NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones

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NMDA receptors are present at glutamatergic synapses throughout the brain, and are important for the development and plasticity of neural circuits. Their subunit composition is developmentally regulated. We have investigated the developmental profile of functional synaptic NMDA receptor subunits in dopaminergic neurones of the substantia nigra pars compacta (SNc). In SNc dopaminergic neurones from rats aged postnatal day (P)7, ifenprodil inhibited NMDA-EPSCs with an estimated IC_{50} of 0.36 μM and a maximum inhibition of 73.5 $\pm$ 2.7% (10 μM), consistent with a substantial population of NR1/NR2B-containing diheteromeric receptors. UBP141, a novel NR2D-preferring antagonist, inhibited NMDA-EPSCs with an estimated IC_{50} of 6.2 μM. During postnatal development, the maximum inhibitory effect of 10 μM ifenprodil significantly decreased. However, NMDA-EPSCs were not inhibited by Zn^{2+} (200 nM) or potentiated by the Zn^{2+} chelator TPEN (1 μM), and the effect of UBP141 did not increase during development, indicating that NR2B subunits are not replaced with diheteromeric NR2A or NR2D subunits. The time course of the decay of NMDA-EPSCs was not significantly changed in ifenprodil at any age tested. Together, these data suggest that diheteromeric NR1/NR2A or NR1/NR2D receptors do not account for the ifenprodil-resistant component of the NMDA-EPSC. We propose that NR1/NR2B/NR2D triheteromers form a significant fraction of synaptic NMDA receptors during postnatal development. This is the first report of data suggesting NR2D-containing triheteromeric NMDA receptors at a brain synapse.

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Dopaminergic neurones of the substantia nigra pars compacta (SNc) project to the striatum and are required for the normal physiology of the basal ganglia; this dopaminergic projection is lost in Parkinson's disease (PD). Excitatory afferents to SNc dopaminergic neurones activate NMDA glutamate receptors (Mereu *et al.* 1991; Wu & Johnson, 1996) to induce burst firing (Johnson *et al.* 1992; Johnson & Wu, 2004), and NMDA receptors are required for the induction of long-term potentiation at these synapses (Bonci & Malenka, 1999). NMDA receptors may contribute to excitotoxic death of dopaminergic neurones (Doble, 1999; Blandini *et al.* 2000), and NMDA receptor antagonists have therapeutic potential in PD (Hallett & Standaert, 2004; Paoletti & Neyton, 2007).

The functional and pharmacological properties of NMDA receptors are determined by their subunit composition (Stern *et al.* 1992; Wyllie *et al.* 1996; Vicini *et al.* 1998; see Cull-Candy & Leszkiewicz, 2004 and Paoletti & Neyton, 2007 for review). NR2 subunits are the main determinants of NMDA receptor functional diversity, and the four NR2 subunits, NR2A–NR2D, each confer different properties. In brain neurones, diheteromeric NR1/NR2A and NR1/NR2B receptors predominate; NR2C subunits are mainly expressed in the cerebellum and NR2D subunits have been sparsely reported (Hrabetova *et al.* 2000; Vicini & Rumbaugh, 2000; Cull-Candy *et al.* 2001; Brickley *et al.* 2003; Cull-Candy & Leszkiewicz, 2004; Jones & Gibb, 2005; Borgland *et al.* 2006). Triheteromeric NMDA receptors (Sheng *et al.* 1994) composed of NR1 subunits and more than one type of NR2 subunit show unique properties (Chazot & Stephenson, 1997; Cheffings & Colquhoun, 2000; Chazot *et al.* 2002; Brickley *et al.* 2003; Cull-Candy & Leszkiewicz, 2004; Hatton & Paoletti, 2005), providing scope for further diversity of function, but triheteromeric

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NMDA receptors have not been widely reported in CNS neurones (Brickley *et al.* 2003).

In some brain regions, NMDA receptor subunit expression changes during postnatal development (Monyer *et al.* 1994; Wenzel *et al.* 1997). For example, in cortical and hippocampal pyramidal neurones NR2B subunits initially predominate but are later replaced or joined by NR2A subunits (Williams *et al.* 1993; Flint *et al.* 1997; Okabe *et al.* 1998; Stocca & Vicini, 1998; Tovar & Westbrook, 1999; Nase *et al.* 1999). This is important for the development and possibly for the functional plasticity of neuronal circuits (Katz & Shatz, 1996; Liu *et al.* 2004*a*,*b*; Lozovaya *et al.* 2004; Massey *et al.* 2004; but see Berberich *et al.* 2005).

Although SNc dopaminergic neurones express functional synaptic NMDA receptors (Mereu *et al.* 1991; Wu & Johnson, 1996), their subunit composition during postnatal development is not known. NR2D subunit mRNA and protein is expressed in the developing and adult rat brain, particularly in brainstem and diencephalon structures, including the SNc (Monyer *et al.* 1994; Buller *et al.* 1994; Dunah *et al.* 1996). NR2A and NR2B subunit proteins are present in low levels in the SNc of adult rats (Albers *et al.* 1999). Recently, we found that functional NR2B and NR2D subunits form somatic NMDA receptors, possibly as triheteromeric receptors, while no somatic NR2A subunits are present in SNc dopaminergic neurones from rats aged postnatal day (P)14 (Jones & Gibb, 2005). The absence of NR2A subunits was intriguing, as they are present by the second postnatal week in cortex, hippocampus and cerebellum (Monyer *et al.* 1994).

Following this work, we asked whether SNc dopaminergic neuronal synapses express possible triheteromeric combinations of NR2B and NR2D subunits but no NR2A subunits. We also asked whether NR2 subunit expression is regulated in SNc dopaminergic neurones during early postnatal development, as postnatal changes are seen in other brain regions, and differentiation of dopaminergic neurones continues through the first three postnatal weeks in rats (Prakash & Wurst, 2006). Therefore, the objective of this study was to determine which NR2 subunits form synaptic NMDA receptors in SNc dopaminergic neurones, and whether synaptic NR2 subunit expression is developmentally regulated.

Methods

Brain slice preparation

Brain slices were prepared from rats aged between P6–P22. Three age groups have been studied: rats aged around P7 (P6–P8), P14 (P13–P15) and P21 (P20–P22); the notations P7, P14 and P21 are subsequently used for clarity. Rats were killed by decapitation under halothane anaesthesia. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and with local ethical approval. Brains were rapidly removed into ice-cold sucrose-based Ringer solution of the following composition (mM): sucrose 206, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 1, $MgCl_2$ 5, and D-glucose 25. Horizontal slices (300 μm) containing the midbrain were prepared (DTK-1000 microslicer, Dosaka, Japan) and held after cutting in a submersion chamber containing artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 119, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 2.5, $MgCl_2$ 6, D-glucose 25, at 30°C and saturated with 95% O_2 and 5% CO_2.

Electrophysiological recordings

Slices were transferred to a recording chamber 1–6 h after preparation and continuously perfused at 2–3 ml min^{-1} with ACSF (as detailed above but containing 1.3 mM $MgCl_2$ and 10 mM glucose) at 29–31°C, saturated with 95% O_2 and 5% CO_2. Slices and individual cells were visualized using an Olympus BX51W microscope with differential interference contrast optics. A bipolar stainless steel electrode (Frederick Haer and Co., USA) was placed just rostral to the SNc, and stimuli (100 μs duration; amplitude 100–400 μA) were applied at 10 s intervals. Whole-cell patch-clamp recordings were made using pipettes of 1–3 MΩ resistance when filled with intracellular solution (mM): caesium methane sulphonate 117, NaCl 2.8, $MgCl_2$ 3, Hepes 20, MgATP 2, Na_3GTP 0.3, EGTA 5, $CaCl_2$ 0.5, pH 7.2. Dopaminergic neurones comprise ∼90% of the neuronal population in SNc (Margolis *et al.* 2006), and were identified in this study by the presence of a time-dependent, hyperpolarization-activated inward current in response to voltage steps from −60 mV to between −100 and −120 mV (Johnson & North, 1992; Jones & Gibb, 2005). Excitatory postsynaptic currents (EPSCs) recorded in SNc dopaminergic neurones were amplified using a HEKA EPC9 amplifier, low-pass filtered at 3 kHz and digitally sampled to a PC at 20 kHz using a Micro1401 interface (Cambridge Electronic Design, Cambridge, UK). The amplitude of the EPSC was measured at the peak unless otherwise stated, using the computer program Spike 2 (Version 4, Cambridge Electronic Design, Cambridge, UK).

The AMPA : NMDA ratio was determined by measuring the amplitude of the AMPA receptor-mediated EPSC (recorded in the presence of D-AP5) and the amplitude of the NMDA-EPSC (obtained by subtracting the AMPA-EPSC from the total EPSC), as shown in Fig. 1 (the average of 10 EPSCs in each condition was used for the calculation), and compared using one-way ANOVA. To determine the change in amplitude following addition of all other drugs, EPSCs were normalized to pre-drug control amplitudes. EPSC amplitudes before and after

drug within experimental groups were compared using a paired t test, while for comparisons between experimental groups, EPSC amplitude values (measured during a 10 min plateau between 10 and 30 min after drug addition) were compared using one-way ANOVA (with *post hoc* tests if $P < 0.05$) or an unpaired t test (with Welch's correction if the variances between the groups were unequal). All statistical tests were performed using GraphPad Prism (Version 4, San Diego, CA, USA). Antagonist inhibition data were fitted with a single site hyperbola (in GraphPad Prism) of the form:

$$y = (y_{\max} - y_{\min})\frac{\mathrm{IC}_{50}}{[I] + \mathrm{IC}_{50}} + y_{\min}$$

For measurement of the time constant of NMDA-EPSC decay, 10–20 EPSCs were averaged and the decay from the peak of the response to the plateau was fitted with a two-component exponential decay function (in GraphPad Prism):

$$I(t) = A_1 e^{-\frac{t}{\tau 1}} + A_2 e^{-\frac{t}{\tau 2}}$$

The weighted time constant (τ_W) was calculated as:

$$\tau_W = \tau_1\left(\frac{A_1}{A_1 + A_2}\right) + \tau_2\left(\frac{A_2}{A_1 + A_2}\right)$$

All combined data are shown as mean ± standard error (S.E.M.), the 'n' values reported refer to the number of

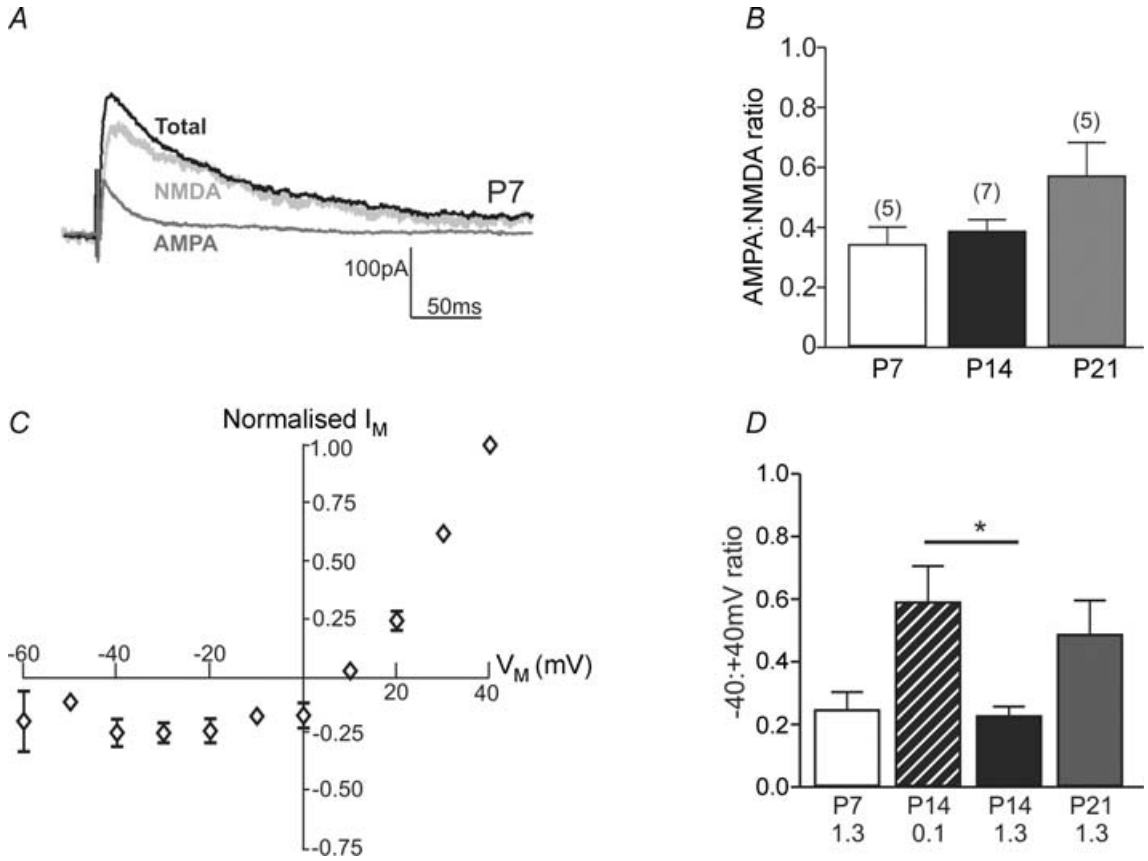

Figure 1. NMDA-EPSCs throughout early postnatal development
A, example recording of EPSCs at +40 mV from a SNc dopaminergic neurone in a slice from a rat aged P7. Traces are the average of 10 EPSCs. 'Total' EPSC (black trace) was recorded in picrotoxin (50 μM) and glycine (10 μM), 'AMPA'-EPSC (dark grey trace) was recorded in the presence of 50 μM D-AP5, and 'NMDA'-EPSC was obtained by subtracting the AMPA-EPSC from the Total EPSC. *B*, bar graph showing the ratio of the AMPA-EPSC to the NMDA-EPSC (obtained by measuring the responses shown in *A* at the three developmental stages tested. There was no significant difference during the first 3 weeks of postnatal development (ANOVA $P > 0.05$; 'n' in parentheses). *C*, current–voltage relationship for NMDA-EPSCs recorded in 1.3 mM extracellular Mg^{2+} and normalized to the response at +40 mV (rat aged P7). *D*, bar graph showing the ratio of the NMDA-EPSC recorded at −40 mV to that recorded at +40 mV in slices from rats aged P7, P14 and P21. Data for P14 slices are shown at 1.3 mM and 0.1 mM extracellular Mg^{2+}. ANOVA revealed a significant difference ($P < 0.05$), and the *post hoc* tests revealed a significant difference only between 0.1 and 1.3 mM Mg^{2+} at P14 (*$P < 0.05$, Bonferroni's multiple comparison *post hoc* test; 'n' in parentheses).

slices, and a critical P value of $P < 0.05$ was considered significant for the statistical tests used throughout this study.

Materials

All standard laboratory salts were obtained from BDH Laboratory Supplies (Poole, UK). Picrotoxin, DNQX (6,7-dinitroquinoxaline-2,3(1H,4H)-dione), D-AP5, $ZnCl_2$, TPEN (*N,N,N′,N′*-tetrakis-[2-pyridylmethyl]-ethylenediamine) and ifenprodil were obtained from Sigma-Aldrich (Dorset, UK). NVP-AAM077 ([(R)-[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)methyl]-phosphonic acid) was a gift from Dr Y. Auberson, Novartis Institutes for BioMedical Research, Basel, Switzerland.

Results

NMDA-EPSCs are present throughout postnatal development

In order to determine the contribution of NMDA receptors to excitatory synaptic transmission during postnatal development, evoked EPSCs were recorded from individual SNc dopaminergic neurones in midbrain slices from rats of different postnatal ages (P7, P14 and P21), and the NMDA receptor antagonist D-AP5 (50 μM) was applied (Fig. 1*A*). The AMPA : NMDA ratios at each postnatal week were: P7, 0.34 ± 0.06 ($n = 5$); P14, 0.39 ± 0.04 ($n = 7$); P21, 0.57 ± 0.11 ($n = 5$). The AMPA : NMDA ratio did not significantly change in SNc dopaminergic neurones during the three developmental periods (ANOVA $P > 0.05$; Fig. 1*B*).

NMDA receptor-mediated EPSCs in SNc dopaminergic neurones showed the expected sensitivity to Mg^{2+} ions. The current–voltage relationship for NMDA-EPSCs at P7 is illustrated in Fig. 1*C*, showing the characteristic negative slope conductance in the presence of Mg^{2+}. The ratio of the NMDA-EPSC recorded at −40 mV relative to the NMDA-EPSC recorded at +40 mV gives an indication of the voltage sensitivity of Mg^{2+}-dependent block and is shown in Fig. 1*D*; there was no significant difference in the ratio at the three ages.

NR2B subunit expression is developmentally regulated

To determine the contribution of NR2B subunits to synaptic NMDA receptors during postnatal development of SNc dopaminergic neurones, the NR2B subunit-selective antagonist ifenprodil (0.1–10 μM) was used. At selective concentrations, ifenprodil causes a maximum inhibition of 80–90% of diheteromeric NR2B receptors (Williams, 1993; Neyton & Paoletti, 2006). In slices from P7 rats, ifenprodil caused a concentration-dependent inhibition of NMDA-EPSCs; the effect of a maximal concentration of ifenprodil (10 μM; $73.5 \pm 2.7\%$ inhibition; $n = 6$; $P < 0.0001$) is shown in Fig. 2*A–D*. At P14, the effect of 10 μM ifenprodil in inhibiting NMDA-EPSCs ($55.1 \pm 2.7\%$ inhibition; $n = 17$; $P < 0.0001$) was significantly less than at P7 ($P < 0.01$); no further significant change occurred between P14 and P21 (Fig. 2*B* and *C*). These data suggest that there may be a reduction in the contribution of NR1/NR2B-containing diheteromeric receptors to NMDA-EPSCs during early postnatal development. The estimated IC_{50} values for ifenprodil at the three developmental ages, calculated from the fit of the inhibition data shown in Fig. 2*D*, were: P7, 0.36 μM; P14, 0.75 μM; P21, 0.94 μM. At lower ifenprodil concentrations, differences between age groups are not significant because the effect of ifenprodil is smaller while the variance between experiments is fairly constant.

NR2A subunits are absent throughout postnatal development

In cortical and hippocampal neurones, NR2A subunits replace or join NR2B subunits during early postnatal development (Williams *et al.* 1993; Flint *et al.* 1997; Stocca & Vicini, 1998; Tovar & Westbrook, 1999; Nase *et al.* 1999). The reduction in the inhibition by ifenprodil may be due to a concomitant increase in the contribution of NR2A subunits during postnatal development of SNc dopaminergic neurones. To address this, the effect of the NR2A subunit-preferring antagonists Zn^{2+} (Paoletti *et al.* 1997) and NVP-AAM077 (Auberson *et al.* 2002; but see Neyton & Paoletti, 2006; Frizelle *et al.* 2006), and the Zn^{2+} chelator, TPEN were investigated in slices from rats aged P13–P22. TPEN (1 μM) caused no significant potentiation of NMDA-EPSCs ($101.7 \pm 5.6\%$ of baseline, $n = 8$; $P = 0.59$; Fig. 3*B*, *D*), indicating either that Zn^{2+} ions are not present in our solutions or that the concentration of endogenous Zn^{2+} ions is not sufficient to inhibit NMDA-EPSCs. Addition of Zn^{2+} (200 nM) had no effect on NMDA-EPSCs when applied alone ($-8.3 \pm 10\%$ inhibition, $n = 6$; $P = 0.13$; Fig. 3*B*, *D*) or immediately following application of TPEN ($3.9 \pm 6.7\%$ inhibition, $n = 11$; $P = 0.21$). In contrast, Zn^{2+} (200 nM) inhibited NMDA-EPSCs recorded from hippocampal pyramidal cell layer neurones in slices from rats aged P20–P22 ($72.7 \pm 11.8\%$ inhibition, $n = 4$), serving as a positive control for Zn^{2+}. These data suggest that NR2A subunits do not contribute to functional synaptic NMDA receptors in SNc dopaminergic neurones. Application of low concentrations of NVP-AAM077 inhibited NMDA-EPSCs in a manner consistent with their predicted effect on synaptic NR2B-containing receptors (Frizelle *et al.* 2006), with 10 nM causing $15 \pm 2\%$ ($n = 9$;

$P < 0.001$) and 30 nM causing $38.7 \pm 3.8\%$ inhibition ($n = 10$; $P < 0.001$). These data are summarized in Fig. 3*C*,*D*.

NR2D subunits form functional synaptic NMDA receptors

We have previously reported that NR2D-containing NMDA receptors are present at extrasynaptic sites in SNc dopaminergic neurones (Jones & Gibb, 2005). In order to determine whether NR2D subunits also contribute to synaptic NMDA receptors, and whether the contribution of NR2D subunits is developmentally regulated, the NR2D subunit-preferring antagonist UBP141 (1–10 μM) was applied. Reported K_D values for UBP141 at NMDA receptor subtypes are: NR2A, 14.2 μM; NR2B, 19.3 μM; NR2C, 4.2 μM; NR2D, 2.8 μM (Morley *et al.* 2005). At a concentration (3 μM) which should have minimal effect on NR1/NR2B receptors, UBP141 inhibited the peak of NMDA-EPSCs in slices from P7 rats by $33.7 \pm 6.8\%$ ($n = 6$; Fig. 4*A*), and the slow NMDA-EPSC component (~200 ms after the stimulus) by $46.1 \pm 8.3\%$ ($n = 6$; $P = 0.28$ compared with peak). In slices from rats aged P14 or P21, the amount of inhibition with 3 μM UBP141 was not significantly different to that seen at P7 (Fig. 4*A*). The estimated IC_{50} values for UBP141, obtained from the fit of the inhibition data shown in Fig. 4*B*, were: P7, 6.2 μM; P14, 9.6 μM; P21, 9.7 μM. Together, these

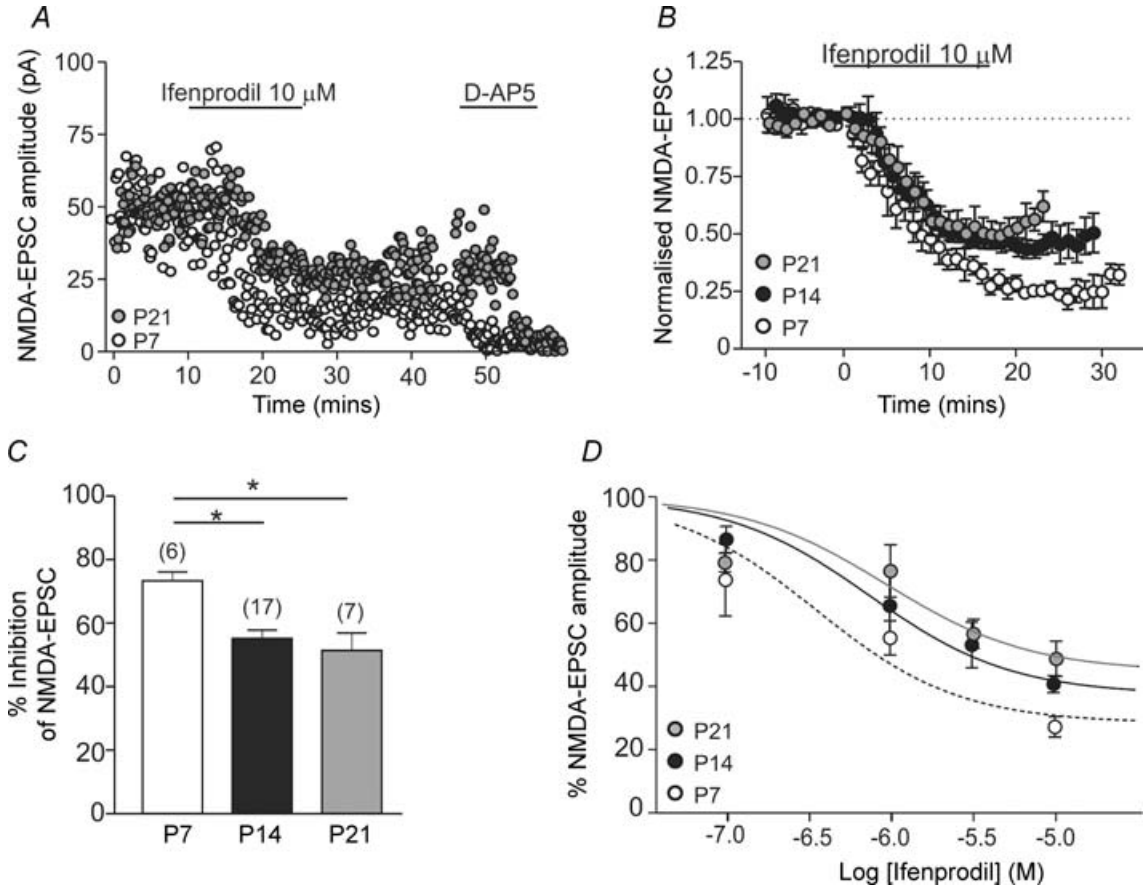

Figure 2. Developmental change in functional NR2B receptors
A, example recordings of NMDA-EPSC amplitude (pA) over time in a slice from a P7 rat (open circles) and a slice from a P21 rat (grey circles). Ifenprodil (10 μM) was added after a 10 min stable baseline was recorded, D-AP5 (50 μM) was added at the end of each experiment. *B*, combined data of NMDA-EPSCs over time showing the effect of ifenprodil (10 μM) at the three developmental stages tested (open circles, P7, $n = 6$; black circles, P14, $n = 17$; grey circles, P21, $n = 7$). In all three age groups ifenprodil caused a significant inhibition. *C*, bar graph comparing the mean inhibition (%) induced by 10 μM ifenprodil at P7 (measured 20–30 min post-drug), P14 and P21 (both measured 10–20 min post-drug). Significant difference detected with ANOVA; $^*P < 0.01$, Bonferroni's multiple comparison *post hoc* test ('*n*' in parentheses). *D*, inhibition curves showing the effect of increasing concentrations of ifenprodil on NMDA-EPSCs at the three developmental stages. The fit of the data (see Methods) was used to obtain the IC_{50} values reported in the text. Numbers of slices at each concentration and age are as follows: P7 (open circles) 0.1 μM, $n = 4$; 1 μM, $n = 5$; 10 μM, $n = 6$; P14 (black circles) 0.1 μM, $n = 6$; 1 μM, $n = 5$; 3 μM, $n = 6$; 10 μM, $n = 17$; P21 (grey circles) 0.1 μM, $n = 4$; 1 μM, $n = 5$; 3 μM, $n = 4$; 10 μM, $n = 7$.

data suggest that NR2D subunits form synaptic NMDA receptors in SNc dopaminergic neurones, and that the functional contribution of NR2D-containing receptors to NMDA-EPSCs is not obviously developmentally regulated.

NR2B and NR2D subunits may form triheteromeric synaptic NMDA receptors

Rather than NR2B subunits being replaced with NR2A subunits or more NR2D subunits, the pharmacological properties of NMDA-EPSCs suggest that NR2B and NR2D subunits may increasingly form triheteromeric receptors that show a decrease in the maximum inhibition by ifenprodil. Previously, we have reported evidence for triheteromeric NR1/NR2B/NR2D receptors at extrasynaptic sites in P14 SNc dopaminergic neurones (Jones & Gibb, 2005). To investigate this further, we have looked at two properties of NMDA-EPSCs.

Firstly, we have measured the time constant of the decay of NMDA-EPSCs before and after the addition of ifenprodil. If the sizeable ifenprodil-insensitive component at P14–P21 was dominated by NR2A receptors, the rate of decay should accelerate in ifenprodil, while if NR2D subunits dominate the rate of decay should be slowed by ifenprodil (Hestrin, 1992; Flint *et al.* 1997; Vicini *et al.* 1998; Stocca & Vicini, 1998; Cull-Candy & Leszkiewicz, 2004). Figure 5*A* shows example NMDA-EPSCs at P14 recorded in control conditions and in the presence of ifenprodil (10 μM), scaled for comparison. At all three ages, the weighted decay time constant of control NMDA-EPSCs was not significantly different in the presence of ifenprodil (Fig. 5*B*), suggesting that the ifenprodil-insensitive

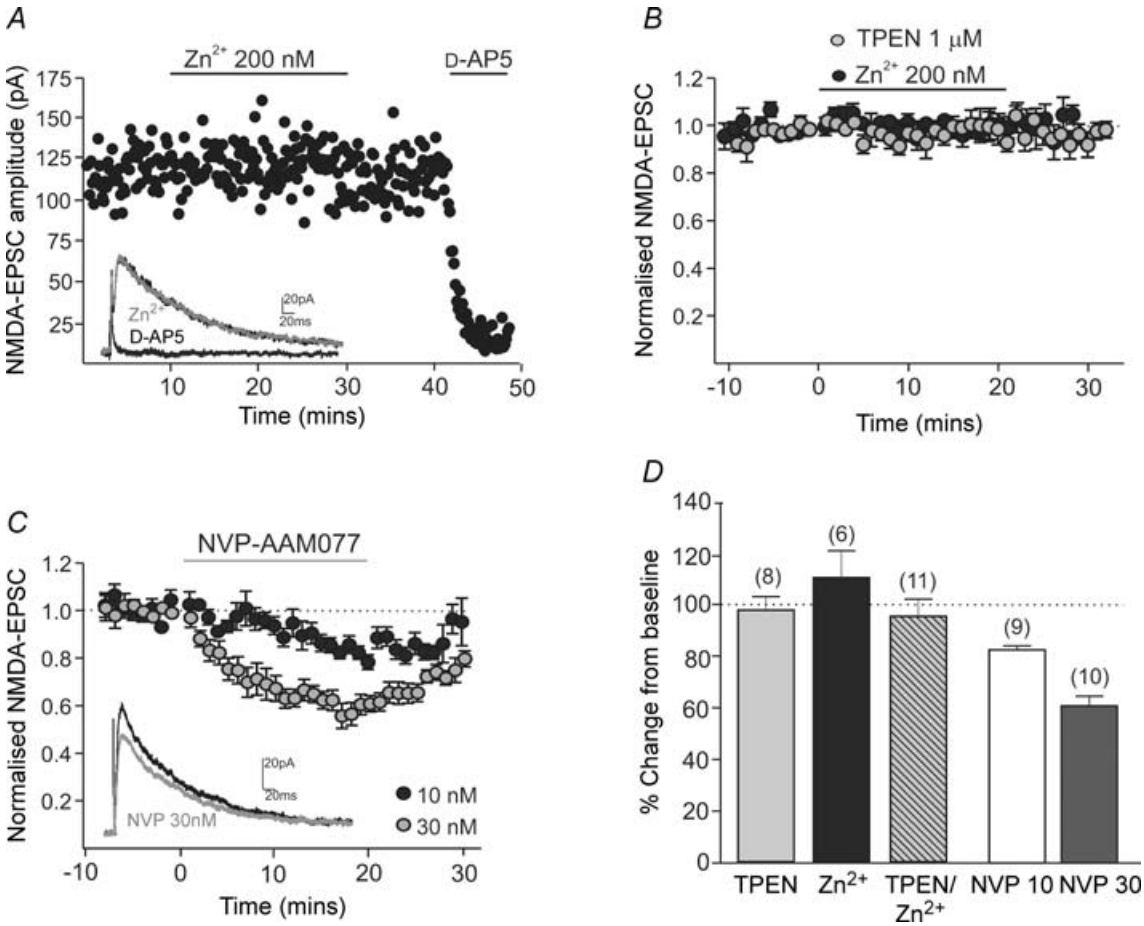

Figure 3. NR2A-containing receptors are absent during early postnatal development
A, example recording of NMDA-EPSC amplitude (pA) over time in a slice from a P14 rat. Zn^{2+} (200 nM) was added after a 10 min stable baseline, D-AP5 (50 μM) was added at the end of the experiment. Inset shows NMDA-EPSCs in control, Zn^{2+} and D-AP5. *B*, combined data of NMDA-EPSCs over time showing the lack of effect of TPEN (1 μM; grey circles, $n = 8$) and Zn^{2+} (200 nM; black circles, $n = 6$) in slices from rats aged P13–P22. *C*, combined data of NMDA-EPSCs over time showing the effect of NVP-AAM077 (10 nM; open circles, $n = 9$; grey circles, 30 nM, $n = 10$) in slices from rats aged P13–P22. Inset shows NMDA-EPSCs in control and 30 nM NVP-AAM077. *D*, bar graph summarizes the lack of effect of TPEN and Zn^{2+} alone or Zn^{2+} following a baseline in TPEN, and the modest effects of NVP-AAM077, suggestive of an effect on NR2B-containing receptors.

NMDA-EPSC at P14 is mediated by a similar NMDA receptor population to the total NMDA-EPSC. Consistent with this, the weighted decay time constant of NMDA-EPSCs remaining in 3 μM UBP141 at P14 (115 ± 38 ms, $n = 5$) was not significantly different to control values (106 ± 26 ms, $n = 5$, $P = 0.55$, paired t test). On the other hand, there was a trend towards a decrease in the weighted decay time constant of control NMDA-EPSCs during development, with a significant difference between P7 (152 ± 23 ms, $n = 6$) and P21 (68 ± 8 ms, $n = 6$; $P < 0.05$, Bonferroni's multiple comparison *post hoc* test).

Secondly, we have examined the current–voltage relationship to obtain the ratio of the NMDA-EPSC at -40 mV relative to $+40$ mV (an indicator of Mg^{2+} sensitivity) before and after application of ifenprodil, because NR2A and NR2B receptors have a higher Mg^{2+} sensitivity and a steeper voltage-dependent Mg^{2+} block than NR2C and NR2D receptors (Monyer *et al.* 1994; Kuner & Schoepfer, 1996; Qian & Johnson, 2006). No significant change was observed (Fig. 5*C* and *D*), indicating that the ifenprodil-insensitive component has a similar Mg^{2+} sensitivity to the total NMDA-EPSC.

Discussion

We have investigated the developmental profile of functional synaptic NMDA receptors present in SNc dopaminergic neurones. We propose that NR2B and NR2D subunits but not NR2A subunits are present throughout early postnatal development, and that the properties of synaptic NMDA receptors are developmentally regulated. Our data suggest that triheteromeric NMDA receptors composed of NR1/NR2B/NR2D subunits are important during postnatal development.

A developmental change in synaptic NR2B subunit expression

Ifenprodil is a non-competitive antagonist of NR2B-containing NMDA receptors that acts by enhancing proton inhibition (Mott *et al.* 1998). Ifenprodil has a higher affinity for recombinant receptors containing NR2B subunits and, at the concentrations used here (0.1–10 μM), has little effect on recombinant diheteromeric receptors composed of NR1 and NR2A, NR2C or NR2D subunits (Williams, 1993, 1995, 2001; Cull-Candy *et al.* 2001; Paoletti & Neyton, 2007). In SNc dopaminergic neurones, 0.1–10 μM ifenprodil reduced NMDA-EPSCs at all stages of postnatal development, indicating that NR2B-containing receptors are persistently present. However, ifenprodil caused a greater maximal effect in the first postnatal week; subsequently, the maximum effect of ifenprodil decreased, suggesting either that NR2B-containing receptors were replaced with NMDA receptors composed of other subunits, or that during development NR2B subunits form different proportions of triheteromeric receptors that show a lower maximal inhibition with ifenprodil (Chazot & Stephenson, 1997; Tovar & Westbrook, 1999; Chazot *et al.* 2002; Hatton & Paoletti, 2005).

The persistent presence of NR2B subunits during development could contribute to slow NMDA

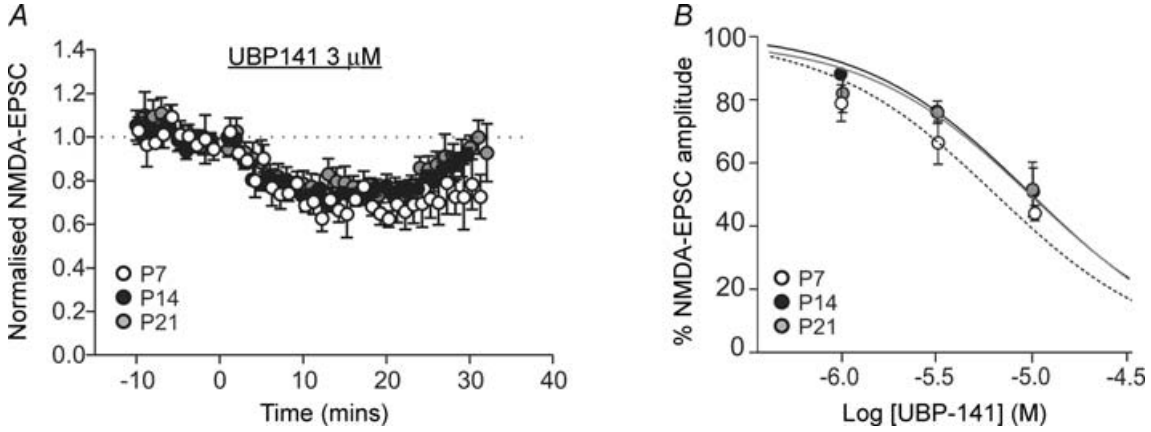

Figure 4. NR2D receptors are present throughout early postnatal development
A, combined data of NMDA-EPSCs over time showing the effect of UBP141 (3 μM) at the three developmental stages tested (open circles, P7, $n = 6$; black circles, P14, $n = 5$; grey circles, P21, $n = 4$). In all three age groups UBP141 caused a significant inhibition. *B*, inhibition curves showing the effect of increasing concentrations of UBP141 on NMDA-EPSCs at the three developmental stages. The fit of the data was used to obtain the IC_{50} values reported in the text. Numbers of slices at each concentration and age are as follows: P7 (open circles) 1 μM, $n = 6$; 3 μM, $n = 6$; 10 μM, $n = 4$; P14 (black circles) 1 μM, $n = 5$; 3 μM, $n = 5$; 10 μM, $n = 4$; P21 (grey circles) 1 μM, $n = 5$; 3 μM, $n = 4$; 10 μM, $n = 4$.

receptor-dependent signalling and coincidence detection (Nevian & Sakmann, 2004) until at least the third postnatal week, and may also be important for NMDA receptor-mediated excitotoxicity (Zhou & Baudry, 2006), as NR2B-selective NMDA receptor antagonists protect SNc dopaminergic neurones against neurotoxicity in experimental models of PD (Blanchet *et al.* 1999; Nash *et al.* 1999; Steece-Collier *et al.* 2000; Nash *et al.* 2000; Hallett & Standaert, 2004).

Functional synaptic NR2A subunits are absent

NMDA-EPSCs in SNc dopaminergic neurones were not affected by NR2A-selective pharmacological agents, Zn^{2+} ions and the Zn^{2+} chelator TPEN (Paoletti *et al.* 1997; Rachline *et al.* 2005). On the basis of these data, we conclude that NR2A subunits are absent in SNc dopaminergic neurones. This is consistent with our previous study of extrasynaptic NMDA receptors (Jones & Gibb, 2005). NVP-AAM077 is a NR2A-preferring

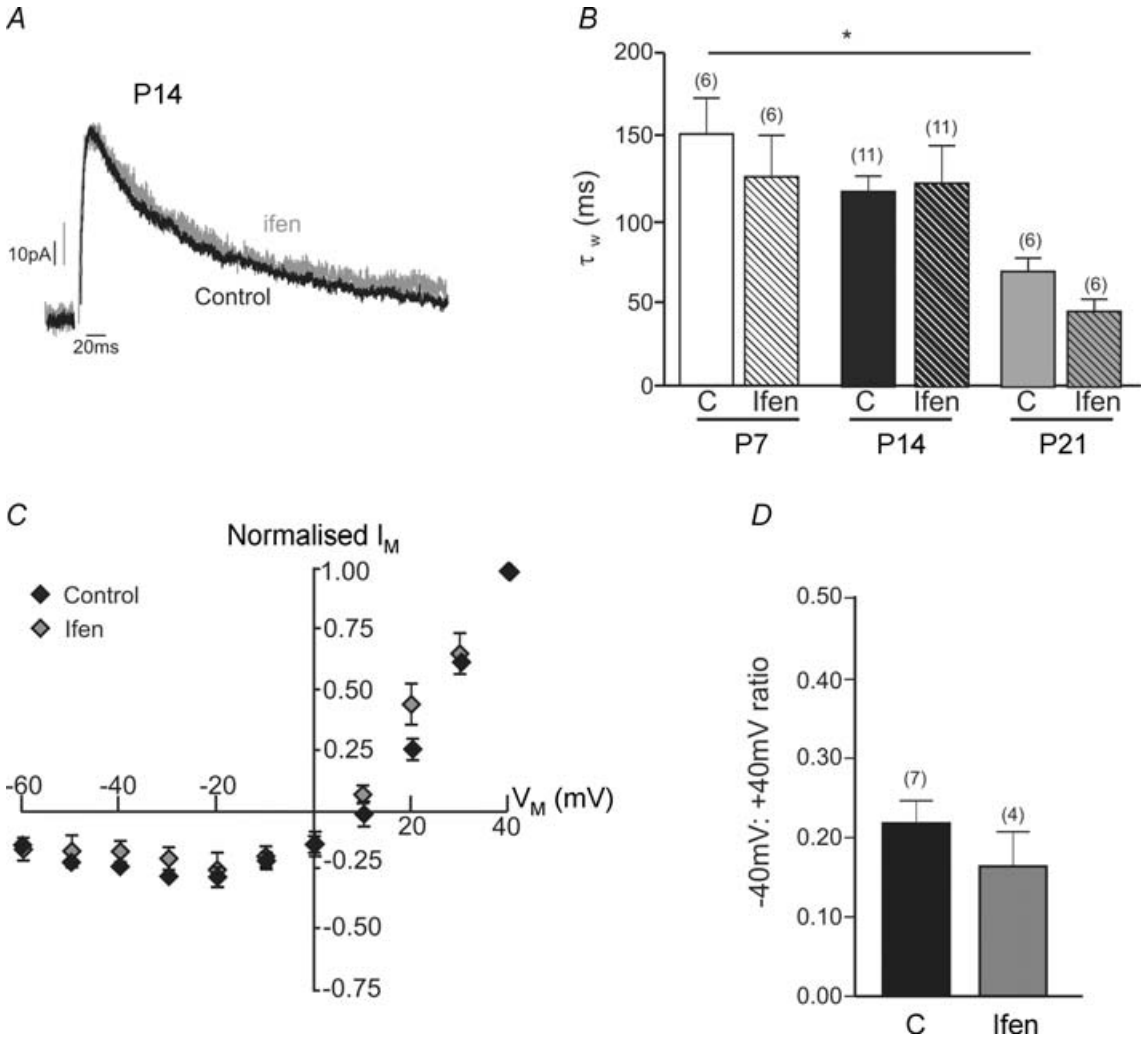

Figure 5. NR2B and NR2D subunits may form triheteromeric NMDA receptors
A, example recording of NMDA-EPSCs at +40 mV from a SNc dopaminergic neurone in a slice from a rat aged P14. Traces are the average of 20 NMDA-EPSCs. 'Control' EPSC (black trace) was recorded in picrotoxin, glycine and DNQX (10 μM), 'Ifenprodil' EPSC (grey trace; Ifen) was recorded in the presence of 10 μM ifenprodil, and the two traces have been scaled for comparison of the decay. *B*, bar graph showing the time constant of the decay (weighted from a two-component exponential fit of the control NMDA-EPSC at P7, P14 and P21 and of the NMDA-EPSC component remaining in the presence of 10 μM ifenprodil at each age. ANOVA revealed a significant difference ($P < 0.05$); *post hoc* tests revealed a significant difference between control τ_W at P7 and P21 (*$P < 0.05$, Bonferroni's multiple comparison *post hoc* test; '*n*' in parentheses). *C*, current–voltage relationship for NMDA-EPSCs recorded in 1.3 mM extracellular Mg^{2+} (normalized to the response at +40 mV) in control conditions ($n = 7$) and in the presence of 10 μM ifenprodil ($n = 4$) in slices from P14 rats. *D*, bar graph showing the ratio of the NMDA-EPSC recorded at −40 mV to that recorded at +40 mV in control and ifenprodil.

Table 1. Fit of observed block of synaptic currents at P7, P14 and P21 to a model assuming synaptic receptors are a mixture of diheteromeric NR1/NR2B receptors, triheteromeric NR1/NR2B/NR2D receptors, and diheteromeric NR1/NR2D receptors

	P7			P14			P21		
	Predicted % of current	% EPSC block by ifenprodil	% block by UBP141	Predicted % of current	% EPSC block by ifenprodil	% block by UBP141	Predicted % of current	% EPSC block by ifenprodil	% block by UBP141
NR2B diheteromer	46.6	42.0	6.3	21.4	19.2	2.9	5.2	4.7	0.7
NR2B/NR2D triheteromer	53.4	26.7	18.6	78.6	39.3	27.4	92.6	46.3	32.3
NR2D diheteromer	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	1.1
Predicted block	—	68.7	24.9	—	58.6	30.3	—	51.0	34.1
Observed block	—	73.5	33.7	—	55.1	24.6	—	51.4	23.4

The model assumes that diheteromeric NR2B receptors are inhibited by 90% (Williams, 1993), triheteromeric NR2B/NR2D receptors are inhibited by 50% (Hatton & Paoletti, 2005), while diheteromeric NR2D receptors will be inhibited by 2% by 10 μM ifenprodil (Williams, 1995). The model assumes that 3 μM UBP141 will block diheteromeric NR2B receptors by 13.5% and diheteromeric NR2D receptors by 51.7% (K_d values of 19.3 and 2.8 μM respectively; Morley *et al*. 2005) and triheteromeric NR2B/NR2D receptors by 34.9%. The model was fitted to the data by minimizing the sum of squares of the differences between predicted and observed block.

antagonist at concentrations < 100 nM (Auberson *et al.* 2002; Neyton & Paoletti, 2006); 10 nM is predicted to cause $> 50\%$ inhibition of synaptic NR2A-containing receptors and $< 20\%$ inhibition of synaptic NR2B-containing receptors (Frizelle *et al.* 2006). In SNc dopaminergic neurones, 10 nM NVP-AAM077 caused only about 15% inhibition of the NMDA-EPSC, suggestive of an effect on NR2B- but not NR2A- containing receptors. NVP-AAM077 (50 nM) caused ~27% block of NMDA responses mediated by NR2B receptors expressed in HEK293 cells, and 19% inhibition of NMDA-EPSCs in hippocampal slices from mice lacking NR2A subunits (and therefore expressing only NR2B subunits; Berberich *et al.* 2005), effects smaller than that observed in our experiments. NVP-AAM077 also inhibits NR2D-containing receptors, with higher affinity than NR2B-containing receptors (Feng *et al.* 2004), and therefore NVP-AAM077 acting at NR2D-containing di- or triheteromeric receptors (see below) could contribute to the inhibitory effect in SNc dopaminergic neurones.

NR2D-containing synaptic NMDA receptors

Current commercial pharmacological antagonists have limited selectivity for NR2C or NR2D compared with the other subunits; however, when measured in electrophysiological assays against agonist binding to subunits expressed in *Xenopus* oocytes (with both agonist and antagonist binding to NMDA receptors at equilibrium), UBP141 shows 7-fold selectivity for NR2C and/or NR2D-containing receptors over NR2B-containing NMDA receptors and 5-fold selectivity over NR2A-containing receptors (Feng *et al.* 2004; Morley *et al.* 2005). NMDA-EPSCs in P7 SNc dopaminergic neurones were inhibited by UBP141 with an IC_{50} close to K_D values for NR2C- or NR2D-containing receptors. It should be noted that all of our experiments test antagonists against synaptic currents; thus, the binding of glutamate is not at equilibrium, which will cause an apparent increase in potency where the drug is a competitive antagonist. NR2D subunit mRNA (Monyer *et al.* 1994) and protein (Dunah *et al.* 1996) is expressed at high levels in the brain stem, while NR2C expression is mainly limited to the cerebellum; therefore, the presence of NR2D seems more likely given that we have previously found evidence that NR2D subunits form the low conductance NMDA receptors present at extrasynaptic sites in SNc dopaminergic neurones (Jones & Gibb, 2005). Reports of NR2D subunits at brain synapses are sparse (Hrabetova *et al.* 2000; Vicini & Rumbaugh, 2000; Cull-Candy & Leszkiewicz, 2004; Borgland *et al.* 2006).

The presence of NR2D subunits in synaptic NMDA receptors might be expected to confer a slow decay time constant (~1.5–4 s at room temperature; Monyer *et al.* 1994; Wyllie *et al.* 1998; Cull-Candy & Leszkiewicz, 2004), whereas the decay time constants at all ages in SNc dopaminergic neurones were substantially faster. If the proportion of NR2D-containing receptors was less than 30% then their low open probability (0.04; Wyllie *et al.* 1998) and slow activation ($\tau_{act} \approx 45$ ms; Wyllie *et al.* 1998) would mean that the NMDA-EPSC may be dominated by the NR2B-containing receptors. At room temperature, NR1/NR2B receptors have a weighted decay time constant of 287 ms; (Vicini *et al.* 1998); assuming a Q_{10} of 3.5, the weighted decay time constant for NR1/NR2B receptors at 30°C (the temperature for recordings in this study) would be 96 ms, similar to our measured values.

Interestingly, in most brain regions, levels of NR2D mRNA peak after the first postnatal week and then decrease (Monyer *et al.* 1994), but in SNc dopaminergic

neurones, the effect of UBP141 did not change with development.

Functional synaptic NMDA receptors in SNc dopaminergic neurones may be triheteromeric receptors composed of NR1, NR2B and NR2D subunits

One possible interpretation of our data is that NR2B and NR2D subunits form triheteromeric receptors, and that the proportions of triheteromers change during postnatal development. The interaction of ifenprodil-type ligands with the NMDA receptor is affected by the presence of other NR2 subunits in the complex, including NR2A (Chazot & Stephenson, 1997; Tovar & Westbrook, 1999; Chazot *et al.* 2002; Hatton & Paoletti, 2005) and NR2D (Brickley *et al.* 2003), and triheteromeric receptors have been shown to exhibit a lower maximum inhibition with ifenprodil than their diheteromeric counterparts. If all of the receptors (at any age) were triheteromeric, we would expect a maximal inhibition by ifenprodil of around 50% (Tovar & Westbrook, 1999; Hatton & Paoletti, 2005; although a triheteromeric NR1/NR2B/NR2D receptor has not been directly tested in this way). However, ifenprodil causes ∼74% inhibition at P7, making it likely that a combination of diheteromers and triheteromers contribute to the NMDA-EPSC at this age since if all receptors were diheteromeric NR2B receptors, we would expect a maximum of ∼90% inhibition by ifenprodil (Williams, 1993). At P14–P21, our maximum inhibition with ifenprodil was 50–55%, which is more consistent with a triheteromeric receptor (Tovar & Westbrook, 1999; Hatton & Paoletti, 2005). The decrease in the maximum effect of ifenprodil could also be explained by a decrease in triheteromers, if triheteromers are replaced by non-NR2B containing diheteromers. However, there is no apparent increase in NR2A-containing or NR2D-containing diheteromers, and so we feel that this is unlikely. Table 1 presents the results of a global least-squares fit of our proposed scheme to our data. The results suggest that there are few, if any, diheteromeric NR1/NR2D receptors at these synapses even though the proportion of these has been allowed to vary freely during fitting. In contrast, the proportion of diheteromeric NR1/NR2B receptors is predicted to decrease with development, while the proportion of triheretomeric NR1/NR2B/NR2D receptors increases from around 50% at P7 to 90% by P21.

The shapes of the current–voltage relationships for control and ifenprodil-insensitive NMDA-EPSCs are similar at P14, suggesting that the ifenprodil-insensitive NMDA-EPSC is not mediated by diheteromeric NR1/NR2D receptors with lower Mg^{2+} sensitivity. It should be noted that the reversal potential for NMDA-EPSCs was typically around +10 mV and therefore there is an uneven driving force on the inward currents at −40 mV compared with the outward currents at +40 mV such that the true Mg^{2+} sensitivity is underestimated in this study. In addition, the ifenprodil-insensitive component at P14 and P21 does not have a significantly accelerated or slowed decay time constant, suggesting that NMDA receptors in control and ifenprodil have similar kinetics and are composed of similar subunits.

There is no unequivocal test for triheteromeric receptors in native neurones, and so we cannot state with absolute certainty that triheteromers are present and that their expression is developmentally regulated. However, it is known that native triheteromeric NMDA receptors can arise from NR1, NR2B and NR2D subunits in rat brain (Buller *et al.* 1994; Buller & Monaghan, 1997; Dunah *et al.* 1998; Brickley *et al.* 2003; Cull-Candy & Leszkiewicz, 2004), and it has been proposed that the rat midbrain does not contain NR1/NR2D diheteromeric NMDA receptors but instead has NR2D subunits present as triheteromeric receptors (Dunah *et al.* 1998); thus, we propose that NR1/NR2B/NR2D-containing triheteromeric NMDA receptors may be present at all ages tested.

In summary, we have shown that NMDA receptors at excitatory synapses on SNc dopaminergic neurones contain NR2B and NR2D subunits, and exhibit properties that may reflect a triheteromeric molecular composition, a relatively rare synaptic NMDA receptor. The observed developmental changes in synaptic NMDA receptors may have important consequences for the function and plasticity of the dopaminergic circuitry.

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