J. Pharm. Pharmacol. 1990, 42: 171–174
Received October 11, 1989

In-vivo Radiolabelled Oxiracetam Binding to Rat Brain

TIZIANA MENNINI, CARLO TADDEI, ALFREDO CAGNOTTO AND FRANCA PONZIO*

*Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy, and *ISF Laboratories for Biomedical Research, Via Leonardo da Vinci 1, 20090 Trezzano S/N, Milan, Italy*

Abstract. The in-vivo binding of [^{3}H]oxiracetam has been studied in brain areas of rats examined 30 min after i.c.v. injection. Soluble radioactivity accounted for more than 90% of total radioactivity in all the structures considered and was not affected by co-injection with a 1000-fold excess of unlabelled oxiracetam. Both total and bound radioactivity showed a marked regional distribution, with highest concentrations in the septum, followed by the hippocampus; the cerebral cortex, striatum and cerebellum had the lower concentrations of radioactivity. Computer-assisted quantitative autoradiography with [^{14}C]oxiracetam confirms these findings. Analysis of [^{3}H]oxiracetam bound to membranes indicated that, after co-injection with a 1000-fold excess of unlabelled oxiracetam, there was a significant reduction of binding only in the septum, hippocampus and cerebral cortex. These results suggest that in those cerebral structures oxiracetam binds to saturable sites.

Oxiracetam is a nootropic drug of the 2-pyrrolidinone class (Banfi & Dorigotti 1986). The positive effect of these compounds in learning and memory processes has been suggested by a variety of behavioural tests (Banfi & Dorigotti 1986; Spignoli & Pepeu 1987). Oxiracetam exerts its pharmacological activity directly in the central nervous system, since it antagonizes scopolamine amnesia after direct administration into the brain lateral ventricles of conscious, freely moving rats (Ponzio et al 1989). It has recently been shown that oxiracetem crosses the blood–brain barrier and preferentially localizes in selected brain areas (Ponzio et al 1989). With the aim of extending knowledge on oxiracetam's localization in brain structures, in the present study we determined the in-vivo interactions of labelled oxiracetam with the two major pools (membrane-bound and soluble, either free or bound to cytosolic proteins) responsible for drug accumulation (Mennini et al 1985; Barone et al 1985; Gobbi et al 1989).

In-vivo oxiracetam binding to rat brain was studied by biochemical and autoradiographic techniques, for the best possible anatomical resolution and to avoid artifacts arising during homogenization and processing of brain tissue.

Materials and Methods

Animal protocol

Male Wistar (Charles River, Italy) rats, 200 ± 20 g (mean ± s.e.m.) permanently implanted with polyethylene cannulas under tribromoethanol anaesthesia (200 mg kg^{-1} i.p.) were injected i.c.v. with 5 μCi of [^{3}H]oxiracetam, labelled in position 4 of the ring (spec. act. 21·72 Ci mmol^{-1}, SKF Welwyn, UK), dissolved in 5 μL of 0·9% NaCl (saline) (final concentration). Thirty minutes after the injection, the rats were decapitated and the brain ventricles perfused with 200 μL of saline to eliminate the extracellular excess of [^{3}H]oxiracetam.

Correspondence to: T. Mennini, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

Determination of [^{3}H]oxiracetam pools

Brain areas of interest (cerebellum, hippocampus, septum, striatum and cerebral cortex) were then rapidly dissected and homogenized in 50 volumes of Tris HCl, 50 mM, pH 7·4, using an Ultra-Turrax TP 1810 (20 s). The total radioactivity present in the areas was measured in two 0·5 mL samples of whole homogenate which were put directly into two scintillation vials each containing 10 mL of scintillation cocktail (Filter Count, Packard), and counted for radioactivity in a Beckman LS-7500 liquid scintillation-spectrometer with counting efficiency of 45%.

The radioactivity bound to the membrane fraction was determined in two 0·5 mL samples of the whole homogenate immediately filtered under vacuum through Whatman GF/C filters. The filters were then washed twice with 5 mL of ice-cold buffer and counted for radioactivity in 10 mL of Filter Count, as above.

Nonspecific binding was determined: (a) in-vitro, by incubating parallel homogenate samples for 3 h at 4°C in presence of 1 mM unlabelled oxiracetam, to allow dissociation of the in-vivo bound [^{3}H]oxiracetam, and (b) in-vivo, by co-injecting i.c.v. labelled oxiracetam (5 μCi/rat = 0·041 μg/rat) with 1000 fold excess of unlabelled oxiracetam (41 μg/rat) (Barone et al 1985; Gobbi et al 1989). Soluble radioactivity was calculated as the difference between total and bound radioactivity, and represents the amount of labelled drug trapped inside the brain structures, either free or bound to cytosolic protein (Mennini et al 1985; Barone et al 1985).

Protein content in the homogenates was determined by Peterson's method (Peterson 1977) using bovine serum albumin as standard.

Autoradiography

For autoradiographic studies, [^{14}C]oxiracetam ([pyrrolidone-2-^{14}C]oxiracetam, 18·8 μCi mg^{-1}, Amersham, UK) was diluted with unlabelled oxiracetam to a final concentration, 8·73 μCi mg^{-1}; the solution was injected intra-arterially (i.a.) at the dose of 100 mg kg^{-1}/2 mL. Thirty minutes after injection the rats were perfused through the heart with saline solution at 37°C, and the brains were removed and frozen.

Table 1. In-vivo [^{3}H]oxiracetam distribution.

	Total radioactivity (fmol (mg prot.)$^{-1}$)		Bound radioactivity (fmol (mg prot.)$^{-1}$)		Soluble radioactivity (fmol (mg prot.)$^{-1}$)	
	0·04 μg/rat	41 μg/rat	0·04 μg/rat	41 μg/rat	0·04 μg/rat	41 μg/rat
Septum	980±137	1023±249	57±6	46±22	924±131	977±229
Hippocampus	689±179	671±133	35±11	31±11	653±169	640±123
Cerebellum	393±91	367±75	28±8	27±4	365±87	340±72
Striatum	300±57	320±21	19±3	20±6	280±54	300±18
Cer. cortex	260±33	215±31	25±6	18±9	235±28	197±27

Data are presented as fmol (mg protein)$^{-1}$ and are mean±s.d. of 4 animals per group. Rats were injected i.c.v. 30 min before with 5 μCi of [^{3}H]oxiracetam dissolved in 5 μL of 0·9% NaCl in the absence (0·4 μg/rat) or presence (41 μg/rat) or a 1000-fold excess of unlabelled oxiracetam. Total and bound radioactivity were determined as described in Materials and Methods, soluble radioactivity was calculated as the difference between total and bound.

Thick coronal tissue sections (54 μm) (Paxinos & Watson 1982) were cut, thoroughly dried under vacuum and exposed to Hyperfilm Bmax (Amersham, UK) for 14 days. The [^{14}C] micro-scale standard (Amersham, UK) containing known amounts of non-volatile labelled compound was exposed to the film with the tissue sections. Films were analysed using a computerized image-analysis system (RAS 1000, Amersham) which provides quantitative values of [^{14}C]oxiracetam binding densities.

Results

Total [^{3}H]oxiracetam distribution

Thirty minutes after i.c.v. injection of [^{3}H]oxiracetam, the total radioactivity was measured in different rat brain regions (Table 1). From the total radioactivity per area, calculated as counts min^{-1} in the total homogenized volume, the total radioactivity present in the brain can be established: 679 700 and 623 000 counts min^{-1}/brain, respectively, in the absence or presence of a 1000-fold excess of unlabelled oxiracetam. These values, representing 13% of the total radioactivity injected in the rat, indicate that total [^{3}H]oxiracetam distribution is proportional to the injected dose (0·041 and 41 μg/rat).

The regional distribution of total [^{3}H]oxiracetam was as follows: septum»hippocampus»cerebellum > striatum > cerebral cortex (Table 1). More than 90% of [^{3}H]oxiracetam is present in a soluble pool and is not affected by co-injection of unlabelled oxiracetam (Table 1).

[^{3}H]oxiracetam binding

Radioactivity bound to brain membranes, measured by filtering a sample of homogenate, is also shown in Table 1. The regional distribution of bound [^{3}H]oxiracetam showed that the value in the septum (57 fmol (mg prot.)$^{-1}$) was higher than in the other brain regions: hippocampus (35 fmol(mg prot.)$^{-1}$) > cerebellum (28) > striatum (19) and cerebral cortex (25).

Table 2 shows the ratios of bound to total radioactivity obtained in two seperate experiments and analysed by ANOVA, randomized blocks. Bound [^{3}H]oxiracetam was significantly less in septum, hippocampus and cerebral cortex after co-injection with a 1000-fold excess of unlabelled oxiracetam, indicating that in these brain regions there is a saturable, though small, component of [^{3}H]oxiracetam binding. In-vitro dissociation of [^{3}H]oxiracetam bound in-vivo was therefore tested on parallel homogenate samples from hippocampus and striatum incubated for 3 h at 4°C with 1 μM of unlabelled oxiracetam (Fig. 1). Dissociation of [^{3}H]oxiracetam binding was obtained in the hippocampus but not in the striatum of rats injected with the tracer dose. No significant reduction of [^{3}H]oxiracetam binding was observed in either the hippocampus or striatum of rats co-injected with a 1000-fold excess of unlabelled oxiracetam.

Table 2. In-vivo [^{3}H]oxiracetam binding.

	% Total bound/Total radioactivity	
	[^{3}H] Oxiracetam	[^{3}H]oxiracetam +1000-fold excess unlabelled oxiracetam
Septum	5·7±0·4	4·6±1·2**
Hippocampus	5·8±1·4	4·7±0·9**
Cer. cortex	10·5±1·7	9·2±2·9**
Cerebellum	8·3±2·0	9·0±1·9
Striatum	7·3±0·9	7·0±1·7

Data are presented as % total bound/total radioactivity and are the means±s.d. of 8 animals per group. Rats were injected 30 min before with 5 μCi of [^{3}H]oxiracetam dissolved in 5 μL of 0·9% NaCl in the absence or presence of 1000-fold excess of unlabelled oxiracetam. Total and bound radioactivity were determined as described in Materials and Methods. * $P=0·13$, ** $P<0·05$ ANOVA and Tukey's test.

Specific [^{3}H]oxiracetam binding in hippocampus calculated considering the non-specific binding determined by dissociation in-vitro, was equal to the value calculated considering as non-specific the [^{3}H]oxiracetam bound in-vivo after co-injection of unlabelled oxiracetam, and represented 1·2% of total radioactivity present in the homogenate and 25% of total binding.

[^{14}C]oxiracetam autoradiography

Table 3 gives a quantitative picture of [^{14}C]oxiracetam distribution in brain regions after i.a. injection of 100 mg kg^{-1}. In these experimental conditions the largest amount of [^{14}C]oxiracetam was again in septum. Intermediate densities were seen in hippocampus and ventricles, followed, to a lesser degree, by pineal gland, hypothalamus and periaqueductal grey. Densities were low in cortex (external layers and

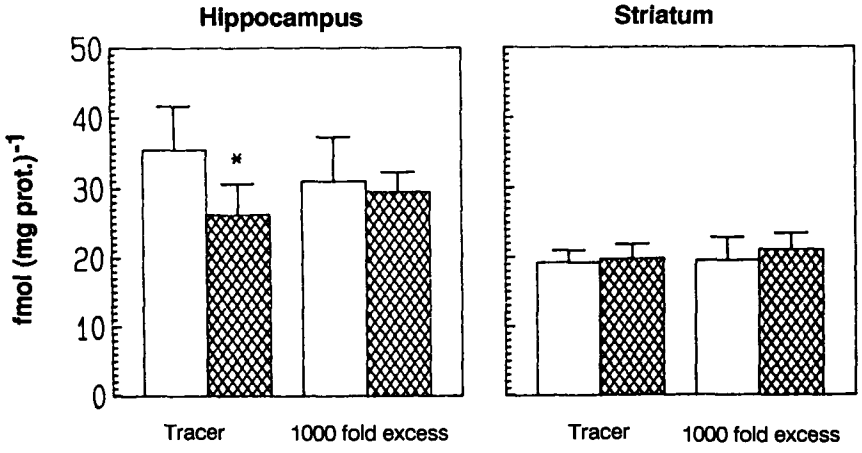

FIG. 1. Bound [^{3}H]oxiracetam was dissociated in-vitro by incubating homogenate samples for 3 h at 4°C with 1 μM oxiracetam, as described in Methods. The [^{3}H]oxiracetam present on the filters after dissociation was considered "non-specific bound"; the non-dissociated was considered "total bound". Data are mean ± s.d. of 4 animals per group ("tracer" received 5 μCi/rat of [^{3}H]oxiracetam alone, "1000 fold excess" were co-injected with 41 μg/rat unlabelled oxiracetam). * $P < 0.05$ ANOVA and Tukey's test.

Table 3. Autoradiographic distribution of in-vivo [^{14}C]oxiracetam binding.

	nmol g^{-1} tissue ± s.e.
Septum	1012 ± 89
Ventricles	459 ± 46
Hippocampus	322 ± 36
Pineal gland	291 ± 27
Hypothalamus	289 ± 44
Periaqueductal	212 ± 52
Cortex*	158 ± 26
Brainstem	118 ± 16
Striatum	54 ± 6

Data are mean ± s.e. of 3 animals.
* Cortical regions showing [^{14}C]oxiracetam binding were the external layers (1–2) and cingulate cortex.

cingulate cortex), brainstem and striatum. A representative picture is given in Fig. 2.

Discussion

We herein describe the in-vivo binding of [^{3}H]oxiracetam in brain areas of rats examined 30 min after i.c.v. injection. Previous studies indicate that in such experimental conditions total radioactivity corresponds to unmetabolized oxiracetam (Ponzio et al 1989). Total radioactivity distribution was proportional to the two doses injected in all tested areas, and 13% was recovered. This suggests that in the wide dose range from 0·04 to 41 μg/rat oxiracetam distribution from cerebrospinal fluid to brain structures could increase linearly. Soluble radioactivity accounted for more than 90%

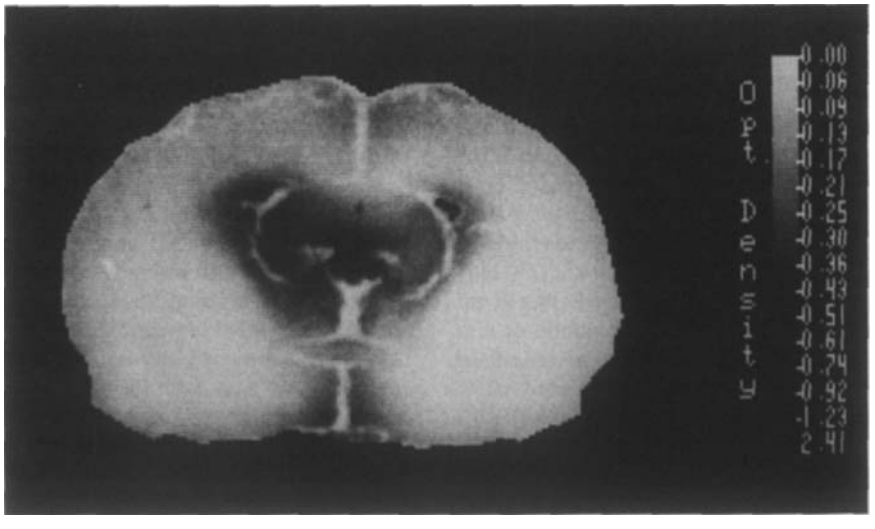

FIG. 2. Representative autoradiogram of [^{14}C]oxiracetam bound in-vivo to rat brain coronal section at the level of the septum.

of total radioactivity in all the structures considered and was not affected by co-injection with a 1000-fold excess of unlabelled oxiracetam.

Both total and bound radioactivity showed marked regional distribution, with highest concentrations in the septum, followed by hippocampus; the cerebral cortex, striatum and cerebellum had the lowest concentrations of radioactivity.

To avoid artifacts arising during tissue homogenization and processing, we compared the biochemical data with the findings of autoradiography where no drug redistribution can occur during sample manipulation. The distribution of oxiracetam in brain areas was again uneven, with preferential accumulation in the septum. Moreover, since brain perfusion before killing removes the extracellular radioactivity, the autoradiographic data represent labelled oxiracetam which is trapped in the cells, either free or bound to membranes and soluble proteins. The uneven regional distribution of labelled oxiracetam is not related to the i.c.v. injection route, used for biochemical studies, as it was confirmed when oxiracetam was given by the general route, employed for autoradiographic studies. These results confirm previous findings describing the selective regional distribution of labelled oxiracetam after i.c.v., i.a. or oral administration (Ponzio et al 1989).

Analysis of [^{3}H]oxiracetam bound to membranes indicated that, after co-injection with a 1000-fold excess of unlabelled oxiracetam, there was a significant reduction of binding only in septum, hippocampus and cerebral cortex. These results suggest that in these cerebral structures oxiracetam binds to saturable sites. To verify the specificity of [^{3}H]oxiracetam binding to membranes, we also studied the dissociation of [^{3}H]oxiracetam bound in-vivo by in-vitro incubation with 1 μM unlabelled oxiracetam (Mennini et al 1985; Barone et al 1985). There was a significant dissociation in hippocampus but not in striatum of rats injected with the tracer solution, confirming that oxiracetam binds to specific and saturable sites in the hippocampus, representing 25% of total [^{3}H]oxiracetam; while binding in the striatum is to non-specific, non-saturable sites. The fact that no dissociation in-vitro could be obtained in the hippocampus homogenates from rats co-injected with a 1000-fold excess of unlabelled oxiracetam further supports the hypothesis that [^{3}H]oxiracetam bound in-vivo in such experimental conditions represents non-specific binding to membrane constituents.

In conclusion, the present findings confirm and extend previous reports about the brain regional distribution of oxiracetam after in-vivo injection and its marked localization in soluble pools.

We also demonstrated that a small, but significant, amount of oxiracetam binds in-vivo to saturable sites on membrane fraction present in the septum, hippocampus and cerebral cortex.

Acknowledgements

The authors thank Angela Uslenghi and Ernesto Donini for excellent technical assistance. Labelled oxiracetam was synthetized by Dr D. Saunders, SKF, UK.

References

Banfi, S., Dorigotti, L. (1986) Experimental behavioral studies with oxiracetam on different types of chronic cerebral impairment. Clin. Neuropharmacol. 9 (Suppl. 3): S19–S26

Barone, D., Luzzani, F., Assandri, A., Galliani, G., Mennini, T., Garattini, S. (1985) In vivo stereospecific 3H-spiperone binding in rat brain: Characteristics, regional distribution, kinetics and pharmacological properties. Eur. J. Pharmacol. 116: 63–74

Gobbi, M., Taddei, C., Mennini, T. (1989) In-vivo (+)-[^{3}H]fenfluramine binding to rat brain: biochemical and autoradiographic studies. J. Pharm. Pharmacol. 41: 253–256

Mennini, T., Barone, D., Gobbi, M. (1985) In vivo interaction of premazepam with benzodiazepine receptors: Relation to its pharmacalogical effects. Psychopharmacology 86: 464–467

Paxinos, G., Watson, G. (1982) The Rat Brain in Stereotaxic Coordinates. Academic Press, Sidney

Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83: 346–356

Ponzio, F., Pozzi, G., Banfi, S., Dorigotti, L. (1989) Brain entry and direct central pharmacological effects of the nootropic drug oxiracetam. Pharmacopsychiatry. Suppl. 22: 111–115

Spignoli, G., Pepeu, G. C. (1987) Interactions between oxiracetam, aniracetam and scopolamine on behavior and brain acetylcholine. Pharmacol. Biochem. Behav. 27: 491–495