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SYNAPTIC MECHANISMS

Functional heterogeneity of NMDA receptors in rat substantia nigra pars compacta and reticulata neurones

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Abstract

The nigra substantia nigra pars compacta (SNc) and substantia pars reticulata (SNr) form two major basal ganglia components with different functional roles. SNc dopaminergic (DA) neurones are vulnerable to cell death in Parkinson's disease, and NMDA receptor activation is a potential contributing mechanism. We have investigated the sensitivity of whole-cell and synaptic NMDA responses to intracellular ATP and GTP application in the SNc and SNr from rats on postnatal day (P) 7 and P28. Both NMDA current density (pA/pF) and desensitization to prolonged or repeated NMDA application were greater in the SNr than in the SNc. When ATP levels were not supplemented, responses to prolonged NMDA administration desensitized in P7 SNc DA neurones but not at P28. At P28, SNr neurones desensitized more than SNc neurones, with or without added ATP. Responses to brief NMDA applications and synaptic NMDA currents were not sensitive to inclusion of ATP in the pipette solution. To investigate these differences between the SNc and SNr, NR2 subunit-selective antagonists were tested. NMDA currents were inhibited by ifenprodil (10 μM) and UBP141 (4 μM), but not by Zn^{2+} (100 nM), in both the SNr and SNc, suggesting that SNc and SNr neurones express similar receptor subunits; NR2B and NR2D, but not NR2A. The different NMDA response properties in the SNc and SNr may be caused by differences in receptor modulation and/or trafficking. The vulnerability of SNc DA neurones to cell death is not correlated with NMDA current density or receptor subtypes, but could in part be related to inadequate NMDA receptor desensitization.

Introduction

Substantia nigra (SN) neurones form two important components of the basal ganglia. Dopaminergic (DA) neurones in the SN pars compacta (SNc) project to the striatum, and their dopamine output is essential for correct modulation of the flow of information through the basal ganglia. Neurones in the SN pars reticulata (SNr) form one of the output nuclei, providing GABAergic afferents to the thalamus, superior colliculus, and brainstem (Williams & Faull, 1985; Blandini *et al.*, 2000; Bolam *et al.*, 2000). SNc DA neurones degenerate early in Parkinson's disease (PD), whereas degeneration of SNr neurones occurs in the later stages of the disease and is less extensive (Hardman *et al.*, 1996). Although the processes underlying SNc DA neurone degeneration are not fully understood, NMDA receptor (NMDAR)-mediated excitotoxicity may be a contributing factor (Choi, 1988; Blandini *et al.*, 2000; Hallett & Standaert, 2004). Excitatory afferents, capable of activating synaptic NMDARs in the SNc and SNr (Mereu *et al.*, 1991; Wu & Johnson, 1996), originate from several regions, including the subthalamic nucleus, and these are overactive in PD and in animal models of PD, implying that there is increased excitatory drive to the SN in PD (Bergman *et al.*, 1994; Obeso *et al.*, 2000). Furthermore, NMDARs may boost Ca^{2+} transients generated by action potentials (Putzier *et al.*, 2009), and so contribute to Ca^{2+}-induced metabolic stress of DA neurons (Chan *et al.*, 2009; Surmeier *et al.*, 2010).

NMDARs are hetero-oligomeric membrane proteins, with each receptor being composed of four subunits (Cull-Candy & Leszkiewicz, 2004). Three subunit families have been identified: NR1, NR2 (NR2A, NR2B, NR2C, and NR2D) and NR3 (NR3A and NR3B). Subtypes of NMDARs can be distinguished by their functional properties, such as single-channel properties, kinetic behaviour, and sensitivity to different drugs and modulators, with, for example, NR2A and NR2B having higher Ca^{2+} permeability than NR2C and NR2D (see Cull-Candy & Leszkiewicz, 2004; Paoletti & Neyton, 2007 for review). In addition, the mechanisms controlling receptor trafficking and thereby the density of receptors on the cell surface are dependent on NR2 subunit composition (Pérez-Otaño & Ehlers, 2005; Groc *et al.*, 2006; Lau & Zukin, 2007). NR2-dependent interactions with scaffolding proteins control the targeting of NMDARs to the postsynaptic density (Newpher & Ehlers, 2008; Stephenson *et al.*, 2008), whereas the extrasynaptic receptor population is an important determinant of the response to excitotoxic insults (Soriano & Hardingham, 2007; Martel *et al.*, 2009) and the kinetics of exchange with synaptic receptors (Groc *et al.*, 2006; Newpher & Ehlers, 2008).

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We have found evidence for NR2B-containing and NR2D-containing NMDARs at both extrasynaptic (Jones & Gibb, 2005) and synaptic (Brothwell *et al.*, 2008) sites of SNc DA neurones. A proportion of these receptors appear to have an unusual triheteromeric NR1–NR2B–NR2D subunit composition, which is developmentally regulated. Given that the SNc and SNr have functionally different roles in health and disease, the aim of this study was to investigate the properties of NMDARs in the two SN nuclei [at postnatal day (P) 7 and P28].

Materials and methods

Brain slice preparation

Sprague-Dawley rats (at P7 or P28) were deeply anaesthetized with isoflurane, and decapitated in accordance with the Animals Scientific Procedures Act, UK (1986); the brain was then removed into ice-cold oxygenated slicing solution containing: 100 mM sucrose; 62.5 mM NaCl; 2.5 mM KCl; 1 mM $CaCl_2$; 4 mM $MgCl_2$; 25 mM $NaHCO_3$; 1.25 mM NaH_2PO_4; and 25 mM glucose (pH 7.4 when saturated with 95% O_2/5% CO_2). In some preparations from P7 and P28 rats, kynurenic acid (0.26 mM), taurine (0.02 mM) and pyruvic acid (2 mM) were added to the slicing solution. Coronal or horizontal brain slices (300 μm thick) were prepared using a Dosaka DTK-1000 Vibroslicer (Kyoto, Japan). Slices containing the midbrain SN region were kept in oxygenated solution containing 125 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 25 mM $NaHCO_3$, NaH_2PO_4 and 25 mM glucose at room temperature for 1–6 h before use.

Whole-cell recordings from SN neurones

Slices were placed in a recording chamber on the stage of a Zeiss Axioskop FS upright microscope (Oberkochen, Germany), and viewed at a final magnification of × 600 with infrared Nomarski differential interference contrast optics. During whole-cell current recordings from coronal slices, slices were continuously bathed in the same solution used for storing the slices (without added $MgCl_2$ and with 10 μM bicuculline, 10 μM strychnine and 100 μM tetrodotoxin from Ascent Scientific, Bristol, UK) at room temperature (22–24°C) while synaptic currents were recorded from horizontal slices in the absence of tetrodotoxin and with 1.3 mM $MgCl_2$ at 30°C. Patch pipettes were made from thick-walled borosilicate glass (GC150F; Harvard Apparatus, UK), fire polished to a final resistance of 4–7 MΩ when filled with a solution containing 140 mM CsCl, 10 mM HEPES, 10 mM EGTA, 10 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 29.53 mM CsOH (pH 7.2), with 2 mM Na_2ATP and 0.3 mM NaGTP (all from Sigma, Sigma-Aldrich, Dorset, UK).

Recordings were made from visually identified neurones in the SNc and SNr. SNc neurones were identified on the basis of the presence of a clear, time-dependent, hyperpolarization-activated inward current (I_h) in whole-cell mode (Fig. 1A) in response to a voltage step from −60 to −120 mV, as described previously (Johnson & North, 1992; Lin & Lipski, 2001; Jones & Gibb, 2005). P28 SNc cells had an average I_h relaxation amplitude of 325 ± 25 pA and activation time constant (τ) of 742 ± 42 ms (at P7, amplitude = 224 ± 36 pA, and τ = 867 ± 78 ms). Although some cells classified as SNr neurones on the basis of the absence of significant I_h may have been located in the SNc, these are likely to be few in number, as ∼90% of I_h-positive neurones in the rat SNc are DA (Margolis *et al.*, 2006).

Cells were voltage-clamped, and whole-cell currents (at −60 mV) or synaptic currents (at +40 mV) were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA), filtered at 1 kHz, and digitized at 10 kHz using a Micro 1401 interface (Cambridge Electronic Design, Cambridge, UK). Cell membrane capacitance and series resistance were estimated using the series resistance compensation circuit of the Axopatch 200B. Series resistance compensation between 70 and 90% was used. Whole-cell NMDA currents were evoked by adding NMDA (10, 50 or 200 μM; Sigma) and glycine (10 μM; Sigma) to the solution perfusing the recording chamber, and rapid (rise time, ∼5 s) NMDA responses were obtained by first 'cleaning' the cell body as described by Edwards *et al.* (1989), and then, after obtaining the whole-cell configuration, switching to agonist solution for 15 s (Fig. 1B), using a solenoid valve system (c-Flow; CellMicroControls, VA, USA) regulating solution flow to a theta-glass applicator placed 500 μm above the surface of the slice. These responses had 10–90% rise times of 3–5 s. Synaptic currents were evoked with a bipolar stainless steel electrode (Frederick Haer and Co., USA) and stimuli (100 μs in duration; amplitude, 100–400 μA) applied at 5-s intervals. The subunit composition of NMDARs was investigated by adding ifenprodil (Sigma), which is a non-competitive NR2B-preferring antagonist (Williams, 1993; Mott *et al.*, 1998), or UBP141, which shows a five-fold to seven-fold selectivity for NR2D vs. NR2A and NR2B (Morley *et al.*, 2005), to the agonist solution. After a slice had been exposed to NMDA, antagonist, or NMDA plus antagonist, the slice was discarded.

Electrophysiological measurements

NMDA currents were recorded using WinEDR v. 2.5.9 software (Dempster, 2001) (V2.2.3 available at http://spider.science.strath.ac.uk/PhysPharm/showPage.php?pageName=software_ses). Whole-cell mean NMDA current (I_{NMDA}) was measured during the steady-state response to NMDA or to NMDA with antagonist. Synaptic currents were acquired and analysed using Spike 2 (Version 4) software (Cambridge Electronic Design). To obtain the amount of the NMDA component relative to the AMPA component of synaptic currents, D-AP5 (50 μM; Sigma) was added to the perfusate and the remaining AMPA receptor-mediated excitatory postsynaptic current (EPSC) was subtracted from the total evoked current. To obtain a measure of the desensitization of the NMDAR-mediated EPSC with repeated stimulation, at 0.2 Hz the amplitude of the slow component of the total EPSC (recorded > 30 ms after the peak, and which is blocked by D-AP5) was measured at the start of the recording and then after 150 s. The decrease in the amplitude (desensitization) of the slow EPSC during this 150 s period is expressed as initial amplitude/amplitude at 150 s × 100%. In order to quantify desensitization during prolonged agonist application (500 s), I_{NMDA} at 300 s was measured as a fraction of I_{NMDA} at 30 s. To quantify desensitization in response to two consecutive agonist applications (of 60 s each), the fractional decrease in I_{NMDA} between the first (N1) and second (N2) NMDA application was measured [i.e. 1–(N2/N1)]. To quantify antagonist inhibition, two consecutive agonist applications (of 60 s each) were made, the second in the presence of the antagonist ($N2_{antag}$). The corrected fractional inhibition was calculated after taking the desensitization into account using

$$Fractional\ inhibition = 1 - \frac{N2_{antag}}{N1_{antag} \times \frac{N2_{control}}{N1_{control}}}$$

Data are expressed as mean ± standard error; the *n*-values refer to the number of slices. In this correction, the variance of

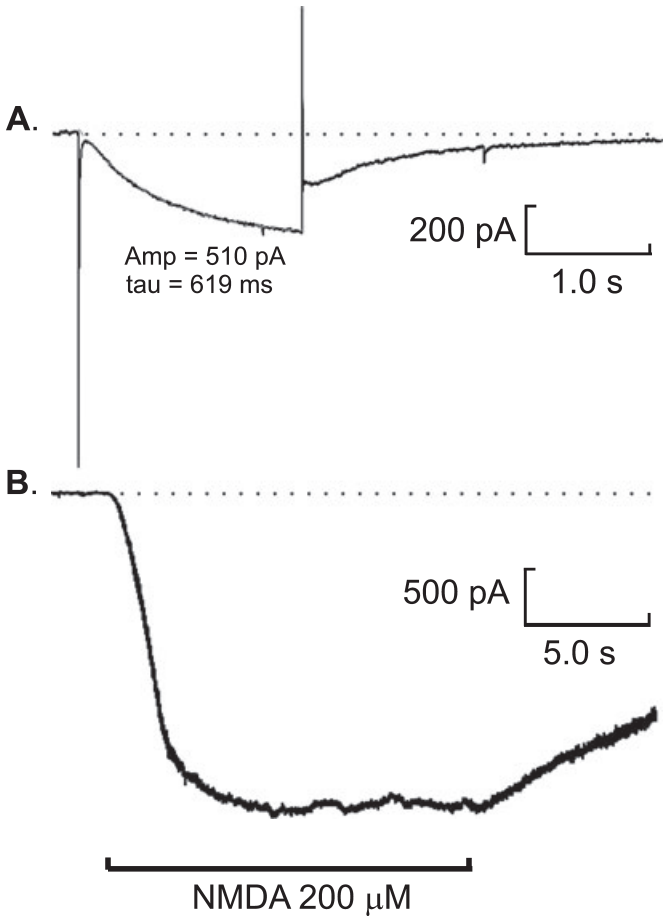

FIG. 1. Whole-cell I_h and rapid NMDA responses in SNc neurones. (A) Sample I_h response of a neurone in the SNc region of a brain slice from a P28 rat. Cells were voltage-clamped at −60 mV, and the membrane potential was stepped to −120 mV for 1.8 s in order to activate the I_h current. Superimposed (grey dashed line) is the fit of a single exponential that was used to determine the I_h amplitude and time constant. (B) Sample response of a P28 SNc neurone to rapid application of NMDA (200 μM for 15 s). In order to allow rapid access of the agonist, neurones were 'cleaned', as originally described by Edwards *et al.* (1989), before the whole-cell recording configuration was obtained.

$N2_{control}/N1_{control}$ contributes to the variance of the fractional inhibition. For comparisons of two paired groups of data, the *t*-test was used. For three or more groups of data with two variables, two-way ANOVAs with Bonferroni *post hoc* tests were used.

Results

In order to investigate the properties and subunit composition of NMDARs in SNc and SNr neurones and their sensitivity to ATP and GTP supplementation in the pipette solution, we used whole-cell patch clamp recordings of synaptic currents or responses to the agonist NMDA. Single prolonged agonist application (50 μM NMDA and 10 μM glycine; 500 s in duration), briefer consecutive agonist applications (200 μM NMDA and 10 μM glycine; 60 s in duration, 300-s interval) or rapid agonist applications to the cell body region in the slice (200 μM NMDA and 10 μM glycine; 15 s in duration, 300-s interval) were performed. Differences between the SNc and SNr were investigated at P7 and P28 with or without ATP (2 mM) and GTP (0.3 mM) in the pipette solution (supplementation with ATP and GTP is denoted as 'ATP' in the figures).

NMDAR current density

The NMDA sensitivity of SN neurones was initially tested at an approximate half-maximal agonist concentration ($[A]_{50}$) of NMDA (50 μM with 10 μM glycine, applied for 500 s; Fig. 2A and B). NMDA current per unit of capacitance (current density; pA/pF) in SNc and SNr neurones is illustrated in Fig. 2Ci and ii. Cell membrane capacitance was significantly greater in SNc neurones than in SNr neurones (P7 SNc, 53.5 ± 2.63 pF, $n = 44$; P7 SNr, 33.8 ± 1.41 pF, $n = 67$; P28 SNc, 49.4 ± 2.03 pF, $n = 35$; P28 SNr, 30.9 ± 1.78 pF, $n = 27$). Current densities measured at 10, 50 and 200 μM NMDA are plotted as concentration–response curves in Fig. 2B; when these are fitted with $[A]_{50}$ values and Hill coefficients (25 μM and 1.22, respectively) constrained to be the same for NMDA in both cell types and at both ages, the data suggest that, in P7 SNc neurones, NMDA receptor density was 66% of that in SNr neurones (Fig. 2B), whereas at P28 it was 56% (not shown). There was a significant difference in mean NMDA current density at P7 and at P28 ($P = 0.001$ and $P = 0.007$, respectively, ANOVA). Bonferonni's multiple comparisons test revealed that NMDA current density was lower specifically at P7 for SNc neurones with no ATP than for SNr neurones with ATP ($P < 0.05$), and for SNc neurones with ATP than for SNr neurones with ATP ($P < 0.01$), and at P28 for SNc neurones with no ATP than for SNr neurones with ATP ($P < 0.05$). Thus, current density was generally higher in SNr neurones, and this pattern was consistently observed at all three NMDA concentrations tested (10, 50 and 200 μM) in whole-cell recordings. The effect of intracellular ATP and GTP supplementation was measured on synaptic currents (EPSCs) from P28 SNc neurones. ATP had no significant effect on the AMPA/NMDA current ratio (Fig. 2D), a measure of the NMDA contribution to the EPSC. Thus, NMDAR-mediated whole-cell and synaptic currents in P28 SNc neurones show a similar lack of sensitivity to intracellular ATP and GTP supplementation.

NMDAR desensitization during prolonged activation

In order to investigate whether the time course of the response to prolonged NMDA application differed between the two neuronal populations, we measured the average NMDA current in 25-s intervals over a period of 400 s following the peak of the response to bath application of 50 μM NMDA (Fig. 3). These data demonstrate that, during prolonged NMDA application, although there was a modest desensitization of the response of P7 and P28 SNr neurones and P7 SNc neurones (Fig. 3Ai, Bi and Bii), there was no significant desensitization of P28 SNc neurones (Fig. 3Aii). There was a significant effect of ATP on the desensitization of the NMDA response only in P7 SNr neurones (Fig. 3Bi). We quantified the NMDA current amplitude at 300 s as compared with 30 s (Fig. 3C). In P7 SNc DA neurones with no ATP supplementation, NMDA current amplitude at 300 s was significantly less than at 30 s (0.76 ± 0.08, $n = 6$; $P < 0.05$, paired *t*-test), whereas when ATP was added there was no significant decrease (Fig. 3Cii). In P28 SNc neurones, NMDA responses did not desensitize (Fig. 3Ci).

In P7 SNr neurones, NMDA current amplitude at 300 s was significantly less than at 30 s (0.73 ± 0.04, $n = 8$; $P < 0.01$, paired *t*-test), whereas no significant decrease occurred when intracellular ATP was added (Fig. 3Bi and Ci). In P28 SNr neurones, the current amplitude significantly decreased during the NMDA response, to 0.63 ± 0.08, even with added ATP (Fig. 3Cii). There was a significant difference in mean NMDA current at 300 s as compared with at 30 s in SNc and in SNr neurones ($P = 0.042$ and $P = 0.021$, respectively, ANOVA). Two-way ANOVA followed by Bonferonni's multiple

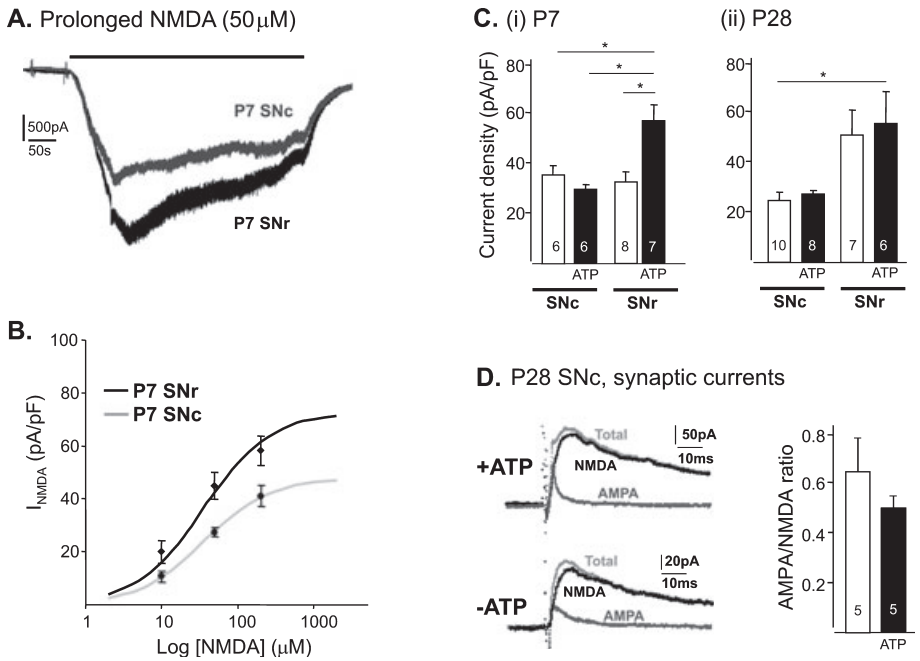

FIG. 2. NMDAR current density in SNc and SNr neurones. NMDA (50 μM) and glycine (10 μM) were applied for 500 s to midbrain slices, and current responses were recorded from SNc and SNr neurones. (A) Sample traces recorded from P7 SNc and SNr neurones (both with intracellular ATP supplementation), illustrating that current density is greater in SNr neurones. (B) Concentration–response curves for NMDA-activated currents (expressed per unit of capacitance, pA/pF) for SNc (black) and SNr (grey) neurones at P7 (left) and P28 (right). Solid lines show fits to the Hill–Langmuir equation, assuming that the two cell types have the same $[A]_{50}$ for NMDA [$[A]_{50}$ value of 25 μM, and Hill coefficient of 1.22 at both P7 and P28 (not shown)]. The ratios of the predicted maxima were 1.5 at P7 and 1.8 at P28. Experiments were performed with ATP in the pipette solution. (C) SNr neurones had significantly greater current density than SNc neurones at P7 and P28, as indicated by $*P < 0.05$ (Bonferroni multiple comparison *post hoc* tests following two-way ANOVA). (D) Examples of synaptic currents in P28 SNc neurones ± ATP. The AMPA receptor component of the current was recorded in the presence of 50 μM D-AP5. The AMPA/NMDA current ratio was not significantly different with or without ATP (unpaired *t*-test).

comparisons test demonstrated that when the decrease in NMDA current was stratified by SN nucleus, there was significantly less desensitization in SNc neurones at P28 than at P7 when ATP was not added ($P < 0.05$), and in SNr neurones (P7 vs. P28, added ATP, $P < 0.01$). When stratified by age, the decrease in NMDA current amplitude at 300 s was significantly different in SNc neurones than in SNr neurones, with or without added ATP, at P28 ($P < 0.05$) but not at P7.

Our observations suggest that in SNc and SNr neurones at P7, during prolonged receptor activation, NMDAR desensitization is greater at low intracellular ATP levels (Fig. 3C). Furthermore, at P28, SNr neurones showed more pronounced desensitization during prolonged NMDAR activation than SNc DA neurones. These data suggest that whereas SNr neurones develop NMDAR desensitization as they mature, these mechanisms are absent in DA SNc neurones, which may indicate that when ATP levels are compromised, SNc neurones lack a potentially protective mechanism.

NMDAR desensitization to consecutive agonist application

The dependence of NMDAR desensitization on NR2 subunit composition has been investigated with the use of heterologous expression of recombinant receptors: receptors containing NR2C and NR2D typically do not show glycine-independent desensitization, whereas those containing NR2A and NR2B do (Köhr *et al.*, 1994; Monyer *et al.*, 1994; Krupp *et al.*, 1996; Villarroel *et al.*, 1998; Wyllie *et al.*, 1998; Dravid *et al.*, 2008). The differences observed in NMDAR desensitization in SNc and SNr neurones described above might be explained by the presence of different NMDAR subunits in the two neuronal populations. Using a pharmacological approach to test this hypothesis, we performed paired agonist applications or agonist followed by agonist + antagonist applications to SNc and SNr neurones (see Materials and methods). ATP and GTP were included in the pipette solution for all subsequent experiments unless otherwise indicated.

In the first set of experiments, a near-maximal concentration of NMDA was applied (200 μM) with glycine (10 μM), either by bath application for 60 s or by rapid application to the cell soma for 15 s (N1); following a washout period of 5 min, a second NMDA + glycine application was made (N2). Sample NMDA current responses in P7 SNr neurones are shown in Fig. 4A, and responses to rapid application of NMDA to P28 SNc neurones are shown in Fig. 4C. When NMDA was applied by bath application for 60 s, N2 was significantly less than N1 in SNc and SNr neurones at both P7 and P28 (Fig. 4B; $P < 0.05$, paired *t*-tests). In P28 neurones, responses to brief NMDA applications showed no significant difference between N1 and N2 (Fig. 4D). When 60-s NMDA applications were made, there was a significant difference in the mean N2/N1 ratios ($P < 0.0001$, ANOVA). Bonferroni's test revealed a significant difference between P7 SNr neurones and P7 SNc neurones ($P < 0.001$), and between P7 SNr neurones and P28 SNc neurones ($P < 0.001$). Thus, the most prominent decrease in N2/N1 was observed in SNr neurones at P7. These results contrast with responses to rapid and relatively brief NMDA application, which showed no significant desensitization between two brief applications of 200 μM NMDA, 5 min apart, and no significant effect of ATP supplementation in the pipette solution in either SNc or SNr neurones ($P = 0.47$, ANOVA). Like the responses to rapid NMDA application, the amplitude of the slow (NMDAR-mediated) synaptic current changed by < 10% during repetitive stimulation, and was not sensitive to ATP supplementation ($P = 0.6$, unpaired *t*-test; Fig. 4E and F).

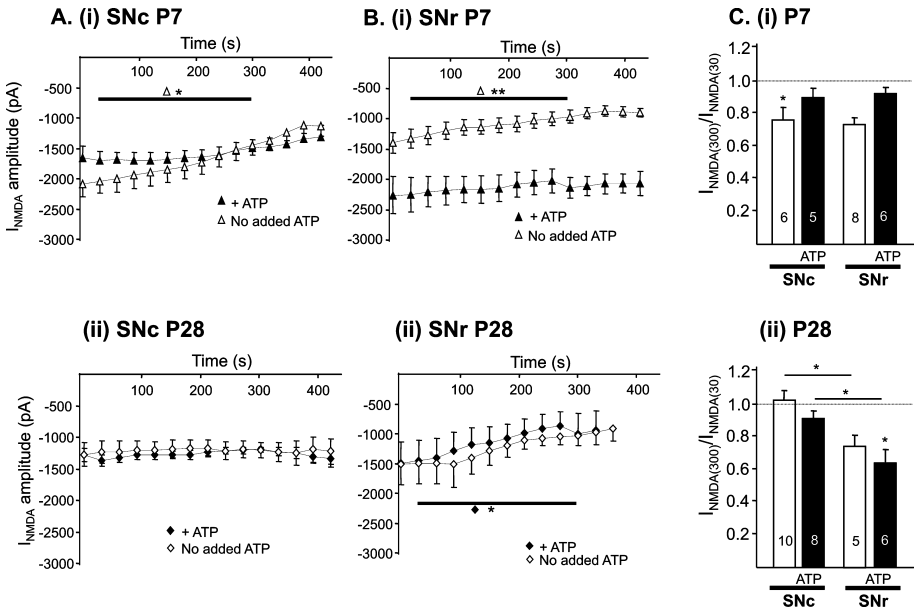

FIG. 3. NMDAR desensitization to prolonged agonist application: amplitude of responses in SNc neurones (A) and SNr neurones (B) to NMDA (500-s application) at P7 (i) or P28 (ii). Open symbols represent recordings made without ATP supplementation, and filled symbols represent recordings made with added intracellular ATP. In paired *t*-tests, there was a significant difference in the NMDA current amplitude at 300 s as compared with peak current in P7 SNc neurones with no added ATP (open triangles), P7 SNr neurones with no added ATP (open diamonds), and P28 SNr neurones with added ATP (filled diamonds). *$P < 0.05$ (paired *t*-test). (C) The change in NMDA current at 30 s as compared with NMDA current at 300 s was used to estimate NMDAR desensitization during prolonged agonist application. NMDA responses in P28 SNc neurones (Cii) did not significantly desensitize. At P28, SNr neurones desensitized more than SNc neurones (Cii). There was a significant decrease in desensitization between P7 and P28 (no ATP) in SNc neurones. In SNr neurones, there was a specific increase in desensitization between P7 and P28 when ATP was added (ANOVA with Bonferroni multiple comparison *post hoc* tests; *$P < 0.05$).

NR2B and NR2D but not NR2A are expressed in SNc and SNr neurones

In the second set of experiments, we used paired applications of NMDA for 60 s, spaced 300 s apart, to investigate the subunit composition of NMDARs in SNc and SNr neurones by including an antagonist in the second application. In order to separate the effect of the antagonist from the effect of desensitization, we used the ratios between NMDA responses evoked in two conditions: agonist (N1) followed by agonist (N2), and agonist ($N1_{antag}$) followed by agonist + antagonist ($N2_{antag}$) (see Materials and methods) to calculate the proportion of the current reduction that can be ascribed to receptor block.

There was no significant difference in the mean inhibition with ifenprodil (10 μM; Fig. 5A) across the groups ($P = 0.27$, ANOVA). At P7, percentage inhibition was 57.6% ± 8.2% ($n = 21$) in SNc neurones and 42.7% ± 7.0% ($n = 29$) in Snr neurones, whereas at P28, percentage inhibition was 48.1% ± 7.6% ($n = 7$) in SNc neurones and 68.5% ± 6.2% ($n = 6$) in SNr neurones. There was no significant difference in the mean inhibition with the NR2D-preferring antagonist UBP141 (4 μM; Fig. 5B) across the groups ($P = 0.57$, ANOVA).

In other rat brain regions, the expression of NR2A subunits increases between the first postnatal week and maturity. In order to test for this possibility, we investigated the Zn^{2+} sensitivity of NMDA responses at P28. In P28 SNc DA neurones, the average current response to NMDA (50 μM) in the presence of the Zn^{2+} chelator TPEN (1 μM) was −1303 ± 110 pA, and in the presence of $ZnCl_2$ (100 nM) it was −1263 ± 107 pA ($n = 7$; not shown). These data are consistent with our previous studies indicating that NR2A receptors are absent from SNc DA neurones during postnatal development (Jones & Gibb, 2005; Brothwell *et al.*, 2008). In P28 SNr neurones, the average current response to NMDA (50 μM) in the presence of TPEN (1 μM) was −1508 ± 144 pA, and in the presence of $ZnCl_2$ (100 nM) it was −1408 ± 122 pA ($n = 7$). Overall, our data are consistent with the presence of NR2B and NR2D but not NR2A subunits in SNc and SNr neurones at P7 and P28.

Discussion

Prolonged or excessive NMDAR activation is a potential mechanism contributing to cell death in SNc DA neurones. Both synaptic and extrasynaptic NMDARs are probable contributors to Ca^{2+} influx into the neurone, depending on the frequency of excitatory synaptic inputs, the degree of spillover of synaptic glutamate, and the degree of coincidence between tonic action potential firing of DA cells and synaptic current activation. We have investigated the properties and subtypes of synaptic (Brothwell *et al.*, 2008) and extrasynaptic NMDARs in SNc DA neurones as compared with SNr neurones. SNc DA neurones had a lower NMDA current density than SNr neurones, and there was less desensitization of NMDA responses in SNc neurones, either to sustained or to repeated NMDA applications. On the basis of their pharmacology, the subunit composition of NMDARs in SNc and SNr neurones was similar.

NMDAR current density in SNc vs. SNr neurones

NMDA current density was higher in SNr neurones than in SNc neurones, particularly when ATP was added. These data suggest that the NMDAR-mediated current in SNr neurones may more effectively contribute to Ca^{2+} entry and membrane depolarization than NMDARs in SNc neurones.

SNc DA neurones fire action potentials at 0.1–10 Hz in the absence of synaptic input, and this pacemaking activity maintains basal

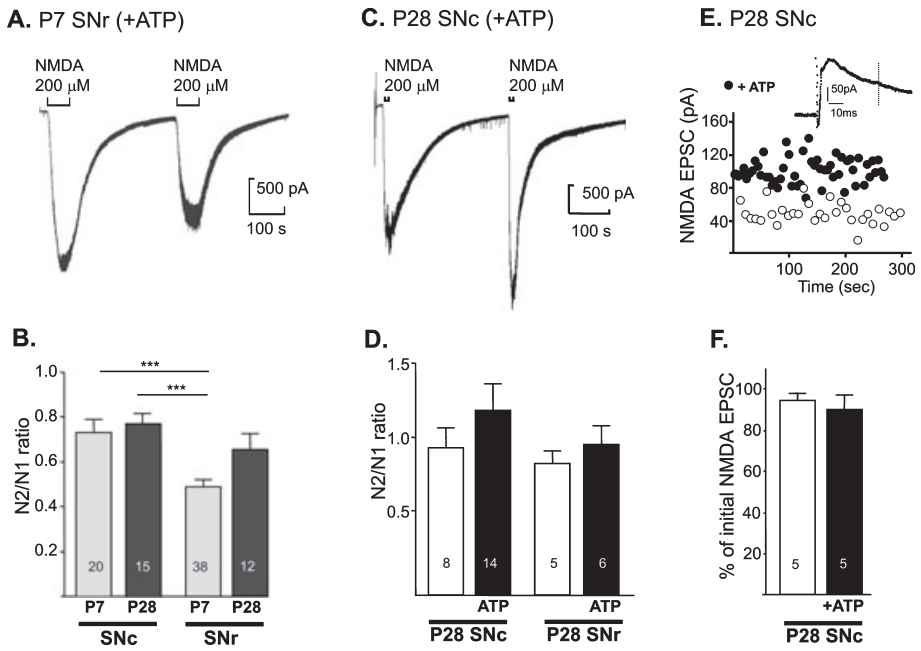

FIG. 4. NMDAR desensitization to consecutive NMDA applications. (A) Sample recording of the response to paired applications of NMDA (200 μM, 60 s indicated by bar, 5 min between each application) recorded from SNr neurones at P7. (B) Bar graphs show the ratio of the second and the first response to NMDA. (C) Sample recording of the response to brief (15 s), rapid application of NMDA recorded from an SNc neurone at P28. (D) Adding ATP did not significantly change the N2/N1 ratios of responses to rapid application. Ages and numbers of cells are indicated below and on the bars, respectively. ***$P < 0.001$. (E) Slow (NMDAR-mediated) EPSCs recorded from P28 SNc neurones show little evidence of desensitization with repeated activation at 0.2 Hz, with or without added ATP. (F) NMDA EPSC amplitude after 150 s of repetitive stimulation, as a percentage of the initial amplitude; there is no significant effect of adding ATP (unpaired *t*-test).

dopamine levels in the striatum (Grace & Bunney, 1984a,b). During excitatory synaptic stimulation, SNc DA neurones fire in phasic bursts of up to 100 Hz. SNr GABAergic neurones may fire at frequencies of > 200 Hz (Hikosaka & Wurtz, 1985; Hikosaka *et al.*, 2000; Ibanez-Sandoval *et al.*, 2007). These high-frequency depolarizations will very effectively relieve the voltage-dependent Mg^{2+} block of any active NMDARs. A higher current density in SNr than in SNc neurones may result from differences in receptor modulation or trafficking mechanisms. Our data suggest that SNc NMDARs are less affected by intracellular processes that are dependent on ATP than are SNr NMDARs.

Excitotoxic neuronal death is associated with overactivation of NMDARs, and NMDAR-mediated excitotoxicity is implicated in some neurological disorders, including PD (Blandini *et al.*, 2000; Kemp & McKernan, 2002; Gardoni & Di Luca, 2006). A higher density of NMDARs in a neurone might lead to a greater influx of Ca^{2+} and therefore increased vulnerability to excitotoxicity or metabolic stress (Chan *et al.*, 2009; Surmeier *et al.*, 2010). If NMDAR-mediated excitotoxicity is a contributing factor in the loss of SNc DA neurones in PD, our measurements of higher peak current density in SNr than in SNc neurones do not readily explain the preferential survival of SNr neurones; rather, our results suggest that SNr neurones might be more vulnerable to NMDAR-mediated excitotoxicity.

However, changes in glutamatergic drive to SNc DA neurones must also be considered, for example, glutamatergic excitatory synaptic input to SNc and SNr neurones from the subthalamic nucleus, which shows increased activity in PD patients (Blandini *et al.*, 2000; Obeso *et al.*, 2000). SNc DA neurones may respond unfavourably to this increase in excitatory drive and NMDAR activation, as compared with SNr neurones; this has not been investigated. Furthermore, Ca^{2+} influx depends not only on peak current but also on the duration of NMDAR activation, which is determined by the receptor kinetics and, during prolonged or repeated receptor activation, by desensitization. The fact that we found the receptor pharmacology to be not significantly different between SNc and SNr neurones suggests that synaptic receptors in SNc and SNr neurones may be similar, but this need not be the case, because the whole-cell currents measured in this study encompass both synaptic and extrasynaptic receptors. Prolonged NMDAR activation, combined with impaired Ca^{2+} buffering and/or mitochondrial dysfunction and/or metabolic stress resulting in compromised intracellular ATP levels, could lead to exacerbated susceptibility of SNc neurones to excitotoxic damage (Chan *et al.*, 2009).

NMDAR desensitization in SNc vs. SNr neurones

NMDAR excitotoxicity has been extensively studied in hippocampal neurones, and sensitivity to excitotoxic damage is likely to depend on a complicated range of factors, including NMDAR density, NMDAR subtypes, Ca^{2+} buffering within the neurone, and NMDAR trafficking mechanisms (Choi, 1988; Hardingham & Bading, 2003; Villmann & Becker, 2007; Soriano *et al.*, 2008; Forder & Tymianski, 2009). In cultured hippocampal or spinal cord neurones, NMDAR responses desensitize during prolonged or repeated NMDA application in a manner that has been shown to depend partly on the supply of intracellular ATP (MacDonald *et al.*, 1989; Rosenmund & Westbrook, 1993; Wang & Salter, 1993) and also on intracellular Ca^{2+} levels (Rosenmund *et al.*, 1995). We investigated NMDAR desensitization during prolonged application of NMDA (50 μM for 500 s) or during two consecutive 60-s applications of 200 μM NMDA. Brief (15-s) applications of 200 μM NMDA did not evoke significant desensitization when spaced 300 s apart.

During prolonged application of NMDA, our observations suggest that, in SNc and SNr neurones at P7, lack of intracellular ATP may promote NMDAR desensitization. We speculate that, in conditions of low intracellular ATP, for example, when SN neurones are

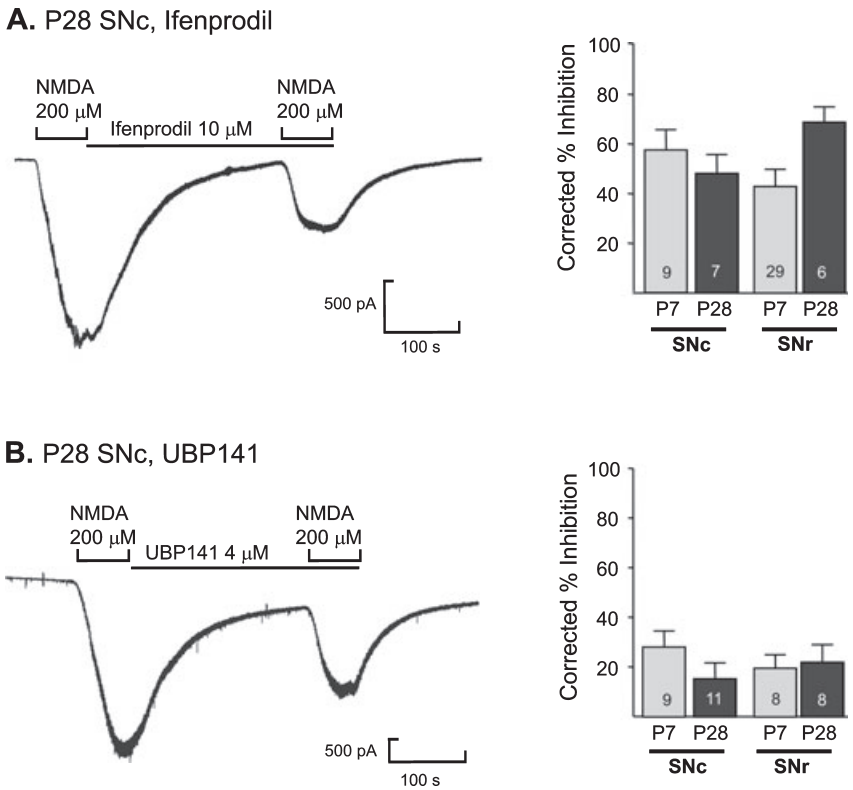

FIG. 5. Pharmacology of NMDARs in SNc and SNr neurones. Sample traces in response to paired applications of NMDA (200 μM, 60 s indicated by bar, 5 min between each application) recorded from SNc neurones at P28, with N2 recorded in the presence of (A) 10 μM ifrenprodil or (B) 4 μM UBP141. ATP was added for all recordings. Bar graphs show the percentage inhibition of NMDA currents (corrected for control N2/N1 as described in Materials and methods). Ages and numbers of cells are shown below and on bars, respectively. There was no significant difference in the mean inhibition with ifenprodil or with UBP141 between the different groups (ANOVA).

metabolically compromised, NMDARs show significant desensitization in response to prolonged activation, and furthermore that this may be a protective mechanism to limit Ca^{2+} influx. Strikingly, then, in P28 SNc DA neurones, this potentially protective mechanism for limiting Ca^{2+} entry is no longer apparent; NMDARs do not desensitize when ATP is not added. During excessive glutamate release or compromised glutamate re-uptake, the lack of desensitization could contribute to the vulnerability of SNc neurones to excitotoxicity. In contrast, the NMDAR desensitization observed in SNr neurones at both P7 and P28 (notably when ATP was not added) may serve to counterbalance the higher NMDA current density and consequent Ca^{2+} entry in SNr neurones.

In this study, we found significant differences between the response of neurones to: (i) repeated activation of synaptic NMDARs; (ii) repeated brief activation (15 s) using a near-maximal NMDA concentration; (iii) repeated but slower activation (60 s) using a near-maximal NMDA concentration; and (iv) prolonged activation (500 s) using a near-$[A]_{50}$ NMDA concentration. One plausible explanation for these differences is that brief application of a high NMDA concentration produces a transient high concentration of intracellular Ca^{2+}, which may be effectively buffered between applications, whereas prolonged application of a lower NMDA concentration produces a sustained but lower rise in intracellular Ca^{2+}. On the basis of our results, we can speculate that the latter situation, perhaps via activation of a relatively high-affinity Ca^{2+} target such as the phosphatase calcineurin, may be responsible for the desensitization seen in P7 SNc and P28 SNr neurones, but not in P28 SNc neurones,during prolonged receptor activation.

Pharmacology of NMDARs in SNc vs. SNr neurones

The differences in desensitization between the two SN nuclei in response to prolonged or repeated NMDAR activation could arise from differences in NMDAR subunit composition. However, only one apparent difference in NMDAR pharmacology was evident in this study. At P7, the ifenprodil sensitivity of SNr neurones was less than that of P28 SNr neurones, although this difference was not statistically significant (Fig. 5A). One possible explanation for this is that P7 SNr neurones express a greater proportion of NR1–NR2D receptors than SNc neurones. However, this is not apparent in the inhibition measured for UBP141. UPB141 caused approximately 30% inhibition in both cell types, and at both P7 and P28, with no significant difference between SNc and SNr neurones being seen at either age, suggesting that the proportion of NR2D-containing NMDARs is similar in the two populations of neurones.

An alternative explanation for the reduced effect of ifenprodil in P7 SNr neurones is that these neurones express more triheteromeric NR1–NR2B–NR2D receptors than SNc neurones (Dunah *et al.*, 1998; Jones & Gibb, 2005), as the efficacy of N-terminal domain ligands such as ifenprodil is reduced 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), NR2C (Hatton & Paoletti, 2005), and NR2D (Brickley *et al.*, 2003). Triheteromeric receptors have been shown to exhibit lower maximum inhibition with ifenprodil than their diheteromeric counterparts (Tovar & Westbrook, 1999; Hatton &

Paoletti, 2005), and so would be predicted to be less sensitive to ifenprodil (Hatton & Paoletti, 2005), but to perhaps have a sensitivity to the glutamate site antagonist UBP141 that is similar to that of NR1–NR2D diheteromers. Unfortunately, the pharmacology of recombinant triheteromeric NR1–NR2B–NR2D receptors has not been studied, leaving a number of significant uncertainties in interpreting the pharmacology data. In particular, the effectiveness of ifenprodil in blocking these triheteromers is uncertain. In addition, we do not know the affinity of NMDA for these triheteromeric receptors, and so cannot predict the effect of UBP141 on steady-state responses to NMDA.

Some qualitative conclusions can be drawn regarding NMDAR subtypes in SN neurones; ifenprodil (10 μM) gives < 90% blockade, indicating that not all receptors are diheteromeric NR2B (Williams, 1993). Around 60% blockade by ifenprodil in SNc neurones indicates that the majority of receptors contain NR2B subunits. The 30% blockade by UBP141 means that the data cannot be explained by a combination of NR1–NR2B and NR1–NR2B–NR2D receptors, but that some NR1–NR2D diheteromers must also be involved, because 4 μM UBP141 will probably produce only about 10% blockade of NR2B diheteromers and 20% blockade of NR2B–NR2D triheteromers when 200 μM NMDA is used.

Comparison of whole-cell and synaptic NMDAR currents in SNc neurones

Surprisingly, intracellular ATP did not affect the size of the whole-cell or NMDA synaptic current in P28 SNc neurons, and there was no evidence of desensitization with repeated stimulation. NMDAR-mediated synaptic currents in P7 SNc neurones (Brothwell *et al.*, 2008) have a similar or slightly greater ifenprodil sensitivity to that observed for whole-cell currents in this study. During postnatal development, the synaptic current shows a small but significant decrease in ifenprodil sensitivity, which may result from an increasing proportion of triheteromeric NR1–NR2B–NR2D receptors being present at the synapse, as the sensitivity of the synaptic current to UBP141 is unchanged during development (Brothwell *et al.*, 2008). In contrast, no developmental change in ifenprodil sensitivity was seen in whole-cell responses to NMDA in SNc DA neurons. One possible explanation for these results is that, with development, there may be active trafficking of triheteromeric receptors to the synapse.

In conclusion, the different properties of NMDARs in SNc and SNr neurones in response to high concentrations of NMDA are therefore probably caused by differences in receptor modulation and/or trafficking. Thus, the greater vulnerability of SNc DA neurones to cell death is not correlated with NMDA current density or receptor subtypes, but may, in part, result from inadequate desensitization when intracellular ATP levels are compromised.

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Abbreviations

$[A]_{50}$, half-maximal agonist concentration; DA, dopaminergic; EPSC, excitatory postsynaptic current; I_h, hyperpolarization-activated inward current; I_{NMDA}, whole-cell mean NMDA current; NMDAR, NMDA receptor; P, postnatal day; PD, Parkinson's disease; SN, substantia nigra; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; τ, activation time constant.

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