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European Journal of Pharmacology 473 (2003) 35–40
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In vivo receptor occupancy of mGlu5 receptor antagonists using the novel radioligand [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine)

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Received 20 March 2003; received in revised form 23 May 2003; accepted 27 May 2003

Abstract

In vivo receptor occupancy of mGlu5 receptor antagonists was quantified in rat and mouse brain using the mGlu5 receptor selective antagonist [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine) ([^{3}H]methoxy-PEPy). Administration of [^{3}H]methoxy-PEPy (50 μCi/kg i.v.) to mGlu5 receptor-deficient mice revealed binding at background levels in forebrain, whereas wild-type mice exhibited 14-fold higher binding in forebrain relative to cerebellum. Systemic administration of the mGlu5 receptor antagonists 2-methyl-6-(phenylethynyl)pyridine (MPEP) and 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) reduced the binding of [^{3}H]methoxy-PEPy in rats and mice, reflecting mGlu5 receptor occupancy by these compounds. MPEP (10 mg/kg i.p.) and MTEP (3 mg/kg i.p.) maintained >75% receptor occupancy for 2 h in rats, while in mice MPEP and MTEP achieved >75% occupancy for only 30 and 15 min, respectively. Compound levels in plasma were substantially lower in mice suggesting species differences in receptor occupancy result from differences in absorption or metabolism of the compounds. These findings demonstrate that [^{3}H]methoxy-PEPy is useful for determining the occupancy of mGlu5 receptors in the brain.

Keywords: Metabotropic glutamate receptor; Receptor binding; Occupancy; mGlu5

1. Introduction

Metabotropic glutamate (mGlu) receptors are G-protein coupled receptors which play an important role in mediating glutamate neurotransmission in the brain (Conn and Pin, 1997). There are presently eight subtypes of mGlu receptors, which are subdivided into three groups based on sequence homology, signaling pathways, and agonist selectivity (Pin and Duvoisin, 1995). The mGlu5 receptor subtype is a member of the Group I family of mGlu receptors which initiate cellular responses through $G_{q/11}$ protein coupling to phospholipase C and stimulation of phosphoinositide hydrolysis.

mGlu5 receptors are present in high densities in many forebrain regions (e.g. striatum, hippocampus, and frontal cortex) and are present along key points of the pain neuraxis (e.g. thalamus, the dorsal surface of the spinal cord, and dorsal root ganglion). mGlu5 receptor agonists and antagonists have a broad range of neurobiological effects, and antagonists have been considered for several therapeutic indications. In vivo stimulation of mGlu5 receptors by intracerebral or intrathecal administration of the mGlu1/5 receptor agonist 3,5-dihydroxyphenylglycine (DHPG) or the mGlu5 receptor agonist 2-chloro-5-hydroxyphenylglycine (CHPG) can lead to increases in locomotor activity and seizures (Camon et al., 1998; Chapman et al., 2000), neurotoxicity (Camon et al., 1998), and behaviors consistent with increased nociception (Fisher and Coderre, 1996; Karim et al., 2001). In contrast, mGlu5 receptor antagonists have shown activity in animal models of anxiety (Brodkin et al., 2002b; Spooren et al., 2000; Tatarczynska et al., 2001), depression (Tatarczynska et al., 2001), Parkinson's disease (Breysse et al., 2002), pain (Bordi and Ugolini, 2000; Walker et al., 2001), and seizures (Chapman et al., 2000).

The behavioral effects of mGlu5 receptor antagonists have been principally demonstrated in rats and, to a lesser extent, in mice. It is not known, however, if there are

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doi:10.1016/S0014-2999(03)01935-6

inherent differences between these two species with respect to activities of mGlu5 receptor antagonists. In addition, with the availability of mGlu5 receptor knockout mice, as well as the development of other relevant transgenic and knockout mice that may be utilized in mGlu5 receptor research, it becomes important to have a clear understanding of the actions of mGlu5 receptor antagonists in mice.

We have recently shown that [^{3}H]methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine ([^{3}H]methoxymethyl-MTEP), a potent and selective mGlu5 receptor antagonist, is useful for labeling mGlu5 receptors both in vitro and in vivo (Anderson et al., 2002). Similarly, the structurally related mGlu5 receptor antagonists, [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine ([^{3}H]methoxy-PEPy) (Cosford et al., 2003) and [^{3}H]2-[(3-methoxyphenyl)ethynyl]-6-methylpyridine ([^{3}H]M-MPEP) (Gasparini et al., 2002), have both been shown to selectively label mGlu5 receptors in vitro. Here we demonstrate that [^{3}H]methoxy-PEPy is also useful for labeling mGlu5 receptors in vivo and for determining functional brain penetration and receptor occupancy of unlabeled mGlu5 receptor antagonists following systemic administration. Our results demonstrate key differences in the time course of receptor occupancy between rats and mice that may provide useful guidelines to follow for selecting doses and time lines for behavioral assessments of mGlu5 receptor activities in these two species.

2. Materials and methods

2.1. Animals and compounds

Male Sprague–Dawley rats (175–225 g) were purchased from Harlan (San Diego, CA) while mGlu5 receptor knockout mice (20–25 g) (Lu et al., 1997) and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Wild-type litter mates served as controls for the knockout mice. All animals were group housed on a 12-h light/dark cycle with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with *The Guide for the Care and Use of Laboratory Animals*. MPEP, MTEP, and [^{3}H]methoxy-PEPy were synthesized at Merck Research Laboratories.

2.2. Time course of in vivo binding of [^{3}H]methoxy-PEPy

Rats and mice were gently restrained in a plastic cone and the tail was warmed briefly to facilitate vessel dilation. [^{3}H]Methoxy-PEPy (30 μCi/kg; 1 ml/kg injection volume in isotonic saline) was then administered through a lateral tail vein. At the appropriate time, animals were euthanized and brain tissue was rapidly dissected on a cooled dissecting tray. Hippocampus and cerebellum (in the case of rats) or forebrain and cerebellum (in the case of mice) were immediately weighed and homogenized in 10 volumes of ice-cold buffer (10 mM potassium phosphate, 100 mM KCl, pH 7.4) using a Polytron. Homogenates (400 μl) were then filtered over GF/B membrane filters (Whatman) and washed twice with 5 ml ice-cold homogenization buffer to separate membrane bound from free radioactivity (Anderson et al., 2002; Atack et al., 1999). Filters were then counted for radioactivity using a Beckman counter. The hippocampus was utilized since it is a region with high density of mGlu5 receptors, while cerebellum was used as a reference region because it has a low density of mGlu5 receptors. Forebrain was utilized in mice to provide sufficient mGlu5 receptor-rich tissue.

2.3. Binding of [^{3}H]methoxy-PEPy in mGlu5 receptor deficient mice

In vivo binding in mGlu5 receptor deficient mice (and wild type controls) was performed by administering [^{3}H]methoxy-PEPy (50 μCi/kg; 5 ml/kg injection volume in isotonic saline) through a lateral tail vein. Mice were euthanized 1 min later and forebrain and cerebellum were rapidly dissected, homogenized, and filtered as described above.

2.4. In vivo receptor occupancy

For studies to determine the in vivo receptor occupancy of unlabeled compounds, animals were dosed i.p. with

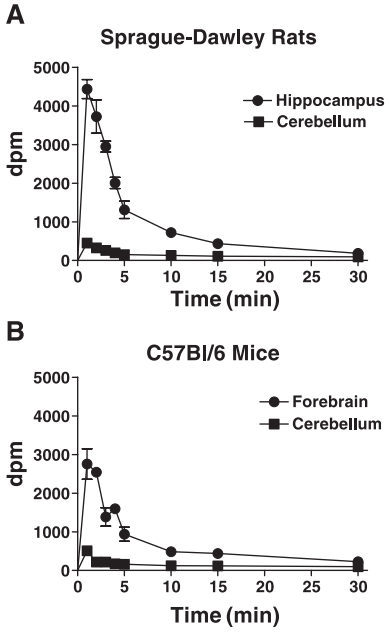

Fig. 1. Time course of the binding of [^{3}H]methoxy-PEPy in brain in Sprague–Dawley rats (A) and C57Bl/6 mice (B) following intravenous administration of the radioligand (30 μCi/kg). The dpm indicated represent membrane-bound radioactivity in tissue homogenates which have been filtered over GF/B membranes. Values shown represent the mean ± S.E.M. (n = 3–4 animals/time point).

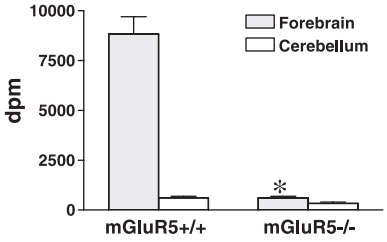

Fig. 2. In vivo binding of [^{3}H]methoxy-PEPy in mGlu5$^{+/+}$ and mGlu5$^{-/-}$ receptor mice. Mice were administered 30 μCi/kg [^{3}H]methoxy-PEPy intravenously and euthanized 1 min later. The membrane bound counts in homogenates from forebrain and cerebellum were determined. Values indicate the mean ± S.E.M. ($n = 8–10$ mice/group). *$P < 0.05$ versus forebrain by analysis of variance and Dunnett's t test.

unlabeled compound (dissolved in 50% polyethylene glycol 400 (2 ml/kg injection volume)). One minute prior to tissue collection, [^{3}H]methoxy-PEPy was administered (30 μCi/kg) through a lateral tail vein. Animals were then euthanized and hippocampus or forebrain was rapidly dissected, homogenized, and filtered as described above.

Nonspecific in vivo binding of [^{3}H]methoxy-PEPy was estimated by measuring radioactivity in washed filters following administration of a saturating dose of MPEP (50 mg/kg i.p.).

2.5. Determination of compound levels

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes and centrifuged at 2500 × g for 10 min at 10 °C, after which plasma was removed and frozen at − 70 °C until analysis. Plasma was assayed by a specific and sensitive liquid chromatographic method with tandem mass spectrometric detection (LC-MS/MS) assay. A standard curve ranging from 0.02 to 20.5 μM was prepared in rat or mouse plasma. The lower limit of quantitation of the assay was 0.02 μM.

2.6. Data analysis and statistics

The percent receptor occupancy was calculated by first expressing the specific binding value from each animal as a percentage of specific binding of the vehicle group, then subtracting that value from 100 (i.e., % occupancy = 100 − % vehicle binding). Values expressed are the arithmetic means ± standard error of the mean (S.E.M.). Differences between treatment groups were assessed by analysis of variance followed by either Dunnett's t-test or Student–Neuman–Keuls test to identify specific group differences. Nonlinear regression analysis was used to calculate the ED_{50} values and 95% confidence limits for the dose–response receptor occupancy studies (GraphPad Prism).

3. Results

3.1. Time course of in vivo binding of [^{3}H]methoxy-PEPy

The binding of [^{3}H]methoxy-PEPy in both Sprague–Dawley rats and C57Bl/6 mice was maximal 1 min post-tail vein dosing (Fig. 1), producing 4435 ± 245 dpm in rats ($n = 4$) and 2755 ± 401 dpm ($n = 3$) in mice. In addition, the level of binding in hippocampus relative to cerebellum was approximately 10:1 for rats, and the level of binding in forebrain relative to cerebellum in mice was approximately 8:1. Because maximal binding in hippocampus or forebrain was observed at 1 min, in all subsequent experiments, [^{3}H]methoxy-PEPy was administered 1 min prior to tissue collection.

3.2. Receptor occupancy in rat hippocampus and rat forebrain

Since the receptor occupancy of mGlu5 receptor antagonists was examined in rat hippocampus and mouse forebrain, we determined whether the occupancy was similar between hippocampus and forebrain in rats. The mGlu5 receptor antagonist MTEP (5 mg/kg i.p.) exhibited approximately 96% receptor occupancy in both hippocampus and whole forebrain in rats at 1 h post-treatment (data not shown). Hence, any differences in receptor occupancy between the two species are likely not due to the use of different brain tissues. Also in rats, the receptor occupancy of MTEP was similar in hippocampus ($ED_{50} = 1.1$ mg/kg,

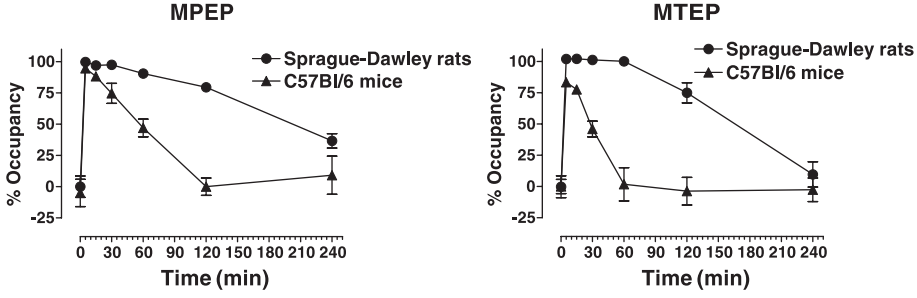

Fig. 3. Time course of the receptor occupancy of the mGlu5 receptor antagonists MPEP and MTEP in rats and mice. Animals were injected with MPEP (10 mg/kg i.p.) or MTEP (3 mg/kg i.p.) and received an i.v. injection of [^{3}H]methoxy-PEPy 1 min prior to tissue collection at the indicated times. Rat hippocampus or mouse forebrain was dissected, homogenized, and filtered. Values represent the mean ± S.E.M. ($n = 4–6$ rats/group).

95% confidence intervals 0.5–3.0 mg/kg), striatum (ED_{50} = 0.9 mg/kg, 95% confidence intervals 0.5–1.7 mg/kg), and frontal cortex (ED_{50} = 0.9 mg/kg, 95% confidence intervals 0.5–1.8 mg/kg), suggesting similar occupancy of mGlu5 receptors in brain regions with a high density of these receptors.

3.3. [^{3}H]Methoxy-PEPy binding in mGlu5 receptor deficient mice

The binding of [^{3}H]methoxy-PEPy was also examined in mGlu5$^{+/+}$ and mGlu5$^{-/-}$ receptor mice 1 min following intravenous administration. As shown in Fig. 2, binding of [^{3}H]methoxy-PEPy was 14-fold greater in forebrain than in cerebellum in the mGlu5$^{+/+}$receptor mice. In mice lacking mGlu5 receptors, however, forebrain binding of [^{3}H]methoxy-PEPy was comparable to the background levels of binding observed in the cerebellum (Fig. 2).

3.4. Time course of receptor occupancy of mGlu5 receptor antagonists in rats and mice

The receptor occupancy of MPEP and MTEP was examined over time following i.p. dosing in Sprague–Dawley rats and in C57Bl/6 mice (Fig. 3). Both MPEP (10 mg/kg) and MTEP (3 mg/kg) rapidly achieved full (100%) receptor occupancy in Sprague–Dawley rats, which was sustained for at least 1 h. By 4 h post-dosing in rats, the receptor occupancy of MPEP and MTEP decreased substantially. In mice, 10 mg/kg MPEP achieved full receptor occupancy 5 min post-administration, but the occupancy declined steadily thereafter returning to control levels by 2 h post-dosing. Similarly, MTEP (3 mg/kg) reached 75% receptor occupancy at 5 and 15 min post-dosing in mice, but by 60 min the occupancy was at baseline. The shorter time course of receptor occupancy in mice may be due to either a more rapid metabolism or a lower absorption of the compounds in mice relative to rats. As shown in Table 1, the plasma levels of MPEP and MTEP following i.p. dosing are highest in both rat and mouse at the initial time point of sampling (5 min), then steadily declined throughout the study. Absolute plasma levels for each compound, however, are significantly lower in mice (compared to rats) and by 60 min the levels are below the limit of quantitation in mice (0.02 μM).

Table 1
MTEP and MPEP plasma levels in rats and mice

Time (min)	MPEP		MTEP	
	Rats	Mice	Rats	Mice
5	11.7 ± 2.8	3.8 ± 0.4	4.8 ± 1.4	0.8 ± 0.1
15	9.3 ± 3.4	1.2 ± 0.2	3.8 ± 0.5	0.4 ± 0.1
30	3.8 ± 0.3	0.7 ± 0.2	2.9 ± 1.0	0.1 ± 0.02
60	1.7 ± 0.2	<LLQ	1.2 ± 0.3	<LLQ
120	0.5 ± 0.1	<LLQ	0.4 ± 0.1	<LLQ
240	0.2 ± 0.1	<LLQ	<LLQ	<LLQ

Values are mean ± S.E.M.; n = 3–7 per group.
LLQ (lower limit of quantitation) = 0.02 μM.

Table 2
MTEP and MPEP occupancy in rats and mice

	ED_{50} (mg/kg, i.p.)	
	MPEP	MTEP
Rats, 60 min	2.3 (1.5–3.6)	1.1 (0.4–2.9)
Mice, 30 min	1.6 (0.5–5.3)	2.8 (2.0–4.0)
Mice, 60 min	10.1 (3.0–34.0)	17.3 (3.3–92.0)

Each value is the ED_{50} determined from 4–5 doses of each compound with 3–7 animals per dosing group. The 95% confidence intervals are given in parentheses.

Dose–response studies were conducted and ED_{50} values and 95% confidence intervals were calculated for MPEP and MTEP in rats (60 min) and mice (30 and 60 min) (Table 2). There was a significant shift (5–15 fold) to the right in the ED_{50} for both compounds at 60 min post-administration in mice. Effective ED_{50} doses in mice at 30 min were similar to those at 60 min in rats, consistent with the shorter time course of receptor occupancy in mice.

4. Discussion

An in vivo receptor occupancy assay for determining the functional brain penetration and receptor binding of mGlu5 receptor antagonists has importance for developing these compounds for neurological and psychiatric disorders. Receptor occupancy assays rely on the selective labeling of a receptor by a specific radioligand and a reduction in radioligand binding results from occupancy of the receptor by the unlabeled compound. Systemic injections of unlabeled mGlu5 receptor antagonists MPEP and MTEP reduced the binding of [^{3}H]methoxy-PEPy in both rats and mice reflecting occupancy of mGlu5 receptors by these compounds. The time course of receptor occupancy, however, was of markedly shorter duration in mice compared to rats for both MPEP and MTEP. Mice also exhibited significantly lower plasma levels of MPEP and MTEP following i.p. dosing suggesting either more rapid metabolism or reduced absorption in mice. There were no differences in binding affinity of MPEP and MTEP between rat and mouse brain membranes in vitro (unpublished observations), hence the species differences in receptor occupancy are likely due to differences in metabolism or absorption of the compounds.

Although full receptor occupancy was sustained for at least 1 h in rats for both compounds, the plasma levels decreased throughout the 1-h sampling interval. Hence, threshold plasma levels of MPEP and MTEP necessary to produce 100% brain receptor occupancy in the rat were in the low μM range (1.7 μM for MPEP and 1.2 μM for MTEP at 60 min). Threshold plasma levels of MPEP were achieved in mice but only at the 5-min time point (which corresponded to 95% occupancy). In contrast, threshold plasma levels of MTEP were not achieved in mice, even 5 min post-

administration. This likely explains why in mice MTEP consistently showed slightly lower receptor occupancy than MPEP, while the opposite was observed in rats. Species differences in drug metabolism are well documented in the literature (Collins, 2001; Martinez, 1998) and differences in organ physiology and metabolism between rats and mice are becoming better understood (Rao and Verkman, 2000). Nevertheless, more rapid metabolism and shorter time course of receptor occupancy of MPEP and MTEP in mice should be considered when examining these compounds in mice.

Whether the behavioral responses produced by MPEP and MTEP track closely with the time course of receptor occupancy has not been examined in detail. Most published reports utilized a single pre-treatment time for MPEP of 60 min or less in rats and mice (Brodkin et al., 2002b; Chapman et al., 2000; Spooren et al., 2000), times at which, depending on the dose, MPEP should show substantial receptor occupancy. Some studies reported in mice seem to suggest that higher doses of MPEP are required to achieve anxiolytic effects in this species than in rats. For example, MPEP at 1 mg/kg i.p. (60 min pre-treatment) increased the number of shocks accepted in the conflict drinking test of anxiety in rats, while efficacy in the murine four-plate test of anxiety (60 min pre-treatment) required 30 mg/kg i.p. (Tatarczynska et al., 2001). A recent report examined the time course of the effect of MTEP on stress-induced hyperthermia in wild-type and mGlu5 receptor deficient mice (Brodkin et al., 2002a). In this study, MTEP (16 mg/kg s.c.) significantly attenuated the rise in body temperature produced by injection stress in wild-type mice (but not in mGlu5 receptor deficient mice). This effect was apparent up to 39 min post-administration, which coincided with the time frame of full receptor occupancy produced by this dose of MTEP (unpublished observations). Although further studies are needed to more fully address the temporal relationship between receptor occupancy and behavior, it is likely that rapid elimination of currently available mGlu5 receptor antagonists in mice limits the apparent efficacy in behavioral models.

[^{3}H]methoxy-PEPy rapidly entered the brain following intravenous dosing in both rats and mice. The brain penetration of this radioligand enables its use in an in vivo (as opposed to ex vivo) receptor occupancy assay. [^{3}H]methoxy-PEPy also selectively labeled mGlu5 receptors as demonstrated by its lack of binding in forebrain in mGlu5 receptor knockout mice, the 10-fold greater degree of binding in hippocampus versus cerebellum in Sprague–Dawley rats, and the eightfold higher binding in forebrain compared to cerebellum in C57Bl/6 mice. The cerebellum is relatively devoid of mGlu5 receptors while the forebrain, and hippocampus in particular, has a high expression level of mGlu5 receptors (Romano et al., 1995; Shigemoto et al., 1993). Selectivity of a radioligand is an important characteristic in determining its usefulness in occupancy assays. The present findings are in agreement with previous experiments with this radioligand which also demonstrated selectivity for mGlu5 receptors (Patel et al., 2003) and are consistent with our recent results with a structurally related mGlu5 receptor antagonist radioligand, [^{3}H]methoxymethyl-MTEP (Anderson et al., 2002).

In summary, the present results describe a rapid and reliable method for determining the functional brain penetration and receptor occupancy of mGlu5 receptor antagonists using an in vivo binding technique. This procedure can be used in both mice and rats, and is useful for determining the appropriate doses and time points for behavioral assessments in these two species.

Acknowledgements

The authors wish to acknowledge the excellent work of Ashok Chaudary in the synthesis of [^{3}H]methoxy-PEPy.

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