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Melatonin and N-Acetylserotonin Stress Responses: Effects of Type of Stimulation and Housing Conditions

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The effects of housing condition and type of stimulation on serum melatonin and N-acetylserotonin (NAS) were investigated. Male rats were housed under a 12/12-hour light-dark cycle, with ad libitum food and water, either individually or in groups of four. At the start of the light phase, separate groups were sacrificed at rest or subjected for 3 minutes to the stimulation of cold water, noise, novel environment, or ether vapour and then decapitated at 0, 5, 15, 30 or 60 minutes after the end of stimulation. Melatonin was measured by a modified radioimmunoassay and NAS by a specific radioimmunoassay. Melatonin levels responded to stimulation with an increase, while NAS levels responded with a decrease. Housing condition had no effect on hormone response. However, the pattern of response for each of the two hormones differed greatly among the stimuli. For melatonin, cold water was the most potent stimulus, followed by noise, novel environment, and ether. NAS responded most to ether, fleetingly to cold, and in a bimodal manner to noise. The data are interpreted as suggesting that separate mechanisms regulate serum melatonin and serum NAS is response to environmental stimulation and that under appropriate control conditions melatonin from the pineal is very responsive to environmental stimuli, in a manner similar to that of pituitary hormones.

Key words: melatonin, N-acetylserotonin, stress, stimulus, housing, rat, pineal

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INTRODUCTION

Hormonal changes in serum have been used to characterize the response of the hypothalamic-pituitary system to stressful stimulation. It has been demonstrated that corticosterone and prolactin responses to four different types of stimulation have markedly different time courses [Seggie and Brown, 1982; Seggie et al., 1982]. Pineal melatonin is also responsive to environmental stimulation in the rat [Lynch et al., 1973, 1977] as are pineal N-acetyltransferase, N-acetylsertonin, and melatonin [Friedman and Yocca, 1984; Welker and Vollrath, 1984]. However, there are inconsistencies in the direction of response, and these are considered in the discussion. N-acetylserotonin, a precursor of melatonin in the pineal, has been demonstrated to be present in the circulation [Pang et al., 1981] and to be regulated in serum in a manner different from melatonin [Brown et al., 1983a]. These data suggest that N-acetylserotonin may be biologically active in its own right, and preliminary data indicate it may be responsive to certain types of stimulation [Seggie et al., 1982].

The present study was undertaken to investigate and map the response profile of serum melatonin and N-acetylserotonin following 3 minutes of stimulation by exposure to a novel environment, cold water, noise, or ether vapor.

MATERIALS AND METHODS

Subjects

Male Wistar rats weighing 250–300 g at the start of the experiment were housed in light-, sound-, and temperature-controlled rooms on a lighting cycle of 12 hours dark:12 hours light (lights-on noon to midnight) with free access to rat chow and water. Animals were given 2 weeks to adapt to the environment prior to experimentation. Each experimental treatment had eight subjects randomly assigned to it.

Housing Conditions

Animals were housed either individually or in groups of four to a cage. At the time of experimentation, group-housed animals were subjected to simulation on an individual basis. All members of a group were simultaneously treated so animals housed in groups were only separated for the duration of their stimulation. For each cage of group-housed animals, one of the four animals was assigned to each of the four different stimulation conditions.

Types of Stimulation

Separate batches of animals from each housing condition were subjected to each of four types of stimulation consisting of novel environment, noise, cold, and ether vapour. In addition, a separate batch of animals from each housing condition was sacrificed immediately upon entering the housing room at the start of the experiment for acquisition of blood in the resting or baseline condition. All exposures to stimulation were applied for a period of 3 minutes.

Novel environment stimulation consisted of removing an animal from its home cage and placing it in a plexiglass mouse cage with a mesh lid.

Noise stimulation consisted of placing the animal in a large cardboard drum 2 ft. in diameter with a smoke detector alarm sounding. The electronic beep produced by the device rated 85 decibels. The drum was cleaned between trials.

Cold stimulation consisted of placing the animal in an ice-water slurry in a steep-sided container with the slurry of such a depth that the animal could just touch the bottom but was not required to swim.

Ether vapour stimulation consisted of placing the animal in a sealed container with a volume of 0.2 cubic feet along with 2 cc of ether. This procedure was done in a fume hood, and the container was cleaned and aired between trials. This concentration of ether was such that animals became limp within 2 minutes but none died.

Procedure

Animals were randomly assigned to experimental groups. All experimentation was done in the time period 1–4 hours after the lights came on. No one was allowed access to the housing room for a minimum of 8 hours prior to the start of experimentation. Those animals that were assigned to resting conditions were sacrificed within 30 seconds of entering the housing room on any day. In order to avoid the possibility of a sequential sacrificing such as is apparent in corticosterone and prolactin responses when large numbers of animals are sacrificed [Seggie et al., 1974; Seggie and Brown, 1976] no more than two animals were sacrificed in the resting condition on any one day. The remaining animals were then subjected to their assigned stimulation for 3 minutes, and then sacrificed at one of five times consisting of: (1) immediately, (2) 5 minutes after (3) 15 minutes after (4) 30 minutes after and (5) 60 minutes after the end of stimulation. Sacrifice was done by decapitation, and trunk blood was collected in chilled tubes, centrifuged, and serum frozen and stored at −20°C for future hormone assay. Animals that were not killed immediately after stimulation were returned to their home cage for the assigned waiting period prior to sacrificing. All experimentation was done in a separate room from the housing room.

Assays

Melatonin radioimmunoassay was substantially modified [Grota et al., 1981] from that described by Pang et al. [1977]. For this assay, a new antiserum was used (R158, August 13, 1976). This antiserum which has greater specificity than that described by Pang et al. [1977], was produced using the identical methodology, and has undergone additional cross-reactivity studies of naturally occurring indoleamines. 6-Hydroxymelatonin showed 0.95% cross-reactivity; O-acetyl-5-methoxy-tryptophol, 0.9%; N-acetylserotonin, 0.008%; and other indoles, less activity. A series from N-caproyl to N-propronyl-(5-methoxytryptamine) was also tested for cross-reactivity. Cross-reactivity was inversely related to chain length of the N substitution, with N-propionyl-5-methoxytryptamine having the highest cross-reactivity (0.84%).

An improved extraction procedure was used that is relatively simple and effective: 0.5 or 1 ml of serum is extracted with pesticide-grade dichloromethane (Fisher, 1:5 ratio), and the phases are separated by centrifugation at 3,000g. The aqueous layer is aspirated and the tubes placed in dry ice for 40 minutes to freeze the remaining water and interphase to the side of the tube. The organic phase is then decanted and evaporated to dryness either in a Savant concentrator or under nitrogen in a 37°C water bath. The residue is reconstituted in buffer (pH 6.5, 0.01 M phosphate buffer containing 0.1% gelatin), and 2,000 cpm of ^{3}H-melatonin (New England Nuclear) and melatonin antiserum (1:39,000 final dilution) are added for a final volume of 0.65 ml. Following a 5-day incubation at 4°C, globulins and bound ligand are precipitated by adding an equal volume of saturated ammonium sulfate. Following centrifugation the supernatant is decanted, the residue redissolved in 0.55 ml of deionized water, of which 5.0 ml is pipetted into scintillation vials and counted.

Parallelism of various aliquots of rat serum extracted by the above method was obtained. Recovery of labelled melatonin added to rat serum was 84.5 ± 0.43% (n = 6). Intra-assay coefficient of variation for a rat serum pool was 6.7% for a sample of 30 pg/ml (n = 5). Inter-assay coefficient of variation for another pool was 17.4% for a sample of 143 pg/ml (n = 5). The least detectable concentration was 10 pg/ml, and the midrange of the assay was 70 pg/ml. Melatonin levels assayed in six rat serum samples by this radioimmunoassay and by gas chromatography-mass spectrometry (GC-MS) [Lewy et al., 1978] showed a correlation of 0.983 [Grota et al., 1981].

Two separate studies of pinealectomized rats have been performed 3 and 7 weeks after surgery, respectively. Radioimmunoassay of serum melatonin in animals sacrificed in groups of 6 to 12 every 4 hours for 24 hours showed levels of melatonin that were uniformly below the limit of detection. Data obtained with this assay are thus in agreement with studies using GC-MS which showed a virtual disappearance of serum melatonin [Lewy et al., 1980] and of urinary 6-hydroxymelatonin [Markey and Buell, 1982] in the rat following pinealectomy. Using the previous assay system [Pang et al., 1977] measurable "melatonin" was detected following pinealectomy [Yu et al., 1981]. We conclude that those earlier results are invalid and that disappearance of melatonin from the circulation of pinealectomized rats should be one criterion for a valid melatonin radioimmunoassay.

N-acetylserotonin was assayed using the radioimmunoassay of Pang et al. [1981] with the only difference that the R228 antibody was replaced with antibody R238 that has been prepared in the same way. The validation data for antibody R238 has been published elsewhere [Brown et al., 1983b]. For the current NAS assay, the intra-assay coefficient of variation for a rat serum pool taken in the middle of the light phase (N = 9, mean value 212 pg/tube) was 5.6%, and the inter-assay coefficient of variation was 6.9%.

RESULTS

Serum samples from 42 experimental groups were assayed for melatonin and N-acetylserotonin. The hormone data were subjected to a three-

way analysis of variance for the main effects of housing condition, type of stimulation, and time and all interactions of these factors. In no instance did the type of housing have any effect on hormone levels, nor were there any significant interactions with this factor (F = .90 and .22; df = 1,333 and 1,353; P = .34 and .64 for main effect of housing on melatonin and N-acetylserotonin, respectively). Therefore, the data were reanalyzed using a two-way analysis of variance with data collapsed across housing conditions. The results of this analysis appear in Table 1. Data are shown in Figure 1 (melatonin) and Figure 2 (N-acetylserotonin). The response pattern of both hormones were significantly affected by the type of stimulation and time ($P < .001$). Furthermore, there was a significant interaction of these two factors for each hormone ($P < .001$). Further investigations of data were done using one way analysis of variance, Duncan range tests and t-tests. Investigation of the significant stimulation × time interactions are shown in Table 2. Only responses that differed significantly ($P < .05$) from the baseline resting level are included.

Melatonin responded to stimulation with an increase in levels while N-acetylserotonin responded with a decrease; however, the magnitude of the response differed greatly depending on the type of stimulation. For melatonin, all stimuli elicited a significant response; however, cold water elicited the largest and longest-lasting elevation with levels still maximal 30 minutes after stimulation; and although it returned toward baseline, the response was still significantly elevated over baseline 60 minutes after the end of 3 minutes' exposure to cold water. Noise elicited the next largest response, which was apparent at 0, 5, and 15 minutes after stimulation. Novel environment elicited a rapid response that was apparent at 0 and 5 minutes after 3 minutes of stimulation, but the levels had returned to baseline by 15 minutes after end of stimulation. Ether elicited a transitory but significant elevation evident immediately after removal from the vapor, but this effect was over by 5 minutes after the end of ether vapour stimulation, when melatonin levels had returned to resting values.

N-acetylserotonin evidenced a different pattern of response to stimulation in that it responded to all stimuli except novel environment. Response to noise and ether was most rapid and seen at 0 minutes after stimulation.

TABLE 1. Analysis of Variance Results

Hormone Factor	F	df	P
Melatonin			
Type of stimulation	753	3,333	<.001
Time	18.9	5,333	<.001
Stimulation × time	5.33	15,333	<.001
N-acetylserotonin			
Type of stimulation	9.57	3,353	<.001
Time	8.08	5,353	<.001
Stimulation × time	1.80	15,353	<.001

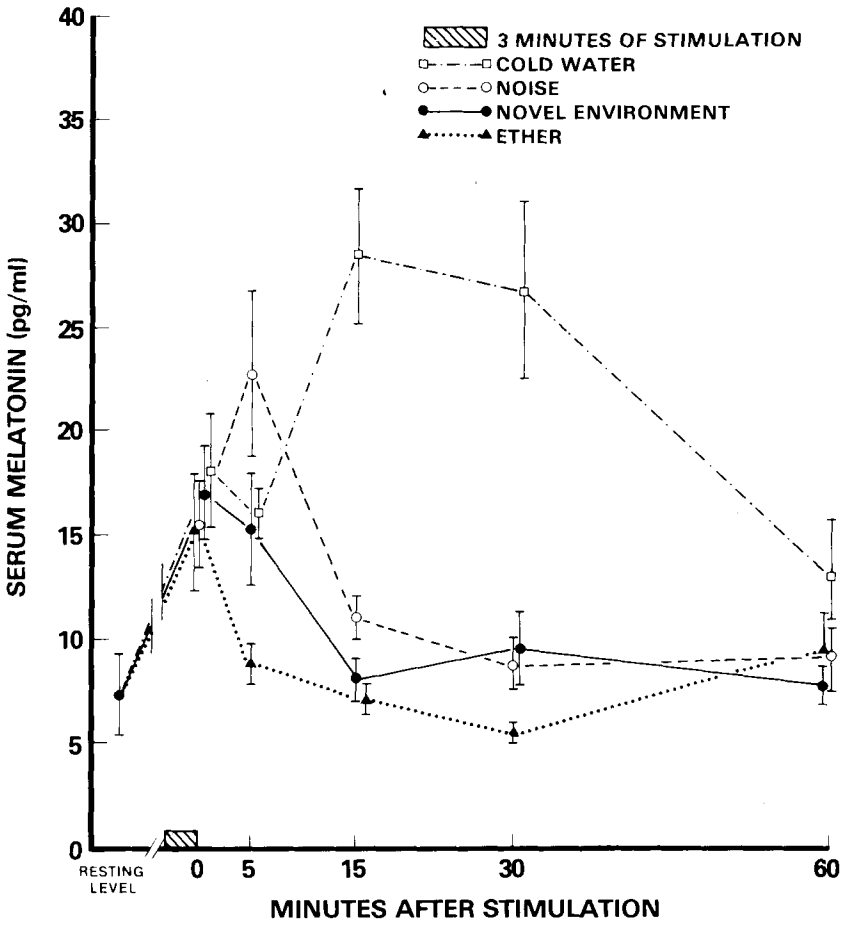

Fig. 1. Serum melatonin levels at rest and during the first hour following 3 minutes of stimulation by exposure to novel environment, cold water, noise, or ether. The N per point is 14–16. Data points represent the means ± standard error.

TABLE 2. Effect of Type of Stimulation on Magnitude of Hormone Response[1]

Sample time (min)	Melatonin	Nas
0	Noise = cold = novel = ether and all > baseline	Noise = ether Both < baseline
5	Noise = cold = novel and all > baseline	Cold = ether Both < baseline
15	Cold > noise Both > baseline	Ether < baseline
30	Cold > baseline	Ether < baseline
60	Cold > baseline	Ether = noise Both < baseline

[1]Only responses that differ significantly ($P < .05$) from resting level (baseline) are included. = means the two stimuli elicited responses that did not differ significantly; < means less than with $P < .05$; > means greater than with $P < .05$.

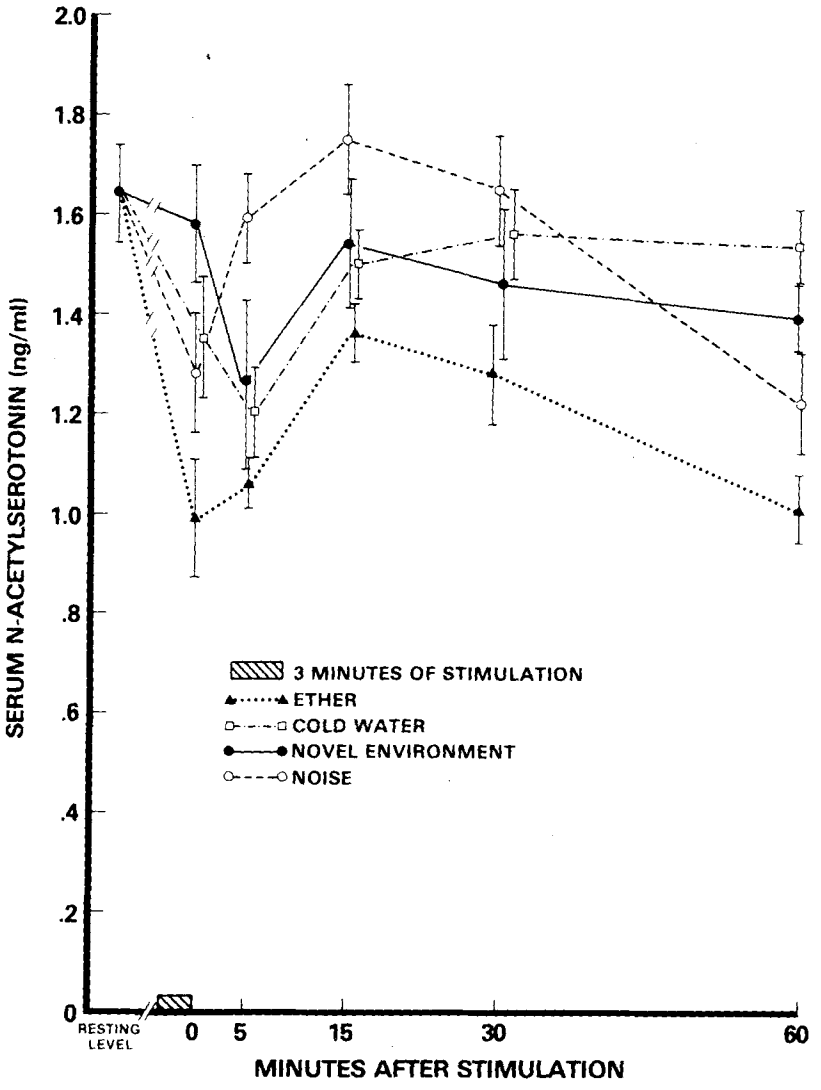

Fig. 2. Serum N-acetylserotonin levels at rest and during the first hour following 3 minutes of stimulation by exposure to novel environment, cold water, noise, or ether. The N per point is 15–16. Data points represent the mean ± standard error.

Noise stimulation produced an immediate fall, which was followed by a rapid return to resting baseline levels between 5 and 30 minutes, followed by a second significant decline that was evident 60 minutes after stimulation. Ether evoked the largest drop in N-acetylserotonin level in that it was significantly less than resting level at all time points. Cold water elicited a short-lived response that was significant only at 5 minutes after stimulation.

DISCUSSION

Serum melatonin and N-acetylserotonin were found to be responsive to environmental stimulation, immediately after a 3-minute exposure to stimu-

lation: The former with a rise, the latter with a fall in levels. Housing of animals individually or in groups had no effect on these normal response patterns. However, the various types of stimuli used resulted in very different responses pattern for the two hormones indicating that the control mechanisms for melatonin and N-acetylserotonin are differentially sensitive to the stimuli of cold water, novel environment, noise, or ether vapour.

There is considerable evidence that melatonin in the serum originates in the pineal. Following pinealectomy there is a virtual disappearance of melatonin from the circulation [Lewy et al., 1979] and of its major metabolite from urine [Tetsuo et al., 1982]. Furthermore, under various lighting conditions, designed to alter 24-hour patterns, serum melatonin, pineal melatonin, and pineal N-acetytransferase (NAT) rhythms are in phase [Ho et al., in press].

Dissociation of serum N-acetylserotonin and melatonin responses to stimulation is not surprising. There is increasing evidence that circulating N-acetylserotonin is regulated independently of melatonin and that it does not originate from the pineal gland [Brown et al., in press]. In animals on short photoperiods, the 24 hour rhythms of serum N-acetylserotonin and melatonin are markedly different, with the peaks separated by several hours [Brown et al., 1983a]. In response to scheduled feeding, the serum NAS crest is synchronized with feeding, while the serum melatonin crest still occurs during darkness [Ho et al., 1983]. In pinealectomized animals, serum NAS levels are not substantially altered [Yu et al., 1981]. This finding has recently been confirmed (Harvey and Brown, unpublished) using a specific NAS radioimmunoassay [Pang et al., 1981]. The site of origin of circulating NAS is currently unknown.

There are several possible explanations for the transient drop in serum N-acetylserotonin. It could be related to increased conversion of serum N-acetylserotonin to melatonin by erythrocytes [Rosengarden, 1972] or other factors. However, the 200-fold difference in levels of circulating N-acetylserotonin and melatonin make this interpretation unlikely. A second possibility is that it is related to the redistribution of blood compartments following stress so that the pool that is being sampled has a lower N-acetylserotonin content. Yet another possibility is that synthesis and/or release of NAS is altered. The effectiveness of ether, a metabolic stress, in eliciting an NAS response in the present study supports this idea. Further studies will be essential to resolve this issue.

With respect to melatonin, the finding that during light hours serum melatonin rises following acute environmental stimulation is in agreement with several other reports measuring other indices of pineal activity. Pineal N-acetyltransferase (NAT) activity in rats increased following insulin induced hypoglycemia or acute immobilization [Lynch et al., 1973], ether [Vaughan et al., 1978; Yu et al., 1981], and swimming [Parfitt and Klein, 1976; Welker and Vollrath, 1984]. In the mouse, cold exposure leads to a rise in dense-core vesicles in the pinealocyte [Matsushima and Morisana, 1980]. In the rat, cold exposure increases locomotor activity, which in turn elevates cyclic GMP in the pineal [Meyerhoff et al., 1979].

In animals on alternating light and dark cycles, the pineal response appears to be critically dependent on the time of day. In the rat, a fourfold

increase of pineal N-acetyltransferase (NAT) in response to ether was found *early in* the light phase, but not late in the light phase or in the middle of the dark phase [Vaughan et al., 1978]. During the dark phase, immobilization resulted in a decrease in pineal NAT and melatonin [Friedman and Yocca, 1984].

The present study was done early in the light phase under alternating light and dark conditions. Exposure of subjects to continuous light or dark prior to stimulation also leads to changes in pineal responsiveness compared to testing under alternating light conditions [Lynch et al., 1973, 1977; Welker and Vollrath, 1984].

In contrast to the present data, a recent report [Welker and Vollrath, 1984], concluded that during daytime, the rat pineal is not responsive to environment stimuli including cold, heat, immobilization, noise, hunger, thirst, or hypoglycemia. Unfortunately, these authors did not include an *undisturbed* control group for comparison with their stimulated group. Their report of decreased pineal NAT levels within their study time is more readily interpreted as the normal decline or return toward baseline levels following an elevation in response to stress. The sample times in the study were such that the response was just about over before observation began. A similar problem exists with the Vaughan et al. [1978] study, which concluded there is no melatonin stress response in humans. There was no undisturbed control group and the observation began late. The high daytime melatonin levels and the significant drop over the observation period seen in this study suggest that melatonin levels were in fact elevated by something, *before* the start of the study, possibly by transport and anticipation of the upcoming procedures. In the present study, the data suggests the melatonin response to stimulation in addition to being stimulus dependent, is also very dynamic and short lived. Characterization of pineal responsivity will require methodology similar to that developed for study of the pituitary axis.

It has been suggested that the pineal activation following stimulation is mediated by increased circulating catecholamines, resulting in increased pineal NAT activity and, hence, increased synthesis of melatonin. This was because the effects of hypoglycemia or immobilization were blocked by propranolol but potentiated by destruction of pineal sympathetic nerve terminals with 6-hydroxydopamine [Lynch et al., 1973]. However, the increase following immobilization is prevented by bilateral adrenalectomy but not by superior cervical ganglionectomy [Lynch et al., 1977]. Friedman and Yocca [1984] postulate that pineal response to stress is mediated by sympathetic neuronal activity, but not plasma catecholamine elevations.

In view of the apparent sensitivity of the pineal gland and the existence of non-sympathetic nerve input into the pineal via the posterior and habenular commissures [Karasek, 1983], the possibility that the pineal, like the pituitary, is a target organ for the brain cannot be ignored.

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