Putative Melatonin Receptors in a Human Biological Clock

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In vitro autoradiography with ^{125}I-labeled melatonin was used to examine melatonin binding sites in human hypothalamus. Specific ^{125}I-labeled melatonin binding was localized to the suprachiasmatic nuclei, the site of a putative biological clock, and was not apparent in other hypothalamic regions. Specific ^{125}I-labeled melatonin binding was consistently found in the suprachiasmatic nuclei of hypothalami from adults and fetuses. Densitometric analysis of competition experiments with varying concentrations of melatonin showed monophasic competition curves, with comparable half-maximal inhibition values for the suprachiasmatic nuclei of adults (150 picomolar) and fetuses (110 picomolar). Micromolar concentrations of the melatonin agonist 6-chloromelatonin completely inhibited specific ^{125}I-labeled melatonin binding, whereas the same concentrations of serotonin and norepinephrine caused only a partial reduction in specific binding. The results suggest that putative melatonin receptors are located in a human biological clock.

THE FUNCTION OF MELATONIN IN human physiology is a mystery. Melatonin is a hormone produced rhythmically by the vertebrate pineal gland (*1*). On the basis of studies in rodents (*2*) and nonhuman primates (*3*), the melatonin rhythm in mammals is thought to be generated by a biological (circadian) clock in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The rhythm is entrained (synchronized) to the 24-hour period by the daily light-dark cycle, with hormone levels increased at night (*1–3*). In humans, the melatonin rhythm appears to be regulated similarly and is expressed prominently in blood and cerebrospinal fluid (CSF) (*4*).

Exogenously administered melatonin can entrain circadian rhythms of some species of lizards (*5*), birds (*6*), and mammals (*7*). Melatonin can also alter the entrainment of human circadian rhythms. Melatonin administration can change the phase of the endogenous melatonin rhythm (*8*), alleviate the symptoms of jet lag (*9*), and facilitate reentrainment of some rhythms (*10*). Thus, melatonin may modulate the entrainment process in humans and be useful for treating circadian rhythm disorders.

To better understand how melatonin functions, it is necessary to delineate where in the brain the hormone acts. By using the physiologically active radioiodinated ligand of melatonin, ^{125}I-labeled melatonin (*11*), reasearchers can study putative sites of melatonin action in humans. This ligand is a potent melatonin agonist both in vivo (*12*) and in vitro (*13*). In vitro autoradiography with ^{125}I-labeled melatonin has revealed a discrete distribution of melatonin binding sites in the brains of adult and fetal rodents (*12, 14*); the SCN of the rodent hypothalamus are consistently labeled. We report autoradiographic localization of specific ^{125}I-labeled melatonin binding to the SCN of adult and fetal humans in postmortem material.

Binding of ^{125}I-labeled melatonin was examined in hypothalamic specimens obtained 9 to 24 hours postmortem [14.8 ± 1.9 hours (mean ± SEM)] from brains of 11 adult human subjects (two female, nine male) (*15*). Slide-mounted hypothalamic sections were incubated with ^{125}I-labeled melatonin (*16*) either alone or in the presence of unlabeled melatonin (*17*). Autoradiographs were generated by apposition of the processed sections to LKB Ultrofilm for 1 to 3 weeks. Sections were stained with cresyl violet or toluidine blue and examined by light microscopy to confirm the localization of anatomical structures. Quantitative densitometry of autoradiographs was performed with a digitized image analysis system (*18*).

To locate ^{125}I-labeled melatonin binding sites in human hypothalamus, serial coronal sections (*20* μm) from two specimens were examined at 200-μm intervals throughout the hypothalamus (from the rostral boundary of the anterior hypothalamus to the mammillary bodies). Visual assessment of the autoradiographs revealed specific ^{125}I-labeled melatonin binding (displaced by 1 μ*M* melatonin) only in a bilateral structure in the suprachiasmatic region (*19*). Examination of the Nissl-stained sections by one of us (E.G.S.) unaware of the autoradiographic results led to histological identification of the SCN (Fig. 1A, upper panel), which were then found to correspond to the sites of specific binding (Fig. 1A, middle panel). A low level of nonspecific binding (not displaced by 1 μ*M* melatonin) was observed throughout the hypothalamus; that is, silhouettes of the sections were visible above film background (Fig. 1A, lower panel).

Next, the anterior hypothalamic regions of the nine other brains were examined at 200-μm intervals. Visual assessment of these autoradiographs revealed specific binding over the SCN in all but one brain (*20*). Visual assessment of the SCN image on the autoradiographs and independent assessment of the SCN region by light microscopy of the Nissl-stained sections showed that specific binding was consistently localized to the caudal two-thirds of each nucleus; there was no apparent difference in binding throughout the dorsal-ventral or medial-lateral extent of the SCN. Quantitative densitometry of autoradiographs showed that SCN binding was similar among the different hypothalami and did not vary systematically according to the age of the individual or to postmortem interval.

The binding of ^{125}I-labeled melatonin was also examined at 40-μm intervals in serial coronal sections (20 μm) of hypothalami obtained from four fetuses (two male, two female; 18 to 19 weeks of gestation) within 12 hours after therapeutic termination of pregnancy. Specific binding was clearly evident over the SCN in all fetal hypothalami (Fig. 2). The higher optical density of the autoradiographic image over fetal SCN relative to comparably exposed autoradiographs from adults appeared to be due to the greater cellular density of the SCN during fetal development (a comparison is shown in Figs. 1A and 2A, upper panels). A low level of specific binding was also found in the surrounding fetal hypothalamus (hypothalamic silhouettes above film background were eliminated with 1 μ*M* melatonin).

After defining the autoradiographic boundaries of the SCN in adult and fetal hypothalami, intervening sections were used to more fully characterize ^{125}I-labeled melatonin binding in SCN. Competition experiments with varying concentrations of melatonin (10^{-11} to 10^{-7} *M*) were performed in two adult and two fetal brains. Image analysis of the resultant autoradiographs revealed monophasic competition curves with mean half-maximal inhibition (IC_{50}) values for melatonin of 150 and 110 p*M* for the adult and fetal SCN, respectively (Figs. 1B and

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2B). Next, we tested three compounds for their ability to inhibit specific ^{125}I-labeled melatonin binding in six adult brains. 6-Chloromelatonin, a potent melatonin agonist in vivo (*21*) and in vitro (*13*), completely displaced specific ^{125}I-labeled melatonin binding at 1 μM (Fig. 1C). Serotonin and norepinephrine, however, caused only a 33 and 21% reduction, respectively, in specific SCN binding at 1 μM concentrations (Fig. 1C); a comparable inhibition of specific binding occurred at much lower concentrations of melatonin (that is, <100 pM) (Fig. 1B). A similar pattern of binding inhibition by these three compounds was also observed in two fetal brains.

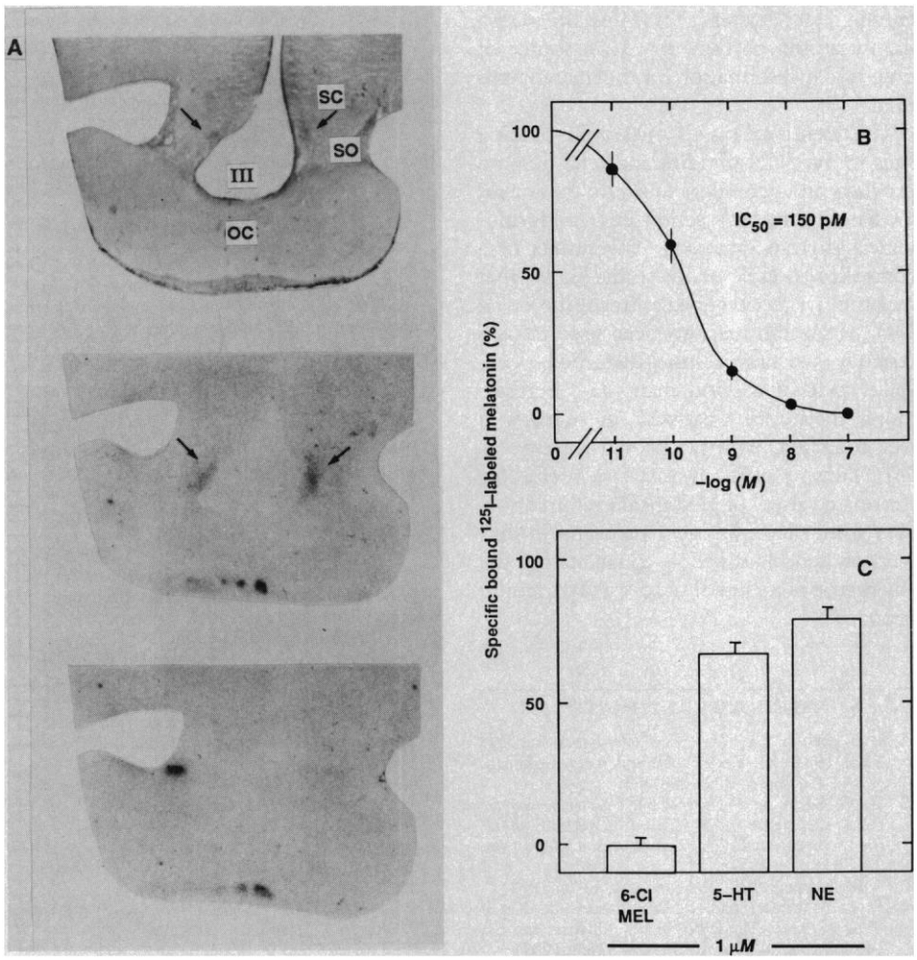

Fig. 1. Anatomic specificity and pharmacologic characteristics of ^{125}I-labeled melatonin binding in the SCN of adult humans. (**A**) Localization of specific ^{125}I-labeled melatonin binding to SCN by in vitro autoradiography. A 60-μm section of hypothalamus (not used for autoradiography) was stained with cresyl violet to show location of the SCN (photomicrograph, upper panel). On an adjacent 20-μm section, ^{125}I-labeled melatonin binding over the SCN was apparent as dark densities (arrows) (autoradiograph, middle panel). SCN binding was eliminated when melatonin was added to the incubation solutions (autoradiograph, lower panel). Autoradiographic exposure, 11 days, Magnification, ×2.9. Abbreviations: OC, optic chiasm; SC, suprachiasmatic nucleus; and SO, supraoptic nucleus. (**B**) Competition for binding between ^{125}I-labeled melatonin and melatonin in adult SCN. Slide-mounted hypothalamic sections were incubated with ^{125}I-labeled melatonin (100 pM) either alone or in the presence of increasing concentrations of melatonin (10^{-11} to $10^{-7}M$). The incubation baths contained 0.1% ascorbic acid and 0.025% ethanol (a necessary diluent for melatonin). Three to six sets of serial sections were analyzed with each series of concentrations throughout the rostral-caudal extent of the SCN for each brain. Nonspecific binding was 14% of total binding. Values were corrected to 100% of total specific binding (^{125}I-labeled melatonin alone) for each brain to allow comparison between brains. Because only two brains were examined, the results were combined. The values depicted are mean ± SEM; IC_{50} was 150 ± 23 pM, as determined by computer with a nonlinear regression program (*23*). (**C**) Inhibition of ^{125}I-labeled melatonin binding by 1 μM concentrations of 6-chloromelatonin (6-Cl MEL), serotonin (5-HT), or (±)norepinephrine (NE). Slide-mounted hypothalamic sections were incubated with ^{125}I-labeled melatonin (100 pM) either alone or in the presence of 1 μM concentrations of one of the three drugs. Just before incubations, 6-chloromelatonin (Eli Lilly Laboratories) was dissolved in ethanol, and serotonin and norepinephrine were dissolved in deionized water; drugs were then diluted with buffer to the appropriate concentration. Both incubation solutions contained 0.1% ascorbic acid and 0.025% ethanol. Serial sections were examined such that drug-incubated sections were always bracketed by sections incubated only in ^{125}I-labeled melatonin for each of the six brains examined. Nonspecific binding was 15% of total bound. Serotonin and norepinephrine inhibited specific ^{125}I-labeled melatonin binding ($P < 0.05$, Mann-Whitney U test).

Our results show that the human SCN contain melatonin binding sites. The anatomic specificity and pharmacologic characteristics of these binding sites suggest that they may be functioning melatonin receptors. Specific binding is localized to the SCN and is not apparent in other hypothalamic regions; such a restricted pattern of binding does not occur for other known neurochemical or hormonal receptors or receptor subtypes (*22*). The pattern of binding inhibition by 6-chloromelatonin, serotonin, and norepinephrine is also consistent with the characteristics of a putative melatonin receptor (*13*).

Specific ^{125}I-labeled melatonin binding in SCN is of high affinity. Melatonin competition curves are monophasic with similar IC_{50} values in the adult and fetus. A comparable melatonin competition curve and a dissociation constant of 10 pM have been reported for ^{125}I-labeled melatonin binding to membranes from rat hypothalami (*14*). Thus, at the ^{125}I-labeled melatonin concentrations that we used (about 100 pM), the calculated IC_{50} for melatonin probably underestimates the actual affinity of melatonin for its receptor in the human SCN (*23*). An affinity in the picomolar range is well within the physiological range of melatonin concentrations normally found at night in the blood and CSF of humans (*4*).

Evidence suggests that the SCN are the site of a biological clock in humans, as they are in other mammals. The human SCN are neurochemically similar to the SCN of rodents and nonhuman primates and contain the same topographical distribution of various neuropeptides (*24*). Furthermore, light is a potent entraining stimulus for human circadian rhythms (*25*) and a direct tract from retina to SCN has been demonstrated in human postmortem tissue (*26*). In addition, tumors of the anterior hypothalamus that presumably damage or destroy the SCN and that are associated with the disruption of various daily rhythms have been described in humans (*27*).

An entraining effect of melatonin on mammalian circadian rhythms that is mediated by the SCN has been shown in rats (*28*), a species in which melatonin binding sites are consistently observed in the SCN (*14*). Our finding of melatonin binding sites in the human SCN provides evidence that the reported effects of melatonin on human circadian rhythms (*29*) are also mediated by direct action on a hypothalamic biological clock. Thus, one function of melatonin in adult humans may be to modulate the entrainment process (*30*).

The finding of melatonin binding sites in

the SCN of the human fetus suggests that melatonin is an important source of entraining information for the biological clock during fetal life. In rodents a circadian clock oscillates in the fetal SCN before the retinohypothalamic pathway has innervated the SCN (*31*). During this early stage of development, the maternal circadian system entrains the timing of the fetal SCN to the prevailing light-dark cycle (*31*). Recent studies show that melatonin administration to pregnant hamsters with SCN lesions (rendering them incapable of communicating time-of-day information to the fetus) can entrain fetal hamsters (*32*). Thus, the maternal melatonin rhythm may be a source of time-of-day information for the human fetus (*33*).

The identification of melatonin binding sites in the SCN of adult and fetal humans provides an anatomical substrate that could mediate melatonin's action in treating biological rhythm disorders. The results of a controlled double-blind study show that melatonin is an effective treatment for jet lag (*34*). Melatonin has also been used to successfully synchronize the disturbed sleep-wake cycle of a blind man (*35*). Furthermore, melatonin treatment in humans is well tolerated, with no known toxicity (*8, 36*). Thus, melatonin may be useful for treating a variety of biological rhythm disorders, from alleviating sleep problems in shift workers and blind people to facilitating the emergence of a diurnal sleep-wake pattern in neonates.

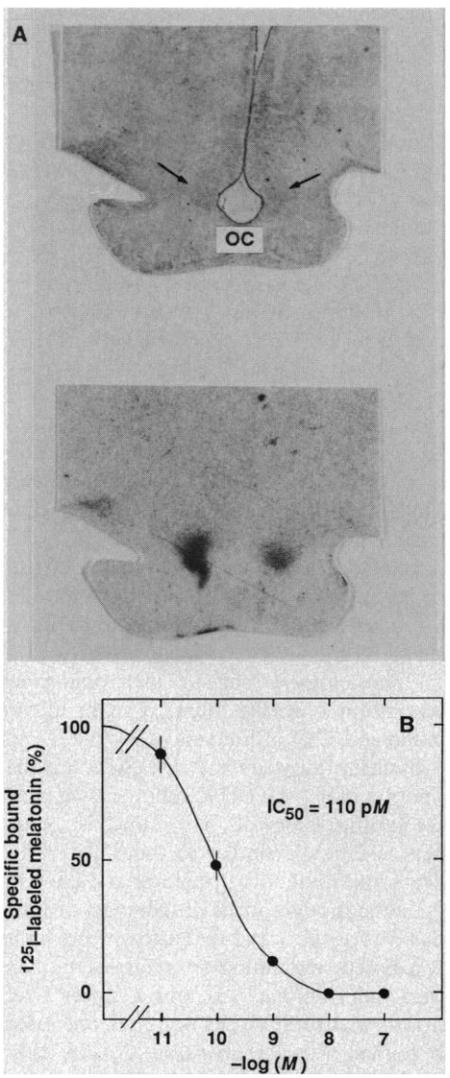

Fig. 2 Specific ^{125}I-labeled melatonin binding in the SCN of the human fetus. (**A**) Localization of specific ^{125}I-labeled melatonin binding to SCN by in vitro autoradiography. The section used to generate the autoradiograph was stained to show the location of the SCN (photomicrograph, upper panel). Specific ^{125}I-labeled melatonin binding was apparent in the autoradiograph as dark densities over the SCN (lower panel). Autoradiographic exposure, 12 days. Magnification, ×5.5. (**B**) Competition for binding between ^{125}I-labeled melatonin and melatonin in fetal SCN. Slide-mounted sections were incubated as described in Fig. 1B. Four to five sets of serial sections were analyzed with each series of melatonin concentratins throughout the rostral-caudal extent of the SCN for each of two brains. Nonspecific binding was equal to film background. Values were corrected to 100% of total specific binding (^{125}I-labeled melatonin alone) for each brain. The results from both brains were combined. The values are mean ± SEM; IC_{50} was 110 ± 43 p*M*.

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15. Hypothalamic specimens were selected from subjects at routine autopsy from Massachusetts General Hospital, Tufts New England Medical Center, and Brigham and Women's Hospital, Boston. The mean age at death was 66.5 ± 4.0 years with a range from 47 to 85 years. Two individuals died with central nervous system disease, one died with a history of dementia, and the other with staphylococcal meningitis. The hypothalami were blocked (3 cm by 3 cm by 2.5 cm) and frozen in 2-methylbutane (−30°C), and serial coronal sections of 20 μm were cut in a cryostat. Sections were thaw-mounted on gel-coated slides, air-dried, and stored at −70°C.
16. ^{125}I-labeled melatonin was prepared by a modification of the method of Vakkuri *et al.* (*11*). The reaction product was purified (>90%) by high-performance liquid chromatography (HPLC) with a C-18 column and a solvent system of isopropanol and water. The column was eluted for 30 min at 1 ml/min with a linear gradient from 10 to 40% isopropanol. Specific activity of HPLC-purified ^{125}I-labeled melatonin was about 2000 Ci/mmol.
17. Slide-mounted hypothalamic sections were allowed to thaw and air dry for 10 min, incubated in 0.02*M* phosphate-buffered saline with 0.1% bovine serum albumin for 1 hour, and then incubated in the same buffer containing ^{125}I-labeled melatonin (60 to 100 p*M*) for 1 hour at room temperature. After incubations, slides were washed in ice-cold buffer and dried on a hot plate. For competition slides, unlabeled melatonin (1 μ*M*) was added to both incubation buffers. This autoradiographic procedure has been validated in rat brain by identifying the incubation time, temperature, and wash duration that maximize specific binding (D. R. Weaver, S. A. Rivkees, S. M. Reppert, in preparation).
18. Image analysis was performed with Drexel University Image Processing Center "Brain Software Package" run on an IBM AT computer. Optical density data were obtained from films in which the values were nonsaturated and within the linear range of the film, as determined by examining the densities of radioactive standards. For calculations, optical density values were converted to radioactivity levels.
19. Thin-layer chromatography of the radioactivity in the incubation bath and bound to tissue showed that ^{125}I-labeled melatonin was stable throughout the autoradiographic procedure.
20. The specimen without specific SCN binding was from a 56-year-old male subject who died of hepatorenal syndrome; there was no apparent pre- or postmortem explanation (for example, drugs, clinical course, or postmortem interval) for the lack of specific ^{125}I-labeled melatonin binding in the hypothalamus of this individual.
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28. Although pinealectomy has little effect on the period or phase of free-running circadian rhythms in this species, exogenously administered melatonin can clearly entrain free-running locomotor activity rhythms in constant light (7). Two lines of evidence suggest that the entraining effects of melatonin in rats are mediated by the SCN. First, the entraining effect of melatonin requires intact SCN [V. M. Cassone, M. J. Chesworth, S. M. Armstrong, *Physiol. Behav.* **36**, 1111 (1986)]. Second, melatonin alters SCN metabolic activity when administered at the proper circadian time for entrainment [V. M. Cassone, M. H. Roberts, R. Y. Moore, *Neurosci. Lett.* **81**, 29 (1987)].

29. In a limited study, melatonin administration to individuals with free-running circadian rhythms who were subjected to fractional desynchronization reinforced the entraining strength of a low intensity light-dark cycle for the circadian rhythm of self-rated fatigue or alertness (*8*). Also, administration of melatonin to volunteers can phase shift the endogenous melatonin rhythm in some individuals (*8*), presumably by an action on a biological clock.
30. A function of melatonin in mammals that breed seasonally is regulation of the dramatic changes in reproduction that occur throughout the course of the year [B. D. Goldman and J. M. Darrow, *Neuroendocrinology* **73**, 386 (1983); F. J. Karsch *et al.*, *Recent Prog. Horm. Res.* **40**, 185 (1984); L. Tamarkin, C. J. Baird, O. F. X. Almeida, *Science* **227**, 714 (1985)]. Melatonin has been suggested as having a role in the initiation or timing of puberty in humans, although firm evidence of seasonal reproductive rhythms or an effect of melatonin on gonadotropin secretion in humans is lacking (*4*) [F. Waldhauser *et al.*, *Neuroendocrinology* **46**, 125 (1987)]. It is possible that melatonin binding sites are expressed in other hypothalamic nuclei at specific development stages (for example, puberty) or at extrahypothalamic sites not examined in the present study.
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34. Melatonin treatment alleviated the symptoms of jet lag after eastward travel over eight time zones in all subjects examined (*9, 10*), although only a small number of individuals was studied. The melatonin-treated individuals had decreased sleep latency and better sleep quality and were more alert than placebo-treated subjects (*10*). Melatonin also facilitated reentrainment of the endogenous melatonin and cortisol rhythms, implicating an effect of the hormone on a biological clock (*10*).
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Postsynaptic Calcium Is Sufficient for Potentiation of Hippocampal Synaptic Transmission

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Brief repetitive activation of excitatory synapses in the hippocampus leads to an increase in synaptic strength that lasts for many hours. This long-term potentiation (LTP) of synaptic transmission is the most compelling cellular model in the vertebrate brain for learning and memory. The critical role of postsynaptic calcium in triggering LTP has been directly examined using three types of experiment. First, nitr-5, a photolabile nitrobenzhydrol tetracarboxylate calcium chelator, which releases calcium in response to ultraviolet light, was used. Photolysis of nitr-5 injected into hippocampal CA1 pyramidal cells resulted in a large enhancement of synaptic transmission. Second, in agreement with previous results, buffering intracellular calcium at low concentrations blocked LTP. Third, depolarization of the postsynaptic membrane so that calcium entry is suppressed prevented LTP. Taken together, these results demonstrate that an increase in postsynaptic calcium is necessary to induce LTP and sufficient to potentiate synaptic transmission.

INTRACELLULAR FREE Ca^{2+} ACTIVATES an array of cellular processes, and thus Ca^{2+} functions as a critical and ubiquitous second messenger. Increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]$) occur either by activation of voltage-dependent Ca^{2+} channels (*1*) or by release from intracellular stores (*2*). In neurons, Ca^{2+} passes through a special type of ligand-gated ion channel linked to the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor (*3, 4*). NMDA receptors are found in high concentrations in a variety of brain regions (*5*) and appear to mediate the initiation of several forms of synaptic plasticity (*6*). The induction of LTP in the hippocampus, the most extensively studied model for memory in the vertebrate brain, requires NMDA receptor activation (*7*). LTP is an enhancement of synaptic transmission lasting many hours in response to brief repetitive activation of excitatory synapses. It has been suggested that Ca^{2+} entering through the NMDA channel acts as a second messenger to trigger LTP (*8*). Despite the fundamental importance of this step to our understanding of LTP, and perhaps other forms of synaptic plasticity, the evidence linking Ca^{2+} to LTP is remarkably limited (*9, 10*). To address this issue directly we have performed three types of experiment in hippocampal pyramidal cells, two of which have relied on nitr-5, a photolabile nitrobenzhydrol tetracarboxylate Ca^{2+} chelator (*11*).

We used standard procedures to prepare and maintain hippocampal slices (*12*). Microelectrodes were filled with nitr-5 (100 m*M*) loaded with Ca^{2+} (50 m*M*) dissolved in 1*M* CsCl to block K^+ conductances that might be activated upon raising intracellular $[Ca^{2+}]$. We impaled CA1 pyramidal cells in

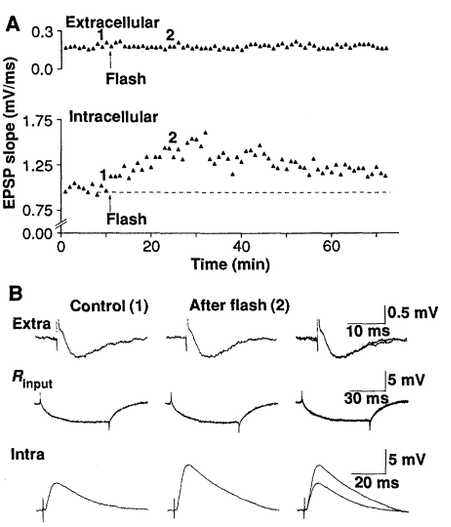

Fig. 1. Photolysis of intracellularly injected nitr-5 enhances synaptic transmission. (**A**) Graphs of the slope of the extracellular EPSP recorded in a stratum radiatum (upper) and the slope of the simultaneously recorded intracellular EPSP (lower) (*12*). Each point represents the average of six slope measurements. The cell was penetrated 15 min before time 0 on the graph. At the time marked by the arrow (flash) the slice was exposed to ultraviolet light for 25 s. (**B**) Sample records obtained at the times indicated by the numbers 1 and 2 in (A) (records are the average of six sweeps). (Upper records) The extracellularly recorded EPSP (Extra). (Middle records) The response to a constant current hyperpolarizing pulse (0.11 nA) used to monitor the input resistance (R_{input}). (Bottom records) The intracellularly recorded EPSP (Intra). The right-hand column shows superimposed records before and after the flash.

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