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Research report

The effects of lesions of the thalamic intergeniculate leaflet on the pineal metabolism

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Abstract

The aim of the present work was to study, in rats, the effects of lesions of the thalamic intergeniculate leaflet (IGL) and the deep pineal/lamina intercalaris region (DP) on the diurnal profile of *N*-acetylserotonin (NAS) and on the nocturnal pineal reactivity to acute retinal light stimulation (1 or 15 min). The 24-h experiment shows that there is no phase-shifting on the diurnal NAS curve of groups of rats with bilateral IGL lesion compared to the controls. On the other hand there is a significant reduction on the amplitude of pineal NAS content observed in every nocturnal point of the curve. The pineal glands of IGL-lesioned rats, after 1 min of retinal light stimulation, keep their NAS content equal to the lesioned dark-killed rats. Nonetheless, after 15 min of photostimulation, the pineal NAS content is reduced to nearly zero equally to the control animals. DP lesion does not modify the content of NAS in the pineal gland of rats killed in the dark. However, the pineal photo-inhibition process induced by 1 min of light exposure is impaired. These results suggest that: (1) the intergeniculate leaflet has a role in regulating the amplitude of the diurnal rhythm of pineal NAS production rather than its phase entrainment to light–dark cycle. This effect is not dependent on the direct geniculo–pineal connections. (2) The nocturnal pineal photo-inhibition phenomenon could be decomposed in two processes. One, triggered by short pulses of light and totally dependent on the IGL and partially dependent on the direct monosynaptic pathway between this structure and the pineal gland. Another one, brought into action by longer lasting light stimulation that is neither dependent on the IGL nor on any direct central neural connections to the pineal gland.

Keywords: Intergeniculate leaflet; Pineal gland; *N*-acetylserotonin; Deep pineal; Lamina intercalaris; Circadian rhythm

1. Introduction

In almost all vertebrates the pineal gland metabolism is under control of daily and seasonal environmental illumination cycles [41]. In mammals, light stimulus, acting through retina can entrain the daily nocturnal peak of activation of the rate-limiting enzyme arylalkylamine *N*-acetyltransferase (NAT) and the resulting increase in the synthesis of *N*-acetylserotonin (NAS) and melatonin [13,16]. Moreover, acute photostimulation during the scotophase immediately brings the NAT activity to basal levels with the consequent shut down in the production and release of NAS and melatonin [8,9,18].

The neural pathway involved in the visual control of the metabolism of the mammalian pineal gland originates in the retina projecting through the retino-hypothalamic tract to the perichiasmatic hypothalamus [11,21,48,49] mainly to the suprachiasmatic nucleus (SCN). The connection between this anterior hypothalamic area and the pineal gland, is made through a not completely well established neural system [20,29] that involves the paraventricular hypothalamic nucleus and ultimately the spinal intermediolateral cell column and the cervical sympathetic nervous system. In addition to this peripheral sympathetic innervation, direct central projections to the pineal gland have recently been unequivocally demonstrated [5,19,22,24–28,30,42,57], originating from several sources among which a conspicuous projection from the intergeniculate leaflet of the lateral geniculate thalamic complex (IGL).

IGL has been proposed to be one of the most important

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structures in the organization of the circadian system [31]. The IGL is an intercalated zone between the dorsal and the ventral lateral geniculate nuclei extending the entire length of the lateral complex [33]. Its major neural input comes from the retina, seemingly from collateral of retinal ganglion cells projecting to the suprachiasmatic nucleus [39]. Among its efferent connections the one that is mainly involved in rhythmic control is the geniculo-hypothalamic tract (GHT) projecting to the ventrolateral portion of the SCN, on the same neuronal population that receives the retino-hypothalamic projection. In addition, the GHT projects to the adjacent anterior hypothalamic area, the subparaventricular zone, the retrochiasmatic area and the lateral hypothalamic area [55]. Moreover, the IGL seems to have a direct access to the pineal gland through its connections to the deep pineal gland and pineal stalk probably making synaptic contacts with pinealocytes processes [22,24]. The IGL cells projecting to SCN and pineal gland are neuropeptide Y-GABA-containing neurons [3,27,32–34,57]. The action of NPY on the pineal metabolism seems to be either excitatory or inhibitory [6,37,43,47,52] depending on the experimental paradigm and on the place of action (pre or postsynaptic), whereas GABA seems to be always inhibitory to the noradrenaline stimulation of NAS and melatonin production [1,45].

The aim of the present paper was to study the role of IGL and its projection routes on the control of the diurnal profile of NAS production and on the reactivity of the pineal gland to acute nocturnal retinal light stimulation.

2. Material and methods

2.1. Subjects

Male (3 month old) albino rats ($n = 214$), were housed (for at least 20 days) in a sound-attenuated, temperature controlled (21 ± 2°C) room, under a 12 h:12 h light–dark cycle (200 to 300 lux at cage level, white fluorescent bulbs:Kodak red 1A filter, 0.5 to 1.0 lux), with lights on at 06:00 h. The animals were maintained with water and food ad libitum.

2.2. Lesions and experimental procedures

Animals were anesthetized with sodium pentobarbital (45 mg/kg b.wt. i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, California, USA) and small holes were drilled in the skull to allow penetration of the electrode or the syringe needle. The lesions were aimed at one or several of the following coordinates [38]: AP = 4.1 to 4.8 mm posterior to bregma, 3.9 mm from midsagittal sinus and 3.9 to 4.8 bellow dural surface, for IGL and AP = 4.8 mm posterior to bregma, 3.7 bellow dura at sagittal plane, for DP.

An anodal DC current of 2 mA was passed for 10 to 20 s (DC LM5 lesion maker, Grass Instruments Co., Quincy, MA, USA), in each point, through epoxylite insulated stainless steel insect pin 00 (0.3 mm of bare tip), with the cathode attached to the skin of the back.

Neurochemical lesion was done by injection of the excitotoxin ibotenic acid (Sigma, 10 μg/μl, 0.6 μl per 10 min, dissolved in phosphate buffer) using a 30 gauge needle, a 10μl Hamilton syringe and a motor driven pump (355 syringe pump, Sage Instruments, Orion Res. Inc., Boston, MA, USA). The needle was left in place for 10 min after injection to avoid backflow of toxin up the needle tract.

One week after the lesion, rats were submitted to one of the several experimental conditions. In the nocturnal photostimulation experiments the animals were removed from the vivarium room and submitted to a 500 lux white fluorescent light for the time needed (1 or 15 min).

All the animals were immediately decapitated either in the dark (red lights, Kodak 1A filter) or in plain light (± 10 s of the scheduled illumination time) and their pineal glands were rapidly removed, individually frozen in dry ice and stored at −70°C until assayed.

2.3. Histology

The brains were removed from the skulls, placed in 10% formalin for 1 week, and placed in 20% sucrose formalin for 48 h before sectioning.

Lesion placement and extents were determined from serial cut at 30 μm on a sliding freezing microtome and stained with thionin. The neural structures were identified using Paxinos and Watson, 1986 atlas [38]. Lesions were identified as areas of neuronal loss and/or gliosis. In the case of ibotenic acid lesion the number of cells in the region of the IGL was counted on Nissl staining. It was included in the analysis only the rats bearing at least 80% of cell loss.

2.4. Chromatography

NAS content in the pineal gland was determined by high-performance liquid chromatography (HPLC) with electrochemical detection. The chromatographic system (Waters, Milford, MA, USA) composed by an isocratic 510 HPLC pump, a Resolve 5 μm spherical C18, 3.9 × 150 mm steel column, and a 464 electrochemical detector operated in DC mode, was controlled by the 820 Maxima Chromatography Software through a System Interface Module.

Each gland was sonicated (Microson XL 2005, Heat System Inc., Farmingdale, NY, USA) for 10 s in 120 μl of ice cold 0.1 M perchloric acid containing 0.02% EDTA and 0.02% sodium bisulfite. Protein and cell debris were removed by centrifugation (14,000 × g, 2 min) (Eppendorf 5415C Centrifuge, Brinkmann Instruments Inc., Westbury, NY, USA). The clear supernatant (60 μl) was injected into

the system through a syringe loading injector (20 μl loop, Mod. 7125, Rheodyne Inc., California, USA).

The chromatographic system was operated with the following mobile phase at room temperature (21°C ± 2°C): 0.1 M sodium acetate, 0.1 M citric acid, 0.15 M EDTA, 12% methanol, pH 3.7 at a constant flow rate of 1.0

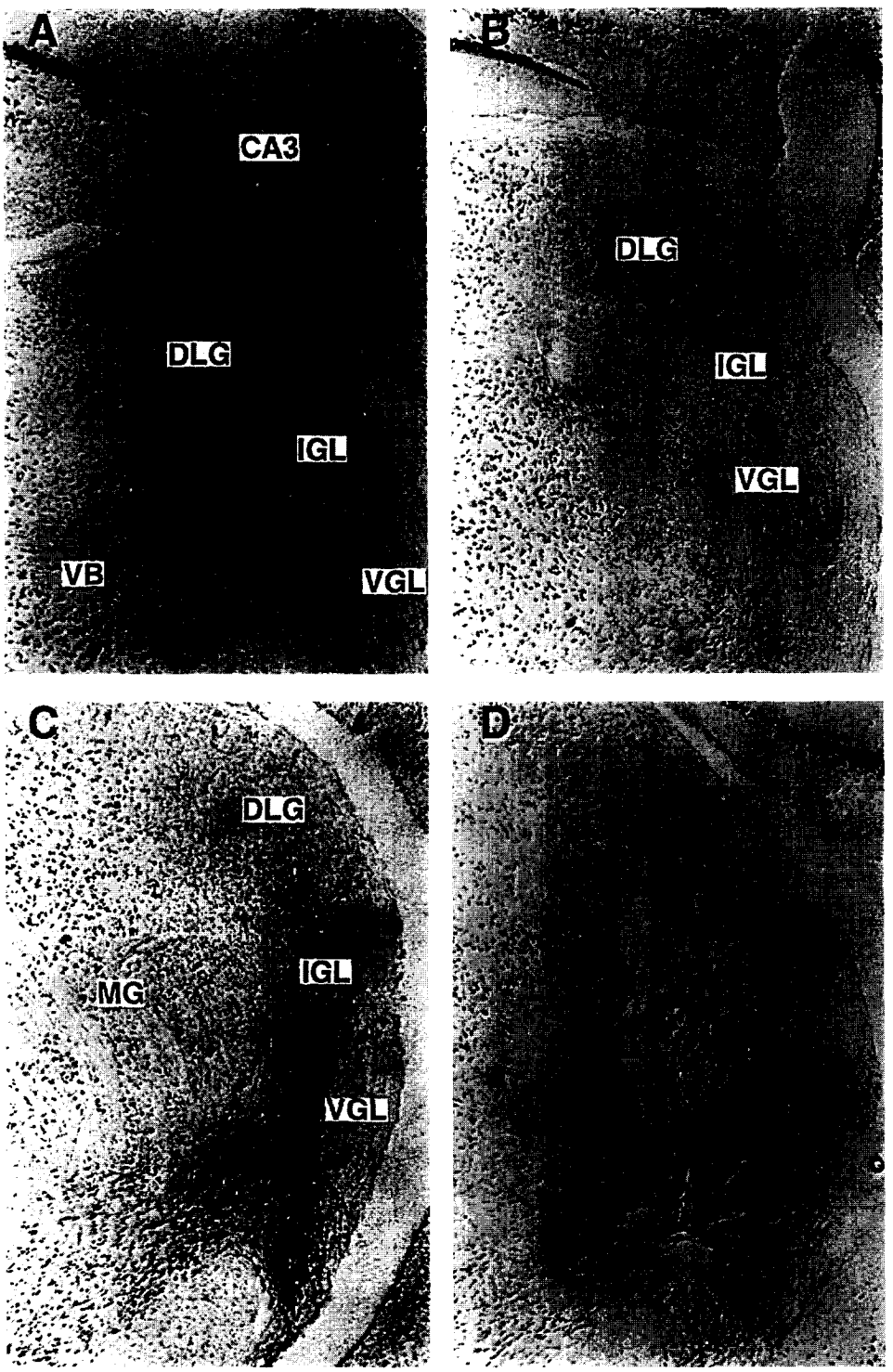

Fig. 1. This figure shows the type and extent of the lesion aimed at the intergeniculate leaflet of the lateral geniculate nuclear complex. The lesioned area was delimited under light microscopy in Nissl-stained tissue. The lesion hit the intergeniculate leaflet most completely. In the case of ibotenic acid lesion there was at least 80% reduction in the IGL number of cells. In a more or less extent the lesion hit as well the dorsal and the ventral lateral geniculate nuclei, the ventrobasal complex, the medial geniculate and the CA3 of the dorsal hippocampus. A, B and C (anterior to posterior) are pictures that represent the most extensive ibotenic acid lesion (10 μg/μl, 0.6 μl stereotaxically injected during 10 min) case of the IGL-lesioned group. D is a representative case of electrolytic lesion. IGL = intergeniculate leaflet, DLG = dorsal lateral geniculate nucleus, VLG = ventral lateral geniculate nucleus, MG = medial geniculate nucleus, VB = thalamic ventrobasal complex.

ml/min. The detector potential was adjusted to a steady value of +920 mV (vs. Ag/AgCl reference electrode). The total run time was 10 min and typically NAS was eluted at 8 min and 30 s.

2.5. Statistics

Results (ng/gland of NAS) are expressed as mean ± standard error of the mean (S.E.M.), and were computed using INSTAT statistical package (Instat V2.04, Graphpad Software, San Diego, CA, USA). The data were analyzed using Kruskal–Wallis non-parametric analysis of variance followed by Dunn's test and when appropriate comparison between independent groups was done using the Mann–Whitney *U*-test.

3. Results

3.1. Histology

Almost every lesion aimed at the intergeniculate leaflet region hit the lateral geniculate nuclear complex (Fig. 1). In spite of not being possible to completely define the IGL from histological sections stained for cell bodies [33], it is possible to say that in the majority of cases the lesion included the intergeniculate leaflet in its most extent, and, in varied magnitude and in different animals, the other following structures: the dorsal and the ventral lateral geniculate nuclei, the intermediate and the subgeniculate nuclei and the dorsal hippocampal CA3 field. In very few cases the acoustic radiation and the intramedullary thalamic area were included in the lesion and very sparingly the thalamic ventrobasal complex and the medial geniculate nucleus.

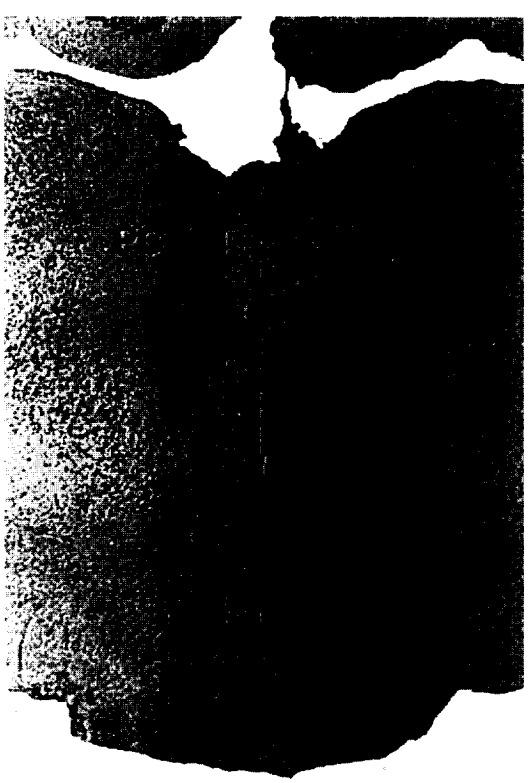

Fig. 2. This figure shows the type and extent of the lesion aimed at the deep pineal/lamina intercalaris region. This intercommissural region was totally lesioned. In addition, the posterior and habenular commissures, and partially the precommissural nucleus and the periventricular gray were lesioned. PCN = precommissural nucleus, PVG = periventricular gray, SM = supramammillary nucleus.

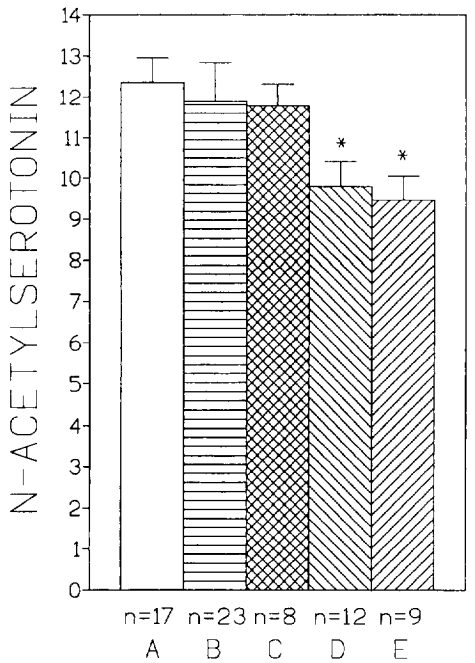

Fig. 3. *N*-acetylserotonin (ng/gland, mean ± S.E.M.) measured by HPLC with electrochemical detection in pineal glands at 24:00 h in rats submitted to a light–dark cycle 12 h:12 h (lights on at 06:00 h). A: intact controls. B: rats with lesion that spared the IGL bilaterally. C: rats with unilateral electrolytical IGL lesion. D: rats with bilateral electrolytical IGL centered lesion. E: rats with bilateral IGL ibotenic acid lesion. D and E are statistically different from A, B or C ($P \leq 0.007$) and equal to each other ($P = 0.52$). There is no statistical difference between A, B and C. n = number of rats.

The lesion aimed at the deep pineal/lamina intercalaris region was very restricted to that area as shown in the Fig. 2 including the habenular and the posterior commissures and partially the precommissural nucleus and the periventricular gray.

3.2. Lesion of the intergeniculate leaflet

Lesions that spared the IGL (and included at least one of the following structures: the dorsal lateral geniculate nucleus, the intramedullary thalamic area, the lateral posterior thalamic nuclei, the posterior nuclear and the ventrobasal thalamic complex and the hippocampus) (Fig. 3) did not change the amount of NAS produced by the pineal gland of rats killed during the night in the dark (11.84 ±

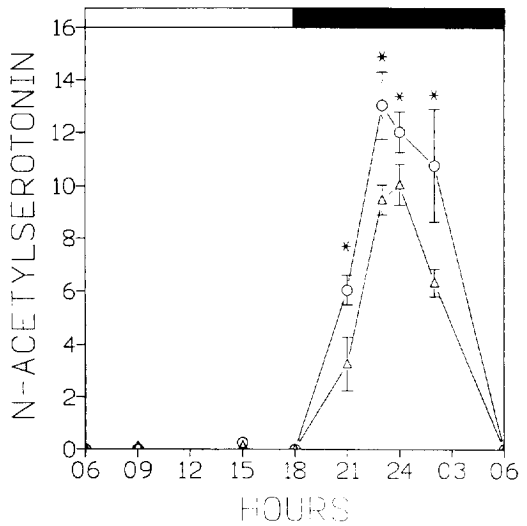

Fig. 4. Diurnal profile of the pineal *N*-acetylserotonin (ng/gland, mean ± S.E.M.) in intact (open circles) and IGL-lesioned (open triangles) rats. * indicates significant statistical difference at level of $P < 0.05$. There are at least six animals per group at each time point.

0.77 ng/gland, $n = 23$ and 12.37 ± 0.59 ng/gland, $n = 17$, for intact rats, $P = 0.73$). Bilateral lesions that include the IGL reduce the amount of NAS in the nocturnal pineal gland of rats killed in the dark (9.48 ± 0.58 ng/gland, $n = 9$, for ibotenic lesion, 9.8 ± 0.62 ng/gland, $n = 12$, for electrolytic lesion, $P \leq 0.007$). Considering similar lesions there is no difference if they were induced by electrical current or by ibotenic acid ($P = 0.52$). Moreover, the pineal NAS content is not different in rats bearing unilateral lesion when compared to dark-killed controls (11.89 ± .95 ng/gland, $n = 8$, $P = 0.63$).

The 24-h experiment, in which rats were killed at several time points (06:00, 09:00, 15:00, 18:00, 21:00, 23:00, 24:00 and 02:00 h, at least 6 animals in each group per time point), shows that there is no phase-shifting on the diurnal NAS curve of groups of rats with large bilateral lesion centered on IGL when compared to the controls. On the other hand, there is a significant reduction on the amplitude of pineal NAS content observed in every nocturnal point of the curve ($P < 0.05$) (Fig. 4).

In experiments done at 21:00 h (Fig. 5), intact animals show a significant reduction on the pineal NAS content after either 1 min or 15 min of light stimulation (6.03 ± 0.56 ng/gland, $n = 9$, for dark control, 2.37 ± 0.51 ng/gland, $n = 9$, for 1 min photostimulated controls and 0.38 ± 0.38 ng/gland, $n = 9$, for 15 min photostimulated controls, $P < 0.001$ in either case). On the other hand, the pineal glands of ibotenic acid-IGL-lesioned rats, killed after 1 min of light stimulation, keep its NAS content equal to the lesioned dark-killed rats ($P = 0.93$). Furthermore, 15 min of photostimulation brings the pineal NAS content of the lesioned group to nearly zero as it does to control animals (3.27 ± 1.03 ng/gland, $n = 9$, for IGL-le-

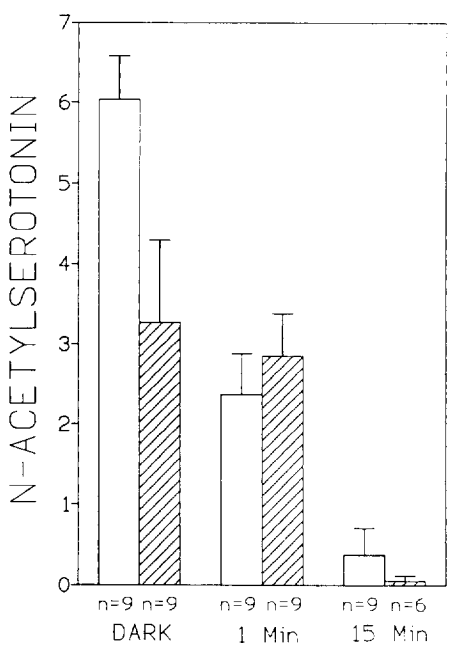

Fig. 5. Pineal *N*-acetylserotonin (ng/gland, mean ± S.E.M.) of intact (open bars) and IGL-lesioned (hatched bars) rats killed in the dark (DARK) or after 1 minute (1 Min) or 15 minutes (15 Min) of 500 lux, white light exposure at 21:00 h (LD 12 h:12 h, lights on at 06:00 h). There is no statistical difference between the rats bearing IGL lesion when killed in the dark or immediately after 1 min of retinal light stimulation ($P = 0.93$). n = number of rats.

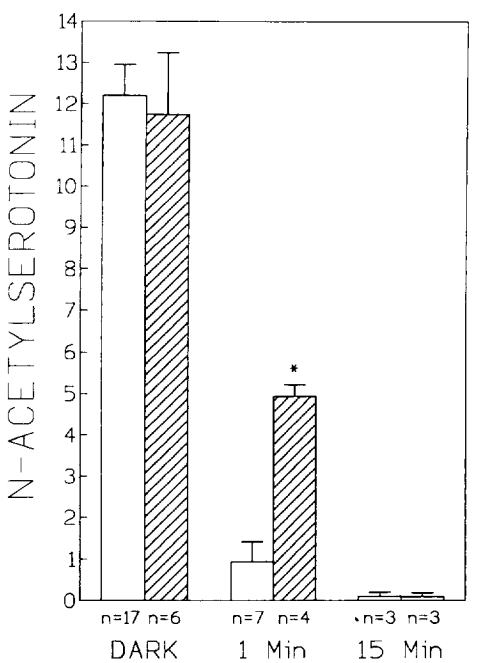

Fig. 6. Pineal *N*-acetylserotonin (ng/gland, mean ± S.E.M.) of intact (open bars) or deep pineal/lamina intercalaris lesioned (hatched bars) rats killed in the dark (DARK) or immediately after 1 minute (1 Min) or 15 minutes (15 Min) of 500 lux, white light stimulation at 24:00 h (LD 12 h:12 h, lights on at 06:00 h). * indicates a statistical difference between the lesioned rats and the controls after 1 min of light exposure ($P = 0.006$); n = number of rats.

sioned dark-killed, 2.85 ± .53 ng/gland, $n = 9$, for 1 min photostimulated IGL-lesioned, and 0.06 ± 0.06 ng/gland, $n = 6$, for 15 min photostimulated IGL-lesioned).

3.3. Lesion of lamina intercalaris region

In experiments done at 24:00 h (Fig. 6), the lesion of the terminal field of the geniculo-pineal direct pathway, i.e., the deep pineal/lamina intercalaris region, does not modify the content of NAS in the pineal gland of rats killed in the dark (12.20 ± 0.74 ng/gland, $n = 17$, for controls and 11.74 ± 1.5 ng/gland, $n = 6$, for DP-lesioned, $P = 0.75$). However, the pineal photo-inhibition process induced by short-term (1 min) light exposure is impaired since the lesioned group has its NAS content reduced much less than the intact animals (0.93 ± 0.47 ng/gland, $n = 7$, for controls and 4.93 ± 0.28 ng/gland, $n = 4$, for DP-lesioned, $P = 0.006$). After 15 min of retinal light stimulation both groups behave equally reducing the NAS pineal production to zero.

4. Discussion

The present data show that IGL lesion changes the metabolic capacity of the nocturnal rat pineal gland.

It has been shown that IGL lesion in hamsters produces an alteration of the entrainment phase angle of circadian rhythms [10,40]. In this way, it could be argued that the reduction we observed in the NAS content in IGL-lesioned dark-killed rats is a consequence of a phase shifting of its diurnal curve. However, in both intact and IGL-lesioned rats the peak of pineal NAS content occurs at approximately the same time and differences in magnitude are observed in every nocturnal sampled point. Therefore, these results indicate that, as far as the diurnal rhythm of NAS production by the pineal gland is concerned, the IGL is regulating its amplitude rather then its phase entrainment to light–dark cycle. IGL seems to play a part in the circadian system of melatonin production strengthening the rhythm and contributing to the maintenance of its amplitude what might be important in aging processes.

Alternatively it might be postulated that the nocturnal pineal NAS reduction observed in the IGL-lesioned animals would be due to an increase on the inhibitory NPY-GABA-mediated control from the IGL over the pineal metabolism released by an unbalance between left and right residual pools of cells [56] or even by a selective neurochemical lesion induced by the ibotenic acid [46]. The first possibility is discarded since the unilateral IGL-lesioned rats show a nocturnal pineal NAS content similar to the intact animals, and the second possibility is ruled out since both ibotenic acid and electrolytical induced lesions produce the same results.

The lesion of the terminal field of the geniculo-pineal projection on the lamina intercalaris does not reproduce the amplitude reduction effect of the IGL-lesioned rats indicating that the putative IGL amplitude modulation effect on diurnal pineal NAS and consequently on melatonin production, is not dependent on the direct IGL–pineal neural connections. This fact points to the possibility that this effect might be dependent on the IGL projections upon the classical hypothalamic-spinal cord-sympathetic-pineal controlling system. The geniculo-hypothalamic tract and its projection to the suprachiasmatic nucleus are the most likely candidate [4]. However, Klein and Moore [14] showed that the complete optic tract lesion on the lateral region to the suprachiasmatic nucleus ('bilateral postchiasmatic optic tract transection'), that would affect the IGL-SCN projection, did not alter the *N*-acetyltransferase activity of the pineal gland. These results indicate that the intergeniculate leaflet would control the nocturnal production of pineal NAS and melatonin via its projections to other hypothalamic structures like the retrochiasmatic area and the paraventricular–subparaventricular complex [23,33,35,36,44,55]. The retrochiasmatic area receives projections from the IGL, SCN and retina and projects to a neural system that by itself may potentially be involved in the control of the sympathetic nervous system, e.g., the precommissural nucleus, the solitary tract nucleus and the thoracic spinal cord [36,50]. In addition, it projects substantially to the paraventricular hypothalamic nucleus and the subparaventricular zone [44]. These are key structures in the control of the circadian fluctuation of melatonin production and mediate several photoperiodic phenomena dependent on the pineal function [17,36]. Therefore, it seems that IGL is able to modulate the circadian rhythm of pineal metabolism in a relay station that is downstream to the clock itself.

Considering the intensity (500 lux) and wave length (white) used in these experiments, the centrally IGL-deafferented pineal gland seems to be less responsive than the intact one, to the acute inhibition of NAS production induced by short-term (1 min) nocturnal photostimulation. On the other hand, the IGL-lesioned rats had no impairment on the ability to block the NAS production after 15 min of light stimulation.

In the photo-inhibition process of the pineal metabolism, several parameters of the stimulus seem to be important: intensity and duration of the light pulse [2,8,9,54]; the onset and offset of the light pulse [51]; the wave length and time of the day [7,12] and the duration plus the time after stimulus onset [18]. In the present work the used wave length and duration of the light pulse are enough to block the NAT activity and suppress NAS and melatonin synthesis [2]. In addition, regarding the inhibition of NAS synthesis in the control rats, our data showed that the 1 min light pulse given at 24:00 h is more effective than the one given at 21:00 h, in accordance with the data of Honma et al. and Kanematsu et al. [7,12]. Nevertheless, in our experimental setup was not possible to separate the possible effect dependent on the duration of light pulse

itself or the moment that the animals were killed after stimulus onset. However, for the sake of reasoning, the animals in our both experiments on nocturnal light stimulation, can be considered as killed 1 min or 15 min after light stimulation onset. In this way, we demonstrated that the IGL lesion affects the early process of pineal inactivation and does not affect the long-lasting pineal inhibition process probed by the biochemical analysis of glands collected 15 min after the starting of the light exposure.

Moreover, it should be stressed, that the pineal metabolic inhibition showed after either 1 min or 15 min probably includes both the immediate biochemical process of NAT inactivation due to thiol-dissulfide exchange, as proposed by Klein and Namboodiri [15] and the longer-lasting inactivation due to protein modification as suggested by Vanecek and Illnerova [53]. Therefore, the differences in the two phenomena, i.e., short-term and long-term pineal inhibition, is more likely to be due to different neural processes leading primarily to a reduction in the level of pineal stimulation.

Another possibility to be considered is that the loss of reactivity of the pineal gland to short-term nocturnal photostimulation in IGL-lesioned rats, would be a consequence of a reduced light sensitivity and/or a reduction in the rate of the response to light of the neural system that controls pineal metabolism. The reduction in sensibility due to retina or SCN deafferentation by retrograde cell lesion is unlikely since the effect was shown in ibotenic acid lesioned rats. Nevertheless, it is not possible to exclude in the present work the possibility that the IGL is modulating the sensitivity or the response rate of the central neural system mediating the inhibitory pineal response.

DP-lesioned rats showed an impairment in the 1-min inhibitory phenomenon, keeping intact the process of photo-inhibition to 15 min of light exposure. Therefore, it can be concluded that the photo-inhibition of the pineal gland, observed after 1 min of nocturnal illumination, is totally dependent on the IGL and partially dependent on the neural system that includes the lamina intercalaris region. The likely anatomical substrate would be the retino-geniculo-pineal direct projections, probably releasing neuropeptide Y and GABA, that are able to inhibit the noradrenergic neurotransmission in the sympathetic terminals and to block the on going stimulating activity of noradrenaline on pineal metabolism [1,37,45,47].

We demonstrated that the intergeniculate leaflet is not involved in the long-term photo-inhibition (15 min) of pineal metabolism. In accordance, Klein and Moore [14] showed that in their preparation with postchiasmatic optic tract transection (that cuts all the direct retinal connections to IGL) 30 min of photostimulation resulted in an almost complete (95%) blocking of NAT activity. Moreover, the presence of the retino-hypothalamic pathway was sufficient to block pineal nocturnal NAT activity due to long-term light stimulation. Furthermore, we showed that the lesion of the lamina intercalaris region did not disturb the blockade of NAS synthesis by 15 min of nocturnal illumination, ruling out, therefore, the putative importance of any other central neural direct connections to the pineal gland in this process. So, in addition to exclude the role of IGL in this long-term photo-inhibitory process we should emphasize its strict dependence on the classical peripheral connections of the pineal gland.

In conclusion, our results showed that the IGL is involved in the regulation of the amplitude of the diurnal curve of NAS and, probably, melatonin production. Moreover, it was shown that the nocturnal pineal photo-inhibition phenomenon may be decomposed in two different processes mediated by two neural systems. A rapid process, shown after 1 min of light stimulation, totally dependent on the IGL and partially dependent on the direct monosynaptic pathway between this structure and the pineal gland. Another one, occurring later on after light onset, not dependent on any direct connections between the central nervous system and the pineal gland and probably dependent on the classical inhibition of the pineal sympathetic neurotransmission, through a more complex system involving the retino–hypothalamic pathway.

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