Lesions in suprachiasmatic nuclei simulate effects of pinealectomy on prolactin release in ovariectomized and sulpiride-treated female rats

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Received 2 December 1988; accepted 19 April 1989

Summary. Previous studies indicate that the pineal gland alters prolactin secretion, and it was suggested that at least part of the effect of the pineal hormone melatonin on prolactin release may be mediated by the hypothalamic structures. In this study, pinealectomy and lesions of the suprachiasmatic nuclei were found to alter serum levels of prolactin in the same direction, an effect that was counteracted by daily afternoon melatonin administration. Melatonin, but not other pineal indoles, also prevented sulpiride-induced prolactin secretion in pinealectomized or suprachiasmatic nuclei-lesioned and ovariectomized rats, which suggested that the pineal gland can modulate prolactin secretion by acting through a dopamine mechanism independent of hypothalamic suprachiasmatic structures.
Key words. Pinealectomy; suprachiasmatic nuclei lesions; ovariectomy; melatonin; prolactin; sulpiride-treated rats.

A relationship has been shown to exist between the pineal gland and certain reproductive functions in mammals, indicating that both antigonadotrophic and counter-antigonadotrophic effects of melatonin are present[2]. In relation to prolactin (PRL) secretion, previous studies often yielded conflicting results, suggesting that pinealectomy (Px) decreased serum PRL levels[3] or left them unchanged[4], whereas melatonin produced PRL-releasing and PRL-inhibiting effects when administered to rats[5,6]. Recent data have shown that the pineal gland exerts a potent inhibitory effect on PRL cells, which may involve changes in the hypothalamic secretion of controlling factors and/or changes in pituitary sensitivity to those factors[7]. Daily afternoon melatonin injections produce an inhibitory effect on PRL, which can be blocked by the dopamine antagonist pimozide[8]. There is also evidence that the pineal gland itself contains both PRL-releasing and inhibiting factors[9].

The pineal gland can infuence PRL secretion at hypothalamic levels either by directly modulating the sensitivity or PRL cells to dopamine or thyrotrophin-releasing hormone (TRH), or by altering dopamine or TRH secretion from the hypothalamus[7,10]. Moreover, this is a likely site for melatonin action, since binding data[11] and autoradiographic studies[12] using ^{125}I-melatonin have shown the occurrence of membrane and cytoplasmic melatonin receptor sites in several parts of the hypothalamus. In the present work, we compared the effects of pineal indole treatment on PRL release in suprachiasmatic nuclei-lesioned (SCNx) or Px and ovariectomized (OVx) rats rendered hyperprolactinemic by sulpiride treatment, in an attempt to determine whether this hypothalamic structure is required for the pineal modulation of PRL release.

Female Wistar rats weighing 180–200 g were used. The animals were kept throughout the study on 12 h light-dark cycle (lights on at 07.00), and housed 3–4 per clear plastic cage with food and water ad libitum. Rats were anesthetized with Equi-Thesin and subjected to different surgical manipulations as follows: Px or sham-pinealectomy (SPx), according to the method of Hoffman and Reiter[13]; and OVx or sham-ovariectomy (SOVx), by a

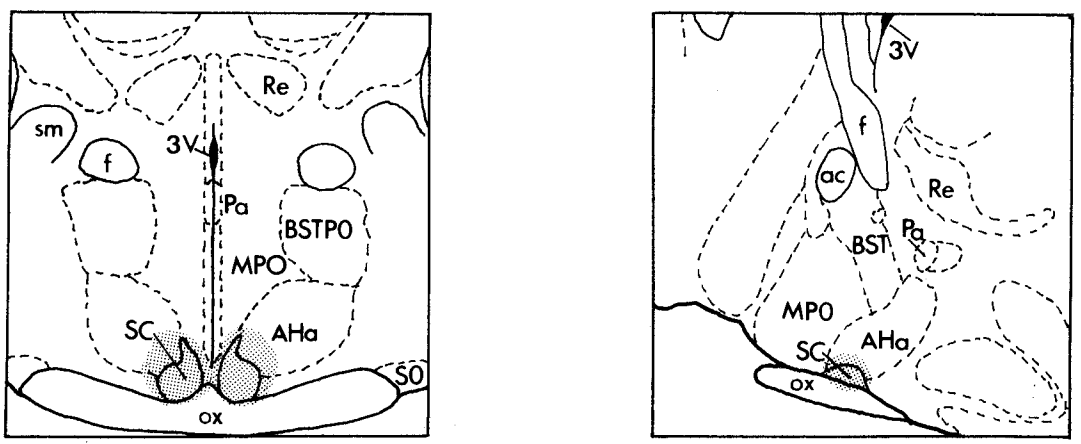

Figure 1. Schematic diagram of coronal (left) and sagittal (right) sections of a rat hypothalamus showing the extent of bilateral, selective lesions (stippled area) of the suprachiasmatic nucleus (SCN). Re, reuniens thalamus nu; sm, stria medularis thalamus; f, fornix; 3V, third ventricle; Pa, paraventricular hypothalamic nu; BSTPO, bed nu stria terminalis, preoptic; MPO, medial preoptic area; SC, suprachiasmatic nu; AHa, anterior hypothalamic area; ox, optic chiasm; SO, supraoptic hypothalamic nu; ac, anterior commisure.

classic lumbar approach. Biltateral extereotaxic lesions of the SCN (fig. 1) were made with a monopolar stainless-steel electrode, insulated except for 0.25 mm at the tip. Stereotaxic coordinates for the lesions were anterior 7.7 mm, lateral 0.2 mm, and 0.9 mm below the dura, according to the atlas of Paxinos and Watson[14]. These coordinates, as well as intensity (3 mA) and duration (7 s) were determined in trial rats. In sham-lesioned animals (SSCN), the electrode was inserted but the lesioning current was not applied. At the end of the experiments, the animals were perfused with 10% formalin and the brains were cut on a cryostat at 50 µm for histological examination of the lesions.

Animals were assigned to one of the following experimental groups: 1) PX, SCNx or OVx rats, studied at 10, 20 and 35 days post-surgery; 2) PX or SCNx rats treated with pineal indoles from day 1 to day 9 post-surgery and killed on the 10th day; and 3) Px or SCNx and OVx rats injected with pineal indoles from day 1 to day 9 post-surgery, rendered hyperprolactinemic with sulpiride and killed on the 10th day. Indoles were dissolved in 95% ethanol, diluted with physiological saline to make work solutions, and daily administered s.c. in the afternoon, at 6 p.m. Sulpiride (10 mg, s.c.) was administered on the 10th day following surgery at 0900 h, i.e. one hour before sacrifice. Serum PRL levels were measured by RIA procedures (NIAMDD) and values were expressed in ng/ml of rat prolactin RP-1. Interassay variance was obtained by including a standard plasma in each of six assays and was found to be 17.0%. Intra-assay variance was 13.6% (n = 5). Analysis of variance and Student's t-test (SPSS/PC+ program) were employed in the statistical analyses. Px in intact rats resulted in a significant increase in PRL at 10, 20 and 35 days post-surgery ($p < 0.001$), an effect which was similar to those recorded in SCNx rats during the same intervals (fig. 2). As expected, OVx significantly depressed PRL levels in all groups ($p < 0.001$, fig. 2). In Px rats, the daily afternoon administration of melatonin during 9 days reversed the effects of pineal removal, in a non dose-dependent manner, as the results were similar at doses of 200, 400 and 800 µg/kg body weight (fig. 3 A). Likewise, melatonin was also capable of reversing the effects of SCNx on PRL levels (fig. 3 A). The afternoon administration of 5-methoxy- and 5-hydroxytryptophol to Px rats during 9 days at doses of 400 µg/kg b. wt had no effect in either Px or SCNx rats (fig. 3 B).

In an attempt to clarify whether these melatonin effects were mediated by a dopamine or dopamine-like mechanism, Px or SCNx and OVx rats were injected with melatonin, 5-methoxy- and 5-hyroxytryptophol at doses of 400 µg/kg b. wt from 1 to 9 days post-surgery, and treated with a single dose of sulpiride on day 10 post-surgery. Figure 4 shows that Px (left) or SCNx (right) performed in OVx rats partially counteracted the effects of OVx alone on PRL levels. Melatonin administration in Px

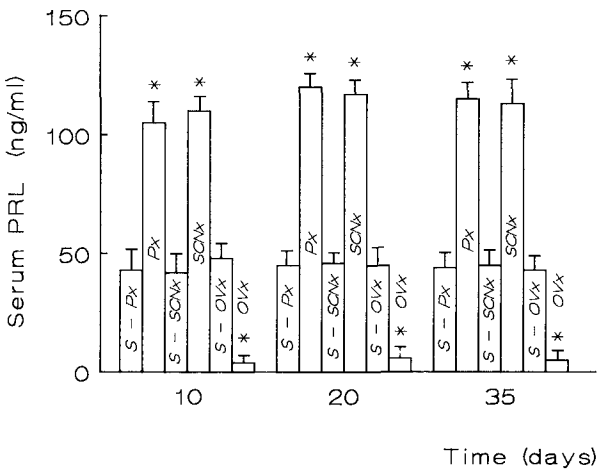

Figure 2. Mean (± SE) serum levels of PRL in Px, SCNx and OVx and their respective sham-operated animals at 10, 20 and 35 days post-surgery. * $p < 0.01$.

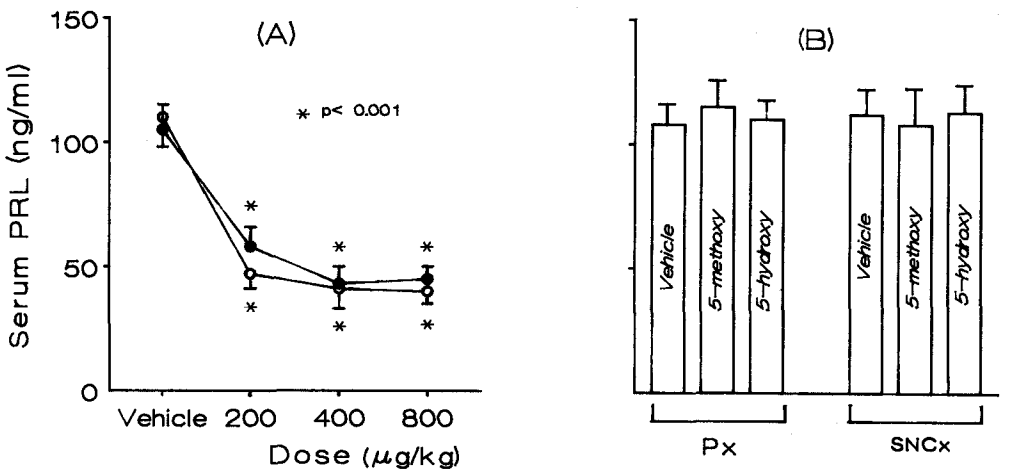

Figure 3. Mean (± SE) serum levels of PRL in Px or SCNx rats. *A* Effects of s.c. injections of various doses of melatonin in PX (○—○) and in SCNx (●—●) animals. Significance was estimated by comparing with control. *$p < 0.001$. *B* Effects of s.c. injections of indoles in Px and in SCNx rats at a dose of 400 µg/kg b. wt.

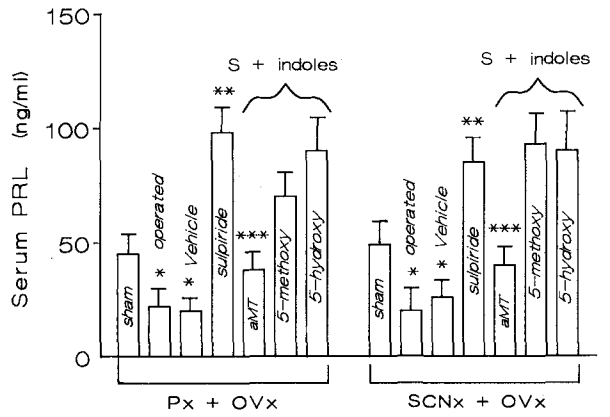

Figure 4. Mean (± SE) serum levels of PRL in Px plus OVx (left) and in SCNx plus OVx (right) rats treated with sulpiride. (S + indoles): sulpiride plus indole injected. Indoles were injected s.c. at a dose of 400 µg/kg b. wt. *$p < 0.005$ vs to sham-operated rats. **$p < 0.001$ vs operated or vehicle-treated rats. ***$p < 0.001$ as compared to sulpiride-treated rats. Samples were collected 1 h after sulpiride injection.

(fig. 4, left) or SCNx (fig. 4, right) and in sulpiride-treated rats was able to lower PRL levels, although it was unable to completely reverse the effect of sulpiride on PRL at the doses used; the other indoles tested failed to modify this sulpiride effect.

The results of the present study indicate that the pineal gland, by means of its hormone melatonin, is indeed involved in the modulation of PRL secretion independently of the hypothalamic SCN, as the effects caused by melatonin administration are the same as those seen in Px or SCNx rats. This suggests alternative mechanisms for melatonin control of PRL secretion, namely a hypophyseal melatonin-mediated and/or a hypothalamic steroid-mediated pathway[6], since lesion studies have provided evidence that frontal afferent fibers to the medial basal hypothalamus are indispensable for the occurrence of steroid-induced surges of PRL[15]. The latter possibility is more likely, as melatonin binding sites in other areas than SCN (i.e. median eminence and medial basal hypothalamus) have been described at the hypothalamic level[11,12].

Previous reports indicate that melatonin administration in the form of a daily afternoon injection, either alone or with simultaneous melatonin beeswax pellets[16], is not able to modify the lowered PRL levels brought about by OVx. However, melatonin at the doses used here inhibited the raising of PRL levels by sulpiride in Px or SCNx and OVx rats, which indicates that the presence of an intact pineal or suprachiasmatic nucleus does not contribute to the action of melatonin by decreasing serum PRL levels. It is important to consider whether the effect brought about by the various doses of melatonin was physiological or pharmacological. As shown earlier[17], the percentage of melatonin injected which reaches the pineal gland is reported to be 0.02%. According to this ratio, at the lower dose used here, i.e. 200 µg/kg (40 µg/rat), 8–10 ng of this dose of melatonin would have reached the pineal gland; since the melatonin content of this gland shows a circadian rhythm which, in rats, varies from 0.5 to 7 ng/gland[17], 200 µg/kg may be regarded as within the physiological range. Moreover, the effect of melatonin seen here suggests that this indole can interfere with dopamine in controling PRL synthesis and/or secretion[7], possibly potentiating the inhibitory effect of dopamine. However, an effect mediated by TRH or by some neurotransmitters involved in hypothalamic-pituitary neurosecretory mechanisms cannot be ruled out, as recent data[18] indicate that melatonin and the superior cervical ganglion regulate GABAergic receptors in the central nervous system of the rat, which in turn control PRL secretion[19].

The inhibitory effects of melatonin administration on PRL secretion may complement the increase in melatonin synthesis observed after prolactin administration in rat pineal organ cultures[20], thus establishing a feedback mechanism between the two hormones. Our results further suggest that acute sulpiride-induced hyperprolactinemia does not occur in rats previously treated with melatonin. The failure of 5-methoxy- and 5-hydroxytryptophol to cause noticeable effects in sulpiride-treated rats parallels the lack of effects on rats with Px or SNCx alone, indicating that these hormones are not significantly involved in the pineal regulation of PRL secretion, at least at the doses used here. Since Px alters not only PRL secretion but also pituitary PRL content, in a manner that varies under different photoperiods[10,21], further investigations with Px and/or SCNx animals sampled throughout the day should be conducted to elucidate the role of these structures in PRL physiology.

1 The authors thank Ms Karen Shashok for revising the English style. This work was supported in part by a grant GG85-0168 from the 'Comisión Asesora de Investigación Cientifica y Tënica'. The NIAMDD, through the National Pituitary Agency, supplied the radioimmunoassay materials for prolactin determinations.

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Gradient distribution pattern of muscarinic receptors in N1E 115 mouse neuroblastoma cells

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Received 24 February 1989; accepted 5 May 1989

Summary. The distribution pattern of muscarinic receptors in N1E 115 mouse neuroblastoma cells after linear and non-linear gradient centrifugation was investigated. In untreated cells, at least two forms of the receptors, with different densities, were found.
Key words. Muscarinic receptors; N1E 115 neuroblastoma cells; gradient distribution; concanavalin A.

It has been suggested that receptors for catecholamines are localized in the plasma membrane, and that treatment with agonists causes the internalization of these receptors. This has been demonstrated for β-receptors in frog erythrocytes[1,2], human astrocytoma cells[3,4], mouse lymphoma cells[5] and C6 rat glioma cells[6]. These results were in most cases obtained using either differential[1,2] or gradient centrifugation techniques, combined with incubation in a medium containing concanavalin A[3-6].

As far as the muscarinic receptor is concerned, few data are available. Harden et al.[7] reported the presence of muscarinic receptors in the plasma membrane of human 1321N astrocytoma cells, and the formation of a 'light vesicle' fraction containing muscarinic receptors after carbachol stimulation. To our knowledge, no such data are available for neuroblastoma cell lines. Since these cells are frequently used for the investigation of the interaction of agonists with muscarinic receptors in neuronal cells, we studied the cellular localization of the muscarinic receptors in the N1E 115 mouse neuroblastoma cells. These cells contain a well-defined muscarinic receptor[8] predominantly of the M1 subtype[9]. Using centrifugation techniques comparable to those used for β-receptors, it was found that in non-stimulatory conditions, two forms of the muscarinic receptor, characterized by a difference in density, are present in N1E 115 mouse neuroblastoma cells.

Materials and methods

Reagents. Tritiated quinuclidinylbenzylate (QNB, 36 Ci/mmole) and N-methylscopolamine (NMS, 72 Ci/mmole) were obtained from Amersham. All other reagents were from commercial sources and of the highest purity available.

Cell culture conditions. Mouse neuroblastoma cells, clone N1E 115, passage numbers 45 to 52, were cultured as described before[10]. Briefly, the cells were grown as monolayer in DMEM medium, containing 10% fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were kept in 100% relative humidity at 37 °C in 5% CO_2. The cells were subcultured when they had grown to confluency (between days 6 and 10), at a ratio of 1 to 5 or 1 to 10 in disposable plastic culture vessels of 175 cm^2 surface, using 0.025% trypsin in 0.9% NaCl solution. The cells were fed every other day.

The glioma cell line C6, passage numbers 82 to 85, was cultured in DMEM with the same additives as for the neuroblastoma cells. Subculturing at a ratio 1 to 10 was done by transferring the loosely attached cells to new culture vessels.

Pre-incubations and tissue preparations. Cells were harvested when confluent, which took 6 to 10 days. In all cases, they were rinsed twice for 5 min in 0.9% NaCl at room temperature before further manipulations.

Three different tissue preparations were used.

The *lysate* was prepared by rinsing the cells in 1 mM Tris buffer pH 7.4[6]; the cells were subsequently lysed in the same buffer during 20 min at 4 °C. The lysate was centrifuged at 300 g for 5 min and the supernatant was used for gradient centrifugation (see further).

The *microsomal pellet* was prepared by scraping the cells in 0.9% NaCl from the culture surface with a policeman.