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Changes in Serotonergic Receptors in the Suprachiasmatic Nucleus

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Chapter 1

Background and Significance

Importance of Circadian Rhythms

Organisms must maximize their chances of survival by acclimating themselves to their environment. Because the earth follows a twenty-four rotation period with corresponding periods of light and dark, the body's internal oscillation must closely mimic this time frame in order to entrain stably to its environment (Dunlap et al., 2004). Biological processes or activities that repeat consistently approximately every 24 hours and continue in a constant environment are termed circadian rhythms (Refinetti, 2000).

These circadian bodily oscillations are innate because animals raised from birth in constant light or dark exhibit free-running periods that approximate 24 hours even though they have never experienced light and dark cycles. Organisms' free-running periods, circadian rhythms exhibited in a constant environment, vary from about 23 to 25 hours, which again indicates that these endogenous rhythms are innate and caused by some genetic factors rather than acquired learning. Species living in the same environments as one another have different and internally consistent circadian rhythm periods, which reinforces the idea of genetic cause. This was observed in mutant hamsters with the *tau* allele, who experienced a phase advance for every mutant allele that they had. So, normal hamsters had a period of 24 hours, those with one *tau* allele had a 22 hour period, and homozygote *tau* mutants had a free-running period of 20 hours (Dunlap et al., 2004).

There are three defining criteria of circadian rhythms. The first one has just been described as a free-running period between 23 and 25 hours. The next criterion is that circadian rhythms must be temperature compensated so that at different ambient temperatures the length of

the oscillation should remain stable. This is necessary so that the clock does not run faster or slower following temperature changes. The final criterion is that these circadian rhythms must be able to entrain to 24-hour environmental cues such as light-dark cycles, and temperature cues (Dunlap et al., 2004). While there has not been enough research done yet, it is often believed that circadian rhythms are ubiquitous and present in all species. It has been established however, that not all species are as precise as others, and specifically nocturnal animals are more consistent in their circadian rhythm periods than diurnal ones (Dunlap et al., 2004).

The importance of an internal oscillator is seen in hibernating animals because their compulsory physiological processes must continue in a regular and predictable rhythm even when not exposed to a daily environmental time cue such as light-dark cycles (Dunlap et al., 2004). Circadian rhythms within an organism do not free run separately; sleep-wake cycles, temperature cycles, and metabolic cycles remain in synch with each other. For example, sleepiness is associated with the trough in the daily temperature cycle. This decrease in internal temperature and onset of sleepiness occur before any decrease in the level of motor activity (Refinetti, 2000).

The endocrine system also displays circadian rhythms that oscillate with the sleep-wake cycle in organisms. Prior to waking, the hypothalamo-pituitary-adrenal axis is activated to raise the levels of corticosteroids released by the adrenal gland. The level of corticosteroids peaks in the early morning and steadily declines until nighttime, and thus high levels are associated with activity. In nocturnal rodents, high levels of corticosteroids are seen in the early night, which is in keeping with these particular animals' onset of heightened activity. During circadian nighttime, nocturnal and diurnal animals' pineal gland releases the hormone melatonin, which among other activities regulates seasonal reproductive status (Redfern & Lemmer, 1997).

Circadian rhythms, combined with ovarian timers, have also been observed in the estrous cycle of mammals. Because female rodents produce viable eggs in a cyclical manner, there is a period during the cycle in which reproductive success is the highest. To maximize the chance of having viable offspring, the rodents must have appropriate cycling of their ovulation and sexual activity. In hamsters, the females ovulate every fourth day at around midday and the ruptured follicle releases progesterone, which enhances sexual activity during the following night. This is necessary because nocturnal female and male hamsters have the most chance of contact at night. Thus, the light-dark entrainment of the circadian pacemaker allows this process to occur consistently (Dunlap et al., 2004).

The importance of circadian rhythms is also seen in the cell division cycle (CDC). Cyclic AMP may play a coupling role between certain steps in the CDC and particular phases in the circadian cycle. This idea may explain why certain drugs that interfere with the cell division cycle are more effective at certain times in an organism's circadian period. The sensitivity of cells to many cytostatic drugs and ionizing radiation is "highly cell-stage specific" (Redfern & Lemmer, 1997). Varying drug administration throughout the 24 hour circadian period can promote the well-being of the patient while reducing his or her risk factors. The tolerance of the patient to the medicine is maximized through a "temporal shielding of normal, healthy tissues" (Redfern & Lemmer, 1997).

For humans, traveling across time zones can cause jet lag, which also calls attention to circadian rhythms. When traveling to regions with a delayed or advanced light-dark cycle, the cycles of body temperature, feeding and drinking, and activity entrain to the new time at separate rates. It has been determined that on average, it takes slightly less than one circadian day to re-entrain the body following travel across each time zone. While the most common treatment used

for jet lag is simply waiting for the gradual phase shift caused by the new light-dark cycle to occur, other methods have been investigated for use when this natural process must be expedited. Light pulses can phase shift the circadian pacemaker rapidly so that it synchronizes with its new environment. Alternatively, melatonin can be administered before the desired sleep time to induce sleepiness and shift the clock (Refinetti, 2000).

The Suprachiasmatic Nucleus

The circadian pacemaker controls all the circadian rhythms in an organism. The circadian clock in mammals has been located to the Suprachiasmatic Nucleus (SCN) due to several reasons. Removal or permanent damage to the SCN leads to a permanent loss of most overt free running circadian rhythms. If part of the SCN is removed and at least ten-percent of it remains intact, normal rhythmicity is maintained (Dunlap et al., 2004). When the SCN is disconnected from the rest of the brain, the nuclei continue their rhythmic electrical activity (Redfern & Lemmer, 1997). If an adult animal rendered arrhythmic due to SCN removal has SCN tissue from a donor fetus transplanted into its brain, the circadian rhythmicity characteristic of the donor animal's genotype is restored in the recipient. Individual SCN cells isolated *in vitro* under cell culture conditions display free-running circadian rhythms for some time (Dunlap et al., 2004). These cells display a range of periods, while the SCN exhibits a period that is the average of its cells (Refinetti 2000). The rhythmic release of peptidergic secretion and electrical activity by the SCN cells *in vitro* also continues and can be phase shifted by environmental cues (Redfern & Lemmer, 1997).

The two ovoid nuclei of the SCN are located in the ventral hypothalamus on either side of the third ventricle's base, directly above the optic chiasm (Redfern & Lemmer, 1997; Dunlap et al., 2004). "Their morphology is conserved across mammals, including man" (Redfern &

Lemmer, 1997). The approximately 16,000 SCN neurons are very small compared to those in most of the rest of the brain. Structurally the SCN consists of a shell that lies dorsal to a core, which is in a ventrolateral position (Dunlap et al., 2004). Peptidergic neurons are a large proportion of those in the SCN, including neurons immunoreactive for Arginine vasopressin (AVP) in the shell, Gastrin-releasing peptide (GRP), Vasoactive intestinal polypeptide (VIP) in the core, somatostatin and neurotensin (Redfern & Lemmer, 1997). Which of these neurons are essential to the workings of the pacemaker has not been established, however, "successful SCN grafts always contain some VIP-ir and AVP-ir" cells, leading to the possibility that these neurons are, or are closely related to, the ones critical to SCN function (Redfern & Lemmer, 1997). There is also a dense concentration of astrocytes, which are more immunoreactive for glial fibrillary protein (GFAP) than those in the rest of the hypothalamus. Astrocytes exhibit a circadian cycle in which they are more condensed and defined in early subjective night. Throughout the cycle, the astrocytes go through stages in which they define the borders of the SCN. While it has not been established, this circadian cycle exhibited by the astrocytes may be associated with the metabolic cycle of the SCN, which utilizes more glucose and oxygen during the subjective day. These astrocytes are in all parts of the nucleus but the immunoreactive (ir) neurons are arranged such that the ventrolateral portion of the SCN contains mostly VIP-ir and GRP-ir cells, while the SCN's dorsomedial section has AVP-ir neurons. For the majority of SCN neurons, in addition to having peptidergic transmitters, they are also GABAergic (they react with the neurotransmitter GABA) (Redfern & Lemmer, 1997).

Several genes have been identified that play a role in circadian rhythm expression. The expression of *period* (*per*) gene mRNA oscillates with a circadian period. The PER protein translated from the *per*-mRNA then forms a feedback loop with the *per* gene to down regulate its

expression. This *per*→PER loop may play a role in the pacemaker's oscillator mechanism, an idea that is supported by the fact that depletion in PER protein inhibits the SCN's function (Redfern & Lemmer, 1997; Refinetti, 2000). The homologs of the *per* gene in humans are *Per1*, *Per2*, and *Per3,* whose RNA levels display circadian rhythmicity in the SCN. During subjective night the levels of *Per1*-mRNA and *Per2*-mRNA are increased following photic stimulation (Refinetti, 2000).

In mice there is a semidominant gene called *clock,* that, when mutated, can lengthen the circadian period from the normal 23.3 hours. When one mutant allele is expressed, the circadian period is extended to 24.4 hours and if the mouse is a homozygote for the mutant gene, it has a circadian period of about 27.3 hours (Refinetti, 2000). Comparison of the major genetic components that contribute to the circadian clock shows high conservation between both vertebrates and invertebrates (Refinetti, 2000).

Afferent Connections to the SCN (Refer to Figure 1)

Three major afferent connections to the SCN can regulate its activity and shift the circadian clock. The primary input to the SCN comes from its only photic sensor, the retina, by way of the retinohypothalamic tract (RHT). Photic entrainment of the SCN is primarily regulated by the RHT input (Refinetti, 2000; Dunlap, et al., 2004). From the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) in the thalamus, there is a projection called the Geniculo-hypothalamic tract (GHT), which also terminates in the SCN's core (Refinetti, 2000; Redfern & Lemmer, 1997). The GHT also contributes some retinal input that has been modified in the geniculate body to the circadian clock. This retinal input could increase the sensitivity of the SCN to light input from the primary photic relay the RHT. The neurotransmitter associated with the GHT is Neuropeptide Y (NPY), and its Neuropeptide Y –

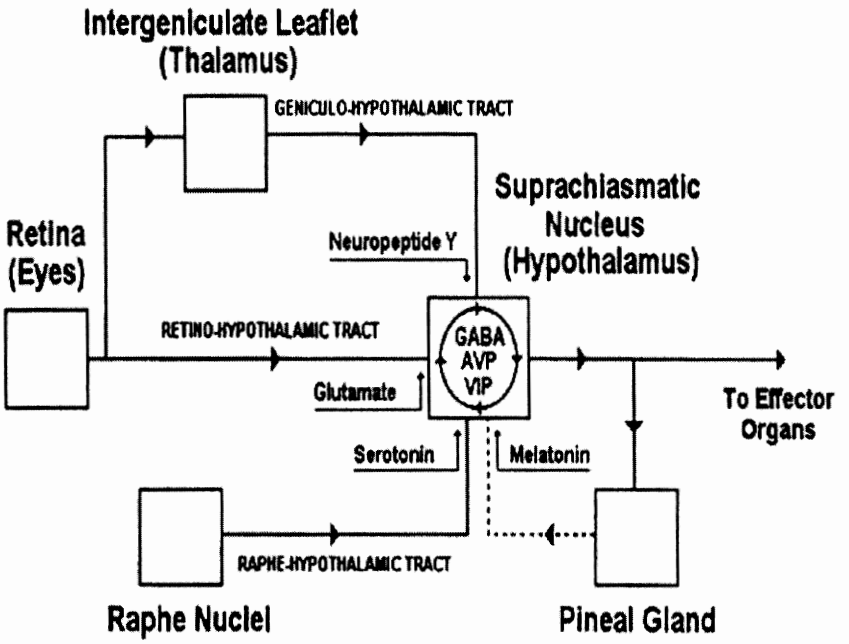

Figure 1: Afferent Connections to the SCN (From Refinetti, 2000)

immunoreactive fibers and terminals overlap in the ventrolateral core of the SCN with some of the afferent direct retinal and serotonergic afferents. There is evidence to suggest that this neurotransmitter is involved in non-photic entrainment of the animal (Redfern & Lemmer, 1997; Dunlap et al., 2004). Nonphotic cues all involve changing the arousal state of the animal (Redfern & Lemmer, 1997). Examples of nonphotic entraining cues for animals include forced exercise, running wheel availability (for exercise), and handling stress (Refinetti, 2000; Redfern & Lemmer, 1997).

The serotonergic afferents to the pacemaker come from the Raphe Nuclei in the midbrain. There is also a projection from the raphe nuclei to the intergeniculate nucleus so that the raphe may modify the photic information that the IGL carries (Dunlap et al., 2004). As with the RHT and geniculo-hypothalamic tract, the raphe input also projects to the core of the SCN. The retinal input to the raphe nuclei is secondary to the raphe nuclei's role in relaying information on the general arousal state of the animal. The raphe nuclei use the neurotransmitter serotonin to accomplish this task. Serotonergic fibers and terminals are present in the SCN at a higher density than in the surrounding hypothalamus. While the raphe nuclei are not necessary for circadian rhythms to occur, they can modulate the clock's activity through entrainment to non-photic arousing stimuli. It may also modulate certain photic responses of the pacemaker (Redfern & Lemmer, 1997). Serotonin agonists have been found to "attenuate the electrophysiological responses of the SCN to stimulation of the optic nerve, and the light-induced phase advances of hamster activity rhythms are reduced if the animals are active during the interval of illumination" (Redfern & Lemmer, 1997). Interaction between the afferent inputs to the SCN is apparent.

Efferent Connections to the SCN

The SCN makes connections throughout the brain to control various circadian rhythms. For instance, the sleep-wake cycle and daily temperature cycle are closely linked so that the trough of the temperature cycle correlates with sleep. Neural connections exist between sleep centers of the midbrain's reticular formation, in the pons and medulla, and the SCN. The reticular formation projects to the spinal cord and has a role in regulating muscle tone, reflexes, and transmission of sensory information. Reticular formation input to the forebrain influence consciousness and the pattern of sleep. The regulation of body temperature exhibits the clock's control over the balance between heat loss and generation through neural connections. Regulating the association between the sleep-wake cycle and the body temperature cycle is controlled in part by changes in basal metabolic rate, which suggests that the circadian pacemaker communicates with parts of the autonomic nervous system that regulates metabolism (Dunlap et al., 2004).

Many hormonal release cycles are also controlled in part by the SCN. Through a neural connection to another region of the hypothalamus, the SCN controls the production of corticotropic-releasing hormone. The daily release of this hormone influences the anterior pituitary's circadian release of adrenocorticotropic hormone. The rhythmic secretion of glucocorticosteroids and minerocorticosteroids from the adrenal cortex is in turn regulated by adrenocorticotropic hormone. Many mammalian physiological functions depend on these steroid hormones (Dunlap et al., 2004).

Entrainment of the SCN

The act of imposing an external oscillation frequency on the internal pacemaker is entrainment. "Entrainment of the circadian clock provides an internal estimate of external local time" (Dunlap et al., 2004). The physiological time that an organism experiences is only indirectly an effect of the current external time, and conversely is directly a result of its own timekeeper. To accurately estimate local time, the internal pacemaker entrains to the 24-hour external cycle of the earth's rotation. There are two criteria used to determine whether an external time cue has actually entrained the internal pacemaker. The first is that the period of the entraining agent's rhythm must correlate with that of the SCN. The second decisive factor states that even after returning to an environment with constant conditions, the animal will free-run beginning with a circadian period that compares to the entraining agent's (Dunlap et al., 2004).

Light pulses administered to an animal have been found to phase shift the circadian rhythm, they cause "delays during early subjective night and advances during late subjective night" (Refinetti, 2000). The neurotransmitter involved in photic entrainment is glutamate. Stimulation of the optic nerve leads to a release of glutamate in the SCN and dose dependent phase shifts, similar to those seen following a light pulse. Conversely, glutamate antagonists (external agents that stop the cell from responding to glutamate) block these phase shift responses. Retinal terminals in the SCN have also been found to be immunoreactive for glutamate (Redfern & Lemmer, 1997; Refinetti, 2000).

Photic stimulation has also been found to phosphorylate and activate the *c-fos* immediate early gene to produce the Fos protein in the SCN. The expression of *fos* gene is mediated by cyclic adenosine monophosphate response element (CREB), which is phosphorylated and activated when the organism experiences light pulses that induce phase shifts of the circadian

clock. After synthesis, the Fos protein can enter the nucleus to regulate various late response genes' transcription (Refinetti, 2000).

The geniculo-hypothalamic tract (GHT) that runs from the intergeniculate leaflet of the thalamus to the core of the SCN in the hypothalamus relays photic information that it receives from the retina. The GHT however, is not necessary to entrainment the internal pacemaker to light-dark cycles. Neuropeptide Y is the pancreatic polypeptide hormone produced by the GHT neurons, and seems to be involved in the entrainment of the clock through nonphotic stimuli (Refinetti, 2000; Dunlap et al., 2004). Neuropeptide Y administration causes phase advances of the endogenous clock, presumably through the activation of protein kinase-C during the subjective day (Prosser, 2000).

Serotonergic Input to and Entrainment of the SCN

The raphe input to the SCN may also contribute to the entrainment of the clock to nonphotic stimuli. The raphe nuclei are not necessary for expression of circadian rhythms, yet seem to play a role in stabilizing and reinforcing the clock's actions (Dunlap et al., 2004; Prosser, 2000). It has been found that raphe nuclei lesions cause a dampening of the amplitude and regularity of wheel running rhythms and in constant darkness shorten the period of this activity. An increase in the aforementioned activities also probably increases the serotonergic activation in the forebrain (Redfern & Lemmer, 1997). The raphe acts largely to set the general arousal state of the animal through the release of serotonin (5-hydroxytryptamine; 5-HT) (Dunlap et al., 2004). The level of arousal is directly correlated with the level of activity seen in the serotonergic cells of the raphe nuclei (Redfern & Lemmer, 1997). In the SCN, 5-HT agonists (external agents that mimic the effects of 5-HT) administration causes phase shifts

characteristic of nonphotic stimuli; conversely, depletion in 5-HT levels inhibits the usual shortening of the circadian period caused by running wheel availability (Refinetti, 2000). Providing 5-HT or 5-HT agonists to the SCN *in vitro* during mid subjective day causes two to three hour advances (Refer to Figure 2 and Figure 3) in the period of the pacemaker, while this delays the clock when presented during subjective night (Prosser, 2003b; Prosser, 2000). The SCN neurons respond to 5-HT, in that they are more responsive to 5-HT during subjective night rather than during subjective day (Refinetti, 2000).

The actions of 5-HT (as well as other inputs to the clock) can be monitored using brain slices of the SCN. "The ability of the SCN pacemaker to continue generating circadian rhythms when isolated in a brain slice preparation or as dispersed cells underscores its endogenous nature" (Prosser, 2003b). Thus, the SCN continues to exhibit regular circadian rhythms and spontaneous neuronal electrical activity *in vitro*, with a peak in activity observed about six hours after lights-on (Prosser, 2003b; Prosser, 2000). *In vivo*, the mouse SCN free-runs with a period of about 23.5 hours and its period *in vitro* closely matches this at 24 hours (Prosser, 2003b). Through work on such slices, a signal transduction model has been established. Because 5-HT agonists such as (+)8-hydroxy-2-(di-n-propylamino) tetralin (DPAT) and quipazine cause phase advances that mimic those caused by 5-HT, and the 5-HT antagonist metergoline opposes these advances, these compounds are used in *in vitro* experiments. However, these chemicals do not produce an effect during nighttime administration so they can only be used to observe daytime phase advances (Prosser, 2000). It was found that cyclic-AMP (cAMP) and cAMP agonist administration cause phase advances of the clock that emulate those caused by 5-HT (Prosser, 2000). Likewise, the peak sensitivity of cAMP is during the mid subjective day just like 5-HT (Lovenberg et al., 1993). These similarities in activities point to a relationship between 5-HT

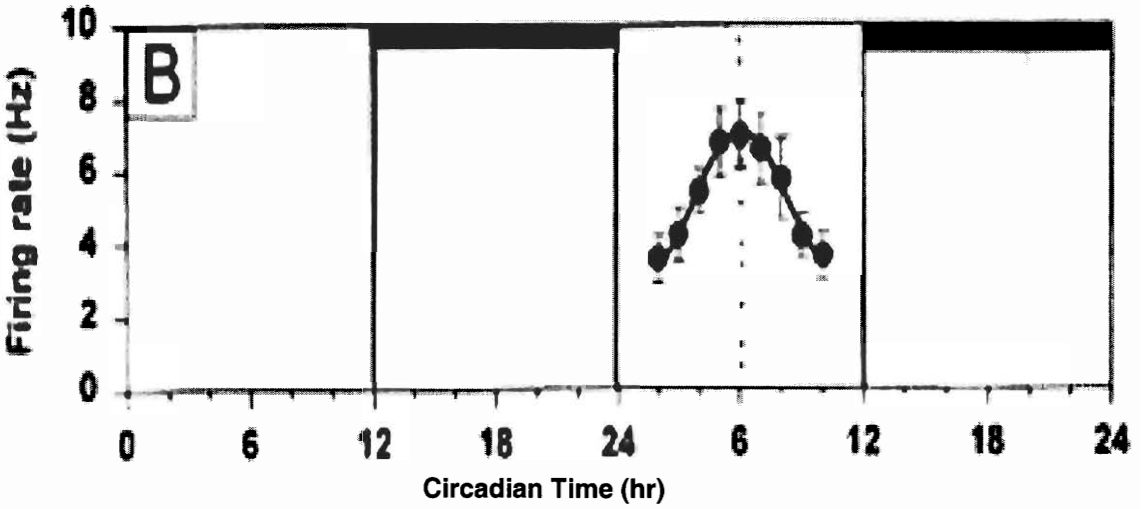

Figure 2: Firing Rates of Neurons in the SCN (From Prosser, 2000)

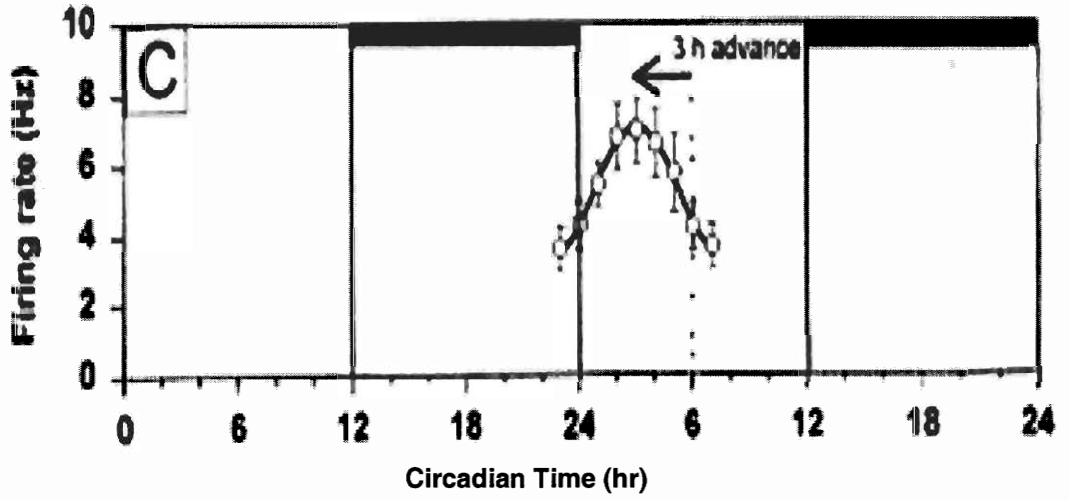

Figure 3: Firing Rates of Neurons in the SCN Following 5-HT Administration During Mid Subjective Day (From Prosser, 2000)

induced phase advances and increases in the levels of cAMP. If the activation of protein kinase-A (PK-A), inhibition of photodiesterases, and the opening of K^+ and Ca^{2+} dependent K^+ channels are blocked, the effects of 5-HT agonists are lost (Lovenberg et al., 1993; Prosser, 2000). This information was used to form a signal transduction model to describe the cellular effects of 5-HT. Serotonin agonists activate adenylate cyclase, which catalyzes the production of cAMP. Cyclic-AMP can then activate PK-A, which phosphorylates proteins. Protein kinase-A has also been linked to initiation of the *c-fos* immediate early genes that causes the transcription of other genes. This may not be involved with the phase advances but the levels of *c-fos* mRNA increases following SCN exposure to 5-HT (Prosser, 2000).

It is not clear yet whether 5-HT acts directly on pacemaker cells in the SCN, or indirectly through interneurons that synapse onto pacemaker cells. By allowing 5-HT to bind to its receptor but inhibiting synaptic interactions that would occur between interneurons and pacemaker cells, by supplying TTX which blocks action potentials, and Mg^{2+} which blocks neurotransmitter release, the location of the serotonin receptors can be ascertained. These experiments did not block 5-HT induced phase advances so the receptors are probably on the clock cells themselves. However, these treatments do not inhibit interactions between gap junctions so the possibility remains that 5-HT exerts its effects through gap junctions either between glial cells pacemaker cells, or between pacemaker cells (Prosser, 2000).

There are several 5-HT receptors in the mammalian brain. In the SCN, large concentrations of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{5A}, and 5-HT_7 are found as well as some 5-HT_{1C} and 5-HT_2 (Prosser, 2000). The 5-HT_7 receptors are located throughout the thalamus and hypothalamus with a high concentration in the SCN (Gannon, 2001; Lovenberg et al., 1993). About 20-40% of the 5-HT receptors are 5-HT_7 while the remaining are mostly 5-HT_{1A} (Gannon, 2001). At first it

was thought that the 5-HT_{1A} receptor controlled the 5-HT induced phase advances in the clock because 5-HT agonist activity was blocked following treatment of the SCN with NAN-190, an antagonist specific for the 5-HT_{1A} receptor. Increasing evidence however indicates that the 5-HT_7 receptor may in fact be responsible for the characteristic phase advances. Both receptors are pharmacologically similar in that they have high affinities for the same agonists and antagonists. However, 5-HT_7 is positively coupled to adenylate cyclase, which catalyzes the production of cAMP, while 5-HT_{1A} is negatively coupled to adenylate cyclase, which leads to a decrease in the levels of cAMP. In addition, the antagonist pindolol is specific for the 5-HT_{1A} receptor and cannot block the phase advances following 5-HT agonist exposure, while the antagonist ritanserin specific for 5-HT_7 does block the shifts. Thus, the biochemistry of the 5-HT induced phase advances mimics those caused following activation of the 5-HT_7 receptor rather than activation of the 5-HT_{1A} receptor (Prosser, 2000).

The 5-HT_7 receptors are located near cells immunoreactive for c-Fos, GABA, vasopressin and VIP (Gannon, 2001). Raphe nuclei input synapses on the same neurons onto which Neuropeptide Y, and retinal input synapse. There is an interaction between the various afferent connections to the SCN so that they influence each other's actions. For example, NPY inhibits the production of cAMP, and so hampers serotonergic phase advances. Serotonin however, does not act to discourage NPY's actions. Melatonin also inhibits 5-HT induced phase advances through the inhibition of cAMP, and enforces a phase advance in the pacemaker similar to that following exposure to NPY (Prosser, 2000).

There is an interaction between the retina and raphe input to the SCN to transmit photic information. This interaction is a modulatory one such that raphe stimulation attenuates the phase shifts caused by photic stimulation through the release of 5-HT, while 5-HT antagonists

can enhance this same input (Refinetti, 2000). Both 5-HT_{1B} and 5-HT_7 aid in the inhibition of photically induced phase advances at night. The 5-HT_{1B} receptors on retinal terminals block glutamate release, while 5-HT_7 receptors antagonize glutamate's postsynaptic effects (Prosser, 2003b). Serotonin can block the postsynaptic effects of glutamate by inhibiting the rise in Ca^{2+} levels that usually follows glutamate administration (Gannon, 2001).These effects may occur because the serotonergic receptors in the SCN synapse on the same cells that receive input from the retina. The connection between the two afferent inputs is apparent in hamsters. A photically induced phase shift in the activity rhythm of the hamster is lessened when the animal is active during the period of light. This activity may increase the concentration of serotonin present, among other places, in the SCN (Redfern & Lemmer, 1997). Glutamate can also interfere with serotonergic phase advances through the actions of AMPA-like receptors (Prosser, 2000).

The aforementioned effects that 5-HT has on the SCN have not been observed equally *in vivo* and *in vitro.* "Direct clock phase resetting by 5-HT...has been seen more consistently *in vitro* than *in vivo*" and several possible reasons could explain why this is so (Prosser, 2003a). That all afferent connections are severed when the SCN is removed from the brain for *in vitro* experimentation, may cause phase shifts uncharacteristic of living animals. When examining the influences of 5-HT on the SCN clock *in vitro,* neurotransmitters such as NPY, melatonin, and glutamate which would inhibit serotonin's effects are removed, the result being that the administered 5-HT is free to exert its full effects. In addition, when the SCN tissue is maintained *in vitro* the tissue may spread out slightly, creating more extracellular space. This might allow entraining chemicals to interact with the SCN neurons more easily and perhaps exert larger effects on the pacemaker than they could *in vivo.* The sensitivity of the 5-HT receptors may also be enhanced following separation of the SCN from the brain because there is a decrease in the

available 5-HT *in vitro*. Thus, the SCN may respond more robustly to 5-HT following a period of detachment from the raphe nuclei because the 5-HT receptors have become more sensitive to their ligands presence. If the 5-HT toxin *p*-chlorophenylalanine (PCPA) is injected into the SCN *in vivo* given to the pacemaker cells *in vivo*, the cells exhibit a larger phase advance when exposed to the 5-HT agonist DPAT than in control conditions (Prosser, 2003b). Correspondingly, if *in vivo* SCN cells can be sensitized to the presence of 5-HT following a depletion in 5-HT using PCPA, supplying *in vitro* SCN cells with a constant supply of 5-HT following slice preparation should desensitize the cells and cause no phase shift following a burst of 5-HT administration. This was indeed found in previous literature and "these results are consistent with the hypothesis that 5-HT receptor sensitivity (or downstream processes) normally increases *in vitro* due to the severing of 5-HT afferents during slice preparation" (Prosser, 2003a). These experiments indicate that 5-HT receptor sensitivity is mediating the different effects 5-HT has on the periodicity of the SCN *in vitro* and *in vivo.*

Because of this information I used immnuocytochemistry to investigate changes in the sensitivity of serotonin receptors in mouse brain slices. I hypothesized that the density of these receptors is correlated with the presence or absence of serotonin. When serotonin is plentiful the number of receptors is lower because there is so much serotonin to bind easily. However, when serotonin levels are low, the density of receptors should increase because serotonin is scarce. In mouse brain slices, the SCN has effectively been cut off from endogenous serotonin release. To test whether the density of serotonin receptors in the SCN (particularly 5-HT_7) changes in response to different levels of serotonin stimulation, I planned to perform immunocytochemical analysis of serotonin receptors in the SCN from brain slices exposed to different amounts of serotonin. Such investigation should help reveal whether serotonin causes a more robust

response *in vitro* because its receptors' sensitivity has been heightened due to deprivation from the endogenous serotonin input it received *in vivo*.

Chapter 2

Immunocytochemistry

Brain slice preparation: Coronal brain slices (500 μm) containing the SCN were prepared during the daytime from adult, male C57BL/J6 mice housed in a 12:12 light-dark cycle as reported previously (Prosser, 2000). Slices were maintained at the interface of a Hatton-style brain slice chamber, where they were perfused continuously with warm (37°C), oxygenated (95% O2 / 5% CO2), glucose/bicarbonate-supplemented Earle's Balanced Salt Solution (pH=7.4) (Sigma, St. Louis, MO). Slices were maintained in vitro for approximately 30 hours. Slices were then transferred to cold (4°C) 2% paraformaldehyde for 2-3 hours. Slices were then transferred to cold phosphate buffered solutions until sectioning.

Vibrotome sectioning was done to make 40-50 μm slices of the mouse SCN. The sections were rinsed with 0.1M Phosphate Buffer (PB) (pH=7.4) three times for ten minutes each time. Next, the slices were quenched in 0.3% H_2O_2 / 0.1M PB for 30 minutes at 4°C. After quenching, sections were put through serial washing with TritonX-100 (Sigma, St. Louis, MO) / 0.1M PB (pH = 7.4). Subsequently, the slices were blocked by 2% normal goat serum (Sigma, St. Louis, MO) (pH = 7.4-7.5). Then the sections were incubated with 5-HT_7R antibody (1:5000) (ImmnuoStar, Hudson, WI) (raised from rabbit) for 24 hours. The next day, after rinsing, the sections were incubated with anti-rabbit IgG conjugated with peroxidase (1:600) (Sigma, St. Louis, MO) at room temperature for one hour. For visualization, sections were reacted with 0.025% DAB, 0.01% $CoCl_2$, and 0.0003% H_2O_2 for 5-7 minutes. After several washings with dH_2O, the "stained sections were floated on to a gel-coated slide and mounted with coverslips with Permount (Fisher Scientific, Houston, TX)" (Prosser et al., 2003). For data analysis an Optronics Camera manufactured by Generic was used to take pictures under an

Olympus BX40 Microscope. And then, image files were captured by the PictureFrame version 2.1 program. Had strong positive staining consistently resulted, optical density measurements would have been conducted to determine the density of the 5-HT_7 receptor in the SCN. To accomplish this, the density of color in the SCN would be compared to the density of color in nearby areas of the hypothalamus that experienced nonspecific staining, and the ratio of the two densities would provide the relative optical density. Slices exposed to different amounts of 5-HT could all be analyzed in this way. For the full Immunocytochemical protocol refer to Appendix A.

Chapter 3

Results

5-HT_7 receptors are mainly expressed in the hippocampus, thalamus, hypothalamus, and suprachiasmatic nucleus. For the purpose of this research, I focused on the changes in the 5-HT_7 receptor (5-HT_7R) in the SCN. To observe changes in the 5-HT_7R in the SCN caused by variation in availability of serotonin, I did immunocytochemistry on mouse brain slices using 5-HT_7R Ab (ImmunoStar, Hudson, WI) from rabbit as the primary Ab and anti-rabbit goat IgG conjugated with peroxidase (Sigma, St. Louis, MO) as the secondary Ab.

Positive staining in the SCN for 5-HT_7R in the SCN was found using a 5-HT_7R Ab concentration of 1:5000 and a secondary Ab concentration of 1:600. This can be seen in Figure 4, which seems to show a clear contrast in staining between the SCN and surrounding tissue. The SCN section in this image was bathed in 0.05M Tris-HCl buffer during the DAB reaction that occurs during the visualization step. These results are ambiguous however because using the same concentrations of antibodies as before, weak staining was observed as shown in Figure 5. The difference in these two experiments is the buffer used during the visualization step. The tissue in Figure 5 was exposed to 0.1M Phosphate Buffered Saline instead of 0.05M Tris-HCl buffer during the visualization step. When the concentration of 5-HT_7R was varied from 1:5000 to 1:1000, and concentration of secondary Ab held constant at 1:600, slightly weaker staining of the SCN was found than in Figure 5. These new concentrations of antibodies produced staining as seen in Figure 6. Again the buffer used during the visualization step was 0.1M Phosphate Buffered Saline. While clear distinctions in staining strength were not seen following varied concentration of primary Ab administration, they were observed when different

buffers were used. Specifically, coronal sections buffered with 0.05M Tris-HCl buffer during the visualization step provided much clearer staining of the 5-HT_7R in the SCN.

Again however, these results are not absolute because nonspecific staining of the tissue was also found when the slices were exposed to a primary Ab concentration of 1:5000 and a secondary Ab concentration of 1:600, using 0.1M Phosphate Buffered Saline during visualization. Thus, under the same Ab concentration and buffering conditions, both weak staining in the SCN, and nonspecific staining throughout the tissue was found.

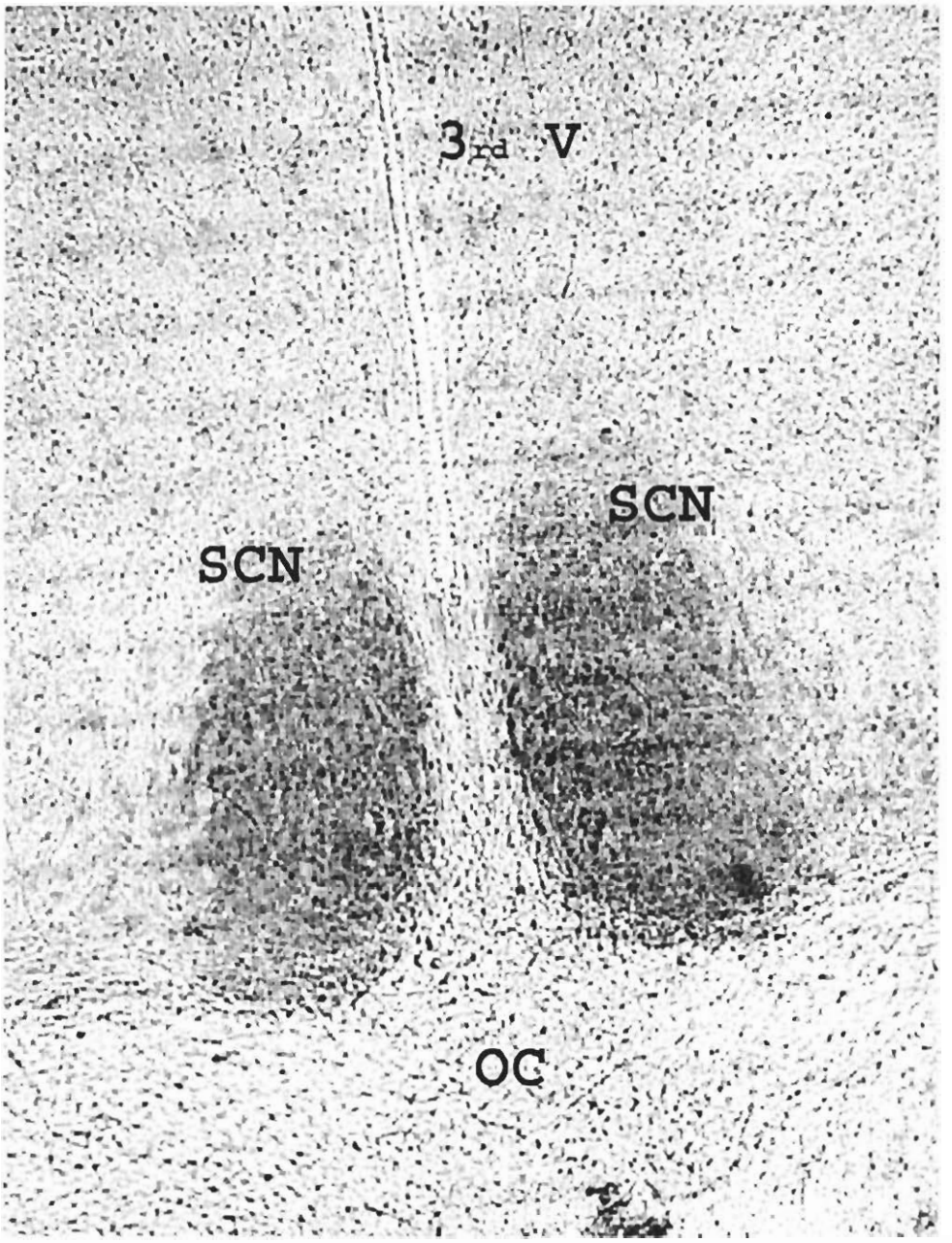

Figure 4: Positive Staining of 5-HT$_7$R in the SCN.

This is an image of a 50μm mouse coronal section of the SCN. To observe the 5-HT$_7$R in the SCN, I used anti-5-HT$_7$R Ab (1:5000) from rabbit and anti-rabbit goat 2° Ab (1:600). During the DAB reaction required for visualization, 0.05M Tris-HCl buffer was used. Abbreviations are: 3rd V-Third Ventricle, SCN-Suprachiasmatic Nucleus and OC-Optic Chiasm.

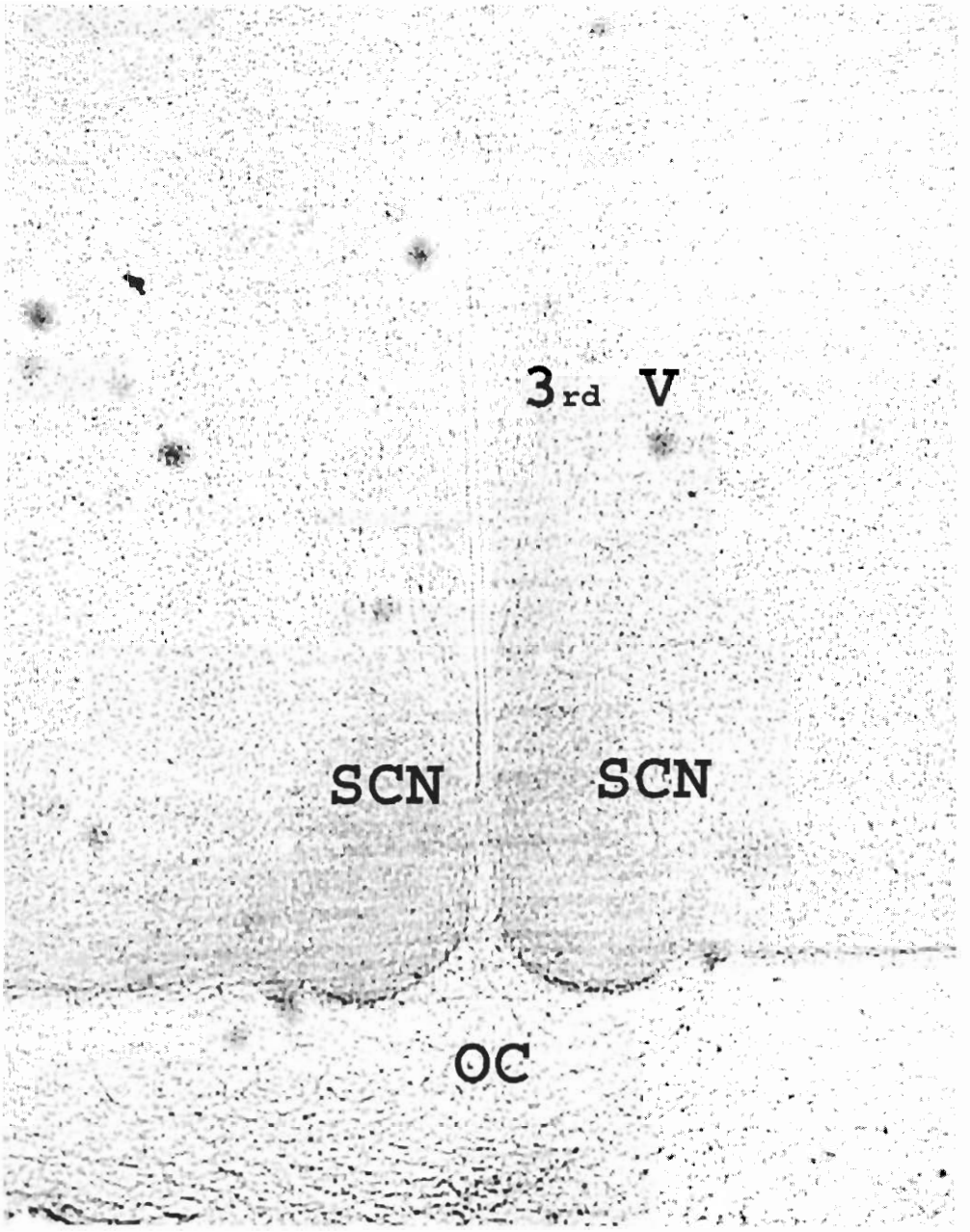

Figure 5: Weak Staining of 5-HT$_7$R in SCN.

This is an image of a 50μm mouse coronal section of the SCN. To observe the 5-HT$_7$R in the SCN, I used anti-5-HT$_7$R Ab (1:5000) from rabbit and anti-rabbit goat 2° Ab (1:600). During the DAB reaction required for visualization, 0.1M Phosphate Buffered Saline was used. Abbreviations are: 3rd V-Third Ventricle, SCN-Suprachiasmatic Nucleus and OC-Optic Chiasm.

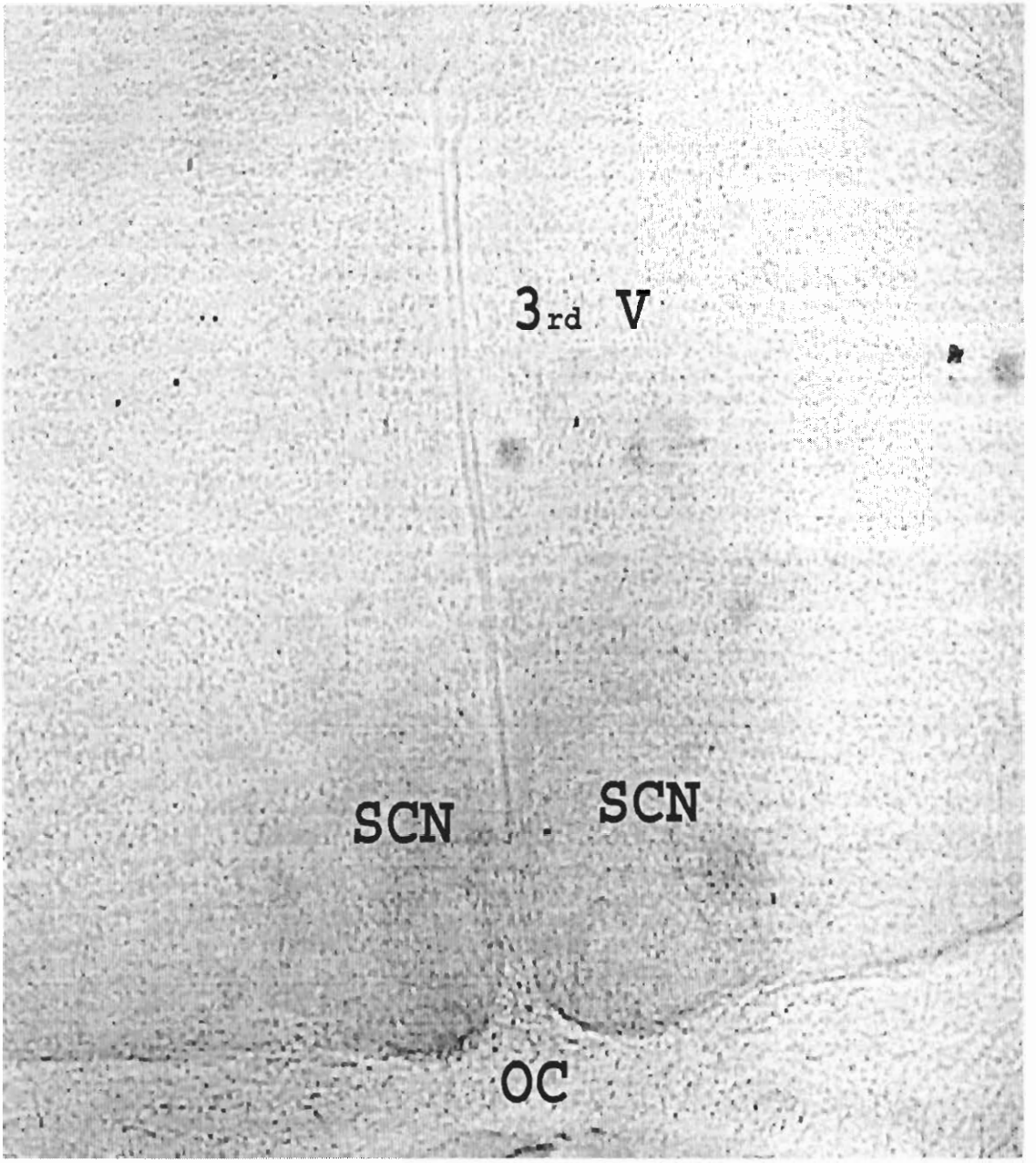

Figure 6: Weak Staining of 5-HT$_7$R in SCN.

This is an image of a 50μm mouse coronal section of the SCN. To observe the 5-HT$_7$R in the SCN, I used anti-5-HT$_7$R Ab (1:1000) from rabbit and anti-rabbit goat 2° Ab (1:600). During the DAB reaction required for visualization, 0.1M Phosphate Buffered Saline was used. Abbreviations are: 3rd V-Third Ventricle, SCN-Suprachiasmatic Nucleus and OC-Optic Chiasm.

Chapter 4

Discussion

In immunocytochemistry there are four main factors to consider. They are antibody (Ab) ratio, pH, H_2O_2 concentration, and section condition. The correct balance of each factor must be attained for optimal results. Strong positive staining was not found in my experiments and there are several reasons that this could be so, all dealing with the aforementioned issues. To begin with, the Ab ratio of both primary and secondary antibodies must be considered. The affinity of the Ab for the tissue antigen is important and inversely correlated with incubation time required for equilibrium (the point when the available binding sites on the antibody have become saturated). For this reason it might be beneficial to pick a primary Ab with a high affinity for its antigen so that more intense staining can be obtained in a shorter amount of time. Also, antibody-antigen interactions are reversible so a high affinity Ab must be utilized because during immunocytochemistry repeated washings and rinses can loosen a weak complex. Dynamics that could change the strength of this antibody-antigen complex are high temperature, low pH, and high salt concentrations. However, incubating the specimens with a higher concentration of the primary Ab for a shorter duration could enhance nonspecific background staining. "Gentle agitation helps to reduce background staining" (Boenisch, 2004a).

The maintenance of proper pH of all solutions and materials used in the immunocytochemistry protocol is also necessary for proper positive staining to occur. This is because antibodies and buffers work optimally at pH of 7.4-7.8. Allowing the sections to be bathed in acidic or basic environments can skew results because the effectiveness of all agents is lowered.

In addition, during the immunocytochemistry I performed on the mouse coronal sections of the SCN, I found that the buffer used during the visualization step had an effect on the clarity of staining obtained in the SCN. Using 0.05M Tris-HCl buffer in the visualization step appeared to give more positive staining than the 0.1M Phosphate Buffered Saline did. Boenisch stated that "of the diluents tested, Phosphate Buffered Saline, although widely used as a diluent for primary antibodies, was found to suppress the reactivity of most...antibodies tested" (Boenish, 2004c). This finding agrees with the weak results I observed using Phosphate Buffered Saline, however does not explain why 0.05M Tris-HCl buffer utilization during visualization led to stronger positive staining.

Another factor that must be controlled for is the concentration of H_2O_2 used during the visualization step because an excess of H_2O_2 can increase the dark background staining that would inhibit unambiguous analysis of the findings.

During sectioning the SCN slices can become crushed or ripped which has a detrimental effect on the quality of positive staining that can result from the immunocytochemistry. Care must be taken to keep sections in top condition so that antigens in question remain in the area of the slice in which they would naturally be. Poor sections can result in excessive nonspecific background staining.

Other factors that exacerbate potential background staining include diffusion of antigens from its original site throughout the surrounding tissue, ingestion of antigens by phagocytes in tissue outside the target area, and cross-reactivity. Diffusion of antigens could occur if they are also in the blood plasma and had already perfused the tissue prior to the experiment. Phagocytes outside the area of interest could also have ingested the antigen, in this case 5-HT_7R, and so could be capable of interacting with the primary Ab. Cross-reactivity could also occur in which

the Ab when the target protein is similar to proteins outside of the target cells. If this similarity is strong enough the primary Ab could bind to several antigens to produce nonspecific staining (Boenish, 2004b). Many factors must be taken into consideration when attempting to perform a successful experiment using immunocytochemistry. Less than desirable balancing of any combination of these factors could have caused the relative absence or irregularity of positive staining seen in my experiment. Careful balancing of these aspects of the immunocytochemistry protocol is necessary to obtain clear and unambiguous staining in future research.

Had expected results been obtained, specifically strong positive staining in the SCN following exposure to a single burst of 5-HT_7 Ab, further experimentation would have been desired. These cells could represent the optical density of cells not exposed to continuous supply of 5-HT preceding a phase-shifting dose of 5-HT (10 μM), or similar immunocytochemistry could be performed using 5-HT instead of 5-HT_7 Ab. A control set of SCN tissue slices would not be exposed to any 5-HT following excision from the animal, which should result in no staining. These slices would be used as a baseline to compare all other optical density ratios obtained. As reported previously (Prosser, 2003a), slices continuously bathed in a non-phase shifting dose of 5-HT (0.05 μM), and then administered a phase-sifting dose of 5-HT (10 μM), exhibited no phase shift. SCN tissue under these same conditions should likewise show no positive staining in the SCN for 5-HT_7R. A fourth trail would keep these conditions the same, however, the non-phase shifting dose of 5-HT would be kept at 0.01 μM, and the resultant staining should be intermediate between that seen in samples treated with no continuous supply of 5-HT preceding a phase-shifting dose of 5-HT (10 μM), and that seen in slices exposed to a constant non-phase shifting dose of 5-HT (0.05 μM) and then a phase-shifting dose of 5-HT (10 μM). Further research would hopefully incorporate these ideas.

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Appendix A

IHC (ver. 3.1) for 5-HT7R

Consideration> section, removing bubbles in egg yoke block, incubation time of 1° Ab, all Ab ratio, Ab condition, pH

Fixing tissue (for 5-HT7)
Put slices (on filter paper) in 2% paraformaldehyde/0.1% glutaraldehyde for 2hr and transfer slices to 0.1M PB for overnight (in cold room)

1. Fix egg yoke block at platform of vibratome
2. Pour PB up to soaking block in bath

Note> Free floating method

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3. Cut brain slices each 50um
4. Transfer them to PB

Note> No dehydration step
Do not dry

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5. **Rinse 3x10min in 0.1M PB** (pH 7.4~7.8)
6. **Quenching: Incubate 30min in 0.3% H2O2 in PB**
7. Rinse 3x5min in 0.1M PB

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8. Rinse 2x10min in 0.5% Triton X-100 in 0.1M PB (pH 7.4-7.8)
9. Rinse 2x10min in 0.1% Triton X-100 in 0.1M PB (pH 7.4-7.8)
10. Incubate 30-60min in 0.1% Triton X-100 in 0.1M PB with normal serum **at R.T.** (final concentration 1~10%, **usually 2%**)

Note> Normal serum generally is raised from 2° Ab animals (e.g. if 2° Ab is rabbit anti-XXX, use normal rabbit serum (NRS) or if 2° Ab is goat anti-XXX, use normal goat serum (NGS))
When negative control is done, use opposite normal serum, vice versa

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11. Rinse 2x5min in 0.1% Triton X-100 in 0.1M PB
12. **Incubate it with targeted 1° Ab (adjust final concentration of Ab, diluted in PB with normal serum) overnight (24h~48h) in the cold room**

Note> Keep cold condition step 5 ~ 11 (except step 10)

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13. Equilibrate at room temperature for 30min
14. Rinse 3x10min (or 3x5min) in 0.1% Triton X-100 in 0.1M PB
15. **Incubate for 1hr 2° Ab IgG peroxidase at room temperature (Incubation time depends on Ab ratio)**

Note> The ratio of **2° Ab and PB with normal serum** can be adjusted (1:1000, 1:500, **1:600**, 1:400 etc.)

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16. Rinse 3x10min in 0.1% Triton X-100 in 0.1M PB
17. Rinse 2x10min in 1X PBS
18. React with 0.025% DAB, 0.01% CoCl2 in **0.1M *PBS*** (or 0.05M Tris buffer) for 20 min ***in dark room,*** room temperature.

19. Add H_2O_2 (final concentration 0.0003%~0.0006%) - can be adjusted) to DAB solution and react until desired staining
20. Rinse 3x10min in dH_2O (rinse them as fast as you can)

Note> **DAB is strong carcinogen Wear gloves and Carefully handle it.**

In step 18 and 19, immediately use DAB/H_2O_2 solution (Do not mix them before using)
In the final washing step (step 20), do not over-react staining with remained DAB/H_2O_2 solution

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21. coverslipping with permount

Note> If it may be necessary, do counterstaining before step 22

Supplement for Step 18 and Step 20

Step 18> React with 0.05% DAB, 0.02% $CoCl_2$ in **0.1M *PBS*** for 20 min ***in dark room,*** room temperature (5mg DAB+100ul 2% $CoCl_2$ in 10ml PBS) / rinse with 0.05M Tris-HCl(pH 7.4) to prevent $CoCl_2$ from over-precipitating.

Alternative visualization method

1. → Mix 5mg DAB in 10ml PBS (0.05% DAB) and incubate it for 5min
2. → Transfer the slide and briefly rinse it in 0.05M Tris-HCl (pH7.4)
3. → Add 50ul of 2% $CoCl_2$ to DAB/PBS solution and incubate it for 5min
4. → Transfer the slide and briefly rinse it in 0.05M Tris-HCl (pH7.4)
5. → Transfer the slide to DAB/$CoCl_2$ solution and incubate it for 5min
6. → Transfer the slide and briefly rinse it in 0.05M Tris-HCl (pH7.4)
7. → Repeat 5 and 6
8. → Remove DAB/$CoCl_2$ solution and add fresh 10ml of 0.1M PBS
9. → Add 1ul of 30% H_2O_2 and react until desired staining

Step 20> Rinse 3x10min in dH_2O (rinse them as fast as you can)

Method *** **USE "COLD dH2O"** and **fastest** ***

→ After reaction, rinse with tap water (**dH2O**) (by using water bottle)
*** **Do not directly** pour water on the tissue surface ***
→ Briefly rinse it in COLD dH2O
→ Rinse with tap water (dH2O)
→ Briefly rinse it in COLD dH2O
→ Rinse with tap water (dH2O)
→ Rinse it in COLD dH2O for 5min
→ Rinse it in COLD dH2O for 5min
→ Rinse it in COLD dH2O for 10min

→ this rinse method is optional.