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Iodinated melatonin mimics melatonin action and reveals discrete binding sites in fetal brain

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Iodinated melatonin was used to study melatonin sites of action in brain. Iodomelatonin mimicked the effects of melatonin on reproductive development in Djungarian hamster fetuses. ^{125}I-melatonin injected into the dam was recovered from fetal brain. In vitro autoradiographic studies revealed a remarkably discrete distribution of competitive ^{125}I-melatonin-binding sites in the fetal brain, with binding in median eminence/arcuate nucleus area > suprachiasmatic nucleus > pineal gland >> anterior pituitary gland >> preoptic area. ^{125}I-melatonin promises to be a useful tool for understanding the sites and mechanism of action of melatonin.

Melatonin; Circadian rhythm; Photoperiodism; Reproduction; (Hypothalamus, Suprachiasmatic nucleus)

1. INTRODUCTION

The pineal hormone melatonin regulates the dramatic changes in reproductive function that occur in seasonally breeding mammals [1-3]. Melatonin is produced rhythmically, with levels being elevated at night. The melatonin rhythm is generated by a biological clock in the suprachiasmatic nucleus (SCN) and is regulated by the daily light-dark cycle [4]. In several species, the nightly duration of melatonin secretion is a critical parameter in mediating its effects on reproduction [1-3]. Although much is known about how the melatonin rhythm is regulated [4], very little is known about where in the brain melatonin acts to exert its potent biological effects.

We recently discovered in Djungarian hamsters that the pattern of melatonin experienced by the fetus in utero profoundly affects postnatal reproductive development [5,6]. The nightly duration of melatonin produced by the dam's pineal gland communicates day-length information to the fetus. The developing animal compares this prenatal signal with the duration of melatonin experienced postnatally and adjusts the rate of reproductive development accordingly. Because the fetus is clearly sensitive to melatonin late in gestation and the fetal nervous system is less complex than that of the adult, this system is potentially useful for probing sites of melatonin action.

Vakkuri et al. [7,8] recently showed that the indole ring of melatonin can be directly iodinated. Thus, a radioligand (2-^{125}I-melatonin) with high specific activity now exists for investigating melatonin sites of action [9-11]. We report here that iodinated melatonin is a physiologically active ligand, mimicking the effects of melatonin on reproductive development in the hamster fetus. Furthermore, in vitro autoradiographic studies with ^{125}I-melatonin demonstrate a remarkably discrete distribution of putative melatonin-binding sites in the fetal brain.

We dedicate this paper to Dr Lawrence Tamarkin, a friend and colleague, who has been an inspiration for our work on melatonin receptors

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2. EXPERIMENTAL

2.1. *Animals and infusions*

Pinealectomized pregnant Djungarian hamsters (*Phodopus sungorus*) were outfitted with indwelling subcutaneous cannulae for infusions, as in [5]. The infusion apparatus allowed each dam to move about her cage during infusion. Food and water were always available. All animals were housed in 14 h of light per day (lights on at 05:00 h) throughout the prenatal and postnatal periods. At 34 days of age, reproductive development was assessed in the male pups by measuring paired testicular weights.

2.2. *Preparation and purification of iodinated melatonin*

^{127}I-melatonin was prepared by the method of Vakkuri et al. [8]. The reaction product was purified by high-pressure liquid chromatography (HPLC) using a μPorasil column (Waters Associates, Milford, MA) and a solvent system of isooctane/ethyl acetate (1:1, fig.1). The column was eluted for 40 min at 3 ml/min. The elution positions of melatonin and ^{127}I-melatonin were monitored by ultraviolet absorption at 280 nm. The fractions containing ^{127}I-melatonin were pooled and the solvent evaporated under nitrogen. Using this HPLC system, several milligrams of ^{127}I-melatonin were purified.

^{125}I-melatonin was prepared and purified by thin-layer chromatography (TLC) using a modification (Reppert, unpublished) of the method of Vakkuri et al. [7]. The TLC-purified radioactive material was analyzed using the HPLC system described above and shown to be greater than 90% ^{125}I-melatonin; specific activity was about 2000 Ci/mmol.

2.3. *Autoradiographic methods*

Two pregnant dams and their fetuses (average weight 1.5 g) were decapitated within 48 h of birth in mid-afternoon (14:00–16:00 h). Fetal brains were either removed from the skull ($n = 4$) or left in the skull to allow for examination of the pituitary gland ($n = 2$). Tissues were immediately frozen in cooled 2-methylbutane (−20°C), and stored at −70°C until serial coronal sections (20 μm) were cut in a cryostat (−20°C). Atlases of the entire fetal brain were generated by collecting on alternate sets of slides 3 sections for reaction with ^{125}I-melatonin and 3 for competition (see below). Sections were thaw-mounted on gel-coated slides, air-dried (10 min), and stored at −70°C. Slides were thawed for 10 min, preincubated in 0.02 M phosphate-buffered saline with 0.1% bovine serum albumin (PBS/BSA, pH 7.4) for 1 h, and incubated with PBS/BSA containing ^{125}I-melatonin (65–100 pM) for 1 h at room temperature. Following incubation, slides were washed in ice-cold PBS/BSA (15 min), PBS without BSA (15 min), and dried on a hot-plate. For competition slides, the pre-incubation and incubation media also contained 1 μM melatonin (Sigma, St. Louis, MO). Autoradiographs were generated by apposition of sections to LKB Ultrofilm for 1 and 3 weeks. Sections were stained with cresyl violet and examined by light microscopy to confirm the location of anatomical structures.

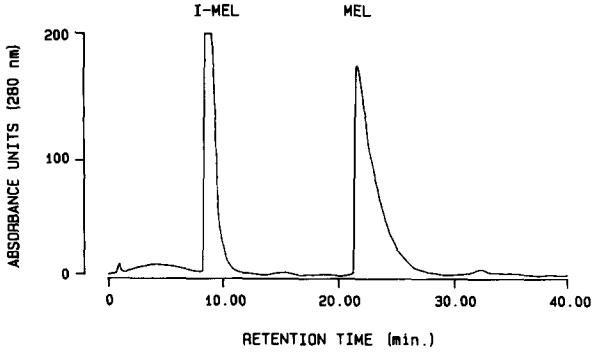

Fig.1. HPLC purification of iodomelatonin. I-Mel, ^{127}I-melatonin; Mel, melatonin.

3. RESULTS

The duration of ^{127}I-melatonin infusion during gestation determined testicular weights in the pups. Compared to either vehicle infusions (not shown) or no infusion (fig.2), infusions of ^{127}I-melatonin (50 ng/0.2 ml isotonic saline containing 0.025% ethanol) into pinealectomized dams over 10 h per night late in gestation caused a consistent, 6-fold increase in testicular weights of the offspring. When the same dose of iodomelatonin was delivered for a comparable number of nights, but over a 6 h duration, testicular size on day 34 was significantly lower than in the 10-h infusion group ($p < 0.01$, Mann-Whitney U-test). While the testes of pups receiving prenatal 6-h iodomelatonin infusions were larger than those of pups receiving pre-

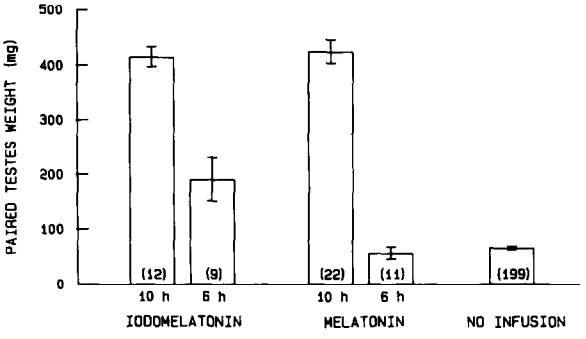

Fig.2. Iodomelatonin mimics melatonin action on reproductive development. Iodomelatonin (50 ng) was infused into pinealectomized dams over either 10 or 6 h per night for the last 4–7 nights of gestation. Paired testicular weights of the offspring were assessed at 34 days of age. Data from similar melatonin infusions during pregnancy (50 ng over either 10 or 6 h per night) are shown for comparison; the melatonin data were derived from [5,6]. Numbers in parentheses denote the number of animals in each group.

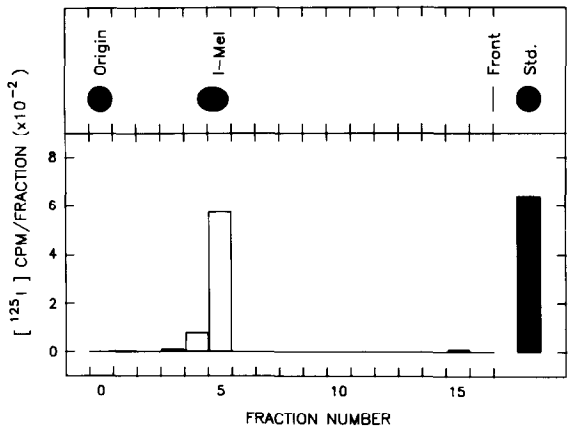

Fig.3. TLC of chloroform-extractable radioactivity from fetal brain after maternal injection of ^{125}I-melatonin. ^{127}I-melatonin (I-Mel, 20 nmol) was added to the chloroform extract. After chromatographic development, I-Mel was detected with short-wavelength UV light for comparison with the elution position of radioactivity. Seventeen 1 × 5 cm parallel sections of the gel were analyzed for radioactivity. Fraction number increases with distance from the origin. Std., recovery standard.

natal 6-h melatonin infusions, there was clearly a duration-dependent effect on testicular development for each compound (see fig.2). The difference between the 6-h infusion groups could be due to a difference in potency or pharmacokinetics between iodomelatonin and melatonin.

Since iodomelatonin mimics melatonin in the ability to influence reproductive development of the offspring when infused into pregnant dams, we next determined whether iodomelatonin reaches the fetus, as does melatonin [12,13]. ^{125}I-melatonin (20 μCi, 10 pmol in 0.2 ml isopropanol vehicle) was injected s.c. into a pregnant dam, and 15 min later she was killed by decapitation. The fetal brains (n = 5) were removed from the skull, pooled and homogenized by sonication in 0.45 M sodium borate buffer, pH 10. Virtually all of the chloroform-extractable radioactivity recovered from the fetal brain homogenate co-migrated with ^{127}I-melatonin standard, as determined by TLC using

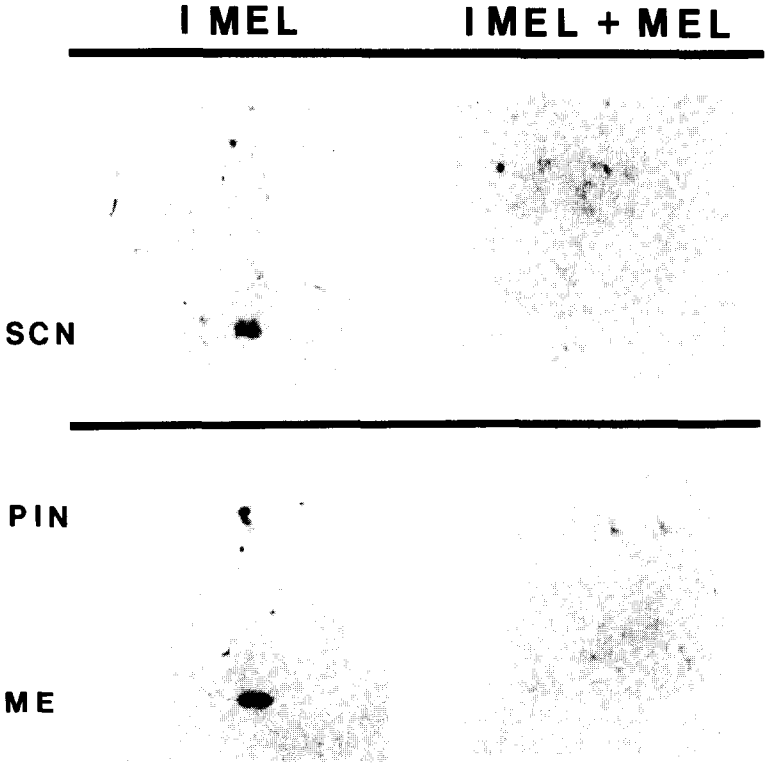

Fig.4. Film autoradiographs of ^{125}I-melatonin binding in fetal hamster brain. ^{125}I-melatonin (IMEL) binding was apparent as dark densities over the suprachiasmatic nucleus (SCN), pineal gland (PIN), and median eminence/arcuate region (ME) (left-hand panels). Binding was not detectable in these structures when melatonin (MEL, 1 μM) was added to the preincubation and incubation solutions (right-hand panels). Autoradiographic exposure for 3 weeks; magnification, 5.6×.

silica gel plates with ethyl acetate as solvent (fig.3). Thus, iodomelatonin injected into the dam reaches the fetus and could potentially interact with melatonin receptors in fetal brain.

We next used in vitro autoradiography to localize ^{125}I-melatonin binding sites in fetal hamster brain (fig.4). After 1 week film exposure, a remarkably discrete distribution of binding was observed. Competitive binding of ^{125}I-melatonin (displaced by 1 μM melatonin) occurred only in the median eminence/arcuate nucleus area, SCN, pineal and anterior pituitary glands. After exposure for 3 weeks, competitive binding significantly above background was also discernible in the preoptic area of the hypothalamus. A low level of noncompetitive binding (not displaced by 1 μM melatonin) was observed throughout the brain at 3 weeks (e.g., silhouettes of the sections were visible above film background, fig.4). Image analysis (Drexel University Brain Software Package) of competitive binding sites within individual fetuses showed a consistent rank order of film optical densities as follows: median eminence/arcuate nucleus area > SCN > pineal >> anterior pituitary >> preoptic area.

4. DISCUSSION

These results demonstrate that iodomelatonin is an effective melatonin agonist in vivo, as the reproductive responses of hamsters treated with either of the two compounds in the late prenatal period are similar. ^{125}I-melatonin is recovered from the fetal brain after systemic injection into the dam, and ^{125}I-melatonin autoradiography reveals discrete binding sites in the fetal brain.

We are confident that the autoradiographic procedure used in these studies identified the major sites of ^{125}I-melatonin binding in the fetal hamster brain. The ability to visualize sections above film background after 3 weeks exposure suggests that any site binding significant levels of ^{125}I-melatonin should have been visible. However, it is conceivable that a different set of reaction conditions could reveal a slightly different distribution of binding sites. It is also possible that the distribution or level of binding varies with time of day. It seems unlikely that we have inadvertently detected binding for some neurotransmitter or neuromodulator other than melatonin (e.g., serotonin or norepinephrine), as the localization of ^{125}I-melatonin-binding sites is much more restricted than that observed for known neurochemical receptors [14].

The pattern of ^{125}I-melatonin binding in adult male Djungarian hamsters is generally similar to that described for the fetus (Weaver and Reppert, unpublished), suggesting that these sites are important targets for melatonin action throughout development. Interestingly, most of the sites we have identified by in vitro autoradiography have previously been implicated in the regulation of reproduction by melatonin in adult rodents. The anterior hypothalamus (preoptic area and SCN) appears to be a critical site of action of melatonin in the regulation of reproduction [15]. Furthermore, it is tempting to speculate that the precise measurement of melatonin duration involves a circadian clock such as is located in the SCN. The median eminence/arcuate area may also represent a site for the effects of melatonin on reproduction; the gonadotropin-releasing hormone content of axons in the median eminence is influenced by photoperiod [16] and melatonin [17]. In addition, gonadotropin secretion may be affected by melatonin acting directly on the anterior pituitary gland [18]. The significance of ^{125}I-melatonin-binding sites in the fetal pineal gland is unknown.

Our results strongly suggest that ^{125}I-melatonin binds to physiologically active binding sites (putative melatonin receptors) in the hamster brain. This ligand promises to advance significantly our understanding of melatonin sites and mechanisms of action in the mammalian central nervous system.

NOTE ADDED IN PROOF

Vaněček et al. [19] recently reported the use of autoradiography to localize ^{125}I-melatonin-binding sites in the suprachiasmatic nucleus and median eminence of adult rat brain.

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