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DEVELOPMENTAL CHANGES IN PROTEIN CARBOXYLMETHYLATION AND IN THE BENZODIAZEPINE RECEPTOR PROTEINS IN RAT BRAIN

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The intraventricular administration of [^{3}H-methyl]-methionine resulted in a steady increase in carboxyl-[^{3}H]methylated protein formation in the brain of developing rats (2-42 days). The analysis by gel filtration on Sepharose-4B of Lubrol-P_x-solubilized membrane proteins, including benzodiazepine receptor proteins, revealed co-migration of carboxyl-[^{3}H]methylated and [^{3}H]flunitrazepam-binding protein species. The latter displayed a single peak at 2 days and 2-3 peaks subsequently. At 26 days the lighter of the two [^{3}H]flunitrazepam and [^{3}H]muscimol-binding peaks associated with the principal carboxyl-[^{3}H]-methylated protein peak. The findings suggest the participation of carboxylmethylation in the maturation of benzodiazepine receptor proteins.

Although carboxylmethylation is a well recognized post-translational protein modification (1,2) involving exclusively aspartic and glutamic acid residues, little is known regarding its ontogeny in brain tissue. Paik et al. (3) reported a slow increase in activity of protein methylase II (PM II), (EC 2.1.1.24), the carboxylmethylating enzyme, during the first 10 days of life in rat brain, while, in the mouse, Clark et al. (4) reported a 2-3 fold increase in cerebral PM II after gestation day 19 and adult levels by 30 days post-natally. Similarly, Kloog et al. (5) noted that PM II activity of neuroblastoma cells increases in parallel with their differentiation.

In contrast to the meager knowledge about the ontogeny of cerebral protein carboxylmethylation, there is ample information regarding the ontogenetic development of the cerebral benzodiazepine (Bz)-γ-aminobutyric-acid-(GABA)-chloride channel receptor complex (6,7). Ontogenetic studies (8-10) have revealed an early development of Bz-receptors, present at birth, and a differential rate of formation of the associated proteins in different brain regions (8-10). The pharmacologically distinct type 1 and type 2 Bz-receptors

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(11) have also been shown to develop differentially, as has the ability of GABA to modulate the binding of [^{3}H]flunitrazepam to the cerebral Bz-receptor (12).

In view of these facts, a relevant question arose: is carboxylmethylation implicated in the ontogeny of Bz-receptor proteins? Since another neural receptor complex, the nicotinic acetylcholine receptor (13), is an excellent methyl accepting protein *in vitro*, experiments designed to obtain an answer to the above question appeared desirable. In the present study we measured the carboxylmethylation *in vivo* of detergent-solubilized, Bz-receptor-containing rat brain membrane proteins (14) at 4 early post-natal time points and we determined, in parallel, their ability to bind [^{3}H]flunitrazepam and, in one case, [^{3}H]muscimol, as well.

The results indicate that, while at 2 days, the peak of [^{3}H]flunitrazepam binding associated with proteins of low carboxyl-[^{3}H]methyl content, at 17, 26 and 42 days, [^{3}H]flunitrazepam binding associated with proteins of significant carboxyl-[^{3}H]methyl content. In addition, our data show a single, soluble [^{3}H]flunitrazepam-binding protein peak at 2 days and 2 to 3 such peaks at 17, 26 and 42 days.

MATERIALS AND METHODS

Animals and Chemicals

Male rats (Sprague-Dawley) were from Spartan (Haslett, MI). [^{3}H-methyl]-methionine (8-10 Ci/mmole) was from ICN Pharmaceuticals (Irvine, CA), [^{3}H-N-methyl]-flunitrazepam (78 Ci/mmole), [^{3}H-methylamino]-muscimol (9 Ci/mmole) NCS and ACS scintilation cocktail were purchased from Amersham. Sepharose-4B was from Pharmacia (Piscataway, NJ). Nonradioactive flunitrazepam was a gift from Hoffman-La Roche, Inc. All reagent chemicals were from Sigma. The GF/B filters were from Whatman. Polyethylene glycol (8,000) was from Sigma.

Solubilization Procedure

Animals under light ether anesthesia were injected into the right lateral cerebral ventricle with 10-30 uCi of [^{3}H-methyl]-methionine in 10-15 ul of artificial cerebrospinal fluid (Merlis solution). The rats were decapitated one hour later together with an equal number of naive littermates. The two sets of brains (minus cerebellum) were homogenized in 8-10 volumes of ice-cold 10 mM sodium phosphate buffer, pH 6.8, containing 0.2M NaCl and the protease inhibitors listed by Stephenson and Olsen (15) using the Polytron homogenizer (2 x 5 sec bursts, 15 sec apart, setting 5 on the scale of 8). The homogenates were centrifuged at 100,000 x g for 60 min, the resulting pellets were resuspended in the above buffer containing 1% Lubrol-P$_X$ and were allowed to stand at 0°C for 30 min. Subsequent centrifugation at 100,000 x g for 60 min yielded Lubrol-solubilized proteins (fraction S$_L$) as supernatants.

Gel Filtration of Fraction S_L

Immediately after its preparation, the radioactive fraction S_L (30-40 mg protein) was injected on a 2.5 x 50 cm column of Sepharose-4B, equilibrated with homogenization buffer containing 0.2% of Lubrol-P_x, but no bacitracin and benzamidine. 2.8 ml fractions were collected at a flow rate of 30 ml/h and 1-2 ml aliquots were assayed for carboxyl-[^{3}H]methyl containing proteins. The non-radioactive S_L fraction was similarly fractionated and the binding of ligands to the eluate was determined. All chromatographic operations were carried out at 4°C.

Assays

Protein carboxylmethylation was determined according to O'Dea et al. (16). Briefly, 1-2 ml aliquots were precipitated with trichloracetic acid (TCA), the methyl groups were hydrolyzed in alkali and the formed methanol was extracted with toluene: isoamyl alcohol (3:2). The difference between total and nonvolatile radioactivity was used as index of protein carboxylmethylation. Binding of [^{3}H]-flunitrazepam was determined at 5 nM after 30 min incubation at 0°C. The samples were precipitated with polyethylene glycol (8,000) and filtered through GF/B filters under reduced pressure (17). Washed and dried filters were counted in 7 ml ACS with 42-45% efficiency. [^{3}H]muscimol binding was determined by the same method at 50 nM except the incubation time was 15 min. For non-specific binding cold flunitrazepam and GABA in 1000-fold excess were used.

Protein concentrations were determined by a modified Lowry method (18) employing sodium dodecyl sulfate to avoid Lubrol-P_x interference. Bovine serum albumin was used as standard.

RESULTS AND DISCUSSION

Following the intraventricular injection of [^{3}H-methyl]-methionine, its uptake by brain cells and its conversion to [^{3}H-methyl]-S-adenosyl-L-methionine (AdoMet) by cerebral S-adenosylmethionine synthase (EC 2.5.1.6), part of the [^{3}H-methyl] group of AdoMet became transferred to the free carboxyl groups of selected aspartic and glutamic acid protein residues by PM II (1). Figure 1 clearly shows the steady increase in carboxylmethylation of homogenate proteins with age, while Table 1 shows that the proportion of the total homogenate radioactivity converted to protein carboxyl-[^{3}H]methyl residues quadrupled between days 2 and 42. A similar increase is seen in the homogenate as well as in Fraction S_L when the data is expressed as dpm/mg of protein per 1 uCi of radioactivity present in 1 g of brain wet weight (Table 2). Our findings confirm, in the developing rat brain *in vivo*, the recent results of Clark et al. (4) who showed that protein carboxylmethylation, measured in mouse brain minces, keeps increasing up to about 30 days post-natally. *In vitro* measurements of cerebral PM II acting on gelatin yielded similar results (3).

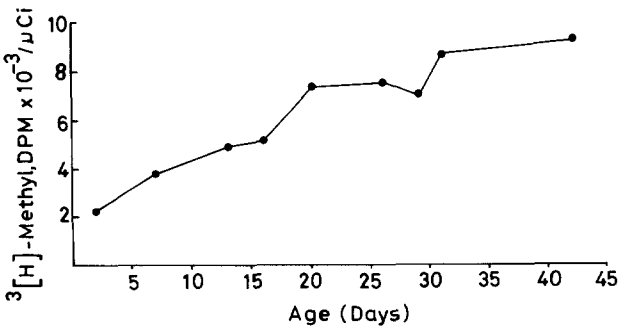

Fig. 1. In vivo carboxylmethylation of rat brain proteins during early postnatal development.
Protein carboxylmethylation in homogenates was determined as described (16). Total homogenate radioactivity was determined on 20 ul aliquots of ten times diluted suspensions by direct counting in 10 ml of ACS. The number of rats was as follows: (age in days in parenthesis) 10 (2), 9 (7), 6 (13), 4(16), 5 (20), 8 (26), 3 (29), 2 (31) and 3 (42). The points represent triplicate measurements for a single experiment for 2, 7, 13, 20, 29, 31 and 42-day old animals and averages of 4 and 5 experiments for 16 and 26-day old animals. The ordinate refers to the radioactivity (DPM) as carboxyl-[^{3}H]methyl groups divided by the total radioactivity in uCi present in the homogenate.

The in vivo carboxyl-[^{3}H]methylated, Lubrol-P_x-solubilized proteins contained in Fraction S_L (Table 3) could be resolved into distinct subpopulations upon gel filtration on Sepharose-4B. Since, however, the main objective of gel filtration was to compare the age profiles of the carboxyl-[^{3}H]-methylated proteins to the corresponding profiles marking Bz-receptor activity, possible differential losses in carboxyl-[^{3}H]methyl groups due to the relatively low acidity of the chromatographic buffer (pH 6.8) were not rigorously controlled and therefore the reported radioactivities represent minimum values. Although several workers (15,17,19-25) have analyzed solubilized Bz-receptor proteins from adult brains, no study has appeared, to our knowledge, in which such proteins were examined during early brain develop-

Table 1. The effect of age on the carboxylmethylation of brain proteins in vivo

Age, Days	Total Radioactivity	Carboxyl- ^{3}[H]Methylated Proteins	
	dpm x 10^{-6}	dpm x 10^{-4}	% of total
2	25.50	2.60	0.103
17	18.90	2.70	0.143
26	11.70	3.86	0.330
42	4.64	1.95	0.420

Table 2. The effect of age on the specific radioactivity of carboxyl-[^{3}H]methylated brain proteins.

Age, days	Homogenate (dpm/mg protein)*	Fraction S_L (dpm/mg protein)*
2	44	60
17	50	85
26	77	88
42	143	200

* The specific activity values shown were normalized by dividing by the total radioactivity/gram wet weight (uCi/g) (see text).

ment. Figure 2 (panel A) reveals a single, rather broad, peak of [^{3}H]flunitrazepam-binding activity and two distinct peaks of carboxyl-[^{3}H]methylated protein at 2 days; two distinct [^{3}H]flunitrazepam-binding peaks became apparent at 17 days, one of which (tubes 37-43) clearly associated with 2 peaks of carboxyl-[^{3}H]methylated protein. At 26 days (panel C), the smaller second [^{3}H]flunitrazepam-binding activity peak again associated with the principal carboxyl-[^{3}H]methylated proteins. [^{3}H]muscimol-binding to the same eluate also had 2 peaks, the second of which clearly associated with the highest carboxyl-[^{3}H]methylated protein peak. At 42 days (panel D) [^{3}H]flunitrazepam-binding activity and carboxyl-[^{3}H]methylated proteins also shared a substantial portion of the eluate, namely, tubes 35-41.

Table 3. Protein carboxylmethylation and TCA-insoluble radioactivity in a 20-day old rat brain after a 1 hour pulse of [^{3}H-methyl]-methionine.*

Fraction	TCA-Insoluble** Radioactivity dpm/mg protein	Carboxyl-[^{3}H]-Methylated Proteins
Homogenate	41,300	172
Fraction S_L	42,700	207
Sepharose-4B peak***	61,400	285

* The injections were 12 uCi per animal. Five brains were pooled and the procedure outlined in Methods was followed.
** Aliquots of fractions were precipitated with 10% TCA, washed twice with 5% TCA, the pellets were digested with NCS and counted after addition of 10 ml ACS.
*** Fractions from Sepharose 4B chromatography with high capacity for [^{3}H]-flunitrazepam were pooled and assayed.

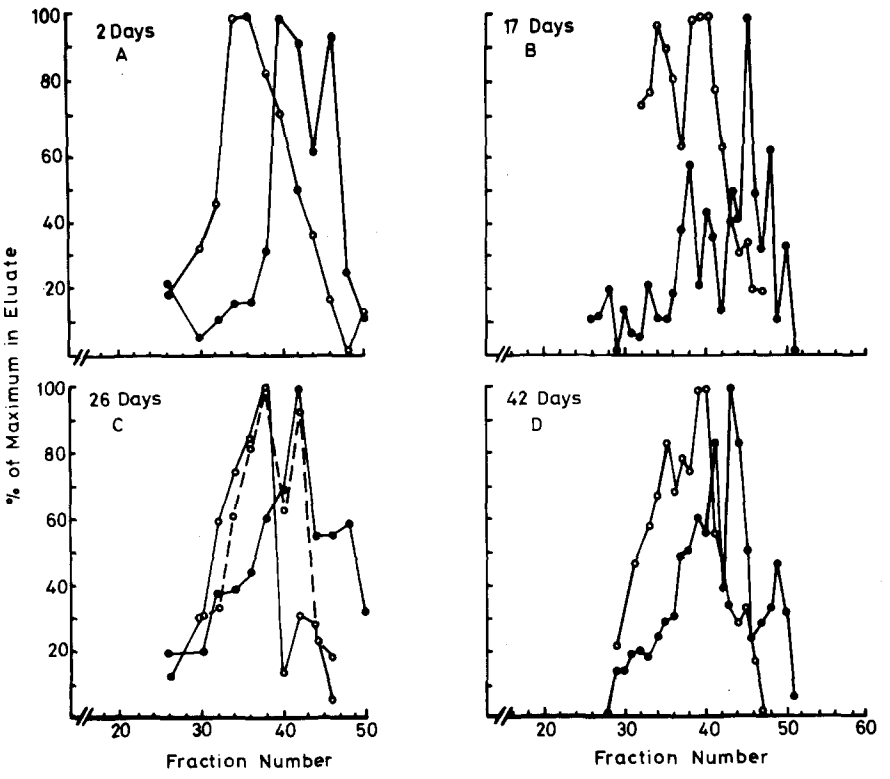

Fig. 2. Sepharose-4B chromatography of Lubrol P_X-solubilized rat brain membrane proteins. Fraction S_L, prepared from brains of 2, 17, 26 and 42 day old rats, half of which were injected intraventricularly with [^{3}H-methyl] methionine (see Methods), were successively passed (radioactive fraction first) through a column of Sepharose-4B, as described in Methods. The ordinates (o----o), carboxyl-[^{3}H]methylated proteins, (o----o), [^{3}H]-flunitrazepam and (o- - o), [^{3}H]muscimol binding are expressed as % of maximum.

The presence of more than one peak of soluble [^{3}H]flunitrazepam-binding activity in the brain of 17, 26 and 42-day old rats and of a single peak in the brain of 2-day old rats, represents a novel finding. Sieghart and Mayer (26) analyzed the post-natal development of Bz-receptors by SDS gel electrophoresis after photoaffinity labelling with [^{3}H]-flunitrazepam. Up to 8 days of age, [^{3}H]-flunitrazepam labelled predominantly the P_{55} and P_{59} polypeptides while, in the second post-natal week the labelling shifted to a lighter, P_{51} polypeptide. Our results are consistent with the notion that, as the Bz-receptor proteins mature, their multiplicity temporarily increases until, in the adult brain, the Bz-receptor complex reaches a definitive size and, hence, exhibits a single molecular weight in soluble form (15,17,19-21,23).

The evidence of an apparent co-migration of the *in vivo* carboxyl-[^{3}H]-methylated proteins with [^{3}H]flunitrazepam-binding proteins at 17, 26 and 42 days but not at 2 days (Figure 2) suggests, without as yet proving, that carboxylmethylation plays a role in the maturation of the Bz-receptor proteins. Given that both PM II activity and Bz-receptor binding are present prenatally and increase dramatically in the first 3 post-natal weeks (4,10) it is attractive to speculate that carboxylmethylation participates in the terminal phase of the receptor's maturation process. At that time, the Bz-receptor complex speciates into pharmacologically diverse subclasses (11) and, in general, acquires both the molecular and physiological characteristics of adulthood (27).

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