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Pharmacological characterization of BNMPA (α-benzyl-*N*-methylphenethylamine), an impurity of illicit methamphetamine synthesis

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Abstract

α-Benzyl-*N*-methylphenethylamine (BNMPA), an impurity of illicit methamphetamine synthesis, has previously been reported to produce convulsions in mice without affecting spontaneous locomotor activity or altering methamphetamine-induced increases in spontaneous activity. In this study the in vitro effects of BNMPA on a variety of neuronal receptor types was determined to better characterize the pharmacological actions of this novel compound. BNMPA and *N*-demethyl-BNMPA fully displaced the dopamine transporter selective ligand [^{3}H]CFT (2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane) from rat striatal membranes with K_i values (mean ± S.E.M) of 6.05 μM ± 0.15 and 8.73 μM ± 1.66, respectively. BNMPA also inhibited [^{3}H]dopamine uptake into striatal synaptosomes with an IC_{50} value of 5.1 ± 1.4 μM. The basal efflux of [^{3}H]dopamine from striatal slices was slightly enhanced by BNMPA only at concentrations ≥ 100 μM. BNMPA had no effect on [^{3}H]norepinephrine efflux from hippocampal slices. BNMPA displaced tritiated paroxetine and prazosin binding from rat cortical membranes with K_i values of 14.5 μM and 11.7 μM respectively. In electrophysiological studies, BNMPA (100 μM) had no significant effects on either $GABA_A$ Cl^- currents in cultured neurons or non-NMDA glutamate receptors expressed in oocytes. However, BNMPA significantly inhibited NMDA-stimulated currents in oocytes expressing the NR1/2A or NR1/2C receptor subunit combinations (IC_{50} values = 24.6 ± 1.8 and 24.0 ± 1.5 μM, respectively). This inhibition was rapid, reversible and voltage-dependent. These results indicate that BNMPA has multiple sites of action in the CNS that could be important in modulating a variety of behavioral effects upon exposure to this synthetic byproduct of illicit methamphetamine synthesis.

Keywords: BNMPA (α-benzyl-*N*-methylphenethylamine); Methamphetamine; Illicit synthesis; Synthetic impurity; NMDA receptor; Dopamine transporter

1. Introduction

Prior to the Controlled Substances Act in 1970, the world medical literature contained only 43 reports of deaths associated with amphetamines in a 35-year period (Kalant and Kalant, 1975). However, since 1980, as clandestinely manufactured compounds have become the primary source of amphetamine/methamphetamine, these compounds have consistently ranked among the 20 most frequently mentioned drugs in emergency room patients as well as medical examiner cases (Annual Emergency Room Data, 1991). Impurities of manufacture are numerous and are characteristic of a particular synthetic method. They have been extensively reviewed elsewhere (Van der Ark et al., 1978; Sinnema and Verweij, 1981; Verweij, 1989; Soine, 1989). These contaminants, of which α-benzyl-*N*-methylphenethylamine (BNMPA; see Fig. 1 for structure) is only one, may be contributing to the apparent increased toxicity of methamphetamine.

The behavioral effects of stimulants like amphetamine and cocaine on locomotor activity appear to result from their interaction with dopaminergic systems (Kuczenski, 1983; Robbins and Sahakian, 1983; Johanson and Fischman, 1989). Seizures induced by stimulants are generally associated with non-dopaminergic systems (Ritz and

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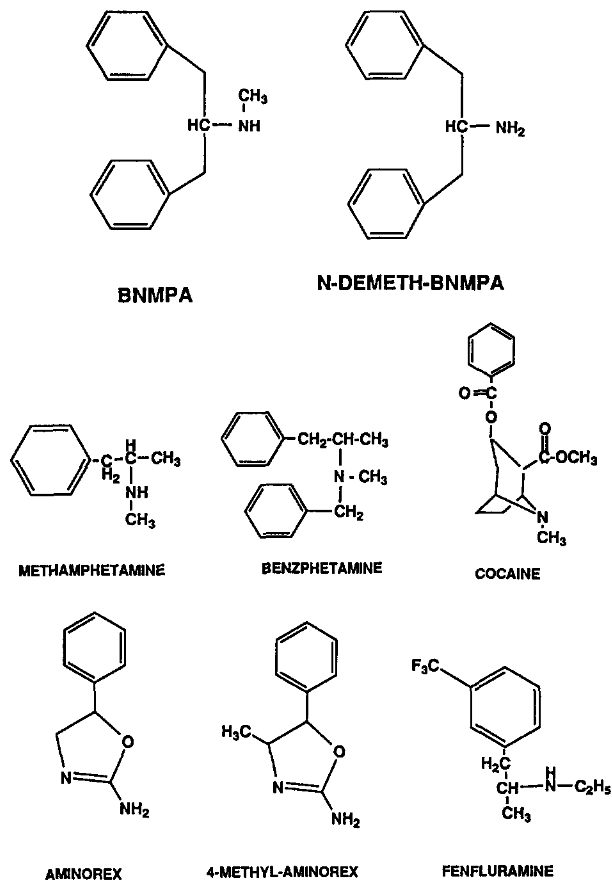

Fig. 1. Structural similarity of BNMPA and *N*-demethyl-BNMPA, to other stimulants/anorexiants.

George, 1993; Bloom, 1985). In a previous study, BNMPA failed to significantly affect locomotor activity or alter methamphetamine-induced increases in spontaneous activity suggesting that BNMPA does not behave as a direct or indirect dopaminergic agonist (Moore et al., 1995a). However, BNMPA produced spontaneous tonic-clonic convulsions at doses lower than those causing lethality (CD_{50} = 41 mg/kg; LD_{50} = 70 mg/kg) (Moore et al., 1995a). Noggle et al. (1985) also reported similar values for the seizure producing effects for BNMPA (CD_{50} = 54 mg/kg) and *N*-demethyl-BNMPA (CD_{50} = 45 mg/kg). In methamphetamine interaction studies, BNMPA appeared to have a small paradoxical neuroprotective effect against low-dose methamphetamine-induced convulsions, but not against high-dose methamphetamine-induced convulsions.

Based on these in vivo observations and BNMPA's structural similarity to benzphetamine, we hypothesized that BNMPA should have a relatively low affinity for dopaminergic systems involved in locomotor activity, while its seizure-producing effects (and paradoxical neuroprotective effect) may involve an interaction with $GABA_A$ receptors, glutamate/NMDA or serotonine receptors/transporters.

The effects of BNMPA on a variety of receptor systems was investigated in the present study in order to better understand the molecular and cellular actions of this compound. The results suggest rather surprisingly that BNMPA, in vitro, has modest affinity for the dopamine transporter and acts as a voltage-dependent blocker of the NMDA receptor.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–300 g) were obtained from Harlan (Dublin, Virginia). Water and food (Rodent Laboratory Chow, Ralston-Purina Co., St. Louis, MO) were available ad libitum. Animals were housed singly in standard cages (18 × 29 × 13 cm) with wood chip bedding in a controlled temperature room (22–24°C) with a 12-h light-dark cycle. All experimental protocols were approved for use by the university IACUC committee and conform to NIH guidelines for use of laboratory animals.

2.2. Chemicals and reagents

[^{3}H]CFT (2-β-carbomethoxy-3-β-(4-fluorophenyl)-tropane; 83.4 Ci/mmol), [^{3}H]paroxetine (16.6 Ci/mmol) and [^{3}H]prazosin (76 Ci/mmole) were obtained from New England Nuclear (Boston, MA). [^{3}H]dopamine and [^{3}H]norepinephrine were obtained from Amersham (Arlington Heights, IL). Cocaine hydrochloride (National Institute of Drug Abuse) and paroxetine hydrochloride hemihydrate (SmithKline Beecham Pharmaceutical) were generous gifts. BNMPA and *N*-demethyl-BNMPA were synthesized as described in a previous publication (Moore et al., 1995b). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

2.3. Dopamine / serotonin transporter assays

2.3.1. [^{3}H]CFT binding

CFT binding to striatal membranes was conducted using the method recently described by Woodward et al. (1995). Briefly, striatal tissue (minimum of 225 mg) was placed in 10 ml of ice-cold buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4). Tissue was homogenized using a Tissumizer (Tekmar Inc.) and centrifuged at 40 900 × *g* at 4°C for 20 min. The supernatant was decanted and 10 ml of buffer was added and the tissue was centrifuged again. After pouring off the supernatant, the tissue was brought up to its reaction volume (25 mg tissue/ml buffer) and introduced into the reaction immediately. Saturation binding assays were performed using increasing concentrations of [^{3}H]CFT (0.1–300 nM) Displacement assays were performed using 30 nM [^{3}H]CFT and increasing concentrations of the various drugs to be tested. Non-specific binding in both assays was determined in the presence of 30 μM (−)-cocaine. After a 2 h incubation at 0°C, tissue

binding was terminated by two rapid washes of ice-cold buffer onto Whatman GF/B filters using a Brandel 24-well cell harvestor. Filters were placed in scintillation vials containing 10 ml of Boise II scintillation cocktail and radioactivity was counted in a Beckman LS 6000 scintillation counter.

2.3.2. [^{3}H]Paroxetine binding

Paroxetine binding was conducted using a modified Habert et al. (1985) and Mann and Hrdina (1992) protocol. Briefly, rat cortical tissue was placed in 20 ml ice-cold buffer (50 mM Tris-HCl, 15 mM NaCl, 5 mM KCl, pH 7.4), homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at $40900 \times g$ at 4°C for 20 min. The supernatant was decanted and the pellet was re-homogenized in ice-cold buffer and centrifuged as before. Supernatant was again decanted, the tissue diluted to its reaction volume (40 mg tissue/ml buffer) and aliquotted into the reaction tubes immediately. Saturation binding was performed using increasing concentrations of [^{3}H]paroxetine (0.015–1.5 nM). Displacement assays were performed using 0.15 nM [^{3}H]paroxetine. Non-specific binding in both cases was determined in the presence of 100 nM paroxetine. After one h incubation at 22°C, tissue binding was terminated by the addition of 2 ml of ice-cold buffer and filtered onto a 0.05% PEI pretreated Whatman GF/B filter using a Brandel 48 well harvester (Gaithersburg, MD). Filters were placed in scintillation vials containing 10 ml Bio-Safe II® scinitillation cocktail (RPI; Mount Prospect, IL) and radioactivity quantitated on a Beckman LS 6000 scinitillation counter. Protein content of tissues was determined spectrophotometrically using the Bio-Rad dye technique for a modified Lowry's protein assay.

2.3.3. Dopamine uptake assay

Dopamine uptake by striatal synaptosomes was determined using the method described by Woodward et al. (1995). Briefly, pairs of striata were homogenized with ten up and down strokes (300 rpm) using a Thomas A teflon to glass homogenizer. The homogenized preparation was centrifuged at $3000 \times g$ for 10 min at 4°C and the supernatant was carefully decanted to a fresh tube. After centrifugation at $12000 \times g$ (4°C), the P2 pellet was resuspended in ice-cold incubation medium (136 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 10 mM glucose, 10 mM Tris, pH 7.65, at room temperature) to a protein concentration of approximately 1 mg/ml (Bradford assay, Bio-Rad Inc.). Calcium was omitted from the incubation medium to reduce release of endogenous dopamine. Synaptosomes were incubated for 10 min at 35°C prior to a 2-min exposure to a solution containing [^{3}H]dopamine (100 nM) and the various drugs to be tested. Uptake was stopped after 2 min by rapid filtration through Whatman GF/B filters followed by two washes with ice-cold incubation medium. Radioactivity remaining on the filters was quantitated by liquid scintillation counting. For each experiment, a control uptake was performed at 0°C to assess non-specific binding of radioisotope. This value averaged approximately 2.5% of control non-drug uptake at 35°C and was subtracted from all values to yield net uptake. All experiments were performed in duplicate and were repeated at least three times.

2.3.4. Neurotransmitter release assay

The release of preloaded [^{3}H]dopamine and [^{3}H]-norepinephrine from striatal and hippocampal slices was determined as described previously (Woodward and Blair, 1991). Briefly, slices loaded with [^{3}H]dopamine and [^{3}H]norepinephrine were washed, loaded into individual nylon mesh-bot tomed baskets (210 μm nylon mesh, Tetko Inc., Elmsford NY) and suspended in mini-vials each containing 3 ml of buffer. Baskets were transferred at two min intervals with continuous bubbling with 95% O_2/5% CO_2 through a series of vials. Slices were exposed to BNMPA (10–300 μM) for 2 min. Following the drug exposure, the tissue was lysed to release all of the radioactivity remaining in the slices. The radioactivity contained in the vials was quantitated by liquid scintillation spectrometry. The fractional release of neurotransmitter in each vial was calculated as a percent of the total neurotransmitter present at that time.

2.4. α_1-Adrenoceptor binding

Whole rat brain was homogenized in 50 ml ice cold cell buffer (50 mM Tris, 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.4) and the protein content was adjusted to 150–200 μg/ml. For initial characterization of brain α_1 binding, [^{3}H]prazosin at various concentrations (0.625–10 nM) were added to 800 μl of rat brain and incubated at 31°C in a shaking water bath for 50 min. Homogenates were filtered onto a 24-well Brandon cell harvester and radioactivity remaining on the filters was determined by scintillation counting. For displacement assays, BNMPA was added to tubes containing 1 nM [^{3}H]prazosin stock and tissue homogenate and processed as above. Non-specific binding was determined using 10 μM phentolamine.

2.5. Electrophysiology studies

Synthesis of mRNA, oocyte preparation, micro-injection, electrophysiological recordings and data analysis were carried out as described previously with minor modifications (Mirshahi and Woodward, 1995). All recordings utilizing NMDA receptors were done in Mg^{2+}-free Ringers with Ba^{2+} as the divalent charge carrier. For the dose response determinations, oocytes were stimulated by switching to a perfusion solution containing NMDA and glycine and various concentrations of BNMPA for 20 sec using a six-port injection valve. Each stimulation was preceded by a washout period of 3–5 min. For the

voltage-dependence studies, oocytes were ramped from −80 to +40 mV in 2 s, and leak currents were subtracted under each condition. All stimulations were done using 100 μM NMDA and 10 μM glycine unless otherwise stated. Experiments were performed at room temperature (20–22°C).

2.6. Data analysis

For binding studies, the K_D, K_i and B_{max} values were determined using the KELL software package EBDA program (Biosoft, Milltown, NJ). Data are expressed as the mean ± S.E.M. Reported means are from 3–5 independent experiments.

Dopamine uptake results and oocyte data were analyzed using oneway analysis of variance (ANOVA), and Duncan's test was used for post-hoc analysis when appropriate. Differences were considered significant at the $P \leq 0.05$ level. IC_{50} values were determined using ALLFIT.

3. Results

3.1. [^{3}H]CFT binding / dopamine uptake and neuro-transmitter release

Binding of radiolabelled CFT to the rat striatal membranes yielded a linear Scatchard plot with a K_D value of 37.95 nM and B_{max} of 5.69 pmol/mg protein. A single population of receptor sites was identified. BNMPA and *N*-demethyl-BNMPA fully displaced CFT binding and yielded K_i values (mean ± SEM) of 6.05 μM ± 0.15 and 8.73 μM ± 1.66, respectively (Fig. 2).

BNMPA also significantly inhibited [^{3}H]dopamine uptake at concentrations that inhibited CFT binding with a calculated IC_{50} value of 5.1 ± 1.4 μM (Fig. 3). Interestingly, low concentrations of BNMPA (0.1 and 0.3 μM) consistently enhanced dopamine uptake by 10–15% as compared to the non-drug control.

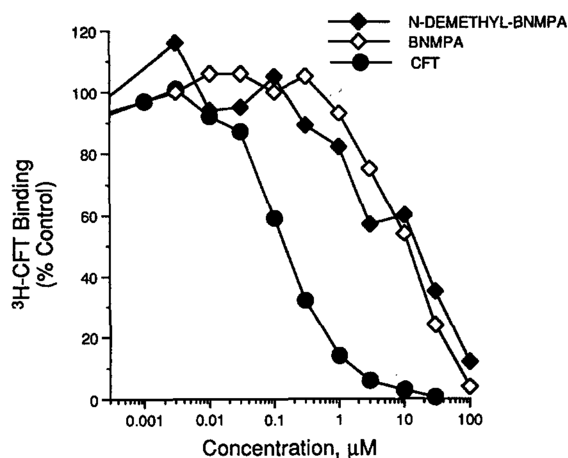

Fig. 2. Displacement of [^{3}H]CFT binding (30 nM) in fresh rat striatum by *N*-demethyl-BNMPA, BNMPA and CFT. Each curve is from one representative experiment ($n = 3$) and represents the mean of triplicate determinations.

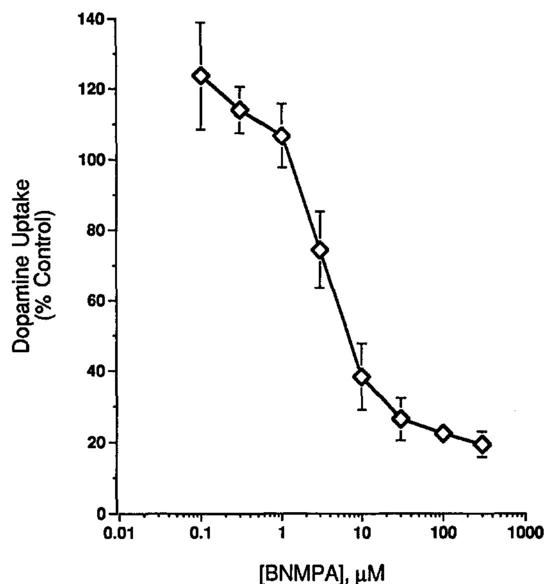

Fig. 3. Effect of BNMPA on synaptosomal DA uptake ($n = 3$). BNMPA (0.1–1 μM) slightly enhanced dopamine uptake with the 0.1 μM value being statistically different from the control ($P \leq 0.05$; Student's *t*-test). Higher concentrations (3–300 μM) significantly inhibited dopamine uptake with an IC_{50} value of 5.1 ± 1.4 μM. Data is expressed as the mean (± S.E.M.) percent of the control uptake.

The effects of BNMPA on the dopamine transporter were further investigated by monitoring [^{3}H]dopamine efflux from striatal slices. The basal efflux of [^{3}H]dopamine from rat striatal slices averaged approximately 2.8% (±0.2) per 2-min period. BNMPA did not significantly alter this efflux at concentrations of 10 and 30 μM. Higher concentrations of BNMPA (100 and 300 μM) increased the basal efflux to 4.5% and 8.7%, respectively. In parallel studies, BNMPA did not significantly alter the efflux of [^{3}H]norepinephrine at any concentration tested (10–300 μM; data not shown).

3.2. [^{3}H]Paroxetine binding

Paroxetine binding to rat cortical membranes yielded a linear Scatchard plot with a mean K_D value of 548 pM and B_{max} of 1.88 pmol/mg protein. The [^{3}H]paroxetine binding to the neuronal membranes from the cortex also indicated a single population of receptor sites. BNMPA displaced [^{3}H]paroxetine binding with a K_i of 14.5 μM ± 6.83. *N*-Demethyl-BNMPA did not fully displace [^{3}H]paroxetine over the concentration range tested (1–100 μM). At the highest concentration tested (100 μM), *N*-demethyl-BNMPA displaced 42% (±2.6) of total [^{3}H]paroxetine binding.

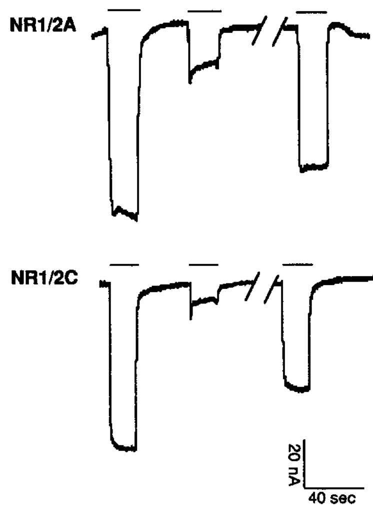

Fig. 4. Effects of 100 μM BNMPA on NMDA-activated currents. Oocytes expressing NR1/2A (top) and NR1/2C (bottom) NMDA receptor subtypes were stimulated with 100 μM NMDA/10 μM glycine for 20 s (indicated by solid bar over tracing). The middle tracing of each panel represents NMDA-induced currents in the presence of 100 μM BNMPA.

3.3. [^{3}H]Prazosin binding

Prazosin binding to rat whole brain membranes yielded a linear Scatchard plot with a mean K_D value of 251 pM and B_{max} of 83.2 fmol/mg (data not shown). BNMPA displaced [^{3}H]prazosin binding with a K_i value of 11.7 μM (data not shown).

3.4. Electrophysiology studies

BNMPA administered alone had no effects on un-injected oocytes or those injected with NMDA receptor subunit mRNA. However, NMDA-induced currents in

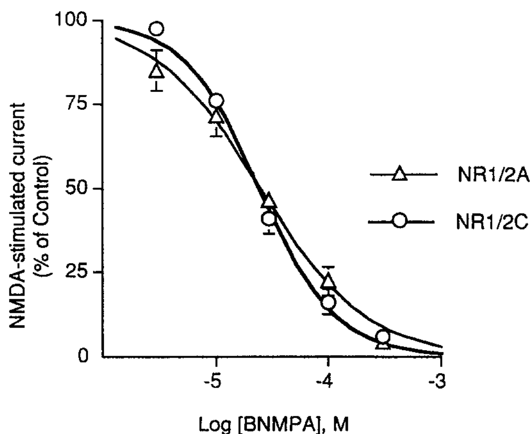

Fig. 5. Dose-response curves of BNMPA-induced inhibition of NMDA-activated currents in oocytes expressing the NR1/2A and NR1/2C NMDA receptor subunits. All values were obtained in the presence of 100 μM NMDA and 10 μM glycine. BNMPA inhibited the NR1/2A and NR1/2C receptor combinations with IC_{50} values of 24.6 ± 1.8 and 24.0 ± 1.5 μM, respectively.

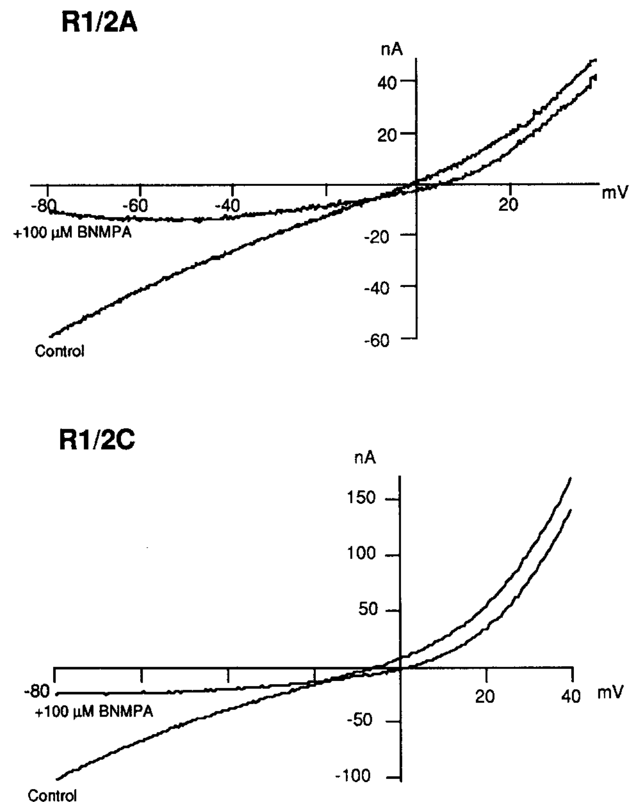

Fig. 6. Current-voltage dependence of the BNMPA block. Oocytes expressing NR1/2A and NR1/2C receptors were activated by application of 100 μM NMDA and 10 μM glycine in the absence or presence of 100 μM BNMPA. Current-voltage curves were generated by 2-s ramps from −80 mV to +40 mV; leak currents were subtracted in each case. BNMPA displayed a voltage-dependent block of the NMDA-activated currents with more blockade at lower potentials. There was also a slight shift of the reversal potentials to a more positive voltage.

oocytes expressing the NR1/2A or NR1/2C receptors subunits were significantly inhibited by BNMPA (Fig. 4). The BNMPA inhibition of the NMDA-activated current was slowly reversible with most of the current recovering after several minutes of washout. Fig. 5 shows dose response curves for the BNMPA inhibition of NMDA-induced currents in oocytes expressing the NR1/2A or NR1/2C subunits. The IC_{50} values for BNMPA in oocytes expressing the NR1/2A and NR1/2C were 24.6 ± 1.8 and 24.0 ± 1.5 μM, respectively. Near maximal inhibition was seen for both subunit combinations at 300 μM BNMPA. The inhibitory effects of BNMPA were not altered by increasing the concentration of NMDA or glycine (data not shown).

The BNMPA induced block of the NMDA-activated current in both subunit combinations displayed a strong voltage dependence (Fig. 6). The inhibition produced by BNMPA was greatest at negative potentials and was reduced, but not abolished, at more positive membrane potentials. In addition, BNMPA slighlty shifted the reversal potentials for both subunits to a more positive voltage.

BNMPA (100 μM) did not alter either GABA-induced

chloride currents in cultured hippocampal neurons (data not shown) or kainate-induced currents in oocytes expressing the GluR3 glutamate receptor (data not shown).

4. Discussion

One of the defining characteristics of psychomotor stimulants (methamphetamine, amphetamine, cocaine etc.) is their ability to elicit increases in spontaneous motor activity. At low doses, these drugs produce an alerting response characterized by increases in exploration, locomotion, grooming and rearing. As the dose increases, locomotor activity decreases and the behavioral patterns become more stereotyped (i.e., a continuous repetition of one or several types of behavior). Evidence suggests that the neurochemical basis underlying increased motor activity involve dopaminergic systems (Johanson and Fischman, 1989). By enhancement of neurotransmitter release/blockade of reuptake, stimulants facilitate catecholaminergic neurotransmission. The 'rewarding effects' (those responsible for psychomotor stimulant abuse) are also thought to result from enhanced dopamine release in the limbic regions such as the nucleus accumbens (Robbins and Sahakian, 1983).

4.1. BNMPA and dopamine

In a previous study, doses of BNMPA ranging from 1 mg/kg to 50 mg/kg failed to significantly alter locomotor activity significantly from controls although a slight depressant effect was noted at lower doses (Moore et al., 1995a). The degree of spontaneous activity when BNMPA was combined with methamphetamine was not significantly different from methamphetamine alone. Based on these observations, it was concluded that BNMPA probably does not act as an indirect dopamine agonist. However, in this study BNMPA fully displaced CFT binding and inhibited dopamine uptake with half-maximal effects occurring at approximately 5–6 μM. At concentrations below 100 μM at which transporter activity would be expected to be significantly inhibited, BNMPA did not significantly enhance [^{3}H]dopamine efflux from striatal slices during a 2-min exposure. This is consistent with previous reports which suggests that basal dopamine efflux from striatal slices is only slightly enhanced in the presence of pure dopamine transporter blockers like cocaine (Woodward and Harms, 1992). Higher concentrations of BNMPA ($\geq$ 100 μM) enhanced basal dopamine efflux, suggesting that BNMPA may also release dopamine in addition to its transporter blocking activity. It should be noted, however, that this enhancement of basal dopamine efflux was much less than that observed with amphetamine (Woodward and Harms, 1992). It is possible that longer incubations of striatal slices with lower concentrations of BNMPA might have resulted in a more significant release.

Although BNMPA's affinity at the dopamine transporter was low relative to CFT, the K_i for (−)-cocaine (0.6 μM) at the dopamine transporter is only tenfold greater than that for BNMPA (Ritz and George, 1993; Woodward et al., 1995). This suggests that BNMPA would be expected to enhance synaptic concentrations of dopamine if levels approach or exceed 10 μM following dosing in an intact animal. Assuming complete distribution in body water, it was estimated that the in vivo concentration of BNMPA in a 20 g mouse would be approximately 366 μM following a 50 mg/kg dose (Moore et al., 1995b). The lack of effect on locomotor activity even at these high doses suggests that either BNMPA does not reach these sites or that other mechanisms may mask the locomotor effects.

4.2. BNMPA and seizures

Despite its inability to enhance locomotor activity, BNMPA does produce convulsions at doses much lower than those that cause lethality (Noggle et al., 1985; Moore et al., 1995a). Seizures, which are often associated with stimulant abuse, tend to occur only at very high doses (Weiner, 1985; Cameron et al., 1992; Ritz and George, 1993). Seizure liability is most often associated with blocking GABA-induced inhibition or enhancing glutamate-mediated excitation (Franz, 1985). In addition, Ritz and George (1993) also demonstrated that the potencies of cocaine and related stimulant drugs in producing seizures were highly associated with drug affinity at serotonin (5-HT) transporters suggesting that drugs with relatively high affinity at the 5-HT transporter will also exhibit potent seizurgenic effects.

The in vitro assays conducted in this study are not fully supportive of these mechanisms as the molecular basis of the proconvulsive activity of BNMPA. BNMPA's affinity for the 5-HT transporter was relatively modest (15 μM) and there was no evidence of any effect of BNMPA with either $GABA_A$ receptors or the AMPA-kainate glutamate receptors.

4.2.1. NMDA receptors

Surprisingly, BNMPA blocked NMDA-activated currents in oocytes expressing either the NR1/2A or NR1/2C subunit combinations. The IC_{50} values for the blockade of the NR1/2A and NR1/2C receptor combinations were not different from one another and were approximately 25 μM. Preliminary investigations into the mechanism of action of BNMPA suggested that the drug did not compete with either the NMDA or glycine binding sites on the receptor. However, BNMPA's blockade of the NMDA-activated current displayed a strong voltage dependence. This blockade was similar to the well characterized Mg^{2+} blockade of the NMDA-activated current although it was slightly less voltage dependent with residual block even at positive holding potentials. In addition, BNMPA slighlty

shifted the reversal potential of the NMDA-induced currents suggesting a complex interaction with the receptor ionophore.

It has been shown that many NMDA receptor blockers possess neuroprotective properties and may serve as effective anticonvulsant agents. Sonsalla et al. (1991) reported that the non-competitive NMDA receptor antagonist, dizocilpine as well as other glutamate receptor antagonists, protect the mouse neostriatal dopaminergic system from neuronal degeneration induced by methamphetamine. Based on the results of the in vitro studies in this report, BNMPA would also be expected to possess anticonvulsant actions in the micromolar concentration range. This conclusion is somewhat consistent with the previous finding that BNMPA was able to suppress low-dose methamphetamine-induced convulsions (Moore et al., 1995b). These findings also suggest that the convulsant actions of BNMPA observed at higher doses are probably not mediated via their actions at the NMDA receptor.

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