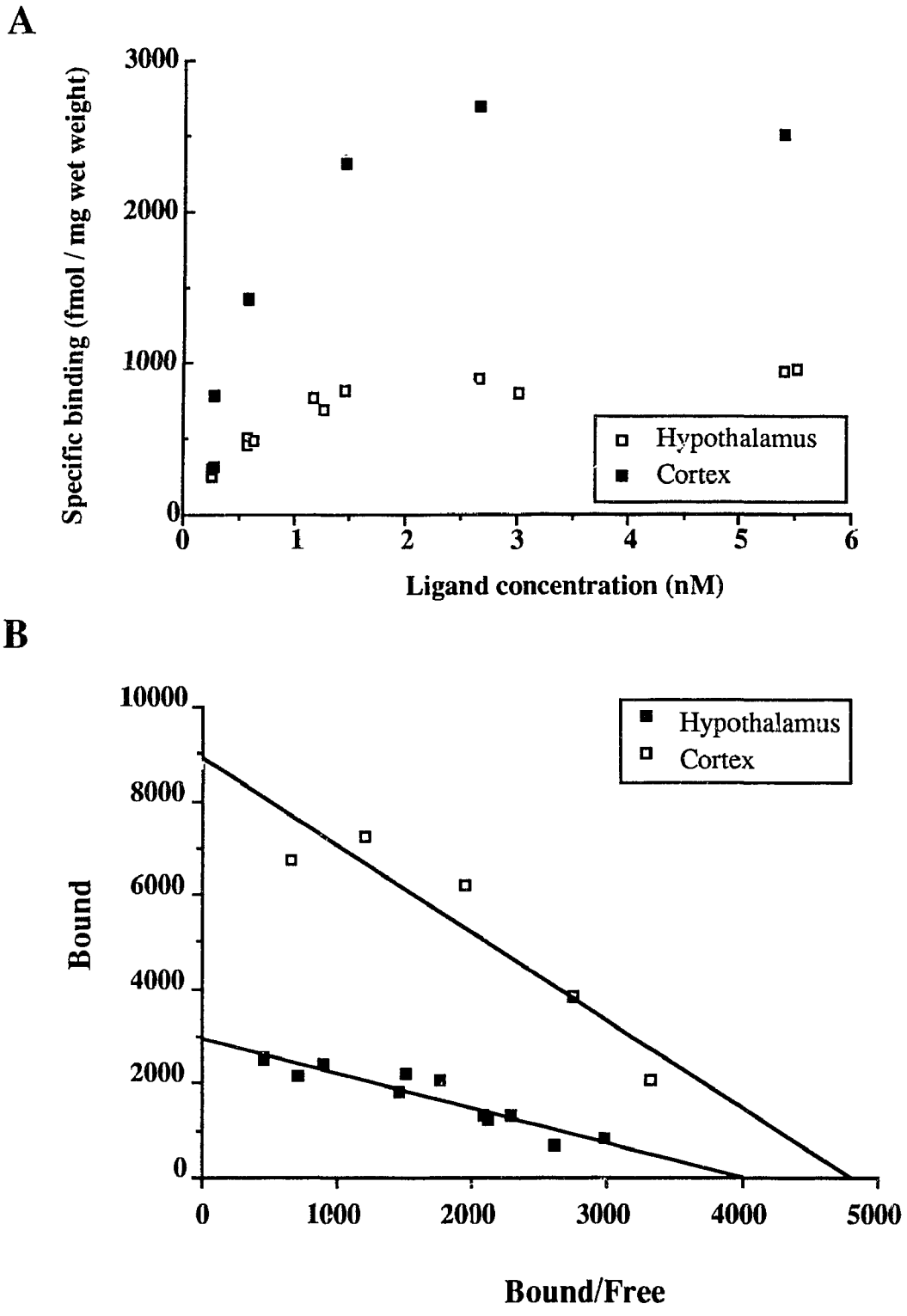
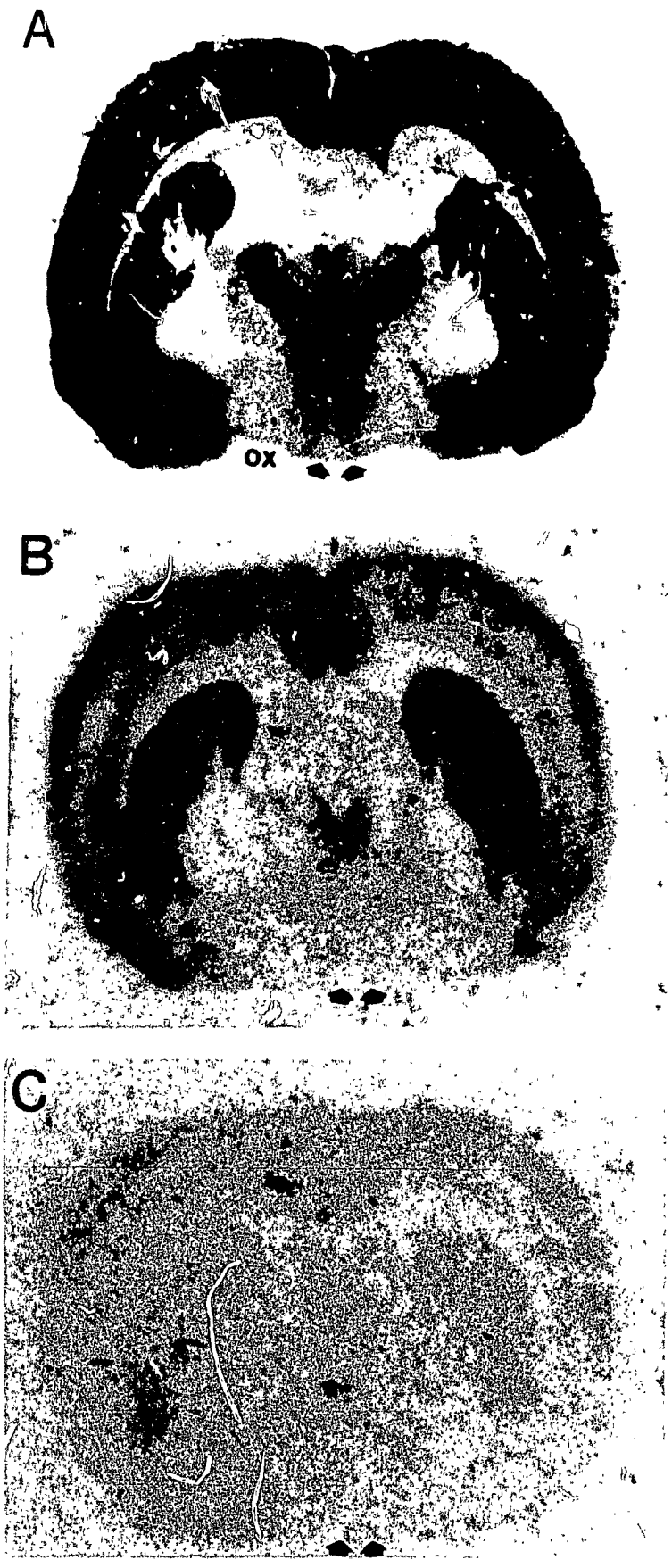

A
Specific binding (fmol / mg wet weight)
3000
2000
1000
0
0
1
2
3
4
5
6
Ligand concentration (nM)
Hypothalamus
Cortex
B
Bound
10000
8000
6000
4000
2000
0
0
1000
2000
3000
4000
5000
Bound/Free
Hypothalamus
Cortex

Figure 14 Autoradiograph of hypothalamic tissue at the level of SCN showing binding of 2nM of [^{3}H]NMS following incubation for 2 hr in the ligand. Panel A shows labelling in the ventrolateral aspects of the SCN. Panel B shows the binding of [^{3}H]NMS following incubation in methoctramine, an M2 specific antagonist, and panel C shows binding of [^{3}H]NMS following incubation in pirenzepine, an M1 specific antagonist. Arrows point to the SCN.

FIGURE 14



CHAPTER III

ANATOMY OF NEURONS PROJECTING TO THE SCN WHICH CONTAIN NERVE GROWTH FACTOR-RECEPTOR

As described in chapter I, nerve growth factor (NGF) is a protein which functions as a trophic factor for the development and maintenance of central cholinergic neurons (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Whittemore and Seiger, 1987). Receptors for NGF (NGF-R) are synthesized in most cholinergic neurons in the basal forebrain (Dawbarn et al., 1988; Eckenstein, 1988; Kiss et al., 1988; Pioro and Cuello, 1990a; 1990b; Schweitzer, 1987; Yan and Johnson, 1988) and they are transported anterogradely to the terminals of these cholinergic neurons located in the hippocampus and cortex. NGF released from the targets of these cholinergic cells in the hippocampus and the cortex binds to the NGF-R located on the terminals of the cholinergic neurons, and is transported retrogradely into the cell body as an NGF/NGF-R complex where NGF exerts its known biological effects.

The SCN exhibits the most intense neuropil staining for NGF-R in the rat forebrain (Sofroniew et al., 1989), but the source of the NGF-R in the SCN is not known. There is evidence that some cholinergic neurons in the basal forebrain project to the SCN (chapter II) and that NGF-R are found in the retina (Carmignoto et al., 1991), a major source of afferents to the SCN (Johnson et al., 1988). I therefore examined the basal forebrain and retina as potential sources of NGF-R in the SCN through a series of experiments described below. First, the extent and location of NGF-R immunoreactivity in the rat SCN was confirmed using a conventional peroxidase-anti-peroxidase (PAP) immunohistochemical method. Second, cells in the forebrain and the retina that contain NGF-R and project into the SCN were identified using injections of a retrograde tracer into the SCN combined with

fluorescence immunohistochemistry to identify NGF-R containing cells. Forebrain neurons identified as being retrogradely labelled from the SCN and as containing NGF-R were also assessed for their immunoreactivity for ChAT. In the third series of experiments, I attempted to identify the projection pathways by which NGF-R immunoreactive (IR) fibres reach the SCN by producing selective deafferentations of the SCN followed by immunohistochemical studies of the levels of NGF-R in the SCN.

METHODS

ANIMALS

Male Sprague Dawley rats weighing 200-250 g, obtained from Charles River Canada (St. Constant, Quebec), were housed in a 12:12 light-dark cycle with free access to food and water.

NGF-R IN SCN

Six rats were deeply anesthetized with an i.p. injection of sodium pentobarbital (65mg/kg), and perfused with 60 ml of 0.1M PB (pH 7.4) followed by 400ml of ice cold fixative comprising 4% paraformaldehyde in PB, at a flow rate of 10ml/min. The brain was immediately removed, blocked and postfixed in 4% paraformaldehyde at 4°C for 4 hr. The brain was cut on a microslicer into 50μm sections. The sections were collected in 0.05M TBS (pH 7.3), rinsed in three TBS washes of 20 minutes each and processed for NGF-R immunohistochemistry using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1979). Specificity of the NGF-R antibody has been previously reported (Taniuchi and Johnson, 1985).

RETROGRADE TRACING

Forebrain

Rats were injected with fluorogold into the SCN, perfused, their brains sectioned and the forebrain and brainstem sections processed for ChAT immunohistochemistry, using a Texas-red conjugated secondary antibody, as described in chapter II. The forebrain

sections were then thoroughly washed in TBS and processed for immunohistochemistry to reveal NGF-R using the fluorescence method, as described below.

Eyes

Both eyes of the rats which received fluorogold injections aimed at the SCN were removed after the perfusions and postfixed in 4% paraformaldehyde for 4 hrs, and then stored in 30% sucrose for 24 hrs. The eyeballs with the retinae attached were rapidly frozen with dry ice and cut on a cryostat into 40 µm thick sections, each containing a ring shaped section of the retina. The sections were collected directly on glass slides and allowed to dry for 15-45 min (to facilitate adhesion to the glass slide). The slides were washed three times in TBS and processed for immunohistochemistry using the PAP method, as described below. Following this the tissue was processed for the immunohistochemical conversion of fluorogold into a non-fluorescent material that could be viewed through the microscope under bright field, in order to visualize NGF-R and FG labelling at the same time. Glass slides containing the sectioned retinae were washed in 0.05 M TBS and then incubated at 4° C for 48 hours in 1:1000 dilution of a polyclonal antibody raised in rabbit against fluorogold (a gift from Dr. H. Chang), in antiserum diluent (ASD - 0.3% Triton X, 2.0% normal goat serum (NGS) and 0.01% sodium azide added to 0.05 M TBS). After this, the sections were washed in TBS and processed for immunohistochemistry using the PAP method. Fluorogold was visualized as a black-purple reaction product, whereas NGF-R was visualized as a brown reaction product (see below).

LESION STUDIES

Knife cuts

In order to study the course of entry of NGF-R containing neurons that project to the SCN, rats were positioned in a Kopf stereotaxic holder with the incisor bar set 5 mm above the interaural line. Cuts were made lateral (knife positioned 1.2 mm anterior, 1.2 mm

lateral to bregma and lowered 9.8 mm ventral to the skull), rostral (2.2 mm anterior to bregma, along the midline and lowered 9.8 mm ventral to the skull) and caudal (0.2 mm anterior to bregma, along the midline and lowered 9.8 mm ventral to the skull) to the SCN, using a Halasz knife, with 1mm long horizontal and vertical dimensions. The cuts placed lateral to the SCN were semicircular and extended unilaterally and those placed rostral and caudal to the SCN were semicircular and extended into both hemispheres. All knife cuts were made using a back and forth rotation of the knife, moving gradually forward until the end of the cut. For lateral cuts the horizontal shaft of the knife was always directed rostrally during insertion and withdrawal from the brain. However, for caudal and dorsal cuts the horizontal shaft was directed to the left side of the brain during insertion and withdrawal. Roof cuts (1.2 mm anterior to bregma, along the midline and lowered 8.8 mm ventral to skull) were made using a 1mm L-shaped knife. Roof cuts were all circular encompassing the entire dorsal aspect of the SCN. The horizontal shaft of the L-shaped knife was directed rostrally during insertion and withdrawal from the brain. Four rats were used for each of the knife cuts. Rats were sacrificed one week after surgery.,

Orbital enucleation

To examine the possibility that some of the NGF-R immunoreactivity in the SCN is located on the terminals of retinal ganglion cells, the eyes of eight anesthetized rats were removed either unilaterally or bilaterally by first cutting the extraocular muscles and then cutting the optic nerve. Following surgery, the orbits were filled with gelfoam and the rats were allowed to survive for one week.

Ganglionectomy

In order to determine whether NGF-R were located on the terminals of sympathetic fibres arising from the superior cervical ganglion, two animals were deeply anesthetized and unilateral superior cervical ganglionectomies performed through an incision made anterolaterally in the neck. The success of the surgery was confirmed by the observation of ipsilateral ptosis. Rats were allowed to survive for one week post-surgically.

Following their respective survival periods, all lesioned animals were perfused as described earlier and their brains processed for immunohistochemistry for NGF-R using the PAP method.

IMMUNOHISTOCHEMISTRY

Sections were washed in 0.05 M TBS and then incubated at 4° C for 48 hours in 1:100 dilution of a monoclonal antibody raised in mouse against NGF-R (MC192, a gift from Dr. R. Lindsay), in ASD (0.3% Triton X, 2.0% normal goat serum (NGS) and 0.01% sodium azide added to 0.05 M TBS). The specificity of the antibody has been documented (Taniuchi et al., 1986). Sections from brains used in the forebrain retrograde tracing studies were processed using a fluorescence method to identify NGF-R, while sections from brains used to study retrograde labelling in the retina and the effects of lesions were processed using a PAP method.

Fluorescence Method

Following incubation in NGF-R antibody as described above, sections were rinsed for 3 x 20 min in TBS and incubated for 1 hr at room temperature in fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Jackson Immunoresearch Labs. Inc., PA) diluted to 1:50 with TBS and 2% NGS.

Sections were mounted on slides, dried overnight, coverslipped with 1:9 concentration of ethylene diamine (Sigma Chemicals Co., MO) in glycerol or Fluoromount and visualized with an Olympus epifluorescence microscope. Fluorogold labelling was assessed using an ultraviolet filter cube. FITC and Texas red fluorescence was assessed using blue and green filter cubes, respectively. Retrogradely labeled cells which were also labeled with NGF-R and/or ChAT were mapped and in selected cases photographed.

Peroxidase-antiperoxidase (PAP) Method

After 48 h incubation in primary antibody, the tissue was rinsed in TBS for 3 x 20 min and incubated in affinity purified goat antimouse IgG (Organon Teknika Corp., PA) diluted 1:300 in TBS containing 2% NGS, for one hr at room temperature. The sections were then washed for 3 x 20 min in TBS and incubated for one hr in 1:300 dilution of mouse PAP (Jackson Immunoresearch Labs. Inc., PA) in TBS. Following 3 x 20 min rinses in TBS, the tissue was incubated for 10 min in 0.05 M Tris-HCl buffer containing 0.02% of the chromogen DAB and 0.6% nickel ammonium sulphate to which 0.006% hydrogen peroxide was added, followed by an additional 5-10 min incubation. This reaction yields a black-purple reaction product.

In the case of the retinal sections, nickel ammonium sulphate was omitted from the last solution in order to get a brown reaction product. To visualize fluorogold in bright field, after incubation in the primary antibody the sections were washed and incubated in goat antirabbit IgG (Jackson Immunoresearch Labs. Inc., PA) diluted to 1:300 in TBS containing 2% NGS, for 1 hr at room temperature. After this the sections were washed for 3 x 20 min in TBS and incubated for 1 hr in rabbit PAP (Jackson Immunoresearch Labs. Inc., PA) in TBS and processed using DAB and nickel ammonium sulphate, as described above.

All sections were rinsed in TBS, mounted on slides, dried overnight, coverslipped with Permount and examined using an Olympus microscope. Photographs were taken using Kodak Tri-X and TMAX film.

Lesion Histology

Location of the knife cuts was initially analyzed by the observation of the knife track in the brain, during sectioning. Following processing for immunohistochemistry for NGF-R the knife cuts became apparent from the accumulation of reaction product on either side of the cut.

RESULTS

NGF-R IMMUNOREACTIVITY IN THE SCN

Strong, diffuse NGF-R immunoreactive neuropil was visualized in the SCN (Figure 15A). This labelling was restricted to the ventrolateral portion of the SCN, with the dorsomedial region of the SCN showing an absence of NGF-R immunoreactivity. Fibres immunostained for NGF-R could be seen entering the SCN through the optic chiasm and extending into the immunoreactive areas. At high magnification, some non-immunoreactive cell bodies could be seen to be surrounded by a coat of reaction product (Figure 15B). Strong labelling was also observed in the basal forebrain, where cell somata were strongly immunoreactive. Diffuse labelling similar to that observed in the SCN was seen in the intergeniculate leaflet of the lateral geniculate complex.

RETROGRADE LABELLING IN THE FOREBRAIN

Injection sites in the SCN were the same as described in chapter II, and they are illustrated in Figure 6A. Retrogradely labelled cholinergic neurons in the forebrain which were also immunoreactive for NGF-R are mapped in Figure 7. Figure 8 shows an example of a neuron in the SI region of the CBC which was retrogradely labelled with fluorogold (Figure 8A) and also immunopositive for both ChAT (Figure 8B) and NGF-R (Figure 8C).

Retrogradely labelled cells immunoreactive for ChAT as well as NGF-R were observed throughout the entire rostro-caudal extent of the CBC. The largest numbers of triple-labelled neurons were located in the SI and the NBM areas of the CBC (Figures 7F and G). A few triple-labelled cells were also observed in the vDBB and hDBB. A few retrogradely labelled cells immunopositive for NGF-R but not for ChAT were observed in the caudal CBC areas. However, no retrogradely labelled neurons were found anywhere in the basal forebrain that were immunopositive for ChAT but failed to show NGF-R immunoreactivity. Neurons retrogradely labelled with fluorogold but immunoreactive for

neither ChAT nor NGF-R were located in the lateral septum, zona incerta, intergeniculate leaflet of the LGN and most of the CBC.

After injections of fluorogold into the SCN, retrogradely labelled retinal ganglion cells were observed which were immunoreactive for NGF-R (Figure 16). In addition, there was a large number of retinal ganglion cells immunopositive for NGF-R but not retrogradely labelled with fluorogold.

LESION STUDIES

Only three animals receiving knife cuts lateral to the SCN had cuts placed along the entire SCN, without entering the SCN. However, in all of these animals the knife cut extended into the optic chiasm at the caudal level. Comparisons of the levels of NGF-R immunoreactivity between the ipsilateral and the contralateral SCN following a unilateral knife cut placed lateral to the nucleus, revealed a reduction in NGF-R immunoreactivity in the ipsilateral SCN as compared with the contralateral side (Figure 17A). Knife cuts placed rostral, caudal or dorsal to the SCN had no effect on the pattern of NGF-R immunoreactivity in the SCN (Figure 17B).

Unilateral orbital enucleation resulted in a reduction in the amounts of NGF-R immunoreactivity observed in the contralateral SCN as compared with the ipsilateral SCN (Figure 17C). Because of the variability in the density of immunoreactivity observed in different brains, small differences in levels of immunoreactivity between operated and control animals could not be reliably detected. As a result, it is not possible to determine whether the ipsilateral SCN showed small reductions in levels of immunoreactivity compared with unoperated controls. Following bilateral orbital enucleation, there appeared to be an equal reduction in NGF-R immunoreactivity on the two sides of the SCN (Figure 17D). Seven days after bilateral enucleation, some NGF-R labelling in the SCN still persisted.

Unilateral ablation of the SCG produced ipsilateral ptosis. However, even 7 days after the surgery, there was no effect on the intensity of NGF-R immunoreactivity in either the SCN or the basal forebrain.

DISCUSSION

Strong NGF-R immunoreactivity was observed in the ventrolateral aspects of the SCN. Insofar as could be determined at the light microscopic level, most of the NGF-R immunoreactivity observed in the SCN appeared to be located on fibre terminals. Some immunoreactivity was also observed on the surface of non-immunoreactive neurons. Whether this immunoreactivity represents NGF-R located intracellularly or on fibre terminals surrounding these cells could not be determined at the light microscopic level. It could also not be determined if these non-immunoreactive cells were neurons or glia. However, NGF mRNA (Ojeda et al., 1991) and NGF precursor-like immunoreactivity (pro-NGF) (Senut et al., 1990) have been localized in the SCN, suggesting that cells in the SCN are capable of synthesizing NGF and/or are involved in the uptake and transport of precursor molecules. It is possible that the cell-surface related NGF-R immunoreactivity seen in the SCN is located on NGF-R-positive fibre terminals making synaptic contacts with neurons that synthesize NGF.

The evidence reported here for NGF-R immunoreactivity in the SCN is in agreement with previous findings (Yan and Johnson, 1989; Pioro and Cuello, 1990). The pattern of NGF-R immunoreactivity in the SCN resembles the pattern of labelling observed in the SCN following a bilateral injection of an anterograde tracer into the eye (Johnson et al., 1988; Figure 3 chapter I), suggesting that at least some of the neuropil labelling for NGF-R is located on retinal terminals in the SCN. The observation of NGF-R immunopositive fibres in the optic chiasm seen entering the immunoreactive zone in the SCN strengthens this suggestion. Ichikawa and Hirata (1986) observed a very dense distribution of ChAT positive fibres in the ventral aspect of the SCN which was similar to the region showing

NGF-R labelling and receiving retinal terminals in the SCN. These findings suggest the possibility that some of the NGF-R are located on ChAT terminals, a correlation which is also observed in the hippocampus (Seiler and Schwab, 1984; Korsching et al., 1985; Korsching, 1986). However, as described in chapter II, although most of the ChAT immunoreactive fibres in the present study were detected in the ventrolateral aspects of the SCN, the pattern of distribution of ChAT immunoreactive fibres in the SCN that I have observed was not the same as that reported by Ichikawa and Hirata and it was also not similar to the pattern of NGF-R labelling in the SCN. Furthermore, it has not been established whether cholinergic retinal ganglion cells bearing NGF-R project to the SCN. There is some experimental evidence arguing against this possibility. Although amacrine cells in the retina have been shown to be cholinergic, there is no evidence for ganglion cells being cholinergic (Massey and Redburn, 1987). Furthermore, it appears that NGF treatment cannot prevent a reduction in the number of retinal ganglion cells expressing nicotinic acetylcholine receptor-like immunoreactivity (probably ganglion cells postsynaptic to cholinergic amacrine cells) following axotomy (Ehrlich et al., 1990). This finding suggests that the neurons in the retina which express nicotinic receptors are not dependent on NGF for their survival and that the NGF-R containing retinal ganglion cells are different from these ganglion cells that contain nicotinic receptors.

The distribution of ChAT immunoreactive neurons in the basal forebrain that were retrogradely labelled following fluorogold injections into the SCN was described in chapter II. Fluorogold labelled neurons that were immunopositive for NGF-R were also located throughout the rostro-caudal extent of the basal forebrain, with the highest densities being found at the level of the SCN, in the SI and NBM regions. Most of the retrogradely labelled NGF-R positive neurons were also ChAT positive. The majority of such triple labelled cells were found in the caudal nuclei of the CBC, namely the SI, MgPA and the NBM areas. A few triple labelled cells were also found in the vDBB and hDBB. About 90% of the ChAT positive neurons in the basal forebrain have been found to be NGF-R

positive (Schweitzer, 1987; Dawbarn et al., 1988; Eckenstein, 1988; Kiss et al., 1988; Yan and Johnson, 1988; Batchelor et al., 1989; Pioro and Cuello, 1990a; 1990b). A few retrogradely labelled NGF-R positive neurons that did not show ChAT immunoreactivity were found in the CBC. Although the neurotransmitter content of these cells is unknown, it is possible that these neurons are GABAergic, since some of the cultured neurons from septal and diagonal band regions which respond to exogenously applied NGF have been shown to express GABA immunoreactivity (Arimatsu and Miyamoto, 1991).

Retrogradely labelled NGF-R immunoreactive neurons were found in the ganglion cell layer of the retina, suggesting that at least part of the NGF-R immunoreactivity seen in the SCN is located on retinal terminals. The presence of NGF-R immunoreactivity in the retina, optic nerve and the superior colliculus, has been observed early in development (Raivich et al., 1987; Eckenstein, 1988; Schatteman et al., 1988; Yan and Johnson, 1988). In the adult retina, NGF-R immunoreactivity is localized in retinal ganglion cells and Müller cells (Carmignoto et al., 1991). The NGF-R in adult retina appears to be functional, since intraocular administration of NGF has been shown to promote the survival of adult rat retinal ganglion cells and optic nerve fibres following optic nerve transection (Carmignoto et al., 1989).

The finding that unilateral enucleation resulted in substantial reductions in NGF-R immunoreactivity in the contralateral SCN supports the conclusion that much of the NGF-R immunoreactivity in the SCN is located on retinal terminals. This finding is consistent with the evidence that 80% of the retinal input to the SCN originates in the contralateral eye in the rat (Johnson et al., 1988; K.G. Bina, personal observations). The hypothesis that retinal ganglion cells contribute NGF-R immunoreactive fibres to the SCN is further reinforced by the evidence that ligation of the optic nerve causes the accumulation of NGF-R immunopositive reaction product on both sides of the ligature, suggesting that NGF-R is transported retrogradely and anterogradely by retinal ganglion cells (Carmignoto et al., 1991). Bilateral enucleation reduced the NGF-R immunoreactivity equally in both SCN,

but, even seven days after bilateral enucleation, some NGF-R immunoreactivity persisted in the SCN, indicating that some NGF-R in the SCN derive from a source other than the retinal innervation. These NGF-R are presumably the ones located on the terminals of cholinergic neurons which project to the SCN from several nuclei in the basal forebrain. However, the presence of NGF-R on intrinsic neurons and/or glial cells in the SCN cannot be ruled out.

Knife cuts placed lateral to the SCN resulted in a reduction of NGF-R immunoreactivity in the SCN ipsilateral to the cut. However, cuts made rostral, caudal or dorsal to the SCN had no effect on this immunoreactivity. These results suggest that fibres from NGF-R containing neurons in the basal forebrain approach the SCN and enter it laterally. Some of the cuts placed lateral to the SCN also extended ventrally into the optic chiasm at caudal levels. A reduction in the ipsilateral side and not the contralateral side, however, suggests that the retinal fibres reaching the SCN were largely unaffected. Moreover, the possibility of severing crossed retinal fibres that have not reached the SCN yet remains a possibility.

Ablation of SCG unilaterally had little or no effect on the NGF-R immunoreactivity in the SCN, indicating that none of the NGF-R immunoreactivity is located on the terminals of the sympathetic neurons which may be present in the SCN.

Taken together, these results suggest a mechanism of action of NGF-R in the SCN similar to the one observed in the basal forebrain-hippocampal system, wherein NGF-R are synthesized in the somata of basal forebrain neurons and transported anterogradely to their terminals in the hippocampus and cortex (Seiler and Schwab, 1984; Taniuchi et al., 1985), areas that contain NGF protein and NGF mRNA. At these terminal sites, NGF binds to the NGF-R, is internalized and forms a NGF/NGF-R complex which is retrogradely transported to the somata of cholinergic neurons (Schwab et al., 1979; Seiler and Schwab, 1984; Johnson et al., 1987), where it exerts its biological effects. By analogy with the basal forebrain-hippocampal system, NGF-R may be synthesized in the somata of retinal ganglion cells and neurons in the basal forebrain and then transported anterogradely to their

terminals in the SCN. These terminals may synapse on cells in the SCN that synthesize NGF. The NGF released from such cells would be retrogradely transported back to the ganglion cells and the basal forebrain as an NGF/NGF-R complex which exerts its biological action on the cholinergic, and perhaps other, afferents to the SCN.

In the SCN, NGF appears to play a role in the modulation of circadian rhythms in rodents, since exogenous NGF administered into the SCN produces phase shifts in the activity rhythms of hamsters (chapter IV). However, the manner in which NGF exerts its biological function in the SCN is unclear. It is possible that NGF acts as a retrograde signal to the basal forebrain cholinergic neurons innervating the SCN in order to increase their activity. Increases in ChAT activity and ChAT mRNA levels have been reported after treatment with NGF (Hefti et al., 1984; Hefti et al., 1985; Mobley et al., 1985; Hatanaka et al., 1988; Cavicchioli et al., 1991). Furthermore, increases in ACh synthesis (48%) and release (175%) in the presynaptic cholinergic cells following NGF treatment have also been reported (Kewitz et al., 1990; Lapchak and Hefti et al., 1991). Another possibility is that binding by NGF to NGF-R affects the availability of cholinergic receptors in the SCN, independently of any trophic effects on cholinergic activity. Such an example is seen in the nodose ganglion, a sensory ganglion which expresses NGF-R, but its growth and survival in culture are unaffected by the presence of NGF. Yet, NGF treatment of the neonatal nodose ganglion has been reported to induce nicotinic cholinergic receptors on its neurons (Mandelzys et al., 1990). Taken together, these findings suggest the possibility that NGF mediates its phase shifting effects through its effects on ChAT activity, ACh synthesis or release, and/or induction of acetylcholine receptors.

Figure 15 NGF-R immunoreactivity in the SCN. The labelling is localized in the ventrolateral aspects of the SCN. A higher magnification (panel B) of the immunoreactive zone in the SCN, shown in panel A (arrow) shows immunoreactive areas surrounding the cell bodies. Most of the labelling appears to be located on terminals. However, whether this represents labelling on the soma and/or on terminals making contact with the soma remains to be clarified using electron microscopy.

FIGURE 15

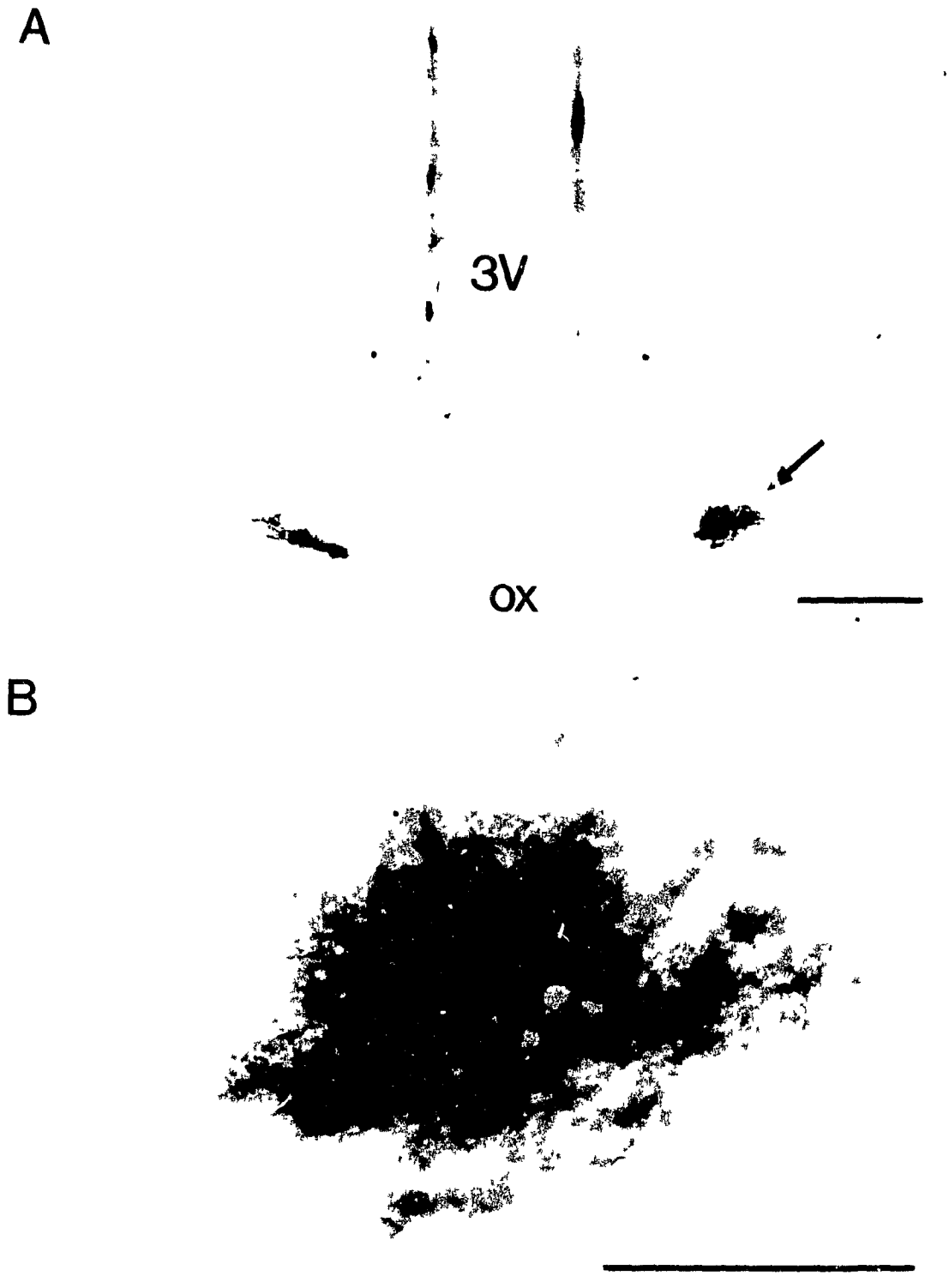


Figure 16 Cells in the retina labelled with the retrograde tracer, fluorogold (black reaction product) and showing immunoreactivity for NGF-R (brown reaction product).

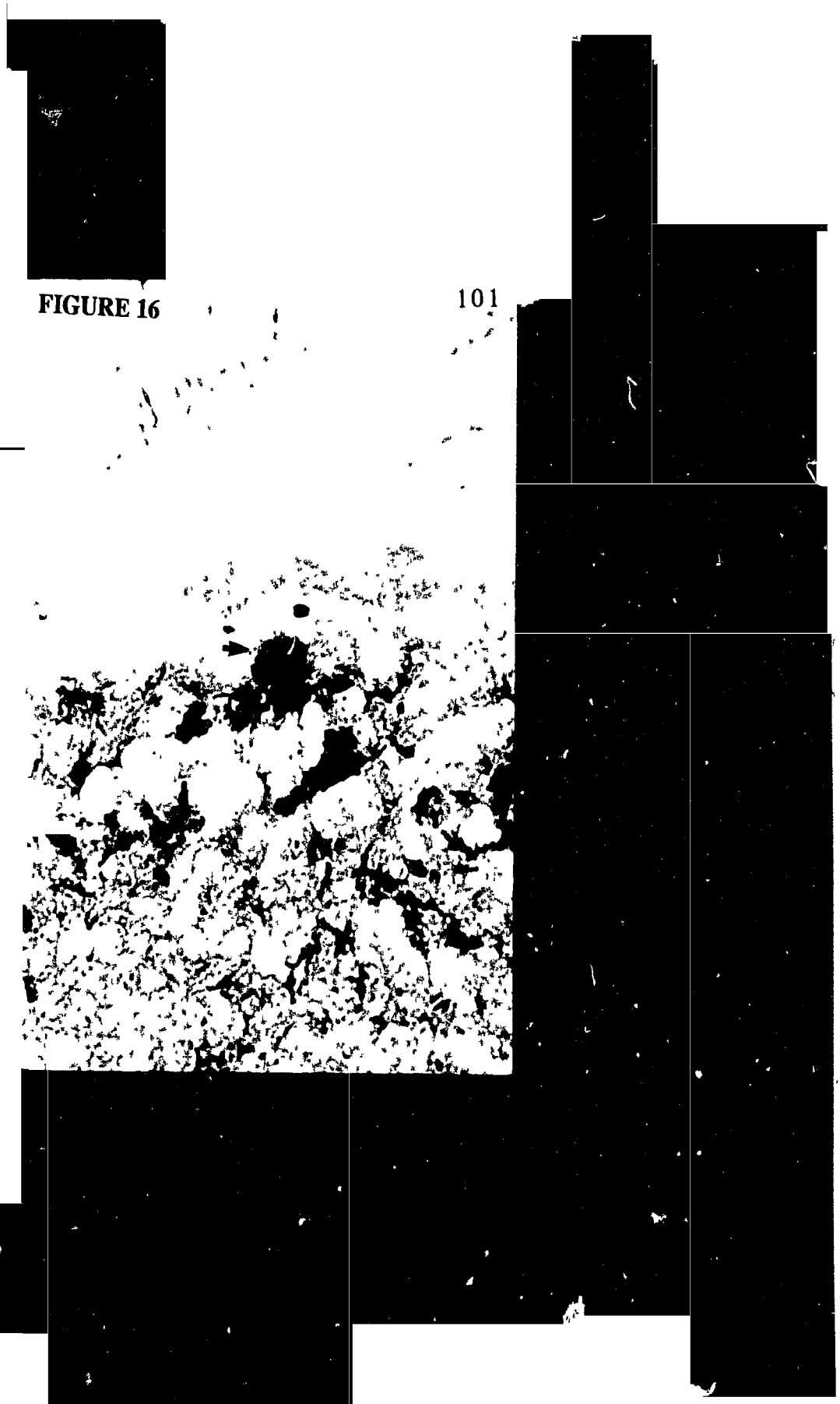
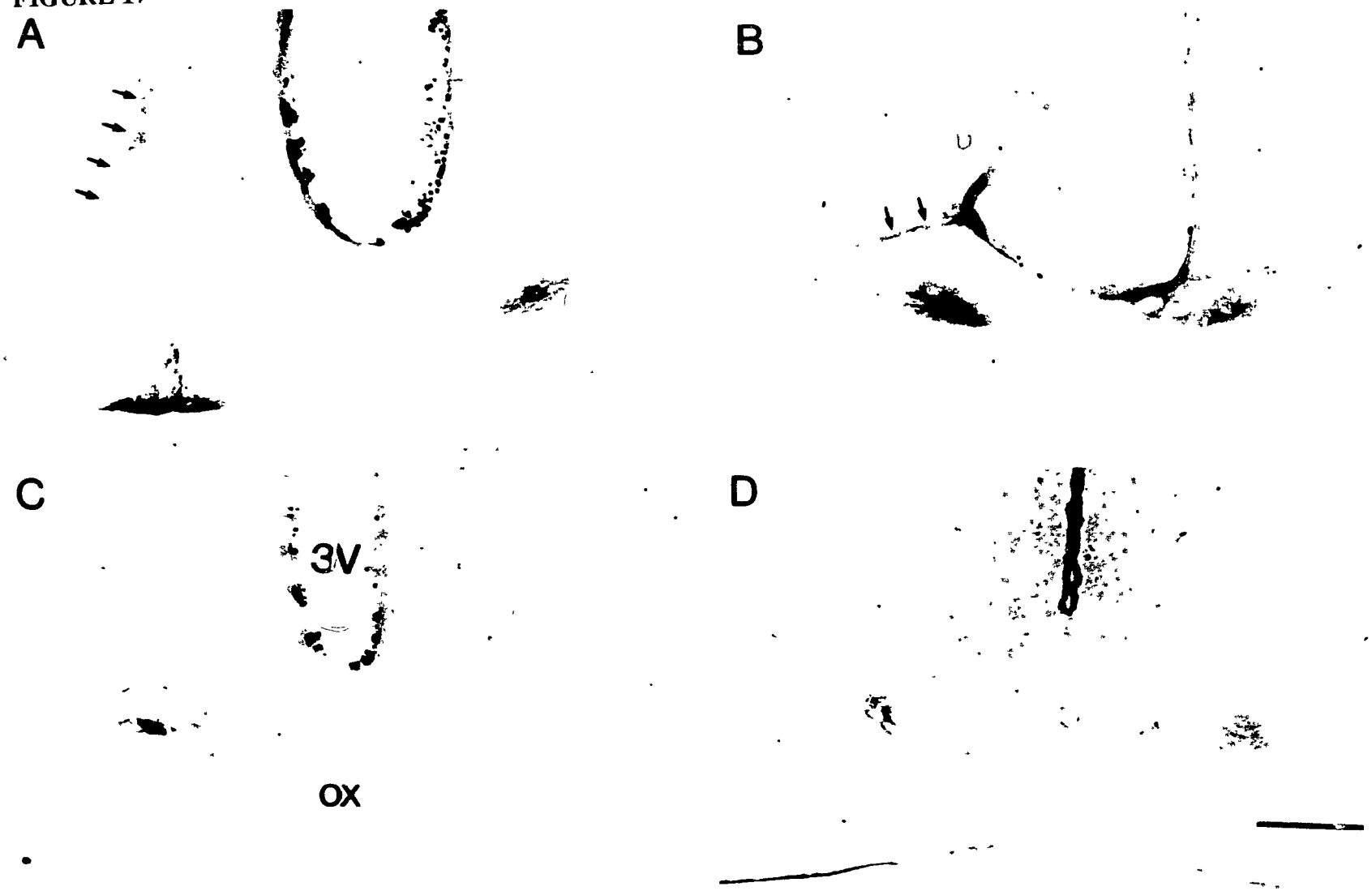


FIGURE 16

Figure 17 Panels A and B show NGF-R labelling in the SCN following knife cuts made lateral and dorsal to the SCN. Cuts made lateral to the SCN decreased the NGF-R labelling ipsilateral to the cut. However, there was no detectable decrease in the total NGF-R following cuts made dorsal to the SCN. Arrows represent knife cut tracks.

Panels C and D show NGF-R labelling in the SCN following unilateral orbital enucleation and bilateral orbital enucleation respectively. Unilateral enucleation leads to a reduction in NGF-R labelling in the contralateral SCN compared with the ipsilateral SCN. Bilateral enucleation resulted in a large decrease in both the SCN. Scale bar represents 100 μm.

FIGURE 17



CHAPTER IV

CHOLINERGIC RECEPTORS MEDIATING CARBACHOL-INDUCED PHASE SHIFTS OF HAMSTER CIRCADIAN RHYTHMS

INTRODUCTION

As described in chapter I, the role of acetylcholine in the entrainment pathway has been investigated by monitoring the effects of injecting carbachol, a non-specific cholinergic agonist, at different circadian times in several rodent species. Carbachol injections have been shown to mimic partially the phase shifting effects of light on locomotor activity rhythms of mice (Zatz and Herkenham, 1981) and hamsters (Meijer et al., 1988; Wee and Turek, 1989), and the pineal SNAT and activity rhythms of rats (Zatz and Brownstein, 1979, 1981; Mistlberger and Rusak, 1986). Furthermore, nicotinic receptor antagonists have been shown to block the phase-shifting effects of light on pineal SNAT activity (Zatz and Brownstein, 1981) and locomotor activity (Keefe et al., 1987). By contrast, muscarinic receptor antagonists fail to block light effects on SNAT (Zatz and Brownstein, 1981). These results imply a role for ACh, acting on nicotinic receptors, in the photic entrainment pathway.

In the present study, I have further investigated the role of cholinergic transmission in phase-shifting rhythms by examining whether the phase-shifting effects of carbachol are mediated through nicotinic or muscarinic receptors in the SCN. By means of antagonists which are differentially effective at these receptors, I have attempted to block the phase-shifting effects of intracerebrally administered carbachol on hamster activity rhythms. In addition, I examined whether these cholinergic antagonists alone would affect activity rhythms.

METHODS

Forty adult male Syrian hamsters (*Mesocricetus auratus*; LVG:lak), weighing 80-100 g, were obtained from Charles River Laboratories (St. Constant, Quebec) and group-housed under a light-dark schedule (LD 14:10) for two weeks. Before the start of the experiment, animals were anesthetized with sodium pentobarbital and implanted with stainless steel indwelling guide cannulae (22 gauge; Plastics One, VA) directed stereotaxically at the SCN (0.5 mm anterior to bregma, 0.3 mm lateral to bregma and 8.4 mm ventral to the skull surface, with the incisor bar 2mm below the interaural line). The cannula was cemented in place using dental acrylic and covered by inserting a dummy inner cannula attached to a dust cap, which helped to keep the aperture open.

After recovery from anesthesia, animals were individually housed in cages equipped with running wheels and maintained under constant dim red light (DD) with free access to food and water. Running-wheel activity was monitored continuously on an event recorder and activity counts were recorded every 10 min using an Apple computer. Event recorder charts were used to measure activity rhythm periods and phase shifts, while the actograms presented in the figures were plotted from computer files. After a stable free-running rhythm was established, hamsters were injected intracranially via the cannula with 2µl of 0.9% saline or a saline solution of one of the following drugs: carbachol (0.01M), a non-selective cholinergic agonist; atropine (0.01M), a cholinergic antagonist at the muscarinic receptor; or mecamylamine (75µg/µl), a cholinergic antagonist at the nicotinic receptor.

To determine whether the phase shifting effects of carbachol are mediated through nicotinic or muscarinic receptors, carbachol was also administered in combination with either atropine or mecamylamine. In such cases, 1µl of atropine or mecamylamine solution (as above) was administered via the cannula ten minutes before a carbachol injection. Each injection was made over 2-3 minutes, using a Hamilton syringe connected via plastic tubing to an inner cannula (28 gauge, Plastics One). The inner cannula was left in place for an

additional minute. Each animal was given one of these injections at circadian time (CT) 6, 14 or 22, with the order of drugs and injection times randomized. These three phases were chosen because carbachol has been shown to produce clear phase shifts at these times (Zatz and Brownstein, 1981; Earnest andTurek, 1985). A minimum interval of two weeks was allowed between injections.

Period lengths were calculated by drawing an eye-fitted line through the activity onsets for ten day intervals before injections and ten day intervals after injections. Post-injection period lengths were calculated for steady state free-running rhythms, after transient cycles (2-3 days) were excluded. Phase shifts were calculated by measuring the difference in minutes between the phases predicted by the two eye-fitted lines extrapolated to the day after the injection (Pittendrigh and Daan, 1976). The phase shifts were calculated by two experienced observers independently, and in the case of a discrepancy in scoring, an average of the two was taken.

At the end of the experiment, hamsters were deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% physiological saline followed by 10% formalin. Brains were sectioned into 40 µm thick sections and stained with Cresyl violet in order to visualize the location of the cannulae. Only animals with their guide cannula in the SCN or within 500 µm of its borders were included in the SCN injection group.

One way analysis of variance was used to determine the significance of various treatment effects and significant differences among the treatments were tested using Fisher's test.

RESULTS

A total of 146 injections were made into the vicinity of the SCN in 30 hamsters. Light microscopic evaluation of cannula placements revealed that 24 of 30 hamsters, representing 132 injections, had cannula placements within 500 µm of the SCN, none of the animals

had placements further from the SCN than 500µm and 6 cannula placements, representing 14 injections, could not be evaluated and the data were discarded.

A phase response curve was constructed using 49 injections of carbachol made at CT6, 14 and 22. Carbachol administered at CT6 and 22 produced mostly phase advances (69.8+/-15.7 min and 83.9+/-24.8 min; mean+/- standard error of the mean) whereas at CT14 it produced mainly delays (-59.7+/-18.2 min). Examples of animals given carbachol, saline or carbachol preceded by one of the antagonists at CT6, CT14 and CT22 are shown in Figures 18, 19 and 20.

Atropine, administered into the SCN 10 min prior to carbachol, produced a significant decrease in the phase shifting effects of carbachol at CT6 and 22 (mean shifts : -11.9+/-4.8 and -20+/-6.6 min respectively; $F_{(5, 60)} = 3.5$; p=0.007 and $F_{(5, 25)} = 5.24$; p=0.002 respectively). At CT14 it also reduced or eliminated phase shifts in response to carbachol in some cases (-23.1+/-16 min; $F_{(5, 29)} = 4.1$; p=0.006), but the blockade was less consistent than at the other phases, and some carbachol injections were still quite effective (Figure 19 and 21). Atropine, administered by itself, did not have any consistent phase shifting effects at the three phases tested (Figure 20 and 21).

Unlike atropine, mecamylamine given prior to the administration of carbachol did not significantly block the phase shifts produced by carbachol, at any of the three phases tested (Figure 21). Mecamylamine administered by itself had no phase shifting effects at any of these phases (Table 1).

Saline injections did not produce phase shifts at any of the phases tested (Figure18). Table 1 summarizes the results for all injections and Figure 22 shows the mean effects of each of the drugs and drug combinations tested.

DISCUSSION

Carbachol injections into the hamster SCN produced phase advances during the late subjective night (CT22) and mid subjective day (CT6) and phase delays during the early

subjective night (CT14). The phase shifts generated by carbachol during the subjective night are in the same direction as those induced by light pulses (Pittendrigh and Daan, 1976), but these treatments had different effects on phase during the subjective day. On average, light pulses cause no net phase shifts when given around CT6 (Pittendrigh and Daan, 1976).

Phase shifts generated in response to carbachol at the three phases tested in this study are similar to those observed in previous studies of mice and Syrian hamsters (Zatz and Herkenham, 1981; Earnest and Turek, 1985). Other studies (on Syrian and Djungarian hamsters) have reported similar phase shifts to carbachol during the early subjective night and subjective day, but, small phase delays (as opposed to the advances observed in this study) during the late subjective night (Meijer et al., 1988; Wee and Turek, 1989). Differences between our findings and the results of these studies may reflect differences in the cholinergic systems of the species and strains used. Animals from different lines and strains have been shown, for example, to have different patterns of binding for cholinergic ligands and different anatomical distributions of ChAT activity (Jenni-Eiermann et al., 1986). The study by Meijer et al. (1988) used Syrian hamsters from a European source, while the study by Wee and Turek (1989) used Djungarian hamsters. Another possible contributor to the observed differences is the use of artificial cerebrospinal fluid or Kreb's Ringer solution as a drug vehicle in these studies, as compared with saline vehicle which was used in our study and in other studies (Earnest and Turek, 1985; Zatz and Herkenham, 1981) showing phase advances in the late subjective night.

Most earlier studies injected carbachol solutions into the lateral ventricles. The phase shifts generated by this method may be attributable to effects at multiple targets in the brain. By making localized injections of carbachol into the SCN, we have attempted to minimize any possible effects of carbachol on other brain regions. Because of the proximity of the injection site to the third ventricle, however, the possibility of leakage into the ventricles or effects on adjacent hypothalamic regions cannot be excluded. This approach, however,

makes it more likely that the observed effects result from the action of carbachol on those cholinoceptive neurons in the SCN that are involved in rhythm generation or which influence SCN pacemaker cells.

Atropine, a muscarinic antagonist, was able to reduce significantly the phase shifting effects of carbachol on locomotor activity at two of three circadian phases tested, suggesting that the effects of carbachol are mediated in large part through muscarinic receptors located in the SCN (see chapter II). Mecamylamine is a nicotinic receptor antagonist which may also affect amino acid transmission in some cases. Its administration had little or no effect on the phase shifts induced by carbachol, indicating a lack of involvement of nicotinic receptors in mediating the effects of carbachol.

Only one study has previously investigated the effects of muscarinic and nicotinic receptor antagonists on the effects of carbachol on circadian rhythms (Zatz and Brownstein, 1981). In that study, D-tubocurarine, a nicotinic receptor antagonist, blocked the acute suppression by intraventricularly administered carbachol of the nocturnal rise in pineal SNAT in rats. Atropine on the other hand, did not block the suppressive effect of carbachol, but seemed instead to potentiate it, making the response to the carbachol/atropine combination more similar to the response to light than was the response to carbachol alone. The values of SNAT obtained in that study (in picomoles SNAT activity/10min/gland) for different treatments were: control: 1720, carbachol: 356, carbachol + atropine: 54, and light: 79 (Table 1, Zatz and Brownstein, 1981). The potentiation of carbachol effects by atropine suggest the possible existence of presynaptic muscarinic receptors that regulate ACh release from nerve terminals somewhere along the pathway from the SCN to the pineal or antagonistic effects of muscarinic and nicotinic receptors on the regulation of pineal SNAT.

There are many differences between the present study and that of Zatz and Brownstein (1981) which may account for the different conclusions reached about the receptors mediating carbachol effects on rhythms. First, different species were studied using

different functional endpoints. Carbachol may affect pineal SNAT in rats via nicotinic receptors and it may affect hamster activity rhythms via a muscarinic site of action. Second, we used localized injections of the antagonists into the SCN, thereby minimizing any potential effects on other circumventricular structures, while injections in the Zatz and Brownstein (1981) study were into the lateral ventricles. It is possible that the antagonists used in the rat study affected cells with cholinergic receptors in other parts of the brain which project to the SCN or affect the pineal gland more directly. Third, the concentration of atropine used in the rat study was 100 fold lower than in this study.

Several lines of evidence have suggested the hypothesis that a single mechanism may mediate the effects of carbachol and light on circadian rhythms, and that both depend on nicotinic receptors (Zatz and Brownstein, 1979; Zatz and Brownstein, 1981; Keefe et al., 1987). First, carbachol and light caused similar phase shifts at some circadian phases. Second, light pulses administered in the middle of the dark phase to rats maintained in an LD cycle resulted in a tripling of ACh concentrations in the SCN within 60 min (Murakami et al., 1984), suggesting that light stimulates an increase in the ACh content in the SCN, induces its *de novo* synthesis or inhibits its degradation. Finally, antagonists putatively active at nicotinic sites are able to block photic effects on pineal and activity rhythms (Zatz and Brownstein, 1981; Keefe et al., 1987). In the former study, α-BTX was used to block the action of light in decreasing nighttime levels of pineal SNAT, and in the latter study mecamylamine was shown to block the phase shifting effects of light on activity rhythms.

It is unclear, however, whether these drugs exert their effects through their actions at cholinergic nicotinic receptors in the SCN. Although, α-BTX binds with very high affinity to nicotinic receptors in the periphery, there is little overlap between its binding sites and those of [^{3}H] nicotine and [^{3}H] ACh in the central nervous system, and particularly in the SCN where [^{3}H] nicotine does not bind at all (Clarke et al., 1985; Miller et al., 1982, 1984; Wonnacott, 1987). Furthermore, other nicotinic antagonists compete poorly for α-

BTX's binding sites (Martin et al., 1989), suggesting that α-BTX binds either to a novel kind of nicotinic receptor, not recognized by conventional nicotinic antagonists, or to a non-cholinergic site. There is also some controversy surrounding the antagonistic actions of mecamylamine. It has also been shown to affect transmission via NMDA receptors (O'Dell and Christensen, 1988; Snell and Johnson, 1989). It is, therefore, possible that some of the effects of mecamylamine are the result of its action on NMDA receptors, which may be very relevant to circadian rhythm phase-shifting.

There is evidence that excitatory amino acids (EAA), including glutamate, are released from the terminals of retinal ganglion cells located in the SCN (Shibata et al., 1986; Moffett et al., 1989; Moffett et al., 1990), and antagonists active at both NMDA and non-NMDA EAA receptors can block photically induced phase shifts (Colwell et al., 1991) and expression of the immediate-early gene *c-fos* in the SCN (Abe et al., 1991; Abe et al., 1992). The present results and the ambiguities associated with previous findings provide little support for nicotinic mediation of the effects of carbachol in phase shifting rhythms. There is also reason to doubt that nicotinic receptors play a critical role in mediating photic effects on rhythms.

The peripheral administration of atropine in earlier tests of muscarinic mediation of photic effects (Zatz and Brownstein, 1981; Pauly and Horseman, 1985) allows for the possibility that actions on muscarinic receptors remote from the SCN counteracted whatever effect atropine might have in the SCN itself. The timing of atropine administration may also be important, but is not reported by Zatz and Brownstein (1981). Pauly and Horseman administered atropine 2 hr before the light pulse, thereby increasing the likelihood of its being metabolized. About 80% of atropine administered peripherally is excreted in the urine within 2 hours. Furthermore, some rodents have a specific enzyme (atropine esterase) that rapidly metabolizes atropine (Katzung,1987). Therefore, previous tests may not have addressed the issue adequately, and it remains plausible that, as with

carbachol-induced phase shifts, light-induced phase shifts of rodent circadian rhythms may involve a role for muscarinic rather than nicotinic receptors.

A simple dichotomy between muscarinic and nicotinic receptors in the CNS may prove to be an inadequate way of discussing cholinergic receptor subtypes. Genes encoding the neuronal nicotinic receptor subunits have been characterized including three α-subunits and one β-subunit (Boulter et al., 1987). The gene encoding the β-subunit is widely expressed in most areas in the brain, but the α-subunits (α2, α3 and α4) are each expressed selectively, but with some overlap, in different brain regions. In the SCN, however, only the β-subunit mRNA and none of the α-subunits are expressed (Wada et al., 1989), suggesting either the absence of nicotinic receptors or the existence of a cholinergic nicotinic receptor subtype which is unlike those known to be present elsewhere in the brain. Based on the present results, however, it appears more likely that the phase shifting effects of carbachol are mediated by a receptor type which more closely resembles the classical muscarinic than the nicotinic receptor.

The exact location of muscarinic receptors in the SCN is not known. Based on the evidence of transport of ACh receptors from the retina to the tectum (Henley et al., 1986) and the lack of transport of nicotinic receptors from the retina to the SCN (Swanson et al., 1987), it is possible that some of the ACh released into the SCN acts on muscarinic receptors located presynaptically on retinal terminals, which may release EAAs onto SCN cells. Thus, exogenously applied cholinergic agonists act presynaptically on the retinal terminals to alter the release of an EAA, which, in turn, acts on pacemaker cells to cause phase shifts. Alternatively, or additionally, carbachol may act on muscarinic receptors located directly on SCN cells. This hypothesis is discussed in further detail in chapter VI.

In summary, the present results indicate that carbachol acts through muscarinic rather than nicotinic receptors, probably in the SCN, to shift the phase of the circadian rhythm in hamsters. Although sensitivity to carbachol resembles that to light at only some phases, it remains possible that muscarinic receptors are involved in mediating photic effects on

rhythms. The failure of atropine to block the effects of carbachol as completely at CT14 as at the two other phases tested may reflect differential sensitivity to cholinergic drugs at these phases, or it may be related to differences in the neurochemical mechanisms mediating phase delays and phase advances (Ralph and Menaker, 1988). A better understanding of the role of ACh in this system will depend in part on elucidating the relations between cholinergic mechanisms and the glutamatergic mechanisms that have been implicated in the transmission of photic information to the SCN.

Figure 18 Free-running rhythm of a hamster maintained in constant darkness. Arrows represent day of administration and black dots represent time of administration of drugs. Numbers next to arrows show the circadian time of administration. AC- atropine+carbachol; C-carbachol; MC-mecamylamine+ carbachol; S-saline. Period lengths were calculated by drawing an eye-fitted line through the activity onsets for ten day intervals before injections and ten day intervals after injections. Phase shifts were calculated by measuring the difference in minutes between the phases predicted by the two eye-fitted lines extrapolated to the day after the injection. Carbachol produced phase advances at CT6. Mecamylamine was unable to block the carbachol-induced phase shifts, whereas, atropine was able to block them. Saline injections at this phase had no effect on the rhythm.

FIGURE 18 **Animal # 7**

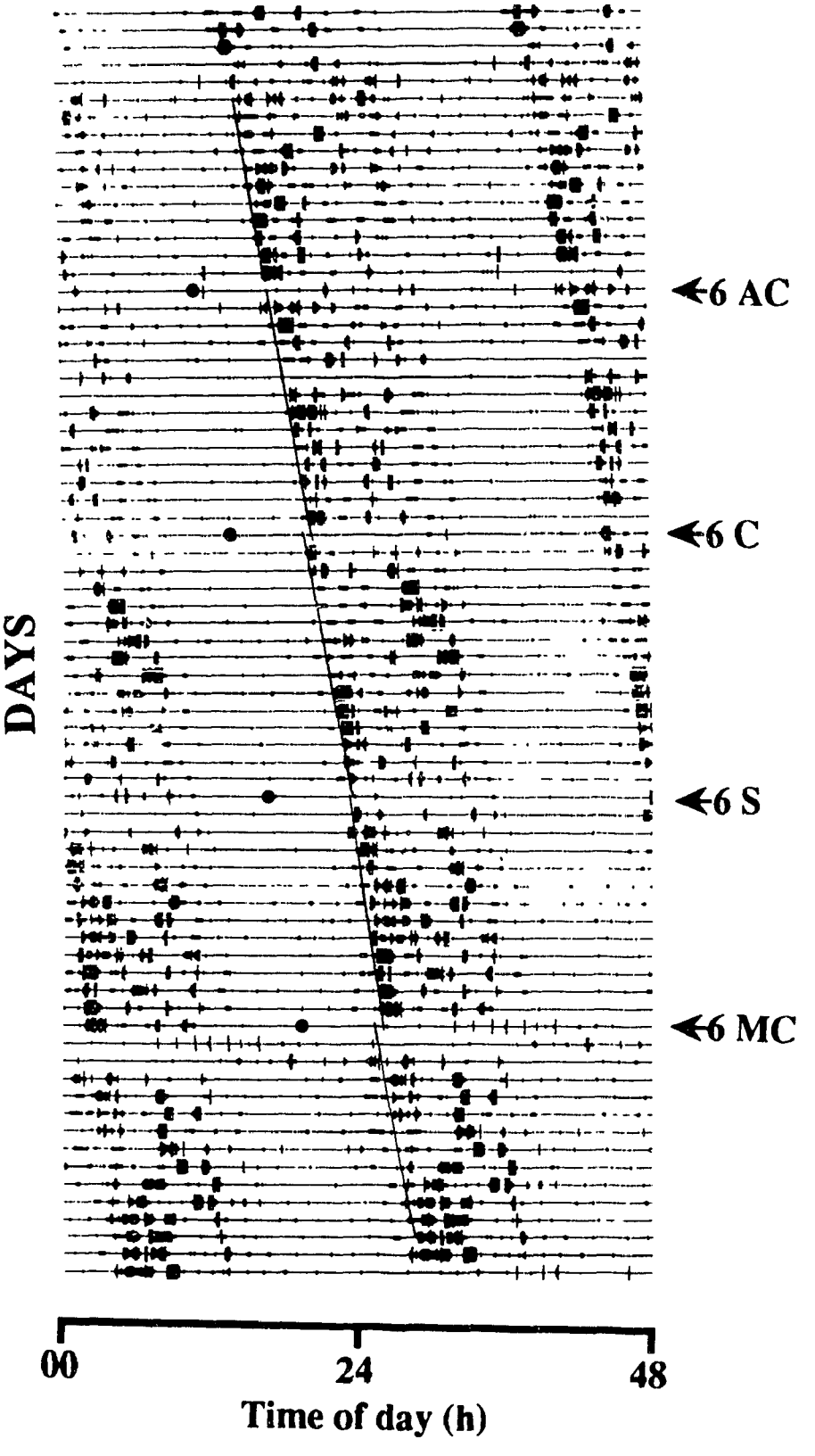


Figure 19 Actogram of an animal administered carbachol, mecamylamine+carbachol and atropine+carbachol during the early subjective night (CT14). Carbachol produced phase delays at this phase. Mecamylamine was unable to block these phase shifts. Atropine, when administered 10 min before carbachol, blocked the phase shifts induced by carbachol.

FIGURE 19

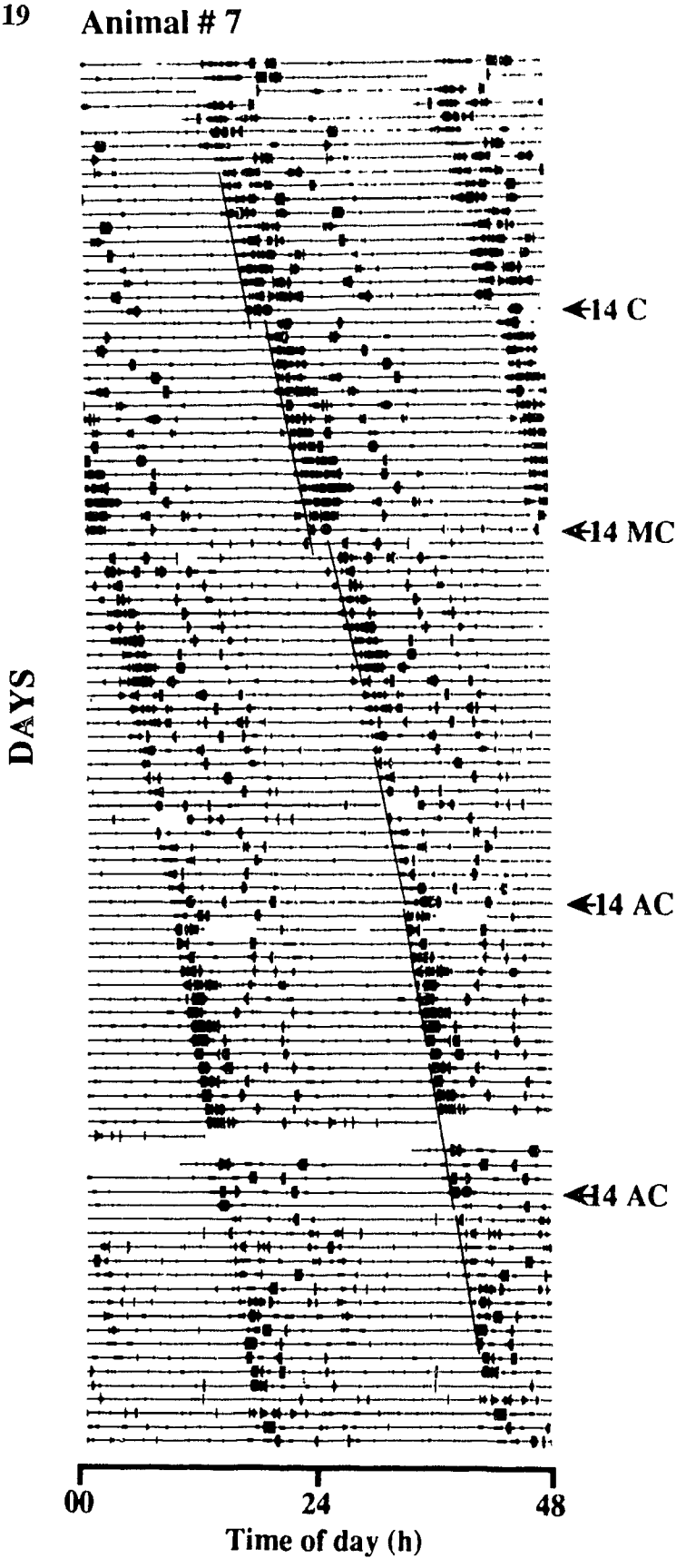


Figure 20 The left panel shows that carbachol produces phase delays at CT14 and phase advances at CT6. Atropine blocked the advancing phase shifts at CT6. Atropine administered alone had no effect on the circadian rhythm. The right panel shows the drugs administered at CT22 (late subjective night). Carbachol produced phase advances at this phase. Mecamylamine was unable to block the phase shifts produced by carbachol, whereas atropine blocked the carbachol-induced phase shifts.

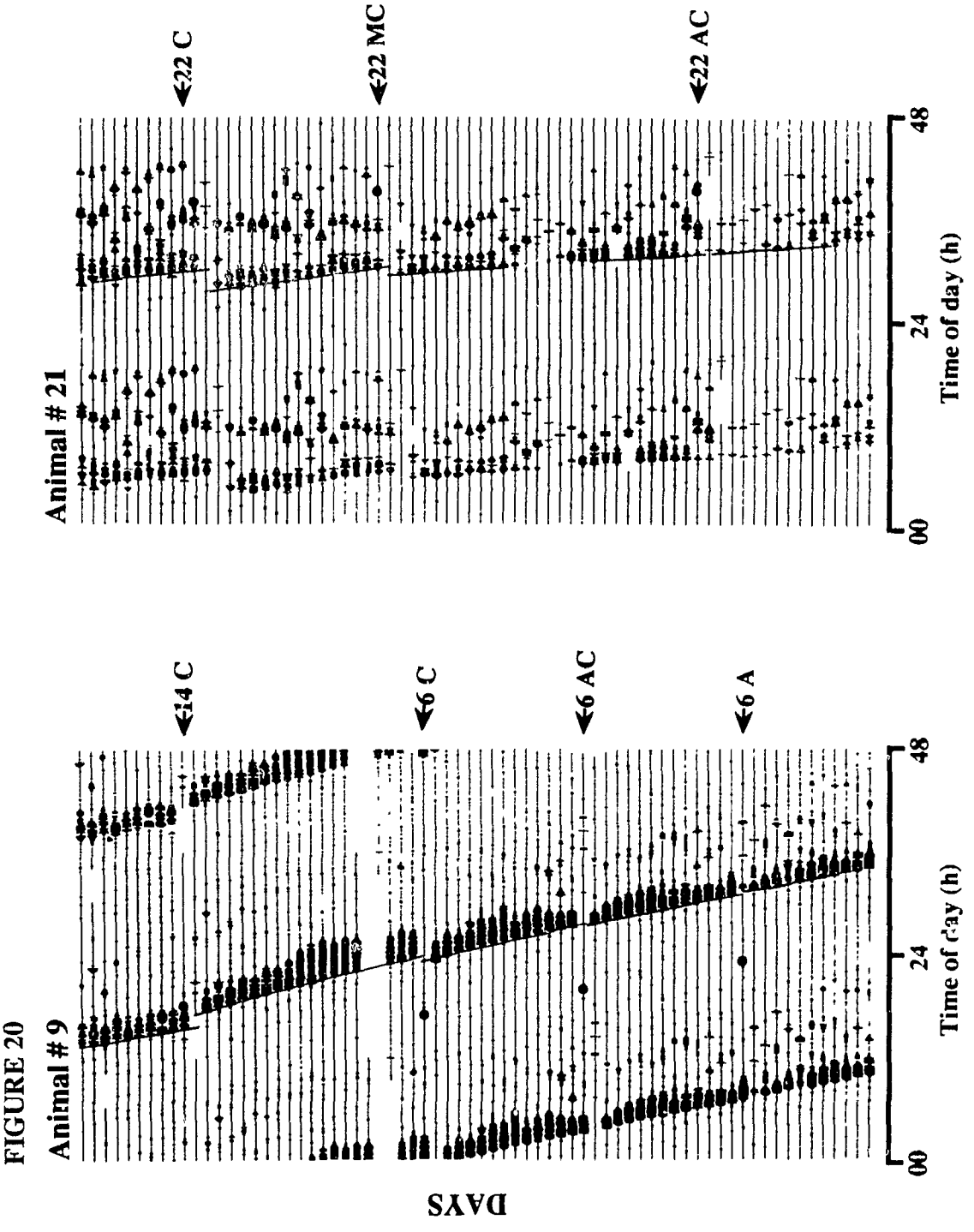
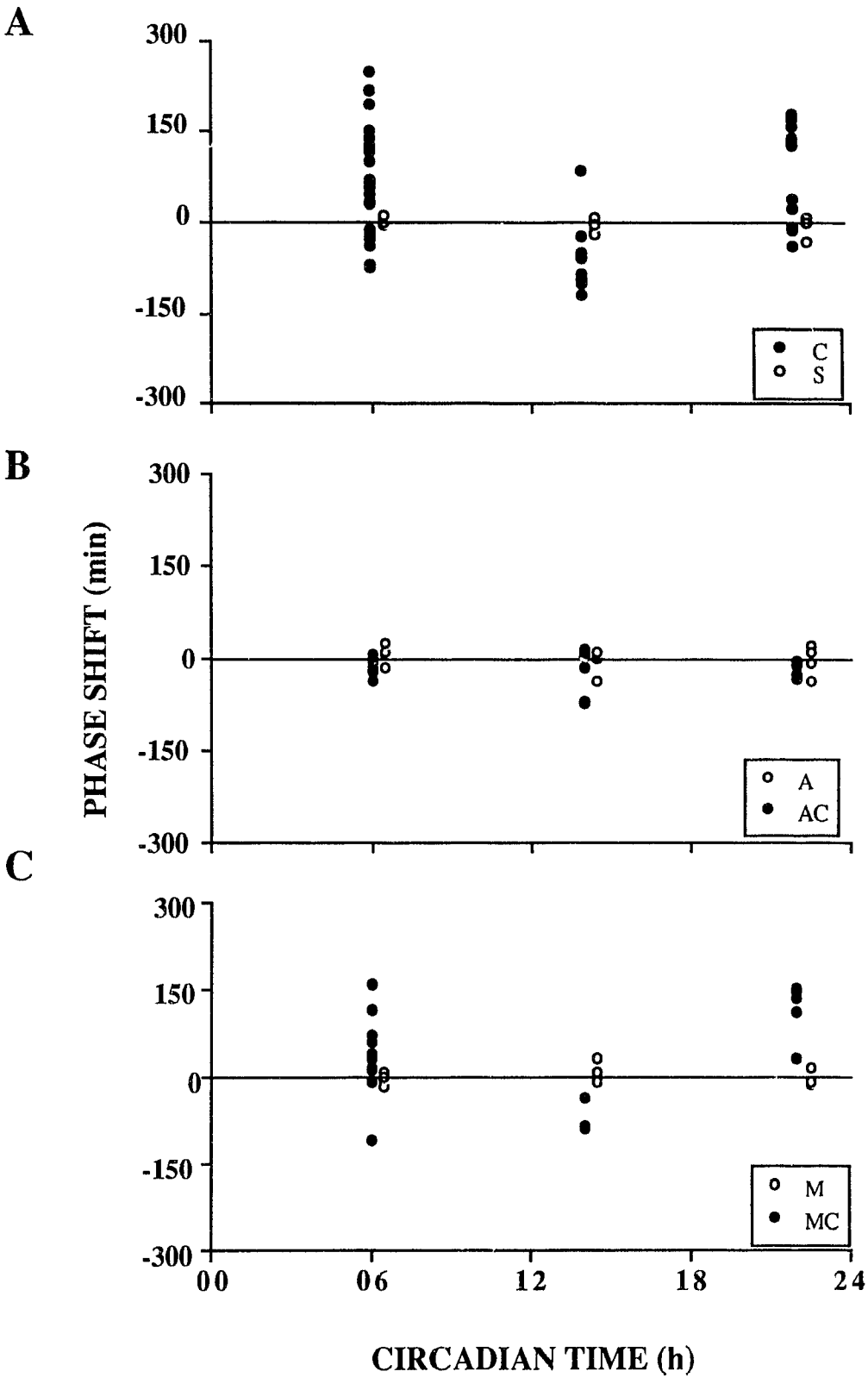

Animal # 21
22 C
22 MC
22 AC
00
24
48
Time of day (h)
Animal # 9
14 C
6 C
6 AC
6 A
00
24
48
Time of day (h)
DAYS

FIGURE 20

Figure 21 Phase shifts induced by various drugs at CT6, CT14 and CT22. Carbachol produced major phase advances at CT6 and CT22, whereas it produced phase delays at CT14 (panel A). Panel B shows phase shifts at CT6, CT14 and CT22 following administration of atropine alone or with carbachol. Atropine administered before carbachol blocked phase shifts at CT6, CT14 and CT22. Atropine alone had no effect on the rhythm. Panel C shows the effect of mecamylamine given with carbachol or by itself. Mecamylamine did not block the phase shifts produced by carbachol, and had no phase shifting effects by itself.

FIGURE 21


A
300
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A
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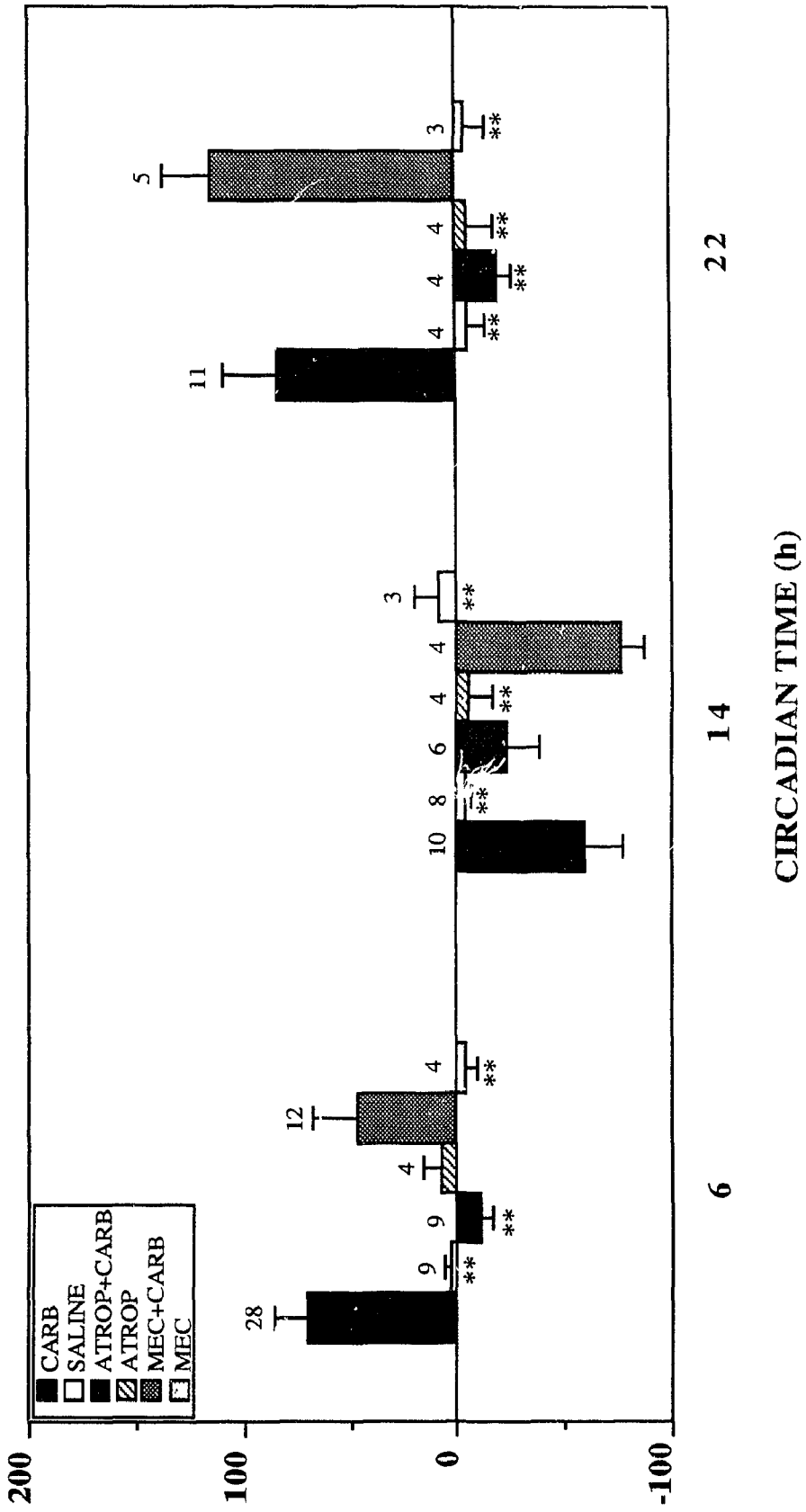
PHASE SHIFT (min)
C
300
150
0
-150
-300
M
MC
00
06
12
18
24
CIRCADIAN TIME (h)

Figure 22 Mean phase shifts produced by carbachol (Carb), saline, atropine, atropine+carbachol, mecamylamine and mecamylamine+carbachol at CT6, CT14 and CT22. Asterisks represent significant differences from the mean phase shift produced by carbachol at a given phase ($p<0.05$). Numbers are the number of tests and bars the standard errors of the mean.

FIGURE 22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SALINE
ATROP+CARB
ATROP
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200
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PHASE SHIFT (min)
6
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22
CIRCADIAN TIME (h)
28
9
9
4
12
4
10
8
6
4
4
3
11
4
4
4
5
3
**

TABLE 1. Effect of carbachol and various cholinergic antagonists on the phase of circadian rhythms. Mean $\Delta\phi$ represents the average phase shift (min) for that circadian time (CT). SEM: standard error of the mean. Stars represent values significantly different ($p<0.05$) from the phase shifts produced by carbachol at their respective CTs.

Drug	CT6			CT14			CT22		
	Mean $\Delta\phi$	SEM	n	Mean $\Delta\phi$	SEM	n	Mean $\Delta\phi$	SEM	n
CARBACHOL	69.8	15.7	28	-59.7	18.2	10	83.9	24.8	11
SALINE	2.0*	3.3	8	3.12*	3.5	8	-6.00*	8.2	4
ATROPINE + CARBACHOL	-11.7*	4.8	9	-23.1	16.1	6	-20.0*	6.6	4
ATROPINE ALONE	6.7	8.2	4	-5.0*	-11.9	4	-5.00*	13.2	4
MECAMYLAMINE + CARBACHOL	45.8	21.8	12	-76.2	12.1	4	114.4	21.7	5
MECAMYLAMINE	-4.7*	5.5	4	-8.3*	11.6	3	-4.60*	8.8	3

CHAPTER V

ROLE OF NERVE GROWTH FACTOR IN INDUCING PHASE SHIFTS OF HAMSTER ACTIVITY RHYTHMS

The presence of NGF and its precursors in the SCN has been demonstrated using blot hybridization techniques and immunocytochemistry (Senut et al., 1990; Ojeda et al., 1991). These observations suggest that cells in the SCN are capable of synthesizing NGF and/or are involved in the uptake and transport of its precursor molecules. NGF-R exist in the SCN on the terminals of retinal ganglion cells and basal forebrain neurons that project to the SCN (chapter III). Taken together, these findings suggest that NGF is released from the cells in the SCN and binds to NGF-R on afferents to the SCN, triggering a series of physiological changes in these neurons in the retina and basal forebrain. While the nature of these changes is not known in this case, NGF in the hippocampus has been shown to increase the synthesis of ChAT (Hefti et al., 1984; Hefti et al., 1985; Mobley et al., 1985; Hatanaka et al., 1988; Cavicchioli et al., 1991) and the synthesis and release of ACh in the basal forebrain (Kewitz et al., 1990; Lapchak and Hefti et al., 1991). These findings suggest that NGF may be altering cholinergic function in cells afferent to the SCN.

Carbachol (a cholinergic agonist) administered into the SCN produces phase shifts of wheel-running activity rhythms in hamsters (chapter IV), and the temporal pattern of sensitivity to carbachol partially mimics the phase shifting effects of light on locomotor activity rhythms in mice (Zatz and Herkenham, 1981), hamsters (Meijer et al., 1988; Wee and Turek, 1989), and rats (Mistlberger and Rusak, 1986), and the pineal serotonin N-acetyltransferase (SNAT) rhythm (Zatz and Brownstein, 1979, 1981) in rats. These observations suggest the possibility that acetylcholine plays a role in the photic entrainment pathway. As discussed in chapter IV, the phase shifting effects of exogenously

administered carbachol may mimic the action of ACh released in the SCN from the terminals of cholinergic neurons in the basal forebrain and the brainstem.

It is, therefore, possible that NGF is involved in the modulation of the phase of circadian rhythms through its action on cholinergic neurons. The present study is designed to test whether NGF administered into the SCN can phase shift the wheel running activity rhythms of hamsters, and if so, whether the effects are phase-dependent and similar to those of carbachol.

METHODS

Fifty adult male hamsters (*Mesocricetus auratus*; LVG:lak), weighing 80-100 g, obtained from Charles River (St.Constant, Quebec) were group-housed under a light-dark schedule (LD 14:10) for two weeks. NGF and Cytochrome-c were obtained from Sigma Chemical Co. (St. Louis, MO). The antibody to NGF (anti-NGF) was obtained from Collaborative Res. Inc. (Bedford, MA)

Before the start of the experiment, the animals were anesthetized and implanted with stainless steel indwelling guide cannulae as described in chapter IV. After recovery from anesthesia, the animals were individually housed in cages equipped with running wheels and maintained under constant dim red light (DD) with free access to food and water. Running wheel activity was monitored as described in chapter IV.

Injections

After a stable free-running rhythm was established, hamsters were injected intracranially with 2 µl of 0.9% saline or a saline solution of one of the following drugs: NGF (20ng/µl saline), Cytochrome-c (Cyt-c; 300ng/µl; physicochemically similar to NGF, but lacks its biological activity (Alleva et al., 1986)), antibody raised against NGF (anti-NGF; 125ng/µl) or normal rabbit serum (to determine if non-specific proteins in the antiserum have any phase shifting effects). To assess further the specificity of the phase

shifting effects of NGF, 1μl of anti-NGF was in some cases administered via the cannula 10 min prior to the administration of NGF.

In addition, in order to assess whether the effects of NGF were mediated by the same mechanisms as those activated by carbachol, 1μl of carbachol was co-administered with 1μl of NGF or 1μl of atropine was administered 10 min prior to the administration of 1μl of NGF in some animals. Injection procedures were as described in chapter IV. Each animal received one of these injections in a random order at circadian time (CT) 6, 14 or 22. These three phases were chosen to compare the phase shifting effects of NGF to those of carbachol which were previously examined at these phases (chapter IV). A minimum interval of two weeks was allowed between injections. Period lengths of hamsters before the administration of NGF were monitored and correlated with the type of phase shift produced. Animals were divided into three groups on the basis of pre-drug baselines: those with short periods (τ smaller than 23.83 hr); intermediate periods (τ around 24 hr, ranging from 23.83 hr to 24.17 hr) and long periods (τ longer than 24.17 hr). Phase shifts were calculated and the results analyzed as described in chapter IV. At the end of the experiment, the hamsters were sacrificed and their cannula placements were verified histologically as described in chapter IV.

RESULTS

In total, 195 injections were made into the vicinity of the SCN in 38 hamsters. Light microscopic evaluation of cannula placements revealed that 31 of 38 hamsters, representing 161 injections, had cannula placements within 500 μm of the SCN, none of the animals had placements further from the SCN than 500μm and 34 injections could not be evaluated because of either an inability to determine cannula placements or highly variable onsets of activity following an injection, and the data were discarded.

A phase response curve was constructed using 72 injections of NGF alone, made at CT6, 14 and 22. NGF administered at CT6 and CT22 produced mostly phase advances

(30.9+/-8.39 min and 36.93+/-11.07 min; mean+/- standard error of the mean) whereas at CT14 it produced mainly delays (-31.26+/-11.7 min) (Figure 23, 24, 25, 26 and 27A and B). At CT14 some of the phase shifts were accompanied by changes in period length, thereby making assessments difficult in some cases. With a few exceptions, hamsters with intermediate and long periods showed phase advances at CT6 and CT22 and phase delays at CT14. However, hamsters with short periods (< 23.83 h) tended to show greater variability in magnitude and direction of phase shifts generated by NGF injections (Figure 28A and B).

Saline injections had no phase shifting effects at any of the phases tested (Table 2). Cyt-c produced little or no phase shift at CT6 (2.5+/-3.5 min) or CT22 (0.5+/-4.5 min), but at CT14 it produced large phase delays (-76+/-22 min). Examples of animals given NGF, saline, Cyt-c or NGF preceded by anti-NGF at CT6, CT14 and CT22 are shown in Figures 23, 24, 25 and 26.

Anti-NGF, administered into the SCN 10 min prior to NGF, decreased the phase shifts induced by NGF at CT6 and 22 (-5.6+/-6.7 and -3.2+/-6.3min respectively) (Figure 29A). At CT14, however, anti-NGF appeared to have little effect on the NGF-induced phase shifts. Anti-NGF administered by itself produced phase shifts at all three phases tested (CT6 43+/-18 min; CT14 -60+/-10 min; CT22 26.2+/-23.7 min). Normal rabbit serum produced no phase shifts when administered at CT6 and CT22 (1.75+/-5.2 min and -4+/-4 min respectively). However, like Cyt-c and anti-NGF, rabbit serum produced phase delays (-20+/-0 min) at CT14.

Analysis of variance performed on phase shifts induced by NGF, saline, Cyt-c, rabbit serum, Anti-NGF and Anti-NGF administered together with NGF revealed that there was no significant differences among groups at CT6 ($F_{(5, 40)}$ =2.22; p= 0.07), CT14 ($F_{(5, 32)}$ =1.9; p=0.12) or CT22 ($F_{(5, 43)}$ =1.14; p=0.34). However, when only animals with long and intermediate period lengths receiving NGF were compared with the other drug effects, significant differences among the various drug applications were observed at CT6 ($F_{(5, 36)}$

=2.77; p= 0.03), CT14 ($F_{(5, 27)}$ =3.25; p=0.01) and CT22 ($F_{(5, 33)}$ =2.46; p=0.05). The lack of significant differences among drug effects when all NGF injections were considered may be attributable to the high degree of variability in the direction of phase shifts observed in animals expressing short periods.

Carbachol co-administered with NGF produced phase advances at CT6 (48.7+/-12.6 min) and CT22 (114.5+/-32 min) and phase delays at CT14 (-33+/-35 min). The phase shifts induced following co-administration of NGF and carbachol were not significantly different from either NGF or carbachol administered alone at CT6 ($F_{(2, 54)}$ = 1.86; p=0.16 and CT14 ($F_{(2, 31)}$ =0.82; p=0.44) (Figure 30B). However, at CT22, the two drugs together produced significantly larger phase shifts than NGF alone ($F_{(2, 43)}$ =3.8; p=0.02), although they were not significantly larger than those produced by carbachol alone.

Atropine administered 10 min before NGF administration produced a significant reduction in the NGF induced phase shifts at CT6 ($F_{(1, 24)}$ =4.3; p=0.04) and at CT22 ($F_{(1, 31)}$ =5.1; p=0.03) (Figure 30A). At CT14, however, atropine had no effect on the phase shifts induced by NGF ($F_{(1, 19)}$ =0.89; p=0.35). The mean phase shifts produced at CT6 and CT22 by NGF after treatment with atropine are shown in Table 3.

DISCUSSION

NGF injections into the hamster SCN produced phase advances during the late subjective night (CT22) and mid subjective day (CT6), and phase delays during the early subjective night (CT14). Although most NGF administrations, at each of the three phases tested, elicited a phase shift in one direction or the other which was consistent within a given animal, there was a degree of variability among animals which appeared to depend on the period length of the animal's rhythm. Administration of NGF in the SCN of hamsters with long periods (τ > 24.17 hr) or intermediate periods (τ between 23.83 hr and 24.17 hr) produced predominantly phase advances at CT6 and CT22, and phase delays at CT14.

However, animals with short periods ($\tau < 23.83$ hr) showed both advances and phase delays at all three phases. The reason for this variability among animals with short period lengths is unclear. In any given animal, the response to NGF at any of the three phases tested remained consistent. On average, animals with short period lengths produced larger phase delays and smaller phase advances compared with animals having intermediate or long periods. The direction of this difference is consistent with that observed in the responses to light pulses of hamsters with different period lengths (Daan and Pittendrigh, 1976). The pooling of data from animals regardless of period lengths probably accounts for the lack of significant differences between phase shifts generated by NGF and those produced by vehicle, Cyt-c or anti-NGF. It was not possible to assess differences among all drugs in animals within each period-length category because there were very few animals with short periods, and thus not all drugs could be tested at all phases in these animals.

The phase-shifting effects of exogenous NGF suggest that NGF may be released physiologically from SCN neurons and bind to NGF-R present in the SCN or elsewhere in the brain to cause phase shifts. The presence of NGF-R on terminals of retinal ganglion cells and basal forebrain cholinergic neurons in the SCN of rats was demonstrated in chapter III. In addition, NGF precursor protein and NGF mRNA have been shown in the rat SCN (Senut et al., 1990; Ojeda et al., 1991). However, whether NGF and NGF-R are present in the SCN of hamsters is not known. Cholinergic cells in the basal forebrain have been shown to project to the SCN of hamsters (Dwyer et al., 1990), suggesting that afferents similar to those in rats exist in hamsters. In addition, preliminary studies using a polyclonal antibody against NGF-R raised in chicken show the presence of NGF-R in the SCN of both hamsters and rats (unpublished observations). The pattern of NGF-R labelling found in the SCN and the basal forebrain in rats using the polyclonal antibody appears to be similar to that observed using the monoclonal antibody to NGF-R, thus suggesting that this antibody recognizes NGF-R. However, this antibody has not been

well characterized, and therefore it is not clear whether it recognizes only NGF-R; it was therefore not considered appropriate for use in anatomical studies in hamsters. Assuming that NGF-R are located on the terminals of retinal ganglion cells and that basal forebrain cholinergic cells project into the SCN of hamsters, the present results suggest that NGF released from cells in the SCN may contribute in some way to phase-shifting the circadian pacemaker.

Saline injections did not produce significant phase shifts at any of the phases tested. On the other hand, Cyt-c, which is physicochemically similar to NGF without its biological activity, produced no phase shifts at CT6 and CT22, while at CT14 it produced large delays. These results suggest that the phase shifting effects of NGF at CT6 and CT22 are through its binding to NGF-R, whereas, its action at CT14 appears to be non-specific and probably attributable to the phase shifting effects of some non-specific proteins common to NGF and Cyt-c solutions.

Anti-NGF administered 10 min before NGF blocked the phase shifting actions of NGF at CT6 and CT22. This blocking effect is presumably a result of binding of the exogenous NGF to anti-NGF, thereby preventing binding to NGF-R. At CT14, although phase shifts were blocked in some of the cases, many hamsters continued to produce large phase delays in response to the combined injections. These results suggest that either anti-NGF does not bind to the exogenous NGF selectively at CT14 (which seems improbable) or that the phase shifting action of NGF at this time is non-specific. The latter possibility is consistent with the effects of Cyt-c (described above), and is further confirmed by the phase delays induced at CT14 following injections of rabbit serum, which was ineffective at other phases. Taken together, the phase shifts induced by NGF, Cyt-c, anti-NGF and rabbit serum at CT14 suggest that the phase shifts induced by these drugs at this phase are non-specific. However, the absence of phase shifts following saline administration at CT14 suggests that the phase shifts induced by the other drugs, although non-specific, are biological, and not a mechanical artifact associated with the injection procedure itself.

Anti-NGF when applied by itself produced phase shifts at all three phases tested, with phase advances at CT6 and CT22 and phase delays at CT14. The phase shifts induced by anti-NGF at all three phases tested were similar in magnitude and direction to those induced by NGF. Possibly the phase shifting effect of anti-NGF results because the epitope on NGF responsible for the phase shifting effects or a functionally similar site is preserved in the anti-NGF molecule, and therefore results in it exerting a biological action similar to that of NGF. Although there is no biochemical evidence for this hypothesis, it is worth investigating further.

By definition, the active site is that part of the NGF molecule which is responsible for binding to its receptor. Since NGF molecules produced by different species are capable of generating identical or very similar results, it suggest that the active site is conserved in all such molecules (Harper and Thoenen, 1980). Thus, the NGF produced by mouse is capable of generating a biological effect in hamster. However, this is not the case with anti-NGF. Anti-NGF raised in reptiles is ineffective in inhibiting the biological effects of mammalian NGF, and the anti-NGF raised in mammals fails to block the biological effects of reptilian and avian NGF (Angeletti, 1971; Levi-Montalcini, 1972; Bailey et al., 1976; Bank and Carstairs, 1977). Anti-NGF binds to NGF made in the same species at a site adjacent to the active site and thereby causes a steric hindrance of the active site resulting in the inability of NGF to bind to NGF-R. However, when anti-NGF from a different species is used, the antibody molecule does not recognize the sites adjacent to the active site due to differences in some of the sequences which are not evolutionarily conserved (Harper and Thoenen, 1980) and, as a result, NGF is able to bind to its receptor and produce a biological effect. A degree of lack of homology in NGF sequence also exists among different mammalian species (Harper and Thoenen, 1980). Since anti-NGF used as the antiserum was raised in rabbit, it is possible that the endogenous NGF in hamster does not bind very well to this molecule. At the same time, it is also possible that anti-NGF raised

in rabbit has sequence homologies with the active site of hamster NGF and thus can bind to NGF-R, which results in the phase shifts generated by anti-NGF.

A second possibility is that, even if the NGF and the anti-NGF are a perfect fit, when large quantities of anti-NGF are administered by itself, the chance that all of it will be bound by endogenous, extracellular NGF in a very short time, before it can have other effects is small. However, when a large quantity of anti-NGF is injected followed by a large quantity of NGF, they are both available in large amounts outside the cells, and quite capable of binding each other up and cancelling any potential biological action.

A third possibility is that anti-NGF, when administered alone, enhances the release of endogenous NGF. Such an enhancement is possible if NGF release is homeostatically regulated such that a threshold level of NGF release sends a feedback signal to the NGF-producing cells, either through the binding of NGF to NGF-R located on the NGF producing cells themselves, or through a signal from their target cells, to inhibit its own release. In the presence of anti-NGF, endogenous NGF may be bound and therefore not available to produce a negative feedback signal, thus resulting in an enhanced release of NGF. This would presumably occur with a small delay, but this delay would still permit NGF release within the range of the circadian phase of sensitivity for NGF. When both anti-NGF and NGF are supplied exogenously, it is likely that they bind to each other and that there is little effect on existing endogenous NGF or newly synthesized NGF. As a result there is no enhanced release of endogenous NGF and no significant phase shift is seen. There is, however, no evidence to support the existence of such regulated feedback mechanisms, and this account of anti-NGF effects must be considered highly speculative for now.

On average, the phase shifts generated by NGF were in the same direction as those induced by carbachol at the three phases tested (chapter IV), and thus, also mimicked the phase shifting effects produced by light (Pittendrigh and Daan, 1976) at two of these phases. Carbachol co-administered with NGF did not produce a consistent synergistic or

additive effect. On the other hand, atropine administered 10 min before the administration of NGF not only blocked the NGF-induced phase advances at CT6 and CT22 but produced phase delays. Atropine in itself does not produce any phase shifts and it produced shifts in a similar direction when given prior to carbachol (Chapter IV). Furthermore, mecamylamine when administered before carbachol tends to produce larger advances at CT22 compared with those produced by carbachol alone at this phase. These observations suggest that carbachol and NGF mediate their phase-shifting effects through their actions on both muscarinic and nicotinic receptors and that muscarinic and nicotinic effects are in opposite directions. The inability of atropine to block NGF-induced phase shifts at CT14 further reinforces the idea that phase shifts at this phase are non-specific and unrelated to the physiological action of NGF. However, the possibility that NGF-induced phase shifts at this phase are induced through a specific, non-cholinergic mechanism cannot be ruled out. Since carbachol has also been shown to mediate phase shifts through its action on muscarinic receptors (chapter IV), it seems likely that both NGF and carbachol mediate their phase shifting effects through a common mechanism.

It is possible that NGF acts as a retrograde signal to the cholinergic neurons located in the basal forebrain, causing them to increase their activity. Increases in ChAT activity and ChAT mRNA following treatment with NGF have been reported (Hefti et al., 1984; Hefti et al., 1985; Mobley et al., 1985; Hatanaka et al., 1988; Cavicchioli et al., 1991). Furthermore, increases in ACh synthesis and release in the presynaptic cholinergic cells of 48% and 175% respectively following NGF treatment have also been demonstrated (Kewitz et al., 1990; Lapchak and Hefti et al., 1991). Taken together, these findings suggest the possibility that NGF mediates its phase shifting effects through its effect on ChAT activity, ACh synthesis or release and/or an induction of acetylcholine receptors.

However, the possibility also exists that NGF acts directly on pacemaker cells located in the SCN to produce the phase shifts. Apart from the induction of late genes whose maximal induction occurs 24 hr later, NGF also induces immediate early genes such as c-

fos (Curran and Morgan, 1985; Morgan and Curran, 1991), NGFI-A (zif 268) (Milbrandt, 1987) and NGFI-B (Milbrandt, 1988). The induction of c-*fos* and both NGFI-A and NGFI-B in the SCN following the administration of a nocturnal light pulse has been demonstrated (Rea, 1989; Aronin et al , 1990; Earnest et al., 1990; Kornhauser et al., 1990; Rusak et al., 1990; Rusak et al., 1992), suggesting that the induction of such genes and their associated transcriptional proteins are early steps in the modulation of pacemaker activity, leading to the generation of phase shifts. Therefore, it is possible that NGF can act directly on pacemaker cells to induce a variety of immediate early genes and thus initiate a cascade of intracellular events which overlap those occurring following a light pulse at some circadian phases.

Figure 23 Effects on the circadian rhythm of wheel-running activity of NGF (N) injected via cannula at CT14 and CT6 into the suprachiasmatic nucleus of a hamster. At CT14 NGF produced phase delays, and at CT6 it produced phase advances. Black dots show times, and arrows indicate the days, of drug administration. Days are plotted from top to bottom and the record is double-plotted in conventional manner to show a 48 hr span on each line. The height of each mark represents the intensity of activity in a 10 min bin.

FIGURE 23

Animal # 28

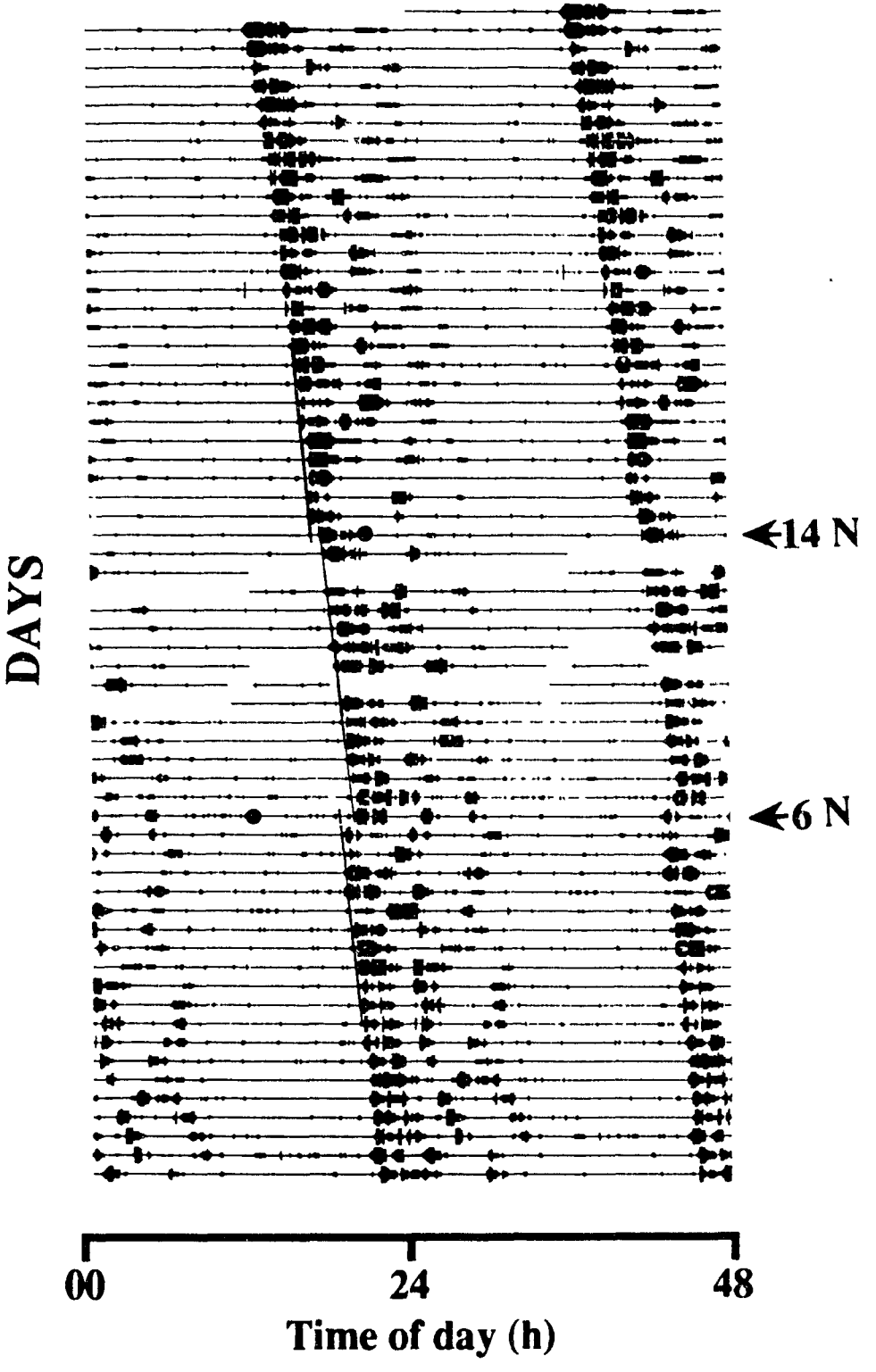

Figure 24 Effects of NGF injections at CT22 and CT14 on the circadian activity rhythm . NGF (N) produced phase advances at CT22 and phase delays at CT14. Saline injections (S) had a very small effect. A combination of Anti-NGF given 10 min before NGF (ANN) at CT22 produced little or no phase advance. See Fig 23 for more details.

FIGURE 24 **Animal # 40**

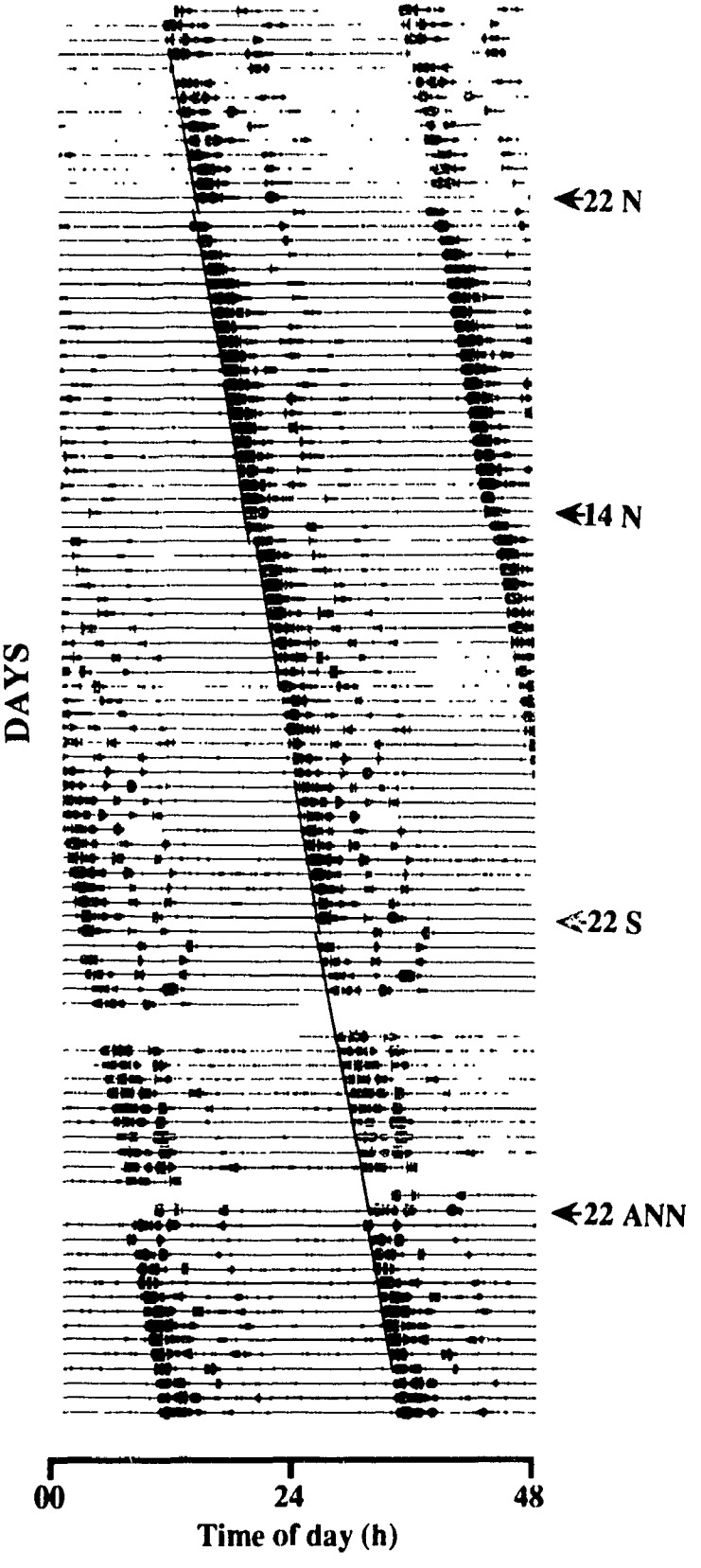

Figure 25 The left panel shows the effects of Cytochrome-c (CYT) at CT6, CT14 and CT22. Cytochrome-c had no phase-shifting effect at CT6 and CT22; however, at CT14 it produced large phase delays. See Figure 23 for more details.

FIGURE 25

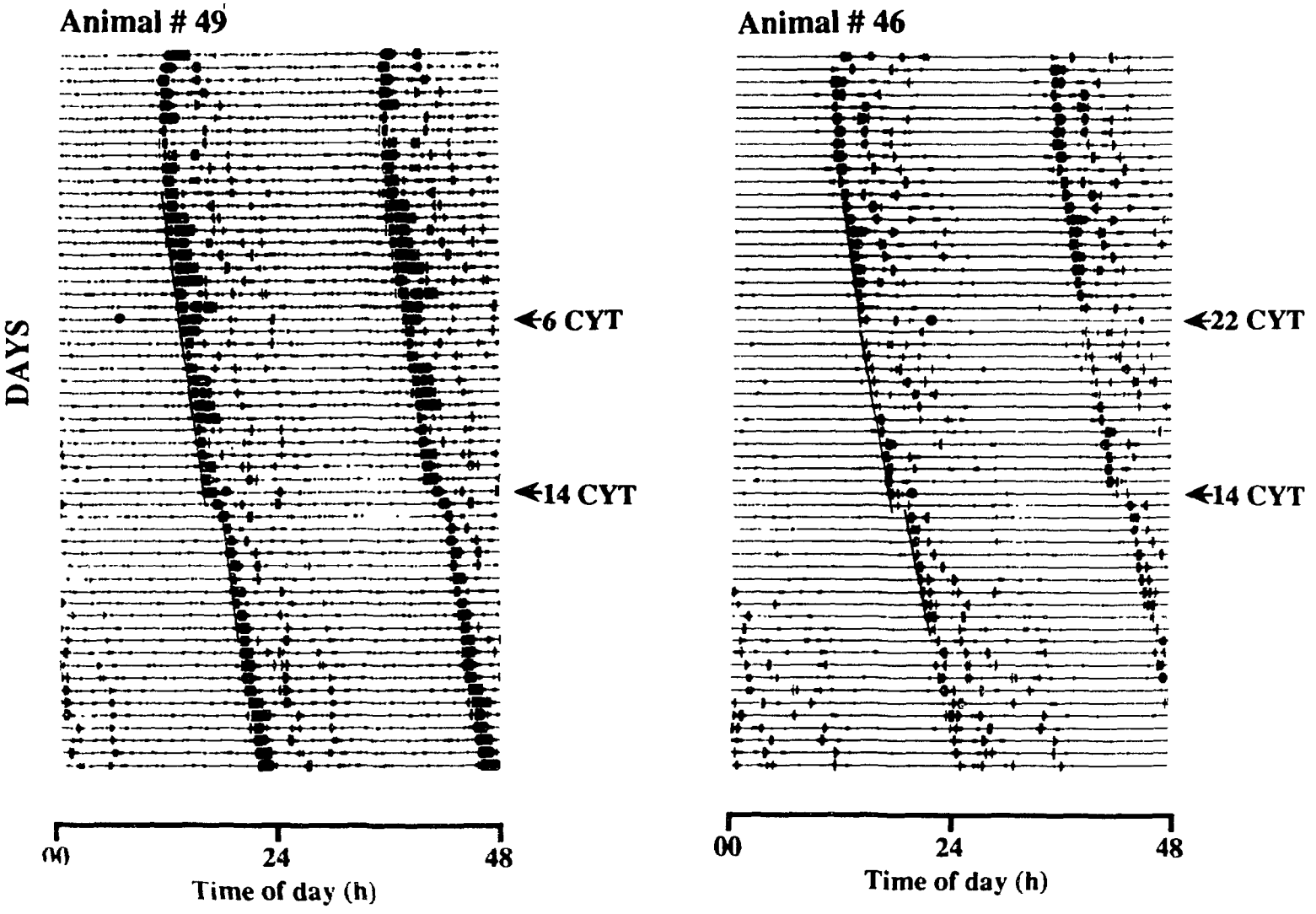
Animal # 49
Animal # 46
DAYS
6 CYT
14 CYT
22 CYT
14 CYT
00
24
48
Time of day (h)

Figure 26 Effects of the antibody to NGF (AN) on the circadian activity rhythm of hamsters. Anti-NGF produced phase advances at CT6 and CT22, whereas it produced phase delays at CT14. See Figure 23 for more details.

FIGURE 26

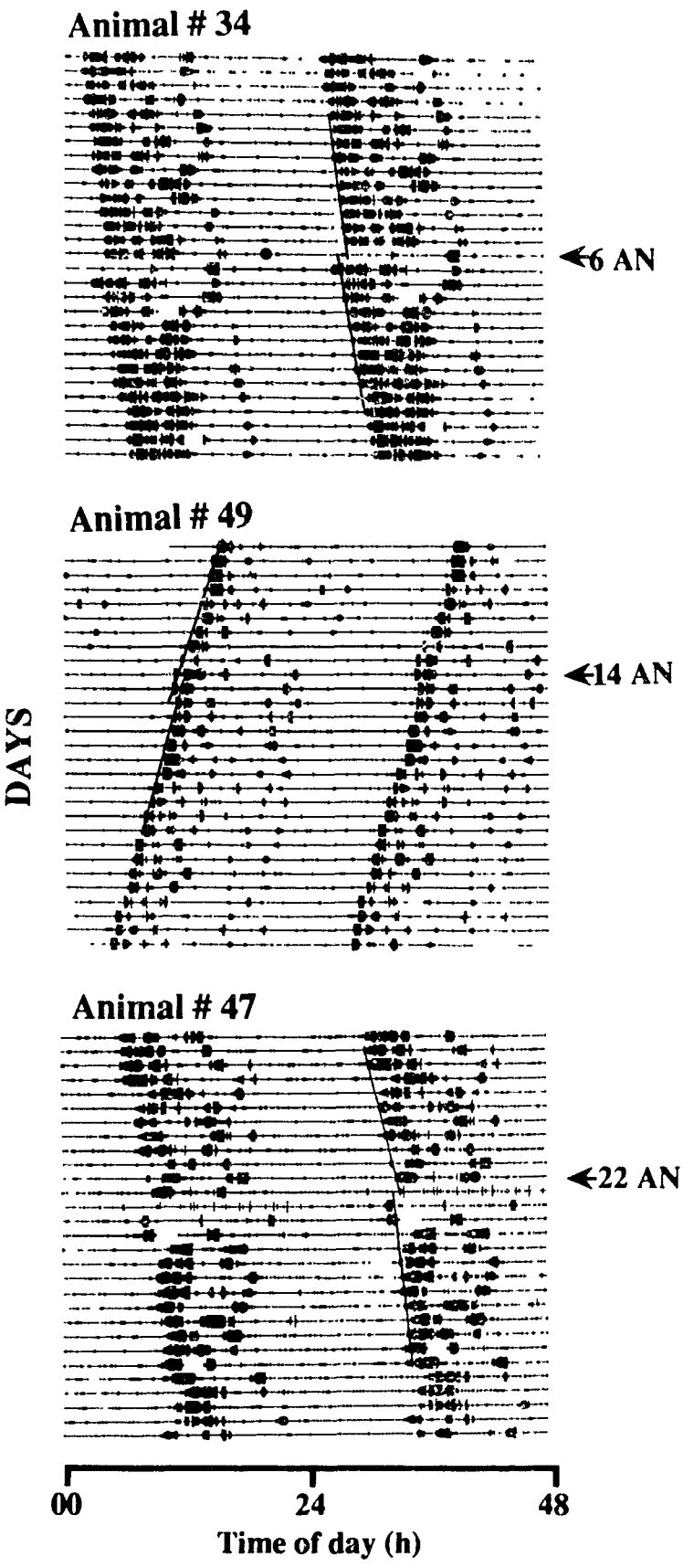
Animal # 34
6 AN
Animal # 49
14 AN
DAYS
Animal # 47
22 AN
00
24
48
Time of day (h)

Figure 27 Panel A shows the phase shifts induced by NGF, saline and Cytochrome-c at CT6, CT14 and CT22. Panel B shows the mean phase shifts produced by these drugs (+/- SEM) at these phases. NGF produced phase advances at CT6 and CT22, whereas it produced phase delays at CT14. Cytochrome-c had no effect when administered at CT6 and CT22, whereas it produced phase delays at CT14. Saline injections had no effect on the rhythm at any of the three phases.

FIGURE 27

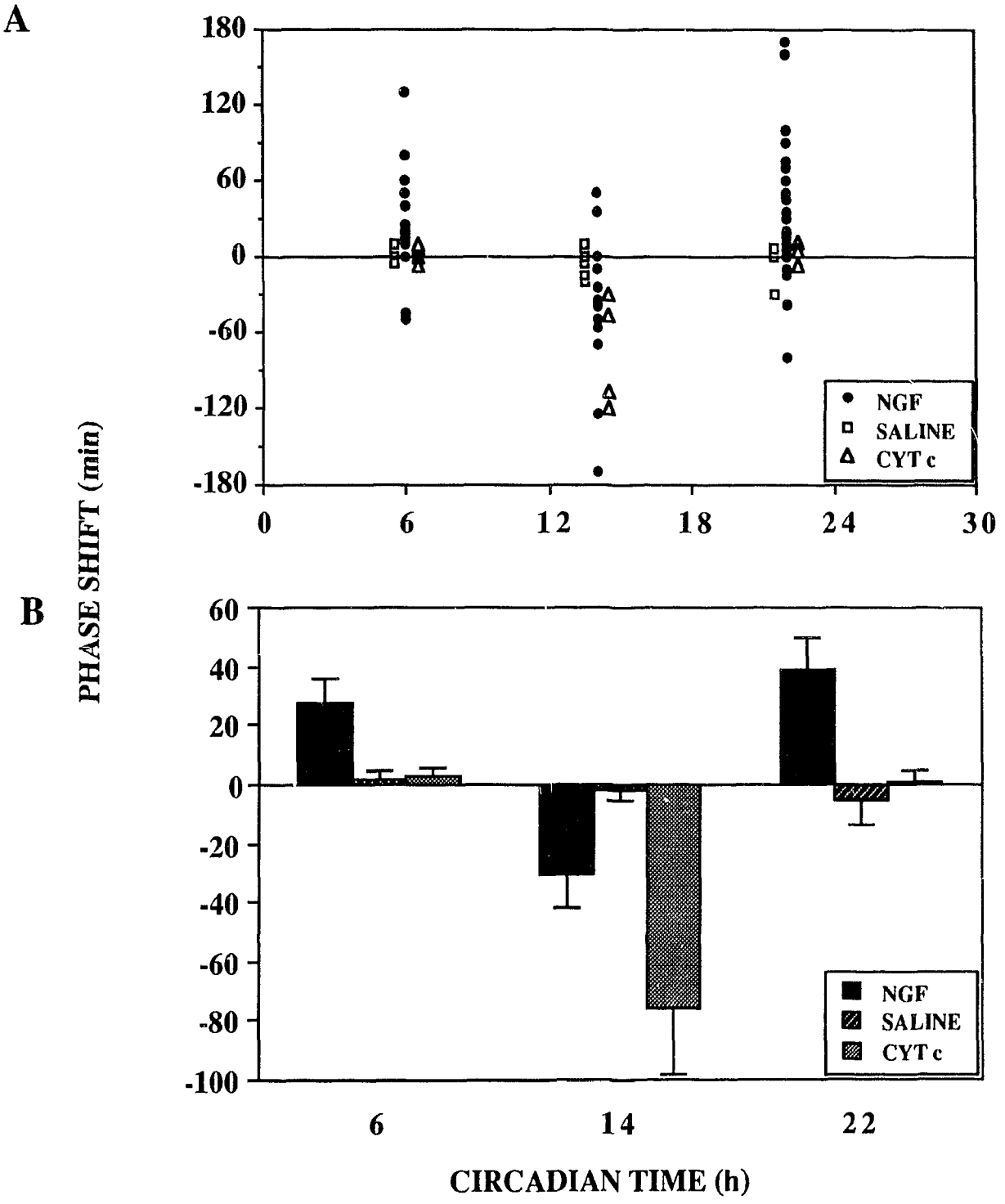
A
180
120
60
0
-60
-120
-180
0
6
12
18
24
30
NGF
SALINE
CYT c
B
PHASE SHIFT (min)
60
40
20
0
-20
-40
-60
-80
-100
6
14
22
NGF
SALINE
CYT c
CIRCADIAN TIME (h)

Figure 28 Effect of pre-injection period length on the phase shifts produced by NGF at CT6, CT14 and CT22. Animals with long τ (greater than 24.17 hr) and intermediate τ (between 23.83 and 24.17hr) showed phase advances at CT6 and CT22 and phase delays at CT14. Animals with short pre-injection period lengths (shorter than 23.83 hr) showed a high degree of variability in the direction of the phase shifts produced.

FIGURE 28

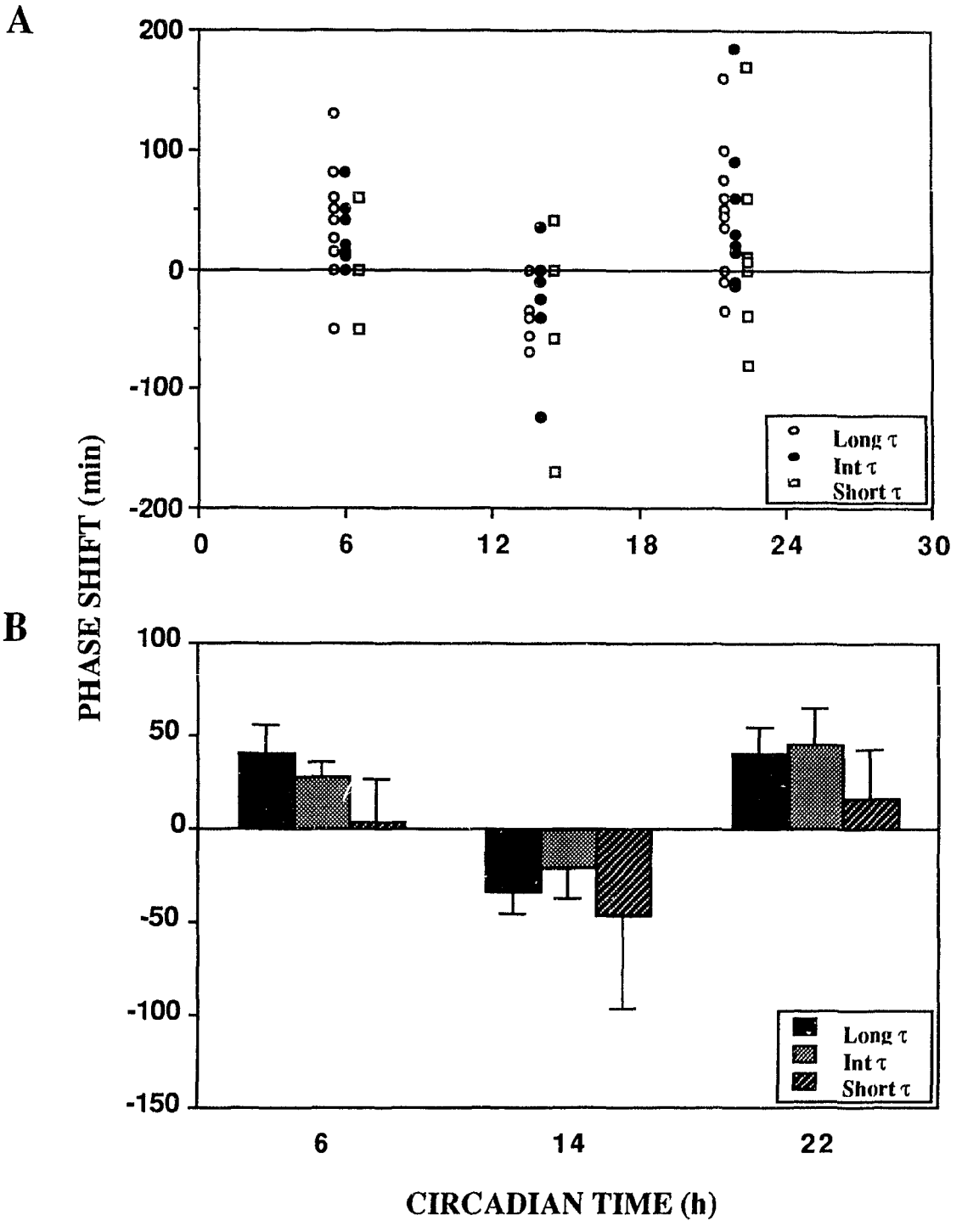
A
200
100
0
-100
-200
0
6
12
18
24
30
Long τ
Int τ
Short τ
B
PHASE SHIFT (min)
100
50
0
-50
-100
-150
6
14
22
Long τ
Int τ
Short τ
CIRCADIAN TIME (h)

Figure 29 NGF-induced phase advances (mean +/- SEM) at Ct6 and CT22 and delays at CT14. Phase shifts induced by NGF were blocked when it was combined with anti-NGF (ANN) at CT6 and CT22. Anti-NGF had little effect on the phase shifts induced by NGF at CT14. Anti-NGF administered alone (AN) produced phase shifts similar to those caused by NGF at all phases. Rabbit serum (RS) produced phase delays at CT14 and had no effect at CT6 and CT22.

FIGURE 29

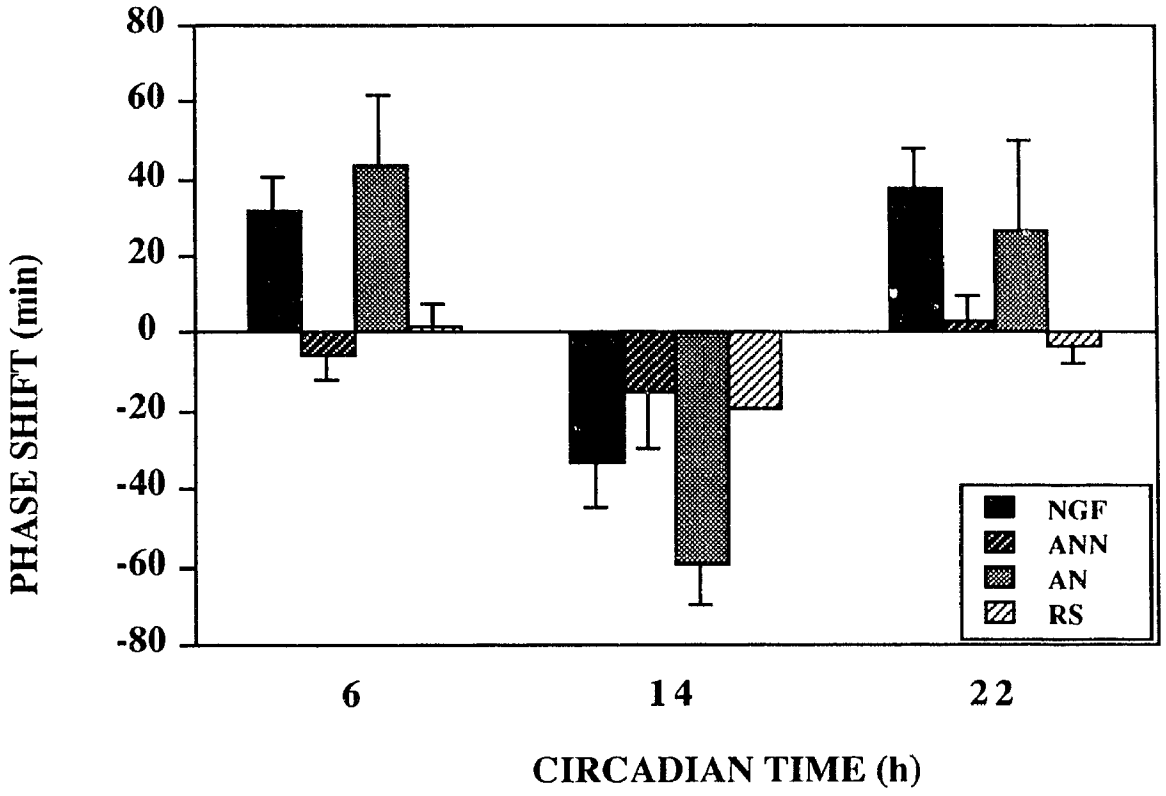
80
60
40
20
0
-20
-40
-60
-80
PHASE SHIFT (min)
6
14
22
CIRCADIAN TIME (h)
NGF
ANN
AN
RS

Figure 30 Panel A shows phase shifts (Mean +/- SEM) induced by NGF alone (NGF) and in the presence of atropine (NAT). Atropine blocked the phase advances normally induced by NGF at CT6 and CT22 and even produced phase shifts in the opposite direction.

Panel B shows the effect of NGF (NGF) and the effect of carbachol (CARB) when each was administered alone, and their effects when administered together (CARB+NGF). The two drugs together did not produce synergistic or additive effects; the phase shifts induced by the combination were not significantly different from those induced by either of the drugs administered alone except for the difference between NGF and NGF-CARB at CT22.

FIGURE 30

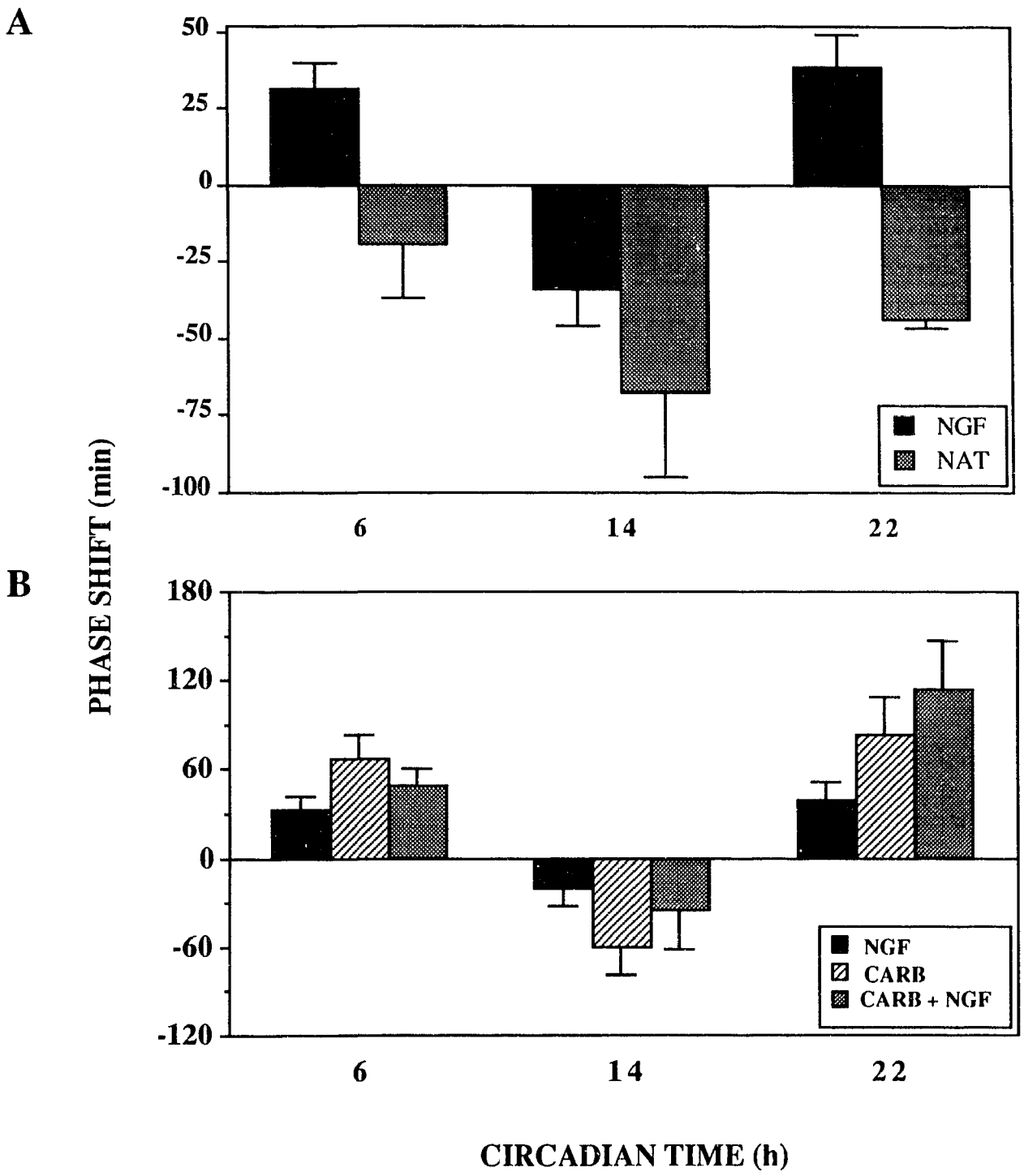
A
50
25
0
-25
-50
-75
-100
NGF
NAT
6
14
22
B
180
120
60
0
-60
-120
NGF
CARB
CARB + NGF
6
14
22
PHASE SHIFT (min)
CIRCADIAN TIME (h)

TABLE 2 : Effect of 2μl of various drugs administered into the SCN. The antagonists, when administered together with NGF were given 10 minutes prior to NGF. Values represent mean phase shifts (Δ ϕ)+/- standard error of the mean. (SEM). n represents the number of animals at each circadian time.

Drug	CT6			CT14			CT22		
	Mean Δ ϕ	SEM	n	Mean Δ ϕ	SEM	n	Mean Δ ϕ	SEM	n
NGF	30.9	8.3	23	-31.2	11.7	19	36.9	11.0	30
SALINE	02.0	3.3	8	3.1	03.5	8	-6.0	08.2	04
CYTOCHROME-C	2.50	3.5	4	-76.0	22.0	4	0.5	4.5	04
RABBIT SERUM	1.75	5.2	4	-20.0	0	3	-4.0	4	03
ANTI-NGF + NGF	-05.6	6.7	3	-15.0	15.0	2	3.2	06.3	04
ANTI-NGF	43.0	18.0	5	-60.0	10.0	2	26.2	23.7	04

TABLE 3 : Effect of 2μl of NGF and carbachol administered singly or together into the SCN. Values represent mean phase shifts (Δ ϕ)+/- standard error of the mean. (SEM). n represents the number of animals tested at each circadian time. Asterisks represent phase shifts significantly different ($p < 0.05$) from the phase shifts produced by NGF at that phase.

Drug	CT6			CT14			CT22		
	Mean Δ ϕ	SEM	n	Mean Δ ϕ	SEM	n	Mean Δ ϕ	SEM	n
NGF	30.9	8.3	23	-31.2	11.7	19	36.9	11.0	30
NGF + ATROPINE	-19.3 *	17.3	3	-67.5	27.5	2	-43.3 *	3.3	3
CARBACHOL	69.8	15.7	28	-59.7	18.2	10	83.9	24.8	11
CARBACHOL + NGF	48.7	12.6	8	-33.0	35.7	5	114.5 *	32.6	06

CHAPTER VI

GENERAL DISCUSSION

The experiments carried out in this thesis were aimed at investigating the potential roles of ACh and NGF in the process of entrainment of wheel-running activity rhythm in rodents. Studies described in Chapter II addressed the issue of the anatomical source of cholinergic input into the rat SCN using immunohistochemical markers for the ACh synthetic enzyme ChAT combined with retrograde and anterograde tract tracing techniques. Cholinergic fibres exist in the SCN and these fibres arise from two groups of cholinergic projection neurons located in the basal forebrain and the brainstem. In the basal forebrain, cholinergic cells that are located in the following nuclei: MS, vertical and horizontal limbs of DBB, MgPA, SI and NBM, project to the SCN. In the brainstem, two strongly ChAT-positive brainstem nuclei, the LDT and the PPT, and a weakly ChAT-positive nucleus, the PBg, provide cholinergic projections to the SCN.

Chapter III investigated the origins of the high density of receptors for NGF (NGF-R) found in the SCN. Immunocytochemical studies indicated that NGF-R are located in the ventrolateral SCN. Combining these studies with injection of a retrograde fluorescent tracer into the SCN and ChAT immunocytochemistry revealed that NGF-R originate in part from cholinergic neurons in the basal forebrain which project to the SCN. Transection of the optic nerves and a combination of retrograde tracing and immunocytochemistry for NGF-R revealed that NGF-R immunoreactive neurons in the retina also project to the SCN and convey NGF-R there. The NGF-R projection from the retina is heavier to the contralateral SCN while that from the basal forebrain is more strongly ipsilateral. Surgical transection studies revealed that NGF-R containing cells in the basal forebrain innervate the SCN through a ventrolateral route.

Chapter IV investigated the role of ACh and its receptors in mediating phase shifts in hamster activity rhythms. Infusions of carbachol, a non-specific cholinergic agonist, into the SCN caused phase advances of the wheel-running rhythm when administered during the late subjective night and during the subjective day. Carbachol produced phase delays during the early subjective night. Atropine blocked carbachol-induced phase shifts at all of these phases, suggesting that these phase shifts were mediated through muscarinic receptors.

Receptor binding studies used N-methyl-scopolamine to identify muscarinic receptors in the hamster SCN. The use of several ligands specific for either M1 or M2 receptors in competition experiments revealed the presence of both muscarinic receptor subtypes in the SCN. These results indicate that the anatomical substrate exists for cholinergic ligands to act via muscarinic receptors to influence SCN activity. Since muscarinic receptors were found in the SCN regions that also receive retinal and NGF-R immunoreactive projections, there is a possibility for such muscarinic activation to affect photic input to the SCN.

In chapter V, studies of the role of NGF in mediating phase shifts were described. Like carbachol, NGF injections into the SCN produced phase advances during the late subjective night and during the subjective day. These phase shifts were blocked by co-injection of the antibody to NGF or by atropine. These observations suggest that phase shifts generated by NGF at these times are specific to its binding to NGF-R and that these depend on activation of cholinergic mechanisms via muscarinic receptors. Control injections of Cyt-c or rabbit serum alone did not cause phase shifts at those phases when NGF-induced shifts were blocked by its antibody and by atropine. Both control injections did, however, cause phase delays in the early subjective night, when both atropine and NGF antibody fail to block NGF-induced delays completely. These findings strongly imply that NGF-induced delays are not specific to the binding of NGF molecules to NGF-R and do not depend on muscarinic activation.

The NGF antibody, when administered alone, was surprisingly potent in mimicking the effects of NGF on rhythm phase. These results imply that an epitope on the antibody molecule can recognize NGF-R or activate biochemical pathways related to phase shifting in other ways. Tests of whether NGF antibodies bind to NGF-R or whether their behavioural effects are also mediated by a muscarinic receptor should in future help to elucidate the mechanisms by which the antibody has its effects.

Taken together, these results suggest an important role for ACh and its associated growth factor, NGF, in mediating phase shifts of activity rhythms in hamsters. Figure 31 shows a schematic representation of possible ways in which ACh and NGF may mediate their phase shifting effects. Acetylcholine released from the terminals of basal forebrain and brainstem cholinergic neurons may act on muscarinic receptors located either directly on pacemaker cells or on interneurons in the SCN that form synapses with pacemaker cells to bring about cholinergically mediated phase shifts. However, what triggers these basal forebrain and brainstem neurons to release ACh is unclear. At least two mechanisms exist which may contribute to ACh release in the SCN.

Since retinal afferents are present in the basal forebrain (Youngstrom et al., 1991; K.G.Bina, unpublished observations), it is possible that photic input reaches the CBC and activates cholinergic cells projecting to the SCN, causing them to release ACh in the SCN. Thus, this indirect input from the basal forebrain, along with the direct retinal input via the RHT and the indirect input via the GHT, may contribute toward inducing a phase shift. Activation of these CBC cells by non-photic inputs may also promote release of ACh into the SCN under other conditions.

Another potential mechanism exists to promote ACh release from brainstem cholinergic cells. The cholinergic LDT nucleus which projects to the SCN may also receive projections from the SCN (Cornwall et al., 1990; but see Semba and Fibiger, 1992). When SCN cells are activated (by photic input, cholinergic forebrain input or other means), they may activate LDT neurons which, in turn, release ACh into the SCN. This cholinergic input to

the SCN may amplify or otherwise modulate the effects of photic input on SCN cells. Because it is still highly speculative this feedback loop is not included in Figure 31.

Another possibility is that cholinergic neurons in the basal forebrain terminate presynaptically on the retinal terminals in the SCN, forming axo-axonic synapses. Activation of basal forebrain neurons (e.g. by retinal or other input) may result in the release of ACh which causes an increased release of neurotransmitter from RHT fibres, most likely a glutamate-like transmitter. This possibility is weakened by the lack of evidence for axo-axonic synaptic connections in the SCN (Güldner, 1978; van den Pol, 1980, however, see Ueda and Ibata, 1989). Furthermore, apart from its action on nicotinic receptors, mecamylamine at some doses has been shown to have antagonistic effects on excitatory amino acid neurotransmission (O'Dell and Christensen, 1988; Snell and Johnson, 1989). The failure of mecamylamine to block carbachol-induced phase shift suggests that carbachol mediates its phase shifting effects independently of both excitatory amino acid and nicotinic receptors. This conclusion makes it unlikely that carbachol acts indirectly by modulating activity in RHT fibres.

The reduction of NGF-induced phase shifts by atropine injections suggests the involvement of cholinergic mechanisms in mediating this effect. Since NGF synthesizing cells are present in the SCN (Senut et al., 1990; Ojeda et al., 1991) and NGF-R are located, in part, on retinal terminals (chapter III), it is possible that photic input received by the retina triggers the release of a neurotransmitter, presumably glutamate, from retinal terminals in the SCN, and that this neurotransmitter triggers the release of NGF from the NGF producing cells. NGF released from such cells would act on NGF-R located on the terminals of basal forebrain cholinergic neurons in the SCN and on the terminals of retinal ganglion cells. NGF acting on the NGF-R located on the terminals of basal forebrain neurons may cause an increase in the release of ACh from these terminals, which, in turn, acts on muscarinic receptors presumably located on pacemaker cells (or cells afferent to them) to increase phase shifts. Increases in ChAT activity and ChAT mRNA following

treatment with NGF have been reported (Hefti et al., 1984; Hefti et al., 1985; Mobley et al., 1985; Hatanaka et al., 1988; Cavicchioli et al., 1991). Furthermore, increases in ACh synthesis and release in presynaptic cholinergic cells following NGF treatment have also been demonstrated (Kewitz et al., 1990; Lapchak and Hefti et al., 1991).

A second possible role for NGF is through its action on NGF-R located on retinal terminals. It is possible that NGF acting on NGF-R on retinal terminals induces an increased release of the neurotransmitter from the retinal terminals which in turn acts on pacemaker cells to induce phase shifts. However, thus far there is no evidence for a role for NGF in modulating the release of neurotransmitters other than ACh in the CNS, and it is unlikely that ACh is released from the terminals of retinal ganglion cells in the SCN since there is no evidence for retinal ganglion cells being cholinergic (Massey and Redburn, 1987).

A third possibility is that NGF released from cells in the SCN acts directly on pacemaker cells to produce phase shifts. The likelihood of such a mechanism is strengthened by evidence that phase shifting light input can induce a variety of immediate early genes, such as c-*fos*, NGFI-A (zif-268), and NGFI-B in SCN cells (Rea, 1989; Earnest et al., 1990; Kornhauser et al., 1990; Rusak et al., 1990; Rusak et al., 1992). It has been hypothesized that such gene activation may mediate intracellular changes in SCN pacemaker cells which ultimately cause phase shifts of circadian rhythms (Rusak et al., 1990). Since NGF can itself induce c-*fos* expression in some mammalian cells (Curran and Morgan, 1985), and since the immediate early genes NGFI-A and NGFI-B were, in fact, discovered because their expression is regulated by NGF (Milbrandt, 1987, 1988), it is very likely that NGF application to SCN cells induces a cascade of changes in gene expression which at least partially overlaps the light-induced response of SCN cells. The viability of this hypothesis will depend, however, on electron microscopic evidence that NGF-R are actually found on SCN cells.

NGF is largely known for its long-acting trophic effects. The acute effect of NGF reported here suggests that NGF is capable of inducing rapid changes in signal transduction mechanisms leading to rapid behavioural changes. NGF has been shown to induce *trk* tyrosine phosphorylation (a possible signal transduction mechanism) within 1 min of treatment (Kaplan et al., 1991). Transient activation of immediate early genes (transcription regulators) such as c-*fos*, c-*jun* and c-*myc* within an hour of NGF treatment in PC-12 cells has also been reported, creating the potential for NGF to alter gene expression which is immediately relevant to short-term behavioural regulation (Wu et al., 1989).

Rapid NGF-induced changes in body weight have been reported within 3 days of NGF treatment (Williams, 1991). Increases in ChAT activity and ACh concentration have also been observed within a day, in the septum, striatum, cortex and hippocampus, following a single injection of NGF in newborn rats (Kewitz et al., 1990). This study did not measure changes in ChAT activity and ACh concentration before 24 hr of NGF treatment, and thus it is difficult to say how rapidly these changes started to occur. NGF has also been shown to inhibit the stimulation-evoked norepinephrine overflow from adrenergic nerve endings of rat mesenteric vasculatures within a few minutes of treatment (Ueyama et al., 1991). NGF effects on ChAT activity, ACh release and norepinephrine release suggest that NGF may have a rapid neuromodulatory effect on cholinergic and noradrenergic cells. The rapidly induced effects of NGF suggest that, apart from its long-acting trophic effects, NGF may also be involved in inducing rapid neuromodulatory effects that may have immediate behavioural consequences.

Entrainment to photic input is most likely not a simple phenomenon, based, for example, on the release of one neurotransmitter from retinal terminals acting on receptors located on pacemaker cells. It is more likely that a combination of some or all of the possibilities listed above, together with the effect of neurotransmitters such as glutamate released from retinal terminals, NPY released from GHT terminals, and GABA released

from interneurons in the SCN, contribute toward the induction of phase shifts by photic and other stimuli.

As described in chapter I, cholinergic neurons in the basal forebrain and the brainstem, together with brainstem aminergic neurons, play a significant role in the regulation of cortical arousal, slow wave sleep and REM sleep. But how the system responsible for the generation of sleep and wakefulness (basal forebrain and brainstem neurons) is linked to the system responsible for the circadian timing of sleep and wakefulness (SCN) is unclear. Figure 31 outlines a very simplistic view of one such mechanism. ACh released from the terminals of the brainstem neurons located in the pontine reticular formation leads to the induction of REM sleep, and the suppression of thalamocortical rhythmical activities associated with slow wave sleep by its action on neurons in thalamic nuclei. The activation of brainstem cholinergic neurons would also result in the release of ACh in the SCN. The ACh released from brainstem neurons gradually activates the aminergic neurons in the dorsal raphe and locus coeruleus. These aminergic neurons activate cholinergic neurons in the basal forebrain to release ACh into the cortex and the SCN. The release of ACh in the cortex results in cortical arousal and that into the SCN presumably causes or modulates phase shifts via actions on pacemaker cells. Some brainstem aminergic neurons (e.g. those in the raphe nucleus and the locus coeruleus) also provide direct projections to the SCN (Fuxe, 1965; Aghajanian et al., 1969; Ajika & Ochi, 1978; Azmitia & Segal 1978; Bobillier et. al., 1979; van den Pol and Tsujimoto, 1985). Serotonin, the neurotransmitter at the terminals of dorsal raphe neurons in the SCN, has been shown to modulate the amplitude of the circadian activity rhythm (Block and Zucker, 1976), and the raphe nuclei have been shown to play an important role in the regulation of rhythm integrity (Rosenwasser, 1989) and may help regulate the phase of entrained rhythms.

Although the SCN receives afferents from structures involved in the regulation of sleep and wakefulness (chapters II and III), there is little or no evidence for a direct feedback

from the SCN to these brain structures. Indirect projections through the PVN or other hypothalamic nuclei may exist, however, and remain to be investigated.

This thesis has only started to address the role of NGF in mediating phase shifts of circadian rhythms. A large number of questions regarding the role of NGF and ACh in phase shifting and in the regulation of sleep and wakefulness remain unanswered. Future studies should include determining the role of ACh in photic entrainment by attempting to block light-induced phase shifts using muscarinic receptor antagonists, and performing double-labelling studies in the retina using a retrograde tracer injected into the SCN combined with immunocytochemistry for ChAT and NGF-R (p^{75} and *trk*). The molecular mechanisms involved in mediating the phase shifting effects of ACh, NGF and excitatory amino acids in the SCN also remain to be clarified. The role of NGF in the regulation of cortical arousal and sleep, and the nature of the efferent pathways by which the SCN connects to the sleep and wakefulness circuitry are also poorly understood. In addition, the results of the behavioural studies reported here suggest some surprising biological activity of NGF antibodies, which warrants further study. Finally, the results imply important differences in the biochemical mechanisms mediating delay and advance phase shifts at different circadian phases, as had been suggested in earlier studies of GABAergic systems in the SCN (Ralph and Menaker, 1985, 1986; 1989). It remains to be determined how the apparent differences in the roles of several neurochemicals in mediating delay and advance phase shifts will ultimately be related to each other.

Figure 31 Schematic representation of the ways in which ACh may affect sleep and wakefulness, and the ways in which NGF and ACh may affect circadian pacemaker phase, and hence the timing of these states. + and - signs at the end of an arrow represent activation or suppression, respectively at the postsynaptic cell. These signs at the base of the arrow represent activation or suppression, respectively of the presynaptic cells. Numbers 1, 2 and 3 represent three hypotheses which might explain how NGF acting in the SCN can promote phase shifting. Number 1 proposes that NGF released from the NGF producing cells in the SCN acts on the terminals of the basal forebrain cholinergic neurons to increase the release of ACh, which in turn (directly or indirectly) acts on pacemaker cells to induce phase shifts. The second hypothesis proposes that NGF released in the SCN acts on the terminals of retinal ganglion cells to induce the release of the neurotransmitter at the RHT which in turn acts on pacemaker cells to produce phase shifts. The third hypothesis suggests that NGF released from the NGF producing cells acts directly on the pacemaker cells to produce phase shifts.

The portions of the model relating to regulation of sleep and cortical arousal are derived from previous models by McCarley and Hobson (1975) and Semba (1991).

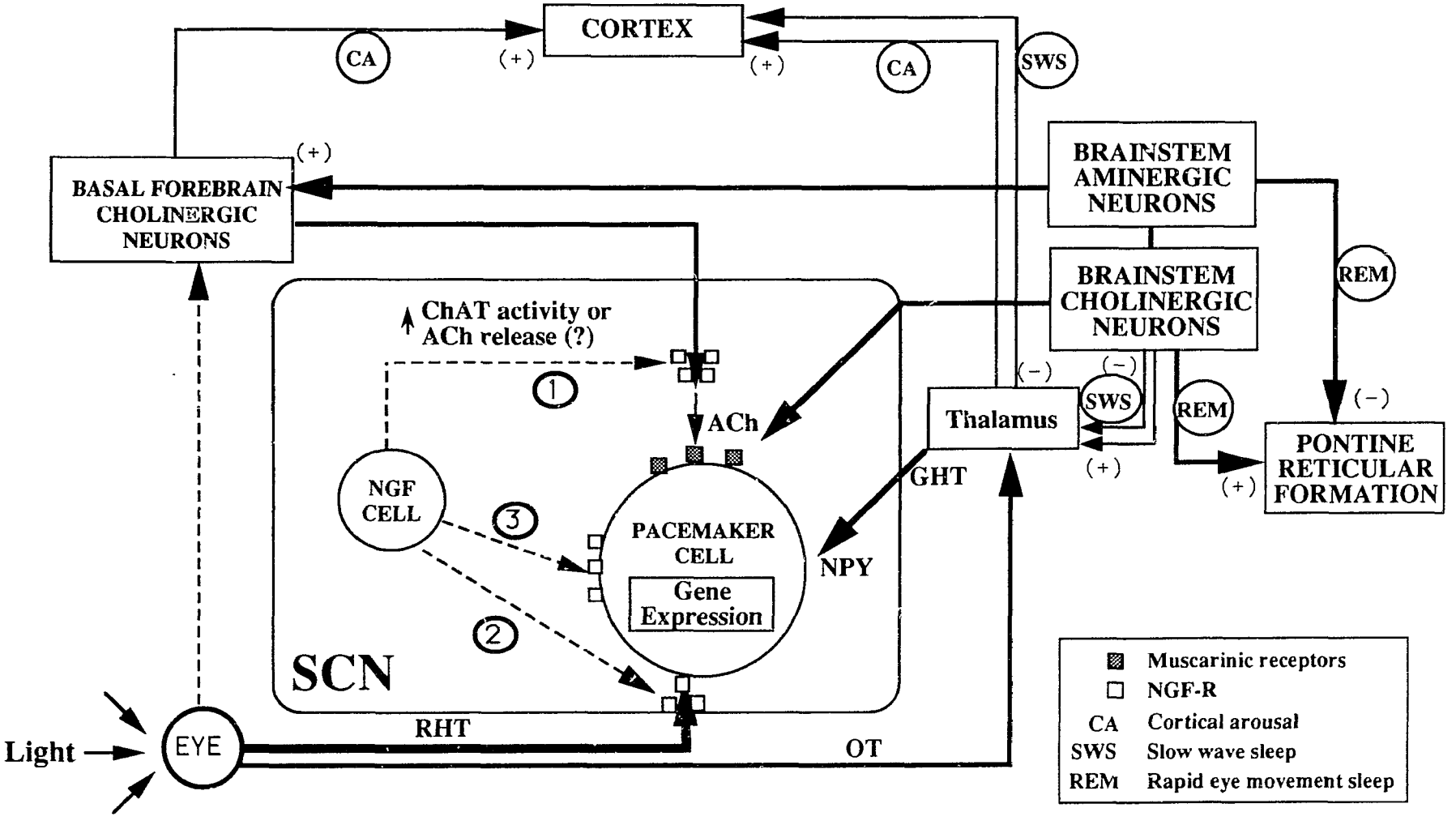
CORTEX
CA
(+)
(+)
CA
SWS
BASAL FOREBRAIN CHOLINERGIC NEURONS
(+)
BRAINSTEM AMINERGIC NEURONS
BRAINSTEM CHOLINERGIC NEURONS
REM
ChAT activity or ACh release (?)
1
ACh
(−)
(−)
Thalamus
SWS
REM
(−)
PONTINE RETICULAR FORMATION
(+)
(+)
GHT
NGF CELL
3
PACEMAKER CELL
Gene Expression
NPY
2
SCN
RHT
OT
Light
EYE
Muscarinic receptors
NGF-R
CA Cortical arousal
SWS Slow wave sleep
REM Rapid eye movement sleep

FIGURE 31

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