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Short communication

Localization of 2-[^{125}I]iodomelatonin binding sites in visual areas of the turtle brain

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Abstract

The hormone melatonin is believed to play an important role in the regulation of both circadian and circannual rhythms. In mammalian vertebrates melatonin receptors are discretely localized, with broader distributions reported in avians and reptiles. To examine the sites at which melatonin may act in the turtle brain, 2-[^{125}I]iodomelatonin binding sites were assessed using quantitative autoradiography. Specific binding sites were primarily restricted to forebrain structures with a wide distribution in visual recipient areas. The distribution of melatonin sensitive sites within the turtle visual system suggests that the ability to transduce received photoperiodic signals in the reptilian brain is broadly distributed within the central nervous system.

Keywords: Melatonin; Receptor autoradiography; (Reptile); 2-[^{125}I]Iodomelatonin; Visual system

1. Introduction

The hormone melatonin is secreted rhythmically by the vertebrate pineal gland, and is believed to play an important role in the regulation of circadian and circannual rhythms. Under normal conditions, these cycles are entrained by environmental cues such as photoperiod and temperature. Light levels critical to the entrainment of many such rhythms are transduced either through the lateral eyes or via extraretinal photoreceptors. The central nervous system must ultimately integrate environmental cues with internally generated rhythms to produce appropriately timed periodic behaviors. Melatonin is thought to play a major role in the synchronization of these periodic changes in light level and the timing of a variety of physiological and behavioral processes (Morgan et al., 1994). The mechanism by which this hormone effects the transduction of photic information to changes in metabolism, activity cycles, reproductive cycles and other periodic behaviors remains largely unknown, but there has been a significant effort made to determine those brain sites at which melatonin might act. Knowledge of the central nervous system distribution of melatonin sensitive receptors provides essential data concerning brain areas in which this transduction process might occur.

In mammals, melatonin receptors are largely restricted to the pineal gland and hypothalamic regions (Morgan et al., 1994). Non-mammalian vertebrates exhibit a wider receptor distribution, with a strong representation in visual areas of the brain in addition to the pineal gland and hypothalamic sites (Siuciak et al., 1991; Wiechmann and Wirsig-Wiechmann, 1992). Thus, non-mammalian vertebrates possess multiple brain sites at which cellular interactions between photoperiod information and rhythmic oscillations in melatonin levels could occur.

Becuase of its unique resistance to anoxic damage, the brain of freshwater diving turtles provides an excellent model in which to examine the cellular mechanisms by which the central nervous system integrates photic information with hormonal (melatonin) levels to ultimately produce periodic behaviors. The turtle cen-

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tral nervous sytem can be maintained in vitro for several days (Hounsgaard and Nicholson, 1990), during which time the responses of discrete brain areas to hormonal, synaptic or photic stimuli can be assessed. For that reason, 2-[^{125}I]iodomelatonin autoradiography was used to localize melatonin receptors in the turtle brain and identify those areas which may serve as substrates for the integration of photic and hormonal information.

2. Materials and methods

All experimental procedures were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals as approved by the Pennsylvania State University College of Medicine Animal Care and Use Review Committee. Experiments were performed on five adult specimens of the freshwater turtle *Chrysemys picta* (carapace length 15–36 cm). Animals were anesthetized by hypothermia (cooled 1–2 h at 5°C until torpid) and decapitated between 09:00 and 10:00 h. The cranium was opened, the dura reflected, and the neuraxis sectioned at the level of the first spinal nerve caudally. The cranial nerves were cut at their point of exit from the skull, and the tissue was gently removed from the cranium. The tissue block was positioned on partially solidified OCT (Tissue Tek, Sigma), rapidly frozen by immersion in 2-methylbutane and subsequently stored at −70°C for less than one week. Serial coronal brain sections (20 μm) were cut on a cryostat (−15°C) and thaw mounted onto gelatin coated slides.

Autoradiography was performed as previously described (Siuciak et al., 1991). Briefly, three alternate sets of sections were generated for the determination of total binding, non-specific binding and histological staining. After air drying for 10 min, slides were stored at −70°C for no more than one week. Slide mounted sections were removed from the freezer and allowed to air dry for 15 min, preincubated in 150 mM Tris-HCl buffer (pH 7.4, 22°C) for 1 h to remove endogenous ligand and then incubated with 100 pM 2-[^{125}I]-iodomelatonin in Tris HCl buffer with (non-specific binding) or without (total binding) 3 μM melatonin for 1 h at room temperature. Slides were rinsed in ice-cold Tris-HCl buffer (3 times for 5 min each) followed by a rapid rinse in ice-cold distilled water to remove buffer. Labeled sections were apposed to Kodak SB5 X-ray film for 14 days. The film was developed using Kodak D19 developer (4°C) for 3 min and fixed for 3 min at the same temperature. The final set of sections was stained with cresyl violet and used to identify labeled areas based on cytoarchitectural landmarks.

Binding densities were quantified from autoradiograms using a charge-coupled camera (NEC TI-324A) and a public domain image processing and analysis program (NIH Image, Version 1.55). Commercially available ^{14}C standards were calibrated for use with ^{125}I binding standards (ARC). For each brain region, mean density readings were calculated from all sections in which that brain area appeared and converted into the amount of radioligand bound using the computed standard reference curve. The protein content in tissue sections was determined from the tissue equivalents supplied by the manufacturer of the ^{14}C standards (ARC). The value of nonspecific binding (binding of 2-[^{125}I]iodomelatonin not displaced by 3 μM melatonin) was subtracted from that of the total binding to produce the value for specific binding reported for each region (Fig. 2).

3. Results

Brain areas demonstrating 2-[^{125}I]iodomelatonin binding are documented in Fig. 2. The greatest extent of 2-[^{125}I]iodomelatonin binding was found within specific regions associated with the visual system. Identical binding patterns were found within all animals examined and representative autoradiograms through some of these visual areas are shown in Fig. 1, in which both specific and total binding levels are demonstrated. Several parallel visual pathways exhibited high levels of binding, and each will be described as a system.

The pineal gland was the most heavily labeled central nervous system structure (Fig. 1B). In all cases, the binding was localized, and did not encompass the entire extent of the gland at any one level. The only hypothalamic nucleus labeled was the supraoptic nucleus (SON, Fig. 1A) which is located immediately dorsal to the optic tracts. The ventral decussation of the supraoptic tract, located at the same level as the suproptic nucleus and slightly rostral to it (DST, Fig. 1A) was also labeled, with a greater binding density than seen in the nucleus (Fig. 2).

Label was found in the habenular nucleus (H, Fig. 1B) and to a greater degree in its efferent target, the interpeduncular nucleus (IP, Figs. 1C and 2). The only other non-visual structures seen to bind 2-[^{125}I]-iodomelatonin were the striatum, represented by high levels in the globus pallidus (GP; Fig. 1A) and lower levels in the paleostriatum augmentatum (PA; Fig. 1A); and the dorsolateral anterior thalamic nucleus (DLA, Fig. 1B).

Specific 2-[^{125}I]iodomelatonin binding sites were distributed throughout the turtle brain with high levels of binding seen in the optic tectum (TO, TI, Fig. 1C). This is a major visual receiving area homologous to the mammalian superior colliculus. The optic tectum sends a projection forward to the nucleus rotundus (ROT, Fig. 1B) of the thalamus via the tectothalamic tract

(TT; Fig. 1B, C). All of these structures showed high levels of binding (Fig. 1B, C). The nucleus rotundus, in turn, projects visual information to the dorsal part of the anterior dorsal ventricular ridge (Fig. 1A). This is a specific subdivision of a multisensory integrating area of reptile and avian brain (Ulinski, 1983) which is known to send projections to the striatum and, via

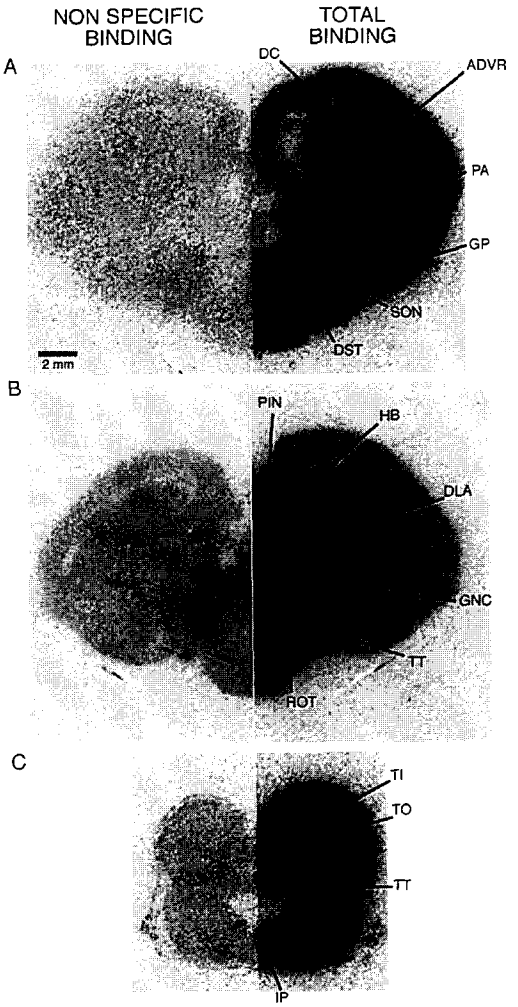

Fig. 1. Autoradiograms of 2-[^{125}I]iodomelatonin binding at three selected coronal levels of the turtle brain. Total binding is shown on the right side and was generally symmetrical (but see text for asymmetries in distribution of label for pineal gland and habenular nucleus). Non-specific binding levels are illustrated on the left side. Binding was focused in telencephalic (A) and diencephalic (B) brain regions. Visual recipient areas also showed strong receptor binding, with the optic tectum exhibiting a pattern of binding which differed between tectal layers (C). No specific binding sites were located in the olfactory bulbs rostrally or the medulla caudally. DC, dorsal cortex; ADVR, anterior dorsal ventricular ridge; PA, paleostriatum augmentatum; GP, globus pallidus; SON, suproptic nucleus of the hypothalamus; DST, ventral decussation of the supraoptic tract; PIN, pineal gland; HAB, habenular nucleus; DLA, dorsolateral anterior nucleus of the thalamus; GNC, geniculate nuclear complex of the thalamus; TT, tectothalamic tract; ROT, nucleus rotundus of the thalamus; TI, optic tectum–internal layer; TO, optic tectum–external layer; IP, interpeduncular nucleus.

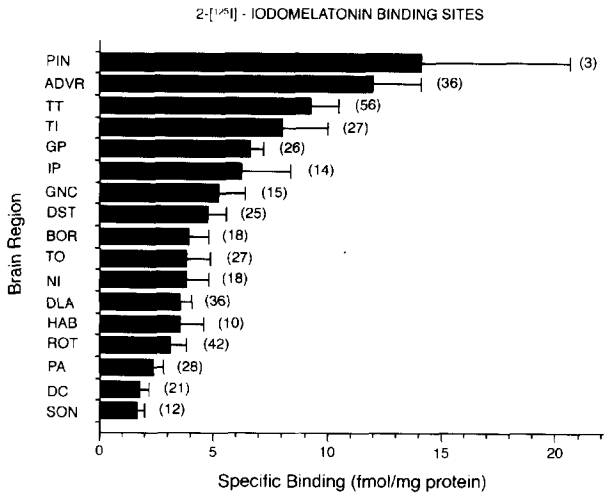

Fig. 2. Bar graph of specific 2-[^{125}I]iodomelatonin binding in turtle brain illustrating relative rank order of binding levels in various regions. Values illustrate mean ± S.E.M. for n independent determinations (shown in parentheses). PIN, pineal gland; ADVR, anterior dorsal ventricular ridge; TT, tectothalamic tract; TI, optic tectum–internal layer; GP, globus pallidus; IP, interpeduncular nucleus; GNC, geniculate nuclear complex of the thalamus; DST, ventral decussation of the supraoptic tract; BOR, basal optic root; TO, optic tectum–external layer; NI, nucleus isthmi; DLA, dorsolateral anterior nucleus of the thalamus; HAB, habenular nucleus; ROT, nucleus rotundus of the thalamus; PA, paleostriatum augmentatum; DC, dorsal cortex; SON, suproptic nucleus of the hypothalamus.

pretectal nuclei, to feed back to optic tectum (Ulinski et al., 1992).

The optic tectum also has reciprocal connections to a caudal mesencephalic nucleus, the nucleus isthmi. This nucleus is the most caudal to show any 2-[^{125}I]iodomelatonin binding above background levels. Despite known projections from the optic tectum to the medulla, no label was found in reticular or raphe nuclei, nor did any rhombencephalic areas exhibit above background levels of binding.

A second thalamic retinal target is the lateral geniculate nucleus. There are relatively high levels of binding in this area (Fig. 1B) and in its principal target, the dorsal (visual) cortex (Fig. 1A).

Finally, direct retinal information reaches the central nervous system through the accessory optic system. Retinal efferents travel in the basal optic root to synapse on the nucleus of that tract at the meso-diencephalic border. Both the nucleus and its tract exhibit 2-[^{125}I]iodomelatonin binding. It has been suggested that this tract transmits information to the pineal gland via a multisynaptic pathway (Moore, 1969).

4. Discussion

The pineal complex evolves from a directly photoreceptive organ to an exclusively secretory one (Quay,

1979) which receives photic information indirectly (Moore, 1969). In all vertebrates studied this complex is involved in the regulation of metabolic and behavioral rhythms which are in some manner driven by both daily and annual changes in light/dark cycles (Morgan et al., 1994; Underwood, 1990). In both reptiles and birds light has been shown to affect the rhythm of melatonin secretion (Underwood, 1990; Vivien-Roels et al., 1988), while melatonin has been shown to play a role in circadian and circannual thermoregulation (Vivien-Roels et al., 1988). The supraoptic nucleus, which exhibits a low number of 2-[^{125}I]iodomelatonin binding sites (Fig. 2), has been reported to receive retinal input in the side-necked turtle (Knapp and Kang, 1968). The presence of 2-[^{125}I]iodomelatonin binding sites in the supraoptic nucleus is consistent with the hypothesis that this area serves to integrate photic and melatonin signals in addition to its more classic role in fluid regulation.

Like birds, the turtle relies primarily upon visually perceived light levels to entrain melatonin secretory rhythms. The maintenance of this oscillatory secretory rhythm may depend upon feedback of the hormone on visual centers which provide the driving input to the pineal gland via multiple pathways. In poikilotherm vertebrates, both temperature and photoperiod play a role in the regulation of patterns of melatonin secretion. The precise role played by temperature varies among species: in turtle, photoperiod controls the duration of secretory bouts while temperature plays a critical role in the amplitude of each secretory phase (Vivien-Roels et al., 1988).

In addition to the visual areas which transmit information to the central nervous system concerning ambient light levels, several non-visual areas likely to be involved in behavioral responses to rhythmic secretion of melatonin show high binding for the hormone. In thalamus, the only non-visual nucleus to label was the dorsolateral anterior thalamic nucleus. This nucleus receives multimodal inputs, though its principal input is likely to be somatosensory (Martinez-Garcia and Lorente, 1990). The projections of thalamic nuclear groups have been studied in emid turtles (Balaban and Ulinski, 1981) and found to project to the forebrain, anterior dorsal ventricular ridge, hypothalamus and striatum. Via its striatal projection the dorsolateral anterior thalamic nucleus may be one pathway in a control loop designed to respond to circadian and/or circannual changes in ambient temperature. The striatum is involved in the control of both food and water intake, and its destruction severely disrupts circadian locomotor patterns (Sándor et al., 1992).

In the turtle, both the habenular nucleus and one of its target nuclei, the interpeduncular nucleus, show modest levels of 2-[^{125}I]iodomelatonin binding. The habenular system is involved in the control of both autonomic and endocrine functions. Projections from this system access hypothalamus, striatum, and large portions of the reticular formation, including those areas involved in arousal (Herkenham and Nauta, 1979). Hormonal control of effector systems influenced by photoperiod may be giving way to neural control systems in mammalian vertebrates which do not exhibit melatonin sensitive sites in these control centers.

The turtle central nervous system shows a pattern of 2-[^{125}I]iodomelatonin binding which is intermediate between that demonstrated in avians and mammals. In both bird and turtle, the level of binding is high in several forebrain visual areas. The turtle does not exhibit melatonin binding sites in the suprachiasmatic nucleus, the brain area which in mammals functions as a circadian oscillator. Thus, the integration of photic and, at least circadian, periodicity is likely fulfilled by multiple visual pathways which provide information not only about ambient light levels, but also about the external visual world. The precise pathways by which this visual information reaches the pineal gland, and the function of the apparent control loop present between these afferent structures and the secretory pineal remain unknown.

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