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IN VIVO OCCUPANCY OF HISTAMINE H_3 RECEPTORS BY THIOPERAMIDE AND (*R*)-α-METHYLHISTAMINE MEASURED USING HISTAMINE TURNOVER AND AN *EX VIVO* LABELING TECHNIQUE

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Abstract—In the brain, the H_3 type of histamine receptor has a pre-synaptic autoreceptor inhibitory role which regulates neuronal release and synthesis of histamine. To examine the interaction of the selective H_3 receptor antagonist thioperamide with H_3 receptors in the brain *in vivo*, we have used a functional and non-functional measurement of H_3 receptor occupancy. In three species (rat, guinea-pig and mouse) peripheral administration of thioperamide caused dose-related increases in histamine turnover in the cerebral cortex (whole brain was examined in the mouse) and, in the same tissues, inhibited the *ex vivo* binding of the selective H_3 receptor agonist [^{3}H](*R*)-α-methylhistamine ([^{3}H]-RAMH). The peak effect of thioperamide to inhibit *ex vivo* binding of [^{3}H]RAMH was observed approximately 30 min after i.p. administration, whilst the maximum increase in histamine turnover did not occur until after at least 100 min. At a pretreatment time of 30 min, the ED_{50} of thioperamide to inhibit *ex vivo* binding of [^{3}H]RAMH binding in the rat, guinea-pig and mouse brain was found to be 2.0 ± 0.2, 4.8 ± 0.6 and 2.6 ± 0.3 mg/kg (mean ± SEM, N = 4), respectively. We have also examined the effect of peripheral administration of RAMH on *ex vivo* binding of [^{3}H]RAMH in rat cortex. Qualitatively and quantitatively similar results to those of thioperamide were observed following i.p. administration of RAMH to rats (ED_{50} = 3.9 ± 0.4 mg/kg, mean ± SEM, N = 4). An effect of RAMH on histamine turnover in rat cortex could not be determined as this compound displayed significant cross-reactivity with the antibodies used in the radioimmunoassay to measure histamine and tele-methylhistamine.

These data indicate that, following peripheral administration, both thioperamide and RAMH penetrate the brain where they can subsequently interact with H_3 receptors. It would appear that binding of thioperamide to H_3 receptors is linked with a concomitant increase in histamine turnover in the brain. In conclusion, the *ex vivo* binding technique, particularly when coupled with measurement of histamine turnover, should provide a valuable means for investigating the ability of any peripherally administered compound to cross the blood–brain barrier and subsequently interact with histamine H_3 receptors.

There is now substantial evidence to support a neurotransmitter role for histamine in the brain [1, 2]. For many years the actions of histamine were thought to be mediated via two receptor types; H_1 receptors, positively linked to phosphatidylinositol turnover, translocation of intracellular Ca^{2+} and cyclic GMP formation, and H_2 receptors, positively linked to cyclic AMP formation (see Ref. 3). However, in 1983 Arrang *et al.* [4] proposed the existence of a presynaptic histamine receptor, the H_3 receptor. The development, in 1987, of a selective agonist, (*R*)-α-methylhistamine (RAMH†), and antagonist, thioperamide, provided a means of investigating more fully the H_3 receptor [5]. In slices of rat cortex, it was demonstrated that RAMH and thioperamide caused a decrease and increase, respectively, in the release and synthesis of neuronal histamine [5]. It is now known that the H_3 receptor also regulates the neuronal release of noradrenaline [6], 5-hydroxytryptamine [7], acetylcholine [8, 9] and non-adrenergic non-cholinergic neurotransmitters [10, 11]: the H_3 receptor thus appears to function as both an auto- and hetero-receptor.

Although the activity of RAMH and thioperamide at H_3 receptors in *in vitro* preparations is well documented (see Ref. 12), less is known about the degree to which these compounds can interact with H_3 receptors *in vivo*. It has been shown that thioperamide causes an increase, and RAMH a decrease, in histamine turnover in rat cerebral cortex and mouse whole brain following peripheral administration [5]. Such an effect of thioperamide is not unexpected since it ought to cross readily the blood–brain barrier owing to its high calculated [13] lipophilicity (log P = 2.95). However, an effect of RAMH in the brain following peripheral administration is surprising as this compound has low calculated [13] lipophilicity (log P = −0.45) and so would not be expected to cross the blood–brain barrier. The principle aim of this study, therefore, was to obtain information about the central

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† Abbreviations: RAMH, (*R*)-α-methylhistamine; TMH, *N*$^\tau$-methylhistamine; RIA, radioimmunoassay; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].

central penetration and duration of action of thioperamide and RAMH in the brain following peripheral administration.

To this end, we have examined the interaction of thioperamide with H_3 receptors in the brain of three species, the rat, guinea-pig and mouse, using both a functional (histamine turnover) and non-functional ([^{3}H]RAMH *ex vivo* binding) measurement of H_3 receptor occupancy. It was not possible to investigate the effect of RAMH on turnover as this compound interfered with the radioimmunoassay (RIA) procedure for measuring histamine turnover. Thus, we only studied the effect of RAMH on *ex vivo* binding.

MATERIALS AND METHODS

Animals. Male Lister-hooded rats (Glaxo bred) weighing 250–300 g, male Dunkin–Hartley guinea-pigs (Porcellus) weighing 250–350 g and male CRH mice (Glaxo bred) weighing 25–30 g were used in these experiments. They were housed in a room controlled at 22 ± 2° with food and water freely available and maintained in an alternating 12 hr light/dark cycle (lights on at 6 a.m.). All experiments were performed between 10 a.m. and 5 p.m.

Chemicals and drugs. HEPES buffer was obtained from the Sigma Chemical Co. (Poole, U.K.). RAMH-dihydrochloride was synthesized by Cookson Chemicals Ltd (Southampton, U.K.) and thioperamide was synthesized by the Chemistry Research Department, Glaxo Group Research Ltd (Ware, U.K.). [^{3}H]RAMH (sp. act. 19.7 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). All other chemicals used were of analytical grade and were purchased from BDH Chemicals (Poole, U.K.). RAMH and thioperamide were dissolved in 0.9% saline and were injected intraperitoneally at the following volumes: rats and guinea-pigs, 0.2 mL/100 g body weight; mice, 0.1 mL/10 g body weight. Doses of all drugs are expressed as the free base.

Tissue removal procedure. Animals were administered thioperamide, RAMH or vehicle (i.p.) and returned to their home cages for the desired pretreatment time. The animals were then administered a lethal dose of pentobarbitone (Expiral, 400 mg/mL, i.p.; rats and guinea-pigs, 0.2 mL/100 g body weight; mice, 0.1 mL/10 g body weight) and the head and upper body region were perfused transcardially with ice-cold HEPES (50 mM, pH 7.4) buffer. A volume of 20 mL was used for rats and guinea-pigs, and 5 mL for mice. Selective upper body perfusion was achieved by shutting off blood vessels supplying the lower body regions with an artery clamp. To minimize risk of contamination of tissues between individual animals, surgical instruments were washed thoroughly between transcardial perfusions. The brain was then removed and samples (approximately 100 mg) of cerebral cortex (rats and guinea-pigs) or whole brain (mice) were taken for measurement of histamine turnover and [^{3}H]RAMH *ex vivo* binding: the weight of each tissue sample was determined accurately prior to further processing. Once weighed, the tissue samples for turnover studies were immediately homogenized in 1 mL ice-cold 0.1 M perchloric acid using a sonicator (MSE Soniprep 150) and then frozen.

Determination of histamine turnover. Tissue homogenates were thawed and then centrifuged (Burkhard "Koolspin") at 10,000 *g* for 10 min at 4°. Levels of histamine and *N*^{1}-methylhistamine (TMH) in the supernatant were measured using RIA kits purchased from Immunodiagnostic Systems Ltd and Pharmacia Ltd, respectively. The acidic nature of the supernatant was neutralized by adding 600 μL 0.15 M Na_2HPO_4 to 200 μL supernatant (the resulting volume of 800 μL was sufficient for both RIAs). The levels of histamine and its metabolite in this solution were determined as described in the RIA kit instructions except that histamine and TMH "standards" were dissolved in the same solution as the samples (see above). Histamine turnover (the ratio TMH: histamine) in drug-dosed animals was expressed as a percentage of that determined in vehicle-dosed animals. Student's *t*-test was used to assess statistical significance of the data.

Ex vivo *binding of* [^{3}H]*RAMH.* The [^{3}H]RAMH *ex vivo* binding assay is based almost exactly on an existing [^{3}H]RAMH binding assay except that in the latter procedure the tissue homogenates were washed twice by centrifugation and resuspension [14]. Such a washing stage could obviously not be used with the *ex vivo* binding procedure as any thioperamide or RAMH present in the tissue might have been washed away. To investigate the effect of omission of this washing stage on the H_3 receptor binding assay, we compared binding of [^{3}H]RAMH to homogenates of washed and unwashed rat cortex. For these experiments, washed rat cortex was prepared as follows: rats were killed by cervical dislocation, the brains removed and the cortices isolated. Approximately 2 g of cortical tissue were homogenized (Ultra-Turrax, 10 sec, full speed) in HEPES buffer (50 mL, 50 mM, pH 7.4) and centrifuged (Sorval RC-5C, 48,000 *g*, 10 min). The supernatant was discarded and the tissue resuspended in the same volume of identical HEPES buffer, homogenized and recentrifuged. The supernatant was again discarded and the tissue finally resuspended in HEPES buffer (7.5 ml/g original tissue weight). Unwashed rat cortex was prepared by pooling tissue from vehicle-dosed rats (see above) and homogenizing in HEPES buffer (7.5 mL/g).

Homogenates of brain tissue from individual animals were prepared by homogenizing (Ultra-Turrax, 10 sec, full speed) in HEPES buffer (50 mM, pH 7.4, 7.5 mL/g tissue). Binding of [^{3}H]RAMH to washed or unwashed homogenates of rat brain cortex was determined (in triplicate) by incubating 100 μL tissue homogenate with [^{3}H]RAMH (0.5 nM) in a final volume of 1 mL at 25° for 1 hr; non-specific binding was defined using thioperamide (10 μM). The ability of thioperamide to inhibit specific [^{3}H]-RAMH binding was determined by including thioperamide (10^{-10}–10^{-5} M) in the incubation medium. Inhibition of binding by RAMH (10^{-11}–10^{-6} M) was investigated in the same way.

Following incubation, samples were filtered through Whatman GF/B filter papers (presoaked in 0.1% polyethylenimine) and washed with 2 × 4 mL of HEPES buffer (4°) using a Brandel 30 sample cell

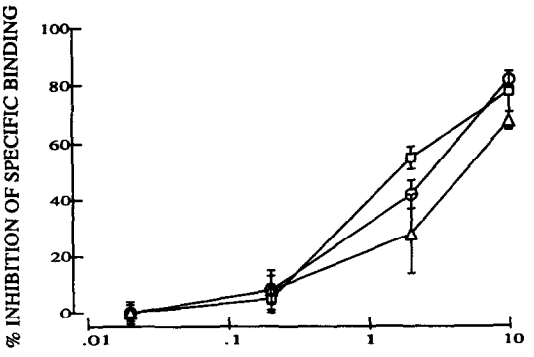

Fig. 1. Effect of 30 min pretreatment with thioperamide (i.p.) on [^{3}H]RAMH *ex vivo* binding to homogenates of rat cortex (□), guinea-pig cortex (△) and mouse whole brain (○). Each point represents data (mean ± SEM) obtained from four animals.

harvester. Emulsifier-safe liquid scintillation fluid (10 mL, Packard) was added to each filter disc and the radioactivity bound to the membranes determined using liquid scintillation counting. Specific [^{3}H]-RAMH binding was calculated by subtracting the value of non-specific binding from that of total binding.

Determination of specific [^{3}H]RAMH binding in drug-treated animals was carried out as above, and was expressed as % inhibition of specific [^{3}H]RAMH binding observed in vehicle-dosed animals. ED_{50} values were calculated using the computer program "Allfit" [15]. Student's *t*-test was used to assess statistical significance of the data.

The tissue homogenization and incubation stages that were necessary for the *ex vivo* binding procedure result in a 75-fold dilution of the tissue. We have not adjusted any values for this dilution factor; all drug doses quoted apply to the actual dose of drug administered.

RESULTS

[^{3}H]*RAMH* ex vivo *binding in vehicle-treated animals*

Specific binding of [^{3}H]RAMH to homogenates of brain tissue was detected in all three species tested (Table 1). Specific [^{3}H]RAMH binding to rat cortex which had been washed prior to the binding assay was found to be greater than that observed in unwashed tissue (Table 1). Inclusion of thioperamide (10^{-10}–10^{-5} M) in the incubation medium resulted in a concentration-related inhibition of [^{3}H]RAMH binding to the brain tissue homogenates. The IC_{50} value for thioperamide to inhibit specific [^{3}H]RAMH binding to homogenates of rat cortex (washed and unwashed), guinea-pig cortex and mouse whole brain ranged from 6.0 to 10.9 nM (Table 1). Inclusion of RAMH in the incubation medium also resulted in a concentration-related inhibition of [^{3}H]RAMH binding to homogenates of rat cortex (Table 1).

[^{3}H]*RAMH* ex vivo *binding in drug-treated animals*

Pretreatment of rats, guinea-pigs and mice with thioperamide 30 min prior to tissue removal resulted in a dose-related inhibition of specific [^{3}H]RAMH binding (Fig. 1). The ED_{50} values (mean ± SEM, N = 4) for thioperamide at this pretreatment time were, rat 2.0 ± 0.2 mg/kg, guinea-pig 4.8 ± 0.6 mg/kg and mouse 2.6 ± 0.3 mg/kg. Similar dose-related inhibition of specific [^{3}H]RAMH binding was seen when thioperamide was administered 1 and 2 hr prior to tissue removal (Fig. 2). In the rat, significant inhibition of [^{3}H]RAMH binding was still observed 8 hr after 10 mg/kg thioperamide (Fig. 2).

Treatment of rats with RAMH 0.5, 1, 2, 4 and 8 hr prior to tissue removal resulted in a dose-related inhibition of specific [^{3}H]RAMH binding similar to that observed for thioperamide (Fig. 3). The ED_{50} value (mean ± SEM) for RAMH at 30 min pretreatment time was found to be 3.9 ± 0.4 mg/kg and inhibition of [^{3}H]RAMH binding was observed 8 hr after 10 mg/kg RAMH (Fig. 3).

Histamine turnover in brain tissue of thioperamide and RAMH-dosed animals

The levels of histamine and TMH in brain tissue of vehicle-dosed rats, guinea-pigs and mice are shown in Table 2. In both the rat and guinea-pig cortex, levels of histamine and TMH were found to be approximately equal whereas in the mouse whole brain, levels of TMH appeared to be some 4-fold higher than those of histamine.

In all three species, administration of thioperamide 1 hr prior to tissue removal resulted in a dose-related increase in levels of TMH in the brain (Fig. 4). The level of histamine in the same tissue remained approximately constant although at the highest dose of thioperamide tested (10 mg/kg) a small decrease

Table 1. Specific binding of [^{3}H]RAMH to washed and unwashed homogenates of rat, guinea-pig and mouse brain

Tissue	Specific [^{3}H]RAMH binding (dpm)	Thioperamide IC_{50} (nM)	RAMH IC_{50} (nM)
Rat cortex, washed	1050 ± 100	10.9 ± 1.3	0.7 ± 0.1
Rat cortex, unwashed	674 ± 55	11.5 ± 1.1	1.5 ± 0.1
Guinea-pig cortex, unwashed	634 ± 38	6.0 ± 1.2	—
Mouse brain, unwashed	696 ± 121	10.8 ± 3.7	—

Results are the means ± SEM of 4–15 separate observations. IC_{50} values were calculated using the computer program ALLFIT [15].

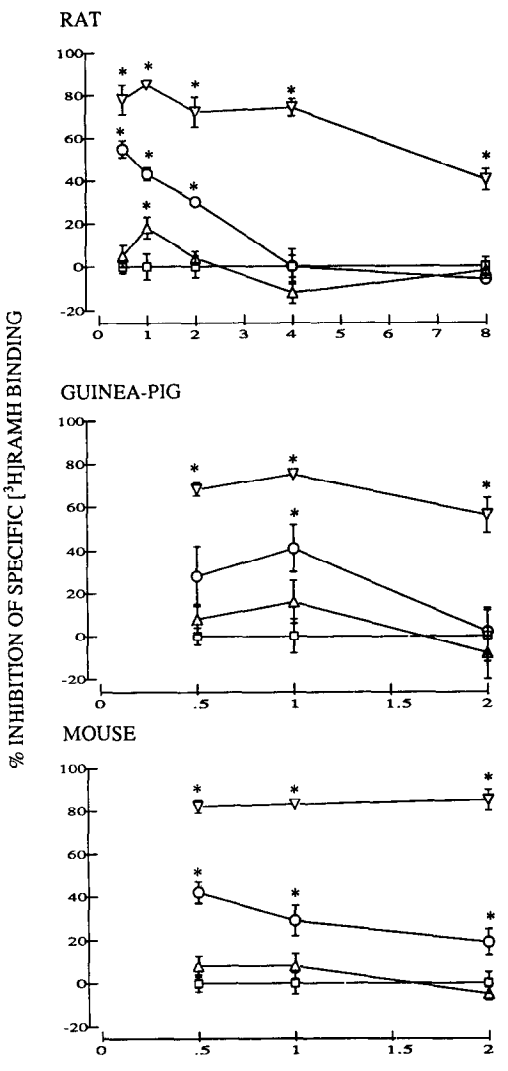

Fig. 2. Effect of thioperamide pretreatment time on [^{3}H]-RAMH *ex vivo* binding to homogenates of rat cortex (top graph), guinea-pig cortex (middle graph) and mouse whole brain (lower graph). Thioperamide was administered peripherally (i.p.) at the following doses: vehicle (□), 0.2 mg/kg (△), 2 mg/kg (○) and 10 mg/kg (▽). Each point represents data (mean ± SEM) obtained from four animals.

in the levels of brain histamine was apparent; however, this did not reach the level of statistical significance except in the mouse (Fig. 4). As a result of these changes in TMH, in all three species administration of thioperamide resulted in a dose-related increase in histamine turnover (calculated as the ratio of TMH: histamine) in the brain (Fig. 5).

DISCUSSION

Ex vivo binding is a technique which has been used previously to obtain information about the central penetration of compounds which interact with several receptor types, for example, opiate receptors [16], dopamine D_2, 5-hydroxytryptamine$_2$ and α_1-adrenoreceptors [17]. The principle behind the technique is that a compound which can penetrate the blood–brain barrier can bind to a particular receptor in the brain, *in situ*, and subsequently inhibit binding of a radioligand to that receptor, *in vitro*. The technique thus detects the presence of, rather than the function of, a particular compound in the brain. As such, *ex vivo* binding can be used to ascertain whether a compound crosses the blood–brain barrier to any extent, and how long it remains in the brain.

In this study, the *ex vivo* binding procedure resulted in a 75-fold dilution of the tissue used in the binding assay. It is evident that any thioperamide or RAMH present in the extracellular space within the tissue will also be diluted by this amount. However, we are not certain whether this dilution factor applies to thioperamide or RAMH already bound to H_3 receptors in the tissue: if these compounds did not dissociate from the H_3 receptor then they would remain bound to the receptor during the binding assay and thus would not undergo dilution. Conversely, if the compounds dissociated very rapidly from the H_3 receptors then a 75-fold dilution factor would apply and the drug dosages could be divided by this amount to give true ED_{50} values. However, we do not know precisely the extent of dissociation of RAMH and thioperamide under our binding assay conditions and hence we do not know the exact dilution factor. We have performed limited *in vitro* experiments (results not shown) which suggest that thioperamide and RAMH would only dissociate to a small and, in any case, equal extent from the H_3 receptor over the time course of the assay. We have therefore not attempted to adjust the drug doses to take into account the dilution factor and so all values quoted apply to the actual dose of drug administered to the animal.

An obvious prerequisite to performing an *ex vivo* binding technique is that an appropriate binding assay should be available. Indeed, such a binding assay, using [^{3}H]RAMH as the radioligand, has already been described for H_3 receptors in brain tissue of rats and guinea pigs [5, 14]; no such binding assay has been reported for H_3 receptors in mouse brain. In this study we have shown that [^{3}H]RAMH binds to homogenates of rat and guinea-pig cortex and to homogenates of mouse brain. Specific [^{3}H]-RAMH binding was detected in all tissues which had been removed using the *ex vivo* binding method (i.e. unwashed), and in homogenates of rat cortex which had been washed prior to incubation as used in the conventional assay [14]. The lower specific [^{3}H]RAMH binding detected in unwashed rat cortex compared with washed rat cortex is presumably due to competition for binding by endogenous histamine which is not removed in the former case: the large amount of histamine present in blood [18] is unlikely to have contributed to this decreased specific [^{3}H]-RAMH binding because the blood is removed from the brain by the transcardial perfusion procedure. The competition by endogenous histamine was presumably also responsible for the lower apparent

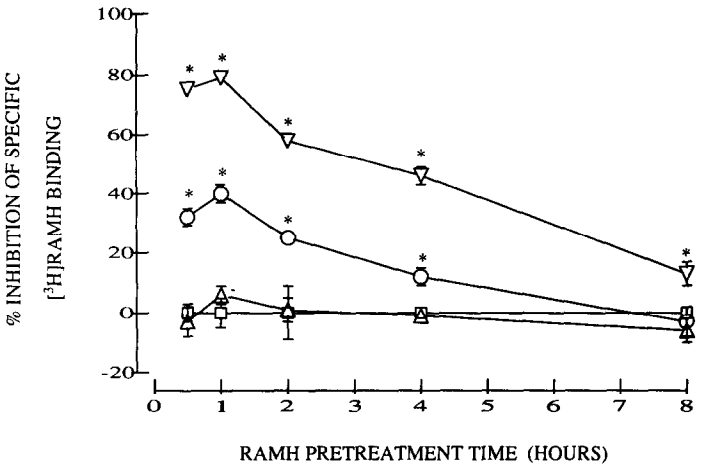

Fig. 3. Effect of RAMH pretreatment time on [^{3}H]RAMH *ex vivo* binding to homogenates of rat cortex. RAMH was administered peripherally (i.p.) at the following doses: vehicle (□), 0.2 mg/kg (△), 2 mg/kg (○) and 10 mg/kg (▽). Each point represents data (mean ± SEM) obtained from four animals.

Table 2. Levels of histamine and TMH in rat, guinea-pig and mouse brain

	Histamine (ng/g tissue)	TMH (ng/g tissue)
Rat cortex	37 ± 3	22 ± 3
Guinea-pig cortex	28 ± 3	29 ± 6
Mouse whole brain	30 ± 2	130 ± 15

Results are the means ± SEM of 12–28 separate determinations. RIA analysis was performed as described in Methods and Materials.

affinity of RAMH detected in unwashed tissues. This was not evident for thioperamide; further study would be required to determine if this is a real effect and, if so, how it relates to the competitive effects of thioperamide.

In this study, any inhibition of [^{3}H]RAMH binding that is observed is presumably due to thioperamide or RAMH (or an active metabolite of either) which has crossed the blood–brain barrier. A contribution to the inhibition of [^{3}H]RAMH binding which might occur due to compound (or metabolite) present in the blood is minimalized by removing the blood from the brain by the transcardial perfusion procedure. The degree to which a peripherally administered compound will inhibit [^{3}H]RAMH binding to H_3 receptors in the brain *in vitro* ought to be dependent upon three factors: first, the ability of that compound to cross the blood–brain barrier; second, the affinity of that compound for the H_3 receptor and, third, the extent to which the compound dissociates from the receptor during the tissue removal/binding assay procedure (i.e. the dilution factor, see above).

We have shown that peripheral administration of thioperamide results in a dose-related and pretreatment time-dependent decrease in specific [^{3}H]RAMH binding to homogenates of brain tissue of all three species. Such a result indicates that in all three species peripherally administered thioperamide is able to penetrate the blood–brain barrier and interact with H_3 receptors in the brain. In the rat we obtained a virtually identical inhibition of [^{3}H]RAMH binding profile for thioperamide and RAMH. However, RAMH has an affinity approximately eight times higher than thioperamide for the H_3 receptor and so, as discussed above, this perhaps indicates that RAMH penetrates the blood–brain barrier less readily than thioperamide. That RAMH appears to cross the blood–brain barrier at all is surprising given its low calculated lipophilicity (log P = −0.45, [13]); histamine, which with the exception of a methyl group is identical to RAMH, is thought not to cross the blood–brain barrier. The *ex vivo* binding technique also shows that thioperamide and RAMH penetrate the brain within 30 min of peripheral administration. In addition, it would appear that, having penetrated the brain, thioperamide and RAMH are cleared slowly such that at the highest doses used in this study (10 mg/kg) both compounds (or active metabolites) are present 8 hr following administration.

In mammalian tissues the major metabolite of histamine is TMH (see Ref. 19). The ratio of the levels of TMH:histamine in the brain gives a measure of brain histamine turnover and this parameter has been used as an index of histaminergic neuronal activity [20, 21]. It has been shown previously [5] that thioperamide and RAMH can modify brain histamine turnover with an increase and decrease observed, respectively. In this study, peripheral administration of thioperamide resulted in a dose-related and pretreatment time-dependent increase in histamine turnover in the brains of all three species. The peak increase in histamine turnover in the rat brain appears to occur at least 1–2 hr later.

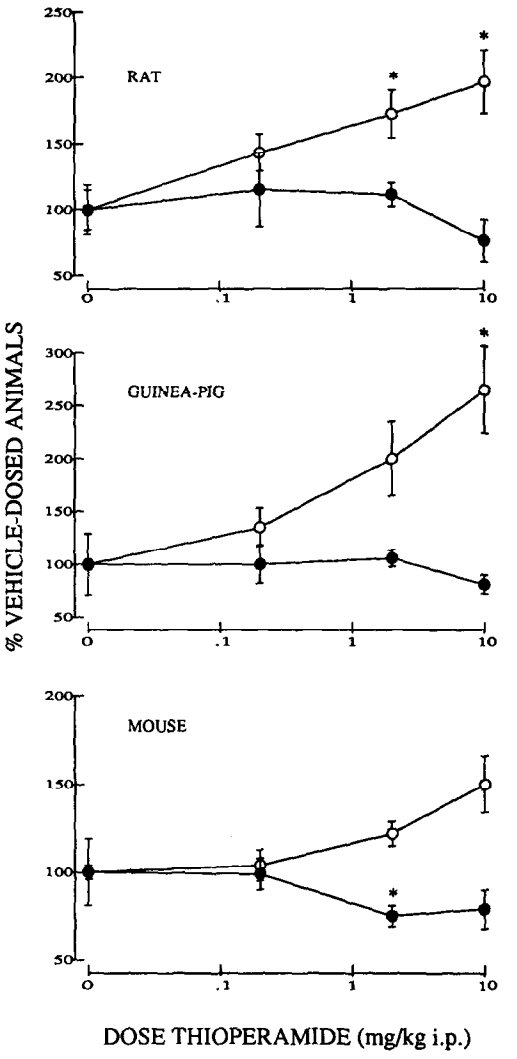

Fig. 4. Effect of peripheral (i.p.) administration of thioperamide on the levels of histamine (●) and TMH (○) in rat cortex (upper graph), guinea-pig cortex (middle graph) and mouse whole brain (lower graph). Each point represents data (mean ± SEM) obtained from four animals.

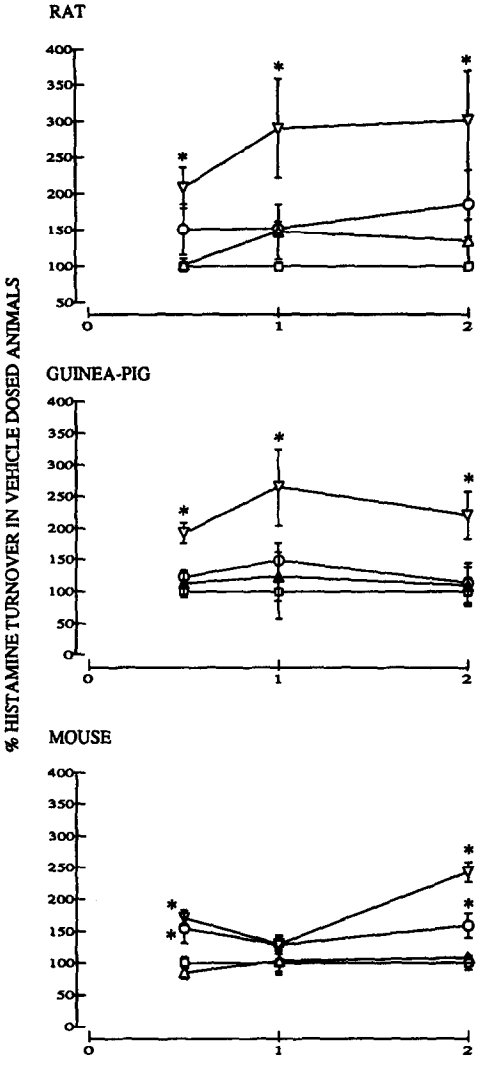

Fig. 5. Effect of thioperamide pretreatment time on histamine turnover in rat cortex (upper graph), guinea-pig cortex (middle graph) and mouse whole brain (lower graph). Histamine turnover was defined as the ratio of the levels of TMH:histamine in the brain tissue. Thioperamide was administered peripherally (i.p.) at the following doses: vehicle (□), 0.2 mg/kg (△), 2 mg/kg (○) and 10 mg/kg (▽). Each point represents data (mean ± SEM) obtained from four animals.

However, from the results obtained using the *ex vivo* binding technique we know that thioperamide can be detected in the brain 30 min following peripheral administration. It would thus appear that there is a time delay between when thioperamide is actually present in the brain and the effect of its presence, i.e. increased histamine turnover. Such a delay might represent accumulation of TMH subsequent to receptor occupancy, the time it takes for thioperamide which has penetrated the brain to diffuse to and interact with H_3 receptors, and/or the effect of compensatory mechanisms which might tend to oppose the effect of thioperamide.

The duration of the increase in histamine turnover mediated by thioperamide seems to reflect the presence of the compound in the brain as measured by *ex vivo* binding. Histamine turnover in the rat brain was found to be elevated by doses of thioperamide similar to those which inhibited *ex vivo* binding. Apart from the delay in reaching the peak increase in histamine turnover, it would appear that the *ex vivo* binding technique and measurement of histamine turnover both give qualitatively the same information concerning the central penetration and duration of thioperamide in the brain.

In summary, we have used a functional (histamine turnover) and non-functional (*ex vivo* binding) measurement of H_3 receptor occupancy to investigate

the degree to which the selective H_3 receptor antagonist thioperamide penetrates the brain following peripheral administration. The results from both techniques support the idea that peripherally administered thioperamide readily crosses the blood–brain barrier. We have similarly used the *ex vivo* binding technique to assess the degree to which RAMH penetrates the brain. Whilst the results from this technique would imply that RAMH can cross the blood–brain barrier, it appears that it does so less well than thioperamide. It would also seem that both compounds have a long (i.e. >8 hr) duration in the brain when administered at high doses. In conclusion, we feel that the techniques used provide a powerful means of assessing the ability of any peripherally administered compound to interact with histamine H_3 receptors in the brain.

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