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THE EFFECTS OF MELATONIN ON HIPPOCAMPAL PHYSIOLOGY

By

Yasir El-Sherif

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

The effects of melatonin on hippocampal physiology

by Yasir El-Sherif

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Department of Biology

The actions of melatonin on hippocampal evoked-potentials from the CA1 region were studied. Melatonin (0.1-2000μM) caused a concentration-dependent attenuation of the population spike (PS) and excitatory postsynaptic potential (EPSP), however did not influence propagation of the action potential as measured by the fiber volley (FV). Melatonin's actions were biphasic, demonstrating a rapid attenuation followed by a delayed recovery/amplification of the potential. The attenuation of the PS by melatonin (10μM) was significantly reduced by the addition of the MT2 melatonin receptor antagonists 4P-PDOT or luzindole. The actions of melatonin were also regulated by factors including: the time of slice incubation, age of mice and environmental lighting. The reduced binding of melatonin to the hippocampal membranes correlated well with the decreasing responsiveness of hippocampal neurons to melatonin, as the slice age increased. Constitutive activation of MT2 receptors by BMNEP mimicked the attenuation of the PS by melatonin. Addition of the charged melatonin receptor ligand TMEPI caused a delayed

(45-60 min) recovery/amplification of the PS. The melatonin-induced depression of the PS could be immediately reversed by application of high frequency stimulation (HFS) commonly used to induce long-term potentiation (LTP). Melatonin was able to convert the inhibition induced by the pairing two pulses with an interstimulus delay of 10-13 ms (paired pulse inhibition - PPI) into facilitation. Melatonin also caused a transient (5 min) conversion of the facilitation caused by the pairing of two pulses with an interstimulus delay of 15-40 ms (paired pulse facilitation - PPF) into inhibition. In conclusion melatonin depressed glutamatergic transmission in the CA1 region of the hippocampus through a receptor-mediated mechanism. These receptor-mediated actions of melatonin may influence learning and memory in the hippocampus. Melatonin as described in this dissertation may act to filter neuronal signals departing from the hippocampus to higher cortical structures.

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Abbreviations:

2-Imel	2-iodomelatonin – Melatonin receptor agonist.
4P	4P-PDOT – Melatonin receptor antagonist with higher specificity for MT2 vs. MT1.
5-HTP	5-hydroxytryptophan
6Cl-melatonin	6-chloromelatonin – Melatonin receptor agonist.
AMPA	Agonist for AMPA receptor, which allows mainly Na^+ ions to flow through.
BMNEP	MT2 agonist, which alkylates to its receptor and permanently, activates it.
CREB	cAMP response element binding protein
comm.	Commissural fibers - fibers arriving from the contralateral hippocampus that join with the Schaeffer collaterals.
DG	Dentate gyrus
EC	Entorhinal cortex
EtOH	Ethanol
EPSP	Excitatory postsynaptic potential - Depolarization of postsynaptic membrane.
FV	Fiber volley - Compound action potential from axonal membrane.
GABA	Major inhibitory neurotransmitter in the brain
HFS	High frequency stimulation – 100 Hz stimulation for one second three times in thirty seconds.
HIOMT	Hydroxyindole-O-methyltransferase
IPSP	Inhibitory postsynaptic potential - Hyperpolarization of postsynaptic membrane.
L/D	Light/dark

LTP	Long-term potentiation – Long-term enhancement of synaptic efficiency.
LFS	Low frequency stimulation – 0.03 Hz stimulation
Luz	Luzindole – Melatonin receptor antagonist with higher specificity for MT2 vs. MT1
Mel	Melatonin
MT1	Type of melatonin receptor
MT2	Type of melatonin receptor
n.s.	Not significant
NMDA	Agonist for NMDA receptor, which allows preferentially Ca^{2+} to enter the neuron.
PPF	Paired pulse facilitation – Short-term synaptic facilitation caused by pairing two pulses with an interstimulus delay in the range of 15-40 ms.
PPI	Paired pulse inhibition – Short-term synaptic inhibition caused by pairing two pulses with an interstimulus delay in the range of 10-13 ms.
PVN	Paraventricular nucleus
PT	Pars tuberalis
PP	Perforant path
PS	Population spike – Combined depolarization of the neuronal membrane and the action potential induced by that depolarization.
SC	Schaeffer collaterals – Axonal projections from CA3 neurons, which synapse onto CA1 neurons.
Serotonin	5-hydroxytryptamine
SNAT	Serotonin N-acetyltransferase
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus

TMEPI	Charged melatonin receptor ligand
UTC	Upper thoracic column of the spinal cord

Chapter 1 - Introduction

Melatonin

The neuronal hormone melatonin, which is synthesized and secreted mainly from the pineal gland (a small neuroendocrine organ whose location varies between species), plays an important physiological role in synchronizing biological rhythms and neuroendocrine functions in vertebrates (Kvetnoy et al. 1997). In all species studied, melatonin levels rise at night and fall to low levels during the day (Reppert et al. 1979; Zawilska et al. 2000; Bubenik et al. 1993). Since light is the most important factor controlling melatonin synthesis (Axelrod et al. 1966; Tomatic and Orias 1967; Zawilska et al. 2000; Zatz et al. 2000) this hormone acts as a chemical messenger by transducing photoperiodic information to the brain. As well as following a circadian rhythm (daily), melatonin also displays a circannual rhythm (yearly; Vivien-Roels et al. 1979; Danilenko et al. 1994; Thrun et al. 1995). In general, melatonin concentrations in the plasma remain elevated longer when daylight is shorter (e.g. the winter). The circadian and circannual rhythms of melatonin have been shown to correlate with the breeding patterns of a number of species (Brinklow and Loudon 1993; Jackson et al. 1990; Bittman et al. 1983).

Melatonin levels rise at night because of an increase in the transcription and translation of serotonin N-acetyltransferase (AA-NAT), the rate-limiting enzyme in melatonin synthesis. Light given during the dark phase of a light/dark (L/D) cycle can suppress AA-NAT, thus reducing melatonin synthesis and release from the pineal gland (Minneman et al. 1974). Melatonin can, in turn, influence circadian rhythms through activation of melatonin receptors in the suprachiasmatic nucleus (SCN) of the hypothalamus, the site of the mammalian circadian pacemaker (Weaver et al. 1993; Dubocovich et al. 1996; Hunt et al. 2001). Exposure to light at different times in the night phase of the L/D cycle can change the periodicity of melatonin synthesis (Reiter 1991). In humans, melatonin supplementation has been used to treat disruptions of photoperiod-induced circadian rhythms such as jet lag syndrome (Arendt and Broadway 1987) and shift work syndrome (Folkard et al. 1993). Both syndromes are expressed as a desynchronization of normal circadian rhythms as well as a lower level of melatonin in the plasma. By supplementation with melatonin a number of these adverse symptoms can be alleviated.

Humans produce relatively low levels of melatonin, which can be found at varying concentrations throughout the

body fluids (Reiter 1986,1993). Physiological levels of melatonin in the blood are in a concentration range of 10-100 pM (30 to 200 pg/ml; Ubeda et al. 1995; Waldhauser and Dietzel 1985). Melatonin levels are highest during early childhood, begin to decrease around puberty and continue to fall into senescence (Garcia-Patterson et al. 1996). In rodents, decreasing the levels of melatonin by pinealectomy has been shown to cause an increase in oxidative damage of macromolecules and tissues (Reiter et al. 1999) over a lifespan. This ability of melatonin to act as an antioxidant has been shown by many researchers (Pierrefiche et al. 1993; Reiter 1996; Konecna et al. 2001; Somova et al. 2001; Lee et al. 2001), leading to a corroboration of research on the oxidative theory of aging and the antioxidative properties of melatonin. The publication of many articles and books describing the potential antioxidant/antiaging properties of melatonin swept the United States in the early/mid 1990's. One of the first books to bring melatonin into the public's attention was published by Pierpaoli and Regelson in 1993 and led to a dramatic public interest in using exogenous melatonin as a "cure" for the symptoms of aging. Currently, the FDA does not regulate the manufacture and distribution of melatonin making it freely accessible for public use. Melatonin has

been used in the treatment of a number of diseases including among others epilepsy and cancer (Lissoni et al. 1987, 1989; Moretti et al. 2000; Molina-Carballo et al. 1997; Fauteck et al. 1999a; Sandyk 1990; Sandyk and Kay 1990; Brzezinski 1991). The most often employed concentration of melatonin for oral use is 3mg/day, which can lead to levels of this hormone in the blood of up to 1-10 nM. In the treatment of cancers, melatonin has been used at a dose of 50mg to 700mg/day (Kane et al. 1994; Gonzalez et al. 1991). In a study by Guardiola-Lemaitre (Guardiola-Lemaitre 1997), ingestion of melatonin by human subjects, at a single dose of 5 mg raised morning melatonin levels (1-10 pM) to almost 10 nM one hour after administration. When 80 mg tablets were used the elevation of melatonin in the plasma was as high as 100 nM, one hour after administration (see **Table 1.1**).

Due to the fact that melatonin is an over-the-counter drug in the U.S., and it can raise levels of circulating melatonin significantly, it becomes important to understand its actions exerted at both physiological, as well as pharmacological concentrations.

The Pineal Gland

The pineal gland is a small secretory organ regulated by either direct exposure to light, or influenced by visual

	Normal (Average)	**5 mg Mel**	**10 mg Mel**	**80 mg Mel**
Morning	*4.3 pM* (Graw et al. 2001; measured from the plasma)	*24,000 pM* (Graw et al. 2001; measured from the plasma)	*90,400 pM* (Dollins et al. 1994; measured from the plasma)	*130,000* pM (Guardiola-Lemaitre 1997; measured from the plasma)
Night	*861 pM* (Graw et al. 2001; measured from the plasma)	*80,200 pM* (Debus et al. 2002; measured from the CSF)	Not data available	No data available

Table 1.1. Physiological and pharmacological concentrations of melatonin in humans.

pathways. In fish and amphibians, regulation of the pineal takes place by direct exposure to light, because of its location on the surface of the brain (Axelrod et al. 1965; Cahill 1996; Iigo et al. 1997a, 1997b). In birds and reptiles, the signal is usually a combination of direct photoreception and light-induced hormonal signals (Veylon 1980; Tosini et al. 2001). In humans, due to the location deep in the midbrain, the pineal glands functions are regulated exclusively by signals arising from the retina (Reiter 1981). Although in rodents the pineal gland is located near the surface of the brain, its regulation is still similar to that of humans.

The neuronal pathways regulating the pineal (**Figure 1.1**) originate in the retina, which projects fibers to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract. From the SCN, the signal passes through the paraventricular nucleus (PVN), follows the medial forebrain bundle and ends in the intermediolateral cell column of the upper thoracic spinal cord (UTC). From there a projection to the superior cervical ganglion (SCG) exists, from which sympathetic neurons (nervii conarii) innervate the pineal (Moller and Baeres 2002). These fibers are the final part of the neuronal pathway between the SCN and the pineal. The main neurotransmitter of the sympathetic innervation is

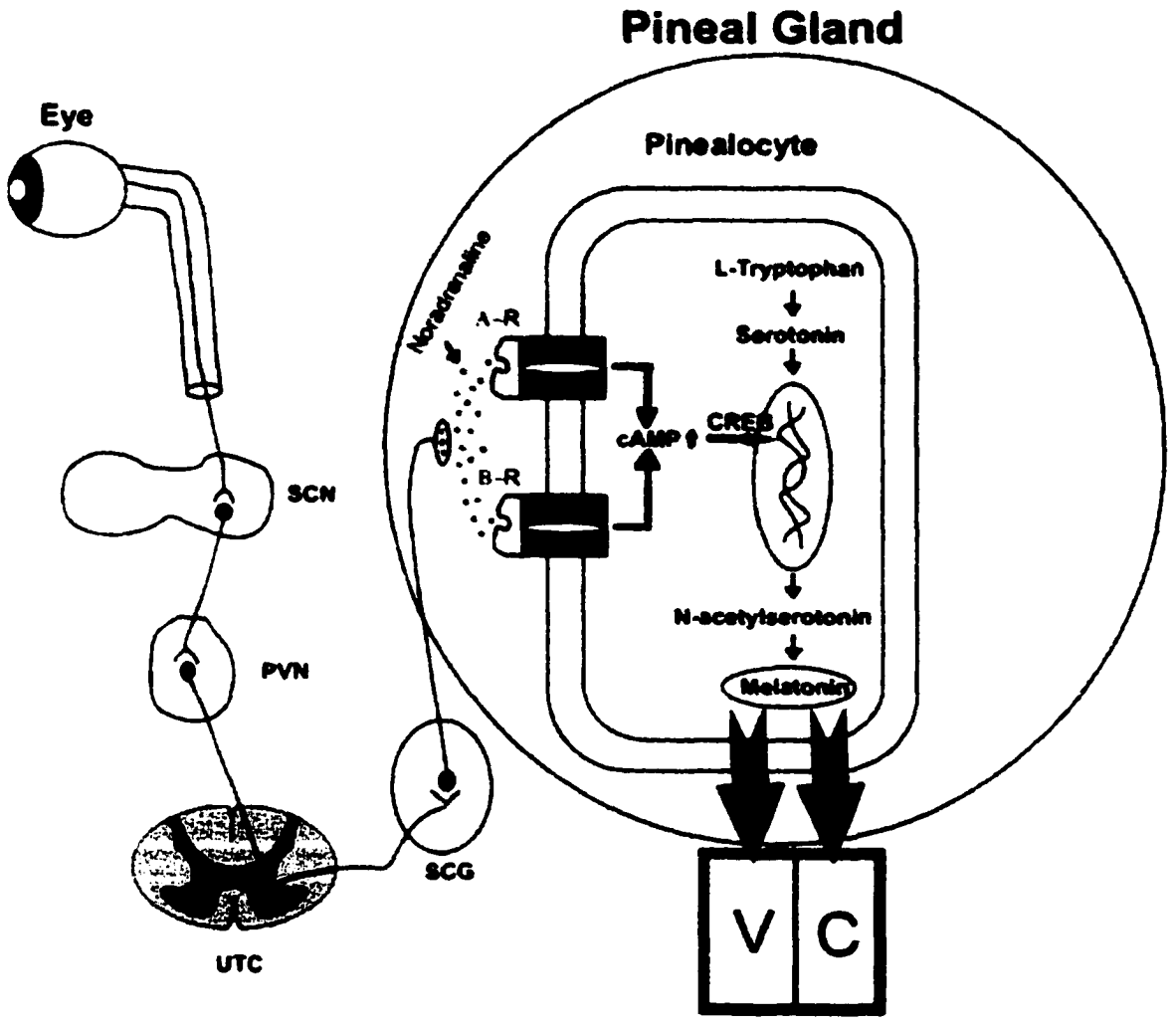

Figure 1.1. Schematic representation of the pathway from the eye to the pineal gland. **SCN**- suprachiasmatic nucleus; **PVN** - paraventricular nucleus; **UTC** - upper thoracic spinal cord; **SCG** - superior cervical ganglion; **A-R** - alpha adrenergic receptor; **B-R** - beta adrenergic receptor; **CREB** - cAMP response element binding protein; **V** - Ventricles of the Brain; **C** - Capillaries. The arrow behind cAMP indicates an increase in its concentration following stimulation of A-R or B-R by norepinephrine.

noradrenaline, which is released in the perivascular space, near the pinealocytes.

The pineal contains a dense vascular network and is thus a well-perfused organ (Hodde 1979a; Hodde and Veltman 1979b). The intensive blood supply assures a rapid transportation of melatonin towards target areas, including the brain. Direct secretion into the cerebrospinal fluid is another important way of melatonin delivery, especially to the brain structures located in the proximity of the ventricles (Rollag et al. 1977; Tricoire et al. 2002; Rousseau et al. 1999).

Melatonin: Metabolism and Distribution

Activation of β-adrenergic and/or α-adrenergic receptors (Figure 1.1; A-R and B-R) on the pinealocytes leads to an increase of cAMP via a G-protein coupled receptor leading to the activation of cAMP response element binding protein (CREB; **Figure 1.1**). CREB activates the transcription of serotonin N-acetyltransferase. Melatonin is synthesized from the essential amino acid L-tryptophan (**Figure 1.2**), which is converted to 5-hydroxytryptophan by tryptophan 5-hydroxlase. 5-hydroxytryptophan (5-HTP) is then converted to 5-hydroxytryptamine (serotonin) with aromatic amino acid decarboxylase. Serotonin N-acetyltransferase (SNAT) then converts serotonin into the

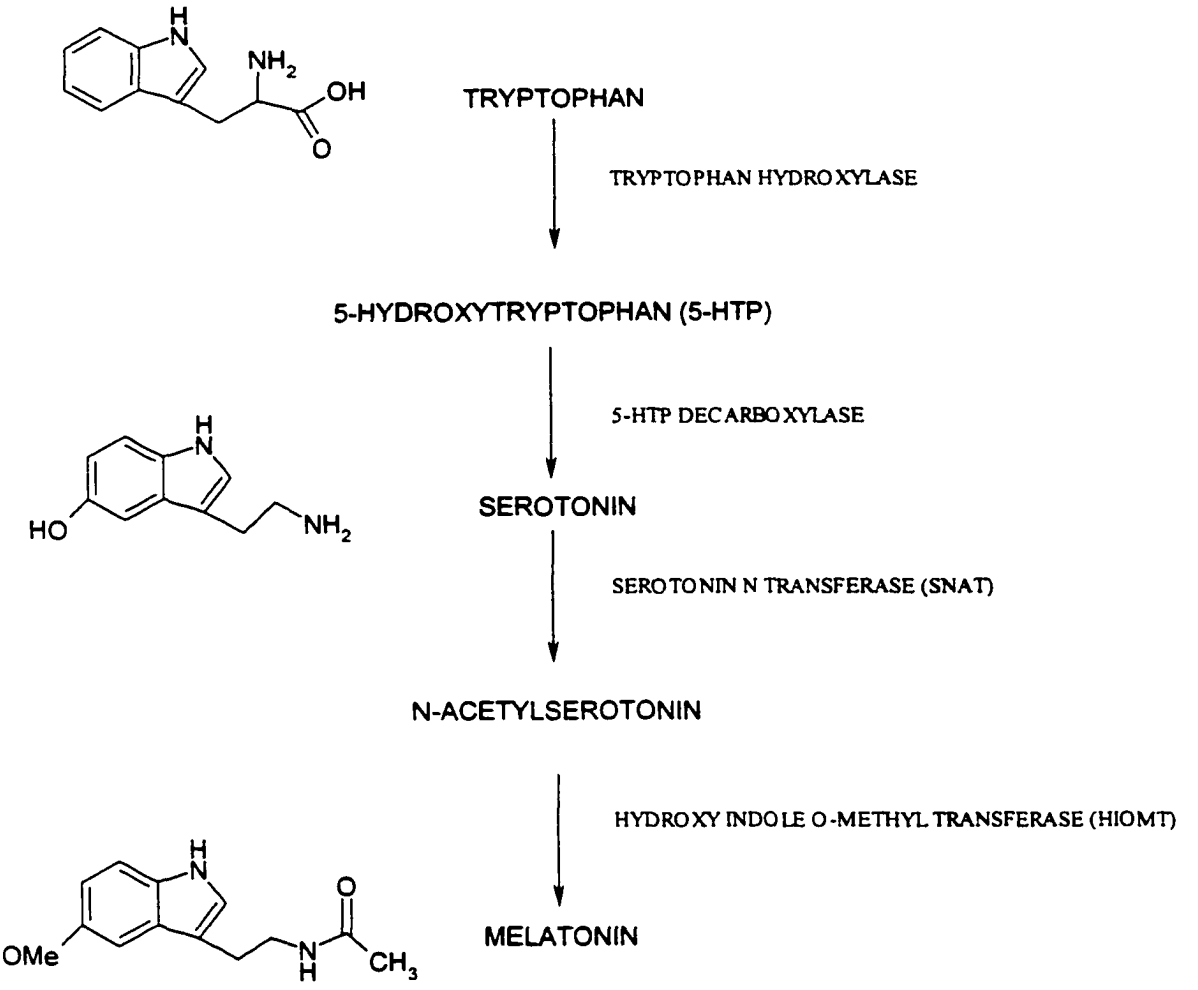

Figure 1.2. Synthesis of Melatonin from L-Tryptophan. L-tryptophan is first converted to 5-hydroxytryptophan by the enzyme tryptophan 5-hydroxlase. 5-hydroxytryptophan (5-HTP) is then converted to 5-hydroxytryptamine (serotonin) with the enzyme Aromatic amino acid decarboxylase. Serotonin N-acetyltransferase (SNAT) converts serotonin into the rate-limiting product for melatonin, N-Acetylserotonin. Melatonin is then synthesized from N-acetylserotonin by the enzyme Hydroxyindole- O - methyltransferase (HIOMT).

rate-limiting product for melatonin, N-acetylserotonin. Melatonin is then synthesized from N-acetylserotonin utilizing the enzyme Hydroxyindole- O - methyltransferase (HIOMT). Because melatonin is lipophilic it then leaves the pinealocytes through passive diffusion into the ventricles (Figure 1.1 - "V") and into the capillaries (Figure 1.1 - "C"). Melatonin can then act on melatonin receptors and/or other non-melatonin receptor binding sites.

The primary sites for melatonin degradation are the liver (the majority of melatonin is hydrolyzed here) and kidneys. Melatonin undergoes 6-hydroxylation followed by the addition of a sulfate or glucuronide group. The two by-products: 6-hydroxymelatonin sulfate (6-sulfatoxymelatonin) and 6-hydroxymelatonin glucuronide are then excreted in the urine (Deacon and Arendt 1994; Arendt et al. 1995). Measurement of the 6-sulfatoxymelatonin has been used as an indicator of circulating levels of melatonin in the plasma (Raynaud et al. 1993; Kennaway et al. 1999; Deacon and Arendt 1994), and is the most widely used method for quantification of circulating melatonin in humans.

Small amounts of melatonin can also be degraded in the brain, where it can be metabolized to either N-acetyl-N-

formyl-5-methoxykynurenamine or N-acetyl-5-methoxykynurenamine (Burkhardt et al. 2001; Tan et al. 2001).

The melatonin concentration in rodents is not uniform and varies depending on the structure studied and the time of day (Pang and Brown 1983; Skinner and Malpaux 1999). While the concentration of melatonin in the plasma was reported to be in the picomolar range, (Cardinali et al. 1997), the brain tissue (neurons and glia) can accumulate melatonin periodically, to a level exceeding 50 times its plasma concentration (Pang et al. 1990; Cardinali et al. 1997). It is speculated that neurons may have the ability to increase their concentrations of melatonin even higher through uptake (Witt-Enderby and Li 2000). The distribution of melatonin within the brain is not uniform and several fold differences in the melatonin content have been reported between brain ventricles (Skinner and Malpaux 1999). This is significant in that the hippocampus (the structure used as a model for this research) is located in proximity to the third ventricle, which receives melatonin directly from the pineal gland via the pineal recess (Tricoire et al. 2002). It was demonstrated that the third ventricle can accumulate melatonin to concentrations of up to 1 nM, while in the pineal recess melatonin

concentrations can be as high as 80 nM (Tricoire et al. 2002). Because the concentrations of melatonin in the third ventricle can be significantly higher then previously reported, structures like the hippocampus bathed by the ventricular cerebral spinal fluid, can be exposed to higher concentrations of melatonin as compared to structures distal to the ventricle.

Melatonin Binding Sites

In the late 1970's binding of tritiated melatonin (^{3}H-melatonin) in the bovine and chicken brain was reported (Cardinali et al. 1979; Cohen et al. 1978; Niles et al. 1979). However due to its low specific activity it was replaced by 2[^{125}I]-iodomelatonin, which showed higher specific activity (Vakkuri et al. 1984). While 2[^{125}I]-iodomelatonin is still used, a more detailed analysis has demonstrated that [^{3}H]-melatonin better resembles the normal binding and dissociation kinetics of melatonin then does 2[^{125}I]-iodomelatonin (Kennaway et al. 1994). Based on the results using 2[^{125}I]-iodomelatonin, the existence of two distinct melatonin receptor sites was shown (Pang et al. 1993; Pickering and Niles 1989; Dubocovich 1995; Sugden et al. 1997). The first was a high affinity site (sub-divided into MT1 and MT2) and the second was a low affinity site (described as MT3).

Some non-receptor binding sites for melatonin were found on both $GABA_A$ receptors (Coloma and Niles 1988) and K^+ channels (Varga et al. 2001), and they will be discussed later. The responses to melatonin can be categorized into two groups: receptor- and non-receptor mediated (discussed below). The classification of melatonin responses can also be differentiated according to the concentration of melatonin used to elicit them. While the effects of low (picomolar) concentrations of melatonin have been considered physiological, the effects observed following application of micro- to millimolar concentrations is usually recognized as pharmacological. While this arbitrary classification is still predominantly used (Tricoire et al. 2002) one has to be aware that high concentrations possibly even in the micromolar may occur normally *in vivo* and still be considered physiological (Reiter 2002; Reiter and Tan 2003).

Receptor-mediated Actions of Melatonin

For an action of melatonin to be categorized as occurring at the melatonin receptor, it must meet the following criteria: 1) the binding of melatonin must be with high affinity and selectivity; 2) melatonin binding should be saturable and reversible, reaching a time-dependent equilibrium; and 3) binding of melatonin must

elicit a biological response. These criteria can be tested experimentally with the use of two specific methods, radioligand binding and cAMP functional assay.

Specific binding using 2[^{125}I]-iodomelatonin has been demonstrated to be at three melatonin receptors: MT1 (formerly Mel1a; Dubocovich et al. 1998), MT2 (formerly Mel1b; Dubocovich et al. 1998) and MT3 (formerly Mel1c and more recently described as quinone reductase 2; Nosjean et al. 2000). Radioligand binding is still one of the most effective methods to demonstrate the presence of melatonin receptors (Duncan et al. 1986; Dubocovich and Takahashi 1987; Siuciak et al. 1991).

Activation of MT1 and MT2 receptors, by low picomolar concentrations of melatonin (1-10 pM) leads to a decrease in adenylyl cyclase activity via a G_i receptor (**Figure 1.3**; Reppert 1997; Reppert et al. 1994; Godson and Reppert 1997; Carlson et al. 1989; Shiu et al. 1989; Conway et al. 1997), while the activation of MT3 initiates hydrolysis of phosphatidylinositide (Eison and Mullins 1993; Blumenau et al. 2001; Mullins and Eison 1994). MT1 receptor mRNA has been found in brain structures including the SCN, pars tuberalis, hypothalamus, cerebellum, hippocampus and cerebral cortex of mammals (Mazzucchelli et al. 1996;

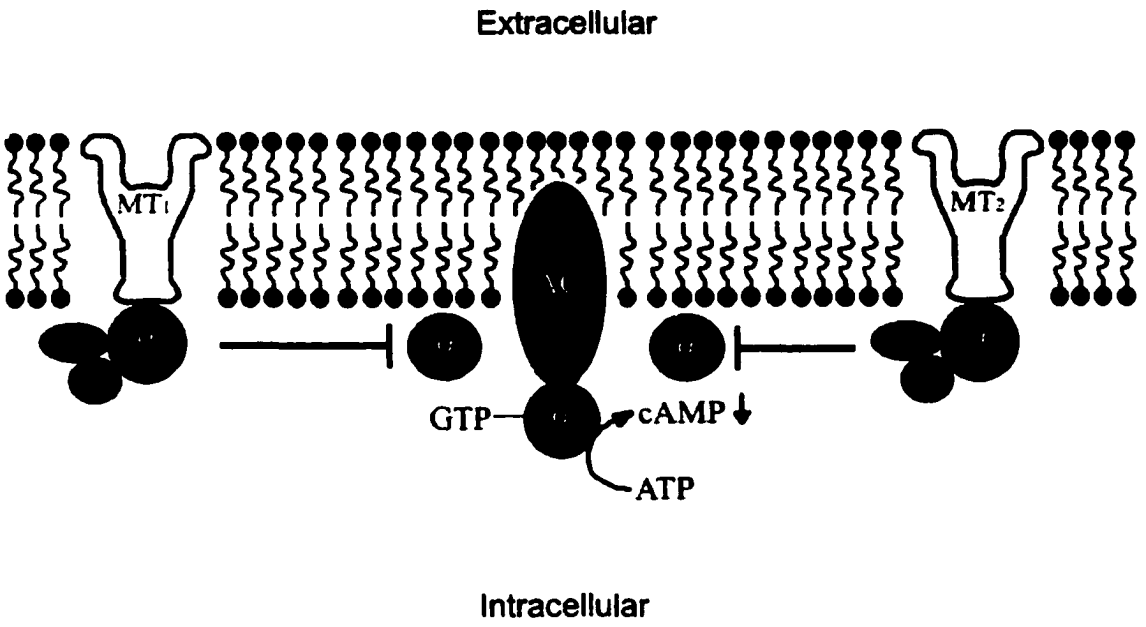

Figure 1.3. The function of MT1 and MT2 melatonin receptors. Both MT1 and MT2 melatonin receptors are shown in the plasma membrane coupled to a G_i-protein. Activation of these receptors by the ligand leads to a decrease in the production of cAMP by adenylate cyclase (AC). alpha (α), beta (β), and gamma (γ) subunits are the functional components of the G-protein. $\dashv$ - inhibition.

Bittman and Weaver 1990). 2-Imel is the best agonist for this receptor followed by melatonin and 6Cl-melatonin (see Appendix A). The MT2 receptor has been localized in the mammalian retina and hippocampus (Mushoff et al. 2002; Dubocovich et al. 1997). The most potent agonist for the MT2 is melatonin followed by 6-ClMel and 2-Imel. The MT3 receptor was originally found in the brain, testes, and kidneys of gerbils (Paul et al. 1999). 2-Imel and 6-ClMel have a higher affinity for the receptor then melatonin itself (Zawilska and Novak 1999). Contrary to MT1 and MT2 receptors, which demonstrate a high affinity binding to melatonin (Kd = 10-200 pM), the ability of MT3 to bind melatonin is much lower (Kd = 3-9 nM). The MT3 has recently been identified as quinone reductase 2 (Nosjean et al. 2000), which functions as an oxidoreductive enzyme. It is currently unclear how MT3 sites, which lead to phosphatidylinositol turnover, can simultaneously function as quinone reductase 2.

Functional cAMP assay

Binding of melatonin fulfills all criteria for binding to a receptor site. It is time- and temperature -dependent, stable, reversible, saturable and specific. The use of functional cAMP assays has led to the functional classification of melatonin receptors in different tissues

throughout the body (Garcia-Perganeda 1999; Nowak et al. 1997). Since melatonin can regulate the levels of cAMP, it could regulate the expression of its own receptor, which is controlled by the levels of cAMP (Hazlerigg et al. 1993).

Regulation of Melatonin Receptors

Melatonin receptors can be regulated by desensitization or downregulation. Desensitization is a decrease in the affinity of the receptor for the ligand, while downregulation is an internalization of the receptor. Comparison of the K_d (the indicator of receptor affinity) or the B_{max} (the indicator of total number of receptors) before and after treatment is generally used to differentiate between these two mechanisms. The results from this type of experiment can be misleading in the case of melatonin since it can cross biological membranes and bind to internalized receptors. Therefore charged melatonin ligands (agonists or antagonists), which cannot penetrate the membrane, are used to measure specific binding to melatonin receptors on the surface of the cell only (Chu et al. 2002).

A daily fluctuation in melatonin receptor mRNA and melatonin receptor protein in the SCN and pars tuberalis (PT) has been shown (Ross et al. 1998). In PT cells, the levels of melatonin receptor mRNA are increased following

an increase in cAMP. During the daylight, when melatonin levels are very low, there is an increase in cAMP and subsequently an increase in melatonin receptor mRNA. During the night when melatonin levels begin to rise, melatonin can act on its receptor and cause a decrease in cAMP and thereby prevent any further melatonin receptor expression. Melatonin has also been shown to cause both desensitization and downregulation of its own receptor by regulating its phosphorylation by PKC and PKA (Barrett et al. 1998; Ross et al. 1998).

Non-receptor binding sites

Any response to melatonin that occurs without meeting all the criteria described above (for classification of melatonin receptors; see p. 9) is considered a non-receptor mediated actions of melatonin. The following section compares the receptor-mediated and non-receptor mediated actions of melatonin.

Melatonin as an Antioxidant

Melatonin has been shown to act as an antioxidant by scavenging the hydroxyl radicals, peroxynitrite anions, peroxyl radicals, and the superoxide anion radical (Poeggler et al. 1993; Marshall et al. 1996) through a non-receptor mediated action. Additionally, the activation of nuclear melatonin binding site (discussed later) increases

mRNA levels of superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, all of which are antioxidative enzymes (Reiter 1998). These antioxidant actions of melatonin are hypothesized to decrease the signs of aging by protecting DNA and mitochondria from oxidative damage (Tan et al. 1993), which may decrease the signs and symptoms of aging.

Melatonin and GABAergic transmission

Melatonin has been shown to bind directly to the $GABA_A$ receptor (Coloma and Niles 1988; Niles and Peace 1990) increasing allosterically its affinity to the agonist (Wu et al. 1999). The allosteric interaction, which occurred at micromolar concentrations, was shown not to be due to binding of melatonin at the steroid or benzodiazepine binding sites (Wu et al. 1999). In amacrine-like cells, melatonin led to a decrease in the amplitude and desensitization kinetics of $GABA_A$ mediated currents (Li et al. 2001). However in rod-dominant bipolar cells melatonin led to a desensitization of the $GABA_A$ mediated current, only (Li et al. 2001). These differential sensitivities were postulated to be due to expression of different $GABA_A$ receptors subunits in each preparation (Li et al. 2001).

While the studies described above are considered non-receptor mediated actions of melatonin, other studies have

shown melatonin receptor-mediated actions on $GABA_A$ receptors. In the SCN, MT1 receptor activation causes an enhancement of $GABA_A$ currents and in the hippocampus activation of MT2 receptors leads to an attenuation of $GABA_A$ receptor mediated currents (Wan et al. 1999). Furthermore when MT1 and MT2 receptors were expressed in HEK 293 cells (Human embryonic kidney 293) there was either an increase (MT1), or a decrease (MT2) in GABA mediated currents. This study in particular implicates the possibility of different pathways activated by MT1 and MT2 melatonin receptors. Because activation of either melatonin receptor has been shown to lead to a decrease in cAMP, this decrease cannot account for the reduction in GABA currents when MT2 receptors were expressed vs. the increase in GABA currents when MT1 receptors were expressed (Wan et al. 1999).

Melatonin and Glutamatergic transmission

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate leads to depolarization of neurons by activating ionotropic (AMPA, kainate, NMDA) or metabotropic (mGluR1, mGluR2, or mGluR3) glutamate receptors (Ireland and Abraham 2002; Semyanov and Kullmann 2001). In the case of ischemia/hypoxia and severe seizures, prolonged activation of glutamate receptors can lead to cell death (Rothman and Olney 1986; Olney et al. 1986). Melatonin has

been shown to protect against damage caused by enhanced glutamate transmission (Cazevieille et al. 1997; Cazevieille and Osborne 1997; Cabrera et al. 2000; Skaper et al. 1998) by interacting with toxic oxygen species generated by glutamate-induced hyperexcitability (Avshalumov and Rice 2002).

Melatonin has also been shown to influence glutamate-mediated transmission. In rat striatal neurons, melatonin significantly attenuated glutamate mediated responses to sensorimotor cortical stimulation (Leon et al. 1998a). Our own studies demonstrated that 1mM melatonin attenuated hippocampal evoked-potentials, generated by stimulation of the glutamatergic synapses (Hogan et al. 2001). The response of hippocampal, evoked-potentials to melatonin were later shown to be mediated by melatonin receptor activation (El-Sherif et al. 2002). While these studies demonstrate a receptor-mediated action of melatonin on glutamate-mediated neurotransmission, they did not determine the specific site of action. Glutamate release, uptake and receptors can all be possible sites for melatonin action. Indeed studies on the golden hamster retina have shown that melatonin can increase [^{3}H]-glutamate uptake and release (Faillace et al. 1996).

Melatonin and Ion Channels

Cell signaling in the brain is mediated in part by ion channel activation. The changes in permeability of voltage-gated Na^+, K^+ and Ca^{2+} channels are responsible for generation of the action potential (AP) and neurotransmitter release. A few neurotransmitters have been shown to modify voltage-gated channels leading to a variety of changes including an increase/decrease in current (Brown et al. 2002; Sun et al. 2001; Imendra et al. 2000; Cantrell et al. 1999), shift in activation/inactivation curves (Xu et al. 2001; El-Sherif et al. 2001; Neusch et al. 2000) as well as a change in the time constants of channel activation/inactivation (White et al. 1994; Camacho and Sanchez 2002).

In mouse ocular tissue, melatonin enhances the activation and inactivation kinetics of TTX-insensitive voltage-gated Na^+ channels (Rich et al. 1999), at concentrations similar to the K_d of the cloned melatonin receptor. At higher concentrations of melatonin (1mM), considered pharmacological, there was an enhancement of the delayed rectifier K^+ channel (Rich et al. 1999). In a study by Huan (Huan et al. 2001), melatonin (1-100μM) was able to reversibly enhance the K^+ current in rat cerebellar granule cells. The effect of melatonin appeared to be receptor mediated, as it was mimicked by lower concentrations of

iodo-melatonin, blocked by pre-incubation of cells with pertussis toxin, and inclusion of GTP-γ-S led to a non-reversible enhancement of the delayed rectifier K^+ channel in the presence of melatonin. Melatonin also blocks specific K^+ channels (compcsed of the Kv1.3 subunit), by directly interacting with the channel (Varga et al. 2001).

Melatonin and Intracellular Molecules

Melatonin has been shown to directly bind to calmodulin *in vitro*, via a non-receptor mediated process (Benitez-King et al. 1991, 1993; Benitez-King and Anton-Tay 1993). Melatonin's binding antagonizes calmodulin's normal physiological effects (Romero et al. 1998), which include activation of Ca^{2+}/calmodulin kinase II (Benitez-King et al. 1996), modulation of nitric oxide synthetase (Leon et al. 2000), and tubulin polymerization (Benitez-King and Anton-Tay 1993). Melatonin also binds to an orphan receptor family RZR/ROR nuclear receptor (Wiesenberg et al. 1995) leading to apoptosis of cancer cells (Winczyk et al. 2001; Ciesla 2001).

The Hippocampus

Background

The hippocampus is one of a group of structures forming the limbic system and is a part of the hippocampal formation, which also includes the dentate gyrus, subiculum, and entorhinal cortex. Different components of the limbic system have been shown to play a critical role in all aspects of emotions, fear, learning and memory (Geinisman et al. 2000a; Geinisman 2000b; McMillan et al. 1987; Cardinal et al. 2002).

The initial insights on the role of the hippocampus came from studies of amnesia in human patients following removal of the hippocampus plus neighboring medial temporal structures (Scoville and Milner 1956). Extensive evidence implicates the hippocampus and related structures in the formation of episodic memories in humans (Reilly 2001; Aggleton and Brown 1999) and in consolidating information into long-term declarative memory (Mumby et al. 1999).

Hippocampal Inputs and Outputs

The hippocampus has direct connections to the entorhinal cortex (via the subiculum) and the amygdala. Outputs from these structures can then affect many other areas of the brain. For example the entorhinal cortex projects to the cingulate cortex, which has a connections

to the temporal lobe cortex, orbital cortex, and olfactory bulb. Thus, all of these areas can be influenced by hippocampal output, primarily from CA1.

The entorhinal cortex is a major source of inputs to the hippocampus collecting information from the cingulate cortex, temporal lobe cortex, amygdala, orbital cortex, and olfactory bulb (Johnston and Amaral 1998). The hippocampus receives inputs via the precommissural branch of the fornix from the septal nuclei. **Figure 1.4** gives an overview of the major inputs and outputs of the hippocampus.

Intrahippocampal Pathways

The main input to the hippocampus (perforant pathway) arises from the entorhinal cortex and passes through to the dentate gyrus. From the granule cells of dentate gyrus connections are made to area CA3 of the hippocampus proper via mossy fibers. CA3 sends connections to CA1 pyramidal cells via the Schaeffer collateral (SC) as well as commissural fibers (comm.) from the contralateral hippocampus. The major neurotransmitter in these three pathways is glutamate. The final output from the two CA fields passes through the subiculum entering the alveus, fimbria, and fornix and then to other areas of the brain. For the purposes of this dissertation I will focus on the synapse between the CA3 to CA1 neuron. **Figure 1.5** is a

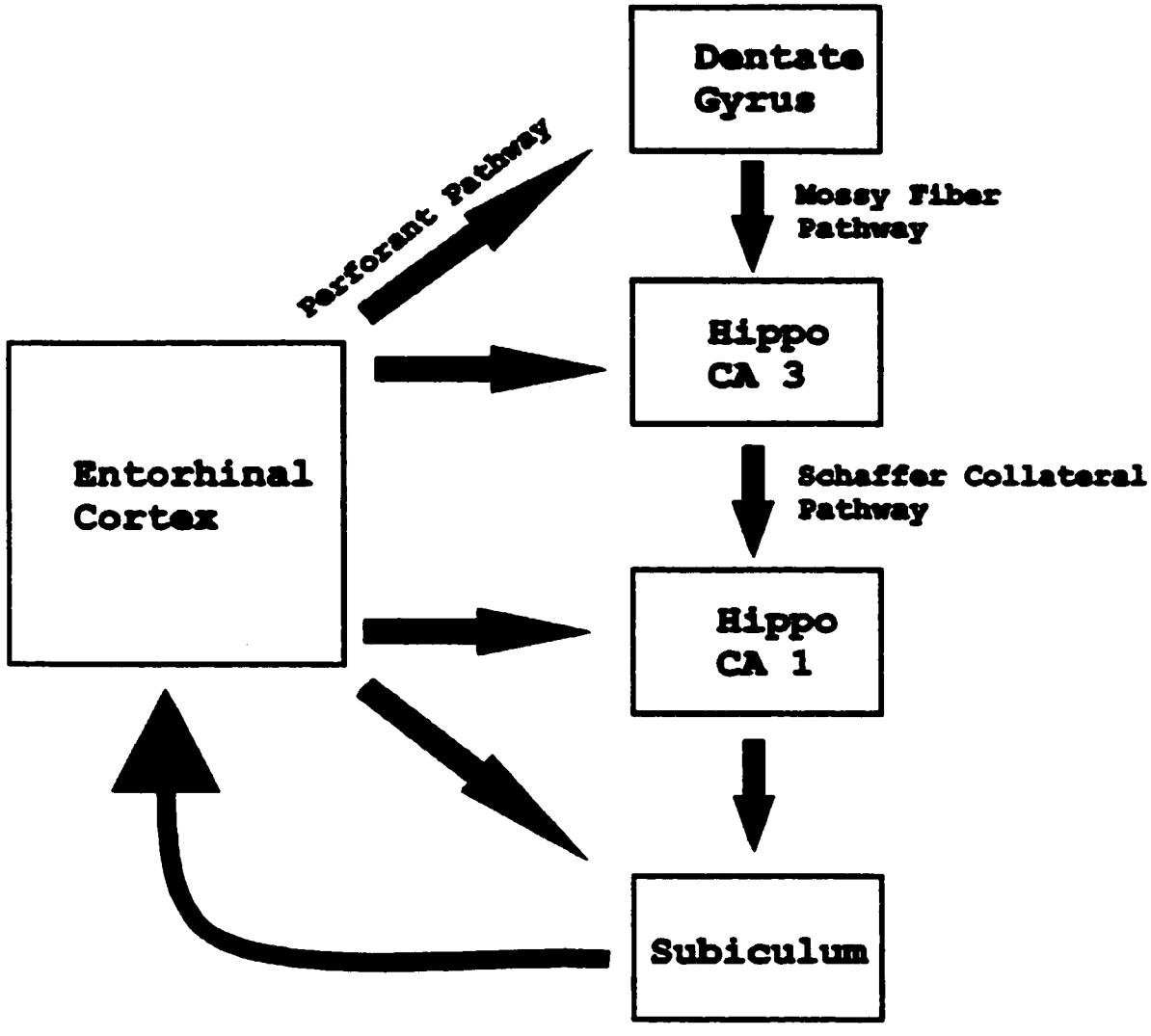

Figure 1.4. Schematic representation of hippocampal connections. Information leaving the entorhinal cortex can enter any of the following layers: CA3, CA1 or the subiculum. Information entering the dentate gyrus predominantly follows the mossy fiber pathway to CA3. Information from CA3 leaves via the Schaffer collateral pathway for the CA1 region. Information from CA1 travels to the subiculum and then to the entorhinal cortex.

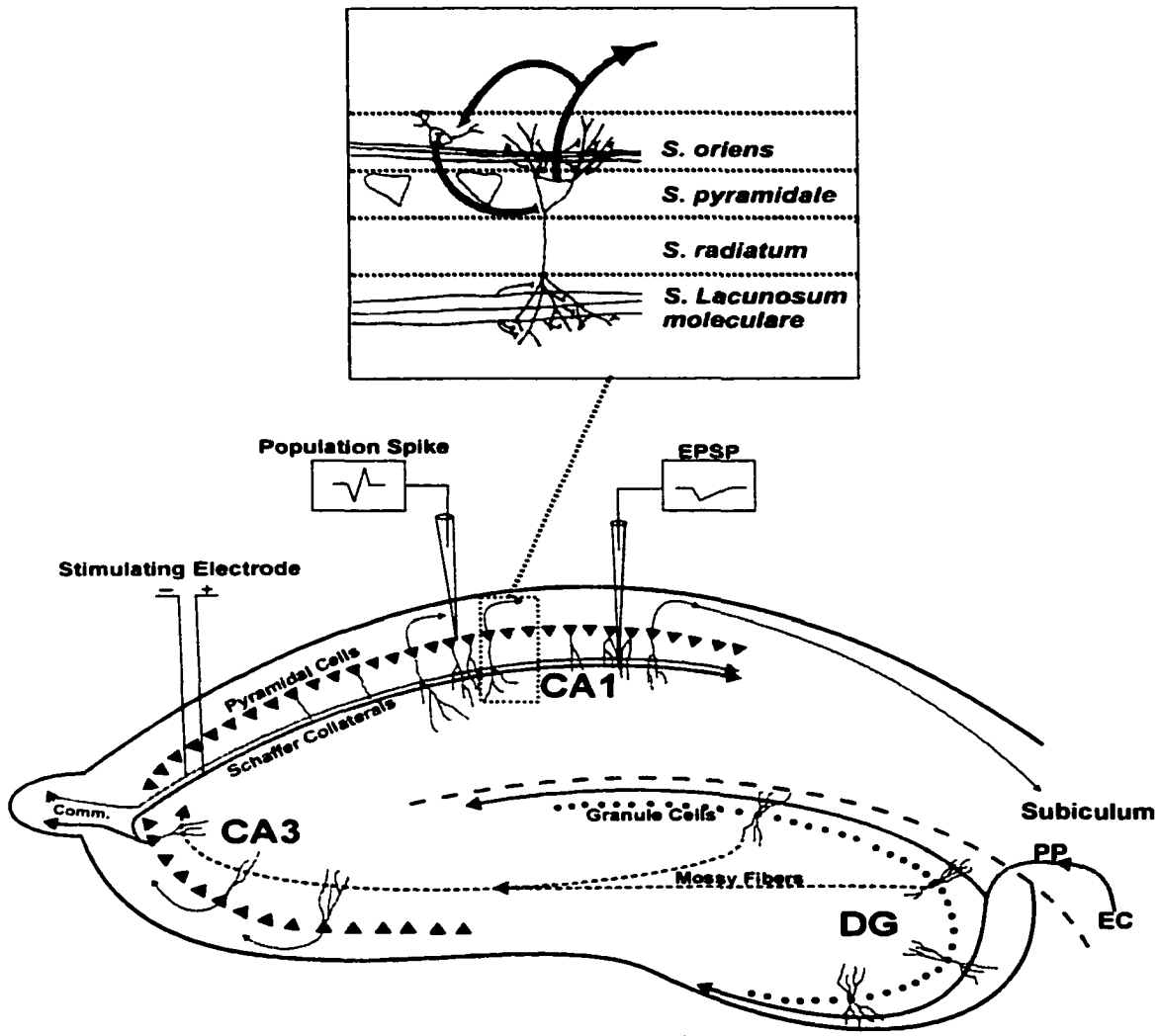

Figure 1.5. Hippocampal pathways and their stimulation
Signals from the entorhinal cortex (EC) enter the dentate gyrus (DG) via the perforant path (PP). From the DG information travels to the CA3 pyramidal neurons via the mossy fibers. From the CA3 neurons the signal leaves via the Schaffer collaterals and joins with the commissural fibers (Comm.) from the contralateral CA3 making connections with CA1 pyramidal neurons. Signals leaving CA1 then travel to neurons within the subiculum.
A bipolar stimulating electrode was placed on the Schaffer collateral and commissural (comm.) fibers. Recording electrodes placed in the dendritic layer and/or the pyramidal layer of CA1 will record an Excitatory Postsynaptic Potential (EPSP) or a population spike (PS) following stimulation, respectively. As discussed in the text the EPSP represents the response at the CA3-CA1 synapse and the PS represents the number of pyramidal cells firing and the contribution of the EPSP at that location.
The top portion of the figure demonstrates the four layers that the CA1 pyramidal neuron lies within (S. denotes Stratum). The small neuron with a letter "I" represents an inhibitory interneuron. The pathway diagramed in the top portion of the figure corresponds to the recurrent inhibitory loop in area CA1.

diagrammatic representation of the pathways entering the hippocampus and the pathways within it.

The highly organized and laminar arrangement of synaptic pathways makes the hippocampus a convenient model for studying synaptic actions *in vivo* and *in vitro* (Andersen et al. 1971).

Electrophysiology of the CA3⇒CA1 Synapses

Extracellular field recordings represent the summed responses from a number of neurons in the vicinity of the recording electrode. Because of the orderly arrangement of the pyramidal neurons and their dendrites, electrical field recordings offer valuable information about the temporal arrangement of responses from apical dendrites to cell bodies. Following stimulation of the SC and commissural fibers (**Figure 1.5** - stimulating electrode), an extracellular recording electrode in the stratum radiatum (**Figure 1.5** - S. radiatum) containing synapses, would record a small negative potential that results from the action potentials generated in the presynaptic fibers (Fiber Volley, FV). Following the FV a slow negative potential, corresponding to the population excitatory postsynaptic potential (pEPSP), would be recorded (**Figure 1.5** shows a representative pEPSP, which will be referred to as an EPSP from now on). The EPSP represents

depolarization at the postsynaptic membrane, indicating that transmission took place at the CA3-CA1 synapse. Placing the recording electrode in the stratum pyramidale (**Figure 1.5** - S. pyramidale) would allow us to record a positive deflection due to current exiting the basal dendrites near the cell body. If the magnitude of the depolarization is sufficient to bring the pyramidal cell to threshold, it will fire one or more action potentials. These action potentials will be recorded as a negative potential overlapping the positive potential. This type of recording is known as a population spike (PS) and is represented in **Figure 1.5**. While the EPSP is affected by changes occurring at the synapse the PS is affected by combination of 3 factors: 1) the amplitude of the EPSP, 2) the passive properties of the CA1 pyramidal cell (from dendrites to axon hillock), and 3) the level of inhibition produced by the GABAergic interneurons innervating the CA1 pyramidal neurons. Changes in the PS give a great deal of information about the number and excitability of neurons involved in the final output from the hippocampus.

The CA1 Pyramidal Neuron

Activation of the CA3 neuron leads to an increase in glutamate release from the nerve terminals of the SC's. Glutamate released in the stratum radiatum and stratum

lacunosum moleculare of CA1 activates either ionotropic or metabotropic receptors. The ionotropic glutamate receptors are classified into three types AMPA, kainite, and NMDA receptors, named after the ligand initially used to characterize them. AMPA and kainite receptors mediate the fast EPSP seen following SC stimulation (Karnup and Stelzer 1999). NMDA receptors mediate slow-rising EPSP's and are thought to be responsible for some forms of long-term synaptic plasticity (Tsien et al. 1996; Kullmann et al. 1996). Metabotropic glutamate receptors, which are located at both the presynaptic and postsynaptic side act to modulate release of neurotransmitter presynaptically (Lie et al. 2000; Baskys and Malenka 1991), and modify postsynaptic responses (Xiao et al. 2001).

The major inhibitory neurotransmitter in the hippocampus is GABA (Roberts 1976). Eliciting a single evoked potential via stimulation of the SC's results in a characteristic sequence of excitation followed by inhibition when recorded from the stratum pyramidale. In rats the excitation typically precedes the inhibition by a few milliseconds. The inhibition arises from feedforward and feedback connections via inhibitory interneurons. The inhibition corresponds to the release of GABA, which initiates two types of inhibitory responses, a fast

inhibitory postsynaptic potential (IPSP) mediated by $GABA_A$ receptors and a slow IPSP brought on by $GABA_B$ receptor activation.

Hippocampal Synaptic Plasticity

The hippocampus exhibits short and long term synaptic plasticity. For the purpose of our discussion plasticity will be defined as a change in the efficiency of synaptic transmission following previous synaptic activity.

Short-term Plasticity

Short-term synaptic plasticity lasting from a few milliseconds to a few minutes can be elicited, among other means by paired pulse stimulation.

Activation of the CA1 pyramidal cell by a single pulse will lead to an action potential sent out of the hippocampus and to inhibitory interneurons within the hippocampus (Top insert in **Figure 1.5**; Arrows leaving S. pyramidale). Activation of inhibitory neurons (**Figure 1.5** - Top insert; Inhibitory interneuron labeled "I" in S. oriens) by the CA1 neurons will lead to the recurrent inhibition of the subsequent response initiated in the CA1 neuron by the second stimulus delivered shortly (10-13 ms) after the first one. This type of pairing of two pulses in rapid succession leads to an inhibition known as paired pulse inhibition (PPI).

Changes in the ratio of the amplitudes of the first and second evoked potentials (in a PPI experiment), can occur through changes in both GABA receptor sensitivity and

GABA release. This has been demonstrated experimentally through, an enhancement of PPI with GABA agonists (Rock and Taylor 1986) and a decrease in PPI by GABA antagonists (Kapur et al. 1989, 1997).

If the stimuli are further apart (15-40 ms) the second stimuli arrives, when the recurrent inhibitory loop has already been inactivated. Therefore, the second response is not inhibited but facilitated due to residual Ca^{2+} increase after the first stimulus. This is called paired pulse facilitation (PPF). Changes in the ratio of amplitudes of first and second potentials are generally accepted as a modification in the presynaptic component of the synapse (Commins et al. 1998; Chen et al. 1996; Gottschalk et al. 1998), although alterations in postsynaptic AMPA receptors have also been reported during PPF experiments (Wang and Kelly 1995-1997, 2001).

Long-term Plasticity

When a change in synaptic efficiency persists for long periods of time (hours to days) it is known as long-term plasticity. In 1973 Bliss and Lomo observed that high frequency stimulation (HFS) of the perforant path in anaesthetized rabbits led to a potentiation of synaptic responses which could last for several hours (Bliss and Lomo 1973). Later many other pathways in the brain

including the SC's in the hippocampus were shown to express similar phenomenon following HFS (see review by Recasens 1995). The general consensus is that induction of LTP at CA1 synapses requires Ca^{2+} entry into the postsynaptic dendritic spine via the activation of the NMDA receptor (Collingridge et al. 1983), however increased Ca^{2+} concentration through NMDA-independent mechanisms may also lead to LTP (Grover 1998; Kullmann et al. 1992). Therefore Ca^{2+} which is directly involved in PPF, also initiates the mechanisms which maintain enhanced synaptic transmission for a long period of time (Impey et al. 1996; Solderling and Derach 2000; Suzuki 1996).

The mechanism responsible for LTP has been an area of intense debate. Some of the more recent mechanisms proposed to explain LTP at hippocampal synapses include: 1) an incorporation of new AMPA receptors into the membrane (Pickard et al. 2001; Hayashi et al. 2000). 2) activation of previously silent synapses (Malinow 1995 and Konerth 1996), and 3) HFS induced splitting of dendritic spines allowing a synaptic response to be amplified (Jontes and Smith 2000).

Although some studies demonstrated a strong correlation between a deficit in LTP and poor spatial memory (Sakimura et al. 1995; Abel et al. 1997), several

reports described normal spatial orientation in spite of impaired LTP (Meiri et al. 1998; Saarelainen et al. 2000; Jun et al. 1998; Bach et al. 1995). These briefly mentioned conflicting results demonstrate some of the difficulties in accepting LTP as the molecular mechanism of memory formation.

Melatonin and Hippocampus

The role of melatonin in the hippocampus

Specific melatonin binding sites exist in the hippocampus of many species (Niles 1987; Laudon et al. 1988; Anis et al. 1989; Stankov et al. 1991, 1993; Deveson et al. 1992; Nonno et al. 1995). As already mentioned, melatonin can bind to many sites on the plasma membrane (MT1, MT2, $GABA_A$ receptors and K^+ channels), and within the cytoplasm (calmodulin, PKA, PKC, free radicals). Therefore identification of these binding sites as plasma membrane melatonin receptors has been difficult.

Cloning of melatonin receptor (Fujieda et al. 1999; Oblap and Olszanska 2001; Wiechmann and Smith 2001) has greatly aided in the identification of melatonin receptors within different tissues. In the vasculature of the human hippocampus specific MT1 mRNA has been immunohistochemicaly localized (Savaskan et al. 2001). In hippocampal pyramidal neurons of humans and rodents, MT1 and MT2 receptor mRNA has been found (Mussoff et al. 2002; Savaskan et al. 2001; Wan et al. 1999). While presence of mRNA for melatonin receptors indicates that the receptors can be expressed, it does not necessarily indicate that they are functional.

Studies demonstrating actions of melatonin or its analogues on hippocampal physiology are common (Carnerio

and Reiter 1998; El-Sherif et al. 2002; Southgate et al. 1998; Arushanian and Beier 1998; Zamorsjii and Pishak 2000; Won et al. 2000; Ortiz et al. 2001 Hogan et al. 2001; Collins and Davies 1997). In the rodent hippocampus, melatonin has been shown to decrease hippocampal activity (Hogan et al. 2001; El-Sherif et al. 2001), and block the induction of LTP (Collins and Davies 1997). In guinea pig hippocampus, melatonin was shown to lower hippocampal excitability and paired-pulse facilitation by modulating the membrane potential (Zeise and Semm 1985). These four studies all looked at the effects of melatonin in the micromolar range. As mentioned previously oral consumption of melatonin can significantly raise levels of melatonin, making these results relevant to the possible effects of melatonin in humans following melatonin ingestion. Therefore pursuing our goal of evaluating the influence of melatonin on the hippocampal neurons, we used a broad range of concentrations of this neurohormone.

Chapter 2 - Materials and Methods

Animals

Four strains of mice: 1) Compton White 2) CD-1 3) C57J/B6 4) Swiss Webster, obtained from Jackson Laboratories, 4-30 weeks old, were kept on a 12 hour / 12 hour L/D cycle, unless otherwise noted. Food and water were provided ad libitum.

For investigation of the influence of light deprivation, siblings of CD-1 mice of the same sex were separated into control and experimental groups. They were kept in separate experiments for 3, 6, 12, 20, or 23 days in normal light/dark cycle (controls) or complete darkness (experimental). Mice were killed by cervical dislocation, both eyes were enucleated, and both hippocampi dissected out. The whole procedure which lasted less than 30 sec, was performed in complete darkness under a 0 lux camera. Slices were then prepared as described below.

Preparation of Hippocampal Slices

The hippocampal slices were prepared according to the procedure used in our laboratory. Following decapitation and brain removal, both hippocampi were dissected out and placed into ice-cold Ringer's solution consisting of (in mM): NaCl 124, KCl 3.1, KH_2PO_4 1.3, $MgSO_4$ 1.3, $CaCl_2$ 3.1, $NaHCO_3$ 25.5, and glucose 10.0. Both hippocampi were cut into

slices (350-400 μm) with a manual tissue chopper, placed in an incubation chamber (33°C) and constantly oxygenated with a 5%/95% CO_2/O_2 mixture. Following a preincubation period, which varied from 1 to 5 hrs, slices were transferred to an interface-recording chamber, maintained at 33°C.

Electrophysiological Recordings

The population spike, which reflects the number of activated pyramidal neurons (Andersen, 1971), was recorded from the pyramidal cell layer following the stimulation of SC and comm. fibers. EPSP's were recorded by placing the recording electrode in the stratum radiatum. The magnitude of the population spike was measured using a computer program, as the average distance between the highest negativity, and preceding and following positivities. The strength of the stimulation in all tested slices was adjusted at the beginning of each experiment to obtain approximately 50-80% of the maximal response. The potential was monitored at this level for at least 10 minutes, and experiments were performed only on slices demonstrating stable responses to Low frequency stimulation (LFS). Each experiment was performed on a separate slice.

High-Frequency Stimulation

A baseline recording was obtained by stimulating the slices with LFS for at least 10 min. Then HFS was applied at 100 Hz for 1 sec, 3 times every 10 sec, and pattern of stimulation was returned back to LFS. Five sweeps taken immediately prior to HFS were averaged and used as the baseline for all calculations of potentiation. The potentiation was measured at 1, 5, and 15 min following HFS, and expressed as a percent of the baseline.

Paired Pulse Stimulation

Paired pulse paradigm experiments employed two consecutive pulses delivered with a frequency of 0.03 Hz. The delay between the two pulses was adjusted for each experiment and varied between 15-40 ms and 10-13 ms, to induce paired pulse facilitation (PPF) or paired pulse inhibition (PPI), respectively. For both experimental paradigms the evoked potentials were evaluated 5 min prior to and 5, 10, 15, 20 and 25 min after the treatment. At each time point the average value of the difference between the first and the second population spike was calculated, and expressed as a percent of control values observed before the treatment. In the case of PPF experiments the negative value indicates that the second potential is smaller than the first one and implies a change of

facilitation into inhibition. The opposite is true for PPI data, where negative values indicate a change from inhibition into facilitation.

Slices were only used during the first 3 hrs following recovery. All potentials were recorded on-line and analyzed using the Neuropro software.

[^{3}H]-Melatonin Binding

For each experiment (n=5) forty slices were incubated for 1 or 4 hr, collected and homogenized in homogenization buffer (20 mM HEPES, 10 mM EDTA, 320 mM sucrose, pH 7.4) 1:10 mg/ml assuming the weight of one slice equal approximately 2 mg. An aliquot of homogenate was taken for protein assay (Biorad Protein Assay). The homogenate was centrifuged at 5,000 x 10 min and then the aspirated supernatant was centrifuged at 33,000 x 10 min. The supernatant was discarded and the pellet was suspended in 1 ml of rinse buffer (50 mM Tris-Base, 4 mM $CaCl_2$, pH 7.4), and centrifuged. The centrifugation and suspension in a rinse buffer was repeated once and the final pellet was resuspended in an assay buffer (20 mM HEPES, 0.1mM EDTA, pH 7.4; 10 mg of original fresh tissue/ml buffer), and kept on ice for further analysis. For the preincubation step each tube contained 100 µl of homogenate and either 50 µl of assay buffer (controls) or 50 µl of cold melatonin (50 µM).

Following 1 hr preincubation at room temperature 50µl of [^{3}H]-melatonin (25 nM) was added to each tube and the samples were incubated for 15 min at room temperature. Immediately after incubation all samples were centrifuged in a refrigerated eppendorf tabletop centrifuge for 4 min, the supernatant was quickly aspirated, the pellet was superficially washed with 1 ml of cold assay buffer and the radioactivity of the pellet was counted following the addition of scintillation cocktail. The final results were expressed as fmoles of melatonin bound/mg of protein. Statistical analysis was performed using the unpaired, two-tailed t-test (SigmaStat software by Jandel).

Preparation of Drugs

Melatonin, its agonist and antagonists were all dissolved in ethanol to obtain stock solutions, which were then diluted with Ringer's to make a working stock. The working stock was added (10-20 µl) directly to the recording chamber to give the desired final concentration. Since solubility of each of these compounds in Ringer's was different, we established the following procedures to prepare the solution of melatonin and used a similar procedure to prepare all agonists and antagonists.

Melatonin (N-acetyl-5-methoxy-tryptamine - Sigma, St. Louis, MO, USA or Regis Technologies) was prepared as a 250

mM stock solution in 100% ethanol and diluted with Ringer's to a working stock of 62.5 mM in 25% ethanol. 16µl of the working solution was added to the chamber to give a final concentration of 1 mM (0.4% ethanol). When testing lower concentrations of melatonin, the original stock (250mM) was diluted in 100% ethanol to the desired concentration of melatonin (e.g. 25mM melatonin in 100% ethanol).

All of the following agonists and antagonists: 6-Chloromelatonin (Sigma), 2-Iodomelatonin (Tocris), Luzindole (Tocris), 4P-PDOT (Tocris), DH-97, BMNEP (gift), TMEPI (gift), were prepared in stock solutions of 100% ethanol and diluted to the desired final concentration in 0.4% ethanol.

Other compounds in this study were purchased either from Sigma: CNQX, AP-5, picrotoxin and kynurenic acid, or CalBiochem: [^{3}H]-melatonin.

Chapter 3 - Results

Effects of Melatonin on Hippocampal Evoked-Potentials

The goal of my dissertation was to determine whether melatonin could influence the physiology of the hippocampus. To achieve this goal I used electrophysiological methods in combination with pharmacological and biochemical approaches.

The effects of melatonin on the Population Spike and EPSP

Melatonin is a hydrophobic molecule and therefore we used ethanol as a vehicle to dissolve and effectively distribute melatonin throughout the tissue. The first part of our research was to determine if ethanol itself had any influence on hippocampal, evoked potentials. Hippocampal slices were prepared from Compton white mice. As shown in **Figure 3.1A**, ethanol added three consecutive times (3 x 0.4%) had no significant effect on the population spike (**Fig. 3.1A**; representative of 12 experiments which all gave similar results) or on the EPSP (n=3, data not shown). In the next phase of our research we evaluated the influence of melatonin on the population spike and EPSP. **Figure 3.1B** and **Figure 3.1C** illustrate the influence of 1 mM melatonin on the population spike and EPSP, respectively.

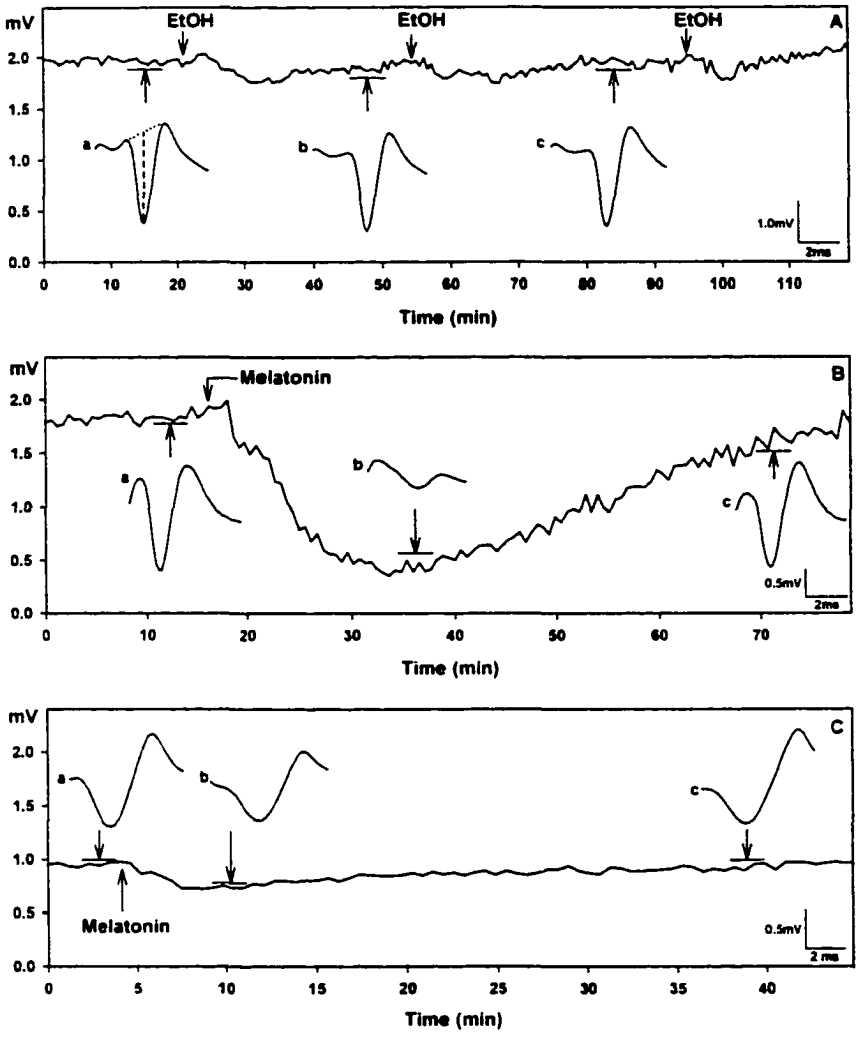

Figure 3.1. The influence of ethanol and melatonin on hippocampal evoked potentials.
The line graph in A, B, and C show the magnitude of the evoked potential (population spike or EPSP) recorded during the whole experiment and the inserts depict the average of 5 potentials recorded during the time period indicated by the horizontal bar at the end of arrows which mark the time of recording.

Calibration marks are shown in the lower right corner of the figure. A - The influence of ethanol (0.4%) on the population spike. The dashed and dotted lines shown on the potential Aa are imaginary lines drawn by the computer to calculate the value of the potential (vertical dashed line). The addition of ethanol is indicated by "EtOH". Note that ethanol added three consecutive times at the concentration of 0.4% had no significant effect on the population spike. B - The influence of 1 mM melatonin on the population spike; addition of melatonin is marked by an arrow; potentials a, b, and c depict the shape of the population spike 10 min before addition of melatonin, at its maximal effect and at the end of recovery phase, respectively. C - The influence of 1 mM melatonin on EPSP. The rest of explanation as in B.

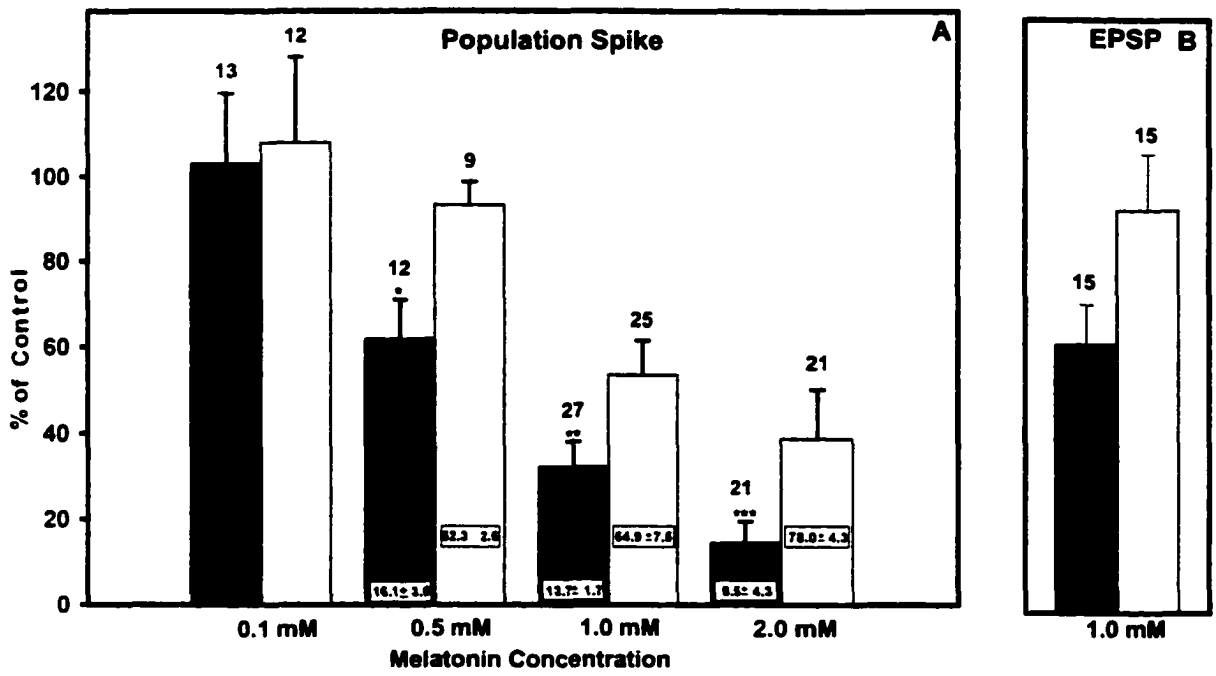

Figure 3.2. The influence of different concentrations of melatonin on hippocampal potentials.
Solid and white bars represent averages during maximal effect of melatonin and at the recovery phase, respectively. A - The concentration-dependent depression of the population spike by 1mM Mel. B - Attenuation of EPSP by 1 mM melatonin. The number at the top of each bar indicates the number of slices tested. The delay (in min) between addition of melatonin and its maximal effect is indicated at the bottom of solid bars; the delay between melatonin addition and maximum recovery is shown at the bottom of white bars; * $p<0.02$, ** $p<0.0004$, *** $p<0.0001$.

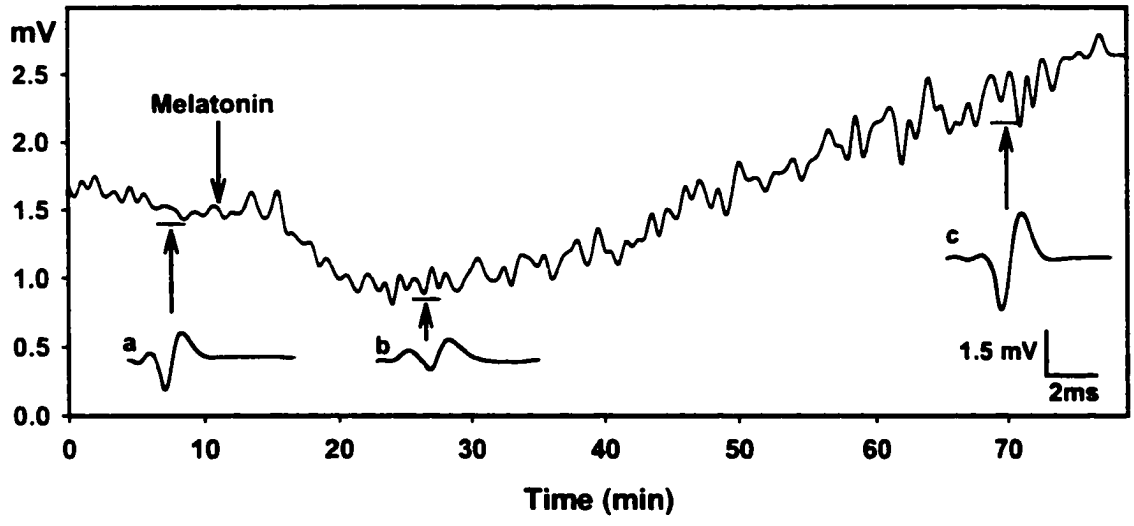

Figure 3.3. The influence of continuous perfusion with 1 mM melatonin on the population spike recorded from hippocampal slices. The potentials marked a, b, and c represents an average of ten potentials recorded before addition of melatonin, and at the maximal depression and amplification triggered by melatonin, respectively. The solid line shows changes in the magnitude of the population spike for the whole experiment. The arrow indicates the start of melatonin perfusion.

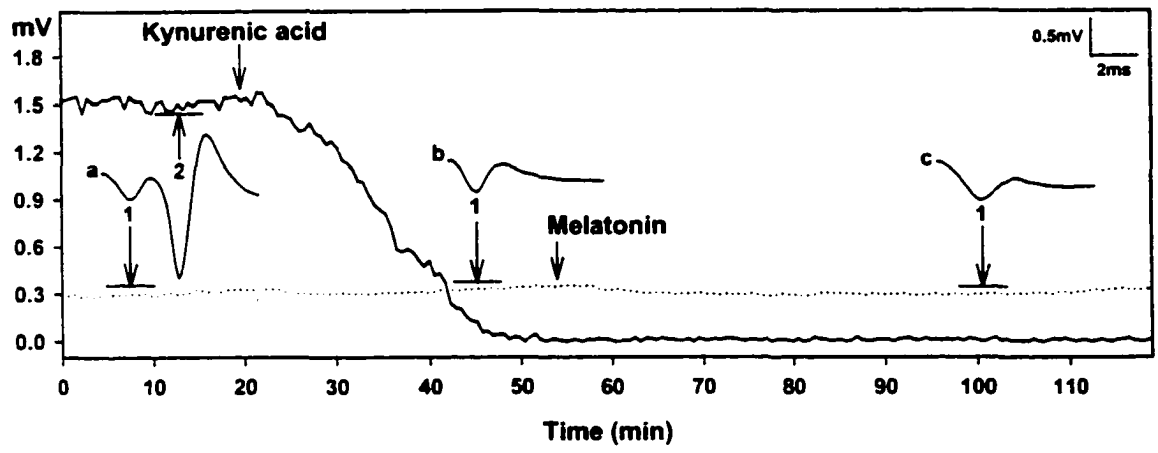

Figure 3.4. The influence of melatonin on the population spike and fiber potential before and after addition of kynurenic acid.
Solid and dotted lines indicate the change in the magnitude of the population spike and fiber potential, respectively. Potential "a" shows the fiber potential (a1) and population spike (a2), respectively. Note that following elimination of the population spike by addition of 2 mM kynurenic acid (marked by an arrow) the fiber potential remains unchanged (compare potentials a and b). The subsequent addition of 1mM melatonin had no influence on the fiber potential (compare potentials b and c). See legends to figure 3.1 and 3.2 for further details. This figure represents one of three experiments which all gave similar results.

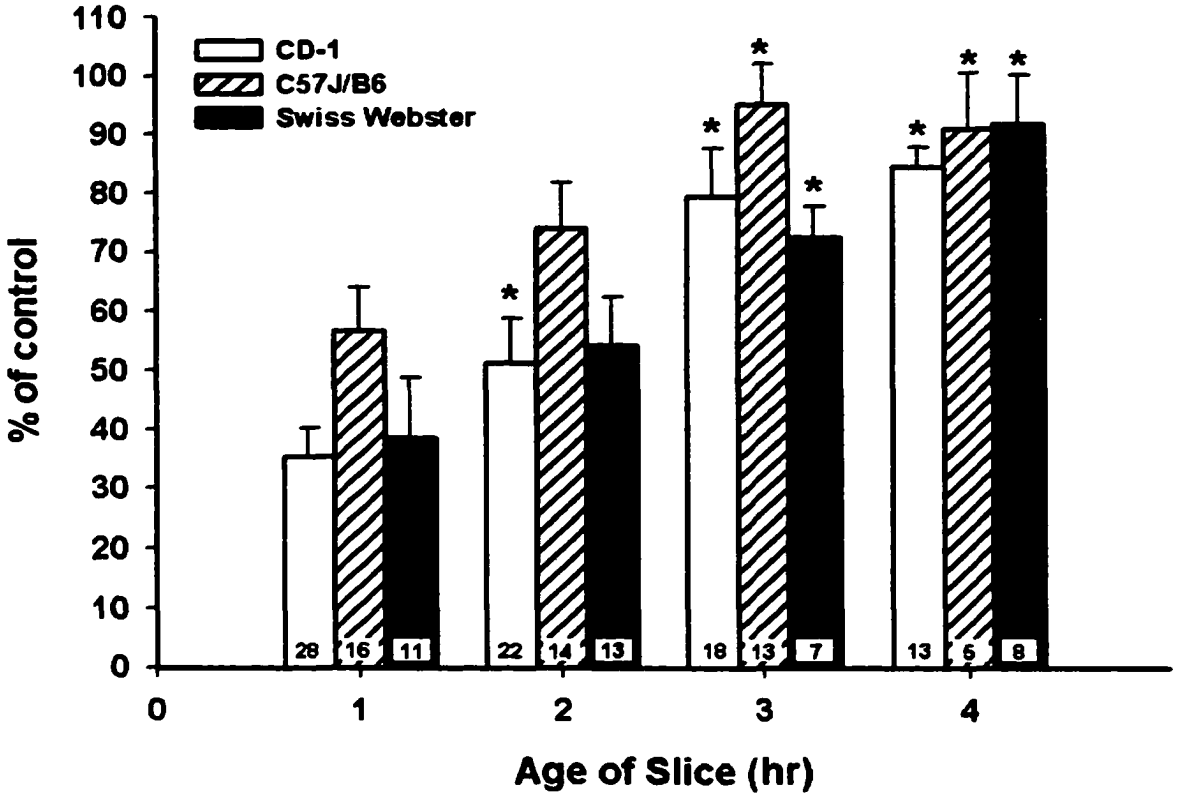

Figure 3.5. The attenuation of hippocampal evoked responses by melatonin in different mouse strains and its relation to the age of the slice.
The percent of control was calculated by comparing the values of the potentials before and after melatonin addition. The asterisks mark the significance ($p < 0.05$) for 2, 3 and 4 hr-old slices vs. 1 hr-old slices within the same strain of mice. Note the parallel decrease in the melatonin-induced inhibition of the population spike in all three strains. In this and the remaining figures, the number of tested slices is indicated inside of each bar and each bar represents averages ± SEM.

Figure 3.2A and **Figure 3.2B** (solid bars) show averaged results for different melatonin concentrations for the population spike and EPSP, respectively. Melatonin applied at 0.1 mM had no apparent effect for the duration of experiments (approximately 60 min). For this reason the magnitude of the population spike recorded in the presence of 0.1 mM melatonin was used as a control for all statistical comparisons (**Figure 3.2**).

Melatonin depressed the population spike in a dose-dependent fashion (**Figure 3.1** and **Figure 3.2**). The population spike was reduced to 61.8±9.1% (n=12; $p<0.02$), 32.2±5.9% (n=27; $p < 0.0004$), and 14.3±5.0% (n=21; $p<0.0001$) following the application of 0.5 mM, 1.0 mM and 2.0 mM melatonin, respectively (**Figure 3.2**). Note that the EPSP seems to be less affected. While melatonin at 1mM reduced the population spike by over 60%, the EPSP was reduced by only 40% in the same experimental conditions (compare **Figure 3.2A** and **3.2B**). The delay between the time of melatonin application and its maximal depressive effect (indicated at the lower part of each solid bar in **Figure 3.2**) was inversely related to the melatonin concentration and lasted 16.1±3 min, 13.7±1 min and 8.5±2 min for 0.5 mM, 1.0 mM and 2.0 mM melatonin, respectively. The melatonin-induced depression of the population spike and EPSP was not

permanent and the potentials recovered either fully or partially depending on the melatonin concentration (**Figure 3.1B** and **3.1C** and **Figure 3.2A** and **3.2B**, crossed bars for the population spike and EPSP, respectively). While full recovery was observed for 0.5 mM melatonin after 62.3±2.6 min (n=9), only partial recovery was recorded following addition of 1.0 mM melatonin (64.9±7.5 min; n=25) or 2.0 mM melatonin (78.0 ± 4.3 min; n=21). Analysis of the potentials that recovered after application of 1.0 mM and 2.0 mM melatonin revealed that they were still significantly lower than controls ($p<0.002$ and $p< 0.01$ for 1.0 and 2.0 mM melatonin, respectively).

As previously described melatonin caused a strong inhibition, which was diminished with time. The decreased concentration of melatonin due to its decomposition in the chamber would be the most appealing and straightforward explanation for the gradual recovery of the potential. To verify this assumption we perfused hippocampal slices with Ringer's solution containing 1 mM melatonin thereby exposing them to a steady concentration of melatonin throughout the entire experiment. Even in these modified experimental conditions the melatonin-induced reduction in the amplitude of the population spike (47.7±4.2%; n=3) was still followed by a recovery/amplification phase

(142.4±33.0%, n=3) in spite of the constant presence of melatonin. The results of one of three experiments are depicted in **Figure 3.3**.

Effects of Melatonin on the Fiber Volley

Blocking synaptic transmission with kynurenic acid (glutamate receptor antagonist) eliminates the EPSP or PS. The remaining inward deflection represents the fiber volley (FV). **Figure 3.4** represents a population spike (a2) and fiber volley (a1) prior and after the addition of 2 mM kynurenic acid. Kynurenic acid completely abolishes glutamatergic synaptic transmission while leaving the fiber volley unaffected (a1). The FV was not influenced by 1mM melatonin (compare potentials a1, b1, and c1 in **Figure 3.4**).

Factors Influencing Melatonin Action

Effects of slice age

To determine if a decrease in sensitivity to melatonin represents a unique property of the melatonergic system in Compton white mice we additionally evaluated the effects of 1mM melatonin on hippocampal slices prepared from CD-1, C57J/B6, and Swiss Webster strains of mice. As illustrated in **Figure 3.5**, the depression of the population spike by melatonin was similar in all three strains and inversely related to the slice incubation time (age of slice).

When the attenuation in 1 hr-old slices was taken as a control, the decrease in the sensitivity to melatonin was statistically significant for 2 hr-old slices from CD-1 animals and for all three strains for 3-, and 4-hr-old slices (**Figure 3.5**). Considering that: a) the response of hippocampal neurons to melatonin was very similar in all three strain of mice studied; b) it was inversely related to the age of the slice; c) CD-1 is commonly used as a model in memory studies and is a reliable breeder in our Vivarium, we continued our studies using CD-1 mice.

Age of Animal

Efficient and timely breeding is extremely important for our project because the sensitivity of hippocampal neurons to melatonin changes not only with the age of the slice, but as reported recently (Fauteck et al., 1999b; Waldhauser et al., 1993; Turek et al., 1999), depends on the age of the animals as well. Our research on CD-1 supports this notion. The age of the CD-1 mice evaluated in our studies ranged from 24 to 193 days and the effects of melatonin were tested on the slices incubated for 1, 2, 3 and 4 hours. We found that while the slices obtained from mature young animals (24-46 days) were losing their sensitivity to melatonin within 60-90 min following preparation (**Figure 3.6**), the slices from older animals

(187-193 days) remained sensitive to melatonin through the entire testing period (**Figure 3.6**).

Melatonin Binding

Since the sensitivity of the hippocampal slices to melatonin was inversely related to the age of the slice, we verified experimentally our assumption that this phenomenon is due to time-dependent modification in binding of melatonin to its receptors. The membranes from hippocampal slices and the binding assays were carried out as described in the Methods section. Membranes were prepared from slices pre-incubated either 1 or 4 hours in the incubation chamber. While the average specific binding of melatonin to the membranes prepared from the slices incubated for 1 hr was 42.93 fmol/mg ± 9.96 (n=6; **Figure 3.7**), the membranes prepared from slices incubated for 4 hr expressed a six fold decrease in melatonin binding (6.94 fmol/mg ± 4.25; n=5; p= 0.013, **Figure 3.7**).

Light Deprivation

Since the results demonstrate a remarkable change in the sensitivity of hippocampal tissue to melatonin, we decided to pursue this issue further and to determine whether light deprivation, which apparently modulates the melatonin level *in vivo* (Gauer et al., 1993), would affect sensitivity of the slices obtained from the light deprived

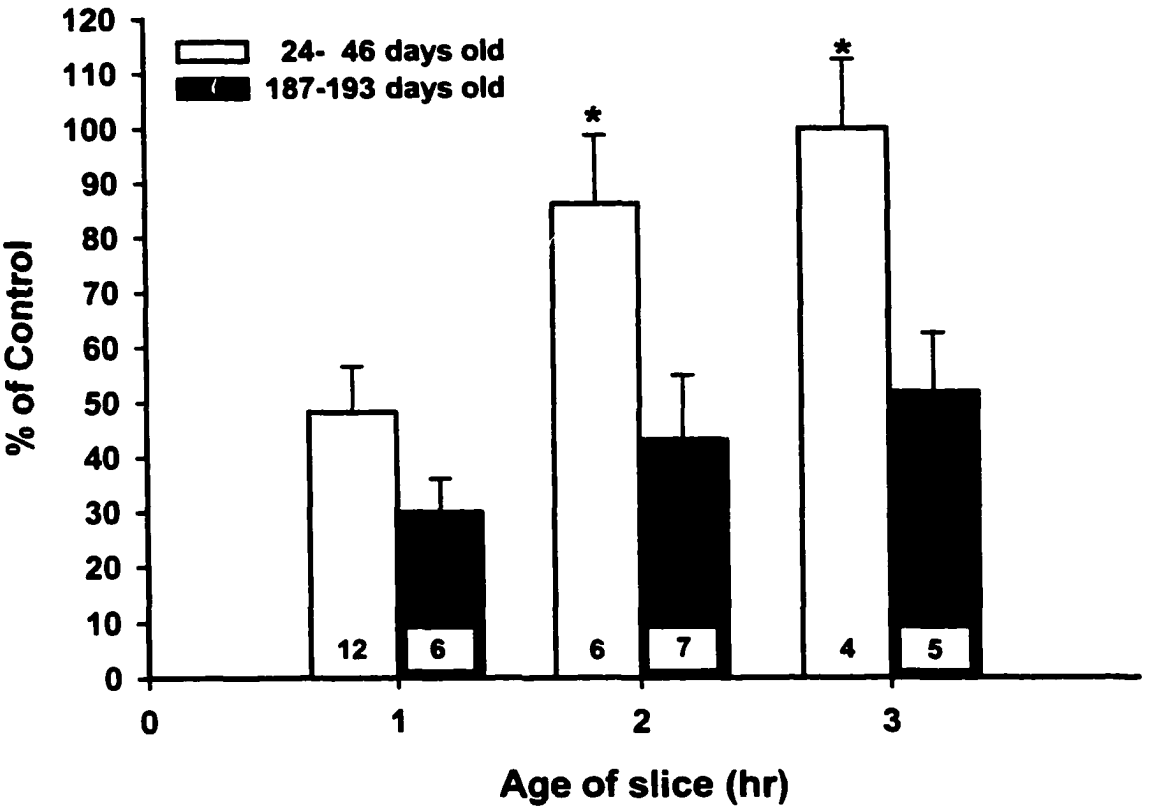

Figure 3.6. The influence of animal age and time of slice incubation (age of slice) on the melatonin-induced inhibition of hippocampal evoked potentials.
Slices were obtained from mature young (24-46 days old) and older (187-193 days old) CD-1 mice. Note that while the sensitivity to melatonin decreases with the time of incubation in slices from younger animals, it remains almost unchanged through the whole incubation period in slices from older animals. The asterisks indicate a statistically significant difference ($p<0.05$) as compared to the results obtained from 1 hr old slices.

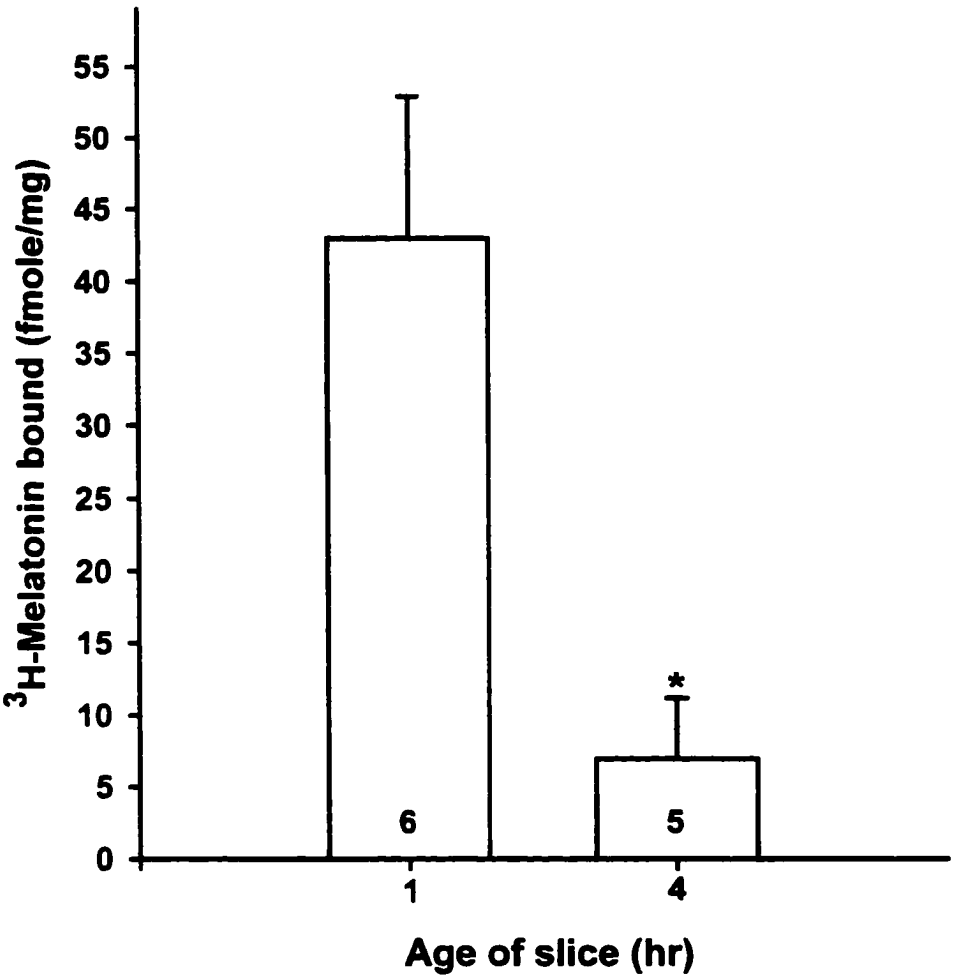

Figure 3.7. Binding of melatonin by hippocampal synaptosomal membranes.
The graph represents the binding of melatonin to its receptors in the hippocampal membranes prepared from slices incubated for 1 or 4 hrs (numbers in bars indicate total number of experiments). The decrease in the binding in 4-hour slices is significantly different from the binding in 1-hour slices (*p=0.013).

mice to melatonin. Experiments were performed as described in the Methods section. While the slices from light-deprived mice were insensitive to melatonin, the potentials recorded from slices obtained from control, light-exposed siblings were depressed by 1 mM melatonin (**Figure 3.8**). Since the duration of the light deprivation (3 - 23 days) had no influence on the slices sensitivity to melatonin, the data obtained from all experimental animals were combined (**Figure 3.8**).

Reassessment of the effective concentrations of melatonin

In the research described above, which has been already published (Hogan et al. 2001) we used melatonin at the micromolar concentrations. However two years ago we noticed that melatonin's effectiveness (stored at -20°C) was not reproducible. We also observed that the solubility and stability in ethanol became much lower. We tested samples from a number of other vendors and discovered that melatonin purchased from Regis Technologies was 1,000 times more effective at attenuating the PS when compared with melatonin from Sigma, our previous vendor (Hogan et al. 2001). We decided to continue our further research using Regis melatonin. **Figure 3.9** shows the inhibition of the PS at different concentrations of melatonin purchased from

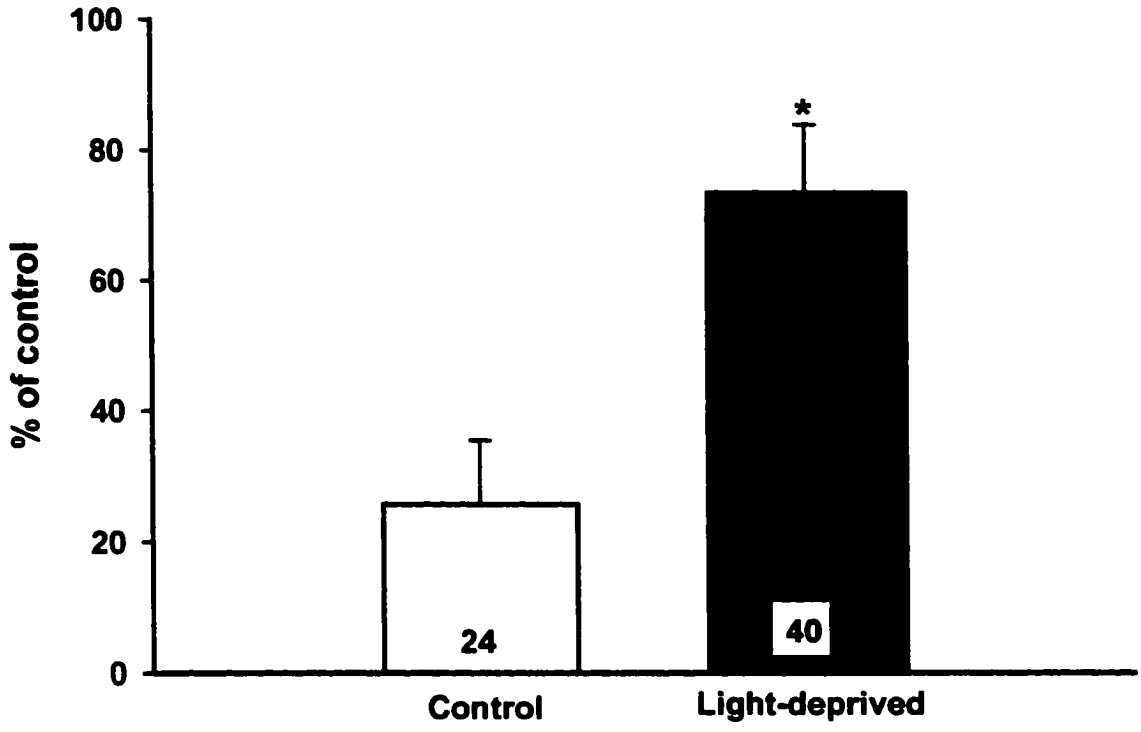

Figure 3.8. The sensitivity to melatonin observed in the slices obtained from control (white bar) and light-deprived animals (black bar).
All experiments were performed on the slices obtained from young animals. The slices were incubated no longer than 1 hour before melatonin addition. The change in the magnitude of the population spike ($p<0.05$) was expressed as percent of control value recorded before melatonin addition. See the text for further details.

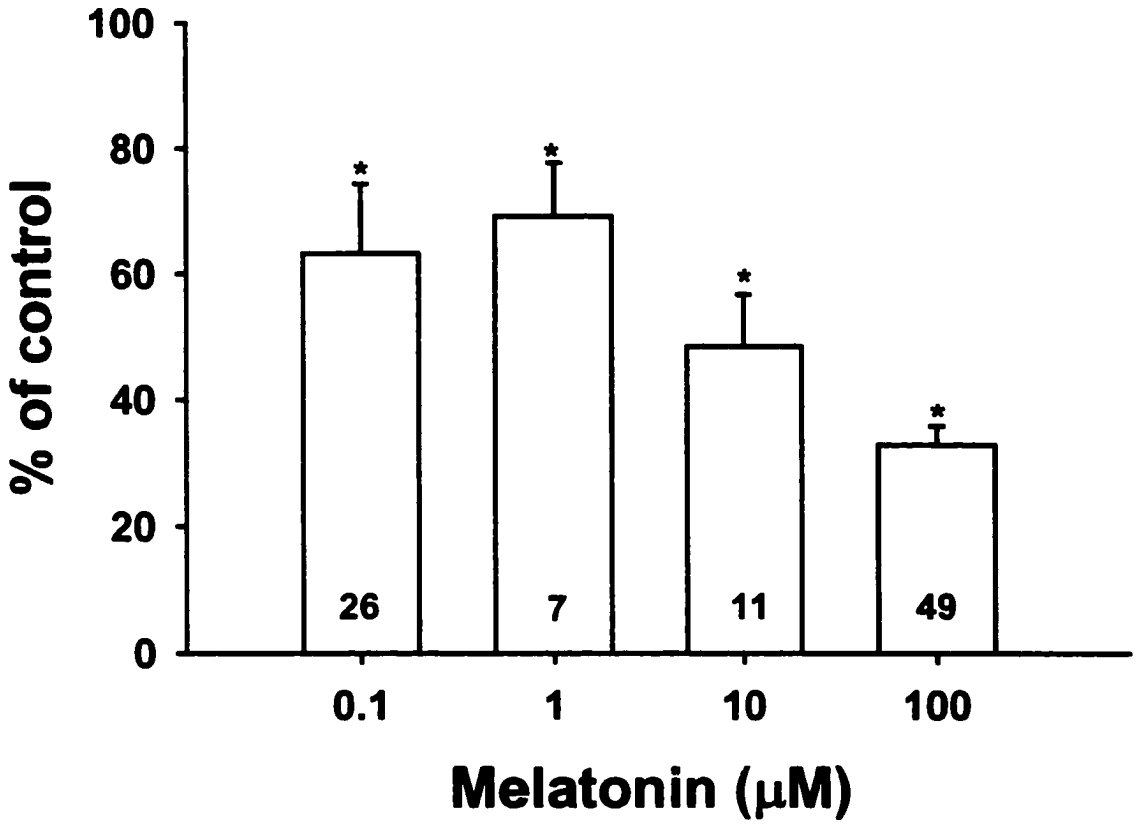

Figure 3.9. The effects of melatonin (Regis Technologies) on the hippocampal population spike.
The average of the potentials recorded from slices exposed to melatonin (0.1, 1, 10, 100μM) were compared with the averages in 0.4% EtOH, alone. Values marked with an (*) indicate a value of <0.01.

Regis Technologies (0.1, 1, 10 and 100μM). These results support our previous work demonstrating a dose-dependent action of melatonin on hippocampal evoked-potentials and the relation between melatonin (10μM) and slice age (**Figure 3.10**).

Melatonin receptor ligands

6-chloromelatonin

The melatonin agonist 6Cl-melatonin (see **Appendix A**) has been shown to be more effective then melatonin at displacing the binding of the radioligand, 2[^{125}I]-iodomelatonin at MT3 sites, equally effective at displacing binding at MT1, and less effective at displacing binding at MT2. At 0.1 mM, 6Cl-melatonin depressed the population spike to 84±10% (n=7; n.s.) and 0.5mM 6Cl-melatonin reduced the population spike to 23.5±7.1% (n=10; $p<0.0007$; **Figure 3.11**). The time period between application of 6Cl-melatonin and its maximal effect was also concentration-dependent and equaled 19.7±4.0 min and 13.9±3.1 min for 0.1 mM and 0.5 mM 6Cl-melatonin, respectively. Recovery was also observed in these experiments. The population spike recovered completely (102±3%; n=4; with a delay of 68.0±12.5 min) and up to 50% (51±15%; n=10; with a delay of 73.0±15 min) following application of 0.1 mM and 0.5 mM 6Cl-melatonin, respectively (**Figure 3.11**).

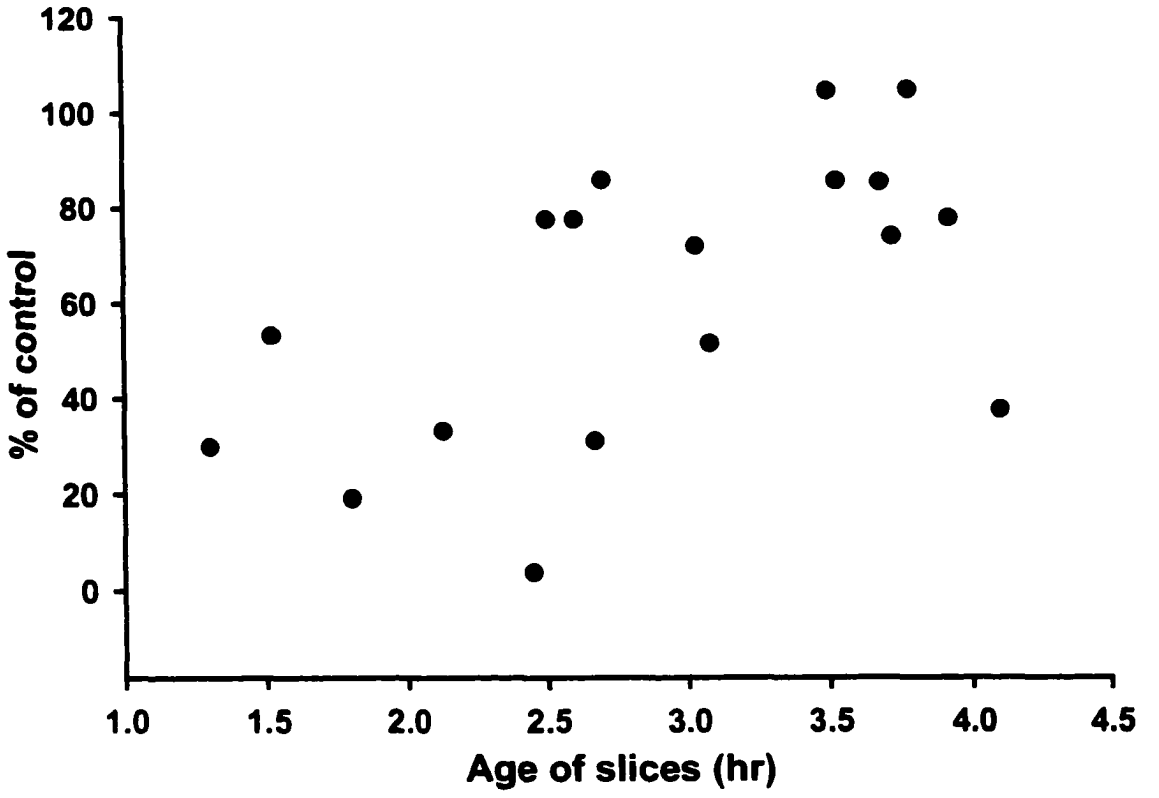

Figure 3.10. The age of the slice-dependent inhibition of the population spike by 10 μM Regis melatonin. Linear regression, $r = 0.6$, $p < 0.01$.

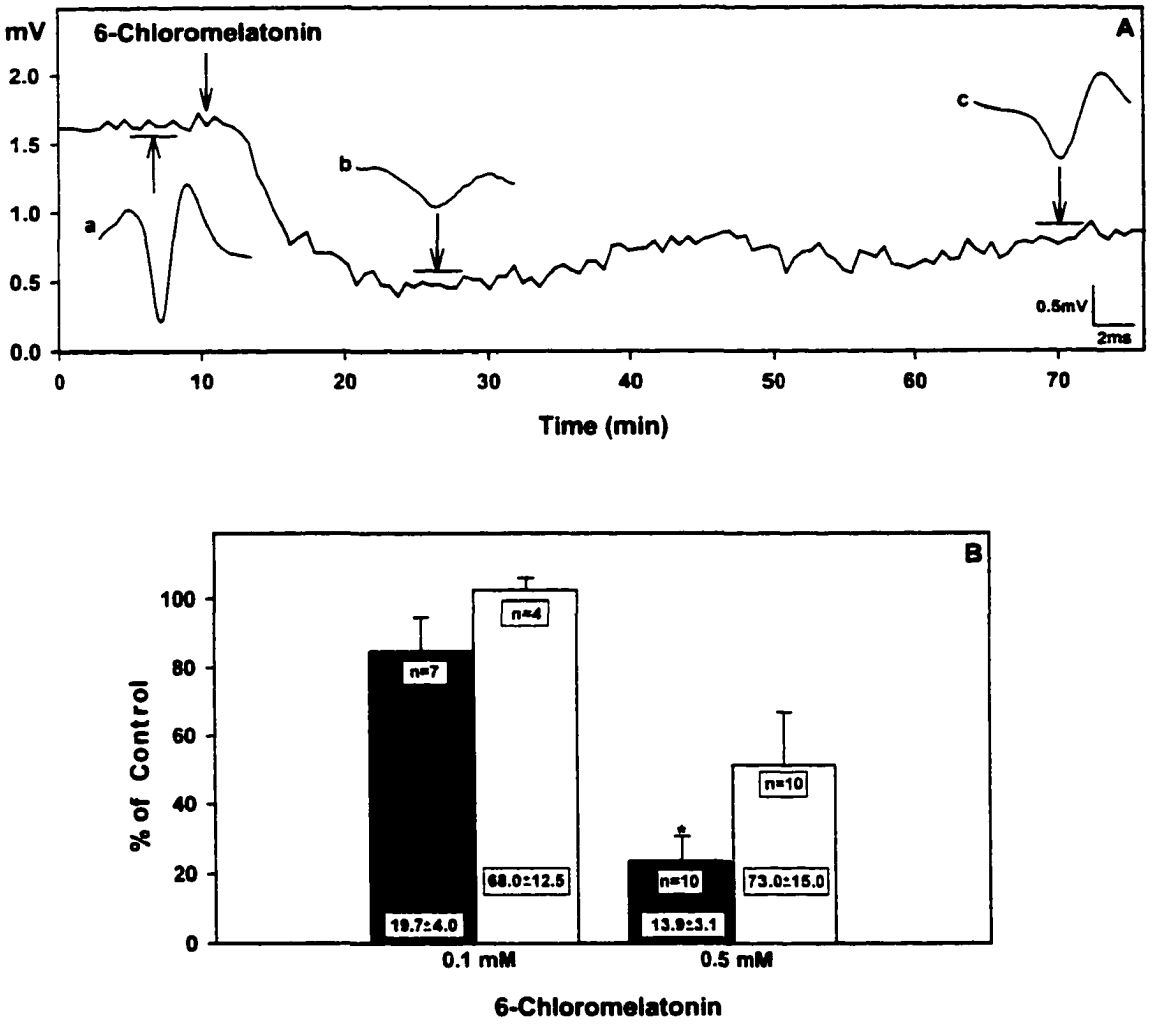

Figure 3.11. The influence of 0.5 mM 6-Chloromelatonin on the population spike.
A - Example of an individual experiment. An arrow marks the addition of 6-Chloromelatonin. **B** - The concentration-dependent depression of the population spike by 6-chloromelatonin - average results; * $p<0.0007$. For further explanation see the legend for Figure 3.2.

Luzindole and 4P-PDOT

There are no melatonin receptor antagonists able to selectively block the action at either MT1 or MT2 receptors, although they show preference towards one type or the other. Moreover, some of them show partial agonist action.

Luzindole is a melatonin receptor antagonist with higher specificity for MT2 then MT1 (Beresford et al. 1998; Dubocovich et al. 1998; see **Appendix A**). At a concentration of 100 μM it reduced the magnitude of the population spike to 61.5±5.8% (n=13) of control. The potential was only slightly reduced further to 52.3±6.4% (n=13, n.s.) by a subsequent addition of 1.0 mM melatonin. The subsequent recovery of the potential is very small (**Figure 3.12B** - crossed bar). **Figure 3.12A** depicts a representative experiment and **Figure 3.12B** provides the averaged data. As indicated in **Figure 3.12C** the pattern of changes in EPSP followed the changes in the population spike, although the EPSP reduced by Luz was further attenuated by the subsequent addition of melatonin. Thus, in the presence of Luz the influence of melatonin on EPSP seemed to be more pronounced than the effect on the population spike.

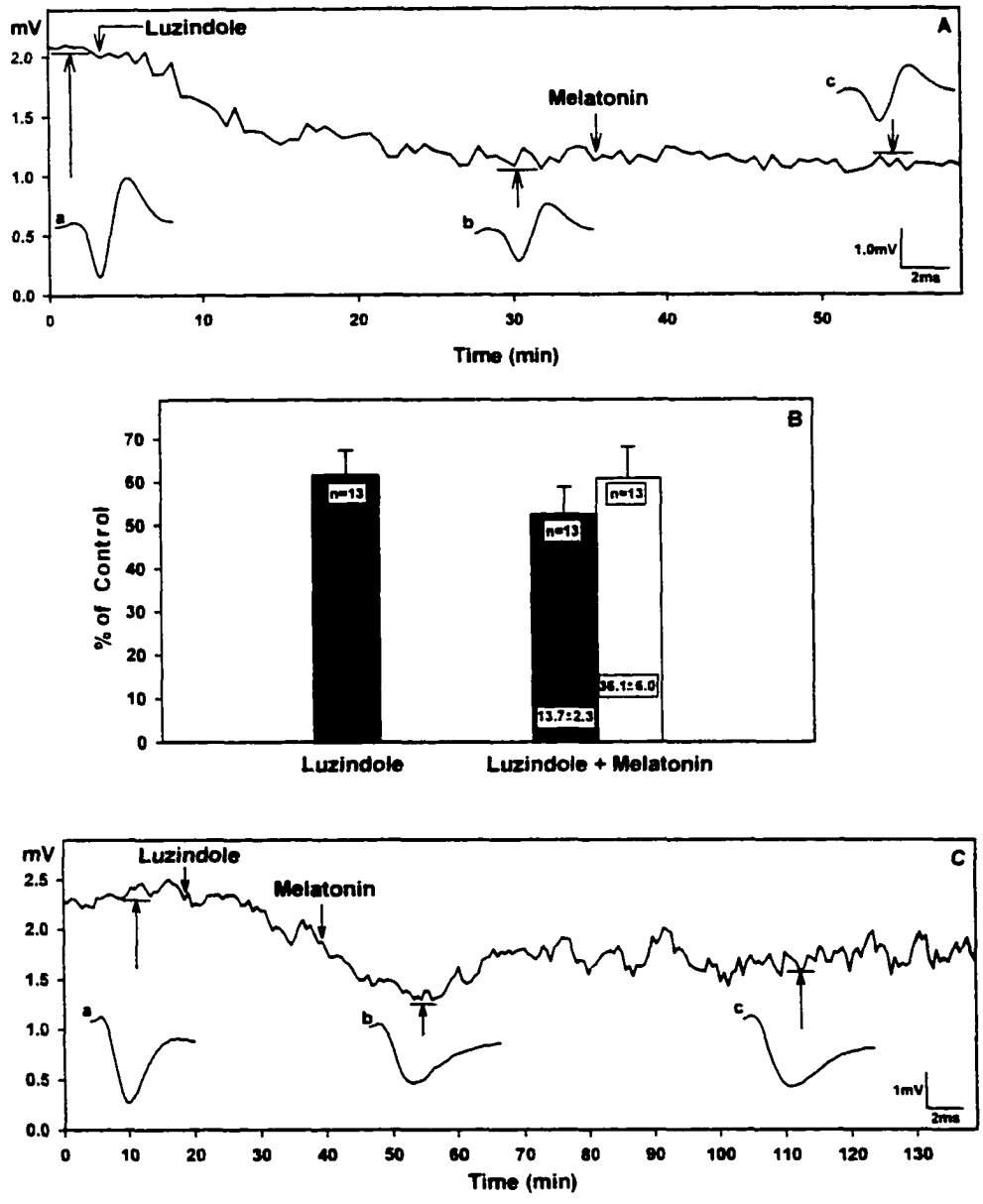

Figure 3.12. Inhibition of the melatonin-induced attenuation of evoked potentials by luzindole.
A - Luzindole (100 μM), although slightly attenuated the population spike by itself, prevented further inhibition by melatonin (1 mM).
B - The average results of luzindole action; solid bars represent the magnitude of the population spike after addition of luzindole alone (left) and after addition of melatonin in the presence of luzindole (right); the white bar depicts the recovery of the potential following addition of luzindole and melatonin. **C** - The influence of melatonin (1 mM) on EPSP in the presence of luzindole (100 μM). Note variations in the size of EPSP following the addition of luzindole.

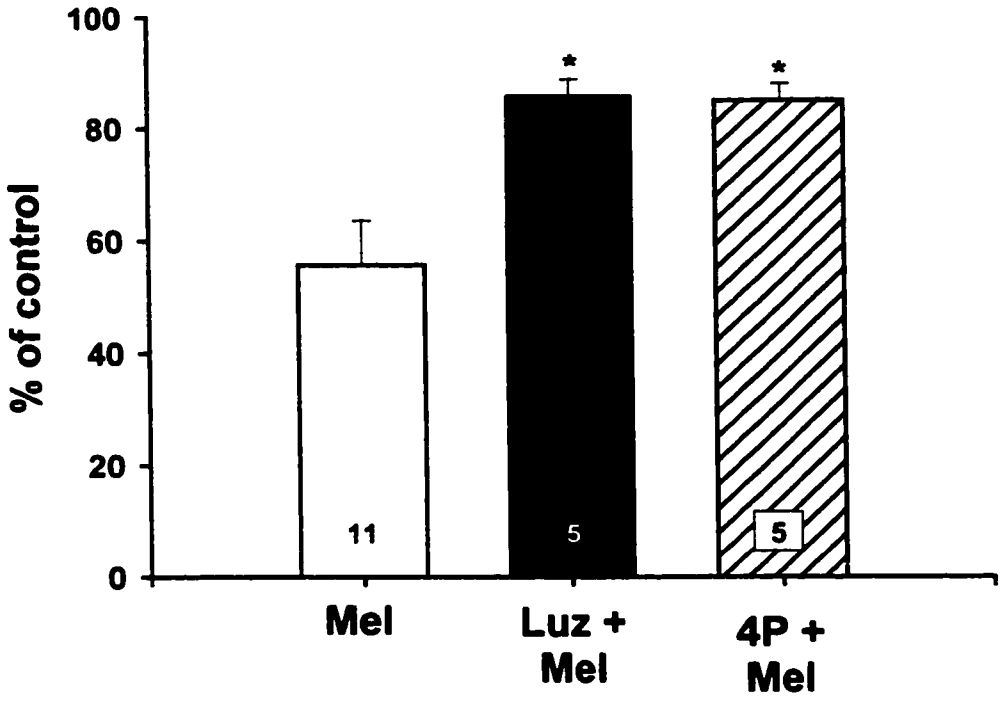

Figure 3.13. Effects of melatonin in the presence of receptor antagonists.
Melatonin (10μM) was added to the slices pretreated with either luzindole (Luz; 10μM) or 4P-PDOT (4P; 10μM) for at least 30 minutes. The melatonin induced attenuation of the PS in the presence of Luz + Mel (solid bar) and 4P + Mel (hatched bar) was significantly smaller then 10μM Mel alone ($p<0.01$; indicated by *).

The small melatonin-induced depression of EPSP was followed by a partial recovery period (**Figure 3.12C**). However, our efforts to investigate this phenomenon further were hampered by the luzindole-induced variability in the size of EPSP.

We re-evaluated the effectiveness of Luz and the effect of 4P against the actions of lower concentrations of melatonin (10μM) from Regis Tech. Luz at 10μM had no influence on the potential (109% ± 8.3; n=5; n.s.). Melatonin (10μM) added 25-30 min after Luz reduced the potential to 85.6% ± 3.1 (n=5) of control. Slices used in these experiments were all taken within the first hour and a half after slice recovery. The effects of melatonin in the presence of the Luz, was significantly lower then that of 10μM melatonin alone (**Figure 3.13,** solid bars; * indicates a $p < 0.001$).

4P is a melatonin receptor antagonist with a higher selectivity for MT2 receptors then for MT1 (Nonno et al. 1999; see **Appendix A**). Addition of 4P (10μM) alone did not affect the size of the PS (108 ± 9%; n=5; n.s.). Following a 30 minute preincubation with 4P, melatonin (10μM) caused a decrease in the amplitude of the PS to 84.95 ± 3% (n=5) of control. When compared to the attenuation in

the presence of 10μM melatonin alone (51.48 ± 8.2; n=11), the two groups were significantly different (**Figure 3.13**, hatched bars).

TMEPI

TMEPI, a charged melatonin receptor ligand, has been shown to display specific binding to melatonin receptors (Li et al. 1997; see **Appendix A**), however its biological activity has not been tested. Following a 10-minute control period slices were treated with either 4μM (**Figure 3.14, solid bars**) or 40μM TMEPI (**Figure 3.14, thatched bars**) for up to 75 minutes. The baseline used to calculate all values was the average of 5 potentials immediately preceding the addition of TMEPI. 100μM melatonin caused no significant change in the amplitude of the PS following TMEPI treatment (data not shown).

BMNEP

BMNEP has been shown to bind specifically and irreversibly to the MT2 receptor (Bordt et al. 2001; Witt-Enderby et al. 1997; see **Appendix A**) and therefore allows us to distinguish between effects mediated either by MT2 or MT1 receptor activation (Witt-Enderby et al. 1997; Witt-Enderby and Li 2000). **Figure 3.15** shows the time-dependent effects of 1μM and 10μM BMNEP. 10μM BMNEP led to a

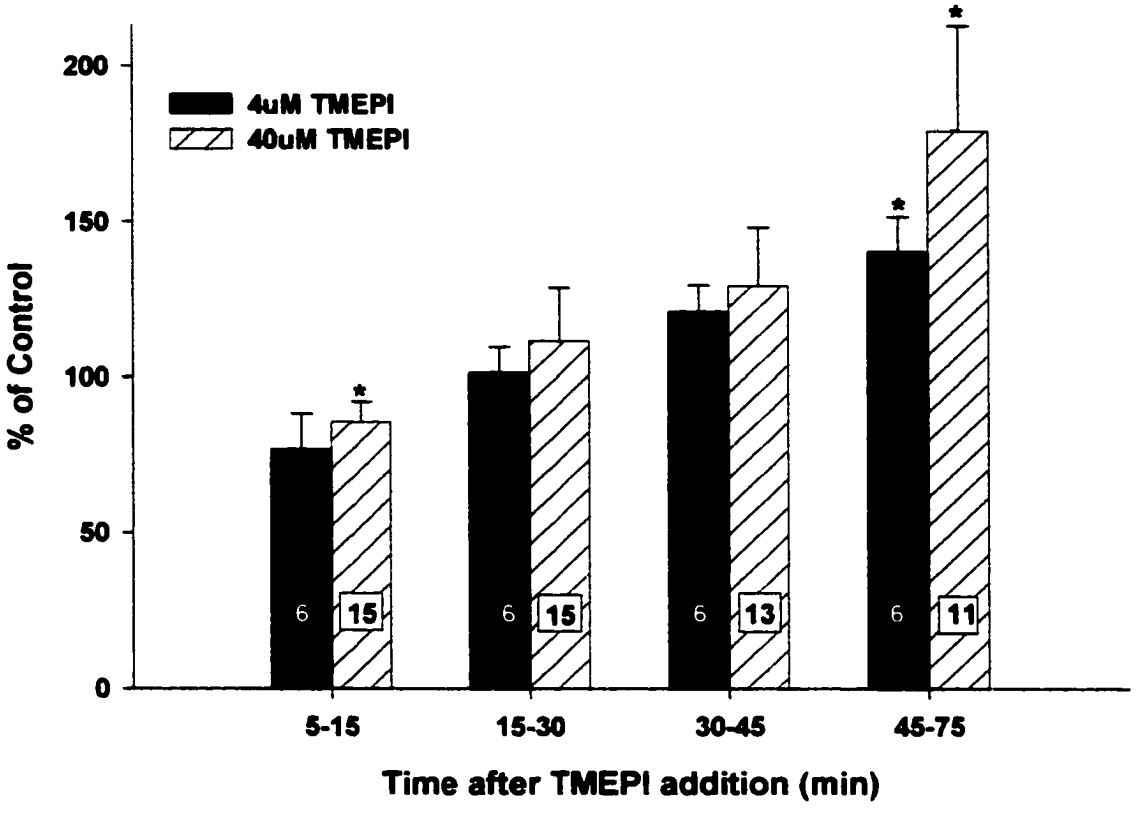

Figure 3.14. The effect of TMEPI on evoked potentials in the hippocampus.
The graph represents the average influence of 4μM (solid bars) and 40 μM TMEPI (thatched) at 5-15, 15-30, 30-45, and 45-75 minutes after addition on the PS. The effect of 40μM TMEPI was significantly different then control at 5-15 and 45-75 minutes after addition ($p<0.01$), while the effect of 4μM TMEPI was only significant at 45-75 minutes ($p<0.01$). Dashed line corresponds to 100% of control.

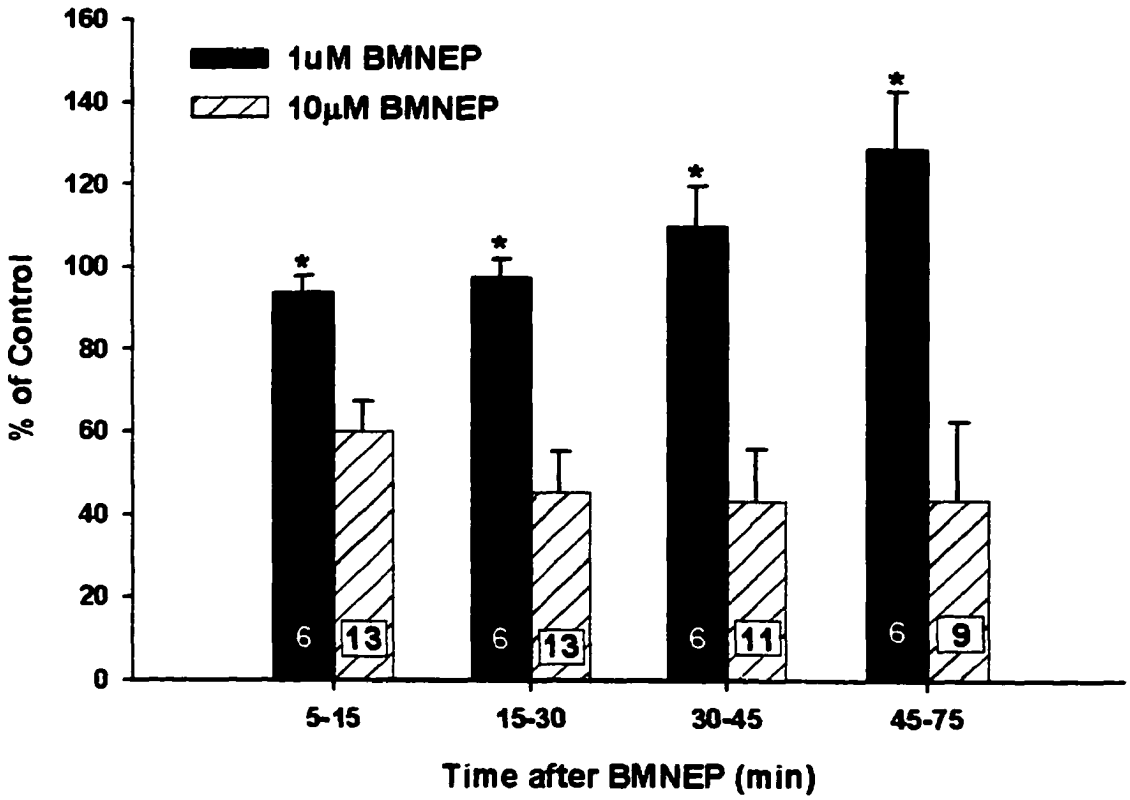

Figure 3.15. The effects of BMNEP on hippocampal evoked potentials. The graph represents the average influence of 1μM (solid bars) and 10 μM BMNEP (thatched bars) at 5-15, 15-30, 30-45, and 45-75 minutes after addition on the PS. The effects of 1 and 10μM BMNEP were significantly different from one another at all time points ($p<0.01$).

significant and permanent attenuation of the PS while 1μM BMNEP caused no significant change in the amplitude of the PS. The effects of 1μM and 10μM BMNEP were significantly different from one another at all time points tested ($p<0.01$, as indicated by an *).

Melatonin and hippocampal neuronal plasticity

Effect of melatonin and ethanol on long-term potentiation (LTP)

A typical experiment testing the influence of melatonin on LTP is shown in **Figure 3.16A**. Following stabilization of the potential (potential "a"), 100μM melatonin was added (arrow "M") to the slice-containing chamber and the potential was followed until the depressive effect of melatonin reached a maximum (potential "b"). LTP was then induced in the slice by application of HFS (arrow "HFS"). In a majority of the tested slices the potential was immediately amplified (potential "c") over the value observed prior to melatonin addition and remained potentiated as is typical for LTP. Since the goal of these experiments was to determine, if and to what extent HFS application can modify the population spike influenced by melatonin, we compared the magnitude of melatonin-attenuated population spike (potential "b") with the size

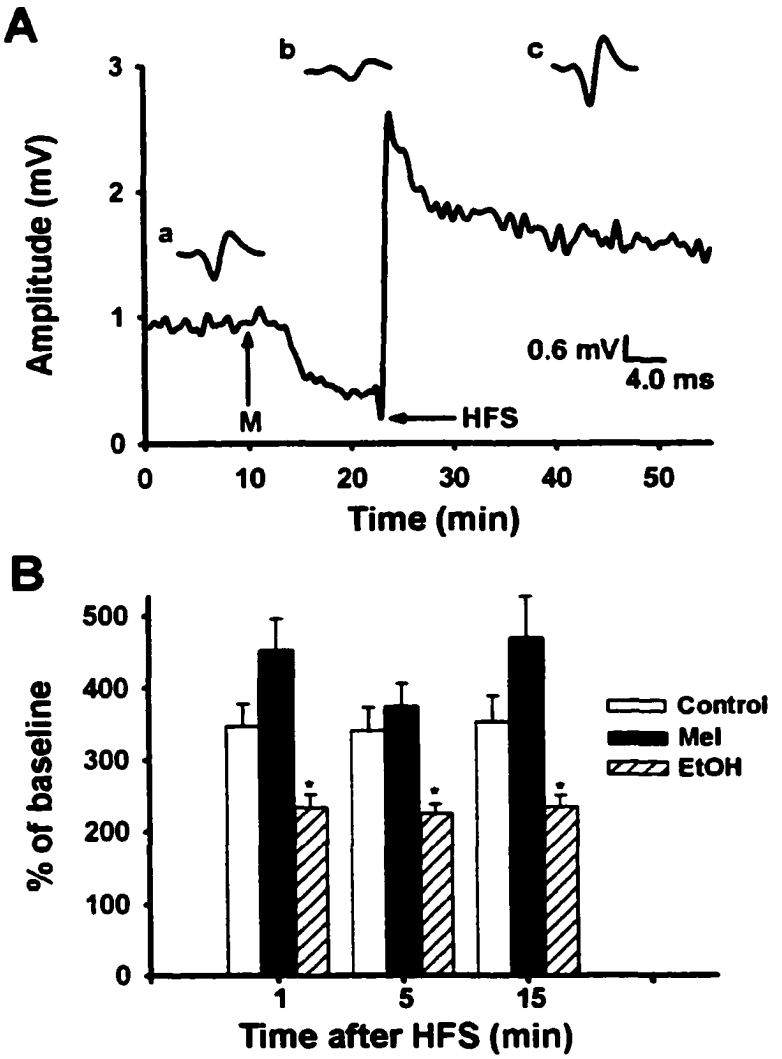

Figure 3.16. The effect of melatonin on HFS-induced potentiation. **A.** A representative experiment demonstrating the depression of the population spike by melatonin (100 μM) and reversal of melatonin's action by HFS. Symbols M and HFS indicate application of melatonin and HFS, respectively. Potentials a, b, and c illustrate an average of 5 potentials recorded before (a), just prior to (b), and 20 min after HFS. The graph illustrates the change in amplitude of the population spike during the entire experiment. Note that the potential amplified by HFS (c) exceeds the control potential (a) recorded before melatonin application. **B.** The magnitude of LTP induced in control slices (open bars, n=18), in slices preincubated with 100 μM melatonin (black bars, n=19) and 0.4% ethanol (hatched bars, n=11). The potentiation was measured at 1, 5, and 15 min after HFS application. The magnitude of LTP induced in the presence of ethanol was significantly lower ($p<0.05$) than in control and melatonin treated slices. In all figures an * indicates a p value of <0.05.

of the potential amplified by HFS (potential "c"). In all experiments the magnitude of the potentiation in control (open bars, **Fig 3.16B**) and in experimental slices (black and hatched bars **Fig 3.16B**) was evaluated 1, 5 and 15 min following HFS application. Melatonin (100μM) was able to increase the magnitude of LTP over control slices in all time intervals tested, although the effect was not statistically different ($p \geq 0.67$). Since melatonin was solubilized in 0.4% ethanol, its influence on LTP was tested in separate experiments. Although 0.4% ethanol had no influence on the PS triggered by LFS, it significantly reduced amplification of the potential induced by HFS at all time intervals (**Figure 3.16B**, hatched bars). The attenuation of HFS-induced potentiation by 0.4% ethanol was blocked in the presence of 100 μM melatonin (**Figure 3.16B**, black bars). While the magnitude of HFS-induced potentiation in the control and 100μM melatonin-treated slices was similar, it was significantly greater than potentiation observed in the presence of 0.4% ethanol alone ($p \leq 0.05$).

Effect of melatonin and ethanol on paired pulse facilitation (PPF)

Two stimuli with an inter-stimulus delay ranging from 15 to 40 ms led to an amplification of the magnitude of a second evoked potential (P2) which exceeded the amplitude of the first potential (P1), (**Figure 3.17A**, upper potentials). Five min following the addition of 100μM melatonin P2 was significantly reduced compared to P1, effectively reversing facilitation into inhibition (see **Figure 3.17A**, middle potentials for an individual experiment and **Figure 3.17B** for the average of 11 experiments). However, as the action of melatonin progressed, the amplitude of P2 stabilized, while the attenuation of P1 markedly accelerated, changing the P2/P1 ratio back to facilitation (**Figure 3.17A**, lower potentials). This trend continued till the end of the experiment. The black bars in **Figure 3.17B** represent the average results, demonstrating this biphasic action of melatonin. These biphasic changes in PPF were apparently modulated by melatonin, since ethanol applied alone had only marginal effects on PPF (**Figure 3.17B**, hatched bars). While P1 remained unchanged during the first 25 min following ethanol application, there was an initial transient, small decrease in the P2 amplitude, which recovered almost completely. The difference in the values of PPF measured in the presence of either ethanol or

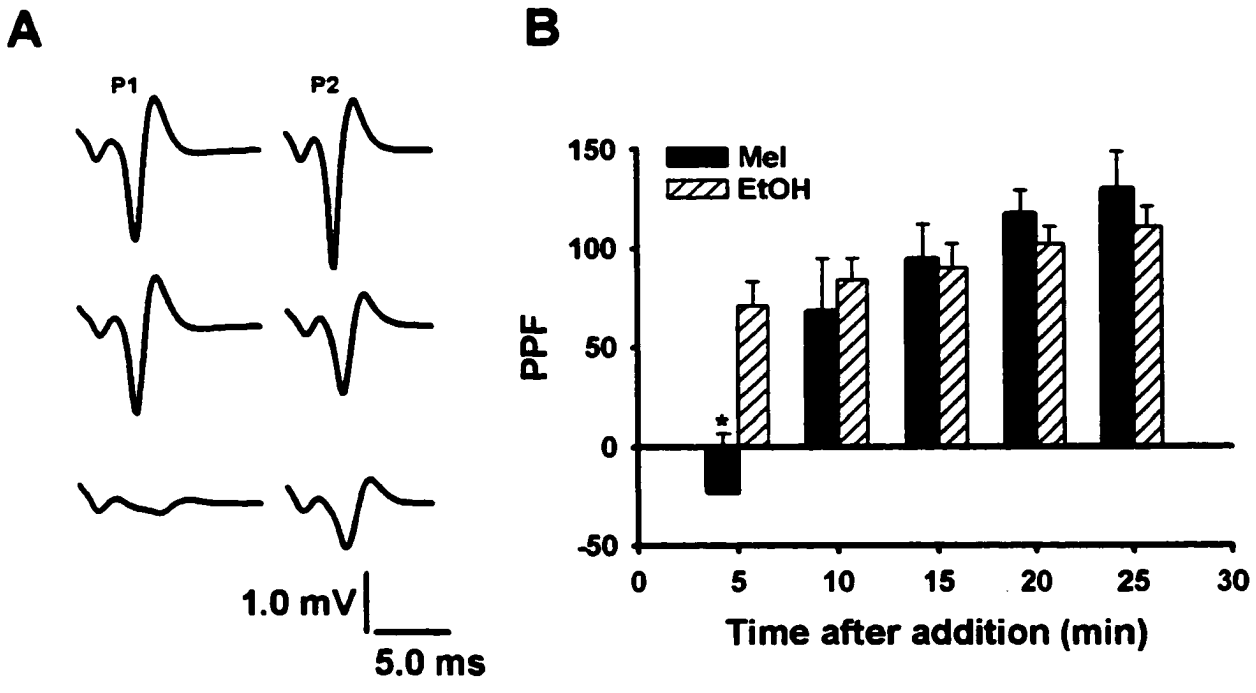

Figure 3.17. The influence of melatonin and ethanol on paired pulse facilitation (PPF). **A.** Potentials marked P1 and P2 correspond to the 1st and 2nd evoked potentials, respectively, and were initiated by two pulses applied with 15 ms delay (see methods). The upper, middle and lower potentials represent PPF recorded 5 min prior to, and 5 and 25 min after 100 μM melatonin addition, respectively. **B.** The average changes in PPF in the presence of 100μM melatonin (black bars, n=11) and 0.4% ethanol (hatched bars, n= 9).

melatonin, were statistically different from each other ($p<0.05$), when evaluated 5 min after the treatment.

Effects of melatonin and ethanol on paired pulse inhibition (PPI)

Two stimuli applied at time intervals ranging from 10 to 13 ms resulted in the inhibition (PPI) of the second potential (P2) as compared to the first one (P1) (**Figure 3.18A**, upper potentials). Addition of melatonin initiated changes in the P2/P1 ratio, which differed from the changes observed in PPF experiments. Although P1 was markedly reduced within 10 min following melatonin application, the decrease in the size of P2 occurred at much slower rate. As this process continued, the PPI changed into PPF (**Figure 3.18A**, lower potentials), which persisted till the end of the experiment (black bars in **Figure 3.18A**). Contrary to the PPF experiments, negative values of PPI, which appear after 15 min and continue through the rest of the experiment indicate facilitation. Addition of ethanol alone did not influence P1, but caused a transient decrease in the amplitude of P2, enhancing PPI (**Figure 3.18A**, hatched bars). P2 returned to the control values within 20 min following ethanol application, re-establishing the original value of PPI observed before ethanol application. The action of ethanol, which transiently affected P2 only,

was very different from the effect of melatonin, which reduced P1 for the duration of the whole experiment. An average of the experiments showing the effects of ethanol on PPI, is shown in **Figure 3.18B** (hatched bars). The difference in the values of PPI measured in the presence of either ethanol or melatonin, were statistically different from each other ($p<0.05$), when evaluated 10, 15, 20 and 25 min after the treatment.

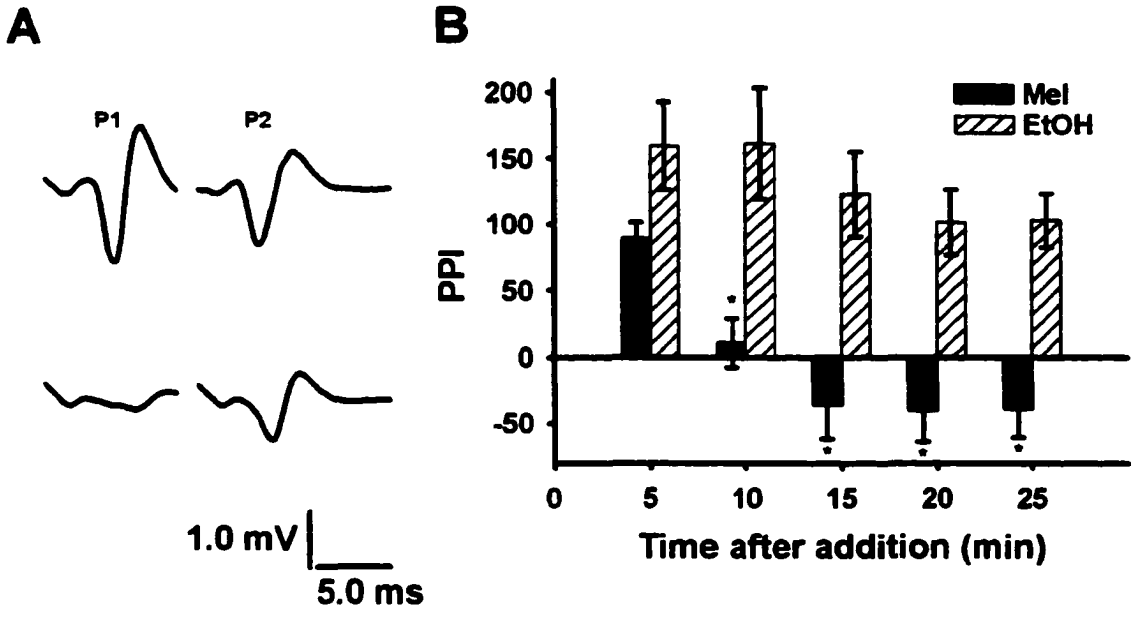

Figure 3.18. The influence of melatonin and ethanol on paired pulse inhibition (PPI). **A.** Potentials marked P1 and P2 correspond to the first and second evoked potentials, respectively, and were initiated by two pulses applied with 10 ms delay. The upper and lower potentials represent PPI recorded 5 min prior, and 25 min after 100 μM melatonin addition, respectively. **B.** Bars represent the average changes in PPI in the presence of and 100μM melatonin (black bars; n=18) and 0.4% ethanol (hatched bars; n=8).

Chapter 4 -Discussion and conclusions

Receptor-mediated actions of melatonin

This dissertation characterizes responses of hippocampal CA1 neurons to melatonin. Of the three types of potentials recorded in our experiments, FV, EPSP and PS, only the last two were influenced by melatonin (**Figure 3.1** and **Figure 3.4**). Consequently, we concluded that the properties of the presynaptic axonal membrane (generation and propagation of the action potential) are not influenced by melatonin. The most likely processes to be affected are either synaptic transmission, postsynaptic membrane depolarization, and/or cell excitability. While the depression of the PS may suggest changes in the efficiency of transmission, attenuation of EPSP reflects changes that occur exclusively at the synapse. Although the PS and EPSP were reduced, the PS was attenuated to a greater degree (**Figure 3.2**). Therefore, one can speculate that although the primary action of melatonin is concentrated at the stimulated synapse (parallel depression of EPSP and PS), other actions of melatonin, expressed as a quantitatively different effect on EPSP and PS, are possible. These could include changes in excitability of the postsynaptic cell, or modulation of inhibition (Velazquez and Carlen 1999; Wan et al. 1999). A decrease in cerebellar and hippocampal

cell excitability (Zeise and Semm 1985), were indeed reported, although at much higher concentrations of melatonin (5 - 10 mM). It is not clear whether these changes were due to a decrease in excitability or an increase in inhibition.

The time necessary to observe melatonin's action was concentration-dependent and ranged from approximately 8 min to 16 min, for higher and lower concentrations of agonist, respectively. Since this time period included diffusion of the tested molecule and its action, we used this short delay as an indicator, that melatonin interacting with its receptor influenced either fast synaptic processes, or the mechanisms directly involved in regulation of cell excitability. The inhibitory action of melatonin was significantly reduced by Luz (**Figure 3.12**), an antagonist of MT2 melatonin receptors (Dubocovich et al., 1997). It should be stressed that Luz exerted partial agonistic action (Nonno et al. 1999) partially depressing the PS on its own, which following subsequent addition of 1 mM melatonin led to no significant response. This overlap between Luz and melatonin actions indicated that melatonin's actions were mediated mostly by MT2 receptors, which have already been described in the mouse hippocampus (Wan et al. 1999; Mushoff et al. 2002). In order to

decrease the partial agonist activity of Luz we tested a ten times lower concentration of Luz (10μM). This concentration demonstrated no significant influence on the potential (**Figure 3.13**). Subsequent addition of 10μM melatonin (Regis Tech.) led to a very small attenuation of the potential (~20%). This response was significantly different then the attenuation in 10μM melatonin alone. The block of melatonin's action by a more specific antagonist of MT2 receptors, 4P (**Figure 3.13**), further confirmed the results with Luz, indicating that the attenuation of the potential is mostly mediated by MT2 receptor activation.

An agonist of MT2 receptors, BMNEP, was originally described in 1997 (Witt-Enderby, 1997) and was shown to bind and permanently activate the MT2 receptor. A response to melatonin and no response to BMNEP would indicate the absence of MT2 and support the presence of MT1 and/or MT3 receptors (Bordt et al. 2001). BMNEP depressed permanently the PS in the hippocampal slice and the recovery/amplification phase normally seen in the presence of melatonin, was absent (**Figure 3.15**). These results support our previous findings indicating that the attenuation of hippocampal potentials by melatonin is an MT2 receptor mediated phenomenon. Thereby, constitutive

activation of the MT2 receptor by BMNEP (10μM) leads to permanent attenuation of the PS. Lower concentrations of BMNEP (1μM) had no influence on the amplitude of the PS (**Figure 3.15**).

MT2 receptors are localized in the retina presynaptically (Dubocovich 1983; Dubocovich et al. 1998) and if this is also the case in the mouse hippocampus, they are in good position to modulate neurotransmitter release. Indeed, melatonin was shown to inhibit Ca^{2+} uptake in brain synaptosomes (Zisapel and Laudon 1983; Vacas et al. 1984), a process essential for the initiation of neurotransmission. This action of melatonin may reduce neurotransmitter release, and subsequently attenuate synaptic efficiency. Melatonin can also shift the balance between glutamate release and uptake in the retina (Faillace et al. 1996) and it may have a similar effect in the hippocampus, where glutamate is a major excitatory neurotransmitter (Wieraszko 1983). It has been demonstrated in the past (Wieraszko 1983) and confirmed recently (El-Sherif et al. 1999) that any disturbance in this glutamate uptake/ release may have profound effects on the efficiency of hippocampal synapses. Melatonin can also modulate glutamate-driven potentials recorded from

hippocampal slices by direct interaction with glutamatergic receptors (Escames et al. 1996).

The recently observed activation of potassium conductance and subsequent neuronal hyperpolarization (Jiang et al. 1995) induced by melatonin could provide an alternative explanation for its inhibitory action. Potassium channels are activated by subunits of pertussis toxin-sensitive G proteins and it is well documented that most of the melatonin receptors are coupled to G proteins (Reppert et al. 1994; Fukawa et al. 1995; Vanecek and Watanabe 1998; Vanecek 1998), which regulate adenylyl cyclase activity and the level of cAMP (Niles and Hashemi 1990; Niles et al. 1991). However, not all melatonin effects have to be related to G protein action, since melatonin receptors have the ability to exist in high and low affinity states, which are G-protein coupled and uncoupled, respectively (Morgan et al. 1994). Indeed, guanine nucleotide-insensitive melatonin binding sites have been described in bovine hippocampus (Nonno et al. 1995). Testing the influence of pertussis toxin may help to distinguish between direct actions of melatonin and effects mediated by G protein-linked melatonin receptors. The inhibitory action of melatonin could also be mediated by an increase in inhibition (Zeise and Semm 1985), however

recently reported decrease of $GABA_A$ currents through MT2 receptor activation in hippocampal neurons by melatonin (Wan et al. 1999) contradicts this suggestion.

Factors regulating the influence of melatonin on hippocampal neurons

The effect of melatonin is expressed in two phases. The first depressive phase, is mediated by the interaction of melatonin with the MT2 receptor, while the recovery phase may involve plasticity of this receptor. Because the amplification of the potential observed in the second phase occurs even in the continuous perfusion (**Figure 3.3**), we ruled out its decomposition (Leon et al. 1998a, 1998b), as a possible cause of this phenomenon, and suggest that plasticity of melatonin receptors might be responsible for the effect. The presence of MT2 melatonin receptors in the bovine, hippocampal pyramidal layer (Nonno et al. 1995) and in the mouse hippocampus (Musshoff et al. 2002), support our notion about the involvement of melatonin receptors, which, like other receptors coupled to second messenger systems (Chuang et al. 1995; Kallal and Benovic 2000; Tsao and Von Zastrow 2000; Waldhauser et al. 1993) might be very dynamic structures in the nervous tissue. Melatonin is involved in regulation of its receptor (Schuster et al. 2001), and a prolonged exposure of these receptors to agonist may result in their subsequent internalization (Faillace et al. 1996; Gauer et al. 1993) diminishing the ability of melatonin to influence hippocampal neurons.

While melatonin as a small lipophilic molecule can cross, cellular membranes (Costa et al. 1995, 1997) and regulate several intracellular neurotransmission-related events (Al Ghoul et al. 1998; Anton-Tay et al. 1998; Benitez-King et al. 1996, 2001; Borjigin et al. 1999; Cardinali et al. 1997; Dubocovich 1995; Huerto-Deladillo et al. 1994), the molecule TMEPI is charged and therefore can act only on extracellular sites. Interestingly the addition of TMEPI led to a delayed amplification of the PS (**Figure 3.14**). The exact mechanisms of TMEPI's actions still remain unknown (agonist or antagonist). Addition of melatonin to slices initially treated with TMEPI failed to elicit any response, indicating either an antagonist action or a competitive agonist action on the melatonin receptors (MT1?). TMEPI may act through MT1 receptors leading to the delayed amplification of the response seen 60 minutes after addition. This hypothesis is attractive in that it implies that MT2 receptor activation leads to a permanent attenuation of the potential, while MT1 activation leads to a slow recovery due to amplification of the potential. Both the actions of BMNEP and TMEPI would be mimicked by the addition of melatonin alone (depression followed by a delayed recovery).

Melatonin also acting on MT1 or MT2 receptors has the ability to interact selectively with the glutamatergic system (Hirata et al. 1974; Leon et al. 1998a). Glutamatergic receptors seem to be especially vulnerable to plastic changes. Those receptors and their location in the hippocampus (dendritic spines) are much more dynamic *in vitro* than originally suspected. Within a few hours following slice preparation the number of dendritic spines and synapses increases by 30-40% in an activity-dependent manner (Kirov and Harris 1999). These observations correlate well with the studies showing continuous, very rapid (minutes), activity-dependent (Lissin et al. 1999; Zhu et al. 2000) endocytosis/exocytosis of glutamatergic receptors (Luscher et al. 2000). The recycling of glutamatergic receptors requires interaction with intracellular carrier proteins, specific for different types of glutamatergic receptors (Hayashi et al. 2000; Lissin et al. 1999; Zhu et al. 2000). Since melatonin has been shown to interact with proteins involved in the regulation of neurotransmission (e.g. calmodulin; Benitez-King et al. 1996; or PKC; Anton-Tay et al. 1998), the proteins regulating the recycling of glutamatergic receptors might also be a potential target for melatonin's action. Thus, one can speculate that during the first

attenuation phase, melatonin or its metabolites, in addition to the mechanisms discussed above, inhibits exocytosis of glutamatergic receptors reducing the magnitude of the evoked responses. That decrease in synaptic activity might stimulate exocytosis of another type of glutamatergic receptors, whose recycling would not be influenced by melatonin, or its metabolites. This process would be expressed as a second, recovery phase, which occurs even in the presence of melatonin. Thus, the mechanisms of the recovery phase, observed in the presence of melatonin could be explained by recycling of glutamatergic receptors.

Another mechanism that can regulate responsiveness of hippocampal neurons to melatonin does not depend on the presence of exogenous melatonin. The pyramidal neurons become gradually less sensitive to melatonin, as slice incubation time lengthens. This trend, important to those studying melatonin's effect on *in vitro* preparations, was much more pronounced in the slices obtained from the younger animals and can be related to the age-dependent flattening of day/night melatonin cycle (Reiter 1994). The binding assay strongly implicates the reduction in binding of melatonin and/or reduction in the number of its receptors as the mechanisms of attenuated responsiveness of

hippocampal slices to melatonin. Since the magnitude of the PS and EPSP (which both are indicators of neuronal response to the endogenous transmitter, glutamate) were not decreasing with the age of the control slice, the attenuation of the responsiveness to melatonin (see **Figure 3.5**) is not related to deterioration of the slice and is specific for the melatonergic system. Interestingly, melatonin binding in quail brain membranes (39.6±2.2 fmoles/mg protein) was similar to the values reported in this study (**Figure 3.7**), and was reduced almost by half in the dark period (Yuan and Pang 1990). A daily change in specific binding of melatonin *in vitro* has been reported by others (Anis et al. 1989; Faillace et al. 1995). Although our results do not provide an unequivocal explanation for this time-dependent attenuation of the sensitivity of pyramidal neurons to melatonin, our data and those reported by others (Anis et al. 1989; Faillace et al. 1995; Yuan and Pang 1990) suggest that the consequences of the oscillatory changes in the melatonin concentration *in vivo* (Pang and Brown 1983) are expressed *in vitro*. Since the diurnal oscillations of some physiological properties of the slices (Liu et al. 2000; Raghavan et al. 1999) can complicate the results, to assure reproducibility of our data, the majority of the slices were prepared at the same time of

day and the experiments were performed within 2-3 hrs following recovery. The interaction of melatonin with the glutamatergic system and changes in the sensitivity of melatonin receptors could occur simultaneously and our electrophysiological experiments would record the final, combined effect of both mechanisms. Additionally, since melatonin can directly interact with intracellular molecules, it may also, when ingested, have an effect on brain structures or even whole organisms devoid of melatonin and melatonergic receptors.

It is interesting to note, that although the precursor for melatonin biosynthesis, serotonin can by itself exert an inhibitory action on hippocampal neurons (Segal 1980), melatonin is unable to displace agonists from the serotoninergic receptor (Bennett and Snyder 1975). Therefore, it is unlikely that activation of serotonin receptors by melatonin contributes in any way to the observed effects.

The most appealing interpretation of the reduced sensitivity to melatonin observed in the slices obtained from the light-deprived animals would be reduced number/affinity of melatonin receptors. One can speculate that long-lasting light deprivation elevates melatonin levels, which in turn facilitate internalization of

melatonin receptors (Faillace et al. 1995). However, although light/darkness cycle is an important factor in synchronizing circadian production of melatonin, the longer periods of the light deprivation (days) leave the animals on a free-run pattern characterized by increased and decreased melatonin levels in subjective nights and days, respectively (Conti and Maestroni 1998; Murakami et al. 1994). Therefore, it seems unlikely that reduced sensitivity to melatonin observed in the slices obtained from these animals results solely from the elevated level of this hormone (**Figure 3.8**). One cannot exclude the possibility that light deprivation is able to reduce the number of melatonin receptors without any involvement of melatonin. Although we are yet unable to provide unequivocal interpretation for this part of our data, the demonstration that external environment (light deprivation) imposed on intact organism is able to modulate subsequent melatonergic-glutamatergic systems interactions *in vitro* seems to be very important and merit further research.

Most of our experiments were performed on the CD-1, outbred strain of mice, which demonstrates morphological changes in the pineal gland, related to circadian rhythmicity (Benson and Krasovich 1977; Krasovich and Benson 1979; Huie et al. 1989; Upson and Benson 1977) and

contains melatonin in its plasma and hippocampus (unpublished results). This strain of mice is becoming increasingly popular in studies of learning and memory (Wall and Flood et al. 1995; Farr et al. 2000; Brodkin 1999) and therefore has been selected as a very suitable animal model for our continuing research.

The actions of melatonin on long and short forms of neuronal plasticity

One of the most remarkable results reported here was an immediate and permanent reversal of the depressive action of melatonin by HFS, which by itself induces a long-lasting amplification of the population spike known as LTP (**Figure 3.16**). Apparently, the mechanisms responsible for LTP remained unaffected in spite of a significant, MT2 receptor induced attenuation of the population spike. The size of the amplified population spike was far above the value of the potential depressed by melatonin and exceeded the value of the initial baseline recorded before addition of melatonin. Undoubtedly, LTP can be induced in slices, whose neurons remain under the influence of the depressive action of melatonin. This contradicts the results of the experiments described by Collins and Davis (1997), and partially challenges the data of Gonzales and Armstrong (1995). While both groups reported a diminished ability of melatonin-exposed, rat hippocampal slices to express LTP, the control potential remained either intact (Collins and Davis 1997), or reduced by melatonin (Gonzales and Armstrong 1995). These discrepancies may arise from different animal species used and from the complex interactions between melatonin and glutamatergic systems,

which might be complicated even further by differences in experimental procedures. We have shown that the sensitivity of the hippocampal population spike to melatonin was inversely related to the time of slice incubation (**Figure 3.5**) and directly correlated with the declining expression of melatonin binding sites in those slices (**Figure 3.7**). These observations agree with the findings showing that the influence of melatonin on spontaneous activity of pyramidal neurons *in vitro* depends on the time of slice preparation (Musshoff et al. 2002). Furthermore, age-dependent, diurnal oscillations in the expression of hippocampal LTP (Harris and Teyler 1983; Raghavan et al. 1999) might further contribute to its regulation by melatonin. While the slices used in our experiments were prepared usually no longer than two hours into the light cycle, Gonzales and Armstrong (1995), and Collins and Davis (1997) prepared their rat hippocampal slices either midway through the dark period, or four hours into the light period, respectively. Since none of those two laboratories provided the time of slice preincubation prior to testing, it is difficult to assess the differences between their and our data. Diurnal oscillation in the level of melatonin (Pang and Brown 1983), its ability to control its receptors (Gauer et al. 1993; Schuster et al. 2001), the changing properties of

hippocampal neurons *in vitro* (El Sherif et al. 2002) and species-related variability in the distribution of melatonin receptors require well-controlled experimental conditions for evaluation of the influence of melatonin on hippocampal neurotransmitter systems.

Although the mechanisms of LTP still remain elusive, the latest experimental evidence implies that an HFS-induced increase in hippocampal cell excitability results from a rapid exocytosis of the AMPA type of glutamatergic receptors and their incorporation into the postsynaptic membrane (Hayashi et al. 2000; Luscher and Frerking 2001; Zhou et al. 2000). One can speculate that these newly recruited AMPA receptors have modified subunit composition, which make them less vulnerable to melatonin action, ensuing in the recovery of the potential. Although melatonin depressed AMPA receptor-mediated neurotransmission before application of HFS, the mechanisms responsible for HFS-induced trafficking of new AMPA receptors to the synaptic region may apparently not be impaired and the population spike still could be amplified. An alternative explanation would imply that while the attenuation of the population spike is mediated by a melatonin-induced activation of GABA-mediated, feed forward inhibitory system (Coloma and Niles 1988; Niles and Peace

1990; Wu et al. 1999), HFS facilitates excitatory, glutamatergic neurotransmission which permanently overwhelms this inhibition.

The induction of LTP in the SC is calcium-dependent (Bliss and Collingridge 1993). While the lowering of intracellular calcium concentration by melatonin (Slanar et al. 2000; Zhang and Zhang 2000) may explain depression of the population spike triggered by LFS, HFS, employed to induce LTP may overcome the depressive effect of melatonin and elevate intracellular calcium concentration to a level essential to express LTP.

One could also speculate that HFS desensitizes melatonin receptors, eliminating their inhibitory influence on the glutamatergic system. Although the mechanism of melatonin receptor internalization, similar to the desensitization of other G-protein linked receptors has been proposed (Witt-Enderby and Li 2000), it is unclear how this process could be initiated by HFS.

While long-lasting changes in neuronal excitability, represented by LTP, are believed to underlie memory (Barnes 1995; Maren and Baundry 1995), short-lasting modifications, occurring during paired-pulse stimulation are also associated with learning (Sinclair and Lo 1986). The first stimulus recruits either excitatory, or recurrent

inhibitory activity (Buzsaki 1984), which initiates the facilitatory or suppressive effect upon the reaction to the second stimulus, respectively. The difference between the sizes of the first and second responses depends on the interstimulus delay. Shorter (10-13 ms), and longer (15-40 ms) delays between pulses results either an inhibition (PPI) or facilitation (PPF) of the second response, respectively. While the effects of PPF are not permanent, they were suggested to play a role in temporary storage of information (Zucker 1989; Buonomano and Merzenick 1995). As reported previously (Zeise and Semm 1985), and observed here, melatonin was able to considerably change the characteristics of PPF. Shortly after its application melatonin reversed the basic property of PPF changing it to PPI (**Figure 3.17**). This transient period was followed by a significant and quickly progressing attenuation of the amplitude of the first conditioning potential, reversing this transient inhibition back to facilitation, which increased with the progress of melatonin's action. Thus, the short period of inhibition, separating PPF into two phases, accentuated the recovery and an increase in PPF occurring later. Similarly, the significance of the HFS-induced amplification of the potential previously attenuated by melatonin is different than LTP occurring

without previous depression. Thus, the pyramidal neurons depressed by the elevated level of melatonin at night, still maintain the ability to selectively enhance their response to frequent activation.

LTP and PPF differ not only in their duration, but also in the neuronal location of their respective mechanisms. Although most of the research supports the view that the mechanism of LTP is postsynaptic (Hayashi et al. 2000; Zhou et al. 2000; Luscher and Frerking 2001), there is also evidence in support of its presynaptic location (Bekkers and Stevens 1990). In contrast to LTP, the majority of studies (Creager et al. 1980; Christie and Abraham 1994; Schulz et al. 1994; Carter et al. 2002), and a recently proposed model for a short-term plasticity (Dittman et al. 2000) implicate a transient elevation in the presynaptic calcium concentration, as a major mechanism of PPF (but see Wang and Kelly 1996, 1997). Therefore, since basic properties of LTP were not influenced by melatonin, one can assume that this hormone modifies neuronal excitability, regulating, like in other systems, presynaptic calcium concentration (Zisapel and Laundon 1983; Vacas et al. 1984; Rosenstein et al. 1991; Faillace et al. 1996; Tsim et al. 1998). The reported influence of melatonin on presynaptic mechanisms of glutamatergic

neurotransmission (Zhang et al. 1999; Escames et al. 2001) supports this conclusion.

Because of the anatomical arrangement of the hippocampal recurrent inhibitory loop, a response activated by two pulses delivered in rapid succession (12 ms in our experiments), has less effect on dendritic response (EPSP) and influences mainly the population spike, which is the best measure of this process (Gutierrez et al. 2001; Sayin et al. 2001). The decline of the second potential is due to release of GABA from the activated interneuron onto GABA receptors on the pyramidal cell body. Addition of melatonin led to a long-lasting change of PPI into PPF (**Figure 3.18**). The most straightforward explanation would be a melatonin receptor-mediated attenuation of the GABAergic interneuron, which would then no longer reduce the activity of pyramidal neurons. However, melatonin was reported to have either a facilitatory or inhibitory influence on $GABA_A$ receptor-mediated currents following activation of MT1 or MT2 receptors, respectively (Wan et al. 1999). Therefore, the final influence of melatonin on hippocampal inhibition could be greatly influenced by the relative distribution of MT1 and MT2 receptors, which are both present in the hippocampus (Nonno et al. 1995; Reppert et al. 1995; Mazzucchelli et al. 1996; Wan et al. 1999;

Musshoff et al., 2002). Future analysis of the distribution of melatonin receptors in the mouse hippocampus would be essential for more unambiguous interpretation of this data.

Melatonin and ethanol

Since melatonin concentrations *in vivo*, vary dramatically, and are significantly elevated in humans following ingestion, we were interested in the effects of melatonin applied at a wide range of concentrations. A number of experiments were performed with relatively high concentrations of melatonin from Sigma-Aldrich. However, due to technical problems we were forced to test multiple sources of melatonin and decided to use melatonin from Regis Technologies (Regis Tech.), which gave us better stability and solubility. Better solubility means enhanced distribution through the tissue and easier penetration to melatonin targets inside the hippocampal slice. Melatonin with lower solubility would require a higher concentration delivered to the chamber in order to achieve an effective concentration in the tissue. Since the pattern of changes induced by either Sigma, or Regis melatonin were analogous (Compare **figure 3.5** with **3.10**), we assume, that the final concentration of melatonin in the slices was similar regardless of melatonin vendor, and that the action of melatonin was exerted through its receptors.

Melatonin and commercially available ligands of its receptors are hydrophobic molecules with limited solubility in water. According to some reports it is possible to

increase the solubility of melatonin in Ringer's (Shida et al. 1994), however we failed to reproduce these findings. We decided to use ethanol as a vehicle to dissolve and distribute melatonin and its analogs throughout the tissue. In the first part of our study we demonstrated that ethanol had no influence on hippocampal LFS evoked-potentials. A number of studies in the rodents have shown that ethanol influences glutamatergic (Costa 2000; Simson 1993) and GABAergic transmissions (Wan 1996;Peoples and Weight 1999). The mechanism of ethanol's action is currently still not well understood. It is believed that the actions of ethanol may actually be heterogeneous, with some cells showing sensitivity and others being much less responsive (Aguayo 2002). It has also been shown that $GABA_A$ receptors from different strains of mice can be differentially sensitive or even completely insensitive to ethanol (Poelchen 2000).

In our study 0.4% ethanol had no significant effect on the PS (**Figure 3.1A**). However, when 100μM melatonin dissolved in 0.4% ethanol was added to the slices, there was a significant attenuation of the PS, which was not present when melatonin was tested at the same concentration dissolved in 0.04% ethanol. We explain this as a concentration dependent solubility of melatonin in ethanol.

Because ethanol helps carry melatonin in aqueous solution, lower concentrations of ethanol may lead to a decrease in the amount of melatonin penetrating the hippocampal slice.

Summary and conclusions

Melatonin receptor activation leads to an attenuation of synaptically evoked potentials in the CA1 region of the hippocampus. This attenuation phase is followed by a recovery/amplification phase, which is not due to the breakdown of melatonin. The attenuation phase was blocked by the use of Luz and 4P, which antagonize the actions of melatonin mostly at its MT2 receptor (at the concentration tested). Permanent suppression of the potential by BMNEP (10μM) suggests that the observed actions of melatonin during the first phase (attenuation of the potential) are mediated by MT2 receptor activation. While the reduction of the potential was rapid, the recovery/amplification phase occurred slower, approximately 60 minutes after melatonin addition. This enhancement was mimicked by the addition of the charged ligand TMEPI, which suggests that this phase of melatonin action occurs through melatonin binding on the surface of the membrane. However, because the exact mechanisms of TMEPI actions are not known, we could not determine whether it functioned as an agonist/antagonist of MT2 or MT1 receptors. Future ligand displacement studies using a radiolabelled form of TMEPI will further allow us to characterize the specificity and selectivity of TMEPI.

While melatonin had no effect on the induction of LTP, the pattern of stimulation used to induce LTP was able to reverse the attenuation of melatonin immediately. In contrast to melatonin's lack of interaction with long-term neuronal plasticity, melatonin had a significant effect on short forms of neuronal plasticity (PPI and PPF). Addition of melatonin led to a fast and short-lasting reversal of PPF into PPI and a fast and long-lasting reversal of PPI into PPF. Melatonin's interaction with both short and long forms of neuronal plasticity shares at least one very important similarity. In the presence of melatonin the PS retained the ability to be potentiated in response to stimuli delivered at a higher then normal rate (PPI, PPF or HFS). During the maximal attenuation of P1 by melatonin in PPI and PPF experiments P2 was always facilitated (compare **Figure 3.17A** and **3.18A**, lower potentials), and delivering pulses at high frequency immediately facilitated the response depressed by melatonin (**Figure 3.16A**).

Circadian rhythms are described as cyclical changes within a 24-hour day and have been shown to regulate many biological events. Some of these include neurotransmitter secretion, regulation of body temperature, and hormone production (Kafka et al. 1986; Wirz-Justice et al. 1980; Touitou et al. 1981; Lincoln et al. 1981; Oren et al. 1995;

Arendt 2000). One of the most studied circadian cycles is the daily fluctuation in levels of the neurohormone melatonin. Levels of melatonin are directly linked to a circadian cycle and are entrained by light (Yu et al. 1981; Wetterberg et al. 1984; Webley et al. 1985; Sharma et al. 1989; Kumar et al. 2000). Furthermore, the regulation of melatonin receptors is directly linked to the circadian rhythm of melatonin.

Since melatonin and its receptors undergo a circadian rhythm, one can speculate that the actions of melatonin throughout the body can also be controlled in a circadian manner. Indeed, it has been hypothesized that circadian rhythms in synaptic activity can follow the rhythm of melatonin (Botev et al. 1992; Semm et al. 1981; Stehle et al. 1989). In the present study melatonin attenuated neuronal responses in mouse hippocampal slices. While all our studies were performed during the morning hours, experiments designed to disrupt the normal circadian rhythms in our mice (through manipulation of the light/dark cycle) manifest as a significant reduction in the action's of melatonin. These results suggest that the response of hippocampal neurons to melatonin is correlated with the exposure to light or lack thereof. These preliminary results implicate a circadian control of melatonin actions

in the mouse hippocampus and corroborate our pharmacological results. We demonstrate that the melatonin receptors in the hippocampus are not stable following removal of the hippocampus from the organism. This instability was demonstrated as a decrease in binding and a loss of responsiveness to melatonin in older slices from young mice.

In conclusion, the modulation of hippocampal activity by melatonin may have far reaching consequences on both normal and pathological states in humans. These changes, coupled with the fact that melatonin fluctuates according to a diurnal cycle, implies a daily interaction of melatonin and hippocampal activity. The observation that melatonin can depress hippocampal activity and that this depression can be overcome by a specific pattern of stimulation implies that melatonin may filter information passing through the hippocampus. Because melatonin levels can also be increased dramatically by oral consumption, understanding its actions in the hippocampus at anytime during the circadian cycle becomes important. While this study does not address the exact mechanisms and specific localization of melatonin receptors in the CA1 region of the hippocampus, it does offer information on the final output of neurotransmission from the hippocampus to

the rest of the brain. The significance of these findings while not immediately apparent has the ability to significantly influence our understanding of how the environment and the hippocampus interact.

Appendix A

Table of melatonin ligands

6-Chloromelatonin (N-[2-(6-Chloro-5-methoxyindol-3-yl)ethyl]acetamide)	Potent melatonin agonist (pK_i values are 9.10 pM and 9.77 pM for human recombinant MT_1 and MT_2 receptors respectively). Displays higher affinity for binding to hamster brain membrane and chicken retina than melatonin.
Luzindole (N-Acetyl-2-benzyltryptamine)	Competitive melatonin MT_1/MT_2 antagonist (18-fold selectivity for MT_2 vs. MT_1.
4-P-PDOT (4-Phenyl-2-propionamidotetralin)	Melatonin receptor antagonist, >300-fold selective for the MT_2 vs. MT_1 subtype.
BMNEP (N-[2-[2- (bromoacetoxy)-7methoxynaphthyl]ethyl]-propionamide)	Melatonin ligand, which binds and alkylates to the MT_2 receptor, leading to constitutive receptor activity. Detailed binding characteristics not known.
TMEPI	Charged melatonin ligand, incapable of penetrating the plasma membrane. Detailed binding characteristics not known.

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