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^{14}C-2-Deoxyglucose Uptake in the Ground Squirrel Brain during Entrance to and Arousal from Hibernation

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Neuronal activity underlying various phases of the mammalian hibernation cycle was investigated using the ^{14}C-2-deoxyglucose (2DG) method. Relative 2DG uptake (R2DGU) values were computed for 96 brain regions across 7 phases of the hibernation cycle: euthermia, 3 body temperature (T_b) intervals during entrance into hibernation, stable deep hibernation, and 2 T_b intervals during arousal from hibernation. Multivariate statistical techniques were employed to identify objectively groups of brain regions whose R2DGU values showed a similar pattern across all phases of hibernation. Factor analysis revealed that most of the variability in R2DGU values for the 96 brain regions across the entire cycle could be accounted for by 3 principal factors. These factors could accurately discriminate the various phases of hibernation on the basis of the R2DGU values alone. Three hypothalamic and 3 cortical regions were identified as possibly mediating the entrance into hibernation because they underwent a change in R2DGU early in entrance into hibernation and loaded strongly on one of the principal factors. Another 4 hypothalamic regions were similarly identified as possibly causally involved in the arousal from hibernation. These results, coupled with characteristic changes in ordinal rank of the 96 brain regions in each phase of hibernation, support the concept that mammalian hibernation is an active, integrated orchestration of neurophysiological events rather than a state entered through a passive process.

The neural mechanisms underlying entrance into, maintenance of, and arousal from mammalian hibernation are poorly understood. The entrance into hibernation appears to be mediated through slow wave sleep with rapid-eye-movement (REM) sleep selectively suppressed (Walker et al., 1977). Below a body temperature (T_b) of 25°C, however, cortical activity is so unusual that conventional electroencephalographic criteria for scoring arousal states can no longer be applied. Depth EEG studies reveal that brain regions become electrically quiescent during entrance into hibernation in a replicable sequence: the cortex becomes isoelectric first, at a T_b around 20°C, followed by the thalamus, then the limbic structures such as the hippocampus (Mihailovic, 1972; Shtark, 1972). Electrical activity is maintained in hypothalamic regions throughout hibernation, whereas intermittent activity is present in the amygdala (Strumwasser, 1959). During arousal from hibernation, this sequence is reversed: activity increases first in the hypothalamus, followed by the hippocampus, thalamus, and, finally, the cortex. The only cellular neurophysiological study of hibernation conducted to date (Krilowicz et al., 1988) recorded from cells with a high arousal state selectivity throughout bouts of hibernation; the results support the concept that torpor is largely homologous with slow wave sleep interspersed with brief periods homologous with wakefulness.

We proposed that brain regions that mediate the changes between euthermia and hibernation should be activated to a greater degree during the dynamic phases of entrance and arousal from hibernation than other, passive brain regions not involved in this regulated physiological change of arousal state. Similarly, during deep hibernation, brain regions involved in various homeostatic regulatory processes should be more active than other neural regions. Identification of these brain nuclei by conventional electrophysiological techniques is problematic because such studies of arousal states can only provide a restricted view of brain activity. To provide a more comprehensive view of brain activity during hibernation, we have previously used the autoradiographic ^{14}C-2-deoxyglucose (2DG) method (Sokoloff et al., 1977) to compare the relative 2DG uptake (R2DGU) of brain regions between the hibernating and nonhibernating (euthermic) states (Kilduff et al., 1982). A similar approach has been taken to study neural metabolic activity during sleep in the monkey (Kennedy et al., 1982; Nakamura et al., 1983), rat (Ramm and Frost, 1983), and cat (Ramm and Frost, 1986). Although some counterexamples exist (Schwartz et al., 1979), direct correlations between electrical activity and 2DG uptake have been demonstrated in both the central (Schoppman and Stryker, 1981; Kadekaro et al., 1985) and peripheral (Yarowsky et al., 1983) nervous systems. Therefore, we think it likely that activation of brain regions during any phase of hibernation would result in an increase in neural metabolic activity and be measurable in terms of R2DGU.

Hibernation, perhaps more so than other changes in arousal state, involves widespread changes in the activity of the nervous system. It has been proposed that other arousal states, such as sleep, are generated by interactions among distributed networks of cells rather than by sleep or wake centers (Hobson et al., 1986). Therefore, data analyses that permit simultaneous examination of multiple brain regions may be the most appropriate treatments for hibernation data.

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We report here patterns of 2DG uptake in the brain of the ground squirrel at various phases of the hibernation cycle. We employed multivariate statistical techniques to identify groups of brain regions whose R2DGU values revealed similar patterns across the hibernation cycle. We have found that the stages of hibernation can be easily distinguished on the basis of recurring patterns of R2DGU common to many brain areas. Two patterns are particularly evident: (1) a relative increase in R2DGU as a function of increasing depth of hibernation, as exemplified by the hypothalamus, and (2) a general and rapid decrease in R2DGU as animals enter hibernation, exemplified by certain cortical and limbic regions.

Materials and Methods

Animals

Golden-mantled ground squirrels (*Citellus lateralis*) were housed at 5°C in a 12:12 light-to-dark photoperiod, with lights on at 0700 and off at 1900. At least 1 week prior to an experiment, each animal was implanted with a subcutaneous thermocouple reentrant tube and a chronic jugular catheter. The catheter was externalized to a latex-covered Plexiglas button that was sutured into the middle of each animal's back. The placement of the button allowed ready access to the catheter as animals assumed the stereotypical curled-up posture of hibernation.

Experimental procedures

A total of 45 incubations were conducted in darkness at an ambient temperature of 5°C. Animals were placed in a clear Plexiglas metabolism chamber to which they had previously been acclimated. A thermocouple was placed into the reentrant tube, and the jugular catheter was connected via polyethylene tubing to a syringe on the outside of the chamber. T_b and, during hibernation, metabolic rate were continuously measured. The ^{14}C-2DG was injected at a concentration of 150 μCi/kg and, after an appropriate incubation period as detailed below, the animal was sacrificed with an overdose of sodium pentobarbital also delivered through the remote syringe and catheter. The brain was rapidly removed, frozen, sectioned, and exposed to Kodak SB-5 X-ray film along with ^{14}C-methyl methacrylate standards (Amersham).

Hibernation cycle experiments. A total of 29 animals were injected with 2DG: (1) at 3 different T_b intervals during the entrance into hibernation, (2) during stable deep hibernation, and (3) at 2 T_b intervals during arousal from hibernation. In all hibernation experiments, the incubation period was increased from the standard 45 min used in euthermic experiments (Sokoloff et al., 1977) because of the effects of reduced T_b on metabolism, as explained previously (Kilduff et al., 1982, 1983). During entrance into hibernation, animals were injected (1) during early entrance ($n = 4$) when T_b had fallen to 30°C, with incubations terminated when T_b reached 25°C; (2) during the midphase of entrance ($n = 4$) at a T_b of 25°C, with incubations terminated when T_b had decreased to 20°C; or (3) during the latter phase of entrance ($n = 6$) at a T_b of 20°C, with incubations terminated at a T_b of 15°C. The 2DG incubation period varied between 82 and 145 min in these groups because T_b decreased at a rate of 2.0–3.6°C/hr. Entrances to hibernation began at different times throughout the day, so the time of injection varied from 0940 to 2320. The majority, however, were in the late afternoon and early evening hours.

Six animals were injected during deep hibernation when T_b was below 8°C. The incubation period for this group was 24 hr. Animals were also injected with 2DG and aroused from hibernation at either a low body temperature (the early arousal group, $T_b < 8$°C, $n = 4$) or a higher body temperature (the late arousal group, $T_b = 25$°C, $n = 5$), and incubations were terminated when T_b plateaued at euthermic levels 48–138 min later. These injections occurred from 0817 to 1950.

Euthermic experiments. Four animals were injected with 2DG during euthermia (nonhibernation) at each of 6 times distributed equally throughout the subjective day (0900, 1300, and 1700) and night (2100, 0100, and 0500). To allow comparison with the various hibernation groups, however, data were used only from the 4 euthermic groups whose time of incubation overlapped with the incubation period of the hibernation groups. Specifically, the euthermic groups used for comparison with the hibernation groups consisted of those animals injected under dim red light at 0900, 1300, 1700, and 2100 ($n = 16$).

Data analyses

On the resultant autoradiographs, optical density (OD) measurements were made using a photographic densitometer (Tobias Instruments, Ivyland, PA) that had a 0.33 mm aperture. (This instrument did not allow superimposition of histology and autoradiographs, as do video-based image analysis systems.) In each brain, ODs were measured over 3–5 sections for each of 96 brain regions. This information enabled calculation of the R2DGU for each brain region within each subject using the average brain OD as a denominator (Ramm and Frost, 1983). This modified technique has been used in previous 2DG studies (Schwartz and Gainer, 1977; Fuchs and Moore, 1980; Reppert and Schwartz, 1983, 1984) and provides controls for slight differences in dose of tracer, section thickness, and film development (Plum et al., 1976; Sharp, 1976; Sharp et al., 1983). The mean R2DGU values were then computed for each brain region for each of the 7 experimental groups.

Four specific goals were established for the data analyses: (1) the description of the patterns of R2DGU for all 96 brain regions across the various phases of hibernation, (2) the identification of brain regions that underwent particularly remarkable changes in R2DGU relative to all other brain regions, (3) the classification of brain regions into groups that had similar patterns of R2DGU across the phases of hibernation, and (4) the construction of short lists of candidate brain regions associated with the initiation of the entrance to and arousal from hibernation. As illustrated in Figure 1, 3 types of data analyses were used to achieve these goals: (1) informal analyses (goals 1 and 2), (2) multivariate descriptive statistical procedures to identify common patterns of R2DGU variation among brain regions (goal 3), and (3) inferential analyses based on a combination of multivariate and univariate statistical procedures (goal 4).

The first goal of the data analyses was met by plotting mean R2DGU values across the 7 phases of the hibernation cycle for all 96 brain regions. Visual inspection of these R2DGU profiles allowed a preliminary classification of brain regions into groups (goal 3). The initial classification scheme was based on subjective criteria (see Results) but provided guidance for the more formal procedures described below.

To meet the second goal, an ordinal rank analysis of the R2DGU values was conduted. Mean R2DGU values of all 96 brain regions were ranked ordinally, from highest to lowest, for each of the 7 experimental conditions. Brain regions whose R2DGU values changed remarkably with respect to all other brain regions were then readily identified by inspection of the ordinal rank values.

The third goal of these analyses was classification of brain regions into groups that had similar patterns of R2DGU across hibernation. From visual inspection of the data, it was apparent that R2DGU values of many of the 96 brain regions covaried across the hibernation cycle. Therefore, exploratory factor analysis was used to reduce the 96 variables to a small set of independent indices (factors) that represented global patterns of R2DGU variation common to many brain regions. The first 6 of these factors were then used as dependent variables in a discriminant analysis of the stages of hibernation, enabling evaluation of the relative strength of the factors in discriminating the stages of hibernation. As a check on the validity of the discriminant functions, classification of animals to the appropriate phase of hibernation was attempted based on their factor scores alone. Finally, brain regions were identified and sorted into groups on the basis of strong ($|r| \geq 0.5$) and consistent correlations with one of the extracted factors across the phases of hibernation.

The fourth goal of the analysis was the compilation of short lists of candidate brain regions most likely to be intimately associated with the initiation of entrance to and arousal from hibernation. As indicated above, the fact that R2DGU values of many of the 96 brain regions covaried suggested the existence of underlying global patterns of R2DGU variation in the data. Therefore, candidate brain regions might be expected to exhibit one of these global patterns and to undergo a change in R2DGU very early in entrance to or arousal from hibernation. Because brain regions involved in mediating the entrance to or arousal from hibernation should therefore be phase-advanced relative to the rest of the brain in undergoing a change in R2DGU, Bonferroni t tests were used to compare R2DGU between euthermia and early entrance and between hibernation and early arousal for each of the 96 brain regions (the latency analyses). Thus, the criteria for assigning a neural structure to the list of candidate brain regions were (1) strong and consistent loading on (i.e., correlation with) one of the derived factors across the phases of hibernation, and (2) a significant change in R2DGU during

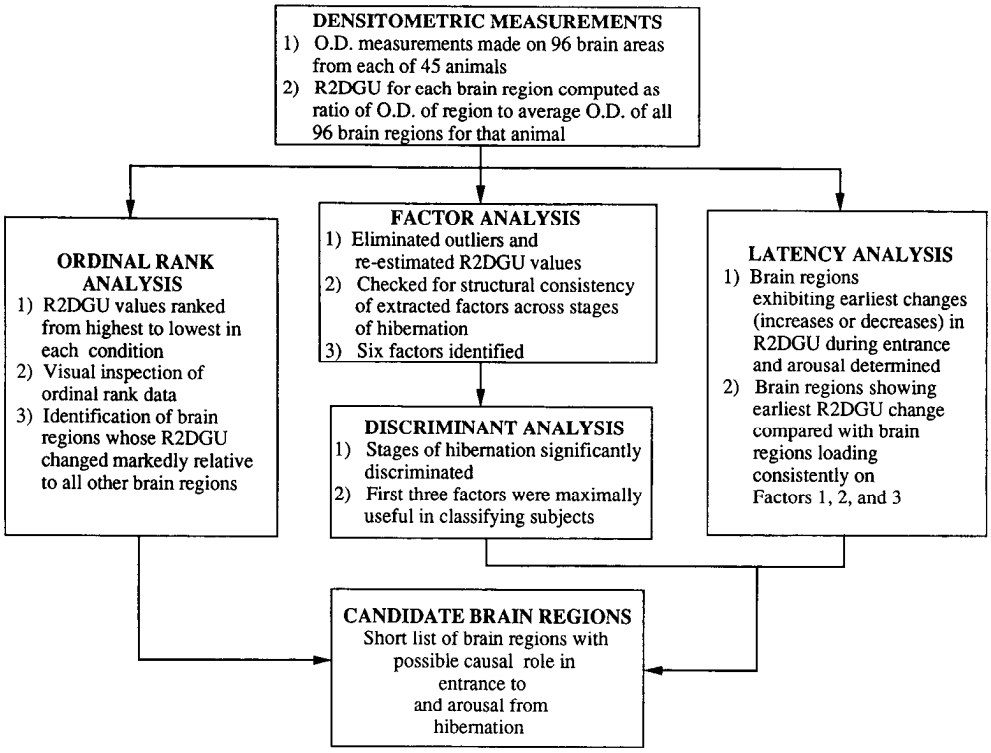

Figure 1. Flow chart of analytical procedures for R2DGU data derived from ^{14}C-2-deoxyglucose autoradiographs of 45 animals distributed across the hibernation cycle.

early entrance to or early arousal from hibernation. The overall strategy for data analyses is summarized in Figure 1.

Results

Goal 1: description of the patterns of R2DGU across the phases of the hibernation cycle

Representative autoradiographs through the diencephalon of the squirrels during each of the 4 major phases of the hibernation cycle are presented in Figure 2. The patterns of R2DGU derived from such autoradiographs during various phases of the hibernation cycle for some selected brain structures are presented in Figure 3. These R2DGU profiles were most readily classified by visual inspection according to associated changes in T_b: brain regions could be classified as undergoing a change in R2DGU in parallel with (Fig. 3, *A, B*) or inversely to (Fig. 3, *C, D*) the change in T_b. The R2DGU profiles of other structures paralleled T_b except for 1 phase of the hibernation cycle (Fig. 3, *E, F*). Still other structures showed no consistent relationship to T_b (Fig. 3, *G, H*). A rough classification of brain regions into these groups was performed by eye and subsequently refined by statistical analyses (see goal 3 below).

Goal 2: identification of brain regions that undergo particularly remarkable changes in R2DGU

Brain regions that markedly increased or decreased their R2DGU values relative to all other brain regions across the hibernation cycle were deemed to be of particular interest. Such changes were readily apparent in the ordinal ranking of R2DGU values. The ordinal ranks of all 96 structures examined in each of 7 phases of the hibernation cycle are presented in Table 1. Each phase of the hibernation cycle had a characteristic "metabolic landscape" as defined by the ordinal ranking of the 96 brain regions within that phase. Contiguous stages of hibernation were characterized by generally similar ordinal rankings.

The ordinal ranking of the majority of the brain regions changed very little or in no consistent pattern across hibernation. Of particular interest were the few brain areas that changed ordinal rank either parallel with or inversely to the change in T_b across the stages of hibernation. Brain regions in the former category included the cerebellar, parafascicular, mammillary and basal amygdaloid nuclei, and lateral preoptic area. The spinal and superior vestibular nuclei and superior olivary nuclei also underwent a consistent decline in ordinal rank as T_b declined, except for the euthermic value, which was lower than or equal to the early entrance rank. Brain regions in the latter category included the paratrigeminal nucleus, locus coeruleus, dorsal raphe nucleus, pontine nuclei, nucleus reticularis pontis oralis, and suprachiasmatic nucleus. The dramatic change in ordinal rank previously described for the paratrigeminal (Kilduff et al., 1988) and suprachiasmatic nuclei (Kilduff et al., 1989) was of particular note in this group.

Goal 3: classification of brain regions into groups that have similar patterns of R2DGU across hibernation

As indicated above, visual inspection of the data suggested that R2DGU values of many of the 96 brain regions covaried. Formal multivariate statistical procedures were used as an objective means of classification of the brain regions into subsets that had similar patterns of R2DGU across the hibernation cycle.

Factor analysis. As a first step, the 96 variables were reduced by exploratory factor analysis to a small set of independent indices (factors) that accounted for the variance in the data. While we are aware that an exact factor analytic solution of this data set was not possible because the number of dependent variables exceeded the number of subjects, a generalized solution was implemented for descriptive purposes and was validated by discriminant analyses (see below). On the basis of the Cattell scree test, consideration of the sorted correlation matrix,

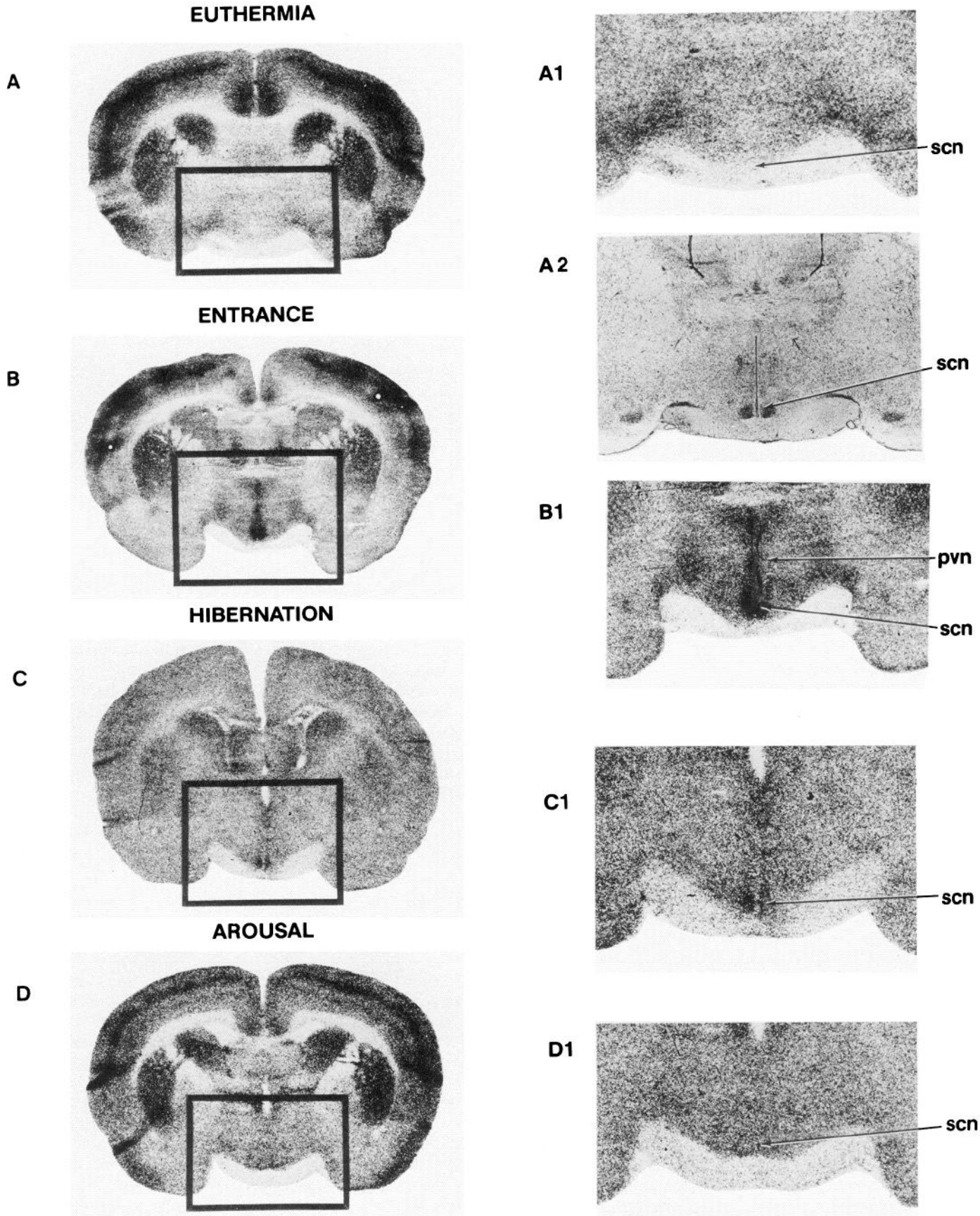

Figure 2. ^{14}C-2-deoxyglucose autoradiographs through the diencephalon of animals incubated during 4 stages of the hibernation cycle. The areas surrounded by the rectangles in panels *A–D* are enlarged on the *right* in panels *A1–D1*; panel *A2* is a Nissl stain corresponding to *A1*. scn, suprachiasmatic nucleus; pvn, periventricular nuclei.

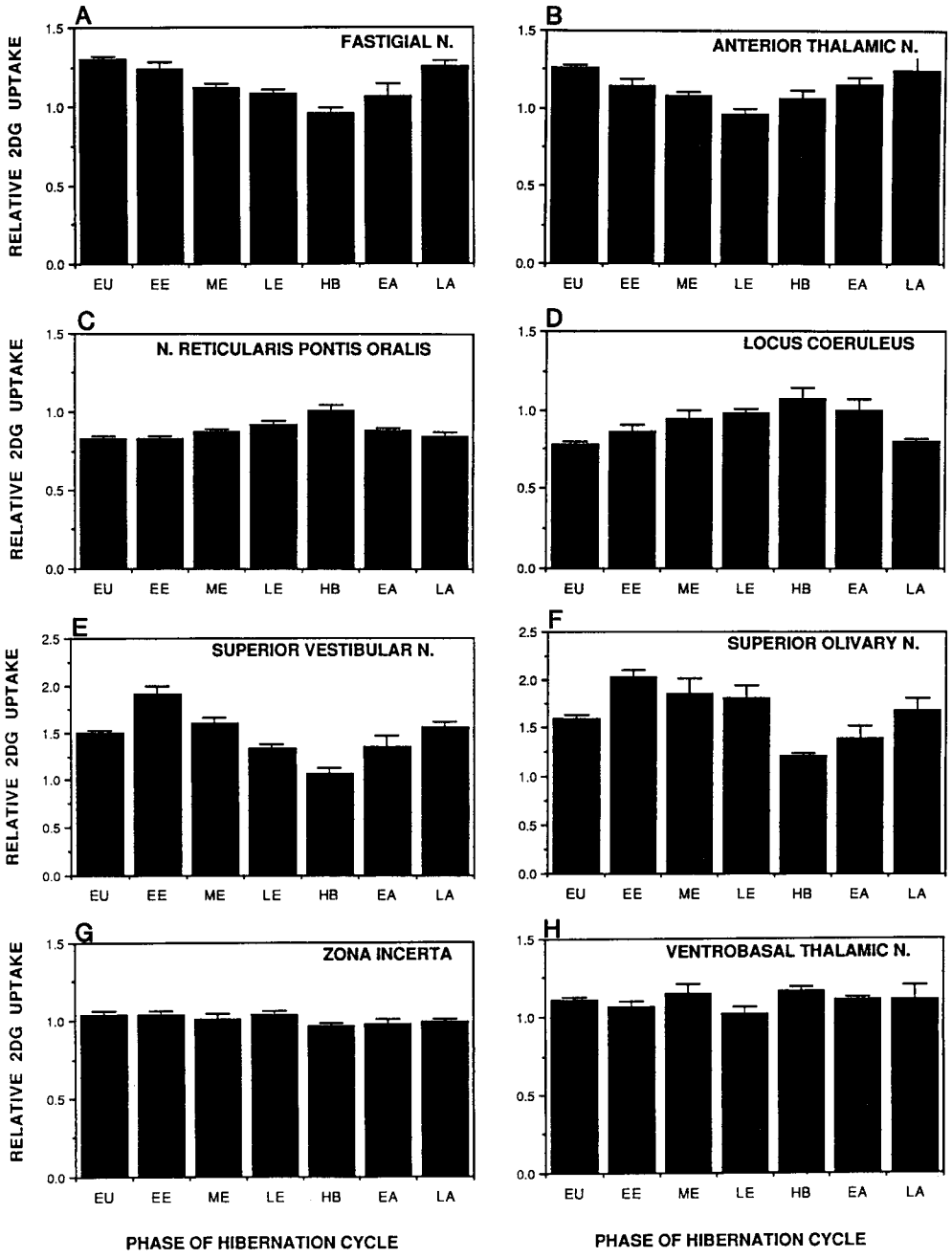

Figure 3. R2DGU values (mean ± SEM) plotted across the hibernation cycle (R2DGU profiles) for some selected brain structures. *A* and *B*, Brain regions whose R2DGU parallels T_b across the hibernation cycle. *C* and *D*, Regions whose R2DGU is inverse to T_b. *E* and *F*, Regions whose R2DGU parallels T_b except for the euthermic value. *G* and *H*, Regions whose R2DGU shows no consistent relationship to T_b. EU: euthermia; EE, ME, LE: 3 T_b intervals during entrance into hibernation; HB: stable deep hibernation; EA, LA: 2 T_b intervals during arousal from hibernation (see Methods).

and the residual correlation matrix, 6 factors accounted for 66% of the variance in the factor space. The 6 derived factors were well defined, distinguishable, and internally consistent. These considerations, in addition to examination of the plots of the rotated factor loadings, suggested that the 6-factor extraction was adequate.

Discriminant analysis. Because factor analysis could only be applied to the data set in an exploratory fashion, it was appropriate to evaluate whether the derived factors could discriminate the stages of hibernation. The extracted factors were therefore employed in a discriminant analysis. Stepwise entry of the dependent variables indicated that Factor 2 exhibited the greatest ability to discriminate the stages of hibernation (F to enter = 20.1, df = 6, 38), followed by Factor 1 (F to enter = 17.8, df = 6, 37), Factor 3 (F to enter = 18.6, df = 6, 36), and Factor 4 (F to enter = 5.2, df = 6, 35). For this number of degrees of freedom, F values in excess of 4.0 are highly significant [$p < 0.01$ for 6 predictor variables in a stepwise analysis of variance (ANOVA)]. It was evident that the first 3 factors were maximally effective as predictor variables. McNemar's repeated measures χ^2 test for change indicated that the classification of individual subjects into stages of hibernation was maximally correct (91.1%) after inclusion of the first 3 factors. Multivariate ANOVA confirmed that the stages of hibernation differed significantly on the 3 factors (F approximation of Wilks's lambda = 19.4, $p < 0.001$). F tests performed on the stages of hibernation indicated that all stages were significantly different from euthermia ($p < 0.05$). Furthermore, all stages were significantly different from each other (21 possible comparisons of the 7 stages), with the exception of the midentrance versus late arousal comparison (F =

Table 1. Ordinal rank of 96 brain regions across 7 phases of the hibernation cycle (regions were ranked ordinally [from highest to lowest] based on their R2DGU values within each phase)

Brain region	Euthermia	Early entrance	Mid entrance	Late entrance	Deep hibernation	Early arousal	Late arousal
Spinal cord							
Dorsal horn	77	72	79	39	42	61	83
Ventral horn	74	81	82	53	86	79	81
White matter	93	94	94	95	94	96	94
Cerebellum							
Medial nucleus	10	13	21	23	60	28	10
Nucleus interpositus	9	14	13	16	46	25	16
Lateral nucleus	11	11	11	17	32	36	14
Paraflocculus	89	87	91	83	41	86	91
Flocculus	53	74	71	67	26	12	63
Nodulus	40	51	22	36	14	22	57
Uvula	48	64	47	62	18	42	67
Medullary layer	95	96	96	94	95	94	95
Posterior hemisphere	83	91	85	86	36	59	90
Anterior hemisphere	86	89	87	76	34	70	92
Brainstem/midbrain							
Nucleus gracilis	68	52	69	49	67	62	74
Nucleus cuncatus	67	62	61	44	77	60	69
Nucleus solitarius	52	21	28	18	51	15	36
Paratrigeminal nucleus	91	55	34	5	1	88	89
Lateral cuneate nucleus	58	30	19	11	29	38	47
Nucleus of spinal V	63	65	48	29	45	44	68
Inferior olivary nucleus	7	7	7	4	11	8	7
Nucleus ambiguus	69	63	53	33	55	23	40
Cochlear nucleus	2	2	3	3	2	2	4
Spinal vestibular nucleus	13	6	6	7	23	9	12
Superior vestibular nucleus	5	4	4	9	22	4	5
Nucleus gigantocellularis	66	59	63	59	65	72	66
Superior olivary nucleus	3	3	2	2	7	3	3
Main sensory nucleus of V	54	50	32	25	31	35	41
Nucleus of lateral lemniscus	6	9	8	6	16	13	6
Locus coeruleus	78	57	54	38	20	41	82
Inferior colliculus	1	1	1	1	9	1	1
Dorsal tegmental nucleus	18	8	10	12	6	5	8
Dorsal raphe nucleus	50	45	43	41	25	37	48
Median raphe nucleus	23	18	15	20	63	32	19
Red nucleus	38	21	24	13	43	18	20
Oculomotor nucleus	17	15	12	14	12	7	9
Interpeduncular nucleus	30	22	23	24	39	11	22
Substantia nigra	61	67	74	47	58	64	45
Subthalamic nucleus	16	12	31	21	62	20	15
Periaqueductal gray	71	70	76	55	61	54	58
Pontine nuclei	91	85	86	73	64	89	86
Pontine tegmental nucleus	60	54	56	27	30	73	53
Zona incerta	45	33	40	31	59	49	42
Nucleus reticularis pontis caudalis	72	53	64	50	15	65	76
Nucleus reticularis pontis oralis	70	69	68	57	37	75	73
Ventral tegmental nucleus of Gudden	26	17	20	15	57	34	23
Deep mesencephalic nucleus/nucleus cuneiformis	42	38	38	30	74	40	27
Anterior pretectal area	37	43	46	37	48	48	33

Table 1. Continued

Brain region	Euthermia	Early entrance	Mid entrance	Late entrance	Deep hibernation	Early arousal	Late arousal
Superior colliculus							
Stratum gr. supf.-lateral	27	19	16	26	5	16	21
Stratum gr. supf.-medial	47	24	25	32	8	19	25
Stratum gr. med.-lateral	59	56	60	80	84	81	71
Stratum gr. med.-medial	65	60	62	79	81	80	78
Stratum gr. prof.-lateral	44	35	35	51	75	50	44
Stratum gr. prof.-medial	56	61	59	69	79	71	64
Thalamus							
Central medial nucleus	32	23	17	22	28	31	17
Parafascicular nucleus	24	34	37	35	50	66	34
Posterior nucleus	46	40	49	40	72	47	35
Lateral posterior nucleus	49	58	80	74	82	74	56
Medial geniculate nucleus	8	16	14	19	21	14	18
Lateral geniculate nucleus	41	29	41	68	38	57	26
Ventrobasal nucleus	31	31	18	34	10	21	24
Ventrolateral nucleus	43	46	44	42	69	53	32
Lateral habenula	14	10	9	10	4	10	13
Medial dorsal nucleus	20	36	42	60	33	46	30
Lateral dorsal nucleus	21	39	66	63	66	51	52
Reticular nucleus	55	73	36	56	73	78	65
Anterior nuclei	12	25	26	48	24	17	11
Paratenial nucleus	33	32	39	52	35	27	28
Hypothalamus							
Mammillary body	4	5	5	8	13	6	2
Dorsomedial nucleus	73	42	52	71	47	67	49
Ventromedial nucleus	88	77	83	87	78	83	75
Paraventricular nucleus	81	68	65	54	54	76	46
Suprachiasmatic nucleus	84	83	50	28	3	39	70
Anterior hypothalamic nucleus	76	84	81	84	70	58	62
Posterior nucleus	82	76	58	72	71	77	54
Lateral hypothalamus	57	75	70	61	89	63	61
Lateral preoptic	51	66	72	75	90	85	60
Medial preoptic	80	80	77	77	52	82	72
Basal ganglia/limbic							
Caudate nucleus	29	37	30	45	27	29	31
Putamen	39	28	29	46	44	26	37
Globus pallidus	87	86	90	89	87	91	89
Dorsal hippocampus	34	44	51	82	83	52	55
Ventral hippocampus	75	82	84	91	88	56	77
Medial amygdaloid nucleus	79	79	89	88	85	90	79
Basal amygdaloid nucleus	64	78	78	85	93	84	84
Medial septal nucleus	62	92	88	90	80	87	80
Lateral septal nucleus	85	90	75	78	19	68	85
Nucleus olfactory tract	35	47	67	58	17	43	50
Cortex							
Cingulate	22	41	55	76	49	33	38
Visual	28	49	57	66	56	69	51
Auditory	19	71	73	63	76	45	43
Somatosensory	25	48	45	81	53	55	59
Frontal	15	27	27	43	40	24	39
Pyriform	36	26	33	65	68	30	29
White matter							
Corpus callosum	94	93	93	93	91	93	93
Internal capsule	92	88	92	92	92	92	88
Optic tract	96	95	95	96	96	95	96

Table 2. Brain regions loading strongly ($|r| \geq 0.5$) on Factors 1, 2, or 3 across the entire hibernation cycle

Loading on Factor 1	Loading on Factor 2	Loading on Factor 3
Positive		
Medial preoptic area[a]	Cingulate cortex[a]	Anterior cerebellar cortex[b]
Anterior hypothalamic nucleus[a]	Medial dorsal thalamic nucleus	Ventral horn
Suprachiasmatic nucleus[a]	Visual cortex	Spinal white matter
Ventromedial hypothalamic nucleus[a]	Lateral dorsal thalamic nucleus	Nucleus gracilis
Corpus callosum	Auditory cortex[a]	Nucleus of spinal V
Lateral septal nucleus	Caudate nucleus[a]	Dorsal horn
Optic tract	Somatosensory cortex	Posterior cerebellar cortex[b]
Globus pallidus	Lateral preoptic area	Nucleus reticularis pontis caudalis
Internal capsule	Nucleus olfactory tract	Nucleus gigantocellularis
Paraventricular nucleus[a]	Frontal cortex[a]	Paraflocculus[b]
Posterior hypothalamic nucleus	Dorsal hippocampus[a]	Cerebellar medullary layer[b]
Medial amygdaloid nucleus[a]	Anterior thalamic nucleus	Uvula
Periaqueductal gray	Medial septal nucleus	Flocculus
Paraflocculus[b]	Parafascicular nucleus	Locus coeruleus
Dorsomedial hypothalamic nucleus[a]	Basal amygdaloid nucleus	Dorsal raphe nucleus[a]
Ventral hippocampus[a]	Lateral posterior nucleus	
Pontine nuclei		
Posterior cerebellar cortex[b]		
Cerebellar medullary layer[b]		
Anterior cerebellar cortex[b]		
Negative		
Medial cerebellar nucleus	External cuneate nucleus	Central medial nucleus
Nucleus interpositus	Nucleus solitarius	Superior vestibular nucleus[b]
Lateral cerebellar nucleus	Paratrigeminal nucleus	Inferior colliculus[b]
Nucleus of lateral lemniscus	Nucleus ambiguus	Mammillary body[b]
Superior vestibular nucleus	Red nucleus	
Inferior colliculus	Nucleus cuneatus	
Ventral tegmental nucleus of Gudden	Pontine tegmental nucleus	
Mammillary body		
Median raphe nucleus		
Superior olivary nucleus		
Superior colliculus: Stratum gr. prof.-lateral		
Subthalamic nucleus		
Medial geniculate nucleus		

Regions are listed in order of the strength of the correlation coefficient.

[a] Brain regions that load consistently on the factor during both euthermia and the deepest phases of hibernation.

[b] Structures that loaded on both Factors 1 and 3 (i.e., exhibited complex structure).

1.04, ns). These considerations strongly suggested the exclusive use of these 3 factors from the original 6 in subsequent analyses.

Figure 4*A* presents the factor scores for the first 2 factors across the phases of the hibernation cycle. Factor 1 is associated with a trend toward increasing R2DGU as a function of depth of hibernation. Factor 1 accounted for 19% of the total variance, with 33 brain regions exhibiting relatively high loading ($|r| \geq 0.5$) on Factor 1, including nearly all hypothalamic regions (Table 2). To illustrate the relationship between the factor score for Factor 1 (the hypothalamic factor) and R2DGU values for a particular brain region, Figure 4*B* presents the R2DGU profile for the suprachiasmatic nucleus, a hypothalamic structure that loaded strongly and consistently on the hypothalamic factor.

In contrast to the hypothalamic factor, Factor 2 is characterized by a rapid decrease in R2DGU early in a hibernation bout (Fig. 4*A*). Factor 2 accounted for 16% of the variance with 23 brain areas exhibiting high loading on Factor 2 (the cortical factor), especially telencephalic and limbic regions (Table 2). Figure 4*C* presents the R2DGU profile for the cingulate cortex, a brain region that loaded strongly and consistently on the cortical factor.

Factor 3 (not illustrated) exhibited a pattern across the stages of hibernation that showed similarity to both the hypothalamic factor (increasing R2DGU with depth of hibernation) and the cortical factor (early decrease in R2DGU). This independent factor accounted for 12% of the variance. Nineteen brain regions loaded on Factor 3 (Table 2), and inclusion of this factor improved the classification accuracy by about 13%. The groups determined here (Table 2) objectively confirm those established by visual inspection (goal 1) and were essentially identical to those determined on the basis of cluster analysis (not shown).

Consistency of factor loadings across hibernation. As indicated above, 75 of the original 96 brain regions loaded strongly on the first 3 factors when the entire data set was considered. To establish the consistency of the factor structure, factor analysis was also performed separately on the euthermic data and the

data from the deepest phases of hibernation. A brain region was considered to be consistent if it loaded strongly ($|r| \geq 0.5$) on 1 of the 3 primary factors across the entire hibernation cycle, during euthermia, and during the deepest phases of hibernation. The 14 brain regions that met these criteria are indicated in Table 2. Whereas 33 brain regions loaded on Factor 1 across the entire hibernation cycle, as indicated above and in Table 2, only 8 hypothalamic and limbic brain regions loaded strongly *and consistently* on the hypothalamic factor. Similarly, only 5 cortical and limbic regions exhibited high and consistent loading on the cortical factor (Factor 2). Only 1 brain region, the dorsal raphe nucleus, loaded on Factor 3 (the raphe factor) with any consistency.

Goal 4: identification of candidate brain regions involved in the entrance to and arousal from hibernation

The ultimate goal of this study was the identification of brain regions that might be causally involved in the entrance to and arousal from hibernation. We hypothesized that such regions would load strongly and consistently on Factors 1, 2, or 3 across the stages of hibernation and undergo a change in R2DGU early during entrance to or arousal from hibernation and would thus be phase-advanced relative to other brain regions in terms of any change in R2DGU. Whereas the criteria for establishing which brain regions load strongly and consistently on the factors have been described above, identification of brain regions that underwent early change in R2DGU was accomplished by the latency analyses.

Latency analyses. As the final step in the identification of brain regions that may play a causative role in entrance into hibernation, the original set of 96 structures was examined specifically for significant positive or negative changes between euthermia and the earliest phase of entrance into hibernation. The Bonferroni *t*-test for groups differing in variance was used for these comparisons, with alpha protected for the multiple tests. Thirty-eight brain structures exhibited a significant change in R2DGU very early in hibernation. These structures were then compared with the structures that consistently loaded positively on Factors 1, 2, and 3 across the stages of hibernation in the factor analysis (Table 2). This comparison resulted in a short list of 6 structures that loaded consistently on either Factor 1 (dorsomedial, ventromedial, and anterior hypothalamic nuclei) or Factor 2 (cingulate, auditory, and frontal cortices) and that underwent a significant change in R2DGU early during entrance into hibernation (Fig. 5*A*). Univariate ANOVA confirmed that these structures exhibited early and consistent changes in R2DGU across the stages of hibernation. Furthermore, the pattern of these changes reflected their respective loadings on Factor 1 or Factor 2; that is, individual brain regions exhibited the same pattern of R2DGU variation across hibernation as did the corresponding global factors (Fig. 4).

A similar analysis was performed on the data obtained during early arousal. Of the 31 structures exhibiting a significant change in R2DGU between deep hibernation and early arousal as revealed by the Bonferroni *t*-test, 4 hypothalamic regions (medial preoptic, suprachiasmatic, paraventricular, and ventromedial hypothalamic nuclei) loaded strongly and consistently on Factor 1 (Fig. 5*B*). No brain regions met these criteria for either Factors 2 or 3. Univariate ANOVA confirmed that these 4 hypothalamic structures exhibited significant change in R2DGU as arousal from hibernation progressed.

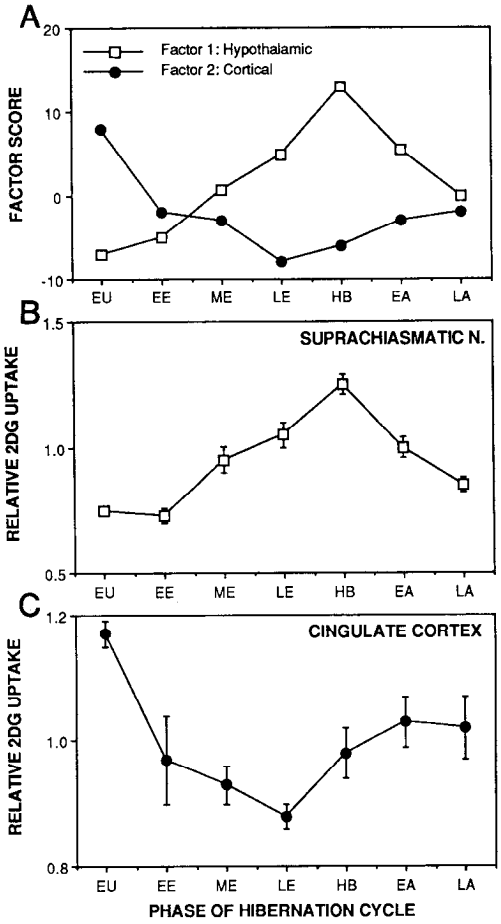

Figure 4. *A*, Factor scores for Factor 1 (hypothalamic) and Factor 2 (cortical) across the phases of the hibernation cycle. *B*, R2DGU profile (mean ± SEM) across the hibernation cycle for the suprachiasmatic nucleus, a representative brain region that loaded strongly on Factor 1. *C*, R2DGU profile (mean ± SEM) across the hibernation cycle for the cingulate cortex, a representative brain region that loaded strongly on Factor 2. *EU*, euthermia; *EE*, early entrance; *ME*, mid-entrance; *LE*, late entrance; *HB*, hibernation; *EA*, early arousal; *LA*, late arousal.

Discussion

The purpose of the present study was to identify categories of brain regions that may be activated during specific phases of the hibernation cycle and may therefore play important regulatory roles in hibernation. The ultimate goal was to identify candidate brain regions likely to be intimately associated with entrance to and/or arousal from hibernation. Our approach to achieving these goals was based on statistical analyses of R2DGU data and assumed that activation of a brain region would be reflected in neuronal metabolic activity and measurable in terms of R2DGU. The direct correlations between 2DG uptake and electrical activity previously demonstrated in the central (Schoppman and Stryker, 1981; Kadekaro et al., 1985) and peripheral (Yarowsky et al., 1983) nervous systems provided the rationale underlying this assumption. However, neuronal inhibition has also been associated with both increases and decreases in neuronal metabolism (Ackerman et al., 1984; Sharp et al., 1988). Therefore, a change in R2DGU of a brain region may indicate a change in the electrical activity of the neurons within that structure but does not unequivocally indicate the relative activation (i.e., net depolarization or hyperpolarization)

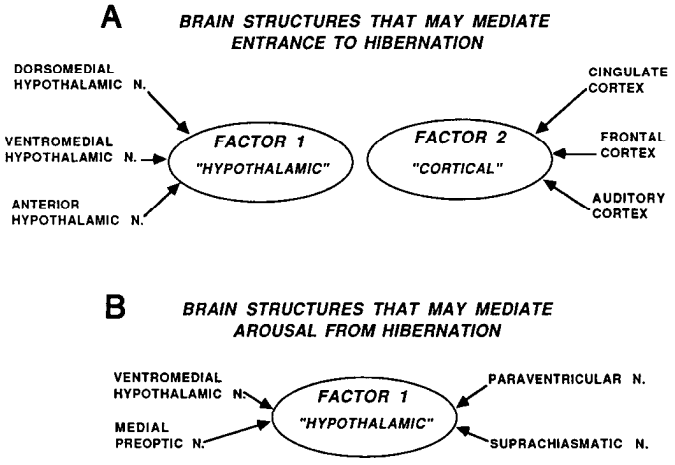

Figure 5. *A,* Schematic diagram illustrating hypothalamic and cortical involvement in the entrance into hibernation. *B,* Schematic diagram illustrating hypothalamic involvement in the arousal from hibernation.

of cells within that brain region. Furthermore, if most brain regions decrease their absolute metabolic rate as entrance into hibernation proceeds, a brain region whose metabolism is highly temperature compensated ($Q_{10} < 2$) may exhibit an increase in its R2DGU and thus appear to be activated during entrance to or during deep hibernation. However, it is likely that neural structures that exhibit this property of temperature compensation in the hibernating brain do so specifically because of the fundamental importance of maintaining their activity during the hibernating state. The 4 steps used to identify brain regions that may play important regulatory roles in the entrance to, maintenance of, or arousal from the hibernating state were described above (see Materials and Methods).

Ordinal rank analysis

The ordinal rank analysis was informative because it provided an index of the R2DGU of a given brain region with respect to every other brain region. If the absolute metabolism of all brain regions was affected to the same degree by the change in T_b, the ordinal rankings would be identical in all phases of hibernation. However, previous results indicated that the ordinal ranks of certain brain regions differ greatly between hibernation and euthermia, suggesting that brain regions may be differentially activated or inhibited during hibernation or, at least, that the Q_{10} effects are differential among brain regions (Kilduff et al., 1982). For the purposes of the discussion below, brain regions that change ordinal rank very slightly across the phases of hibernation are referred to as responding passively to the change in arousal state because they behave as predicted on the basis of Q_{10} effects alone.

When examined across all 7 phases of the hibernation cycle, the ordinal rankings revealed 4 general categories of brain regions: (1) regions whose ordinal rank paralleled T_b across the phases of hibernation, (2) regions whose ordinal rank varied inversely with the change in T_b, (3) regions whose ordinal rank changed slightly, and (4) regions whose ordinal rank changed in no consistent pattern across the hibernation cycle. It is possible that brain regions in category 1 are causally involved in the entrance to or arousal from hibernation. These regions may be undergoing active inhibition during the dynamic phases of the hibernation cycle that may be necessary for hibernation to proceed. Structures assigned to this category were primarily sensory structures that receive input from the statoacoustic nerve (superior and spinal vestibular nuclei, superior olivary nucleus, and inferior colliculus), as well as the cerebellar, parafascicular, and mammillary nuclei.

In contrast to category 1, brain nuclei in category 2 consistently increased their ordinal ranks relative to other brain structures as T_b fell during entrance into hibernation and decreased their ordinal ranks as T_b increased during arousal. Brain regions in this category included a brain-stem structure of unknown function (the paratrigeminal nucleus), 4 reticular formation nuclei (locus coeruleus, dorsal raphe nucleus, nucleus reticularis pontis oralis, and the pontine nuclei), and a hypothalamic structure (the suprachiasmatic nucleus). The changes in ordinal rank characteristic of brain regions in this category could reflect increases in absolute metabolism during entrance and decreases during arousal. However, it is more likely that the ordinal rank changes reflected smaller change in absolute metabolism during these phases of hibernation than in other brain regions, perhaps due to a greater degree of temperature compensation of 2DG uptake in these neural structures relative to the rest of the brain. In either case, this pattern of ordinal rank increase as T_b decreases might well occur in brain regions that play an active role in facilitating the entrance to, maintenance of, and arousal from hibernation. With the exception of the paratrigeminal nucleus, the brain regions in this category have all been implicated in the control of arousal states to some degree (see Hobson et al., 1986, for review), although metabolic studies have failed to document a clear relationship between sleep states and local metabolic rate among reticular formation structures (Kennedy et al., 1982; Nakamura et al., 1983; Ramm and Frost, 1983, 1986). The paratrigeminal nucleus has been shown to project to 2 brain-stem autonomic centers, the nucleus tractus solitarius and the peribrachial region of the dorsolateral pons (Menetrey et al., 1987).

Brain regions in category 3 underwent little change in ordinal rank and, as defined above, therefore exhibited a passive response to the change in T_b across hibernation. Finally, the inconsistent change of ordinal rank observed in some brain regions in category 4 may indicate only transient activation, at best, during specific phases of the hibernation cycle.

The ordinal rank change of structures in categories 1 and 2 reflected the R2DGU profiles: where an increase in ordinal rank was associated with depth of hibernation, the R2DGU profile of that brain region varied inversely with the change in T_b (e.g., nucleus reticularis pontis oralis; see Fig. 3*C*); decreases in ordinal rank were associated with a R2DGU profile that paralleled the change in T_b across hibernation (e.g., fastigial nucleus; see Fig. 3*A*). However, the converse was not true: a particular R2DGU profile was not always associated with a specific change in ordinal rank.

Classification of brain regions into groups

Exploratory factor analysis was used to identify consistent patterns of R2DGU variation across brain regions. Two highly consistent factors were extracted: the hypothalamic factor reflected a pattern of R2DGU that was inversely associated with depth of hibernation, whereas the cortical factor reflected a pattern of rapidly decreasing R2DGU early during entrance into hibernation (Fig. 4). A third factor, the raphe factor, was a hybrid of the hypothalamic and cortical factors. These 3 factors ac-

curately discriminated the various stages of hibernation, supporting their validity.

It is conceivable that the extracted factors represent physiological processes that occur during the hibernation cycle. The hypothalamic factor may represent activation of brain regions that occurs as T_b decreases during entrance into hibernation or, at least, represent a relative sparing of metabolic activity in these regions due to greater temperature compensation, as discussed above. Physiological studies have indicated that the regulator of T_b, located in the preoptic/anterior hypothalamus (POAH), remains active throughout entrance (Heller et al., 1977) and deep hibernation itself (Heller and Colliver, 1974). The cortical factor, on the other hand, may represent inhibition of various brain regions that clearly occurs during entrance into hibernation. Electrophysiological studies have documented that EEG disappears in a reliable sequence during entrance into hibernation: first in the cortex, then in the midbrain reticular formation, thalamus, and hippocampus (Mihailovic et al., 1972; Shtark, 1972).

Brain regions were classified into groups according to the correlation of the R2DGU values with Factors 1, 2, or 3 (i.e, loadings). The classification achieved by this procedure demonstrated a remarkable consistency with regional neuroanatomical organization and suggests that these groupings represent functional systems of neural structures that undergo similar changes in R2DGU across hibernation. As indicated in Table 2, brain regions that loaded on Factor 1 included the hypothalamic, limbic, white matter, and some cerebellar structures. In contrast, brain regions that exhibited high R2DGU values during euthermia, such as several auditory structures (inferior colliculus, nucleus lateral lemniscus, medial geniculate nucleus, and superior olivary nucleus), the cerebellar nuclei, the mammillary body, and the ventral tegmental nucleus of Gudden, loaded negatively on the hypothalamic factor. Cortical, thalamic, and other limbic regions loaded positively on Factor 2, whereas a small set of brain-stem regions were negatively associated with this cortical factor. Although several brain regions loaded positively on Factor 3, many of these also loaded positively on Factor 1, indicating a complex structure for those regions. The dorsal raphe was the only brain region to load consistently on Factor 3 across all phases of hibernation and did not exhibit complex factor structure.

Identification of candidate brain regions involved in the entrance to hibernation

An ultimate goal of these studies was to identify candidate brain regions involved in the entrance into hibernation. A combination of analyses was used to achieve this goal: (1) exploratory factor analysis that reduced the original 96 variables (i.e., R2DGU values of brain regions) to a small set of factors that represent global patterns of R2DGU variation common to many brain regions, (2) a consistency criterion to validate the extracted factors, (3) discriminant analysis to evaluate the ability of these factors to discriminate the stages of hibernation, and (4) latency analyses to identify which brain regions underwent the first significant changes in R2DGU during entrance to and arousal from hibernation.

Neural structures possibly associated with entrance into hibernation were considered likely to be strongly and consistently associated with Factors 1 or 2 and to undergo a change in R2DGU during the earliest phase of entrance into hibernation. Therefore, a conservative procedure (Bonferroni t-test) was used to determine brain regions exhibiting the most rapid increase or decrease in R2DGU in the earliest phase of entrance into hibernation. The resultant group of regions was compared to the group of regions loading heavily and consistently on Factors 1 and 2 (Table 2). The intersection of these groups resulted in a short list of 6 neural structures (dorsomedial, ventromedial, and anterior hypothalamic nuclei and cingulate, frontal, and auditory cortical regions; see Fig. 5) that exhibited the earliest and most consistent and predictive changes in R2DGU.

The 3 hypothalamic structures on this list were associated with Factor 1, indicating a R2DGU profile inversely associated with depth of hibernation. The hypothalamus has a long history of involvement in thermoregulation and has previously been implicated in the entrance into hibernation. Lesion of the posterior hypothalamus prevents hibernation in the European hamster (Malan, 1966a, b), whereas POAH-lesioned squirrels will enter hibernation but will not arouse (Satinoff, 1967). The entrance into hibernation is accompanied by a gradual resetting of the regulator of T_b located in the POAH (Heller et al., 1977), which remains operative throughout a hibernation bout (Heller and Colliver, 1974).

In contrast to the hypothalamus, the 3 telencephalic structures (temporal, frontal, and cingulate cortices) loaded most strongly and consistently on Factor 2. These structures exhibited a decrease in R2DGU early during entrance into hibernation that persisted throughout the hibernation cycle, suggestive of a sustained inhibition of activity in these brain regions. Electrophysiological studies have also suggested that the cortex becomes quiescent very early in hibernation (Mihailovic, 1972). The limbic cortex (specifically, the frontal and cingulate cortices) is highly sensitive, in terms of electrophysiological response, to environmental stimuli, particularly stressful stimuli (Gabriel et al., 1977). Similarly, the limbic cortex is exquisitely sensitive to both conditioned and unconditioned stressors in terms of neurotransmitter metabolites (e.g., dopamine metabolites; Speciale et al., 1986). The pattern of early, strong, and consistent decreases in R2DGU observed in the frontal and cingulate cortices upon entrance into hibernation, in conjunction with their apparent sensitivity to environmental stimuli in euthermic mammals, suggests that the active inhibition of these arousal-sensitive limbic cortical regions may be a prerequisite for the initiation of hibernation.

Taken together, these observations suggest that entrance into hibernation may be mediated through 2 processes. Hypothalamic regions may become activated early during the entrance process and continue to increase their R2DGU as the depth of hibernation proceeds. Second, cortical regions may undergo a reduction in R2DGU early during entrance into hibernation that is maintained throughout a hibernation bout, suggesting, together with electrophysiological observations, an active inhibition of cortical activity. Once entrance into hibernation is initiated, other neural structures, particularly those neural structures that underwent an increase in ordinal rank as depth of hibernation proceeded, may play increasingly important roles.

Identification of candidate brain regions involved in the arousal from hibernation

Another goal of these studies was to identify candidate brain regions likely to be intimately associated with arousal from hibernation. As discussed above with reference to entrance into hibernation, such neural structures were likely to be strongly and consistently associated with Factors 1 or 2 and to undergo

a change in R2DGU during the earliest phase of arousal from hibernation. Brain regions exhibiting the most rapid increase or decrease in R2DGU in the earliest phase of arousal from hibernation were determined using the Bonferroni *t* test and compared to those regions loading heavily and consistently on Factors 1 and 2 (Table 2). The intersection of these groups resulted in a short list of hypothalamic structures (suprachiasmatic, paraventricular, ventromedial hypothalamic, and medial preoptic nuclei) that were associated with early arousal from hibernation. Curiously, all of these structures loaded on Factor 1 and none on Factors 2 or 3. These results are consistent with observations that microinjections of norepinephrine, serotonin, or acetylcholine into the POAH can induce arousal from hibernation (Beckman and Satinoff, 1972) and that POAH-lesioned squirrels fail to arouse from hibernation (Satinoff, 1967). The involvement of hypothalamic, as opposed to cortical, regions in both entrance and arousal from hibernation underscores the functional significance of the hypothalamus in hibernation.

Limitations of the current study

Certain limitations of this data set must be considered. First, the results reported here are correlative and can only suggest causative roles for brain function in hibernation. Future studies could begin to isolate those brain regions that are necessary for hibernation by functional or anatomical lesions, for example, examining the consequences of cortical deactivation or neurotoxic lesions of hypothalamic structures. Second, the analyses presented here were designed to identify brain regions that showed a consistent relationship with T_b across hibernation and were thus predictive of the entire waveform of the hibernation cycle. The combination of ordinal rank analysis, factor analysis, and latency analyses was most likely to detect brain regions that exhibited high correlations (positive or negative) between R2DGU patterns and T_b but that were relatively phase-advanced with respect to the majority of the brain regions. Other patterns are clearly possible. Certain structures might be transiently activated during a specific phase of the hibernation cycle or activated during one phase, such as early entrance, and inactivated during another, such as early arousal. Third, our interpretations are, in large part, based on observations made early during entrance into hibernation, defined as the T_b range between 30°C and 25°C. This temperature range was chosen for practical purposes: it represented the earliest portion of a hibernation cycle during which an intravenous injection of 2DG could be administered without evoking an arousal to euthermia. It is conceivable that any brain region intimately involved in entrance into hibernation may have already exerted its influence earlier during entrance and ceased its activity by the time the animal lowered its T_b to 30°C. Finally, it has been recently estimated that a doubling of impulse flow may be correlated with as little as a 6% increase in local cerebral glucose utilization (Fox et al., 1988). It is not clear whether typical methods for quantification of glucose uptake have the resolution necessary to detect small changes in impulse flow. Furthermore, the rather large changes of 2DG uptake that are most likely to be detected may represent processes other than neural impulse flow (e.g., neuronal glycogen formation, glial metabolism, etc.). Despite these limitations, the present results suggest possible avenues for future experimentation.

Future efforts could also be directed toward distinguishing between the changes in neuronal metabolism that are a result of decreased body temperature (i.e., Q_{10} effects) from those that are specific to hibernation. Rodents can be maintained in induced hypothermia at body temperatures comparable to those of hibernators for up to several days (Volkert and Musacchia, 1976). In contrast to the regulated decrease of body temperature characteristic of hibernation (Heller and Colliver, 1974; Heller et al., 1977), induced hypothermia is an unregulated state in which the animal's heat production capacity is overcome by extreme external conditions (Popovic and Popovic, 1974). Despite this major physiological difference, induced hypothermia may be useful for comparison with hibernation to discriminate changes in neuronal metabolism that are a result of Q_{10} effects from those that are specific to hibernation.

Conclusions

The results presented here support the concept that mammalian hibernation is an actively regulated physiological state and that the events underlying the hibernation cycle involve an integrated orchestration of neurophysiological events. Two physiological processes are suggested as playing a role in the entrance into hibernation: (1) activation of hypothalamic regions (or at least a sparing of the metabolic activity of this brain region relative to other brain areas), and (2) inhibition of cortical regions. In contrast, the hypothalamic nuclei, in particular, play a role in generating the arousal from hibernation. Thus, mammalian hibernation, like mammalian sleep, may be generated by active rather than passive processes. Although there have now been a number of 2DG studies of sleep in mammals (Kennedy et al., 1982; Nakamura et al., 1983; Ramm and Frost, 1983, 1986; Abrams et al., 1988), these studies have been interpreted in "centrist" terms. It may be that an analysis based on systems (Hobson et al., 1986) as opposed to "centers," as exemplified in the analytical procedures of this study, could be profitably applied to the study of mammalian sleep. Furthermore, the procedures for data reduction used here may also be generally applicable for pharmacological studies of local cerebral glucose utilization.

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