Preliminary Study of the Effects of Melatonin Administration on the Release of Endogenous 5-HT and Its Metabolite in Rat SCN

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The effect of melatonin administration on the release of 5-HT (5-hydroxytryptamine, serotonin), 5-HIAA (5-hydroxyindoleacetic acid), DOPAC (3, 4-dihydroxyphenylacetic acid), and HVA (homovanilic acid) in the suprachiasmatic nuclei (SCN) of freely-moving rats was studied by in vivo brain microdialysis coupled to liquid chromatography/electrochemistry (LCEC). The extracellular concentration of 5-HT in the SCN was significantly increased, reaching a maximum dialysate concentration of 4 ng/mL between 60 and 180 min after oral administration of 10-mg melatonin at circadian time (CT) 18. In contrast, release of the 5-HT metabolite 5-HIAA, was significantly reduced by this treatment. Interestingly, those effects were only obtained after nighttime (CT 18) administration of melatonin, but not after daytime (CT 6) administration. These preliminary results suggest that melatonin stimulates the serotonergic system in the rat SCN. That this effect only occurred following nighttime (CT18), but not daytime (CT 6) melatonin administration further suggests that there may be a circadian element to melatonin's influence upon 5-HT. In addition, these experiments illustrate the simultaneous monitoring of rotational behavior and neurochemical changes using the Raturn™ awake animal system coupled to a Pollen-8™ on-line LC injector.

The pineal indoleamine-hormone melatonin elicits a wide variety of physiological actions across vertebrate species (1, 2). An important one is its ability to influence circadian rhythm (3). Redman *et al.* (4) showed that in constant darkness daily injections of melatonin would entrain rat free-running activity patterns when the time of injection coincided with the onset of normal activity. Numerous studies have described the neuroanatomical pathway by which photic information reaches the pineal gland, where melatonin is released (5, 6). Little is known concerning any effect of melatonin on the SCN or its mechanism of action.

The bilateral SCN of mammals, located in the anterior hypothalamus, is a major component of the mammalian biological clock (6). These nuclei are considered to be the main photoperiodic regulator of circadian rhythms responsible for daily patterns in behavior and secretion of neurochemicals (7). Melatonin secretion, although regulated by information from the SCN, is also thought to feedback upon these nuclei to modulate some circadian functions. Recent studies have demonstrated a direct phase-advancing effect of melatonin on electrical activity in SCN slices, and indicated that the phase-shifting effects of melatonin are mediated through the SCN (8, 9). These findings suggest that melatonin modulates the biological clock, either directly or indirectly via neural inputs. This hypothesis is supported by neuroanatomical findings that high-affinity melatonin receptors are present in rat SCN, and their density exhibits a daily rhythm regulated by the light/dark (LD) cycle (10).

The chemical basis for the regulation of SCN-generated rhythms is not well understood. The SCN has been shown to contain a variety of neurochemicals, such as 5-HT, amino acids, and peptidergic neurotransmitters. Many of these have been implicated in the regulation of photoperiodic activity (6, 7). Serotonin is thought to play a primary role in SCN function, since depletion

of brain 5-HT or treatment with specific 5-HT receptor agonists has been shown to have significant effects on circadian rhythms (11). The SCN does receive 5-HT projections from the raphe (12).

Collectively, these observations have led to speculation that serotonergic innervation is part of the mechanism underlying SCN action. Therefore, it is reasonable to hypothesize that exogenous administration of melatonin would directly influence the profile of serotonergic transmission in the SCN.

Microdialysis is now a well-established technique and has been used to sample neurotransmitters in a variety of tissues (13). Recently, this technique has proven useful in circadian studies where amino acids (14), peptidergic (15, 16) and monoaminergic neurotransmitters (17, 18) were monitored in the SCN of animals. The present report describes the use of *in vivo* microdialysis sampling coupled to on-line LCEC to determine the effect of exogenous administration of melatonin on extracellular 5-HT, 5-HIAA, DOPAC and HVA in rat SCN, and on the circadian rotational behavior of the experimental animals.

Materials and Methods

Animals and Microdialysis Procedures

All animal care and treatment procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, USA, 1996). Male Sprague-Dawley rats weighting 300-350 g were used throughout this study. All animals were housed in an animal room under constant conditions of 12-h light/dark with food and water available *ad lib*.

For surgical implantation of brain microdialysis guide cannulae, the animals were anesthetized by an intraperitoneal injection of 1 mL/kg of KX [10 mL ketamine hydrochloride (100 mg/mL) + 1 mL xylazine (100 mg/mL)]. The surgery was performed in a stereotaxic frame with blunt ear bars. The guide cannulae were implanted unilaterally into the SCN (coordinates: A-P +1.2, L-M +0.8, V-D +9.0 from bregma) of rats according to previous reports (15, 17). The guide cannula was secured in place with dental acrylic cement (MD-1300, Bioanalytical Systems, Inc. (BAS) West Lafayette, IN, USA). The rats were allowed to recover from surgery for at least 48 hr prior to initiation of an experiment. On the morning of the experiment, a BAS brain microdialysis probe (0.32 mm ID x 1 mm membrane) was inserted into the pre-targeted guide cannula. The inlet of the microdialysis probe was connected by PEEK tubing (0.65 mm OD, 0.12 mm ID) to a 1.0-mL gas-tight syringe containing Ringer's solution. The outlet was connected to an on-line injection valve (Pollen-8™, BAS). The probe perfusion flow rate was set at 1.0 µL/min. Dialysis samples were not collected for analysis for at least 1 hour following probe insertion and initiation of perfusion. During microdialysis sampling, the awake and unrestrained rat was housed in the Raturn™ interactive awake animal containment system (BAS). The use of the interactive Raturn animal containment system not only prevents the perfusion tubing from tangling during the study, but also allows for the monitoring of rotational behavior of the animal through the optional Raturn data acquisition software.

The Determination of Analytes of Interest by LCEC

The determination of 5-HT, 5-HIAA, DOPAC, and HVA in dialysates was performed by on-line LCEC, using a PM-80 pump and LC-4C electrochemical detector (BAS). The pre-column and analytical columns were UniJet ODS, 3 µm, 14 mm x 1 mm I.D. (MF-8946, BAS) and UniJet PEEK ODS, 3 µm, 100 mm x 2 mm I.D. (MF-8957, BAS), respectively. The columns were maintained at 27.5°C using a LC-22C temperature controller (BAS). The mobile phase consisted of a mixture of 1000 mL buffer (50 mM sodium citrate, 25 mM monobasic sodium phosphate, 10 mM diethylamine hydrochloride, 2.2 mM sodium octylsulfonate and 0.03 mM disodium EDTA, pH 3.2, adjusted with 85% phosphoric acid), 30 mL acetonitrile, 15 mL dimethylacetamide, and was filtered through a 0.20 µm filter. The flow rate was 0.2 mL/min. The back pressure of this system was 3000-3500 psi. Detection was at a glassy carbon working electrode (3 mm; MF-1000, BAS) maintained at a potential of +700 mV vs. Ag/AgCl. Control and data collection/processing were handled through BAS ChromGraph™ software.

Melatonin Administration and Experimental Procedure

After at least a several hour baseline period, 10 mg of melatonin, suspended in 1 mL deionized water, was administered orally at 12:00 (circadian time; CT 6) and 24:00 (CT 18, CT 12 being defined as the onset of darkness) using pipettes. Control data were obtained from rats administered water at the corresponding circadian time. The effect of oral melatonin or sham water administration on the extracellular SCN concentrations of 5-HT, 5-HIAA, DOPAC, and HVA was assessed for at least eighteen hours following administration. Dialysates were automatically injected every thirty minutes using the on-line LCEC. Animal rotational activity was monitored during the pre-treatment baseline period and for at least 48 hours following treatment.

Data Analysis

Concentrations of all analytes are expressed as the mean ± SEM in the dialysates. For statistical analysis, the within group concentration of the analytes collected before administration of melatonin were compared with those collected after administration using Student's *t*-test. For all statistical analyses, a *p* value of 0.05 or less was considered statistically significant. The effect of melatonin administration on the en-

dogenous concentration of 5-HT, 5-HIAA, DOPAC and HVA in the between group brain dialysates were analyzed using ANOVA with repeated measures.

Histology

The day after the experiment, animals were sacrificed by an overdose of sodium pentobarbital. The brains were removed and subsequently fixed with 10% phosphate-buffered formalin solution for two days, after which they were sectioned to confirm the placement of the probe in the SCN. Only data from rats with correctly placed cannulae are reported.

Materials

Melatonin, norepinephrine (NE), epinephrine (E), dopamine (DA), DOPAC, HVA, 5-HT and 5-HIAA were purchased from Research Biochemicals International (Natick, MA, USA). Stock solutions of E, NE, DA, 5-HT, and their metabolites (DOPAC, HVA, 5-HIAA) were prepared at a concentration of 10 mg in 10 mL of 0.1N hydrochloric acid and stored in the dark at -20°C. A standard mixture was prepared fresh daily from the stock solutions diluted to various concentrations (0.04 - 20 ng/mL) with Ringer's solution. Reagents were all of analytical grade.

Results

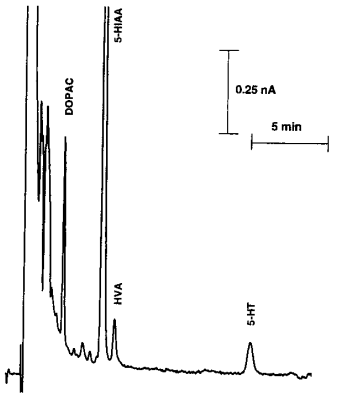
Serotonin, Catecholamines and Their Metabolites

The retention time of the standards were 2.7 min for NE, 3.3 min for E, 3.8 min for DOPAC, 5.9 min for DA, 6.3 min for 5-HIAA, 7.2 min for HVA and 16 min for 5-HT. All compounds of interest were well separated. The detection limits were 2.0 pg injected for DOPAC, HVA and 0.8 pg for 5-HIAA, 5-HT, at a signal-to-noise ratio of three. The calibration curves for those compounds were linear between 2 pg and 800 pg with correlation coefficients of 0.995 - 1.000.

A typical chromatogram for an *in vivo* dialysate sample thirty minutes after administration of melatonin is presented in ***F1***, no interfering peaks were observed. DOPAC, 5-HIAA, HVA and 5-HT in dialysates could easily be identified by comparing retention times and electrochemical characteristics with those of their corresponding standards. The baseline composition and concentrations varied somewhat among different animals. 5-HT was not detectable in control brain dialysates or as a baseline concentration prior to melatonin treatment, while DOPAC, HVA and 5-HIAA were always detected in all tested animals.

F1

Typical chromatogram of in vivo dialysate from rat SCN at 30 minutes after administration of melatonin (10 mg). The peaks of DOPAC, 5-HIAA, HVA and 5-HT have retention times at 3.8 minutes, 6.3 minutes, 7.2 minutes and 16 minutes, respectively.

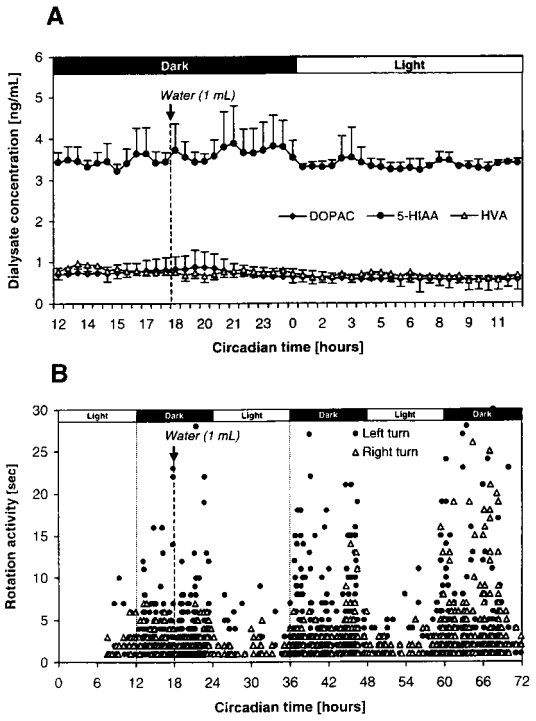


Baseline Levels of Analytes of Interest in Dialysates From SCN of Rats Prior to Melatonin Administration

The profiles of DOPAC, 5-HIAA and HVA in SCN dialysates collected from sham control animals during a 24 hour period in 12/12 hour of dark/light periods, are shown in ***F2***. The pretreatment concentration of 5-HIAA in SCN dialysates from rats administered melatonin was 2.96 ± 0.44 ng/mL (n=3, F3A). For control rats, the mean 5-HIAA level was 3.45 ± 0.34 ng/mL (n=3, ***F2A***). Pretreatment levels of DOPAC and HVA from melatonin treated animals were 0.84 ± 0.16 ng/mL and 0.93 ± 0.21 ng/mL (n=3, ***F3A***), and 0.75 ± 0.20 ng/mL and 0.82 ± 0.14 ng/mL for control rats (n=3, ***F2A***), respectively. These pre-treatment values of 5-HIAA, DOPAC, and HVA did not differ significantly (by ANOVA) between controls and melatonin treated animals. Likewise, no significant differences in these three compounds were observed between nighttime and daytime (by Student's *t*- test) in control animals. And while the rotational activity of control rats did tend to increase in the dark period and level off again in light (***F2B***) this pattern was not significant.

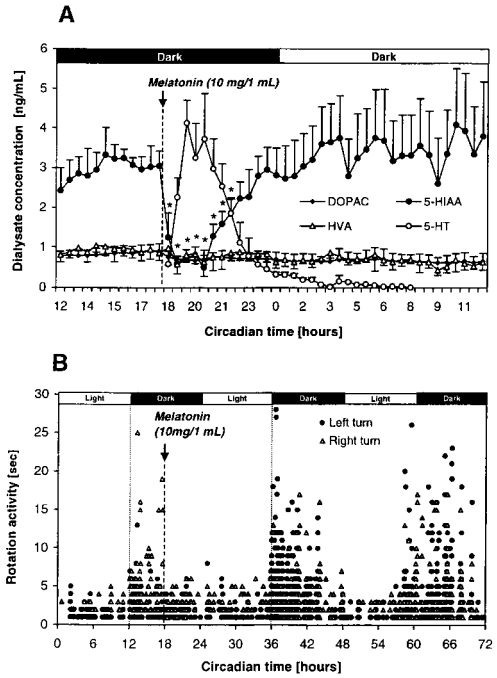
F2

Concentration profiles of 5-HT, 5-HIAA, DOPAC, and HVA in dialysates from the SCN region of rats administered water orally at CT 18 (A) and corresponding rotational activity (B). Arrows indicate the time of water administration. Each point represents mean ± SEM of three animals.



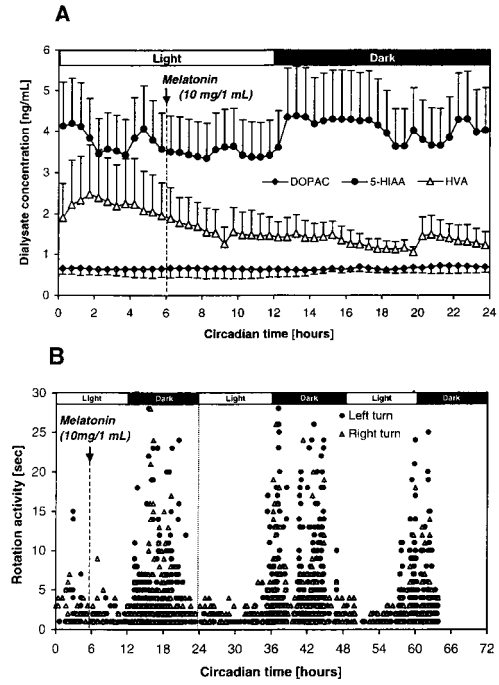
F3

Concentration profiles of 5-HT, 5-HIAA, DOPAC, and HVA in dialysates from the SCN region of rats administered 10-mg melatonin orally at CT 18 (A) and corresponding rotational activity (B). Arrows indicate the time of melatonin administration. Each point represents mean ± SEM of three animals.



F4

Concentration profiles of 5-HIAA, DOPAC, and HVA in SCN dialysates from rats administered 10-mg melatonin orally at CT 6 (A) and the corresponding rotational activity (B). Arrows indicate the time of melatonin administration. Each point represents mean ± SEM of three animals.



Effect of Oral Administration of Melatonin on Levels of 5-HT, 5-HIAA, DOPAC and HVA in Rat SCN Dialysates, and on Rat Rotational Activity

Oral administration of 10 mg melatonin at CT 18 significantly increased the concentration of 5-HT in SCN dialysates, reaching a maximum of over 4 ng/mL between 60 and 180 minutes after treatment (***F3A***), and then gradually decreasing. In contrast, the release of 5-HIAA, the primary metabolite of 5-HT, was significantly reduced during the first two hours after the CT 18 melatonin administration, and started to recover about three hours later ($p < 0.01$; Student's *t*-test, ***F3A***). After six hours, 5-HIAA dialysate concentrations had returned to pretreatment levels. The exogenous melatonin however, did not significantly influence either DOPAC or HVA concentrations. Data in ***F3B*** suggests that the administration of 10-mg melatonin at CT 18 slightly, but non-significantly, reduced the rotational activity of the rat.

Administration of water to rats at CT 18 in the control experiment did not have any significant effect either on the concentration of those metabolites sampled from the SCN region or on rotational activity (***F2***). The profile of 5-HT and monoamine metabolites collected during the water treated sham control experiments at different times (CT 6, 12 and 18) were very similar to the baseline profile and no changes were observed.

Oral administration of melatonin in the daytime (CT 6) did not show significant effects either on the profile or extracellular concentration of 5-HT and monoamine metabolites, or on the rotational activity (***F4A*** and ***F4B***).

Discussion

While the effect of exogenous tryptophan administration on the release of 5-HT in the brain has been previously examined (19, 20), the effects of melatonin administration on SCN 5-HT, the catecholamines, and their respective metabolites, have not been previously reported. The present study is the first report on the effect of melatonin administration on 5-HT and monoamine metabolites in rat SCN by using the combination of *in vivo* brain microdialysis sampling and LCEC.

Tominaga *et al.* (11) indicated that 5-HT plays a major role in the regulation of a variety of SCN-generated rhythms. They examined the wheel-running activity of hamsters following the administration of a 5-HT_{1A} receptor agonist. The administration of 5-HT_{1A} agonists at CT 8 induced a significant phase advance of wheel-running activity under constant light conditions. This phase advance was blocked by pretreatment with a 5-HT_{1A} antagonist. These observations suggested that 5-HT_{1A} receptors in the brain participate in the regulation of circadian rhythms. Al-

though the role of serotonergic innervation of the SCN as regards the circadian photoperiodic function has not yet been established, various relationships between diurnal rhythms and 5-HT concentration (21), uptake, and electrophysiological effects (22) have been reported. Moreover, systemic treatment with the serotonergic agonist quipazine inhibited the discharge of SCN neurons activated by retinal light exposure, while the administration of the 5-HT antagonist metergoline, reversed the effects of quipazine (23). However, no direct evidence showing a relationship between 5-HT and melatonin has previously been reported.

The present study demonstrated that exogenous melatonin markedly increased the extracellular concentration of 5-HT in the rat SCN (***F3A***). This effect is suggestive of a relationship between both melatonin and 5-HT in the modulation of circadian rhythms. Kalen *et. al*. have previously shown that while the concentration of 5-HIAA was reduced, the concentration of 5-HT was elevated by neural depolarization via agents such as KCl (24). A similar inverse relationship between 5-HIAA and 5-HT concentrations in the SCN was also observed in this study (***F3A***). This observation is consistent with the idea that extracellular 5-HIAA levels reflect primarily the intra-neuronal metabolism of an unreleased pool of 5-HT (18). The decrease of 5-HIAA after administration of melatonin at CT 18 might be explained by an alteration in the metabolism of unreleased 5-HT, i.e. inhibition of monoamine oxidase and/or blockage of 5-HT reuptake (25). However, numerous pharmacological investigations have indicated that 5-HIAA may be an unreliable indicator of serotonin release (26-29). Collectively, these observations suggest that one may want to exercise caution if attempting to infer serotonergic neurotransmission based on the measurement of 5-HIAA.

Our experiments showed a dramatic increase in the extracellular concentration of 5-HT in the SCN when melatonin was administered to the rats at night (CT 18, ***F3A***). However, there was no significant difference when the melatonin was administered during the day (CT 6, ***F4A***). This differing response to melatonin administered in the day versus the night is intriguing, and suggests that the SCN serotonergic system's responsiveness to melatonin may be regulated by photic information from the retina through the retinohypothalamic tract.

A major problem in studying 5-HT release from the SCN is the extremely low basal level of 5-HT, which is often close to the detection limit. The use of an uptake inhibitor in such cases may be helpful. However, experiments using an inhibitor may cause unexpected or unknown physiological changes in the target. In this study, we used a microbore column to optimize the LCEC analysis, so we were able to examine the levels of extracellular 5-HT in the SCN without the use of an uptake inhibitor. In addition, the simultaneous monitoring of the rotational activity of rats during *in vivo* microdialysis, or other experimental procedures, may prove advantageous in the investigation of the relationship between long-term rhythmic behavior of freely moving animals and neurochemical regulation.

Conclusion

This preliminary study demonstrates the effects of melatonin administration on the release of 5-HT and monoamine metabolites in the rat SCN by means of *in vivo* brain microdialysis sampling coupled to LCEC. The increased release of 5-HT by exogenous melatonin during the nighttime (CT 18) but not the daytime (CT 6) is taken as evidence to support the involvement of melatonin in the regulation of serotonergic transmission in the SCN. Such regulation may play a key role in modulating circadian rhythms. In addition, the ability to simultaneously monitor behavioral and chemical phenomena using the Raturn is demonstrated.

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References

1. V.M. Cassone, Trends Neurosci 13 (1990) 457-464.
2. A.B. Dollins, I.V. Zhdanova, R.J. Wurtman H.J. Lynch, M.H. Deng, Proc. Natl. Acad. Sci. USA 91 (1994) 1824-1828.
3. S.M. Armstrong, Experientia 45 (1989) 932-938.
4. J. Redman, S. Armstrong, and K.T. Ng, Science 219 (1983) 1089-1091.
5. T.J. Bartness and B.D.Goldman, Experientia 45 (1989) 939-945.
6. A.D.Ramirez, V.D. Ramirez, and D.C.Meyer, Neuroendocrinology 46 (1987) 430-438.
7. S.T. Inouye and S. Shibata, Neurosci Res. 20 (1994) 109-130.
8. R.R. Margraf and G.R. Lynch, Brain Res. 609 (1993) 45-50.
9. S.J. Starkey, M.P. Walker, I.J.M. Beresford and R.M. Hagan, NeuroReport 6 (1995) 1947-1951.
10. M. Masson-Pevet, L. Bianchi, and P. Pevet, J. Neurosci. Res. 43 (1996) 632-637.
11. K. Tominaga, S. Shibata, S. Ueki, and S. Watanabe, Eur. J. Pharmacol. 214 (1992) 79-84.
12. GJ Siegel, BW Agranoff, RW Alpers, SK Fisher, MD Uhler, Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th Edition. Lippencott-Raven Press, Philadelphia. 1999.
13. T.E. Robinson and J.B. Justice, Microdialysis in the Neuroscience. Elsevier, Amsterdan (1991).
14. S. Honma, Y. Katsuno, K. Shinohara H. Abe, and K. Honma, Am. J. Physiol. 271 (1996) R579-R585.
15. A. Kalsbeek, R.M. Buijs, M. Engelmann, C.T. Wotjak, R. Landgraf, Brain Res. 682 (1995) 75-82.
16. M. Kubota, R. Landgraf, and C.T. Wotjak, NeuroReport 7 (1996) 1933-1936.

17. N. Ozaki, D. Nakahara, Y. Kasahara, and T. Nagatsu, J. Neural. Transm. (Gen. Sect.) 86 (1991) 175-179.

18. J.D. Glass, W.W. Randolph, S.A. Ferreira, M.A. Rra, U.E. Hauser, J.L. Blank, and M.J. De Vries, Neuroendocrinology 56 (1992) 582-590.

19. J.D. Schaechter and R.J. Wurtman, J. Neurochem. 53 (1989) 1925-1933.

20. J.D. Glass, M. Selim, G. Srkalovic, and M.A. Rea, J. Biol. Rhythms 10 (1995) 80-90.

21. S. B. Auerbach, M.J. Minzenberg, and L.O. Wilkinson, Brain Res., 499, 281-290.

22. R. Mason, J. Physiol. 377 (1986) 1-13.

23. J.D. Miller and C.A. Fuller, Brain Res. 515 (1990) 155-162.

24. P. Kalen, R.E. Strecker, E. Rosengren, and A. Bjorklund, J. Neurochem. 51 (1988) 1422-1435.

25. W. Wesemann, N. Weiner, Neurobiol. 35 (1990) 1160-1165.

26. L. Hernandez, F. Lee, B. Hoebel, Brain Res. Bull. 19 (1987) 623-628.

27. T. Sharp, G.A. Foster, J. Neurochem. 53 (1989) 303-306.

28. T. Sharp, S.R. Bramwell, D. Clark, D.G. Grahame-Smith, J. Neurochem. 53 (1989) 234-240.

29. H. Shimizu, G.A. Bray, Physiol. Behav. 46 (1989) 799-807.