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Use of a Single Compartment LCEC Cell in the Determinations of Biogenic Amine Content and Turnover

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CO, C., J. E. SMITH AND J. D. LANE. *Use of a single compartment LCEC cell in the determinations of biogenic amine content and turnover.* PHARMAC. BIOCHEM. BEHAV. **16**(4) 641–646, 1982.—Content and specific radioactivity of the biogenic monoamines and content of their precursors and metabolites were simultaneously determined in CNS tissue extracts with a high pressure liquid chromatography system (HPLC). The content of dopamine, norepinephrine, serotonin. 5-hydroxyindoleacetic acid, homovanillic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylethyleneglycol and the turnover rate of dopamine, norepinephrine and serotonin were measured in discrete rat brain regions using a one compartment electrochemical detector cell coupled to a C18-reverse phase HPLC column. The small fluid volume dead space of the cell allows the direct and precise collection of individual peaks for determining specific radioactivites. This method is especially suitable for central nervous system tissue samples from 8–20 mg wet weight and the sensitivity of the system in its routine configuration is approximately 2 pmol. This method for determinations of turnover is appropriate for investigations of animals in sensitive behavioral paradigms.

One compartment LCEC cell Dopamine Norepinephrine Serotonin content
Biogenic amine metabolites Biogenic monoamine turnover HPLC

THERE are several methods for assessing turnover of biogenic monoamines as indicative of rates of activity of their respective neurons in the central nervous system (CNS). The most widely used procedure involves measurement of tissue content of these compounds [6], which frequently does not detect small but possibly relevant changes in the functional pools of neurotransmitters. The second approach involves administration of a drug(s) which blocks the synthesis or degradation of the respective biogenic amine, its precursor(s) or metabolite(s). An example of this technique involves administration of an L-aromatic amino acid decarboxylase inhibitor, with subsequent measurement of the decline in biogenic amine content as a measure of turnover [1,2]. Probenecid has been used to block the transport and excretion of the acid metabolites of the amines, with the rate of their accumulation in the CNS used to calculate biogenic amine turnover rates [8,14]. Unfortunately, these and other drugs with similar modes of action disrupt sensitive behavioral paradigms, making them inappropriate for certain functional and behavioral neurochemical studies. Furthermore, the analysis of the action of certain neuropharmacological agents in the CNS may be obscured by the drug-dependent turnover measurement. Use of radioisotopic techniques to assess turnover [4, 9, 12, 15] circumvent these problems, but require separation of each individual radiolabelled precursor, neurotransmitter and metabolite and compound-specific assays for content.

Several procedures have been reported for the simultaneous measurement of content and specific radioactivities of the biogenic monoamines, their precursors and metabolites. These methods include the use of multiple ion exchange resins to separate the substances, followed by compound-specific fluorometric assays [4, 12, 13]; the use of alumina and thin layer chromatography to separate radiolabelled components into individual fractions [4,12]; or prepurification of the biogenic monoamines by absorption on a cation exchange resin followed by HPLC with electrochemical detection [3,5]. This report presents a method for the direct simultaneous determination of the content and specific radioactivity of norepinephrine, dopamine, serotonin, their precursors and metabolites, which can be used to calculate turnover rates of the biogenic amine neurotransmitters.

METHOD

Adult male Fisher 344 rats were implanted with chronic jugular catheters using a previously described procedure [10,16]. The catheter was filled with saline and placed in a small brass enclosure on the back to prevent access by the rat. After two weeks of post-operative recovery, the rats received an intravenous injection of 0.5 mCi [U-^{3}H]-L-tryptophan (New England Nuclear, Sp. Act. 7.1 Ci/mmol) and 1.0 mCi [2,6-^{3}H]-L-tyrosine (Amersham, Sp. Act. 34 Ci/mmol) in 100 μl saline through the jugular catheter 60 or

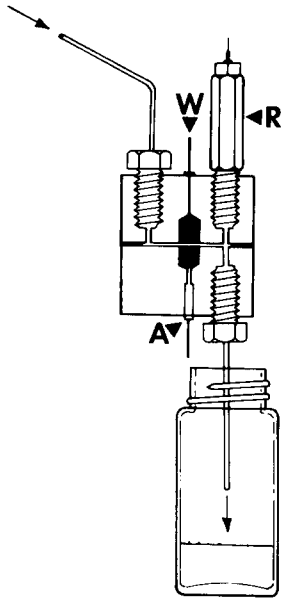

FIG. 1. Diagram of the TL-9A electrochemical detector cell. The glassy carbon working electrode (W), the auxiliary electrode (A) and the reference electrode (R) are contained in a single compartment with a dead space of approximately 5 μl. Scintillation vials can be used to collect the mobile phase at the exit port.

TABLE 1

RETENTION TIMES OF THE BIOGENIC AMINES, THEIR METABOLITES AND PRECURSORS

Compound	Retention Time (min)
DHMA	1.30
DHPG + VMA	1.90
L-DOPA	2.10
NE	2.55
Tyr	2.90
MHPG	3.25
Epi	3.50
DHBA	4.60
NM	5.50
5-HTP	5.95
DOPAC	6.30
DA	7.00
MN	7.70
5-HIAA	12.40
Trp	15.10
HVA	16.40
3-MT	20.00
5-HT	22.00

90 minutes prior to sacrifice. The animals were immersed in liquid N_2 until totally frozen, the brains removed and dissected at −20°C into nineteen discrete areas for analyses. The larger dissected samples were pulverized in liquid N_2 with a mortar and pestel and stored at −70°C until extraction. Tissue samples weighing less than 15 mg were stored at −70°C and directly homogenized with a teflon-glass homogenizer in 20 volumes of 1 N formic acid/acetone (v/v:15/85).

The biogenic amines and their metabolites were extracted from tissue with 20 volumes of ice cold 1 N formic acid/acetone (v/v: 15/85). Tracer levels of ^{14}C-radioactive standards for dopamine (Amersham, Sp. Act. 60 mCi/mmol), norepinephrine (Amersham, Sp. Act. 67 mCi/mmol) and serotonin (Amersham, Sp. Act. 54 mCi/mmol) were added to each tissue homogenate to correct for radioactive recovery (100 pmol of 3,4-dihydroxybenzylamine was also added to each tissue sample as a comparative means to correct for recovery). The tissue homogenates were centrifuged at 1500 × g for 10 minutes at 4°C and the tissue pellets saved for protein determination [7]. Three volumes of heptane/chloroform (v/v: 8/1) were added to the supernatant, mixed, centrifuged and the organic layer and lipid interphase discarded. The aqueous layer was taken to dryness under a stream of N_2 and stored at −20°C until analysis.

Content and specific radioactivity were determined concurrently by HPLC. The HPLC system utilized a reverse-phase 10 micron μBondapak C_{18} column (3.9 mm × 30 cm, Waters Associates, Inc., Milford, MA) coupled with a TL-9A electrochemical detector cell (Fig. 1, Bioanalytical Systems, Inc., West Lafayette, IN). The HPLC mobile phase was 0.1 M citrate-phosphate buffer, pH 3.5, containing 0.01% sodium octyl sulfate (Eastman), which was filtered, degassed under vacuum, and methanol added to a final concentration of approximately 9% (this concentration can vary slightly, depending on several variables like paired ion concentration and age of the column). The dried extract was reconstituted in 40 μl of the mobile phase and 20 μl injected into the HPLC system. The oxidation potential was set at +0.9 volt against the reference electrode, the controller set at 20 nA/V and the recorder at 1.0 volt full scale. The pump flow rate was 2.0 ml/min.

Quantitative amounts of standards [norepinephrine (NE), dopamine (DA), serotonin (5-HT), 4-hydroxy-3-methoxyphenylethyleneglycol (MHPG), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), normetanephrine (NM) tyrosine (Tyr), tryptophan (Trp), 3,4-dihydroxybenzylamine (DHBA), 3,4-dihydroxymandelic acid (DHMA), 3,4-dihydroxyphenylglycol (DHPG), vanilmandelic acid (VMA), L,β-3,4-dihydroxyphenylalanine (L-DOPA), epinephrine (Epi), 5-hydroxytryptophan (5-HTP), metanephrine (MN), and 3-methoxytyramine (3-MT)] were injected into the system. The biogenic amines, their precursors and metabolites were identified by the retention time of the standards (Table 1). The elution profile of these nineteen compounds are shown in Fig. 2. The separation of each compound was achieved (except for DHPG and VMA which co-migrate). However, eight of the compounds are not observed in appreciable amounts in most CNS tissue samples as can be seen by the elution profile of whole brain tissue in Fig. 3. Therefore, only five of the precursors and metabolites are routinely included with the biogenic amines in the external standard mixture.

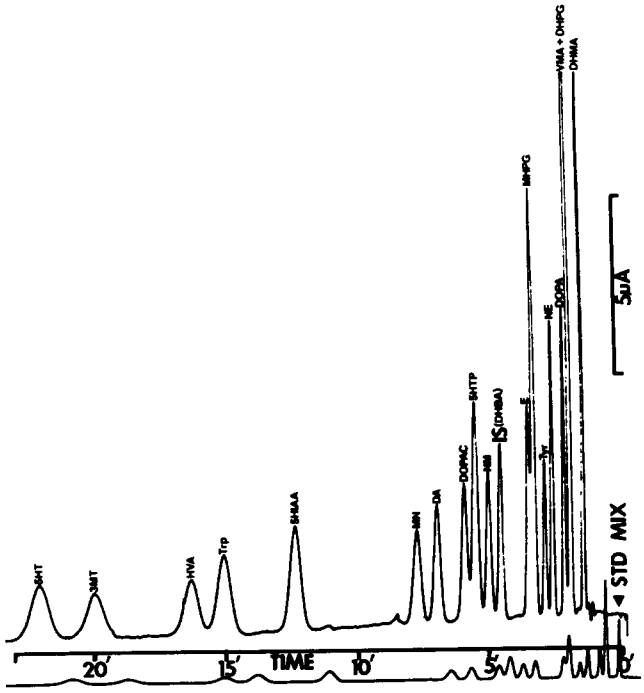

FIG. 2. HPLC elution profile for a mixture of external standards containing 80 pmole each of Tyr and Trp and 40 pmol each of the other compounds.

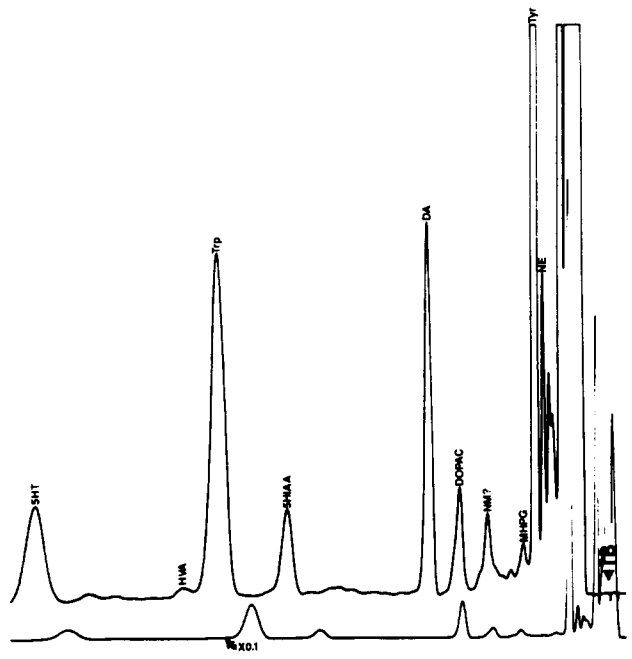

FIG. 3. Elution profile for a tissue extract indicating the compounds of interest. NM? is a peak which co-migrates with authentic normetanephrine.

The content of these eleven compounds of interest was determined by direct comparison of the peak height with the standard and radioactivity incorporated into the biogenic monoamines was measured by collecting each peak in a counting vial (Fig. 1), adding 10 ml of Aquasol-2 (New England Nuclear) and counting the samples in a Searle Isocap Model 6872 liquid scintillation spectrometer using a double-label external standardization quench correction program.

The content and radioactivity values were corrected for recovery and content expressed as pmol/mg protein and specific radioactivity as dpm/mg protein. Turnover rates of the biogenic monoamines were determined, assuming steady state conditions with radiolabel from each neurotransmitter disappearing from a single open pool [4, 12, 17], and are the product of the apparent fractional rate constants (K) and the content values [4, 11, 12]:

$$\text{Turnover}_A = K_A \times \text{content}_A \text{ where } K_A = \frac{\ln 2}{t^1/_2}.$$ Half-life ($t^1/_2$) is extrapolated from a semilogarithmic plot of the specific radioactivity at the two time points (60 and 90 minutes post-injection) on the linear portion of the decay (Fig. 4). Turnover rates are expressed as pmol/mg protein-hour.

RESULTS

The TL-9A electrochemical detector cell has a single compartment which contains the working glassy carbon, auxiliary and reference electrodes (Fig. 1). The dead space for the cell is approximately 5 μl, therefore individual peaks may be collected (without mixing) by monitoring the beginning and end of each peak on a real-time chart recorder and placing a counting vial under the exit port. For the conditions presented in the METHOD, the appearance of the last peak occurs 22 minutes after injection.

The recoveries ranged from 80–90% for all of the compounds, and recoveries based on ^{14}C-radioactive tracers and the DHBA internal standard were in good agreement. The ^{14}C-radioactive tracer added to each tissue sample (approximately 10.0 pmol) does not interfere with measurement of specific radioactivities of the ^{3}H-biogenic amines, but these exogenous amounts of standard are subtracted from the respective contents to obtain the actual values. The content of dopamine, norepinephrine, serotonin and their major metabolites for nineteen discrete brain regions are shown in Table 2. There is a differential distribution of the three biogenic monoamines, with high content of dopamine in the nucleus accumbens and striatum, high content of norepinephrine in the preoptic-diagonal band and medial hypothalamus, and high content of serotonin in the amygdala and lateral septum. The content of the metabolites of the biogenic amines also varied several fold, reflecting the distribution of the respective neurotransmitters. Table 3 shows the turnover rates of the biogenic amines in nineteen discrete brain regions. The turnover rates of DA is highest in the caudate-putamen, preoptic-diagonal band and nucleus accumbens; and intermediate in the amygdala and lateral septum. The turnover of NE is highest in the ventral tegmental area, preoptic-diagonal band region and lateral hypothalamus; and intermediate in the amygdala, hippocampus, ventral thalamus and lateral septum. The turnover rate of 5-HT is highest in the lateral hypothalamus; and intermediate in the ventral tegmental area, amygdala, preoptic-diagonal band region and lateral septum. The turnover of the three biogenic amines was lower in the other regions.

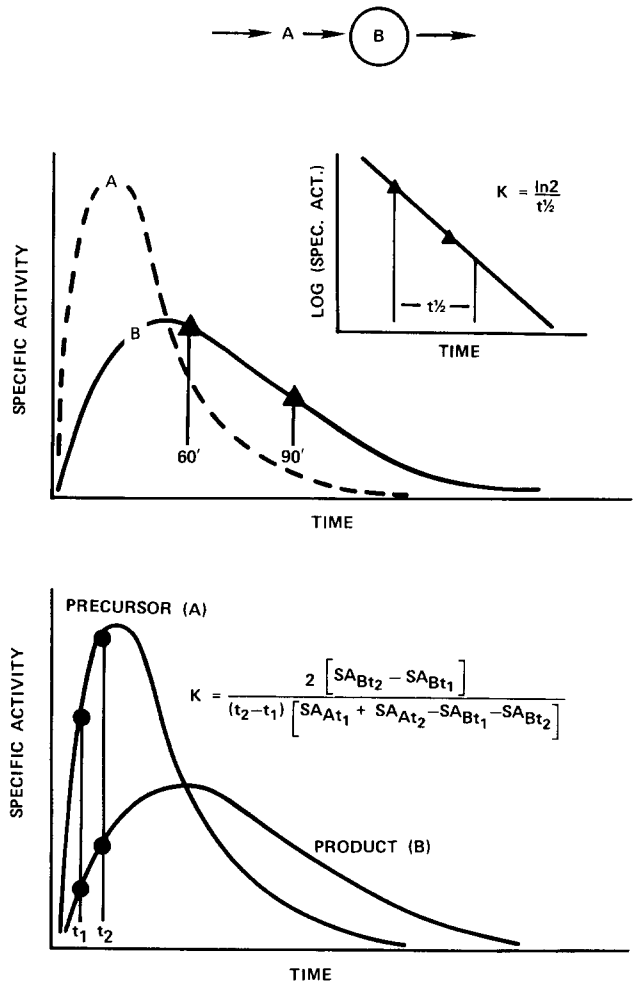

FIG. 4. Procedures for estimation of the apparent fractional rate constant (K) for calculation of the turnover rate of biogenic amines (B). The upper diagram illustrates the procedure used in this investigation, where the specific activities of (B) at 60 and 90 minutes post-injection of radiolabelled precursor (A) were used to calculate (K). The lower diagram illustrates a procedure developed by Neff *et al.* [9] which relies on the specific activities (SA_i) at two time points (t_i). Both procedures assume that (B) exists in one open pool. Turnover (B) = K (B) × Content (B).

DISCUSSION

Since content values of the biogenic amines may not show significant differences in behavioral paradigms (for example [11]), it is necessary to measure turnover rates to assess changes in neuronal activity resulting from the behavioral manipulation. Use of inhibitors of synthesis or degradation to assess turnover are often not recommended in studies of the neurochemistry of behavior since these drugs can result in incapacitation of the animal or in disruption of neuronal feedback or feed-forward mechanisms that may be important in maintaining a viable and sufficient functional pool of each neurotransmitter. Non-invasive radioactive pulse label methods are, therefore, preferable for assessing turnover rates of the biogenic monoamines in behaving animals. Such methods are usually tedious and complicated. This report describes an HPLC method for the simultaneous determination of content and specific radioactivity of the biogenic monoamine neurotransmitters that is both rapid, sensitive and relatively less complicated than existing procedures. The method can be used to determine content and turnover rates of DA, NE and 5-HT in small brain regions (as small as 8 mg wet weight) of animals responding in sensitive behavioral paradigms since it utilizes radioactive pulse labelling techniques. It also permits concurrent determination of the content, collection of the major precursors and metabolites and has a sensitivity of 2 pmol. This sensitivity can be increased by modifying the reconstitution of the tissue extracts and by decreasing the attenuation of the LCEC current monitor. The precursors and metabolites can be collected to determine the relationships between the precursors and the biogenic amines, the metabolites and the biogenic amines, and/or the precursors and the metabolites (for example, Fig. 4).

If Tyr and Trp are not of primary interest, the oxidation potential can be reduced to a lower voltage (circa + 0.75 V), but the retention times of Tyr and Trp must be confirmed and their peaks adequately separated from the other compounds of interest (when incorporation of radioisotopes is being assessed). This lower oxidation potential sustains the sensitivity of the detector cell for a longer period of time. If the chart recorder delivers beginning-peak and end-peak integration signals, or if a timer is available, the sampling could be coupled to a fraction collector. Since the operator must be present to load the samples, and most retention times are short, it is not difficult to collect the peaks manually.

When radiolabelling studies are being performed, one must be aware of a potential problem—peak tailing could result in the sampling of one component in another. For example, one could collect small amounts of radiolabel attributed to the precursor tyrosine in the product norepinephrine (refer to the elution profile in Fig. 2). This would yield erroneous data. To overcome this liability, the user should verify elution patterns by injecting bonafide radiolabelled compounds of interest and collect adjacent peaks, or collect only one half of a symmetrical peak if overlap is suspected.

The apparent fractional rate constants for the biogenic monoamines in the nineteen brain areas ranged from 5-118 percent per hour (Table 3), and yielded rates which agree with previously published values [2, 4, 8, 9, 12, 14]. This method of estimation and calculation of turnover is one of several possible applications. Alternative methods [9,17] can be used for assessing product-precursor relationships (refer to Fig. 4).

This procedure is currently being used in our laboratories to study neurotransmitter turnover in animals exposed to operant and respondent conditioning behavioral paradigms.

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TABLE 2

CONTENT OF BIOGENIC MONOAMINES AND THEIR METABOLITES IN RAT BRAIN AREAS

Brain Area	Content (pmol/mg protein) MHPG	DOPAC	HVA	5-HIAA	NE	DA	5-HT
Frontal Cortex	6.4 ± 2.0	4.1 ± 1.6	ND	4.2 ± 1.2	14.2 ± 2.4	9.6 ± 3.5	29.4 ± 6.3
Pyriform Cortex	ND	29.0 ± 5.9	ND	22.6 ± 2.4	27.1 ± 6.3	85.7 ± 11.3	36.5 ± 5.3
Motor-Somatosensory Cortex	1.6 ± 0.6	12.8 ± 2.4	ND	12.5 ± 1.7	25.0 ± 2.5	14.9 ± 4.2	24.5 ± 2.4
Cingulate Cortex	1.2 ± 0.1	11.7 ± 5.0	ND	11.4 ± 3.3	19.4 ± 3.1	11.6 ± 3.8	28.7 ± 3.8
Entorhinal-Subicular Cortex	1.3 ± 0.6	6.8 ± 1.8	ND	9.9 ± 2.4	23.0 ± 3.7	9.6 ± 2.0	20.8 ± 6.1
Nucleus Accumbens	ND	166.9 ± 20.2	74.2 ± 18.1	22.7 ± 5.1	77.2 ± 15.5	717.8 ± 120.4	47.2 ± 6.6
Caudate-Putamen	ND	67.4 ± 11.8	35.6 ± 5.5	26.7 ± 4.2	7.2 ± 1.1	683.3 ± 98.0	31.5 ± 3.6
Preoptic-Diagonal Band	10.2 ± 5.9	42.8 ± 7.7	11.8 ± 3.9	15.0 ± 4.0	141.1 ± 28.9	282.6 ± 55.3	77.7 ± 12.5
Amygdala	ND	24.1 ± 6.1	ND	33.7 ± 5.3	45.9 ± 8.2	57.3 ± 9.0	87.1 ± 12.5
Globus Pallidus	10.1 ± 2.8	32.8 ± 5.8	24.6 ± 5.1	31.3 ± 4.8	19.5 ± 4.4	81.5 ± 7.2	51.9 ± 9.9
Lateral Septum	ND	63.7 ± 10.6	ND	NC	100.3 ± 7.4	147.6 ± 12.8	84.1 ± 9.6
Hippocampus	1.9 ± 0.4	ND	ND	25.3 ± 4.3	24.7 ± 5.1	2.7 ± 0.8	34.0 ± 3.4
Dorsal Thalamus	13.3 ± 2.2	7.8 ± 1.2	ND	11.6 ± 3.5	18.8 ± 3.2	6.9 ± 2.0	29.0 ± 6.1
Ventral Thalamus	1.6 ± 0.7	9.7 ± 2.0	ND	38.8 ± 6.8	35.2 ± 2.3	7.5 ± 5.2	40.5 ± 8.1
Lateral Hypothalamus	ND	14.8 ± 3.8	ND	49.1 ± 10.7	60.2 ± 8.4	30.0 ± 6.3	81.8 ± 7.6
Medial Hypothalamus	ND	19.7 ± 3.7	ND	23.9 ± 6.2	155.0 ± 21.4	21.7 ± 4.9	67.8 ± 6.8
Substantia Nigra	ND	18.1 ± 3.1	ND	2.6 ± 1.4	26.1 ± 5.2	48.5 ± 6.3	70.0 ± 11.6
Ventral Tegmental Area	17.2 ± 3.1	32.1 ± 3.1	ND	15.5 ± 4.3	104.6 ± 11.3	74.6 ± 5.9	56.6 ± 7.2
Remaining Brain Stem	ND	ND	ND	24.5 ± 2.0	24.5 ± 2.0	34.3 ± 3.8	37.4 ± 5.7

Values represent Mean ± S.D., n=11. In addition, the HPLC chromatograms revealed a peak in some areas, which co-migrated with normetanephrine, and its content ranged from 3.1–15.6 pmol/mg protein; ND—not detected; NC—not calculated, although likely present in a content range circa 10 pmol/mg protein.

TABLE 3

TURNOVER RATES OF DOPAMINE, NOREPINEPHRINE, AND SEROTONIN IN NINETEEN RAT BRAIN AREAS

Brain Area	Turnover Rates (pmol/mg protein-hour) DA	NE	5-HT
Frontal Cortex	2.3 ± 0.7	3.6 ± 0.5	18.0 ± 1.9
Pyriform Cortex	30.9 ± 4.1	6.4 ± 1.9	9.5 ± 1.4
Motor-Somatosensory Cortex	1.7 ± 0.2	5.8 ± 0.6	13.1 ± 1.3
Entorhinal-Subicular Cortex	0.4*	8.9 ± 1.2	12.1 ± 1.1
Cingulate Cortex	0.6*	8.8 ± 1.4	4.4 ± 0.6
Nucleus Accumbens	158.4 ± 16.5	3.8*	3.5 ± 0.5
Lateral Septum	4.7 ± 7.3	15.8 ± 1.6	33.9 ± 0.1
Caudate-Putamen	426.2 ± 60.7	0.5*	11.5 ± 1.0
Preoptic-Diagonal Band	229.2 ± 60.1	52.7 ± 10.6	35.1 ± 6.0
Amygdala	67.0 ± 9.5	25.5 ± 4.2	35.7 ± 4.8
Globus Pallidus	16.0 ± 2.4	1.0*	14.6 ± 2.8
Medial Hypothalamus	6.2 ± 1.3	7.7*	44.1 ± 3.8
Lateral Hypothalamus	10.4 ± 1.6	42.9 ± 5.7	73.7 ± 5.5
Hippocampus	0.6*	20.5 ± 2.0	15.2 ± 1.2
Dorsal Thalamus	0.4*	2.0 ± 0.1	13.7 ± 1.2
Ventral Thalamus	3.8 ± 0.6	16.4 ± 1.0	26.8 ± 3.8
Substantia Nigra	28.0 ± 3.6	1.0*	18.2 ± 3.6
Ventral Tegmental Area	29.9 ± 6.4	107.7 ± 11.4	36.2 ± 6.2
Remaining Brain Stem	3.1 ± 0.6	6.3 ± 0.7	2.0 ± 0.4

Data represent Mean ± S.D., n=6–7.
*Turnover was low (estimate).

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