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Subchronic administration of *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) alters σ_1 receptor binding

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Abstract

BD1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine) is known to bind with high affinity and selectivity to σ sites in vitro. In prior in vivo studies, it has been shown to attenuate the dystonic postures and orofacial dyskinesias that are produced by σ receptor ligands, including the neuroleptic haloperidol. Since abnormal movements, such as dystonic postures and orofacial dyskinesias, are side effects that are associated with many σ-active neuroleptics, compounds such as BD1047 may have therapeutic potential for preventing and treating these unwanted movements. A possible limitation to the therapeutic potential of BD1047, however, is that at least in cell culture and albeit weak, it can be cytotoxic. Therefore, the present study analyzed the possible neurotoxic effects of in vivo subchronic intracerebroventricular infusion of BD1047 (10 nmol/h) or artificial cerebrospinal fluid (CSF) into rat brains using osmotic minipumps for 7 or 14 days. Following a 24 h wash-out period, the animals were killed, the brains removed, and P_2 membranes prepared. Membranes from rats treated for 7 or 14 days with BD1047 showed a marked decrease in [^{3}H](+)-pentazocine binding as compared to membranes from CSF-treated animals, suggesting a loss of σ_1 receptor binding. Histological examination of brain sections processed for Nissl stains and glial fibrillary acidic protein (GFAP) immunohistochemistry excluded the possibility of a cytotoxically induced down-regulation, suggesting possible receptor internalization or desensitization mediated via σ_1 sites. Under the conditions used in our study, BD1047 does not appear to be neurotoxic, and the data, when taken together with other studies, suggest that BD1047 acts as a partial agonist at σ sites.

Keywords: σ Receptor; Cytotoxicity; Regulation; Internalization; (+)-Pentazocine; Desensitization

1. Introduction

σ Receptors were first postulated by Martin et al. (1976) based on the actions of SKF 10,047 and related benzomorphans. Since then, binding studies have demonstrated that σ sites are distinct from opiate, phencyclidine, and dopamine receptors (Su, 1982; Tam and Cook, 1984; cf., Walker et al., 1990). These unique receptors have been shown to bind drugs from various pharmacological classes including neuroleptics (Largent et al., 1988; Tam and Cook, 1984), cytochrome P-450 inhibitors (Klein et al., 1991; Ross, 1991), antidepressants (Largent et al., 1987; Rao et al., 1990; Schmidt et al., 1989), monoamine oxidase inhibitors (Itzhak et al., 1991a,b) and steroids (Su et al., 1988).

Numerous lines of evidence demonstrate a role for σ sites in the control of movement and posture, raising the possibility that they might be involved in some naturally occurring and drug-induced movement disorders (Gold-

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stein et al., 1989; Matsumoto et al., 1990; Walker et al., 1988, 1993). Although initially, almost all σ receptor ligands tested had the ability to induce changes in movement and posture, in more recent studies, the novel aryl ethylene diamine, BD1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine), which binds with high and preferential affinity to σ sites, has been shown to dose-dependently attenuate dystonic postures (Matsumoto et al., 1995) and to decrease orofacial dyskinesias (vacuous chewing and facial tremors) in rats (unpublished observations). The ability of BD1047 to attenuate abnormal movements and postures in rats suggests that it may have future therapeutic potential in humans.

However, a recent study demonstrated that many σ receptor ligands can have cytotoxic effects in cell culture (Vilner et al., 1995). σ Ligands from a wide range of chemical classes cause time- and concentration-dependent alterations in morphology and viability in a number of neuronal and non-neuronal cell lines (Vilner et al., 1995). Although the precise mechanisms underlying this cytotoxicity have yet to be determined, the potencies of σ receptor ligands in producing these changes are known to be roughly correlated with their binding affinities for [^{3}H](+)-pentazocine-labelled sites in C6 glioma cell membranes (Vilner et al., 1995). Somewhat at odds with this report, however, are studies in which σ receptor ligands have neuroprotective effects in in vivo models of neurodegeneration (Contreras et al., 1991; Long et al., 1990; Pontecorvo et al., 1991). Although BD1047 was not tested in these in vivo models, in the in vitro model, it had weak cytotoxic properties (Vilner et al., 1995).

Therefore, in light of the potential therapeutic usefulness of BD1047, the purpose of the present study was to determine whether subchronic administration of BD1047 has cytotoxic effects in vivo. Rats were treated subchronically with BD1047 via osmotic minipumps. Subsequently, brain homogenates or histological sections were prepared and analyzed for σ receptor binding and neuronal loss.

2. Materials and methods

2.1. Drugs

BD1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine) was synthesized as described elsewhere (De Costa et al., 1992). Artificial cerebrospinal fluid (CSF) was made in-house in pyrogen-free sterile water and had the following final ion concentrations (in mM): Na^+ 150, K^+ 3.0, Ca^{2+} 1.4, Mg^{2+} 0.8, PO_4^- 1.0, and Cl^- 155. [^{3}H](+)-Pentazocine (38.3 Ci/mmol) was purchased from Dupont/New England Nuclear (Boston, MA, USA). (+)-Pentazocine was obtained through the National Institute on Drug Abuse Drug Supply System (Rockville, MD, USA). Haloperidol was purchased from Sigma (St. Louis, MO, USA).

2.2. Subchronic intracerebroventricular drug administration

2.2.1. Animals and drug treatment

Male, Sprague-Dawley rats (250–275 g) were purchased from Zivic-Miller (Zelienople, PA, USA). All animal care procedures followed those approved by the University of California Irvine Institutional Animal Care and Use Committee.

Rats were treated for either 7 or 14 days. During this treatment period, each rat received continuous, intracerebroventricular infusion (0.5 μl/h) of either artificial CSF or BD1047 (10 nmol/h) via osmotic minipumps/brain infusion kits (Alza, Palo Alto, CA, USA). Prior to implantation, the minipumps and brain infusion cannulae were filled and weighed to assure correct fill volumes. The minipumps were then immersed in 0.9% sterile saline at 37°C for at least 4 h to equalize the flow rate of the pump at the time of implantation.

2.2.2. Surgical procedure

Animals were anesthetized with 50 mg/kg Nembutal and affixed to a stereotaxic apparatus. After sterile preparation of the head, a 1 cm sagittal incision was made exposing bregma. Brain infusion cannulae were then implanted directly into the left lateral ventricle (1 mm posterior, 4.3 mm ventral, and 1.5 mm lateral, with reference to bregma and the skull surface) and secured to four stainless-steel skull screws with dental acrylic. A subcutaneous, pump-sized pocket was made in the midscapular area of the back, and the osmotic minipumps were inserted. The incisions were then ligated and the animals allowed to recover.

After the specified 7- or 14-day treatment period, the minipumps were removed. For the removal of the pumps, the animals were placed in an induction chamber filled with metofane vapors until they were sedated and anesthetized. A small incision was then made in each rat, the pumps removed, and the incisions closed.

2.3. Ligand binding assays

2.3.1. Membrane preparation

Twenty-four hours after the minipumps were removed, the rats were killed by decapitation and the brains removed. The 24 h wash-out was chosen based on our previous work with BD1047 and reports in the literature describing use of this procedure with other drugs. It was anticipated that with this wash-out period, BD1047 would no longer be bound to the receptor, but that persistent changes induced by subchronic exposure to it would still exist. Membrane preparations were made from groups of at least five pooled brains.

For the analysis of σ binding in whole brain, crude P_2 membrane fractions were prepared. Tissues were homogenized using a Potter-Elvehjem homogenizer and six strokes

of a motor-driven pestle in 10 ml ice-cold Tris-sucrose buffer (0.32 M sucrose in 10 mM Tris-HCl, pH 7.4) per gram wet tissue weight. The homogenates were centrifuged at 4°C at $1000 \times g$ for 10 min and the supernatants saved. The supernatants were then centrifuged at 4°C at $31\,000 \times g$ for 15 min. The pellets were resuspended in 3 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue and allowed to incubate for 30 min at 25°C. Following the incubation, the suspensions were centrifuged at 4°C at $31\,000 \times g$ for 15 min and the pellets resuspended in a final volume of 1.53 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue.

For the measurement of σ receptor binding in brain regions, whole brains were dissected into the following areas: cerebellum, striatum, hippocampus, cortex, diencephalon (minus striatum and hippocampus), mesencephalon (regions inclusive of and underlying colliculi), and myelencephalon. Like brain sections from at least five animals were pooled and crude membrane preparations were then prepared. Using a tissue tearor, the sections were homogenized in 10 ml ice-cold Tris-sucrose buffer

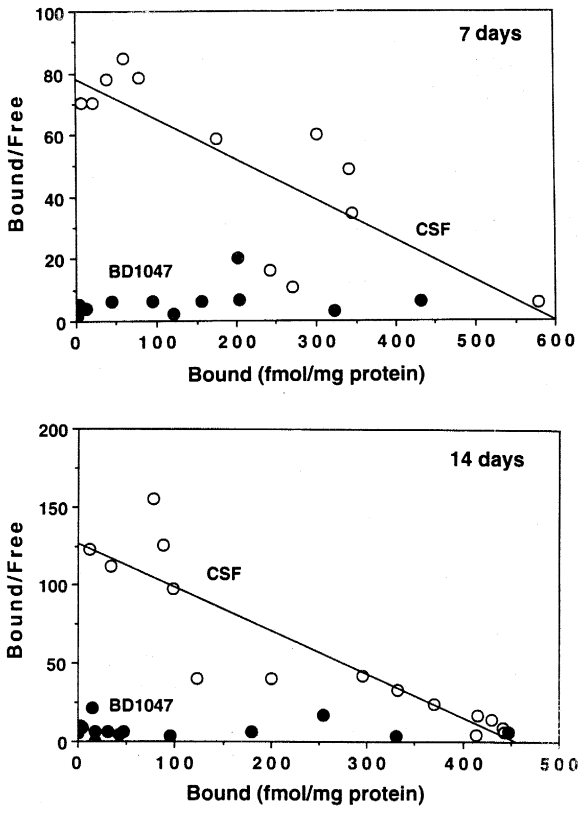

Fig. 1. [^{3}H](+)-Pentazocine binding in rat brain membranes after chronic treatment with CSF or BD1047 for 7 or 14 days. Rats were implanted with intracerebroventricular cannulae connected to osmotic minipumps containing either artificial CSF or BD1047 (10 nmol/h). The solutions were administered for 7 or 14 days at an infusion rate of 0.5 μl/h. The minipumps were then removed and after a 24 h wash-out period, the animals were killed by decapitation and P_2 membranes prepared. [^{3}H](+)-Pentazocine binding was virtually abolished after 7 (upper panel) or 14 (lower panel) days of BD1047.

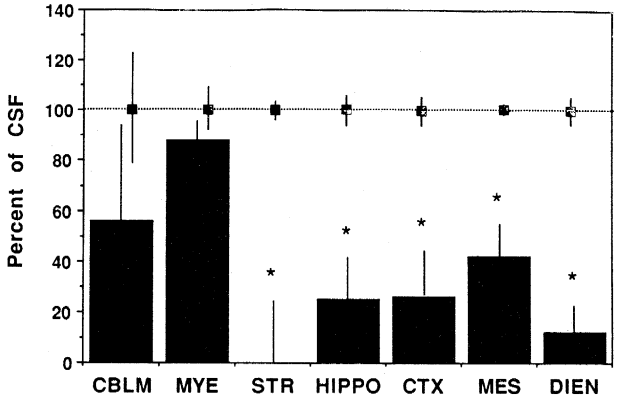

Fig. 2. Regional decreases in [^{3}H](+)-pentazocine binding (8 nM) in rat brain after administration of BD1047 vs. CSF for 7 days. BD1047 (10 nmol/h) or artificial CSF was administered intracerebroventricularly via osmotic minipumps (0.5 μl/h) to rats for 7 days. After a 24 h wash-out period, the animals were killed by decapitation and the brain dissected into the following regions: cerebellum (CBLM), myelencephalon (MYE), striatum (STR), hippocampus (HIPPO), cortex (CTX), mesencephalon (MES) and diencephalon (DIEN). 100% in this figure signifies no change from the level of binding measured in CSF-treated animals; the vertical lines along the 100% line indicate the S.E.M. from the actual bound values in that region of the brain in the CSF-treated animals. BD1047 induced a significant decrease in [^{3}H](+)-pentazocine binding in the striatum, hippocampus, cortex, mesencephalon and diencephalon (* $P < 0.05$).

(0.32 M sucrose in 10 mM Tris, pH 7.4) per gram wet tissue weight. The homogenates were centrifuged at 4°C at $31\,000 \times g$ for 15 min and the supernatants discarded. The pellets were resuspended in 3 ml Tris-sucrose buffer per gram tissue using the tissue tearor. Following a 15 min centrifugation at $31\,000 \times g$ at 4°C, the supernatants were discarded. The pellets were resuspended in 3 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue using the tissue tearor. After a 15 min incubation at 25°C, the suspensions were centrifuged at 4°C at $31\,000 \times g$ for 15 min and the pellets resuspended in a final volume of 1.53 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue.

For all of the membrane preparations, the final suspensions were hand homogenized with five strokes of a Teflon pestle and aliquots stored at −80°C until use. Protein content was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit and lyophilized bovine serum albumin standard (Hercules, CA, USA).

2.3.2. Sigma receptor assays

[^{3}H](+)-Pentazocine was used to label σ_1 sites because the cytotoxic effects of ligands in previous cell culture studies were roughly correlated with binding to this radioligand (Bowen et al., 1993; Vilner et al., 1995). Procedures were performed as described previously (Matsumoto et al., 1996). Briefly, each tube contained 500 μg membrane protein, 50 mM Tris-HCl, pH 8.0, and one of 14 concentrations of [^{3}H](+)-pentazocine (concentration range 0.3–100 nM). Non-specific binding was determined in the presence of 5 μM (+)-pentazocine or 10 μM

haloperidol. The total reaction volume in each tube was 500 μl and the tubes were run in duplicate. After a 60 min incubation at 25°C, the assays were terminated with 5 ml ice-cold 10 mM Tris-HCl, pH 8.0. The samples were vacuum filtered using a Brandel Cell Harvester through glass fiber filters (Schleicher & Schuell, Keene, NH, USA) that were pre-soaked for at least 30 min in 0.5% polyethyleneimine (Sigma). The filters were then washed twice with 5 ml ice-cold buffer. Filters were placed in Ecoscint cocktail (National Diagnostics, Manville, NJ, USA) and counts were extracted for at least 8 h before scintillation counting (Beckman LS 6500, Beckman Instruments, Fullerton, CA, USA).

To label σ_1 sites in specific brain regions, membranes from the brain regions were processed as described above for whole brain except that they were incubated with a single concentration of [^{3}H](+)-pentazocine (8 nM). This concentration was chosen because it approximated the K_d values obtained in full Scatchards.

2.3.3. Statistical analyses

Scatchard analysis was conducted using Prism (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was used to evaluate whether changes in B_{max} or K_d were statistically significant. Student's *t*-tests were used to compare binding, corrected for protein, from specific brain regions from rats treated subchronically with CSF vs. BD1047.

2.4. Histological analysis

Twenty-four hours after removal of the minipumps, the rats were perfused transcardially with saline and 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed and further fixed in a 30% sucrose-paraformaldehyde solution. Coronal sections (50 μm) were taken throughout the brain and alternate sections were processed for Nissl staining and glial fibrillary acidic protein (GFAP) immunohistochemical analyses.

For the GFAP immunohistochemical staining, the sections were processed in a free-floating state. The sections were first washed two times with PBS and then treated with normal goat serum (dilution 1:65) in PBS containing 0.1% Triton X-100 for 60 min. Then, the sections were incubated overnight with polyclonal anti-rabbit GFAP antibody (Chemicon International, Temecula, CA, USA, dilution 1:50). After 3 × 10 min washes with PBS, sections were incubated with anti-rabbit biotinylated secondary an-

Table 1
Extent of neuronal necrosis and glial proliferation after chronic exposure to CSF or BD1047

	7 days		14 days	
	CSF	BD1047	CSF	BD1047
Forebrain				
Olfactory tubercle	N	0	0	0
Ant. olfactory n.	N	N+	0	N
Cortex				
Frontal	N	N++	0	N
Parietal	N	N++	0	N
Cingulate	N	N++	0	N
Piriform	N	N++	0	N
Insular	N	N++	0	N
Entorhinal	N	#	N	N
Occipital	N++	N++	N	N
Temporal	N++	N++	N	N
Thalamus	0	0	0	0
Caudate-putamen	N	0	0	N
Globus pallidus	N	0	0	N
Basal n. Meynert	N	0	0	0
Claustrum	N	N+	0	N
Ventral diagonal band	N	N	++	N
Lateral septal n.	N	N	N	N
Medial septal n.	N	N	++	N
Triangular septal n.	N	0	#	N
Septofimbrial n.	N	0	#	N
Med. preoptic n.	0	0	N	N
Accumbens	N	0	0	0
Arcuate	N	++	0	N
Habenula	N	++	0	N
Amygdala	N	++	0	0
Ant. hypothalamus	0	0	N	0
Hypothalamus	N	0	0	N
Hippocampus	N	N+	N	N
Midbrain				
Central gray	0	0	0	0
Red n.	0	0	0	
Oculomotor (III)	0	0	0	0
Substantia nigra	N++	N++	0	0
Interpeduncular	N	0	0	0
Geniculate, med.	N	N++	0	0
Superior colliculus	N	0	N	N
Subiculum	N	N	N	N
Inferior colliculus	N	N	0	+
Hindbrain				
Raphe	0	N	0	+
Pontine n.		++	N	0
Deep cerebellar nuclei	N	N	N	N
Paraflocculus	N	0	0	N
Olive groups	#	#	N	0
Motor trigeminal (V)	#	#	N	0
Vestibular n.	N	0	0	N
Facial n. (VII)	++	N	0	N+
Spinal trigeminal, oralis	N	0	0	N

Notes to Table 1:
Rats received continuous, intracerebroventricular administration of CSF or BD1047 (10 nmol/h) for 7 or 14 days via osmotic minipumps (0.5 μl/h). After a wash-out period of 24 h, the rats were killed and the brains removed. Brain sections (50 μm) were subsequently processed with cresyl violet or GFAP antibody. The resulting histology was scored as follows: 0, no glial cells present; N, normal glial cells present; +, normal soma with thin processes; ++, disfigured soma with decrease in size and number of processes, some broken processes; +++, significant damage to soma, broken processes; ++++, loss of soma, destruction of processes and clustering of glial cells; and #, sections missing or unsuitable for analysis.

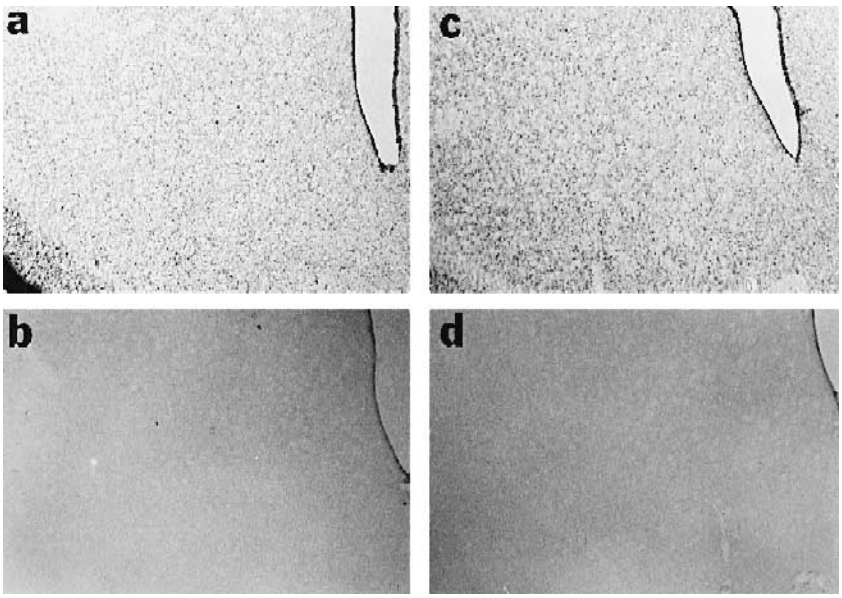

Fig. 3. Nissl and GFAP staining of rat striatum after chronic administration of CSF or BD1047 for 7 days. The solutions were administered under the same conditions as in the binding studies except that instead of preparing brain membranes at the end of the treatment period, the animals were perfused and coronal sections of brain were sliced. Representative sections stained with cresyl violet (a) or GFAP (b) from a rat treated with CSF are shown. There was no significant neuronal loss or glial proliferation in rats exposed to BD1047 as can be seen in striata stained with cresyl violet (c) and GFAP (d). Although not shown, the histology from rats treated for 14 days was virtually identical to these examples.

tibody (Elite kit, Vector Laboratories, Burlingame, CA, USA) for 60 min. Following 5×10 min rinses, sections were treated with avidin-biotin complex (Vector Laboratories) for 2 h. Sections were washed 3×10 min with PBS, then processed for diaminobenzidine reactions. The reactions were terminated after attaining the desired staining intensity, which usually took 3–5 min. Histochemical controls were performed by omitting the primary antibody, and by replacing it with normal rabbit immunoglobulin (IgG). Alternate brain sections were stained with cresyl violet using standard Nissl staining procedures.

Forty-six regions of the brain were examined for neu-

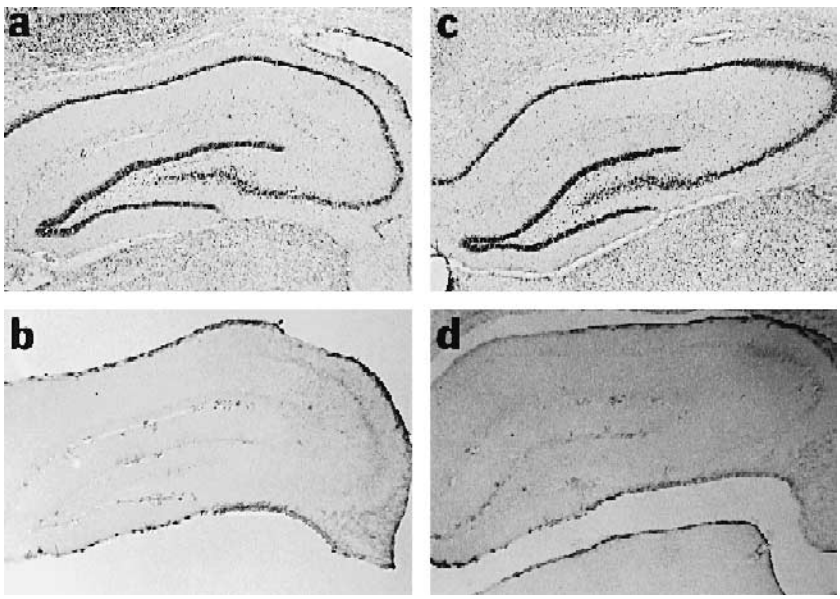

Fig. 4. Nissl and GFAP staining of rat hippocampus after chronic administration of CSF or BD1047 for 7 days. The solutions were administered under the same conditions as in the binding studies except that instead of preparing brain membranes at the end of the treatment period, the animals were perfused and coronal sections of brain were sliced. Representative sections stained with cresyl violet (a) or GFAP (b) from a rat treated with CSF are shown. Hippocampal sections stained with cresyl violet (c) and GFAP (d) from rats exposed to BD1047 can be seen. There was a variable amount of GFAP staining among both CSF- and BD1047-treated animals. The portions of the dorsal hippocampus most likely to be affected, regardless of treatment condition, included the dentate gyrus, hippocampal fissure and CA2 region.

ronal loss and glial proliferation. The histology was scored as follows: 0, no glial cells present; N, normal glial cells present; +, normal soma with thin processes; ++, disfigured soma with decrease in size and number of processes, some broken processes; +++, significant damage to soma, broken processes; ++++, loss of soma, destruction of processes and clustering of glial cells.

3. Results

3.1. Binding assays

3.1.1. Whole brain

Fig. 1 illustrates the marked decrease in [^{3}H](+)-pentazocine binding in rat brain after subchronic administration of BD1047 for 7 or 14 days. The level of binding in the BD1047-treated animals was too low for a Scatchard transformation. The binding parameters for CSF-treated rats were as follows: 7 days $K_d = 8.43 \pm 4.00$ nM, $B_{max} = 689.75 \pm 409.71$ fmol/mg protein; 14 days $K_d = 4.16 \pm 1.03$ nM, $B_{max} = 503.42 \pm 64.69$ fmol/mg protein. The binding parameters from normal, untreated animals that were obtained in separate experiments ($K_d = 11.53 \pm 1.35$ nM, $B_{max} = 482.58 \pm 189.05$ fmol/mg protein) did not differ significantly from the CSF-treated rats (K_d $F(2,7) = 3.32$, $P = 0.10$; B_{max} $F(2,7) = 0.16$, $P = 0.85$).

3.1.2. Brain regions

Fig. 2 summarizes the changes in [^{3}H](+)-pentazocine binding in different brain regions of rats after continuous, intracerebroventricular infusion of BD1047 for 7 days. As compared to CSF-treated rats, there was a significant decrease in [^{3}H](+)-pentazocine binding in the BD1047-treated animals in the following brain regions: striatum ($t = 2.53$, $P < 0.05$), hippocampus ($t = 3.41$, $P < 0.02$), cortex ($t = 3.44$, $P < 0.02$), diencephalon ($t = 5.36$, $P < 0.01$) and mesencephalon ($t = 3.95$, $P < 0.01$). There was no significant change in the level of binding in the cerebellum and myelencephalon.

3.2. Histology

The results from the histological analyses are summarized in Table 1. Since [^{3}H](+)-pentazocine binding was dramatically reduced in the striatum and hippocampus and these areas of the brain are amenable to histopathological analysis, representative histology from these areas of the brain is shown in Figs. 3 and 4. Overall, there was very little pathology after subchronic administration of BD1047 for 7 or 14 days. The only exceptions were the slight, but noticeable, changes in the cortex and certain limbic areas (arcuate, habenula, amygdala) after 7 days of subchronic treatment with BD1047. However, these alterations appear to be transient because after 14 days of treatment, the neurons once again appeared normal.

4. Discussion

The stability of BD1047 in the minipumps, although not measured directly in this study, is thought to be quite good. Structurally, BD1047 itself is quite stable. In our experiments, BD1047 was diluted in a pyrogen-free medium and loaded into the minipumps under sterile conditions. Therefore, no enzymes or solvents were present in the system which could have affected the drug. Since the minipumps are osmotic in nature, with continuous flow of drug out of the reservoir, it is unlikely that things could have entered the pump once it was implanted. Furthermore, others have previously demonstrated that many other drugs, including peptides, are stable in this system. In previous control experiments for cell culture studies using σ receptor ligands that are structurally related to BD1047, 21 days in unchanged culture medium at 37°C resulted in a loss of potency of the compounds in receptor binding studies, as compared to freshly prepared drugs (W.D. Bowen, personal communication). Therefore, if there was loss of BD1047 by degradation, an underestimation of its potency would be expected, but not a qualitative change in the results. However, given the magnitude of the observed changes in the receptor binding portions of the study, it appears that during the time frame of our experiments (7 or 14 days), sufficient quantities of BD1047 remained in the minipumps to produce physiologically relevant effects.

Intracerebroventricular infusion of BD1047 for 7 or 14 days caused a dramatic reduction in [^{3}H](+)-pentazocine binding in membranes prepared from the whole brain of rats. When a single concentration of [^{3}H](+)-pentazocine was used to label σ_1 sites in discrete brain regions, so that a more precise anatomical localization of the regulation could be determined, the data revealed a significant loss of binding in the striatum, hippocampus, cerebral cortex, mesencephalon, and diencephalon of rats treated with BD1047 as compared to those who had received CSF. Despite the virtual loss of [^{3}H](+)-pentazocine binding in many regions of the brain, histological examination of the tissue showed no significant loss of neurons or proliferation of glial cells, excluding the possibility of a cytotoxically induced down-regulation, and suggesting possible desensitization and/or internalization of σ_1 sites.

In terms of the regional differences in the regulation of [^{3}H](+)-pentazocine binding in the brain after subchronic exposure to BD1047, the reason for this region-specific pattern of regulation is unknown, but it cannot be explained by the diffusive properties of intraventricular infusion of BD1047 or the differential representation of different σ receptor subtypes in these various brain areas. After release into the lateral ventricle, BD1047 would be expected to travel caudally with the natural flow of CSF through the third and fourth ventricles, the foramina of Magendie and Luschka, into the subarachnoid space from whence it can flow over the surface of the brain (Rowland, 1985). The myelencephalon and cerebellum, which were

unaffected structures, are thus situated midway in the flow of CSF (i.e., after the midbrain, but before the cortex). Therefore, possible differences in the diffusion of BD1047 into various brain regions cannot account for the variation in the observed effects. Likewise, although regional differences in σ_1 and σ_2 receptor binding in rat brain exist (Leitner et al., 1994), this distribution cannot account for the observed variation in results. For example, in terms of the distribution of σ_1 sites, the hindbrain contains the highest density of sites while the cerebellum contains the lowest density, although both regions of the brain were unaffected after subchronic administration of BD1047. Similarly, although both the hindbrain and cerebellum contain high densities of σ_2 sites, the cortex is also enriched in this subtype, and unlike the hindbrain and cerebellum, the cortex experienced a dramatic loss of [^{3}H](+)-pentazocine binding after exposure to BD1047. One observation that may be relevant in terms of understanding this pattern is that some σ receptor ligands can have opposite actions in the hindbrain vs. forebrain. Haloperidol, for example, appears to act as a σ receptor antagonist in the hippocampus and other forebrain areas (Maurice and Privat, 1996; Monnet et al., 1992), whereas it has actions that are consistent with an agonist in the brainstem (Matsumoto et al., 1995; F.P. Monnet, personal communication). Therefore, the differential regulation of [^{3}H](+)-pentazocine sites, where there was a relative lack of regulation in brainstem areas vs. the loss of binding in other areas of the brain, may be tied in to these other observations.

Despite the fact that [^{3}H](+)-pentazocine binding was virtually abolished in most areas of the brain after subchronic administration of BD1047, histopathological analysis of coronal sections of the brain revealed no significant loss of neurons or glial proliferation in these affected areas. Overall, very few morphological changes were observed after subchronic intracerebroventricular infusion of BD1047. Even in the areas of the brain that were most severely affected, the histopathological abnormalities were extremely mild, and limited to some disfiguring of the soma and a possible reduction in the number and size of processes in select animals. These effects were transient and although no neuronal death was observed, it did appear that in at least some areas of the brain, the neurons were ill for a period of time. Further studies using more sensitive neuroanatomical markers and physiological endpoints are needed to determine whether the functions of these neurons are transiently compromised and whether these changes have physiological consequences for the behavior of the animals. In any event, it should be emphasized that these relatively minor histopathological changes contrast sharply with the dramatic loss of binding that is observed in the receptor binding studies. The histological analyses thus rule out the likelihood of a cytotoxically induced down-regulation and suggest the possibility that the receptors are being internalized (Salisbury et al., 1983; Schwartz, 1995) or desensitized (Creese and Snyder, 1980; Kurlan and Shoulson, 1982).

The phenomena of receptor internalization and desensitization have been reported in a number of neurochemical systems where they appear to represent the consequences of the actions of agonists (Creese and Snyder, 1980; Kurlan and Shoulson, 1982; Salisbury et al., 1983; Schwartz, 1995). Recently, internalization of σ receptors has also been reported in response to the actions of (+)-pentazocine (Yamamoto et al., 1995, 1996), providing further support that these phenomena are relevant for understanding σ receptor function. However, in our early microinjection studies, BD1047 appeared to act as an antagonist at σ receptors because it produced no effects on its own, but it had the ability to attenuate the actions of other σ receptor ligands, including DTG and haloperidol (Matsumoto et al., 1995). Since then, preliminary studies in systems which allow the testing of a wider dose range suggest that BD1047 acts as a partial agonist (W.D. Bowen, personal communication). For example, many σ receptor agonists (e.g., BD737, reduced haloperidol, CB-64D) attenuate KCl depolarization-induced calcium influx into cerebellar granule cells in a dose-dependent manner, giving maximal inhibition by 100 μM (Vilner and Bowen, 1995). In this assay system, BD1047 also inhibits KCl depolarization-induced calcium influx into cerebellar granule cells, a pattern that is similar to other σ receptor agonists. However, the response produced by BD1047 plateaus at about 50% inhibition by the high 100 μM dose. Furthermore, as would be expected of a partial agonist, BD1047 attenuates the responses produced by reduced haloperidol and CB-64D, agonists that possess higher intrinsic efficacies. Since partial agonists are defined as drugs that produce submaximal tissue responses and competitively block the effects of agonists of higher intrinsic efficacies (Kenakin, 1993), the behavior of BD1047 in this assay system is consistent with that of a classical partial agonist. A similar trend has been observed with BD1047 when looking at oxotremorine-stimulated phosphoinositide (PI) turnover, where the compound alone produces submaximal inhibition of the PI response, and also shows partial attenuation of (+)-pentazocine-induced inhibition of the PI response (W.D. Bowen, personal communication). Therefore, when taken as a whole, the ability of BD1047 to produce a mixture of competitive antagonist actions, submaximal agonist responses, and apparent internalization and/or desensitization of receptors, strongly suggests that the drug acts as a partial agonist at σ receptors.

In addition, it should be noted that in the original cell culture studies which reported the cytotoxic effects of BD1047, there is evidence that the drug might be acting as a partial agonist. Similar to other known σ receptor agonists, BD1047 produced some cytotoxicity (Vilner et al., 1995). However, the weak cytotoxic properties that were reported for BD1047 were in a range that was much weaker than would have been expected based on its σ

receptor binding affinity, suggesting that it might possess low intrinsic efficacy (Vilner et al., 1995).

Even when taking into account the possibility that as a partial agonist, BD1047 has weak intrinsic efficacy, the cytotoxic effects of the drug were even weaker under the in vivo conditions than they were in vitro. Based on examination of brain sections processed with Nissl stains and GFAP antibody, BD1047, in fact, appeared to produce no significant changes in neuronal viability or glial proliferation after subchronic in vivo administration (7 or 14 days) directly into the ventricles of the brain. The underlying reason(s) for this difference in vivo vs. in vitro is unknown at this time and could involve any number of factors since subchronic σ receptor ligand binding in vivo would be expected to induce multiple physiological processes, some of which may not exist in cell culture. Since neuroprotective effects of σ receptor ligands have been reported in vivo (Contreras et al., 1991; Long et al., 1990; Pontecorvo et al., 1991), while cytotoxicity is documented in vitro (Vilner et al., 1995), there appears to be additional, unidentified factors under the in vivo condition that provide protection from the cytotoxic mechanisms that are operative in cell culture.

In summary, subchronic intracerebroventricular administration of BD1047 produces a dramatic reduction in [^{3}H](+)-pentazocine binding. Histological examination of brains from rats treated subchronically with BD1047 excluded the possibility of a cytotoxically induced down-regulation, suggesting possible desensitization and/or internalization of σ_1 sites. Taken together with data on other physiological and behavioral effects produced BD1047, the ligand appears to act as a partial agonist at σ sites.

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