Biochemical Pharmacology, Vol. 26, pp. 1569–1572. Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF ETHANOLAMINE-*O*-SULPHATE ON REGIONAL GABA METABOLISM IN THE MOUSE BRAIN

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(*Received* 11 *February* 1977; *accepted* 7 *March* 1977)

Abstract—The distribution of [^{3}H]ethanolamine-*O*-sulphate (EOS) and the regional changes in GABA metabolism after 160 μg (8 mg/kg) intracerebroventricularly and 2 g/kg subcutaneously have been studied in the mouse brain. Over 60 per cent of the injected radioactivity (given i.c.v.) was lost within the first hr. That remaining at 24 hr, however, after subcellular fractionation appeared to be associated with GABA-transaminase (GABA-T). The greatest binding occurred in the hypothalamus, an area with a high endogenous GABA concentration and the highest turnover rate of those areas studied. GABA turnover rates calculated over a 4 hr period for both 160 μg i.c.v. and 2 g/kg s.c. correlated ($P < 0.001$) with the endogenous GABA concentrations. EOS 2 g/kg s.c. was as effective as 160 μg i.c.v. in inhibiting GABA-T in the brain, but has a slower onset of action. The possibility of a larger GABA-T pool in the hypothalamus than in the other areas studied is discussed.

Ethanolamine-*O*-sulphate (EOS) is an active site directed irreversible inhibitor of GABA transaminase (4-aminobutyrate: 2-oxoglutarate amino transferase, E.C. 2.6.1.19.) (GABA-T) both *in vitro* and *in vivo* [1]. EOS does not inhibit glutamic acid decarboxylase [2], succinic semialdehyde dehydrogenase [3], alanine aminotransferase or aspartate aminotransferase [1] and is at present the most specific GABA-T inhibitor available. Upon intracerebroventricular or intracisternal administration to mice or rats, EOS produces raised GABA levels [3, 4] accompanied by behavioural depression and diminished hind limb extensor phase to maximal electroshock [5].

In order to determine whether EOS acts at a specific site in the brain, its distribution and the regional changes in GABA metabolism after intracerebroventricular (i.c.v.) administration were studied. Although it has been stated by Fowler and John [1] that EOS may not be expected to cross the blood/brain barrier, we have also examined the effects of subcutaneously administered EOS on GABA metabolism.

MATERIALS AND METHODS

Preparation and distribution of [^{3}*H*]*ethanolamine-O-sulphate.* [^{3}H]EOS was prepared by the method of Rumpf [6] from [^{3}H]ethanolamine (sp act 3.8 Ci/m-mole). Briefly, [^{3}H]ethanolamine (plus carrier ethanolamine to a final concentration of 16.9 mM), was reacted with an exact double molar excess of H_2SO_4 (33.8 mM) at 140° and then evaporated to dryness under nitrogen. The brown residue was dissolved in 100 μl of distilled H_2O and the [^{3}H]EOS purified and separated from any unreacted [^{3}H]ethanolamine by two dimensional TLC using *n*-butanol:acetic acid:H_2O (120:30:50) as the first solvent and isopropanol:ethanol:*N*-HCl (75:75:50) as the second. Spots were identified by spraying with 0.5 g benzoquinone in 10 ml pyridine and 40 ml *n*-butanol. Ethanolamine and EOS give brown-red spots. Approximately 0.4 mCi [^{3}H]EOS was synthesised from 1 mCi [^{3}H]ethanolamine.

For regional brain distribution studies, 160 μg EOS containing 88,800 dpm (all in 0.02 ml) was injected i.c.v. into groups of 10 female Horne's mice (20 g). After 1, 4 or 24 hr, the mice were killed and six brain areas rapidly dissected onto cardice. The areas were weighed individually on a torsion balance, solubilized and counted by the method of Dent and Johnson [7] DPM were determined by the use of a quench correction curve.

GABA-transaminase assay. Female Hornes mice (20 g) were given EOS 160 μg i.c.v. or 2 g/kg s.c. and whole brain GABA-T activity determined up to 24 hr later. The brains were homogenised in 2 ml 0.4 M borate buffer pH 8.2. GABA-T activity was also determined after 4 hrs in six brain areas. The brain areas from 5 mice were pooled and homogenised at 100 mg/ml (50 mg/ml for hypothalamus) in 0.4 M borate buffer pH 8.2.

Incubations were carried out at 37° for 30 min in 1.5 ml of 0.4 M borate buffer, pH 8.2 containing 50 μmoles GABA, 50 μmoles α-ketoglutarate, 15 μg GSH, 15 μg pyridoxal phosphate and 0.2 ml homogenate. The reaction was stopped by the addition of 0.5 ml 25% TCA. Zero blanks (trichloroacetic acid added before enzyme) were run concurrently. The succinic semialdehyde formed was estimated by the method of Salvador and Albers [8] and standard succinic semialdehyde was prepared by the method of Bruce *et al.* [9].

GABA assay. Mice were killed and the brains frozen in liquid nitrogen. Areas pooled from 5 mice were homogenised in 3 ml 0.5 M perchloric, centrifuged (after standing in ice for 30 min) and the supernatant neutralised with 20% $KHCO_3$. GABA was measured by the formation of NADPH using the GABA-transaminase/succinic semialdehyde dehydrogenase (Gabase) system [19]. The incubation mixture

contained 0.1 M Tris–HCl buffer pH 8.9, 3 2 mM α-ketoglutarate, 0.5 mM NADP, 8 mM 2-mercapto ethanol, 250 μl of neutralised extract (total vol. 1.25 ml) and 'Gabase' to complete the reaction in 60 min. GABA standards (up to 1 mM) and tissue blanks were run in parallel.

Subcellular distribution of [^{3}H]EOS. Mice (20–25 g) were dosed with 88,000 dpm [^{3}H]EOS in 0.02 ml and killed after 24 hr. The brains were removed, washed in 0.32 M sucrose and then pooled. A 10% whole brain homogenate in 0.32 M sucrose was prepared in a glass homogeniser with a Teflon pestle. Subcellular fractions were prepared at 0–4° using the method of Salganicoff and De Robertis [10]. For determination of radioactivity, pellets were rehomogenised in 5 ml of 0.32 M sucrose, and aliquots taken for counting. For estimation of GABA-T activity, 0.5 ml aliquots of pellets rehomogenised in 5 ml of 0.32 M sucrose, or of the soluble supernatant fractions were used in the assay previously described.

Protein content. Protein concentrations were determined by the Miller method [11].

Materials. Labelled materials. [1-^{3}H]Ethan-1-ol-2-amine hydrochloride, 3.8 Ci/m-mole from Radiochemical Centre, Amersham. Gabase in 50% glycerol and NADP from Sigma, London. Ethanolamine-*O*-sulphate (2-amino ethyl hydrogen sulphate) was obtained in crude form from Kodak Ltd., Kirkby, Liverpool, and purified by dissolving in distilled water and precipitating with absolute ethanol.

RESULTS

Distribution of [^{3}H]EOS. Table 1 shows the distribution of [^{3}H]EOS in mouse brain areas for up to 24 hr after i.c.v. administration. Over 60 per cent of the radioactivity was lost within the first hr presumably by leakage from the injection site and into the CSF. The radioactivity remaining at 24 hr appeared to be associated with GABA-T activity ($P < 0.020$) as suggested by the subcellular distribution studies (Table 2). The greatest residual concentration of label occurred in the hypothalamus.

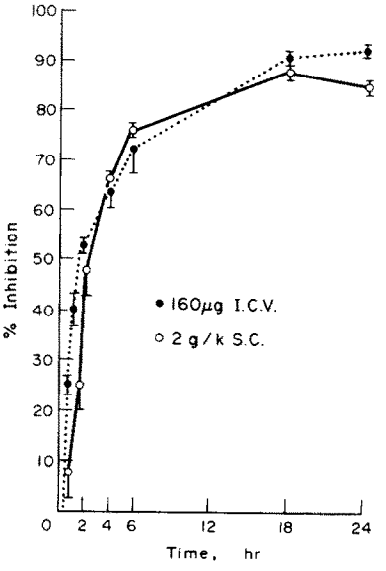

Fig. 1. Inhibition of GABA-T activity by EOS in whole mouse brain. Female Hornes mice were dosed with 160 μg EOS i.c.v. or 2 g/kg s.c. and GABA-T activity measured at various times up to 24 hr. Values are mean ± S.E.M. of 4 animals/time point.

Table 1. Regional distribution of [^{3}H]EOS

Brain region	Time after EOS 1 hr DPM/mg tissue	Ratio	4 hr DPM/mg tissue	Ratio	24 hr DPM/mg tissue	Ratio
Cerebellum	65 ± 3	1.84	72 ± 7	1.57	56 ± 4	1.63
Medulla and pons	96 ± 10	2.58	95 ± 9	2.04	58 ± 4	1.68
Hypothalamus	152 ± 11	4.16	147 ± 13	3.08	107 ± 15	3.12
Midbrain	85 ± 5	2.34	91 ± 7	1.94	58 ± 2	1.69
Striatum	69 ± 9	1.81	71 ± 8	1.61	55 ± 4	1.61
Cerebral cortex	38 ± 5	1	48 ± 5	1	35 ± 3	1

Female Hornes mice (20 g) were dosed i.c.v. with 160 μg EOS containing 88,800 dpm all in 0.02 ml for 1, 4 and 24 h. Values are mean ± S.E.M. of 10 animals. For comparison of distribution ratios, cerebral cortex = 1.

Table 2. Subcellular distribution of [^{3}H]EOS

Fraction	Description	%[^{3}H]EOS*	% GABA-T†	% GABA-T‡
P_1	Nuclear + debris	19.9 ± 3.1	19	16
P_2	Crude mitochondrial	46.3 ± 2.7	64	72
P_3	Microsomal	12.5 ± 0.8	5	2
S_3	Soluble cytoplasm	21.1 ± 1.7	12	7

* Values are mean ± S.E.M. of per cent of total DPM recovered after 24 hr. [^{3}H]EOS (88,800 dpm) was dosed to 6 mice i.c.v. in 0.02 ml. Mice were killed, the brains removed and pooled for centrifugation after homogenisation at 10% in 0.32 M sucrose. Values are mean ± S.E.M. of 5 experiments.

† Values are % of total GABA-T activity determined from brains pooled from 6 mice; $r = 0.987$, $P < 0.020$ with $\nu = 2$.

‡ Values from Waksman *et al.* [18]; $r = 0.982$, $P < 0.020$ with $\nu = 2$.

Table 3. Effect of EOS on mouse brain GABA-T activity

Brain region	Control GABA-T activity (μmoles/mg protein/hr)	Percent inhibition after 160 μg EOS i.c.v. for 1 hr
Cerebellum	0.278 ± 0.020	51 ± 4*
Medulla and pons	0.400 ± 0.038*	41 ± 3
Hypothalamus	0.381 ± 0.071*	55 ± 5*
Midbrain	0.360 ± 0.058*	39 ± 7
Striatum	0.301 ± 0.032*	55 ± 6*
Cerebral cortex	0.220 ± 0.024	40 ± 4

Groups of 5 female Hornes mice (20 g) were given EOS 160 μg i.c.v. for 1 hr. Values are mean ± S.E.M. of 6 experiments.

* $P < 0.050$ when compared with cerebral cortex.

Effect of EOS on GABA-T activity. The inhibition of GABA-T activity by 160 μg i.c.v. and by 2 g/kg s.c. EOS in whole mouse brain is shown in Fig. 1. Both treatments inhibited the enzyme by more than 85 per cent at 24 hr. The inhibition of GABA-T by EOS 2 g/kg s.c. was of a slower onset than 160 μg i.c.v. having no significant effect at 30 min.

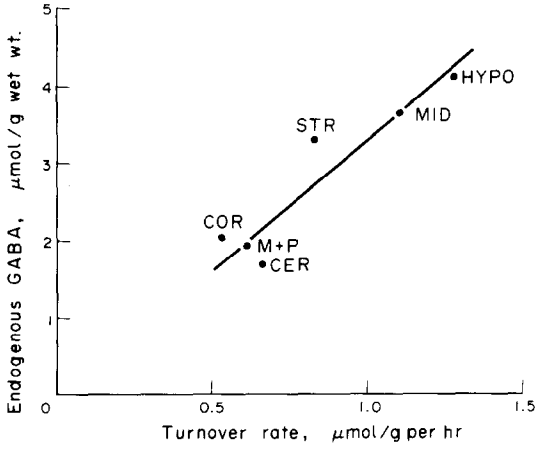

Fig. 2. Correlation between turnover rate as determined after 160 μg EOS i.c.v. and endogenous GABA concentrations in the areas studied. Correlation coefficient $r = 0.933$, $P < 0.010$ with 3 degrees of freedom.

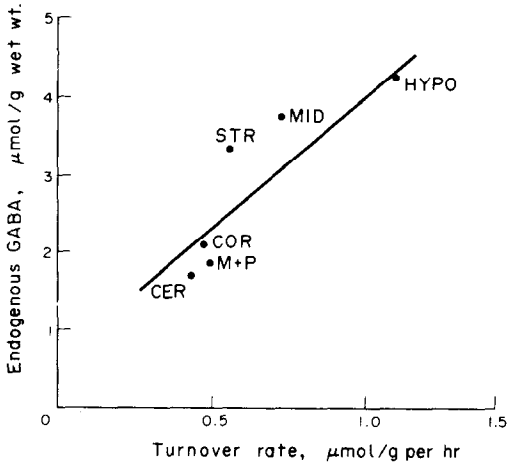

Fig. 3. Correlation between turnover rate as determined after 2 g/kg EOS s.c. and endogenous GABA concentrations in the areas studied. Correlation coefficient $r = 0.865$, $P < 0.025$ with 4 degrees of freedom.

EOS (160 μg i.c.v. for 1 hr, Table 3) did not appear to preferentially inhibit GABA-T to any great extent in any particular brain area. Mean control whole brain GABA-T activity was 0.394 ± 0.014 μmoles succinic semialdehyde produced/mg protein/hr ($n = 12$).

Effect of EOS on GABA concentrations. Both 160 μg EOS i.c.v. and 2 g/kg S.C. (Table 4) caused significant increases in endogenous GABA concentrations in all areas. GABA turnover rates were calculated by exponential rise over the 4 hr period although the rates may only be regarded as apparent, since GABA-T was not completely inhibited at 4 hr. For both treatments, the rates correlated with the control endogenous GABA content of the areas studied (Figs. 2 and 3), with the highest rate occurring in the hypothalamus. The rises in whole brain GABA levels were 240 per cent (160 μg i.c.v.) and 147 per cent (2 g/kg s.c.) with approximately 60 per cent inhibition of GABA-T after 4 hr.

DISCUSSION

The distribution of [^{3}H]ethanolamine-*O*-sulphate given intracerebroventricularly will be dependent upon loss due to leakage from the injection site, loss

Table 4. Effect of EOS 160 μg i.c.v. and 2 g/kg s.c. on mouse brain GABA levels

Brain region	Endogenous GABA levels (μmoles/g/wet wt.) Control	EOS 160 μg i.c.v.	EOS 2 g/kg s.c.	After 160 μg EOS i.c.v. Apparent turnover rate (μmoles/g/hr)	$t_{1/2}$ (h)	k (h^{-1})
Cerebellum	1.75 ± 0.16	8.20 ± 0.57*	4.75 ± 0.35*	0.675 ± 0.050	1.793 ± 0.031†	0.387 ± 0.006†
Medulla and pons	1.87 ± 0.11	7.42 ± 0.42*	5.39 ± 0.32*	0.644 ± 0.033	2.019 ± 0.074‡	0.344 ± 0.013‡
Hypothalamus	4.23 ± 0.58	14.59 ± 0.39*	11.77 ± 0.35*	1.289 ± 0.031†	2.270 ± 0.276	0.314 ± 0.035
Midbrain	3.70 ± 0.20	11.96 ± 0.58*	8.32 ± 0.97*	1.081 ± 0.051†	2.379 ± 0.121	0.293 ± 0.015
Striatum	3.36 ± 0.29	9.10 ± 0.84*	6.48 ± 0.18*	0.818 ± 0.079	2.922 ± 0.472	0.249 ± 0.036
Cerebral cortex	2.08 ± 0.01	6.58 ± 0.31*	5.35 ± 0.49*	0.579 ± 0.025	2.419 ± 0.078	0.289 ± 0.009

Groups of 5 female Hornes mice (20 g) were given EOS either s.c. or i.c.v. for 4 hr. Values are expressed as μmoles/g wet wt. and are mean ± S.E.M. of 3 experiments. The $t_{1/2}$, k and turnover rates were calculated on the basis of an exponential rise in GABA concentrations over the 4 hr period [12].

* $P < 0.005$ cf. control

† $P < 0.001$ cf. cerebral cortex.

‡ $P < 0.025$ cf. cerebral cortex.

into the CSF, upon metabolism and non-specific binding of EOS and its metabolites, and upon irreversible binding to GABA-transaminase. It has been suggested [1] that EOS may undergo β-elimination catalysed by GABA-T and the animoethylene intermediate may bind to inactivate the enzyme. Subcellular distribution studies (Table 2) show a good correlation between binding of ^3H remaining after 24 hr and GABA-T activity, and at 24 hr remaining radioactivity (as [^3H]aminoethylene bound to GABA-T) may be representative of the enzyme pools present in each brain area. There is a good correlation between ^3H binding and the rises in endogenous GABA levels that occur in the different brain areas after EOS, the greatest increase was seen in the hypothalamus and the least in the cortex and cerebellum. EOS did not appear to preferentially inhibit GABA-T in any particular brain area at 1 hr (Table 3) and this may indicate uniform distribution after injection.

After EOS administration, the subsequent rises in GABA levels have previously been shown [12] to follow a biphasic exponential pattern, there being a rapid rise during the first 6 hr after injection and the present calculation of turnover rates is based on the exponential rise. At 4 hr, GABA-T is not completely inhibited (60–70 per cent, Fig. 1), however the subsequently determined turnover rates correlate well with the endogenous GABA levels in the different brain areas (Figs 2 and 3), and since it is known [13] that endogenous GABA levels parallel GAD more so than GABA-T activities, the rates may be indicative of GABA synthesis by GAD. The order of the turnover rates for each area calculated using EOS agree with those published by Collins [14] using [2,3-^3H]GABA. Howoever, since EOS does not completely inhibit GABA-T, the values quoted here must only be regarded as apparent and not true rates.

Our findings show good agreement with other workers [3] in that EOS given i.c.v. inhibits GABA-T and elevates brain GABA. We have extended these observations to show that EOS also acts subcutaneously when a large dose (2 g/kg) is given and sufficient time (2 hr, Fig. 1) is allowed for absorption, thus demonstrating that EOS passes the blood brain barrier.

It has been suggested that the GABA-T pool is located in the mitochondria of the 'small' glutamate compartment and that the morphological identity of this compartment is attributed to glial cells (15–17, 20). Our evidence shows greater binding of ^3H to the hypothalamus (an area which also has a high endogenous GABA level and a high turnover rate), which may suggest the presence in this area, of a larger glial GABA-T pool than in the other areas studied. Another possibility is the existence of a hypothalamic GABA-T enzyme with a higher affinity for EOS, and perhaps therefore a higher affinity for GABA or α-ketoglutarate. Thus although GAD is considered to be rate limiting in the maintenance of endogenous GABA concentrations, at least in the hypothalamus the GABA-T activity, whether a function of glial pool size or relative substrate affinity, cannot be ignored in the regulation of GABA turnover.

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