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Analysis of Serotonin, Dopamine and Their Metabolites in the Caudate Putamen, the Suprachiasmatic Nucleus and the Median Raphe Nucleus of Euthermic and Torpid Deermice, *Peromyscus maniculatus*

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LIN, L.-H. AND E. B. PIVORUN. *Analysis of serotonin, dopamine and their metabolites in the caudate putamen, the suprachiasmatic nucleus and the median raphe nucleus of euthermic and torpid deermice*, Peromyscus maniculatus. PHARMACOL BIOCHEM BEHAV **33**(2) 309–314, 1989.—Deermice, subjected to food rationing and low ambient temperature, were sacrificed in normothermia or during daily torpor. Levels of monoamines and their metabolites in the caudate putamen (CPN), the suprachiasmatic nuclear area (SCN), and the median raphe nucleus (MRN) were quantified through the use of HPLC with electrochemical detection. Significant elevations in levels (pg/mg protein) of the serotonin (5-HT) metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) were noted in torpid individuals in all nuclei examined. The dopamine (DA) metabolite, homovanillic acid (HVA) was significantly elevated in the CPN and MRN of torpid individuals. Moreover, a significant increase in the HVA to DA ratio was also noted in the CPN and the MRN. In the SCN, the concentrations of 5-hydroxytryptophan (5-HTP), 5-HT, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were also increased significantly during torpor. These significant elevations suggest that an increase in the activity of the serotonergic and dopaminergic systems occurs in these nuclei during daily torpor in the deermouse.

HPLC Torpor Hibernation Serotonin Dopamine Deermouse Suprachiasmatic nucleus Caudate putamen Median raphe nucleus

THERE is general agreement that brain monoamines have an important role in the regulation of hibernation. Several investigators suggest that brain serotonin is involved in the control of the entry and the maintenance phases of the hibernation state (2). The serotonin (5-HT) levels in the whole brain or in various regions of the brain are elevated in the hibernating hedgehog (29), ground squirrel (13), and golden hamster relative to awake individuals (7,8). Moreover, the turnover rate of brain 5-HT increases about 14 times at the entry phase and 24 times during hibernation in the golden hamster. However, some investigators report decreased (1) or unchanged (6) brain serotonin levels during hibernation.

The catecholamines, in particular, norepineprhine (NE), have been implicated in mediating the arousal phase terminating individual hibernation bouts. Brain NE has been reported to decrease in the arctic ground squirrel during hibernation and to increase in the early and midarousal period (6). The turnover rates of NE and epinephrine (EP) have been found to be greatly diminished or arrested in hibernating hedgehogs and ground squirrels (5,23). No major change in brain NE, however, has been reported in the European hamster (1). The role of dopamine (DA) in hibernation has not been extensively studied, although Kilduff *et al.* (11) reported that the total tissue concentration of the DA metabolite, homovanillic acid (HVA), in the caudate nucleus of hibernating *Citellus lateralis* is decreased despite increased extracellular DA levels. Salzman *et al.* (21) also reported a decrease in HVA efflux during hibernation in the same species.

In contrast to hibernation, there is a paucity of information regarding the neuroendocrine regulation of the phenomenon of daily torpor. *Peromyscus maniculatus*, the deermouse, displays both spontaneous and induced (food-rationed) daily torpor bouts, attaining minimum body temperatures of 15–20°C and bout durations of 5–12 hr. Through the use of telemetry, Pivorun and Astwood (20) have demonstrated that administration of p-chlorophenylalanine (pCPA), a 5-HT synthesis inhibitor, completely inhibited torpor or reduced the duration and depth of torpor bouts in deermice (20). The administration of apomorphine, a DA agonist, also reduced the incidence, duration and depth of torpor in this species; while administration of butaclamol, a DA antagonist,

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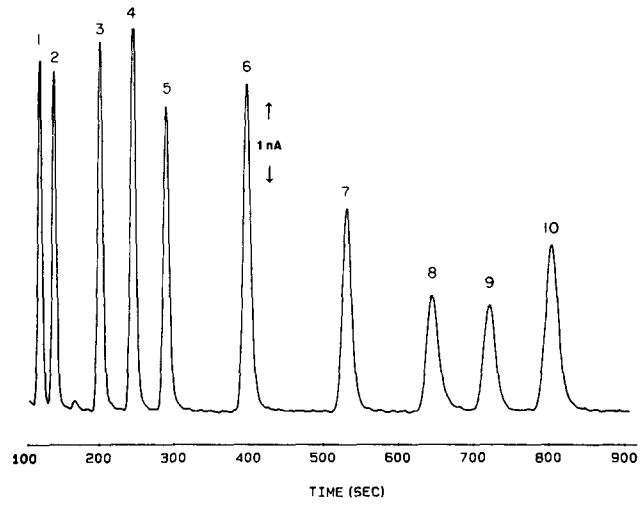

FIG. 1. Representative chromatogram of the standards. 1) NE; 2) EP; 3) 5-HTP; 4) DOPAC; 5) DA; 6) 5-HIAA; 7) HVA; 8) 3-MT; 9) Methyl L-DOPA; 10) 5-HT.

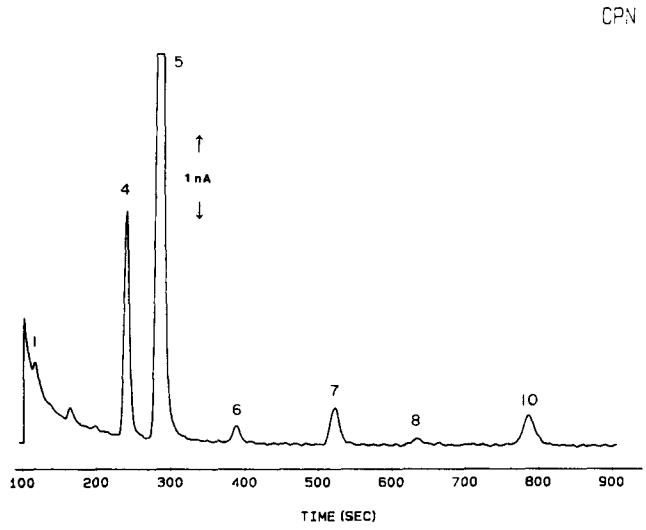

FIG. 2. Representative chromatogram of caudate putamen. 1) NE; 4) DOPAC; 5) DA; 6) 5-HIAA; 7) HVA; 8) 3-MT; 10) 5-HT.

resulted in a significant increase in the duration and depth of torpor bouts.

In the present paper, we examined the changes in levels of biogenic amines associated with daily torpor bouts in *Peromyscus maniculatus* through the use of HPLC with electrochemical detection. Analyses were performed on three areas of the brain: 1) the caudate putamen (CPN)—an area rich in DA nerve terminals and considered to have a role in hibernation (11,21), 2) the suprachiasmatic nuclei (SCN)—nuclei containing a high density of 5-HT terminals and considered as the major pacemaker of the mammalian circadian system (3), and 3) the median raphe nucleus (MRN)—a primary production site of serotonin and implicated in the control of the entrance phase of hibernation (2).

METHOD

Reagents

Norepinephrine hydrochloride (NE), epinephrine bitartrate salt (EP), 5-hydroxytrytophan (5-HTP), dopamine hydrochloride (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), 5-hydroxytryptamine creatine sulfate (5-HT), 1-β-3,4-dihydroxyphenylalanine methyl ester hydrochloride (methyl-l-DOPA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 3-methoxytyramine hydrochloride (3-MT), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxybenzylamine hydrobromide (DHBA), tetrahydrofuran, and monochloroacetic acid were purchased from Sigma Chemical Co., St. Louis, MO. Sodium octyl sulfate was purchased from Eastman Kodak Co., Rochester, NY. Sodium hydroxide, ethylene-diaminetetracetic acid (EDTA) disodium, and HPLC grade methanol were obtained from Fisher Scientific Co., Chicago, IL. The mobile phase consisted of 14.2 g monochloracetic acid, 4.7 g NaOH, 10.0 mg disodium EDTA and 150 mg sodium octyl sulfate in 967 ml of doubly distilled deionized water with 15 ml of methanol and 18 ml of tetrahydrofuran added. The mobile phase was degassed and filtered through 0.20 μm pore size filters before the addition of methanol and tetrahydrofuran. Reference standard solutions (10^6 pg free base/μl) of NE, EP, 5-HTP, DOPAC, DA, 5-HIAA, HVA, 3-MT, 1-DOPA, and 5-HT were made using mobile phase and stored at −20°C. Working standard solutions (50 or 25 pg free base/μl) were prepared each day by serial dilution and were maintained at 4°C.

Animals

First and second generation offspring of deermice *(Peromyscus maniculatus)* that had been trapped in their natural habitats and maintained at room temperatures of 22–25°C were used in the study. Young animals of 4 months to 1 year of age of both sexes were transferred to environmental chambers kept at 10°C under a 9L:15D photoperiod (lights on at 0800 EST). After 4–6 weeks of acclimation to these conditions, individually caged animals were fed a 2.5 g pellet of Purina Rat Chow once daily. This quantity represents approximately 60–75% of the daily ad lib consumption at an ambient temperature of 10°C. Cotton was provided as nesting material and water was available ad lib. After 3–9 days of rationing, animals were sacrificed by cervical dislocation between 1100 and 1200 hr either in a normothermic or torpid state. The body temperature was measured with a computerized thermometer (Model HH-71T, Omega, Stamford, CT) with a needle probe inserted into the abdominal cavity immediately after the cervical dislocation. Torpor was defined as being present in any animal showing a body temperature less than 30°C at time of measurement. All torpid mice displayed body temperatures between 15 to 25°C. The brains were quickly removed, frozen and stored immediately at −80°C until they were analyzed (less than 3 weeks).

Brain Microdissection

A micropunch technique (19) was used to dissect out the CPN, the SCN and the MRN from unfixed, frozen brain sections. Brains were removed from the deep freezer and mounted rostral side up on a specimen stage using a glycol matrix (O.C.T. compound, Lab-Tek Products, Naperville, IL) and sliced coronally every 50 μm to determine the correct location of the respective nuclei using a cryostat (CTF Microtome-Cryostat, International Equipment, Needham Hts, MA) with a chamber temperature of −6°C. Once the correct position was located, 300 μm thick sections were cut and arranged on the surface of the cutting knife (−6°C), and the

TABLE 1

BIOGENIC AMINE LEVELS (pg/μg PROTEIN*) IN THE CAUDATE PUTAMEN OF EUTHERMIC VERSUS TORPID DEERMICE

	DOPAC	DA	5HIAA	HVA	3-MT	5-HT
Euthermia	20.40 ± 2.42 (17)	70.02 ± 4.47 (17)	0.65 ± 0.08§ (17)	4.17 ± 0.48‡ (17)	2.27 ± 0.22† (17)	1.96 ± 0.21 (17)
Torpor	23.82 ± 1.82 (24)	67.14 ± 2.92 (24)	1.17 ± 0.09 (24)	6.25 ± 0.36 (24)	1.64 ± 0.20 (24)	2.26 ± 0.21 (24)

All values are mean ± S.E.M.
Numbers in parentheses indicate sample size.
Comparisons and levels of significance between euthermic and torpid deermice.
*No significant difference between protein content of euthermic (84.0 ± 1.8 μg) and torpid (86.6 ± 3.8 μg) CPN extracts.
†$p<0.05$; ‡$p<0.01$; §$p<0.001$.

CPN, the SCN (bilaterally), and the MRN (unilaterally) were micropunched from consecutive brain sections using thin-walled stainless steel tubing of 0.81 mm i.d. (Small Parts, Inc., Miami, FL) with the aid of a 10× magnifier. A total of 6, 2, and 3 punches were taken respectively for the CPN, SCN and MRN. The atlases of Montemurro and Dukelow (17), Sidman and Angevine (25) and König and Kippel (12) were used as general guides. These frozen samples were pushed into 250 μl polypropylene microcentrifuge tubes by the inner tubing and stored at −80°C until analyzed.

Chromatography

Micropunched samples were homogenized by ultrasonic disruption in 200 μl (CPN) or 100 μl (SCN and MRN) of chilled mobile phase and then centrifuged at 14,000 rpm for 8 min using a microcentrifuge (Model 5415, Eppendorf, Brinkmann Int., Westburg, NY) at 5°C. The supernatant was kept in ice (less than one hour) until injection into the chromatographic system. The chromatographic system consisted of a Bioanalytical Systems LC-4B amperometric detector, PM-48 pump, C-18 reverse-phase column (100 × 3.2 mm, 3 μm ODS), and LC-17 glassy carbon transducer with a LC-22A temperature controller set at 26°C (Bioanalytical Systems Inc., Lafayette, IN). 30 μl of supernatant was injected into a rotary injection valve (Model 7125, Rheodyne, Berkeley, CA) fitted with a 20 μl sample loop. The flow rate was 0.5 ml/min, with the electrode potential maintained at +0.8 volts versus Ag/AgCl (3 M NaCl). The precipitant was resuspended by adding 0.2 N NaOH and the amount of protein was determined according to the method of Lowry *et al.* (15).

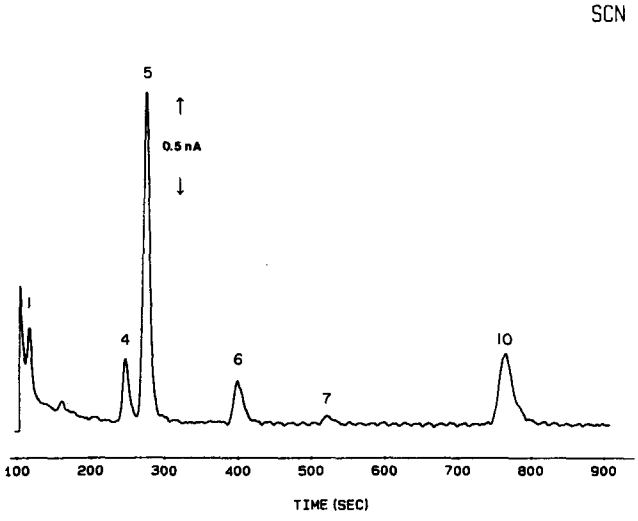

FIG. 3. Representative chromatogram of the suprachiasmatic nucleus. 1) NE; 4) DOPAC; 5) DA; 6) 5-HIAA; 7) HVA; 10) 5-HT.

Calculation and Statistical Analysis

The concentration of amines eluted from the HPLC column was calculated from comparisons between the area of recorded peaks from standards and tissue extracts by using the BASpc scientific workstation software on a IBM PS/2 model 30 microcomputer. DHBA was used as an internal standard to correct for sample loss. The concentration of amines in tissue was expressed as pg/μg protein.

Student *t*-tests (26) and analysis of variance (28) were used to test the significance of differences among mean values. The probability level for accepting the null hypothesis that there were no differences between group means was 0.05.

RESULTS

Caudate Putamen (CPN)

Figures 1 and 2 illustrate the chromatographic runs obtained with standards and a typical CPN extraction. Analysis of the CPN concentration of NE, 5-HT, DA and their metabolites did not reveal any significant differences between male and female deermice (data not shown). As can be seen from Table 1, the concentration of 5-HIAA and HVA increased by 180% ($p<0.001$) and 150% ($p<0.01$) respectively during daily torpor, while 3-MT levels decreased to approximately 70% ($p<0.05$) of euthermic levels. The ratio between HVA and DA was significantly elevated in the torpid state (0.098 ± 0.008) relative to the euthermic state (0.065 ± 0.010) while the 3-MT to DA ratio was significantly lower (0.025 ± 0.003 vs. 0.034 ± 0.003). There were no significant differences noted in the concentrations of 5-HT, DA and DOPAC between the torpid and the euthermic states.

Suprachiasmatic Nuclear (SCN) Area

Since the SCN is a very small nucleus, our micropunched tissue included both the SCN proper as well as small amounts of

TABLE 2

BIOGENIC AMINE LEVELS (pg/μg PROTEIN*) IN THE SUPRACHIASMATIC NUCLEUS OF EUTHERMIC VERSUS TORPID DEERMICE

	NE	5HTP	DOPAC	DA	5HIAA	HVA	5-HT
Euthermia	46.76 ± 5.37 (17)	0.80 ± 0.19† (8)	1.48 ± 0.15§ (17)	4.80 ± 0.88† (17)	3.09 ± 0.34‡ (17)	3.47 ± 1.30 (4)	6.82 ± 1.06† (17)
Torpor	40.11 ± 5.69 (27)	1.28 ± 0.19 (17)	3.14 ± 0.35 (26)	10.44 ± 2.45 (26)	6.55 ± 1.07 (26)	5.53 ± 2.58 (10)	10.88 ± 1.26 (25)

*No significant difference between protein content of euthermic (23.9 ± 1.3 μg) and torpid (23.3 ± 1.7 μg) SCN extracts.
†$p<0.05$; ‡$p<0.01$; §$p<0.001$.

surrounding tissue and is therefore referred to as the SCN area. Figure 3 displays a typical chromatographic run of an SCN extraction. No significant differences in the levels of all the biogenic amines or metabolites in the SCN were noted between male and female mice (data not shown). Significant elevations in levels of DA (220%, $p<0.05$) and 5-HT (160%, $p<0.05$) were observed in the torpid state (Table 2). Moreover, significant increases in the levels of DOPAC (210%, $p<0.001$), 5-HIAA (210%, $p<0.01$) and the 5-HT precursor, 5-HTP, (160%, $p<0.05$) were also associated with torpor. No significant changes in the ratios of metabolites to DA or to 5-HT were noted in the torpid compared to the euthermic states.

Median Raphe Nucleus (MRN)

A typical chromatographic run of a MRN extraction is shown in Fig. 4. There were no differences in the concentration of NE, 5-HT, DA and their metabolites in the MRN between male and female deermice. However, the concentration of 5-HTP in male mice (1.29 ± 0.27 pg/mg protein, n = 24) was 2 times higher than that of female mice (0.64 ± 0.07 pg/mg protein, n = 18, $p<0.05$). The torpid deermice displayed no significant differences in levels of NE, 5-HTP, DOPAC, DA or 5-HT in the MRN compared to the euthermic animals. However, significant elevations of 5-HIAA (170%, $p<0.0001$) and HVA (180%, $p<0.05$) were observed during torpor (Table 3). Moreover, the HVA to DA ratio was significantly elevated in the torpid state (0.605 ± 0.138 vs. 0.264 ± 0.054).

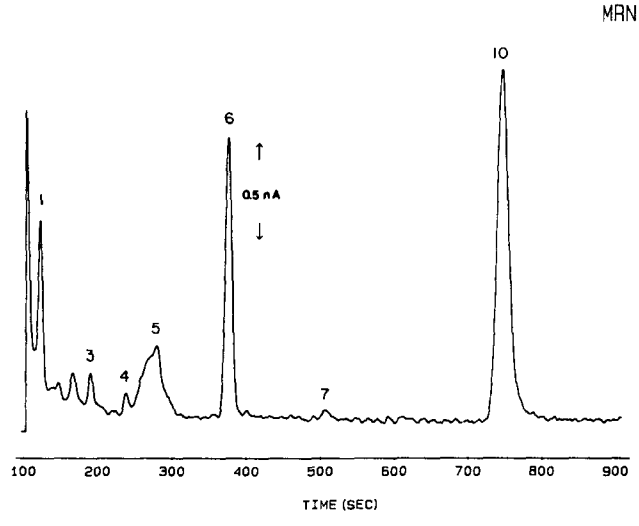

FIG. 4. Representative chromatogram of median raphe nucleus. 1) NE; 3) 5-HTP; 4) DOPAC; 5) DA; 6) 5-HIAA; 7) HVA; 10) 5-HT.

DISCUSSION

CPN

No significant differences in the concentration of DA and its metabolite, DOPAC, were noted in the CPN between the torpid and euthermic states. However, the levels of the other DA metabolite, 3-MT, decreased, while the levels of the end product of DA metabolism, HVA, increased significantly in the torpid state. 3-MT has been implicated as an indicator of DA release (24) and is the result of DA degradation by the action of catechol-O-methyl-transferase (COMT), which is characteristically found in the synaptic cleft (9). Further degradation of 3-MT occurs through the action of monoamine oxidase (MAO) and leads to the formation of HVA. Since there was a significant decrease in levels of 3-MT and a significant depression in the 3-MT to DA ratio in the torpid state, the increase in HVA levels may be the results of an increased activity in MAO present in the synaptic cleft. Another known metabolic pathway leading to the formation of HVA is by the way of DOPAC. However, neither the concentration of DOPAC nor the DOPAC to DA ratio were altered during daily torpor, indicating that the increase in HVA levels in the CPN of torpid deermice may not be the result of the activation of this alternative pathway.

Since HVA is regarded by some authors (18) as a better index of DA release than either DOPAC or 3-MT, the increase in concentration of HVA indicates that the dopaminergic system may be activated during daily torpor. This hypothesis is further strengthened by the fact that a significant elevation in the HVA to DA ratio was characteristic of the torpid state. However, the elevated levels of HVA may not unequivocally represent an activation of the dopaminergic system. Although, DA efflux was elevated during bouts of hibernation in the golden-mantled ground squirrel, Salzman *et al.* (21,22) argued that this increase was not due to enhanced dopaminergic activity, but was a consequence of the saturation and/or inactivation of catabolic and reuptake mechanisms. Moreover, temperature dependent reductions in bulk diffusion, acid transport, and blood flow were also considered contributing factors for the observed increase in DA efflux. In the torpid deermouse the accumulation of HVA may be due to a depression in these temperature dependent clearance mechanisms. Specifically a reduction in transport of the acid metabolites during torpor could result in the accumulation of HVA, even if dopaminergic activity remains stable or actually decreases.

The push-pull perfusion studies of Salzman *et al.* (21) and the post-mortem striatal homogenate analyses of Kilduff *et al.* (11) on hibernating ground squirrels have shown that the efflux and

TABLE 3

BIOGENIC AMINE LEVELS (pg/μg PROTEIN*) IN THE MEDIAN RAPHE NUCLEUS OF EUTHERMIC VERSUS TORPID DEERMICE

	NE	5HTP	DOPAC	DA	5HIAA	HVA	5-HT
Euthermia	8.16 ± 0.62 (17)	0.98 ± 0.14 (17)	1.10 ± 0.14 (17)	2.77 ± 0.61 (17)	5.29 ± 0.54‡ (17)	1.29 ± 0.20† (4)	15.86 ± 1.72 (17)
Torpor	8.15 ± 0.64 (25)	1.03 ± 0.26 (25)	1.91 ± 0.61 (25)	3.92 ± 0.55 (25)	8.95 ± 0.59 (25)	2.32 ± 0.23 (10)	17.44 ± 1.82 (25)

*No significant difference between protein content of euthermic (53.1 ± 2.0 μg) and torpid (47.8 ± 1.9 μg) MRN extracts.
†$p<0.05$; ‡$p<0.0001$.

concentration of HVA in the striatum is decreased during hibernation. Kilduff *et al.* (11) have suggested that the degradation of DA is shifted away from HVA during deep hibernation. Salzman *et al.* (21,22) observed that DA metabolism was shifted from oxidation to reduction and that 3-methoxy-4-hydroxyphenethanol (MOPET) was the primary metabolite of DA produced during the hibernating state. The current results indicate that this may not be the case with daily torpor. An earlier study on the effects of intrahypothalamic administration of biogenic amines on thermoregulation in the euthermic deermouse suggested that the neurohormonal control of body temperature may differ between species that display daily torpor and hibernation (14). This current study also supports the contention that there may be differences in the neuroendocrine regulation of hibernation and daily torpor.

The concentration of the 5-HT metabolite, 5-HIAA, was increased significantly in the CPN during a torpor bout, while the concentration of 5-HT remained unchanged. Since 5-HIAA has been confirmed as an excellent index of 5-HT release (4), our data suggest that 5-HT may undergo rapid turnover in the CPN during daily torpor. However, turnover studies utilizing microdialysis probes or push-pull cannulae are needed to determine if 5-HT turnover rates are indeed elevated during torpor. In contrast, Salzman *et al.* (22) determined that a three-fold decrease in 5-HIAA efflux occurred in the caudate nucleus of the hibernating ground squirrel (*C. lateralis*). These differences again suggest that the neuroendocrine modulation of daily torpor in the deermouse may be different from that of hibernation. Furthermore, the literature on brain 5-HT levels during hibernation has been contradictory with some researchers finding increased brain 5-HT levels during hibernation (7, 8, 13, 29), some noting decreased levels (1,27), and others observing constant levels (6). It has been suggested that central 5-HT metabolism during hibernation is species specific (22).

SCN

Our data indicate that the dopaminergic system in the SCN area may be activated during daily torpor. The concentrations of DA and DOPAC increased to more than 200% above levels characteristic of the euthermic state. An elevation in HVA concentration was also noted during torpor, but these levels were not statistically different from euthermic levels. The fact that the concentration of NE in the SCN remained unchanged, indicates that the noradrenergic system may not participate in modulating daily torpor. However, our data do not provide information on the changes in the levels of NE metabolites. Further investigations are needed to clarify the status of the noradrenergic system during daily torpor.

Our results strongly suggest that the serotonergic system may display increased activity in the SCN during the torpid state. The levels of 5-HT, its precursor, 5-HTP, and the 5-HT metabolite, 5-HIAA, were all elevated in the SCN area during a torpor bout. The uptake of 2-deoxyglucose in the SCN is elevated during the entry and the profound hypothermic state of hibernation, indicating that the SCN is playing an important role during hibernation (10). Our results suggest that the SCN may be an important modulator of daily torpor and further suggest that the increased activity in the SCN is due to the activation of both the dopaminergic and the serotonergic systems.

MRN

In torpid deermice the concentration of NE in the MRN remained stable when compared to euthermic animals. However, the lack of information on levels of NE metabolites precludes evaluate of the role of NE in this region during daily torpor. The levels of DA and DOPAC were not significantly changed during the torpid state, while the concentration of HVA and the HVA to DA ratio increased significantly in the torpid individuals. The elevated HVA levels and HVA to DA ratio indicate that the dopaminergic system may be activated in the MRN during daily torpor.

An increase in the activity of the serotonergic system in the MRN of torpid animals is also suggested by the fact that the level of 5-HIAA was significantly elevated. The MRN serotonergic neurons have been implicated in having an important role in modulating hibernation. In *Citellus lateralis*, inhibition of 5-HT synthesis by injection of p-chlorophenylalanine (pCPA) or destruction of MRN serotonergic neurons by use of electrolytic lesions prevents entrance into hibernation (27). In *Cricetus cricetus*, electrolytic lesions of the MRN also disrupts hibernation (16). However, Canguilhem *et al.* (2) believe that in the European hamster only a specific group of serotonergic neurons of the MRN is involved in the process of modulating the entrance phase of a hibernation bout.

In summary, the alterations in levels of DA, 5-HT and their metabolites in the CPN, SCN and MRN in torpid deermice suggests that both the serotonergic and dopaminergic systems may have a positive role in the modulation of daily torpor. However, turnover and efflux studies are required to determine if alterations in metabolite levels during torpor are a consequence of increased neuronal activity or are the result of a decrease in reuptake, clearance, or catabolic processes. The earlier finding in our laboratory that the administration of pCPA inhibited torpor bouts in the deermouse (20) is consistent with our current conclusion. On the other hand, the present study suggests an opposite role for the dopaminergic system in daily torpor as compared to our earlier findings (20). In this earlier study we found that peripheral injection of apomorphine (DA agonist) inhibited torpor, while the injection of butaclamol (DA antagonist) facilitated torpor. Based on this evidence, we suggested that DA has an inhibitory role in the modulation of daily torpor. One possible explanation for the discrepancy in the results between these two studies is that there

may be specific loci in the brain, which also depend upon DA as a neurotransmitter, but their activation results in inhibition of daily torpor. Direct application of different DA agonists and antagonists into specific regions of the brain may help to clarify these contrasting results.

ACKNOWLEDGEMENT

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