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Differentiation of σ ligand-activated receptor subtypes that modulate NMDA-evoked [^{3}H]-noradrenaline release in rat hippocampal slices

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1 It is now widely accepted that there are two classes of sigma (σ) binding sites, denoted σ_1 and σ_2, and recently σ_3 subtype has been proposed. Selective σ_1 and σ_2 receptor agonists are known to modulate the neuronal response to N-methyl-D-aspartate (NMDA) *in vivo* and *in vitro*. To identify the site of action of a series of recently synthesised high affinity σ ligands, the present *in vitro* series of experiments was carried out on NMDA-evoked [^{3}H]-noradrenaline ([^{3}H]-NA) overflow from preloaded hippocampal slices of the rat.

2 The ligands (+)-cis-N-methyl-N-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl) cyclohexylamine (BD-737) and (+)-pentazocine, considered as the prototypic σ_1 agonists, potentiated the NMDA response from 10 nM to 100 nM. This potentiation faded between 100 nM and 1 μM ligand concentrations. On the other hand, 1,3-di(2-tolyl)guanidine (DTG), a mixed σ_1/σ_2 agonist, at concentrations greater than 100 nM inhibited the NMDA-evoked [^{3}H]-NA release. Spiperone, considered as active on putative σ_3 receptors, was without effect on the NMDA response, or on the potentiating effect of BD-737.

3 The high affinity σ antagonists haloperidol and 1[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD-1063), inactive by themselves on the NMDA-induced response, at concentrations above 30 nM totally prevented the potentiating effect of (+)-pentazocine (100 nM) as well as the inhibitory effect of DTG (300 nM) on NMDA-evoked [^{3}H]-NA release. Whereas haloperidol and BD-1063, at concentrations <1 μM, were inactive on the potentiating effect of BD-737 (100 nM).

4 4-(4-Chlorophenyl)-α-4-fluorophenyl-4-hydroxy-1-piperidinebutanol (reduced haloperidol), N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine (BD-1008), inactive by themselves on the NMDA-evoked [^{3}H]-NA release, failed to reverse the effects of (+)-pentazocine and DTG, but at concentrations of 30 nM to 1 μM antagonised the BD-737-induced potentiation of the NMDA response. Conversely, *N*,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) blocked the effects of (+)-pentazocine as well as those of BD-737, but not those of DTG.

5 The present results provide *in vitro* functional evidence for a σ receptor type preferentially sensitive to BD-737, reduced haloperidol, BD-1008 and also to NE-100, that differs from the already identified σ_1, σ_2 and σ_3 sites.

Keywords: N-methyl-D-aspartate (NMDA); σ receptors; BD-737; BD-1008; NE-100; 1,3-di(2-tolyl)guanidine (DTG); (+)-pentazocine; haloperidol; BD-1063; rat hippocampus

Introduction

The sigma (σ) receptor was originally named by Martin *et al.* (1976) and was initially defined by the behavioural effects of (±)-SKF-10047 (N-allylnormetazocine) (see Walker *et al.*, 1990 for review). Two subtypes of σ receptors, termed σ_1 and σ_2, have since been identified from binding studies and functional bioassays (Hellewell & Bowen, 1990; Walker *et al.*, 1990; Quirion *et al.*, 1992; Su & Junien, 1994). (+)-Pentazocine acts selectively on σ_1 whereas 1,3-di-(2-tolyl)guanidine (DTG) and haloperidol act on both σ_1 and σ_2 receptors. More recently, additional classes of σ sites have been proposed, based on their distinct pharmacological profiles compared to the previously defined σ_1 and σ_2 classes, one differentiating feature being low affinity for DTG. The putative σ_3 receptor subtype, preferentially activated by a series of 1-phenyl-3-amino-1,2,3,4-tetrahydronaphthalenes (1-phenyl-3-aminotetralins, PATs), by haloperidol and by spiperone, has been shown to affect tyrosine hydroxylase activity and dopamine synthesis in rodent striatum (Booth *et al.*, 1993; Myers *et al.*, 1994). Another binding site exhibits high affinity for arylethylene diamine-related compounds, such as (+)-*cis*-N-methyl-N-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl) cyclohexylamine (BD-737) and *N*-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine (BD-1008), moderate affinity for 4-(4-chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol (reduced haloperidol), and low affinity for haloperidol and DTG (Bowen *et al.*, 1995). This site is characterized as a haloperidol-insensitive binding site for the iodo-arylethylene diamine, [^{125}I]-N-[2-(4-iodophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine ([^{125}I]-SH-344), in guinea-pig brain. The extensive cross-reactivity of a variety of classical compounds eliciting high affinity for σ_1 and σ_2 sites as well as a variety of σ-selective arylethylene diamines forms the basis for consideration that this site is related to the previously defined subclasses of σ sites. Both of these additional (putative σ_3 and arylethylene diamine-sensitive) sites require further study and confirmation as members of the σ family.

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Behavioural, neurochemical and electrophysiological studies led to the discovery of the neuromodulatory effects of σ receptor ligands on NMDA(*N*-methyl-D-aspartate)-operated glutamatergic neurotransmission, effects which would account for the neuroprotective actions, amnesic and cognitive effects of σ drugs (Monnet *et al.*, 1990; 1992a; Pontecorvo *et al.*, 1991; Martin *et al.*, 1992; Maurice *et al.*, 1994a, b; DeCoster *et al.*, 1995; Lesage *et al.*, 1995; Lockhart *et al.*, 1995). In an *in vivo* extracellular electrophysiological paradigm, σ ligands that enhance the neuronal response to NMDA were defined as σ agonists [e.g. (+)-pentazocine (Bowen *et al.*, 1993), BD-737 (de Costa *et al.*, 1990; Bowen *et al.*, 1992) and DTG (Weber *et al.*, 1986)], whereas those which, devoid of effects by themselves, reverse the effects of σ agonists were defined as σ antagonists [e.g. haloperidol (Tam & Cook, 1984)] (Monnet *et al.*, 1990; 1992a, b; 1994). Interestingly, in particular conditions, e.g. following $G_{i/o}$ protein inactivation by pertussis toxin, haloperidol fails to reverse the effect of σ agonists on the NMDA-induced neuronal activation (Monnet *et al.*, 1994).

We have also developed an *in vitro* model of [^{3}H]-noradrenaline (NA) release evoked by NMDA from preloaded rat hippocampal slices in which σ_1 agonists [e.g. JO-1784, (+)-N-cyclopropylmethyl- N-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochl-oride] potentiated, whereas DTG inhibited the NMDA response (Monnet *et al.*, 1992a; 1995). The latter response was also blocked by haloperidol, suggesting that DTG was acting in this model as an inverse σ agonist (Monnet *et al.*, 1992a; 1995). Recently, Gonzales-Alvear & Werling (1995), using a similar *in vitro* release model of [^{3}H]-NA evoked by NMDA, have shown that σ_1 agonists [e.g. (+)pentazocine] and BD-737 inhibited the NMDA response, whereas DTG remained inactive. However, methodological differences most likely explain this apparent discrepancy. For example, they used a shorter incubation time for the σ drugs than here (Monnet *et al.*, 1992a; 1995) (10 min *versus* 40 min). In addition, their superfusion medium contained 1 μM desipramine and 1 μM yohimbine to prevent both reuptake and feedback inhibition by the released [^{3}H]-NA which is not the case in the present studies. Moreover, they used repetitive stimulation by NMDA, which is known to induce a long-lasting and profound desensitization of NMDA receptors (Snell *et al.*, 1987; Fink *et al.*, 1989; 1990a, b; Fink & Göthert, 1991). Although the different profiles of action of these specific σ drugs were most likely due to methodological differences between protocols, the observation by Gonzalez-Alvear & Werling (1995) supported the notion that (+)-pentazocine and BD-737, and DTG were acting on σ_1 and σ_2 receptors, respectively. They also showed that the BD-737-induced modulation of the NMDA-evoked [^{3}H]-NA release was insensitive to haloperidol.

The present *in vitro* series of experiments were therefore carried out to investigate the reason for this unusual lack of effect of haloperidol and to identify the receptor by which BD-737 modulates the NMDA-evoked release of [^{3}H]-NA. For this purpose, various specific and high affinity σ drugs, recently identified, such as both isomers of *cis*-*N*-methyl-*N*-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl) cyclohexylamine (BD-737 and BD-738; de Costa *et al.*, 1990; Bowen *et al.*, 1992), *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine (BD-1008), 1[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD-1063) (de Costa *et al.*, 1992; 1993), *N*,*N*-dipropyl-2-[4-methoxy-3-(211phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) (Okuyama *et al.*, 1993) and 4-(4-chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol (reduced haloperidol; Bowen *et al.*, 1990; Klein *et al.*, 1994) have been examined. The prototypic σ_1, σ_2 and/or σ_3 receptor ligands [(+)-pentazocine, DTG and spiperone, respectively] were also tested. Contrary to haloperidol which binds to both σ and D_2-like dopamine sites with similar affinity, its carbonyl-reduced metabolite [4-(4-chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol or reduced haloperidol] binds preferentially to σ sites, where it has been proposed to behave as an antagonist in some systems and as an agonist in others (Bowen *et al.*, 1990; Klein *et al.*, 1994).

Methods

Male Sprague-Dawley rats (200–250 g, $n=84$) were obtained from Iffa Credo (l'Arbresle, France). They were kept at 21°C on a standard 12:12 h light/dark cycle (with lights on at 0700 h) and with free access to water and Purina chow. All experiments were carried out between 1300 and 1800 h.

Brain slice preparation

The rats were killed by decapitation after ether anaesthesia and their brains removed and rapidly dissected, in accordance with the guidelines of the INSERM Animal Use and Care Committee. Hippocampal slices (0.4 mm thick) were prepared with a McIlwain tissue chopper in an orientation corresponding to the lamellar organization of the hippocampus. The slices were then incubated at 37°C for 30 min in Krebs solution containing 0.1 μM [^{3}H]-NA (3,4[7-^{3}H]-noradrenaline; 540.2 GBq mM^{-1} l^{-1}, Isotopchim, Ganagobie-Peyruis, France) and bubbled with a mixture of 95% O_2- 5% CO_2. The composition of the Krebs solution (in mM) was: Na^+Cl^- 118, K^+Cl^- 4.7, Ca^{2+} Cl^-_2 1.3, $Mg^{2+}Cl^-_2$ 1.2, $Na^+H_2PO^-_4$ 1, $Na^+HCO^-_3$ 25, glucose 11.1, Na^+_2 EDTA 0.04 and ascorbic acid 0.06.

Superfusion of the slices

At the end of the incubation period, the slices were washed in oxygenated Krebs solution maintained at 37°C and slices, in twos, were transferred into 2.5 ml glass chambers. The tissue samples were superfused continuously at a rate of 0.5 ml min^{-1} with oxygenated Mg^{2+}-free Krebs solution maintained at 37°C, Mg^{2+} being an inhibitor of the NMDA response (Nowak *et al.*, 1984). Each of the σ ligands BD-737, its negative enantiomer (−)-*cis*-*N*-methyl-*N*-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl) cyclohexylamine (BD-738), DTG, (+)-pentazocine, haloperidol, or 4-(4chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol (reduced haloperidol), BD-1008, BD-1063 or NE-100, and spiperone, were added throughout the superfusion period, i.e. 40 min before the NMDA stimulation and during the following 28 min. The total duration of superfusion was thus 68 min. In a first series of experiments, concentration-response curves were constructed, with 10 nM, 30 nM, 100 nM, 300 nM and 1 μM of each drug. As previously denoted (Monnet *et al.*, 1992a), we considered that σ compounds affecting the [^{3}H]-NA efflux evoked by NMDA were σ agonists. Thereafter, according to the results of the first series of experiments, σ drugs which were inactive by themselves on the NMDA response were tested in the presence of an agonist to assess their respective antagonistic action. Two series of experiments were therefore carried out. First, concentration-response curves of these drugs (10 nM, 30 nM, 100 nM, 300 nM and 1 μM, as above) were established in the presence of a fixed concentration of σ agonists. Second, concentration-response curves of σ agonists (10 nM, 30 nM, 100 nM, 300 nM and 1 μM) were established in the presence of a fixed concentration of each of the inactive drugs. Successive 2 ml/4 min fractions were collected, starting after 32 min of superfusion. After 8 min of collection, superfusion was performed for 4 min with medium containing both the σ ligand(s) and NMDA (200 μM) to evoke [^{3}H]-NA efflux. In all, nine fractions were collected. Only one exposure to NMDA was carried out because a long-lasting desensitization of NMDA receptors occurs in such experimental conditions (Snell *et al.*, 1987; Fink *et al.*, 1989). At the end of the experiments, the hippocampal slices were collected and solubilised in 0.5 ml of Soluene® 350 (Packard Instruments, Rungis, France) and the radioactivity in the slices and superfusate samples was determined by liquid scintillation spectrometry (Packard Tris-Carb 4660).

The amount of tritium per 2 ml sample was expressed as a fraction (%) of the total tissue tritium content at the onset of the respective collection period. The tritium evoked overflow in the presence of σ ligands was compared to that of the corresponding control chambers tested simultaneously. The NMDA-evoked overflow of [^{3}H]-NA was expressed as the increase in tritium content over spontaneous outflow, determined immediately preceding NMDA exposure. This method of determining the tritium amount slightly underestimates the absolute NMDA-evoked [^{3}H]-NA overflow because it is calculated from the extrapolated values of the basal outflow, which decreases constantly for each fraction during the overflow (Snell *et al.*, 1987; Fink *et al.*, 1989; Monnet *et al.*, 1992a). However, since the overflow typically occurred in basal conditions over two to three fractions (8–12 min) when the basal outflow decreases by only about 2–3% [in accordance with previous findings (Roman *et al.*, 1991; Monnet *et al.*, 1992a; Stout & Woodward, 1994)], the degree of underestimation was minimal. The expression 'NMDA-evoked release of [^{3}H]-NA' is used throughout instead of 'NMDA-evoked overflow of tritium from slices preloaded with [^{3}H]-NA'.

Stock solutions of BD-737, BD-738, BD-1008 and BD-1063 were prepared in distilled water and stored at −30°C, and diluted with Mg^{2+}-free Krebs solution to the appropriate concentration before the experiments. Stock solutions of DTG, haloperidol, reduced haloperidol, NE-100 and spiperone were prepared in 0.1 N H^+Cl^-/ethanol 1:1 (vol/vol), stored at −30°C, then diluted with Mg^{2+}-free Krebs solution to the concentration required, H^+Cl^-/ethanol concentration being kept ≤0.5% in the final mixture. (+)-Pentazocine solution was prepared extemporaneously by dissolving the compound in 0.1 N H^+Cl^-/methanol 1:1 (vol/vol) and then diluting it in Mg^{2+}-free Krebs solution to the appropriate concentration, with the H^+Cl^-/methanol concentration kept ≤0.1%. None of the solvents used affected either the spontaneous or the NMDA-evoked release of [^{3}H]-NA from preloaded rat hippocampal slices.

Drugs

The following compounds were used: NMDA (Sigma Chemical Ltd., Saint-Quentin Fallavier, France), DTG (Aldrich, Milwaukee, WI), haloperidol (McNeil Laboratories, Stouffville, ONT), reduced haloperidol, (+)-pentazocine and spiperone (Research Biochemical Inc., Natick, MA, U.S.A.) and [^{3}H]-NA (Isotopchim, Ganagobie-Peyruis, France). BD-737, BD-738, BD-1008 and BD-1063 were provided by Dr W.D. Bowen and Dr B. de Costa (NIH, NIDDK, Bethesda, U.S.A.) and NE-100 was kindly provided by Dr S. Okuyama (Taisho Pharmaceutical Co., Tokyo, Japan).

Statistical analyses

Results are expressed as the mean percentage ± s.e.mean of the NMDA (200 μM)-evoked release of [^{3}H]-NA. Statistical significance was assessed by Student's *t* test with the Dunnett's correction for multiple comparisons. Probabilities smaller than 0.05 were considered as significant.

Results

The 4 min exposure to NMDA (200 μM) produced a release of [^{3}H]-NA over the spontaneous efflux of [^{3}H]-NA which lasted for two to three collections and represented 1.53 ± 0.25% (n=43) of tissue [^{3}H]-NA. This result is in agreement with those previously obtained by Jones *et al.* (1987), Roman *et al.* (1991) and Monnet *et al.* (1992a; 1995). In the absence of NMDA, none of the σ ligands nor spiperone, superfused alone or in association, at concentrations ranging from 10 nM to 1 μM, affected the spontaneous efflux of [^{3}H]-NA. This is consistent with previous findings (Roman *et al.*, 1991; Monnet *et al.*, 1992a; 1995; Gonzalez-Alvear & Werling, 1995).

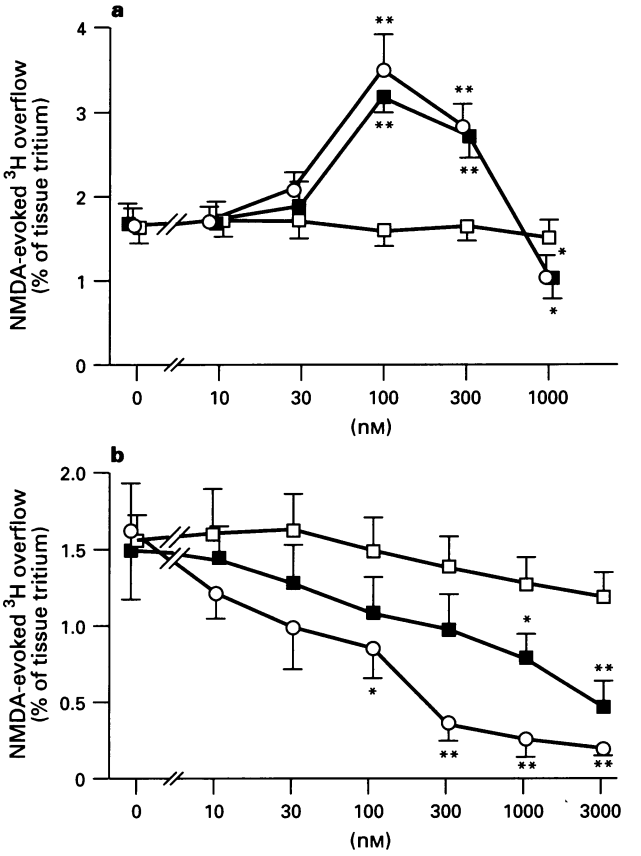

Figure 1 (a) Concentration-response curves for the effect of (+)-pentazocine either alone (○), in the presence of haloperidol (300 nM, □) or reduced haloperidol (300 nM, ■) on the release of [^{3}H]-noradrenaline (NA) evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The σ ligands were added to the superfusion medium from the beginning of the superfusion. (b) Concentration-response curves for the effects of 1,3-di(2-tolyl)guanidine (DTG) either alone (○), in the presence of haloperidol (300 nM, □) or reduced haloperidol (300 nM, ■) on NMDA-evoked release of [^{3}H]-NA in rat hippocampal slices. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); n=7–12. *P<0.05, **P<0.01 compared to basal (NMDA 200 μM) values: Student's *t* test with the Dunnett's correction for multiple comparisons compared to NMDA-evoked release in the absence of σ drug.

Modulation of the NMDA-evoked release of [^{3}H]-NA by (+)-pentazocine and DTG

The selective high affinity σ_1 agonist (+)-pentazocine exerted a biphasic effect on the NMDA-evoked release of [^{3}H]-NA (Figure 1a). Indeed, at concentrations of 100 and 300 nM, (+)-pentazocine significantly potentiated the NMDA response (Figure 1a). At 100 nM, (+)-pentazocine induced a two fold increase of the NMDA-evoked release of [^{3}H]-NA. On the other hand, at a concentration of 1 μM, (+)-pentazocine reduced the NMDA-induced release of [^{3}H]-NA (Figure 1a).

The high affinity σ_1/σ_2 inverse agonist DTG concentration-dependently inhibited the NMDA-evoked release of [^{3}H]-NA (Figure 1b). The threshold concentration of DTG for inducing this inhibition was 100 nM. The maximal inhibitory effect of DTG, which was observed with 3 μM, corresponded to a reduction by 86% of the release of [^{3}H]-NA evoked by NMDA as compared to control conditions.

Effects of haloperidol, reduced haloperidol, BD-1063, BD-1008 and NE-100 on the modulation of the NMDA-evoked release of [^{3}H]-NA by (+)-pentazocine and DTG

Haloperidol, 4-(4-chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol (reduced haloperidol), BD-1008, NE-100 and BD-1063 were inactive on the NMDA-evoked release of [^{3}H]-NA in the concentration range 10 nM–1 μM. Figure 1 shows that haloperidol (300 nM) was very effective in preventing the potentiating effect of (+)-pentazocine (100–300 nM; Figure 1a), as well as the inhibitory effect of DTG (100 nM–3 μM; Figure 1b) on the NMDA-evoked release of [^{3}H]-NA. In addition, as illustrated in Figure 2, haloperidol, BD-1063 and NE-100, from 30 nM to 1 μM, concentration-dependently abolished the (+)-pentazocine (100 nM)-induced potentiation of the NMDA-evoked release of [^{3}H]-NA. In contrast, reduced haloperidol as well as BD-1008 at concentrations below 1 μM were ineffective in antagonizing (+)-pentazocine-induced potentiation of the NMDA response (Figure 2). At 1 μM, both σ antagonists reversed the effect of (+)-pentazocine on the NMDA response (Figure 2). As illustrated in Figure 3, both haloperidol and BD-1063, from 100 nM to 1 μM, concentration-dependently abolished the DTG(300 nM)-induced inhibition of the NMDA-evoked release of [^{3}H]-NA. In addition, reduced haloperidol, BD-1008 and NE-100, between 30 nM and 1 μM, reduced by 60% at most, while haloperidol and BD-1063 in the same concentration range reversed DTG-induced inhibition of the NMDA-evoked release of [^{3}H]-NA (Figures 1b and 3).

Modulation of the NMDA-evoked release of [^{3}H]-NA by BD-737 and BD-738

BD-737, 10–300 nM, potentiated the NMDA-evoked release of [^{3}H]-NA and induced up to a three fold increase of the NMDA response (Figure 4). The negative enantiomer, BD-738, also enhanced NMDA-induced release of [^{3}H]-NA at the same concentrations although to a smaller extent. It is noteworthy that at concentrations ⩾1 μM, BD-737 and BD-738 no longer modified the NMDA-induced release of [^{3}H]-NA (Figure 4), thus exerting a biphasic effect on the NMDA-induced release of [^{3}H]-NA.

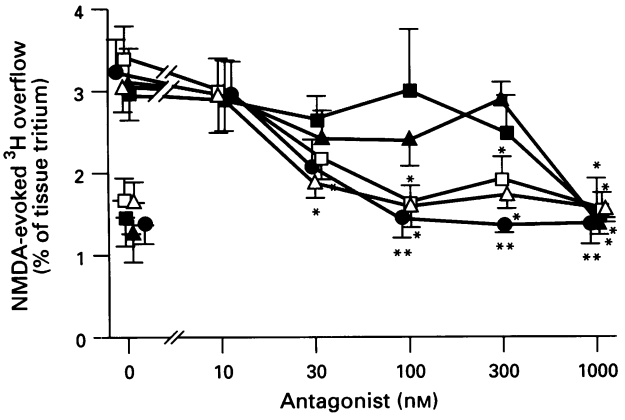

Figure 2 Concentration-response curves for the effects of (+)-pentazocine (100 nM) in the presence of haloperidol (□), BD-1063 (△), reduced haloperidol (■), BD-1008 (▲) and NE-100 (●) on the release of [^{3}H]-noradrenaline (NA) evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The σ ligands were added to the superfusion medium from the beginning of the superfusion. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); $n=7-10$. $^*P<0.05$, $^{**}P<0.01$ compared to basal values (see legend to Figure 1); Student's t test with the Dunnett's correction for multiple comparisons compared to NMDA-evoked release in the absence of (+)-pentazocine (–□–, –△–, –■–, –▲– and –●– for haloperidol, BD-1063, reduced haloperidol [4-(4chlorophenyl)-α-4-fluorophenyl-4-hydroxy-1-piperidinebutanol], BD-1008 and NE-100, respectively).

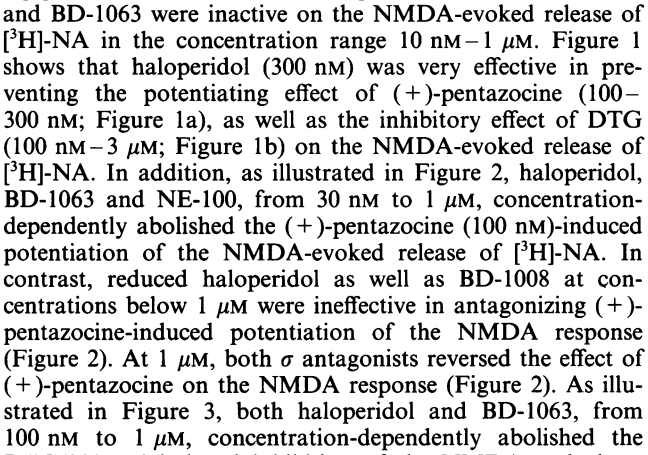

Figure 3 Concentration-response curves for the effects of 1,3-di(2-tolyl)guanidine (DTG; 300 nM) in the presence of haloperidol (□), BD-1063 (△), reduced haloperidol (■), BD-1008 (▲) and NE-100 (●) on the release of [^{3}H]-noradrenaline (NA) evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The σ ligands were added to the superfusion medium from the beginning of the superfusion. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); $n=6-8$. $^*P<0.05$, $^{**}P<0.01$ compared to basal values (see legend to Figure 1); Student's t test with the Dunnett's correction for multiple comparisons compared to NMDA-evoked release in the absence of DTG (–□–, –△–, –■–, – △– and –●– for haloperidol, BD-1063, reduced haloperidol, BD-1008 and NE-100, respectively).

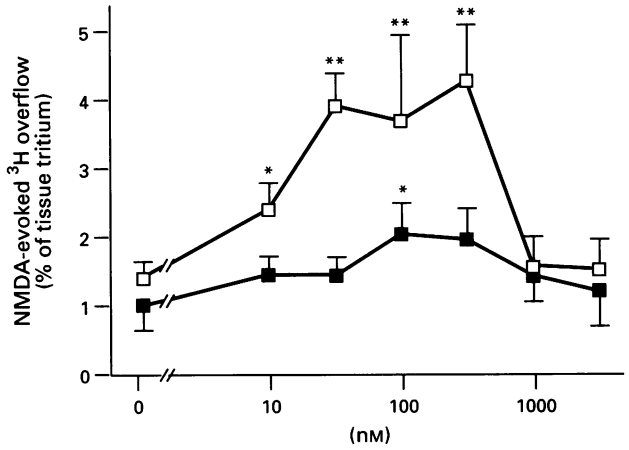

Figure 4 Dose-response curves for the effects of the stereoisomers BD-737 (□) and BD-738 (■) on the release of [^{3}H]-noradrenaline (NA) evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The σ ligands were added to the superfusion medium from the beginning of the superfusion. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); $n=6-8$. $^*P<0.05$, $^{**}P<0.01$ compared to basal values (see legend to Figure 1); Student's t test with the Dunnett's correction for multiple comparisons compared to NMDA-evoked release in the absence of σ drug.

Effects of haloperidol and reduced haloperidol, BD-1008, BD-1063 and NE-100 as well as spiperone on the modulation of the NMDA-evoked release of [^{3}H]-NA by BD-737

When superfused with BD-737 (30 nM – 300 nM), haloperidol decreased the effect of BD-737 by 50% at most (Figures 5 and 6). Similarly, the specific high affinity σ ligand BD-1063 (de Costa *et al.*, 1993), at concentrations ranging from 100 nM to 1 μM, reduced the potentiating effect of BD-737 by ⩽60% (Figures 5 and 6) and shifted the response curve of BD-737 to the right (Figure 5). Reduced haloperidol, BD-1008 and NE-100, which all exhibit selectivity and high affinity for σ sites (de Costa *et al.*, 1992; Bowen *et al.*, 1990; 1992; Okuyama *et al.*, 1993; Klein *et al.*, 1994) concentration-dependently (10 nM to 1 μM) antagonised the BD-737(100 nM)-induced effect (Figure 6).

Spiperone, which is devoid of affinity for σ_1 and σ_2 receptors (see Walker *et al.*, 1990) but is active on putative σ_3 sites (Booth *et al.*, 1993; Myers *et al.*, 1994) did not affect either the NMDA-evoked release of [^{3}H]-NA or antagonize the BD-737-induced potentiation of NMDA-evoked release of [^{3}H]-NA (Figure 5).

Discussion

The effects of the high affinity σ ligands (+)-pentazocine, DTG, BD-737, BD-738, haloperidol, reduced haloperidol, BD-1008, BD-1063 and NE-100 and of spiperone alone or in combination were assessed on NMDA-evoked release of [^{3}H]-NA from preloaded rat hippocampal slices. The effect of NMDA (200 μM for 4 min) on [^{3}H]-NA release was in accordance with results previously obtained in rat brain slices (Fink *et al.*, 1989; 1990a, b; Göthert & Fink, 1989; Hoehn & White, 1990; Roman *et al.*, 1991; Monnet *et al.*, 1992a; 1995; Fink & Göthert, 1992; Malva *et al.*, 1994).

The potentiation by BD-737 and (+)-pentazocine obtained in the present work is consistent with previous observations

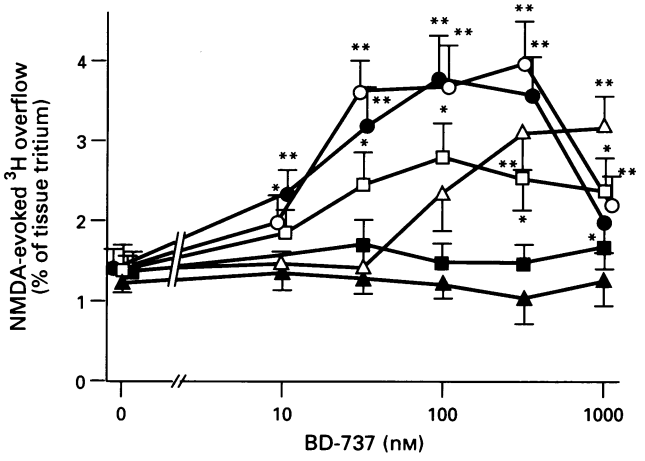

Figure 5 Concentration-response curves for the effects of BD-737 (○) in the presence of haloperidol (100 nM, □), BD-1063 (100 nM, △), reduced haloperidol (100 nM, ■), BD-1008 (100 nM, ▲) and spiperone (100 nM, ●) on the release of [^{3}H]-noradrenaline (NA) evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The drugs were added to the superfusion medium from the beginning of the superfusion. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); $n=7-10$. $^*P<0.05$, $^{**}P<0.01$ compared to basal values (see legend to Figure 1); Student's t test with the Dunnett's correction for multiple comparisons compared to NMDA-evoked release in the absence of σ drug.

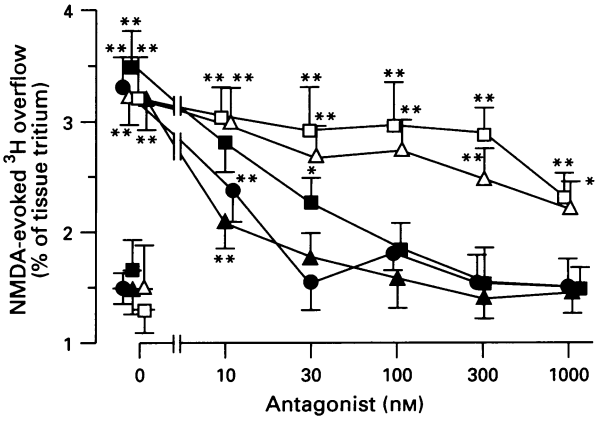

Figure 6 Concentration-response curves of the effects of BD-737 (100 nM) in the presence of haloperidol (□), BD-1063 (△), reduced haloperidol (■), BD-1008 (▲) and NE-100 (●) on the release of [^{3}H]-NA evoked by N-methyl-D-aspartate (NMDA) in rat hippocampal slices. The σ ligands were added to the superfusion medium from the beginning of the superfusion. Each point represents the mean (vertical lines show s.e.mean) of the percentage of the release of [^{3}H]-NA over spontaneous efflux evoked by 4 min exposure to NMDA (200 μM); $n=7-10$. $^*P<0.05$, $^{**}P<0.01$ compared with basal values (see legend to Figure 1); Student's t test with the Dunnett's correction for multiple comparisons compared with NMDA-evoked release in the absence of the drug (–□–, –△–, –■–, – ▲– and –●– for haloperidol, BD-1063, reduced haloperidol, BD-1008 and NE-100, respectively).

from *in vivo* unitary extracellular recordings of rat CA_3 hippocampal pyramidal neurones and microiontophoresis (Monnet *et al.*, 1992b; Bergeron *et al.*, 1995). The biphasic effect of σ agonists on NMDA-evoked release of [^{3}H]-NA has already been shown (i) *in vitro*, in rat hippocampal slices on both NMDA-evoked release of [^{3}H]-NA and of [^{3}H]-acetylcholine (Roman *et al.*, 1991; Monnet *et al.*, 1992a) as well as in a model of NMDA-dependent long-term potentiation (Martin *et al.*, 1992); (ii) *in vivo* in unitary extracellular recordings of rat CA_3 hippocampal pyramidal neurones and microiontophoresis (Monnet *et al.*, 1992b; Bergeron *et al.*, 1993; 1995), and in dizocilpine(MK-801)-induced amnesia and memory impairment in mice (Maurice *et al.*, 1994a, b). Although a satisfactory explanation for this phenomenon is lacking, Bergeron *et al.* (1995) have proposed that such a bell-shaped pattern of action of the σ drugs probably corresponds to the effects of these compounds on different σ receptor subtypes rather than σ receptor desensitization.

The blockade of both the (+)-pentazocine- and DTG-induced effects on the NMDA response by nanomolar concentrations of haloperidol or BD-1063 is consistent with previous findings both *in vivo* and *in vitro*, which have already led to these former drugs being considered to be potent σ_1 and σ_2 receptor antagonists (Monnet *et al.*, 1990; 1992a, b; 1994; 1995; Roman *et al.*, 1991; Quirion *et al.*, 1992; Bergeron *et al.*, 1993; Gonzales-Alvear & Werling, 1995). Furthermore, BD-1063 has been shown to block the motor effects of σ ligands when microinjected into the rat red nucleus (Matsumoto *et al.*, 1995). Surprisingly, haloperidol and BD-1063 superfused even at micromolar concentrations only weakly and partially reduced BD-737-induced potentiation of the NMDA-evoked release of [^{3}H]-NA. The relative insensitivity of σ agonist-induced modulation of the NMDA response to haloperidol has been suggested previously *in vivo* (Monnet *et al.*, 1994) and *in vitro* (Gonzales-Alvear & Werling, 1995), and is in agreement with our present results which clearly indicate that BD-737 potentiates the NMDA response via a σ-like receptor weakly sensitive to haloperidol.

Reduced haloperidol [4-(4-chlorophenyl)-α-4-fluorophenyl)-4-hydroxy-1-piperidinebutanol] has been shown to bind preferentially to σ sites with an affinity 2 to 3 orders of magnitude higher than its affinity for dopamine receptors (Bowen *et al.*,

1990; Jaen *et al.*, 1993; Klein *et al.*, 1994). Bowen *et al.* (1995) have observed K_i values for reduced haloperidol of 10.6 nM at σ_1 sites (vs. [^{3}H]-(+)-pentazocine) and 31.5 nM at σ_2 sites (vs. [^{3}H]-DTG in the presence of dextrallorphan), showing that this ligand potently binds both σ_1 and σ_2 receptors (unpublished observations). In contrast to haloperidol, reduced haloperidol was only partially effective in antagonizing both (+)-pentazocine- and DTG-induced modulations of the NMDA response and only at high concentrations. On the other hand, reduced haloperidol (at concentrations $\geqslant$100 nM) fully antagonized BD-737(100 nM)-induced potentiation of the NMDA-evoked release of [^{3}H]-NA (Figure 6). Thus, despite the high affinity interaction of haloperidol and reduced haloperidol with both σ_1 and σ_2 receptors, these two compounds appear able to differentiate effects mediated by BD-737 compared to (+)-pentazocine and DTG. This suggests that atypical σ sites may also contribute to NMDA modulation by σ ligands. This observation brings further support to the proposal by Jaen *et al.* (1993) that reduced haloperidol interferes with a receptor weakly sensitive to haloperidol, i.e. a σ receptor distinct from the σ_1 and σ_2 receptors.

BD-1008 is considered to be a selective and high affinity ligand for both σ_1 and σ_2 sites (de Costa *et al.*, 1992; He *et al.*, 1993). In the present experiments, it produced no effect on NMDA-evoked release of [^{3}H]-NA but partially inhibited the modulation by (+)-pentazocine and DTG. This is in agreement with the weak antagonistic activity of BD-1008 (100 nM) on (+)-pentazocine(10 μM)-induced modulation of NMDA-evoked [^{3}H]-dopamine release from rat striatal slices (Gonzales-Alvear & Werling, 1994). However, BD-1008 concentration-dependently antagonized (at concentrations $\geqslant$30 nM) BD-737(100 nM)-induced potentiation of NMDA-evoked release of [^{3}H]-NA. This provides additional support for the notion that both BD-737 and BD-1008 act on an atypical σ receptor subtype distinct from σ_1 and σ_2 sites.

NE-100 is another high affinity and specific σ ligand (Okuyama *et al.*, 1993; Chaki *et al.*, 1994). Since by itself, NE-100 has been shown to be devoid of effect in conditioned avoidance response in rats, head-weaving behaviour in dogs, attention capacity in monkeys but dose-dependently suppresses the effects of phencyclidine in the latter models, Okuyama *et al.* (1994) proposed that it behaves as a potent σ_1 antagonist. Interestingly, BD-1008 and NE-100 have been shown to act similarly and equipotently as σ antagonists in both the NMDA-evoked release of [^{3}H]-dopamine from striatal slices (Gonzales-Alvear & Werling, 1994) and the head-weaving behaviour produced by the σ agonist (+)-SKF-10047 (Okuyama *et al.*, 1993; 1994). In our model, NE-100 (>30 nM) antagonized BD-737(100 nM)-induced potentiation of NMDA-evoked release of [^{3}H]-NA (Figure 6), whereas it failed to reverse, even at micromolar concentrations, DTG-induced inhibition of the NMDA-evoked release of [^{3}H]-NA (Figure 3). This suggests that NE-100 has a similar pharmacological profile as reduced haloperidol and BD-1008 in this effect and therefore is also acting on a non σ_1-non σ_2 receptor.

Altogether, our data support the conclusion that BD-737 is an agonist whereas reduced haloperidol, BD-1008 and NE-100 are antagonists of an atypical, non σ_1-non σ_2 receptor which is insensitive or weakly sensitive to haloperidol but sensitive to reduced haloperidol. The stereoselectivity of ligands binding to σ receptors has been considered as a prominent pharmacological characteristic (see Quirion *et al.*, 1992). Therefore, we investigated the effects of the (+)-enantiomer [BD-737] and the (−)-enantiomer [BD-738] of *cis*-*N*-methyl-*N*-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl) cyclohexylamine. The higher potency of BD-737 compared to that of BD-738 (Figure 4) suggests that the atypical σ receptor on which both drugs act is preferentially activated by dextrorotary isomers of this class of compounds.

As outlined above, two additional σ-like sites have been proposed. Booth *et al.* (1993) and Myers *et al.* (1994) have recently proposed that σ_3 receptors exert a powerful control on the tyrosine hydroxylase activity and dopamine synthesis in rodent striatum. A series of 1-phenyl-3-amino-1,2,3,4-tetrahydronaphtalenes (1-phenyl-3-aminotetralins, PATs), haloperidol and spiperone bind with nanomolar affinity to these receptors, which are much less sensitive to DTG and (+)-pentazocine. The affinities of reduced haloperidol and the selective σ ligands utilised in the present study (BD-737, BD-738, BD-1008, BD-1063 and NE-100) at the putative σ_3 receptor site are not yet known. Naturally, spiperone also binds to the D_2 dopamine receptor with very high affinity. However, the observation that spiperone is totally inactive by itself on the NMDA-evoked release of [^{3}H]-NA and fails to block the modulatory effect of BD-737 on the NMDA-evoked release of [^{3}H]-NA, coupled with the relatively weak effect of haloperidol on the BD-737 response (Monnet *et al.*, 1992a, Figure 5) argues against the involvement of the putative σ_3 receptor subtype in the present *in vitro* model. Another possible candidate for the sites mediating these effects is the binding site recently described by Bowen *et al.* (1995). This site was characterized in guinea-pig brain and C_6 glioma cells with [^{125}I]-*N*-[2-(4-iodophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine ([^{125}I]-SH-344), an arylethylene diamine similar in structure to BD-1008. Arylethylene diamine-related compounds such as BD-737, BD-1008 and BD-1063 bind this site with high affinity (K_i values generally <100 nM). It exhibits low affinity for haloperidol (2−3 μM), but moderate affinity for reduced haloperidol and NE-100 (K_i values $\cong$400 and 860 nM, respectively). It also has low to negligible affinity for DTG and (+)-pentazocine (K_i values = 3−9 μM). Thus, this ligand selectivity pattern would be consistent with the observations made in the current study, whereby BD-737 acts at a site which is not affected by (+)-pentazocine or DTG and which has a greater sensitivity to blockade by reduced haloperidol compared to haloperidol.

Further studies will be needed to determine conclusively the nature of the site or sites mediating these effects of σ ligands on the NMDA responses described here. However, the present *in vitro* experiments provide further support to the notion that several types of σ receptors exist in the mammalian CNS, and functional evidence for the existence of an atypical σ receptor subtype. They also indicate that DTG and (+)-pentazocine modulate the NMDA response essentially through haloperidol-sensitive σ receptors, whereas BD-737 potentiates the NMDA response via a haloperidol-weakly sensitive but reduced haloperidol-sensitive σ receptor.

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