GluN2B-containing NMDA receptors as possible targets for the neuroprotective and antidepressant effects of fluoxetine

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Abstract

Accumulating evidence has indicated the involvement of glutamatergic neurotransmission in the pathophysiology of excitotoxicity and in the mechanism of action of antidepressants. We have previously shown that tricyclic desipramine and the selective serotonin reuptake inhibitor fluoxetine inhibit NMDA receptors (NMDARs) in the clinically relevant, low micromolar concentration range. As the different subtypes of NMDARs are markedly different in their physiological and pathological functions, our aim was to investigate whether the effect of antidepressants is subtype-specific.

Using whole-cell patch-clamp recordings in rat cortical cell cultures, we studied the age-dependence of inhibition of NMDA-induced currents after treatment with desipramine and fluoxetine, as the expression profile of the NMDAR subtypes changes as a function of days in vitro. We also investigated the inhibitory effect of these antidepressants on NMDA-induced currents in HEK 293 cell lines that stably expressed rat recombinant NMDARs with GluN1a/GluN2A or GluN1a/GluN2B subtype compositions.

The inhibitory effect of desipramine was not age-dependent, whereas fluoxetine displayed a continuously decreasing inhibitory profile, which was similar to the GluN1/GluN2B subtype-selective antagonist ifenprodil. In HEK 293 cells, desipramine equally inhibited NMDA currents in both cell lines, whereas fluoxetine showed an inhibitory effect only in cells that expressed the GluN1/GluN2B subtype.

Our data show that fluoxetine is a selective inhibitor of GluN2B-containing NMDARs, whereas desipramine inhibits both GluN1/GluN2A and GluN1/GluN2B subtypes. As the clinical efficacy of these drugs is very similar, the putative NMDAR-associated therapeutic effect of antidepressants may be mediated only via inhibition of the GluN1/GluN2B-selectivity of fluoxetine suggests the neuroprotective potential for this drug in both acute and chronic neurodegenerative disorders.

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1. Introduction

In the last 20 years several preclinical and clinical studies have indicated that the glutamatergic system plays an important role in excitotoxicity (Sheldon and Robinson, 2007; Musazzi et al., 2011) and in the pathophysiology of depression (Palucha and Pilc, 2005; Skolnick et al., 2009). In addition, fluoxetine (Chang et al., 2006; Chollet et al., 2011) and several monoaminergic drugs are able to modulate brain plasticity after a stroke and reduce the residual neurological deficit and subsequent disability (Loubinoux and Chollet, 2010). An antidepressant-like effect of competitive and non-competitive NMDA receptor antagonists has been demonstrated in both animal (Layer et al., 1995; Panconi et al., 1993; Papp and Moryl, 1994; Redmond et al., 1997; Rogoz et al., 2002; Trullas and Skolnick, 1990) and human (Berman et al., 2000; Stryjer et al., 2003; Zarate et al., 2006) studies. Therefore, glutamate receptors appear to be promising targets for drug research (Krystal et al., 2002; Palucha and Pilc, 2005; Paul and Skolnick, 2003; Skolnick et al., 2009).

Recently, we have reported that both tricyclic desipramine and SSRI fluoxetine were able to inhibit NMDA-induced currents in cortical cell cultures, although the mechanism of action of the two compounds was different. Inhibition by desipramine was voltage-dependent, and the drug was unable to bind to NMDA receptors that were blocked by Mg2+, whereas the inhibition by fluoxetine was not voltage-dependent, and association with the receptor was still possible in the presence of an Mg2+ block (Szasz et al., 2007a). The observed IC50 values were in the low micromolar concentration range, which has been shown to develop in the brain during antidepressant therapy (Besret et al., 1996; Bolo et al., 2000), implying that the action of antidepressants on NMDA receptors might contribute to the clinical effects of these drugs.

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The subtype composition essentially determines the functional properties of NMDA receptors, as the different subtypes are associated with different intracellular pathways and their subcellular distribution is also dissimilar. Extrasynaptic receptors are predominantly GluN2B-containing, whereas GluN2A-containing receptors are enriched in synapses compared to extrasynaptic locations (Vizi et al., 2010; Vizi and Mike, 2006). This latter difference could be particularly important because based on simulations of transmitter diffusion, it has been argued that nonsynaptic receptors are clearly more affected by both drug treatment and pathophysiological conditions (Vizi et al., 2010). The possible association of a subtype with neuroprotective and/or antidepressant action, therefore, may help us to understand the pathophysiological background of neuroprotection and/or depression. In this work, our aim was to investigate whether the previously observed effects of antidepres- sants were associated with a specific subtype. Using whole-cell patch-clamp recordings in rat cortical cell cultures, we studied the age dependence of inhibition of NMDA-induced currents after treatment with desipramine and fluoxetine, as the expression profile of NMDA receptor subtypes changes during development and depends on the time spent in vitro (Thomas et al., 2006; Zhong et al., 1994). We also compared the inhibitory effects of these anti- depressants on NMDA-induced currents in HEK 293 cell lines that stably expressed rat recombinant NMDA receptors with GluN1a/ GluN2A or GluN1a/GluN2B subunit compositions.

2. Materials and methods

All experimental procedures were approved by the local ethical committee and were in accordance with NIH guidelines. All efforts were made to minimise animal suffering and the number of animals that were used.

2.1. Cortical neuronal cultures

Neuronal cultures were prepared for electrophysiology, as described previously (Szasz et al., 2007a). Briefly, pregnant rats (17–18 day gestation) were anaesthetised with a mixture of ketamine (50 mg/ml) and xylazine (10 mg/ml). The uterus was dissected and placed in a laminar airflow box. From this point, the prepara- tion was performed in a sterile environment. Individual foetuses were isolated, and their whole brains were placed in cold MEM (50 mg/ml) and xylazine (10 mg/ml). Twenty-four hours after plating, the medium was changed to Neurobasal medium (Gibco) containing 25% foetal bovine serum, and placed at a density of 150–300,000/35 mm petri dish (precoated with poly- l-lysine, 2 μg/ml). Twenty-four hours after plating, the medium was replaced with B27 supplemented Neurobasal medium ( Gibco) containing 25 μM 2-mercaptoethanol, 0.5 mM glutamine and 25 μM glutamate. Half of the medium was changed twice a week thereafter with the same (Neurobasal + B27) medium that did not contain glutamate. NMDA-evoked currents were recorded from cortical cells at 6–20 days in vitro (DIV). The co-agonist glycine was present at 10 μM concentration throughout the experiments.

2.2. HEK 293 cell lines

The cell lines that stably expressed rat recombinant NMDA receptors with GluN1a/GluN2A or GluN1a/GluN2B subunit compositions were generated as previously described (Kurko et al., 2005; Nagy et al., 2003). CDNAs encoding the rat GluN1a, GluN2A and GluN2B subunits were obtained from Prof. P. Seeburg (Monyer et al., 1992) and subcloned into edcsyone-inducible eukaryotic expression vectors (pIND(SP1)Hygro and pIND(SP1)Neo). The GluN1a gene incorporating vector included a sequence that coded for hygromycin resistance, whereas the other vectors that incorpo- rated the GluN2 subunit genes contained neomycin resistance genes. The resulting constructs were transfected into EcR 293 cells (HEK 293 cells stably expressing the edcsyone and the retinoid X receptors) using Pfx-7 PerFect Lipid. Resistance to G418 and hygro- mycin was used to screen for potential positive clones. Clonal cell lines expressing rGluN1a/2A and rGluN1a/2B receptors were established from the cells that produced the highest response to NMDA exposure. The cells were maintained in DMEM supplement- ed with 10% foetal bovine serum, G418 (600 μg/ml) and hygromycin (100 μg/ml) in a humidified atmosphere with 5% CO2 at 37°C. One day after plating, the inducing agent MuA (10 μM) and ketamine (500 μM), an NMDA receptor antagonist used to pre- vent cytotoxicity due to receptor activation, were added to the cul- tures. The studies were performed 24–96 h after induction of expression.

2.3. Whole cell patch-clamp recordings of cortical neurons and HEK 293 cells

Transmembrane currents were recorded according to the standard whole-cell patch-clamp technique using an Axopatch 200B amplifier and the pClamp software ( Molecular Devices, Union City, CA). All experiments were performed at room temperature (22–25°C). Borosilicate glass patch pipettes (1.4–3.8 MΩ) were used. The series resistance was compensated to 60–80%. The pipettes were filled with an intracellular solution of the following composi- tion (in mM): NaCl 150, KCl 5, CaCl2 1.4, glucose 10, HEPES 5, tetrodotoxin (TTX) 0.0003, picrotoxin 0.1, strychnine 0.002, glycine 0.01, and NaOH to adjust the pH to 7.3. The composition of the external solution was (in mM): NaCl 150, KCl 5, CaCl2 1.4, glucose 10, HEPES 5, and CsOH to adjust the pH to 7.3. The currents were low-pass filtered at 2 kHz using the built-in four-pole, low-pass Bessel filter of the amplifier and sampled at a rate of 10 kHz.

A pressure-operated, computer-controlled rapid drug application device (DAD-12; Adams and List, Westbury, NY, USA) was used for drug administration. The inlets of the two glass U-tubes were connected to the pressure control unit of the DAD-12 and the outlet to a peristaltic pump to gain precise control of both in- flow and outflow. The drugs were applied by closing the electric valve (controlled by the pClamp software) at the outlet of one of the U-tubes. This arrangement (for details see Szasz et al., 2007) al- lowed the rapid application and removal of the drugs: 10–90% of the solution exchange times were within a 2–10 ms range, as gauged by junction potential measurements, and the rate of re- moval was not dependent on the duration of the drug application. The holding potential was −70 mV in all experiments.

2.4. Statistical analysis

Concentration–inhibition curves were fit using the Hill equa- tion. Curve fittings were performed using either the pClamp soft- ware or the solver function of Microsoft Excel. Statistical signification was determined using ANOVA followed by the Tukey–Kramer multiple comparisons test or a two-tailed Student’s t-test where appropriate. A p value <0.05 was considered signifi- cant. The results are presented as the mean ± SEM.

2.5. Materials

Dulbecco’s modified Eagle’s medium (DMEM), phenol red free DMEM, foetal bovine serum, the EcR 293 cell line, pIND(SP1)Hygro and pIND(SP1)Neo vectors, Pfx-7 Perfect Lipid, muristerone A (MuA), G418 and hygromycin were purchased from Invitrogen Corporation (Carlsbad, California, USA). Desipramine HCl, fluoxetine HCl, strychnine, glycine, and N-methyl-D-aspartate (NMDA)
were purchased from Tocris (Tocris Cookson Ltd., Bristol, UK). Ifenprodil, tetrodotoxin and picrotoxin were obtained from Sigma (Taufkirchen, Germany). NVP-AAM077 was kindly provided by Dr. Yves Auberson (Novartis, Basel, Switzerland). All other chemicals were of analytical grade.

3. Results

3.1. Age dependence of the effect of fluoxetine and desipramine on NMDA-induced currents in rat cortical cell cultures

In the first series of experiments, we investigated whether the inhibition of NMDA-induced currents by these antidepressants was dependent on the age of the cortical cell cultures. During development, the expression of NMDA receptor subunits is dependent on age; the GluN2A subunit appears only at 6–10 days postnatal (Monyer et al., 1994; Sheng et al., 1994). Before this age, only the GluN2B subunit is expressed by the neurons. This developmental pattern can also be observed in cortical cell cultures that are prepared and cultured under similar conditions, as in our study (Zhong et al., 1994). This pattern is characterised by a dominance of the GluN2B subunit during 0–7 DIV, followed by relative amounts of the GluN2A subunit mRNA steadily increasing between 7 and 21 DIV.

The age dependence was investigated by employing a fast drug application system that has been described earlier (Szasz et al., 2007b). Two glass ‘U-tubes’ were placed near the patched cell, and the following drug administration protocol was applied: NMDA (10 μM) for 20 s (A-tube), antagonists (MK-801, ifenprodil, fluoxetine or desipramine) coapplied with NMDA (10 μM) for 40 s (B-tube), and NMDA (10 μM) for 30 s (A-tube). Under these conditions, at a holding potential of −70 mV, the NMDA-induced current was completely blocked by the non-competitive NMDA antagonist MK-801 (1 μM) and Mg2+ (5 mM), which indicated that the response was fully mediated by the NMDA receptors (Fig. 1A). The age-dependence of the inhibition by fluoxetine, desipramine and the GluN1/GluN2B subtype-selective ifenprodil was studied in 6–20 DIV cortical cell cultures (Fig. 1B). The inhibitory effect of desipramine (30 μM) was not dependent on the age of the cultures and varied between 88% and 92%. In contrast, the inhibitory effect fluoxetine (30 μM) monotonically decreased with the age of the cell cultures. In 6 DIV cultures, fluoxetine exhibited a 93.2 ± 6.80% inhibitory effect, which decreased to 33.0 ± 3.08% in 20 DIV cultures. The inhibitory profile of fluoxetine was indistinguishable from that of ifenprodil (10 μM), which showed 95.0 ± 5.0% inhibition at 6 DIV and 23.0 ± 6.51% inhibition at 20 DIV (Fig. 1C).

3.2. The inhibitory effect of fluoxetine and desipramine on NMDA-induced currents in HEK 239 cell lines

In these experiments, we used HEK 239 cell lines that stably expressed recombinant NMDA receptors with GluN1α/GluN2A or GluN1α/GluN2B subunit compositions. We used the same protocol as in the cortical cell cultures (at a holding potential of −70 mV), and the cell lines were validated using pharmacological tools. The GluN2A-containing receptor selective antagonist NVP-AAM077 (Auberson et al., 2002) (10 nM) produced a substantial inhibition (57.0 ± 8.5% inhibition, n = 4) of NMDA currents in the GluN1/GluN2A cell line, whereas it was ineffective in the GluN1/GluN2B cell line (11.6 ± 5.9% inhibition, n = 4) (Fig. 2). In contrast, the GluN2B-containing receptor subtype-selective antagonist ifenprodil (10 μM) almost completely blocked the NMDA-currents in the GluN1/GluN2B cell line (89.7 ± 3.0% inhibition, n = 6) but had no effect on the NMDA currents induced in the GluN1/GluN2A cell line (12.0 ± 5.2% inhibition, n = 6) (Fig. 2). Desipramine was equally effective in both cell lines; the IC50 values for GluN1/GluN2A and GluN1/GluN2B were 5.10 ± 1.25 μM (degree of freedom [DF] = 24, r2 = 0.80) and 3.61 μM (DF = 25, r2 = 0.80), respectively (Fig. 3A and B). In contrast, fluoxetine displayed inhibition only in the GluN1/GluN2B cell line (IC50 = 9.74 μM, DF = 26, r2 = 0.94), whereas it was ineffective (IC50 > 100 μM) in the GluN1/GluN2A cell line (Fig. 3A and C). Each point represents the mean ± SEM of 4–8 independent experiments. The IC50 values were determined by non-linear regression (Prism 5.0).

4. Discussion

The major aim of this work was to study the possible subunit/subtype specificity of the previously observed inhibitory effects of desipramine and fluoxetine on NMDA receptors. As the GluN2C and GluN2D subunits are significantly less abundant, except in some restricted brain regions, and their role in the hippocampus is still largely undetermined, we focused only on the ubiquitously expressed GluN2A and GluN2B subunits. Our observations that fluoxetine, in contrast to desipramine, displayed exactly the same inhibition pattern (Fig. 1A and B) as ifenprodil, a subtype selective GluN2B antagonist (Williams, 1993), suggested that fluoxetine is a selective GluN2B-containing receptor antagonist. This conclusion was verified during the experiments with the HEK 239 cell lines that stably expressed GluN1/GluN2A or GluN1/GluN2B subtypes. Desipramine equally inhibited NMDA-induced currents in both cell lines, whereas fluoxetine proved to be a selective GluN1/GluN2B receptor antagonist (Fig. 3). Taking into account the similarities in the clinical efficacy of desipramine and fluoxetine (Anderson, 2001), our data suggest that if NMDA receptors are involved in the development of the therapeutic response to these drugs, the effect must be at least partly mediated through GluN2B-containing NMDA receptors.

The composition of the GluN2 subunit has a major influence on the functional properties and the subcellular distribution of NMDA receptors (Erreger et al., 2005; Paoletti and Neyton, 2007). Previously, it has been shown that the expression of GluN2A subunits parallels synaptogenesis, and immunocytochemical analyses have indicated an exclusively synaptic localisation of this subtype, whereas GluN2B subunits are present in both synaptic and extrasynaptic locations (Li et al., 1998). Studies have suggested that the majority of extrasynaptic NMDA receptors are GluN2B-containing (Hardingham et al., 2002), but it has also been observed that both subtypes can be expressed at both synaptic or extrasynaptic sites (Liu et al., 2007; Thomas et al., 2006). Nevertheless, it still seems to be valid that most synaptic NMDA receptors are of GluN1/GluN2A, and the extrasynaptic NMDA receptor population is predominated by GluN2B-containing subtypes (Hardingham et al., 2002; Vizi et al., 2010). The subunit composition also determines the signalling pathways. GluN2A-containing and GluN2B-containing receptors play opposing roles in a number of physiological and pathological processes. Activation of the synaptic NMDA (mainly GluN1/GluN2A) receptors induces cAMP response element-binding protein (CREB) activity and the concomitant expression of the neuroprotective brain-derived neurotrophic factor (BDNF), which provides optimal conditions for neuroplastic changes. In contrast, stimulation of the extrasynaptic NMDA (mainly GluN1/GluN2B) receptors activates a dominant CREB shut-off pathway, which blocks BDNF expression and leads to cell death (Duman, 2004; Hardingham et al., 2002; Vanhoutte and Bading, 2003). A subsequent study has demonstrated that, independent of their synaptic or extrasynaptic locations, GluN2A-containing receptors mediate cell survival signalling, whereas the activation of GluN2B-containing receptors results in increased neuronal apoptosis (Zhou and Baudry, 2006). It has been shown...
Fig. 1. Age dependence of the effect of the GluN2B-containing selective antagonist ifenprodil and antidepressants. (A) Drug application protocol and characterization of NMDA-evoked currents. The cells were exposed to NMDA (10 µM) alone for 20 s, then to NMDA plus antagonists or antidepressants at different concentrations for 40 s, finally to NMDA alone again for 30 s. Representative traces show the effect of the NMDA antagonist MK-801 (1 µM) and Mg²⁺ (5 mM). Scale bars represent 10 s time and 50 pA currents. (B) Representative traces showing the effect of three inhibitors (gray bars): ifenprodil (10 µM; upper panel), fluoxetine (FLX, 30 µM; middle panel) and desipramine (DMI, 30 µM; lower panel) on currents evoked by 10 µM NMDA (black bars) in cortical neurons cultured for various amounts times: 6 DIV (left panel), 12 DIV (middle panel) and 20 DIV (right panel). Scale bars: 10 s, 50 pA. (C) Summarised data on the age dependence of the compounds. Desipramine and fluoxetine were applied at 30 µM, and ifenprodil was applied at 10 µM. Each point represents the mean ± SEM of 3–6 independent experiments.
(Chang et al., 2006) that chronic treatment with fluoxetine upregulates hippocampal CREB activation and causes increased BDNF expression that is associated with neuroprotective action. Therefore, it seems likely that overactivation of GluN2B-containing receptors plays a crucial role in these processes (Gogas, 2006). Likewise, inhibition of this subtype (Chang et al., 2006) may enhance neuroplasticity and cellular resilience. Indeed, an essential effect of antidepressants is to promote neurogenesis and synaptogenesis to thereby restore neuronal connectivity (Castren, 2005; Castren et al., 2007; Manji et al., 2003; Maya Vetencourt et al., 2008), which indicates that neuroplasticity may be an important target for the therapy of mood disorders (Krystal et al., 2009). In the last decade, several possible targets of antidepressants have been identified; e.g., it has been shown that monoamine uptake blocker-type antidepressants, including fluoxetine, also inhibit nicotinic acetylcholine receptors (Hennings et al., 1999, 1997; Szasz et al., 2007b) and sodium channels (Lenkey et al., 2006) and exert anti-inflammatory action (Lim et al., 2009) at a clinically relevant low micromolar concentration range (Besret et al., 1996; Bolo et al., 2000; Bymaster et al., 2002). Thus, the therapeutic effect most likely develops through the contribution of a number of drug–target interactions. Nevertheless, due to their pivotal role in cell death pathways, inhibition of GluN2B-containing receptors may be an important mechanism of neuroprotection and antidepressant action.

The unique selectivity of fluoxetine for GluN2B-containing receptors may have further important clinical implications. Any CNS disorder in which neuronal loss is caused by glutamate-induced excitotoxicity (e.g., acute ischemic brain damage or

![Fig. 2. Pharmacological validation of the HEK 293 cell lines that stably expressed rat recombinant NMDA receptor subtypes. The GluN2A selective NVP-AAM077 and the GluN2B selective ifenprodil were applied at 10 nM and 10 μM, respectively. Each bar represents the mean ± SEM of 4-5 independent experiments. Data were analysed by a two-tailed Student’s t-test; **p < 0.01 compared with ifenprodil, ###p < 0.001 compared with NVP–AAM.](image)

![Fig. 3. Inhibitory effect of desipramine (DMI) (A and B) and fluoxetine (FLX) (A and C) on the NMDA-evoked currents in the HEK 293 cells that stably expressed rat recombinant NMDA receptors with GluN1a/GluN2A or GluN1a/GluN2B subunit compositions. (A) Representative traces showing the effect of DMI (30 μM, left panels) and FLX (30 μM, right panels). Scale bars: 5 s, 50 pA. The cells were stimulated by NMDA (10 μM; black bars), desipramine and fluoxetine (gray bars) were washed in and out during the middle section of the agonist pulse as described in the Section 2. (B and C) Dose-response curves for DMI (B) and FLX (C). Each point represents the mean ± SEM of 4–8 independent experiments. The IC_{50} values were determined by non-linear regression (Prism 5.0).](image)
neurodegenerative diseases) has the theoretical potential to be treated by inhibiting NMDA receptor overactivation. However, clinical trials that have employed high-affinity NMDA antagonists, such as phencyclidine or ketamine, have so far failed due to a lack of efficacy and unexpected paradoxical side effects, such as neurodegeneration in certain brain regions (Ellison, 1995). These results can be explained by the opposing role of the two subtypes of NMDA receptors. Whereas inhibition of GluN2B-containing receptors could be beneficial, as it prevents neuronal cell death, the blockade of GluN2A-containing receptors impairs the activation of protective pathways. Therefore, the nonselective NMDA antagonists cannot be effective neuroprotective agents. Nevertheless, the central role of GluN2B-containing receptors in acute and chronic neurodegenerative processes has brought into focus the development of selective GluN2B-containing receptor antagonists, and the field is being actively pursued by leading pharmaceutical companies (Gogas, 2006; Mony et al., 2009).

In this paper, we have demonstrated that fluoxetine, which has been marketed for more than 20 years as an antidepressant, is able to selectively block GluN1/GluN2B receptors. Therefore, we propose that the acute or chronic neuroprotective properties of this drug or its appropriately modified derivatives should be explored. Our conclusion is strongly supported by the observations that fluoxetine employs neuroprotective action (Chang et al., 2006; Kim do et al., 2007; Lim et al., 2009).

During the past decade (Dam et al., 1996; Pariente et al., 2001) and more recently (Acler et al., 2009; Chollet et al., 2011; Zittel et al., 2008), randomised placebo-controlled clinical trials including patients with ischemic stroke and moderate to severe motor deficits showed that fluoxetine enhances motor recovery. In addition, fluoxetine has been shown to improve cognition after traumatic brain injury (Horsfield et al., 2002).

In summary, we have shown that both SSR1 fluoxetine and tricyclic desipramine are able to inhibit the GluN2B subunit-containing NMDA receptors at a clinically relevant, low micromolar concentration range. However, fluoxetine had no effect on the GluN1/GluN2A receptor subtype. As the clinical efficacy of these drugs is almost identical, our data suggest that the GluN2B-containing receptor subtype may be specifically involved in the pathophysiology of depression and the mechanism of action of antidepressants. The selective inhibitory effect of fluoxetine on GluN2B-containing receptors implies an exceptional neuroprotective potential for this drug and may provide a promising new direction for the development of novel neuroprotective agents.

Acknowledgement

This work was supported by a grant from the Hungarian Research Fund (NK 72959).

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