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Short communication

Neuroprotection against *N*-methyl-D-aspartate-induced excitotoxicity in rat magnocellular nucleus basalis by the 5-HT_{1A} receptor agonist 8-OH-DPAT

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Abstract

The present study reports the neuroprotective efficacy of the 5-HT_{1A} receptor agonists 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and ipsapirone against in vivo excitotoxic neuronal injury. Excitotoxic cell death was induced by injections of *N*-methyl-D-aspartate (NMDA) in the rat magnocellular nucleus basalis. The neurodegenerative effects were quantified by image analysis of the axonal density of the nucleus basalis projection to the somatosensory cortex visualized with acetylcholinesterase histochemistry. Pretreatment with 8-OH-DPAT—but not ipsapirone—1 h prior to NMDA infusion showed significant preservation of cortical cholinergic innervation in all doses tested. Furthermore, 8-OH-DPAT exhibited sustained efficacy under homeothermic conditions in which the body temperature was maintained at 36.8 ± 0.1°C. These data indicate that selective 5-HT_{1A} receptor activation by 8-OH-DPAT protects against NMDA-induced excitotoxic neuronal damage, probably as a result of 5-HT_{1A} receptor-mediated neuronal hyperpolarization.

Keywords: Excitotoxicity; NMDA (*N*-methyl-D-aspartate); 5-HT_{1A} receptor; 8-OH-DPAT; Neuroprotection; Homeothermic condition

1. Introduction

Persistent stimulation of NMDA receptors by excessive levels of excitatory amino acids such as glutamate and aspartate and their analogs, causes neuronal damage triggered by Ca^{2+} influx through NMDA receptor channels and subsequent membrane depolarization (Rothman and Olney, 1995). The intracellular Ca^{2+} overload that results from NMDA receptor-mediated Ca^{2+} influx is thought to evoke neuronal cell death both during aging (Verkhratsky and Toescu, 1997) and in the course of different neuropathological conditions such as dementia (Dodd et al., 1994), hypoxia or stroke (Choi, 1995). With regard to excitotoxic brain damage, there is some recent evidence that activation of the 5-HT_{1A} receptor can attenuate the effects of neuronal overexcitation by excitatory amino acids. Prehn et al. (1993) reported reduction of ischemic neuronal damage by 5-HT_{1A} receptor agonists. Furthermore, 5-HT_{1A} receptor agonists were shown to inhibit glutamate-release from nerve terminals (Srkalovic et al., 1994), while Strosznajder et al. (1996) demonstrated that 5-HT_{1A} receptor activation causes a significant reduction of glutamate-induced elevation of the free intracellular Ca^{2+} concentration in rat hippocampal synaptosomes.

Immunohistochemical as well as autoradiographic studies have shown the postsynaptic cholinergic neurons of the magnocellular nucleus basalis to be endowed with 5-HT_{1A} receptors (Khateb et al., 1993; Nyakas et al., 1997). The neurons of the magnocellular nucleus basalis exhibit a

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striking susceptibility to excitotoxic cell damage induced by NMDA (Luiten et al., 1995; Stuiver et al., 1996) and thus may serve as suitable targets to assess the capacity of neuroprotective treatment with 5-HT_{1A} receptor agonists. The present study explores the neuroprotective potential of the full 5-HT_{1A} receptor agonist 8-OH-DPAT and the partial 5-HT_{1A} receptor agonist ipsapirone in an in vivo excitotoxic neurodegeneration model in view of the therapeutic use of these compounds in neurodegenerative conditions where excitotoxic brain damage is eminent.

A conclusive interpretation of the neuroprotective efficacy of these drugs, however, is confounded by evidence for hypothermic effects due to 5-HT_{1A} receptor activation (O'Connell et al., 1992). Since it is generally accepted that hypothermia protects neuronal structures against hypoxic–ischemic insults (Zornow, 1995; Greiner et al., 1998), the efficacy of 5-HT_{1A} agonist treatment was compared in an additional experiment which included the effects of the drug under temperature-controlled homeothermic conditions. Finally, a single experiment in which the effect of the specific 5-HT_{1A} receptor antagonist on excitotoxic cell death was investigated in order to assess the tentative influence of naturally occurring 5-HT_{1A} receptor stimulation by glutamate (Bowen et al., 1992). Part of the current findings were published in a preliminary form elsewhere (Oosterink et al., 1997).

2. Materials and methods

2.1. Materials and subjects

Experiments were carried out on 78 male, 3-month old Wistar rats bred in our own facilities with an average bodyweight of 310 g. At the start and during the experiments the animals were individually housed in perspex cages and kept on standard laboratory rat diet (Hope Farms, Woerden, Netherlands) and tap water ad libitum in an air-conditioned room (21 ± 1°C) with a 12-h daylight cycle (lights on at 0700 h).

Animals were randomly divided into 12 groups which all received treatment 1 h before the NMDA-infusion. All drugs were dissolved in sterile phosphate buffered saline (PBS, 0.01 M and pH 7.4) and injected intraperitoneally (i.p.). The first 10 groups were studied in temperature-free conditions and received: (*1*) vehicle solution injected i.p. ($n = 9$), (*2*) 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, Research Biochemicals International, Natick, MA, USA) in doses of 0.5 ($n = 6$), 1.0 ($n = 6$), 2.5 ($n = 7$), 5.0 ($n = 7$) or 10.0 ($n = 6$) mg/kg dose, (*3*) ipsapirone (a gift from Tropon-Bayer, Cologne, Germany) at doses of 1.0 ($n = 6$), 5.0 ($n = 6$) or 10.0 ($n = 7$) mg/kg, or (*4*) [*O*-methyl-^{3}H]*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexane carboxamide trihydrochloride (WAY-100635, Amersham International, UK) at a dose of 1.0 mg/kg ($n = 6$). Since the initial findings with ipsapirone indicated either no neuroprotection or even neurodegenerative effects the experiment on ipsapirone was limited to the three tested doses. Two additional groups which received the vehicle solution ($n = 6$) or the most effective 2.5 mg dose of 8-OH-DPAT ($n = 6$), were studied under temperature-controlled circumstances using a Harvard homeothermic pad system (Harvard Apparatus, Edenbridge, UK), which kept the body temperature within a close range of 36.8 ± 0.1°C.

2.2. Surgical procedures

Prior to NMDA-infusion, rats were anesthetized with sodium-pentobarbital (Sigma; 24 mg/kg b.w., i.p.) and hypnorm (Duphar; Weesp, Netherlands, 0.4 mg/kg b.w., i.m.). NMDA (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS, pH 7.4). According to Luiten et al. (1995), a 10 μl Hamilton microsyringe was lowered into the brain at coordinates of the magnocellular basal nucleus (AP = −1.5 mm, lat. = 3.2 mm), as defined by the atlas of Paxinos and Watson (1989). A total of 60 nmol NMDA in 1.0 ml solution was slowly (0.1 ml/min) injected at two dorsoventral positions (6.2 mm and 7.0 mm) from the dura. All efforts were made to minimize animal suffering throughout the experiments. Their care and treatment were in accordance with the Dutch National Act on the Use of Live Animals.

2.3. Tissue processing

Animals were deeply anesthetized 12 days after NMDA-infusion by an overdose of sodium pentobarbital and transcardially perfused with 300 ml fixative composed of 2.5% paraformaldehyde, 0.05% glutardialdehyde and 2.0% picric acid in 0.1 M PBS (pH 7.4). The brains were removed and cryoprotected by overnight storage in 30% sucrose in 0.1 M PB for dehydration. Subsequently, the brains were coronally sectioned on a cryostat microtome at a thickness of 20 μm. Brain sections were immediately postfixed by immersion in a 2.5% GA solution in PBS overnight at 4°C. Cholinergic fibers were visualized by staining for acetylcholinesterase according to Hedreen et al. (1985), using a silver nitrate intensification procedure.

2.4. Quantification and statistical analysis

The damage to magnocellular nucleus basalis neurons evoked by the NMDA infusion was quantified by measuring the density of their axonal projections invading the posterior somatosensory cortex by using a Quantimet Q-600HR computerized image analysis system (Leica, Cambridge, UK). Cortical fibre density of the lesioned side was compared to the projections of the contralateral non-lesioned (control) side. Due to the strict unilateral nature of these projections in the cortical area receiving the densest cholinergic innervation from the damaged magnocellular

nucleus basalis (Luiten et al., 1995) the contralateral innervation values served as within-experiment control values. Percentage neuroprotection for each experimental drug treatment was calculated by the ratio of cortical fibre reduction (CFR) after vehicle treatment [X] and after drug application [C], according to the following formula (Stuiver et al., 1996):

$$\%\text{neuroprotection} = 100(1 - (\%\text{CFR}[X]/\%\text{CFR}[C]))$$

One way analysis of variance (ANOVA) followed by post-hoc *t*-test was used to determine the treatment-effects on cortical fibre reduction (MINITAB Release 9.2, 1993, Minitab, State College, PA, USA). The effects of treatment in homeothermic conditions were evaluated similarly. Data were expressed as means ± S.E.M, while a P level of < 0.05 was taken as indicative of statistical significance for the tests.

3. Results

3.1. Neuroprotection by 8-OH-DPAT and ipsapirone

NMDA-infusions in the magnocellular nucleus basalis of vehicle-pretreated animals caused a profound loss of acetylcholinesterase-stained fibres in the ipsilateral somatosensory cortex, showing a cortical fibre reduction of 59.8 ± 4.65% compared to the non-lesioned control-side of the brain (Fig. 1).

The selective 5-HT_{1A} receptor agonist 8-OH-DPAT showed overall protection against NMDA-excitotoxicity (Fig. 1A). Statistical evaluation of the quantitative histochemical data revealed significant effect of 8-OH-DPAT treatment in all pharmacological doses investigated (0.5 mg/kg dose: $F = 6.356$, $P < 0.05$, 29.6% neuroprotection (NP); 1.0 mg/kg dose: $F = 7.541$, $P < 0.05$, 31.9% NP; 2.5 mg/kg dose: $F = 7.983$, $P < 0.01$, 36.8% NP; 5.0 mg/kg dose: $F = 5.110$, $P < 0.05$, 28.3% NP; and 10.0 mg/kg dose: $F = 5.099$, $P < 0.05$, 27.8% NP; Fig. 1A). In contrast to 8-OH-DPAT, the partial 5-HT_{1A} receptor agonist ipsapirone failed to protect against NMDA-induced excitotoxic damage. In fact, the highest dose of ipsapirone even enhanced the neurodamaging properties of the excitotoxin ($F = 11.498$, $P < 0.01$, −35.5% NP; Fig. 1B). Finally, pretreatment with the selective 5-HT_{1A} receptor antagonist WAY-100635 did not elicit any significant change in cortical fibre reduction as compared to the vehicle-treated group (Fig. 1B).

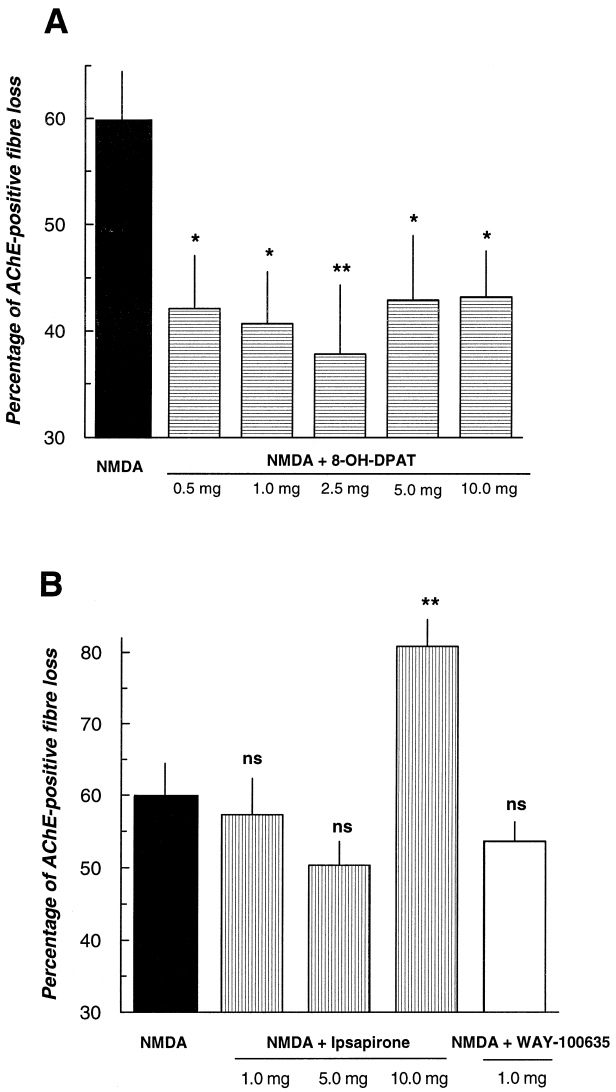

Fig. 1. Acetylcholinesterase (AChE)-positive cortical fibre loss following infusion of 60 nmol *N*-methyl-D-aspartate-(NMDA) into the ipsilateral magnocellular nucleus basalis. The effects of pretreatment with the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (A), and the effects of pretreatment with the partial 5-HT_{1A} receptor agonist ipsapirone and the selective 5-HT_{1A} receptor antagonist WAY-100635 (B). Data are presented as mean ± S.E.M. (* $P < 0.05$; ** $P < 0.01$; ns, non-significant).

3.2. Effect of body temperature on 8-OH-DPAT neuroprotection

Because of the well-documented drop in body temperature as a result of treatments during surgery and application of 5-HT_{1A} receptor agonists, effects of neurodegenerative procedures and neuroprotective treatment were compared with conditions where body temperature was maintained at 36.8 ± 0.1°C. In the currently used experimental set-up body temperature during NMDA injection decreased to 32.8°C, whereas in rats pretreated with 2.5 mg/kg 8-OH-DPAT the body temperature during surgery fell to 29.8°C.

NMDA-infusion in the magnocellular nucleus basalis of vehicle-treated animals in homeothermic conditions yielded a cortical fibre reduction of 57.3 ± 7.62% (Fig. 2). Treatment with the most effective 2.5 mg/kg dose of 8-OH-DPAT (see Section 3.1) showed a cortical fibre reduction of 36.9 ± 4.98% under temperature-controlled circumstances (Fig. 2), which accounts for 35.6% neuroprotection ($F = 5.042$, $P < 0.05$). Statistical analysis revealed no significant differences between either the representative

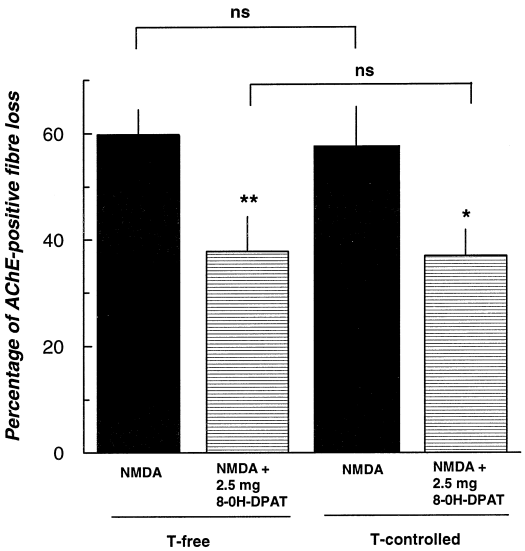

Fig. 2. Histogram comparing the effects of AChE-positive cortical fibre loss in temperature free (T-free) and temperature-controlled (T-controlled) conditions. Pretreatment with 8-OH-DPAT was given in optimal dose. Data are presented as mean ± S.E.M. (* $P < 0.05$; ** $P < 0.01$; ns, non-significant).

sham-treated or 8-OH-DPAT-injected groups (Fig. 2) under different temperature conditions. In fact, 8-OH-DPAT exerts identical neuroprotective potential under homeothermic conditions as compared to the data yielded in uncontrolled temperature experiments.

4. Discussion

By counteracting the effects of NMDA-excitotoxicity, the present model clearly demonstrates the neuroprotective potential of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT. The relatively dose-independent neuroprotective profile suggests a broad therapeutic range for 8-OH-DPAT. While the neuroprotective action was shown to be independent to drug-induced hypothermic effects, the results could be explained by the fact that 8-OH-DPAT is able to hyperpolarize neuronal membranes through a G-protein-mediated pathway leading to increased potassium conductances (Sprouse and Aghajanian, 1987), thus resisting the NMDA channel-induced depolarizations. In this regard, Khateb et al. (1993) provided direct evidence that 8-OH-DPAT is able to hyperpolarize cholinergic neurons of the magnocellular basal nucleus by a direct postsynaptic action. Furthermore, since Kia et al. (1996) by means of immunocytochemistry showed that 5-HT_{1A} receptors are widely expressed on cholinergic neurons, the neuroprotective effects following selective 5-HT_{1A} receptor activation could likely be extended to other cholinergic cell groups.

While the selective 5-HT_{1A} receptor agonist 8-OH-DPAT exhibited high-efficacy neuroprotective properties at all doses tested, the partial 5-HT_{1A} receptor agonist ipsapirone failed to exert significant protection and actually showed neurodamaging properties in combination with excitotoxic procedures at the highest dose. The fact that partial 5-HT_{1A} receptor agonists couple to G-proteins with lower efficacy than selective 5-HT_{1A} receptor agonists does not readily provide an explanation for the observed adverse effects of ipsapirone (Gettys et al., 1994). A likely explanation may be found in the fact that ipsapirone, but not 8-OH-DPAT, metabolizes very rapidly to 1-(2-pyrimidinyl)piperazine (1-PP). 1-PP has been reported to cause release of noradrenaline mediated by a presynaptic antagonistic α_2-adrenergic action (Nocon et al., 1990; Done and Sharp, 1994). In turn, local release of noradrenaline in the magnocellular nucleus basalis may lead to strong depolarizations resulting from postsynaptic α_1-adrenoceptor activation, as shown by Fort et al. (1995). Thus, 1-PP-triggered events may explain both the neuronal damage in the highest ipsapirone dose and the ineffectiveness of the other two doses. Additionally, the 5-HT_{1A} receptor antagonist WAY-100635 did not alter the extent of NMDA-induced neuronal injury. This may be interpreted as a lack of effective blockade of normally occurring serotonergic stimulation of the 5-HT_{1A} receptor in the nucleus basalis. Alternatively, work of Artigas et al. (1996) suggest that WAY-100635 primarily affects 5-HT_{1A} autoreceptors located on dorsal raphe neurons. However, further study on the pharmacological action of 5-HT_{1A} antagonists is required to define the precise action in the suggested neuroprotective mechanisms.

Several reports provide evidence for functional interactions between NMDA channels and 5-HT_{1A} receptors (Ross et al., 1992; Becquet et al., 1993), while Strosznajder et al. (1996) suggest that 5-HT_{1A} receptors serve as mediators of a biological autoprotective mechanism of the brain against the effects of overstimulation by excitatory amino acids. Aging may be one factor that is responsible for failure of this mechanism. This assumption is underscored by a recent study that describes a strong decline of 5-HT_{1A} receptors in selective brain areas of 24- and 30-month old rats when compared to young controls, a decline that was particularly strong in the cholinergic basal forebrain nuclei (Nyakas et al., 1997). Furthermore, human autoradiographic post-mortem studies of patients who died with Alzheimer's disease showed reduction of ligand binding to the 5-HT_{1A} receptors that correlated positively with increasing age (Middlemiss et al., 1986; Bowen et al., 1989). Evidence for a specific decline of functional serotonergic innervation of the magnocellular basal nucleus in Alzheimer's disease is provided by Sparks et al. (1992). In that sense the specific neuroprotective action of 8-OH-DPAT in an animal model of cholinergic cell death—a hallmark of the neuropathology of Alzheimer's disease—suggests a promising therapeutic strategy for treatments of dementia.

In conclusion, from the present studies it may be concluded that the serotonergic system and its associated

5-HT_{1A} receptors are subject to decline during aging or dementia. An age-related reduction of the 5-HT_{1A} receptor system thus may form a reduction of the natural biological protection against neuronal degeneration that results from sustained excitation by excitatory amino acids. Unfortunately, our knowledge on the fate of brain 5-HT_{1A} receptors in vivo is sparse. The recent development of 5-HT_{1A} receptor ligands for positron emission tomography (Pike et al., 1996; Zhuang et al., 1998), however, may provide insight in where and why 5-HT_{1A} receptors disappear and lead to treatment strategies against the assumed receptor loss. In this respect, the currently described effects of selective 5-HT_{1A} receptor activation holds promise for counteracting excitotoxic damage during aging and in different neuropathological conditions.

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