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Effects of trazodone on neurotransmitter release from rat mossy fibre cerebellar synaptosomes

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Abstract

The effects of trazodone and putative sigma (σ) receptor ligands were investigated on KCl-stimulated release of glutamate (Glu) and γ-aminobutyric acid (GABA) from cerebellar mossy fibre synaptosomes. Both trazodone and serotonin (5-HT) inhibited the increase of Glu and GABA release evoked by 15 mM KCl. Trazodone increased the inhibition of Glu release caused by 0.01 μM 5-HT, while it antagonized the inhibition induced by higher 5-HT concentrations. Despite the low affinity of trazodone for both σ_1 and σ_2 binding sites, with a pK_i of 5.9 and 6.0 respectively, two σ receptor ligands, (+)-3-[3-hydroxypheny]-*N*-(1-propyl)piperidine ((+)-3-PPP) and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD 1047) antagonized the effects of trazodone. The putative σ receptor ligand *N*-allylnormetazocine ((+)-SKF 10,047) mimicked the inhibitory effect of trazodone. As with trazodone, (+)-3-PPP and BD 1047 antagonized the activity of (+)-SKF 10,047 but not that of 5-HT. On the whole, these results suggest that trazodone shares a common molecular target with σ compounds distinct from that of 5-HT and is involved in K^+-stimulated Glu and GABA release from mossy fibre cerebellar synaptosomes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: σ Receptor; Glutamate release; GABA (γ-aminobutyric acid) release; Synaptosome; Cerebellum

1. Introduction

Trazodone is a widely used antidepressant drug assumed to act mainly on serotonergic systems (Owens et al., 1997; Pazzagli et al., 1999) and preferentially targeting 5-HT_{2A} receptors, at which it behaves as an antagonist (Haria et al., 1994). In contrast, Marcoli et al. (1998) have suggested that trazodone behaves as a potent full agonist at 5-HT_{2C} receptors mediating inhibition of the *N*-methyl-D-aspartate (NMDA)/nitric oxide cGMP system in cerebellar slices and of the K^+-evoked release of glutamate (Glu) in cerebellar mossy fiber synaptosomes. However, besides its high affinity for 5-HT_{2A} receptors, trazodone has a complex binding profile (high affinity for α_1 receptors and medium affinity for 5-HT_{2C}, 5-HT_{1A} and 5-HT reuptake sites) that could at least partially account for the discrepancies in intrinsic activity observed in different laboratories. Furthermore, in binding studies the distribution of 5-HT_2 receptors in the cerebellum has been found to be remarkably low (Malgouris et al., 1993), though Abramowski et al. (1995), using immunocytochemical techniques, have reported contrasting results. This finding casts some doubt on the relevance of agonism at 5-HT_2 receptors in mediating the inhibitory effect of trazodone on K^+-stimulated Glu release. The cerebellum is highly enriched in sigma (σ) sites (McLean and Weber, 1988; Jansen et al., 1991), and σ receptor ligands mediate the inhibition of K^+-stimulated release of norepinephrine and Glu from rat cerebellar slices (Ellis and Davies, 1994). Pointing to a possible role of σ binding sites in mediating the effect of trazodone on K^+-stimulated Glu release, we have found that trazodone displaces the unspecific σ_1/σ_2 ligand [5-^{3}H]1,3-di-*O*-2-tolylguanidine-di-[*p*-ring-^{3}H] (DTG) in the low micromolar range, while it has no affinity for opioid (human recombinant δ_2, κ and μ) and PCP (phencyclidine) binding sites (unpublished results).

First identified by Martin et al. in 1976 and proposed as belonging to the opiate family, σ sites are now recognized as being independent receptors (Quirion et al., 1992). Moreover, binding studies have demonstrated that σ receptors are heterogeneous and that at least two different

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populations, termed σ_1 and σ_2, exist (Quirion et al., 1992; McCann et al., 1994). In the brain, autoradiographic studies have localized σ receptors in areas relevant for motor and affective functions. Indeed, they are concentrated in brainstem and cerebellum, in certain limbic structures, in some predominantly sensory areas, and in brain nuclei associated with endocrine functions (Walker et al., 1990). Although the physiological role of σ receptors in the central nervous system has not been elucidated, recent functional studies suggest that σ_2 sites may be chiefly associated with the control of movement (Walker et al., 1990; Leitner et al., 1994), while σ_1 sites may exert neuroprotectant activity against ischaemic and Glu induced neurotoxicity (Lysko et al., 1992a,b; DeCoster et al., 1995; Lockart et al., 1995).

Recently, some evidence has been gathered showing that σ receptors localized on presynaptic terminals can modulate neurotransmitter release. With the exception of acetylcholine release, which was increased both in vivo and in vitro by injection or bath application of the σ receptor agonist *N*-allylnormetazocine ((+)-SKF 10,047) (Matsuno et al., 1993; Siniscalchi et al., 1987; Junien et al., 1991), all the available data point to an inhibitory action of σ receptor ligands on brain transmitter release. σ Receptor ligands decrease the potassium-stimulated release of norepinephrine and Glu from rat hippocampal slices (Gonzalez-Alvear and Werling, 1995; Ellis and Davies, 1994), as well as NMDA-stimulated release of norepinephrine and dopamine from striatal, hippocampal and cerebellar slices (Gonzalez-Alvear et al., 1995; Gonzalez-Alvear and Werling, 1994).

This study describes (i) the activity of trazodone and of the σ_1 receptor ligand (+)-SKF 10,047 on K^+-stimulated Glu and γ-aminobutyric acid (GABA) release from mossy fibre cerebellar synaptosomes and (ii) the effect of two more σ receptor ligands with preferential affinity for σ_1 sites, (+)-3-[3-hydroxypheny]-*N*-(1-propyl)piperidine ((+)-3-PPP) and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD 1047), on the responses elicited by trazodone and (+)-SKF 10,047.

It is important to note that, since the endogenous agonist has not been detected so far and its chemical signalling pathway has not been defined, the only operationalistic reason to call the σ site a receptor is the recent cloning and expression of proteins from several species (Hanner et al., 1996; Pan et al., 1998; Seth et al., 1998), including humans (Kekuda et al., 1996), that bind molecules defined as σ receptor ligands. Thus, the agonist or antagonist activity may be at best dependent on the particular preparation used. As a consequence, the same chemical may be described as an agonist or an antagonist, as in the case of haloperidol, which is reported to be a σ receptor agonist (Meyer et al., 1998) and a σ receptor antagonist (Matsuno et al., 1996). In this paper, agonists are defined as σ receptor ligands that inhibit KCl-stimulated Glu release.

2. Materials and methods

2.1. Synaptosomes preparation and release experiments

Giant synaptosomes from mossy fibre endings were prepared according to Maura et al. (1991) following the method described by Israel and Whittaker (1965). Adult male rats (Sprague–Dawley) weighing 200–300 g were used. The cerebellum was rapidly removed and homogenized in 40 volumes of 0.32 M sucrose buffered with phosphate at pH 7.4. The homogenate was centrifuged at $1000 \times g$ for 5 min. The pellet (P1: crude nuclear fraction) was resuspended in an equal volume of sucrose 0.32 M pH 7.4, filtered through a double layer of gauze to remove cell debris, and then centrifuged at $1000 \times g$ for 5 min. The pellet containing the giant synaptosomes (P2) was resuspended in a physiological salt solution with the following composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 22 and glucose 11; pH 7.2–7.4; gassed with 95% O_2 and 5% CO_2 and maintained at 37°C.

Aliquots of the synaptosomal suspension were distributed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri et al., 1974). Synaptosomes were continuously perfused with physiological salt solution aerated with a mixture of O_2 and CO_2 (95%:5%), at a rate of 0.6 ml/min. After 38 min perfusion, during which the synaptosomes were allowed to stabilize, the experiment began and three separate fractions of perfusate were collected during the following 12 min: first basal (B1; 3 min perfusion), stimulated (S; 6 min perfusion) and second basal (B2; 3 min perfusion).

Stimulation of release was induced by perfusing the preparation for 120 s with physiological solution in which 15 mM KCl replaced an equimolar amount of NaCl. Since under basal conditions the efflux of Glu and GABA slowly decreases (data not shown), the Glu and GABA overflows induced by KCl were calculated as the fractional rate (FR) by subtracting the amount of basal release (expressed as the mean of release in the 3 min before and the 3 min after the KCl stimulation) from the amount of Glu and GABA present in the stimulated fraction.

Thus, the FR was calculated according to the formula:

$$\mathrm{FR} = \frac{\mathrm{S(pmol/min)} - \frac{\mathrm{B1(pmol/min)}}{2}}{\frac{\mathrm{B1(pmol/min)} + \mathrm{B2(pmol/min)}}{2}}.$$

All the agonists tested were added together with KCl. Putative antagonists were applied 8 min before KCl and the agonist under study. The effects of test compounds are expressed as percent variation of release compared to the basal values.

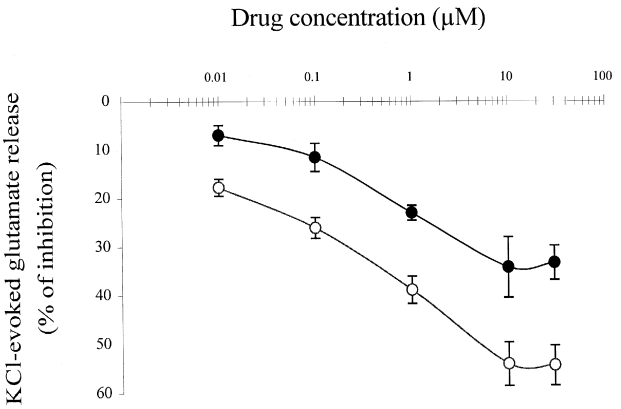

Fig. 1. Effect of 5-HT (○) and trazodone (●) on KCl-stimulated glutamate release from rat cerebellar giant synaptosomes. The compounds were added to the superfusion medium concomitantly with the depolarizing stimulus. Experimental details as in Materials and methods. Each point represents the mean ± S.E.M. of three to six experiments in duplicate.

2.2. Glutamic acid and GABA determination

Endogenous Glu and GABA released from cerebellar giant synaptosomes were quantified by high-performance liquid chromatography (HPLC) according to the method of Tonnaer et al. (1983), with some minor modifications. The method involved precolumn derivatization with *o*-phthaldehyde followed by separation on a C18 reverse-phase chromatography column coupled with fluorescence detection. HPLC was performed with two solvent delivery pumps (Model LC-10AD, Shimadzu, Kyoto, Japan), an autosampler (Model SIL-10A, Shimadzu), a sample cooler (Shimadzu) and a CBM-10A communication module connected to a LC-10 workstation system (Shimadzu) for acquisition and analysis of chromatograms. The column effluent was monitored with a Shimadzu model RF-551 fluorimeter (excitation wavelength 340 nm, emission wavelength 450 nm). Homoserine 2.5×10^{-6} M was added to the samples as the internal standard. A step by step binary gradient was used to separate the amino acids present in the samples. Mobile phase A was composed by 0.1 M acetate buffer (pH 5.8) in 20% methanol (Lichrosolv, Merck, Rahway, NJ), mobile phase B was 0.1 M acetate buffer (pH 5.8) in 80% methanol. The two phases were filtered through 0.22 μm Millipore filters and degassed. The flow rate was 1.2 ml/min. For quantitative analysis of endogenous Glu and GABA, calibration curves were constructed with standard Glu and GABA in the concentration range expected to be found in the samples (6 to 120 pmol), and the amount present in the samples was determined according to the calculated regression line.

2.3. Receptor binding assays

Binding to σ_1 and σ_2 receptors was determined essentially according to the method described by Chaki et al. (1994) with some modifications. The affinity of trazodone for σ_1 and σ_2 sites was determined by incubation of guinea pig brain membranes with the σ_1 receptor ligand [^{3}H](+)-pentazocine or the σ_2 receptor ligand [^{3}H]DTG, at a final concentration of 4.0 nM, in 50 mM Tris–HCl (pH 7.4) at 25°C for 60 min. The reaction was terminated by rapid vacuum filtration onto glass fibre filters. Non-specific binding of [^{3}H]DTG was determined in the presence of 1.0 μM haloperidol and non-specific binding of [^{3}H](+)-pentazocine was determined in the presence of 10 μM (+)-3-PPP. Radioactivity trapped on the filters was measured by liquid scintillation spectroscopy and compared to control values. Under these conditions, the spe-

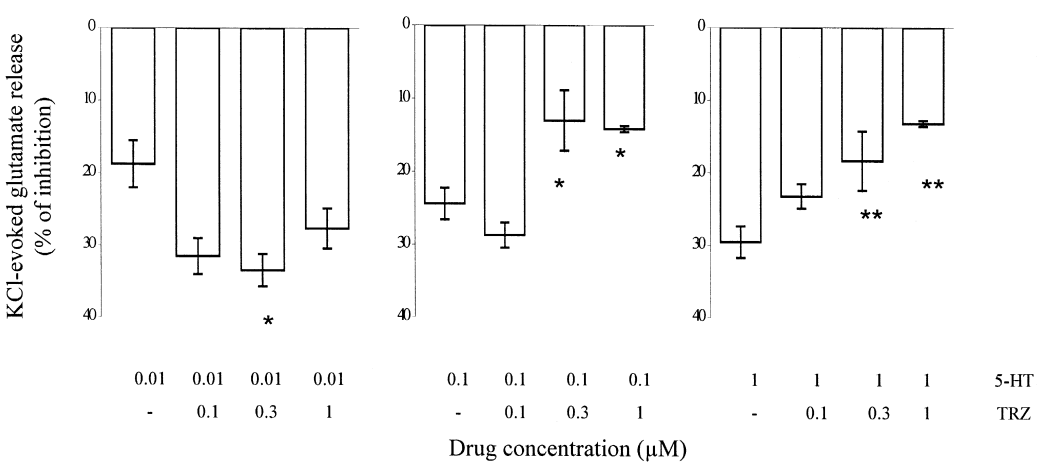

Fig. 2. Concentration-dependent effects of trazodone on 5-HT-mediated inhibition of KCl-stimulated glutamate release from rat cerebellar giant synaptosomes. The compounds were added to the superfusion medium concomitantly with the depolarizing stimulus. Experimental details as in Materials and methods. Each point represents the mean ± S.E.M. of three to six experiments in duplicate. ($^*P < 0.05$; $^{**}P < 0.01$)

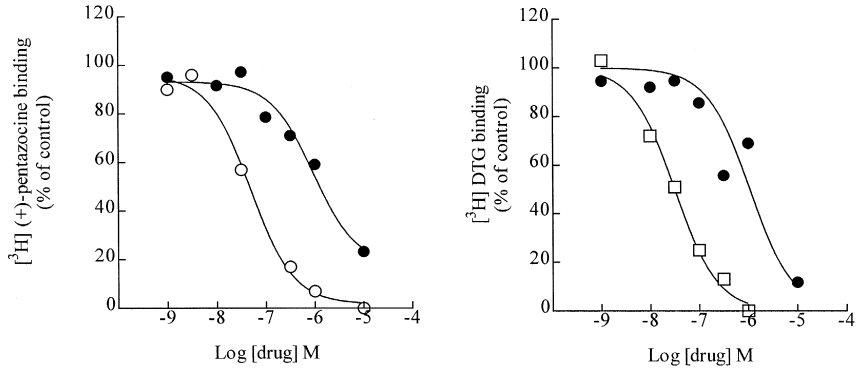

Fig. 3. Inhibitory activity of trazodone (●) in radioligand binding to σ_1 (left panel) and σ_2 (right panel) sites. (+)-3-PPP (○) and haloperidol (□) were used as reference compounds for σ_1 and σ_2 binding sites, respectively. Experimental details as in Materials and methods. The data shown are from a representative experiment performed in duplicate. The calculated K_i are reported in the text.

cific binding of [^{3}H]DTG and [^{3}H](+)-pentazocine was about 80% of total binding.

2.4. Statistical analysis

Means ± S.E.M. of the number of experiments are reported. Analysis of variance followed by Tukey test was used for the analysis of the statistical significance of the difference between means (Stat Soft package, rel. 1993).

2.5. Drugs

The following drugs were used: 5-hydroxytryptamine creatinine sulphate (5-HT) (Sigma, St. Louis, MO), trazodone (ACRAF Angelini, Aprilia, Italy), (+)-SKF 10,047, (+)-3-PPP (RBI Natick, MA) and BD 1047 (Tocris, Langford, Bristol). The compounds were dissolved in water and dilutions were made with the same physiological salt solution used for perfusion of synaptosomes.

3. Results

3.1. Effects of 5-HT and trazodone on KCl-stimulated Glu release

In B1 control samples ($n = 127$; 53 experimental sessions), Glu was released at the rate of 48 ± 1.8 pmol/ml/min. Application of 15 mM KCl increased the efflux of Glu to 102 ± 4.2 pmol/ml/min. The efflux returned to basal level (B2) when the stimulus was removed (40 ± 1.8 pmol/ml/min). Thus, the cumulative FR for 15 mM KCl was a 133 ± 3.1% increase in Glu release.

Fig. 1 shows the effects of 5-HT and trazodone on KCl-stimulated Glu release from cerebellar mossy fibre synaptosomes. As can be seen, both substances antagonized in a concentration-dependent manner (range 0.01–33 μM) the increase in Glu release evoked by 15 mM KCl. However, the maximal effect of 5-HT was greater than that of trazodone: 54 ± 4.1% ($n = 4$) and 34 ± 6.2% ($n = 6$) at 33 and 10 μM, respectively. The EC_{50} values (concentrations causing half-maximal inhibition of the Glu response) were 0.56 and 0.77 μM for 5-HT and trazodone, respectively.

When trazodone was tested against the effect induced by 5-HT on Glu release, its activity depended on the 5-HT concentration (Fig. 2). Thus, trazodone increased the inhibition of Glu release caused by 0.01 μM 5-HT, while it antagonized the inhibition induced by higher 5-HT concentrations. This result may suggest that trazodone behaves as a partial agonist at 5-HT receptors expressed on rat giant synaptosomes. However, other molecular targets cannot be excluded to explain its effects.

3.2. Binding of trazodone to σ receptors and (+)-SKF 10,047 effect on KCl-stimulated Glu release

While it is well known that trazodone has high affinity for several 5-HT receptor subtypes (Haria et al., 1994), in

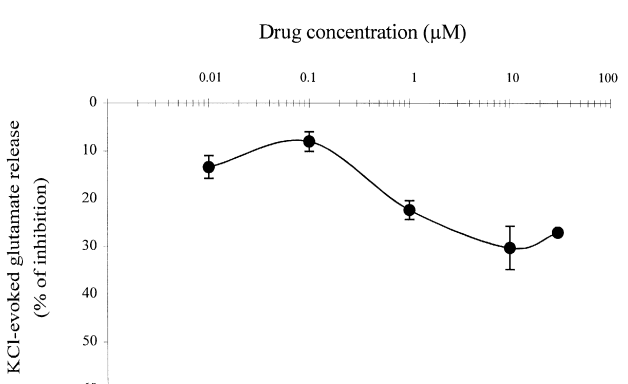

Fig. 4. Effect of (+)-SKF 10,047 (●) on KCl-stimulated glutamate release from rat cerebellar giant synaptosomes. The compound was added to the superfusion medium concomitantly with the depolarizing stimulus. Experimental details as in Materials and methods. Each point represents the mean ± S.E.M. of three to six experiments in duplicate.

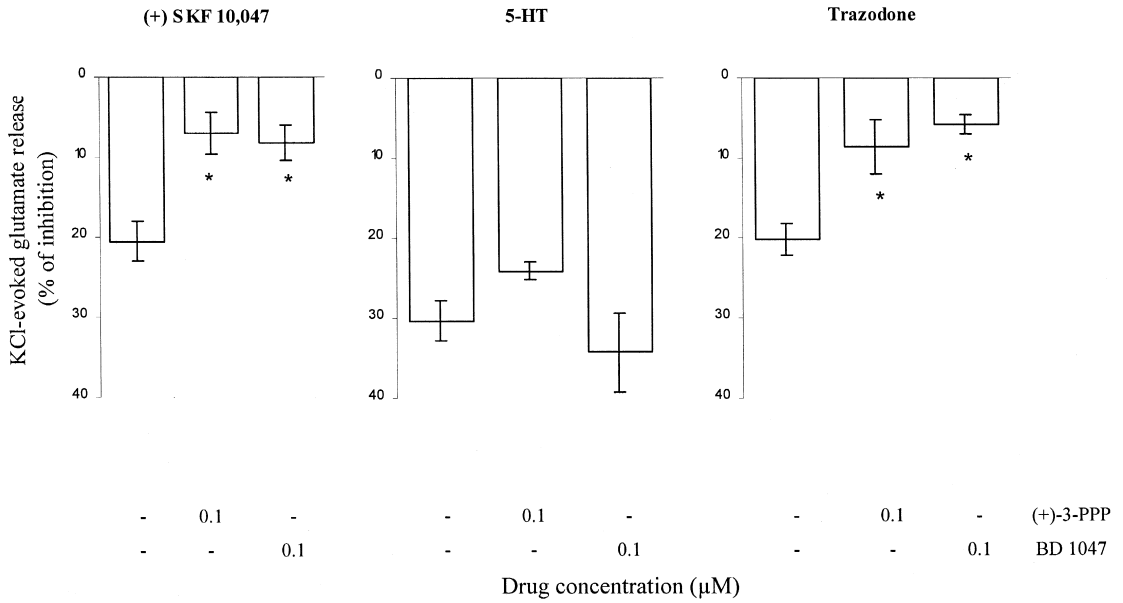

Fig. 5. Effect of 0.1 μM (+)-3-PPP and 0.1 μM BD 1047 on the inhibition by 1 μM (+)-SKF 10,047, 1 μM 5-HT and 1 μM trazodone of KCl-stimulated glutamate release from cerebellar giant synaptosomes. Agonists were added to the superfusion medium concomitantly with the depolarizing stimulus while the inhibitors (+)-3-PPP and BD 1047 were added 8 min before. Experimental details as in Materials and methods. Means ± S.E.M. of three to six experiments in duplicate are presented ($^{*}P < 0.01$).

this study we found that it also binds to σ sites in micromolar concentrations (pK_i 5.93 and 6.03 for σ_1 and σ_2 binding sites, respectively) (Fig. 3).

Therefore, in order to verify if σ receptor ligands have effects similar to those exerted by trazodone on KCl-stimulated Glu release, we studied the activity of the putative σ_1 receptor ligand (+)-SKF 10,047 in the same synaptosomal preparation. (+)-SKF 10,047 dose dependently (range 0.01–33 μM) caused inhibition of KCl-stimulated Glu release with an EC_{50} of 0.58 μM and a maximal inhibition of 30 ± 4.5% ($n = 5$) at 10 μM (Fig. 4).

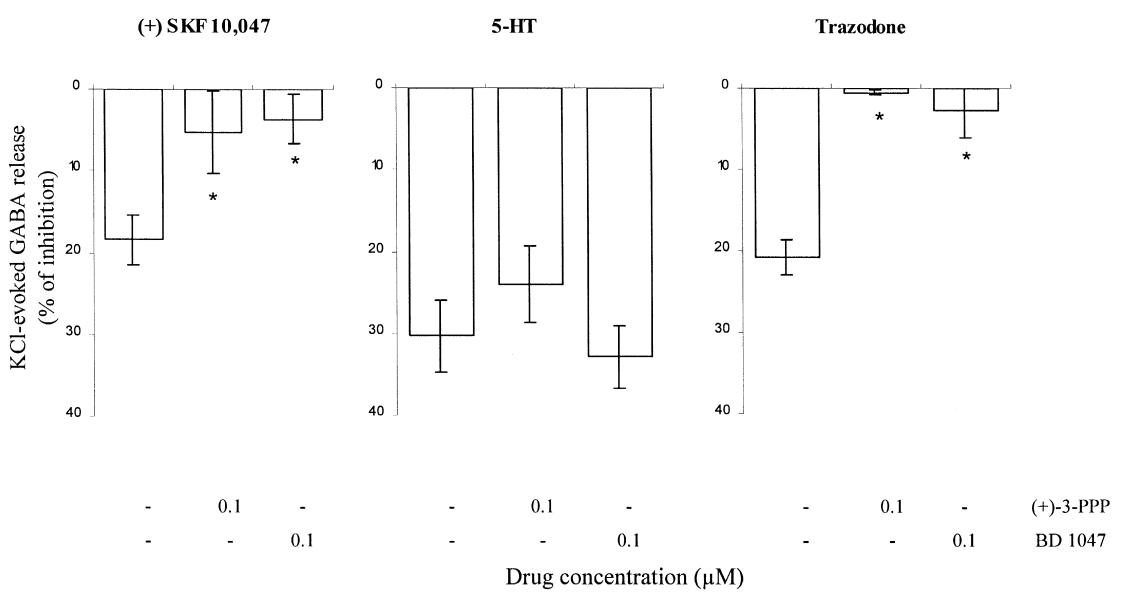

Fig. 6. Effect of 0.1 μM (+)-3-PPP and 0.1 μM BD 1047 on the inhibition by 1 μM (+)-SKF 10,047, 1 μM 5-HT and 1 μM trazodone of KCl-stimulated GABA release from cerebellar giant synaptosomes. Agonists were added to the superfusion medium concomitantly with the depolarizing stimulus while the inhibitors (+)-3-PPP and BD 1047 were added 8 min before. Experimental details as in Materials and methods. Means ± S.E.M. of three to six experiments in duplicate are presented ($^{*}P < 0.01$).

3.3. Antagonism by (+)-3-PPP and BD 1047 of the reduction of KCl-stimulated Glu release induced by 5-HT, trazodone and (+)-SKF 10,047

To further characterize the effects of σ receptor ligands on KCl-stimulated Glu release from cerebellar giant synaptosomes and the possible involvement of σ receptors in the above-described effects of 5-HT, trazodone and (+)-SKF 10,047, we tested two σ ligands, (+)-3-PPP and BD 1047, alone and in combination with 5-HT, trazodone and (+)-SKF 10,047. At the concentration used in the present study (0.1 μM), neither (+)-3-PPP nor BD 1047 had an effect on KCl-stimulated Glu release by itself (percent variation $\leq 2\%$). However, both compounds significantly reduced the inhibitory effect of trazodone and (+)-SKF 10,047 but were ineffective on the 5-HT-induced inhibition (Fig. 5).

3.4. Antagonism by (+)-3-PPP and BD 1047 of the reduction of KCl-stimulated GABA release induced by 5-HT, trazodone and (+)-SKF 10,047

Trazodone, 5-HT and (+)-SKF 10,047 at the concentration 1 μM inhibited KCl-stimulated GABA release (21 ± 2.2%, 29 ± 2.1% and 22 ± 2.0%, respectively). (+)-3-PPP (0.1 μM) and BD 1047 (0.1 μM) antagonized the effects of trazodone and (+)-SKF 10,047, but not that induced by 5-HT (Fig. 6).

4. Discussion

The main result of this paper is the finding that, in the cerebellum, the inhibitory activity of trazodone on KCl-stimulated Glu and GABA release is antagonized by σ receptor ligands. Although it was not the aim of these experiments to provide direct evidence that the effects of trazodone are associated with specific σ receptor subtypes, the results obtained suggest a link with the effects mediated by σ_1 receptors. (+)-SKF 10,047, a putative σ_1 receptor agonist (Quirion et al., 1992), exerted effects similar to those induced by trazodone, and the putative σ_1 inhibitors (+)-3-PPP and BD 1047 both antagonized the effect of (+)-SKF 10,047 and trazodone on Glu and GABA release.

Our results are in agreement with a previous study showing that, in rat striatal slices, haloperidol causes inhibition of KCl-stimulated Glu release by modulation of σ receptors (Ellis and Davies, 1994). However, in the same preparation, these authors failed to observe an inhibition of GABA release and concluded that the effect of haloperidol was selective for Glu transmission. Our results demonstrate that, in synaptosomes obtained from cerebellar mossy fibre terminals, (+)-SKF 10,047 and trazodone inhibited both Glu and GABA release, thus acting as agonists at σ sites. An explanation for these conflicting results could be the use of different preparations (viz. slices versus synaptosomes), different brain regions investigated, or simply that striatal GABA neurons are not enriched with σ sites. However, in the few studies which have addressed the question of the effects of σ receptor ligands on neurotransmission in brain slices, it has been found that agonists, while devoid of effects on basal release, inhibit KCl or NMDA-stimulated release of Glu, norepinephrine and dopamine from different brain areas (Ellis and Davies, 1994; Gonzalez-Alvear and Werling, 1994, 1995; Gonzalez-Alvear et al., 1995). Recently, Marcoli et al. (1998) have suggested that trazodone inhibits the NMDA-stimulated release of Glu by acting as an agonist at postsynaptic 5-HT_{2C} receptors in rat cerebellar slices. Although our results do not rule out the possibility that trazodone at the concentrations tested could also act by modulating serotonergic systems, they suggest that the activity of trazodone is related to its effects on a determinant common to σ compounds. In fact, while (+)-3-PPP and BD 1047 did not antagonize the inhibitory effects of 5-HT, they were able to antagonize the effects of trazodone and (+)-SKF 10,047. It has been reported by Maura et al. (1991) that in cerebellar giant synaptosomes ketanserin, while it does not affect Glu release on its own, blocks the inhibitory effects of 5-HT. In our hands, ketanserin was able to antagonize the effects of both trazodone and (+)-SKF 10,047 on Glu and GABA release (unpublished results). However, it is difficult, on the basis of present knowledge, to understand the mechanism of action of ketanserin, since it binds also to receptors other than 5-HT_2 (i.e. α_1-adrenoceptors, histamine H_1 receptors; Leysen et al., 1981).

Usually, the activity of trazodone is mainly referred to the wiring of serotonergic systems. Indeed, in this study we found that trazodone modulated the inhibitory effect of 5-HT on Glu release according to the concentration of 5-HT itself, suggesting that it behaves as a partial 5-HT receptor agonist. This finding indicates that trazodone may affect Glu release by simultaneously acting on different mechanisms. It is worth noting that the existence of connections between the 5-HT and σ systems has been demonstrated by the recent finding that infusion of the 5-HT uptake blocker fluvoxamine into the red nucleus of rats sensitizes the animals to the dystonic effect of the σ receptor ligand DTG (Faherty et al., 1998).

On the whole, these results suggest that the effects of trazodone are at least partially linked to modulation of σ sites or of a molecular target that is also shared by σ compounds and that such sites are involved in K^+-stimulated Glu and GABA release from mossy fibre cerebellar synaptosomes.

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