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Characteristics of the NMDA receptor modulating hypoxia/hypoglycaemia-induced rat striatal dopamine release in vitro

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Abstract

We investigated the functional characteristics of the NMDA receptor that modulates hypoxia/hypoglycaemia-induced striatal dopamine release. Dopamine release was detected by fast cyclic voltammetry in rat neostriatal slices. Four variables were measured: T_{on} — time from initiation of hypoxia/hypoglycaemia to the onset of dopamine release, T_{pk} — time from onset to maximum, $\delta DA/\delta t$ — rate of dopamine release and DA_{max} — maximum extracellular dopamine concentration. In controls, $T_{on} = 164.9 \pm 1.7$ s, $T_{pk} = 20.9 \pm 0.9$ s, $\delta DA/\delta t = 5.31 \pm 0.44$ μM/s and $DA_{max} = 79.1 \pm 2.5$ μM (means $\pm$ S.E.M., $n = 203$). *Cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid (CGS 19755, 20 μM) lengthened, while *N*-methyl-D-aspartate (NMDA) (100 μM) shortened T_{on}. (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK 801, 1 and 10 μM) and dextromethorphan (10 and 100 μM) increased T_{pk} and decreased DA_{max}. Neither glycine (100 μM), 7-chlorokynurenic acid (50 μM) nor 5-nitro-6,7-dichloro-1,4-dihydroquinoxaline-2,3-dione (ACEA 1021, 100 μM) had any effect although 7-chlorokynurenic acid blocked the effect of NMDA. Increasing $[Mg^{2+}]$ from 1.3 to 3.7 mM, increased T_{pk} and decreased $\delta DA/\delta t$. Dithiothreitol (1 mM) accelerated T_{on} while 5,5-dithio-bis-(2-nitrobenzoic acid) (1 mM) delayed T_{on}. Neither drug affected T_{pk}, DA_{max} or $\delta DA/\delta t$. Neither spermidine (100 μM) nor arcaine (100 μM) affected T_{on}, T_{pk} or $\delta DA/\delta t$ although arcaine decreased DA_{max}. In conclusion, hypoxia/hypoglycaemia-induced dopamine release was influenced by an NMDA receptor although modulation of the glycine recognition site of the receptor was ineffective, as were agents acting at polyamine modulatory zones. These findings highlight differences between recombinant and native NMDA receptors and suggest caution in extrapolating molecular biology to functional studies. © 1997 Elsevier Science B.V.

Keywords: Dopamine; NMDA receptor; Hypoxia; Hypoglycaemia; Striatum; Voltammetry; Brain slice

1. Introduction

Release of neurotoxic neurotransmitters during cerebral ischaemia is thought central to the subsequent neuronal damage: it is well established, for example, that excessive levels of excitatory amino acids play a critical role in the pathogenesis of ischaemic neuronal injury (Benveniste, 1991; Globus et al., 1991). However, although most attention has focused on the amino acids, massive release of monoamines also occurs during an ischaemic episode (Phebus et al., 1986; Brannan et al., 1987; Bhardwaj et al., 1990; Sarna et al., 1990; Richards et al., 1993) and strategies which attenuate this monoamine release are neuroprotective (Weinberger et al., 1985; Clemens and Phebus, 1988; Globus et al., 1987; Buisson et al., 1992).

Dopamine is intrinsically neurotoxic in vitro at the extracellular concentrations attained in vivo following ischaemia (Lieb et al., 1995). Striatal dopamine and its metabolites exert not only direct neurotoxic effects through free radical generation (Lancelot et al., 1995) but also accelerating effects on neuronal damage via dopamine D_2 receptors (Hashimoto et al., 1994). Some of these effects may result from presynaptic interplay between the dopaminergic and glutamatergic systems (Yamamoto and Davy, 1992). The capacity of the dopamine D_2 receptor antagonist haloperidol to attenuate the striatal neurotoxicity of the NMDA receptor agonist quinolinic acid suggests that at least some of the damage caused by 'excitotoxic' glutamate release may be mediated indirectly, via dopamine release (Garside et al., 1996). This strongly infers that modulation of dopamine release/function is a plausible approach to striatal neuroprotection in ischaemia.

One of the main inputs into the striatum, the corticostriatal path, uses excitatory amino acids as neurotransmitters (Young and Bradford, 1986) and the striatum is the site of

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much interplay between glutamatergic and dopaminergic systems. Several in vitro studies have shown that glutamate and *N*-methyl-D-aspartate (NMDA) stimulate dopamine release in the striatum (Roberts and Anderson, 1979; Clow and Jhamandas, 1989; Werling et al., 1990; Lonart and Zigmond, 1991; Overton and Clark, 1991) and work on striatal synaptosomes and brain slices has indicated the presence of presynaptic NMDA receptors on dopamine terminals (Clow and Jhamandas, 1989; Krebs et al., 1991; Wang, 1991). Furthermore, recent in vivo evidence has confirmed that presynaptic glutamate receptors exist not only on striatal dopaminergic nerve terminals, but also on noradrenergic and serotonergic terminals elsewhere and regulate release of these neurotransmitters (Ohta et al., 1994). These findings suggest that ischaemia-induced monoamine release may be modulated via presynaptic NMDA receptors.

The NMDA receptor–ionophore is a complex structure with multiple targets for pharmacological intervention (Wong and Kemp, 1991; Bigge, 1993). Our objective in this paper was firstly to determine whether hypoxia/hypoglycaemia-induced striatal dopamine release was influenced by NMDA receptors and, secondly, to determine the effects of drugs acting at different sites within the NMDA receptor–ionophore complex.

2. Materials and methods

Hypoxia/hypoglycaemia-induced dopamine release was measured by voltammetry in rat neostriatal slices according to the method previously described (Toner and Stamford, 1996). Brief details are given below.

2.1. Preparation of brain slices

Male Wistar rats (300–400 g) were sacrificed by stunning and immediate cervical dislocation. No prior anaesthetic or neuroprotective agents were used. The skull and brain were continuously irrigated with ice-cold (0 ± 1°C) oxygenated artificial cerebrospinal fluid (ACSF) throughout the dissection and removal of the brain from the cranial vault. Slices of neostriatum (350 μm thickness) were prepared using a Campden 752M 'Vibroslice' and transferred to a 'slice saver' where they were incubated for 1–4 h prior to transfer to the slice superfusion chamber. This allowed recovery from the trauma induced by preparation. The slice saver contained continuously-oxygenated (95% O_2/5% CO_2) ACSF maintained at 24°C.

2.2. Imposition of hypoxia / hypoglycaemia

Following recovery in the saver, striatal slices were transferred to a submersion-type recording chamber (Richards and Tegg, 1977), modified according to the specific requirements of our model (Toner and Stamford, 1996), and superfused (400 ml/h) with warmed (34.0 ± 0.2°C) ACSF for 30 min prior to a hypoxic/hypoglycaemic insult.

Unless otherwise specified, the ACSF comprised the following compounds: NaCl (126.0 mM), KCl (4.0 mM), KH_2PO_4 (1.4 mM), $MgSO_4$ (1.3 mM), $CaCl_2$ (2.4 mM), $NaHCO_3$ (26.0 mM), (+)-glucose (4.0 mM) and ascorbic acid (400 μM). The glucose concentration was reduced to 2 mM during the imposition of hypoxia/hypoglycaemia. For the superfusion system, separate flasks of 'maintenance' or hypoxic/hypoglycaemic ACSF were gassed for 30 min (with 95% O_2/5% CO_2 or 95% N_2/5% CO_2 respectively) at 34°C and pumped (400 ml/h) into the chamber by a Watson Marlow 302S peristaltic pump via Marprene™ tubing. The temperature in the inner chamber was adjusted to read 34.0 ± 0.2°C, measured by a thermocouple (RS Components). Each brain slice was subjected only to a single episode of hypoxia/hypoglycaemia, imposed either in the presence or absence of test drugs.

2.3. Detection of dopamine release

Hypoxia/hypoglycaemia-evoked dopamine release was measured using fast cyclic voltammetry, as previously described (Stamford, 1990). Carbon fibre microelectrodes, 8 μm in diameter, (Armstrong and Millar, 1979), were positioned in the dorsolateral quadrant of the striatal slice, under micromanipulator control. Auxiliary (stainless steel) and reference (Ag/AgCl) electrodes were positioned at convenient points in the slice chamber. Voltammetric scans (-1.0 to $+1.4$ V versus Ag/AgCl, 480 V/s) were performed at a frequency of 0.25 Hz with a Millar Voltammetric Analyser (PD Systems, West Molesey).

A 'sample-and-hold' record of current at the peak dopamine oxidation voltage ($+600$ mV versus Ag/AgCl) was displayed on a chart recorder while complete voltammetric scans were recorded on a Nicolet 310 digital storage oscilloscope and saved onto floppy disk. Following digital subtraction of charging current, redox current peaks were analysed either individually at single sample points or, in sequence, as a cascade. At the end of the experiment, the microelectrodes were calibrated by standard flow injection analysis (400 ml/h) in solutions of dopamine (100 μM, 500 μl).

2.4. Data analysis

Four dopamine release variables were measured: T_{on} — time from initiation of hypoxia/hypoglycaemia to the onset of dopamine release (s), T_{pk} — the time from onset of dopamine release to maximum (s), $\delta DA/\delta t$ — the mean rate of dopamine release (μM/s) and DA_{max} — the maximum extracellular dopamine concentration (μM).

As many as 4 slices of neostriatum were taken from each rat. Typically, one would serve as a drug free 'control' while the others would receive different drug treat-

ments. All drugs were tested against control slices (subjected to hypoxia/hypoglycaemia alone) from the same rat to account for inter-animal variability (see Section 3), arising mainly from biological and surgical differences. Comparisons of drug effects with their respective controls from the same brains were made by paired *t*-tests or repeated-measures analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test, as appropriate. Where data pairing was not possible, unpaired *t*-tests were used. Where '*n*' values are quoted, these refer to the number of slices from different animals. No two slices from the same rat received the same treatment.

2.5. Drugs and chemicals

All chemicals used to make the ACSF were of standard AnalaR grade and obtained from BDH Lab supplies, Poole. Glycine, dithiothreitol and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma, Poole. 7-chlorokynurenic acid, arcaine sulphate, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK 801), spermidine trihydrochloride, *N*-methyl-D-aspartic acid (NMDA) and *cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid (CGS 19755) were obtained from RBI, Massachusetts. 5-nitro-6,7-dichloro-1,4-dihydroquinoxaline-2,3-dione (ACEA 1021) was obtained from CoSensys, California.

With the exception of NMDA, all drugs were present in the ACSF from 30 min before the imposition of an hypoxic/hypoglycaemic episode until the end of the experiment. In the experiments involving NMDA, the drug was added solely to the hypoxic/hypoglycaemic ACSF.

3. Results

Imposition of hypoxia/hypoglycaemia (deoxygenation of the ACSF and reduction of the glucose concentration from 4 to 2 mM) caused a distinctive pattern of dopamine release in the dorsolateral striatal quadrant. In controls, dopamine release occurred suddenly and rapidly after a short delay. Control values (means ± S.E.M., $n = 203$) were: $T_{on} = 164.9 \pm 1.7$ s (range: 108–258 s), $T_{pk} = 20.9 \pm 0.9$ s (4–102 s), $\delta DA/\delta t = 5.31 \pm 0.44$ μM/s (0.4–23.0 μM/s) and $DA_{max} = 79.1 \pm 2.5$ μM (20–220 μM).

3.1. The glutamate recognition site

Fig. 1 shows typical chart recordings of hypoxia/hypoglycaemia-induced dopamine release events in the presence of an agonist (NMDA, 100 μM) and antagonist (CGS 19755, 20 μM) at the glutamate recognition site of the NMDA receptor. NMDA (added to the hypoxic/hypoglycaemic ACSF) significantly hastened T_{on} of dopamine release ($P < 0.05$) whilst CGS 19755 delayed DA release ($P < 0.05$). Neither drug significantly affected T_{pk}, $\delta DA/\delta t$ or DA_{max}. Data for the group are summarized in Table 1.

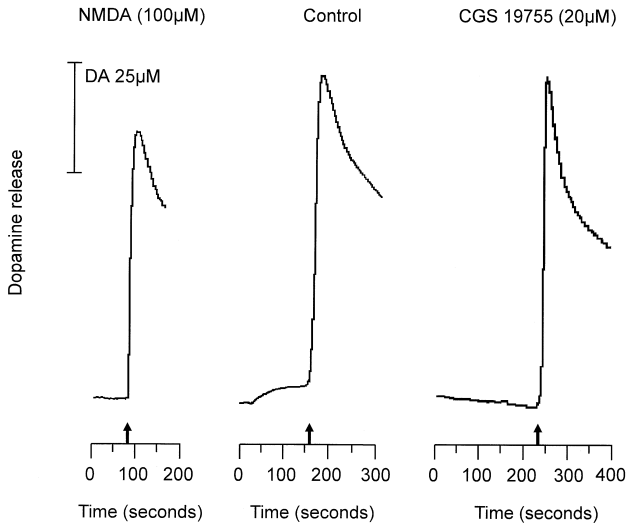

Fig. 1. Effects of stimulation and blockade of the glutamate recognition site on hypoxia/hypoglycemia-induced dopamine release. Representative sample-and-hold records of current at the DA oxidation peak potential (+600 mV versus Ag/AgCl, converted to DA concentration) versus time (s) in a 'control' striatal slice (middle trace) and in ones treated with NMDA (100 μM: left hand signal) or CGS 19755 (20 μM: right hand trace). The horizontal axis shows the time from the onset of hypoxia/hypoglycemia imposed upon the tissue. The vertical arrows show the onset of dopamine release in the slices. NMDA hastened, while CGS 19755 delayed the onset of dopamine release ($P < 0.05$, paired *t*-test).

3.2. The phencyclidine-binding site

Fig. 2 shows cascades of sequential dopamine oxidation peaks obtained during hypoxia/hypoglycaemia in a typical slice treated with MK 801 (10 μM, right panel), and its paired control (left panel). Each constituent line of the cascades represents a single voltammetric scan. MK 801 delayed T_{on} and reduced DA_{max}. The effects on $\delta DA/\delta t$ and T_{pk} are clearer in the group data (Fig. 3).

Fig. 3 shows the effects of dextromethorphan (10 and 100 μM) and MK 801 (1 and 10 μM), non competitive antagonists at the phencyclidine binding site within the ion channel, on hypoxia/hypoglycaemia-induced dopamine re-

Table 1
Effects of stimulation and blockade of the glutamate recognition site on hypoxia/hypoglycemia-induced dopamine release

	T_{on} (s)	T_{pk} (s)	$\delta DA/\delta t$ (μM/s)	DA_{max} (μM)
Control	179±22	17±5	4.0±0.5	90±10
NMDA (100 μM)	84±6[a]	21±2	3.7±0.5	76±5
S.E.M. of difference	23.4	3.3	1.2	13.5
Control	162±12	19±5	5.7±1.4	77±10
CGS 19755 (20 μM)	215±11[a]	21±4	3.9±0.4	85±12
S.E.M. of difference	12.8	7.5	1.3	22.0

All data are means ± S.E.M., $n = 4$.
[a] $P < 0.05$ versus matched controls (paired *t*-test).

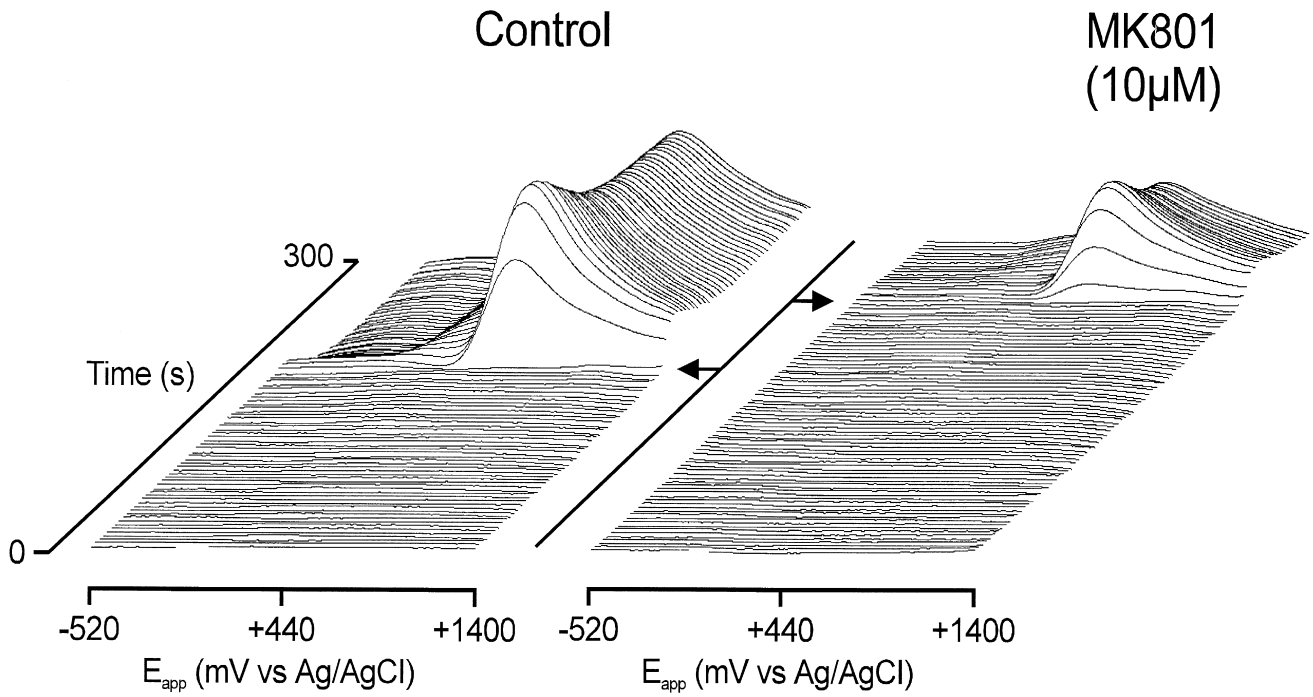

Fig. 2. MK 801 delays and slows hypoxia/hypoglycemia-induced dopamine release in vitro. Cascades of sequential charging current-corrected voltammograms (oxidation scan shown from −520 to +1400 mV versus Ag/AgCl) obtained in a 'control' striatal slice (left hand trace) and in one treated with MK 801 (10 μM: right hand signal). The vertical (y) axis is dopamine oxidation current, the x axis is the applied voltage (E_{app}) and the z axis, into the page, is time. The signals show the release of dopamine as the large peak. Arrows mark the onset of dopamine release in both slices.

lease. Both drugs induced concentration-dependent effects on dopamine release. In both cases, dopamine release was slowed (T_{pk} was increased: $P < 0.05$ for MK 801, $P < 0.001$ for dextromethorphan) and reduced (decreased DA_{max}: both $P < 0.05$). MK 801 also delayed T_{on} at the highest concentration (10 μM).

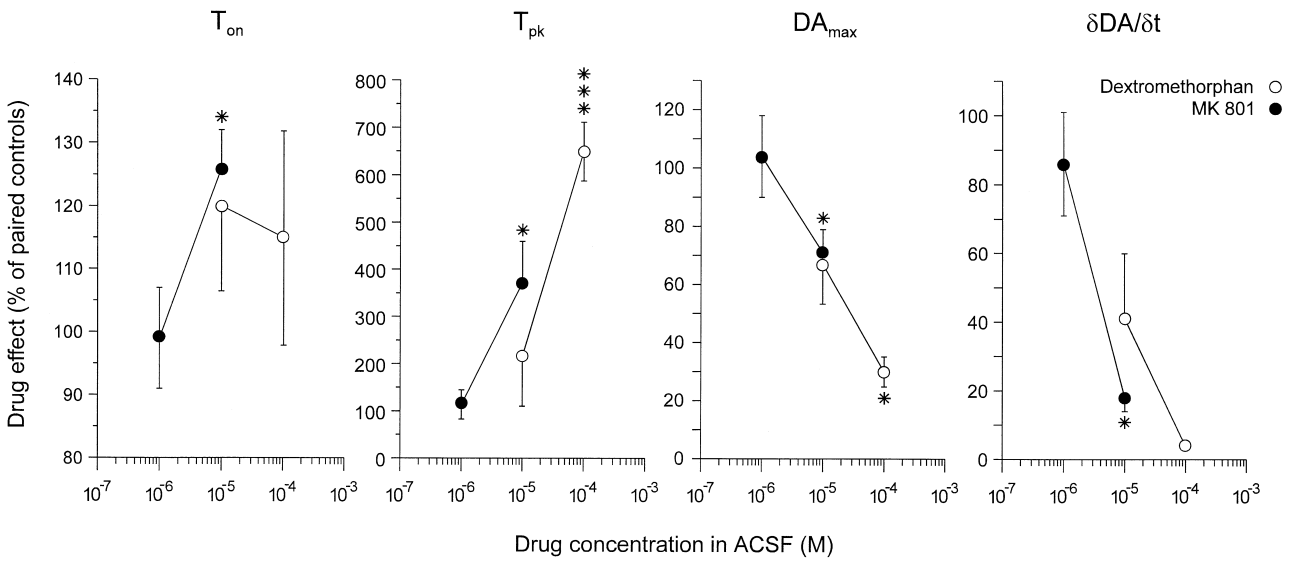

Fig. 3. Effect of dextromethorphan and MK 801 on dopamine release by hypoxia/hypoglycemia. Percentage changes relative to paired controls in time to onset of DA release (T_{on}), time to peak DA release (T_{pk}), peak DA release (DA_{max}) and rate of DA release ($\delta DA/\delta t$) in slices treated with dextromethorphan (open circles) or MK 801 (filled circles). All values are means ± S.E.M. ($n = 4$–8). $^{*}P < 0.05$, $^{***}P < 0.001$ versus control slices (paired t-test).

Table 2
Effects of stimulation and blockade of the glycine recognition site on hypoxia/hypoglycemia-induced dopamine release

	T_{on} (s)	T_{pk} (s)	$\delta DA/\delta t$ (μM/s)	DA_{max} (μM)
Control	174±18	20±8	2.7±0.8	68±20
Glycine (100 μM)	171±11	23±5	3.7±1.2	67±12
S.E.M. of difference	16.5	5.1	0.6	1.9
Control	169±11	16±5	6.2±1.9	76±16
ACEA 1021 (100 μM)	178±13	17±6	6.6±1.3	88±15
S.E.M. of difference	16.4	3.5	2.0	8.2
Control	181±14	17±3	4.2±0.9	60±4
7-chlorokynurenic acid (50μM)	193±16	16±2	3.6±0.5	58±7
S.E.M. of difference	22.3	1.7	0.6	7.6

All data are means±S.E.M., $n=4$ (glycine pairs), 5 (ACEA 1021 pairs) or 7 (7-chlorokynurenic acid pairs).

3.3. The glycine site

Table 2 shows the effects of incubation of striatal slices in glycine and strychnine-insensitive glycine site antagonists. Neither glycine (100 μM) nor the selective glycine site antagonists 7-chlorokynurenic acid, (50 μM) and ACEA 1021 (100 μM) had any effect on T_{on}, T_{pk}, $\delta DA/\delta t$ or DA_{max}.

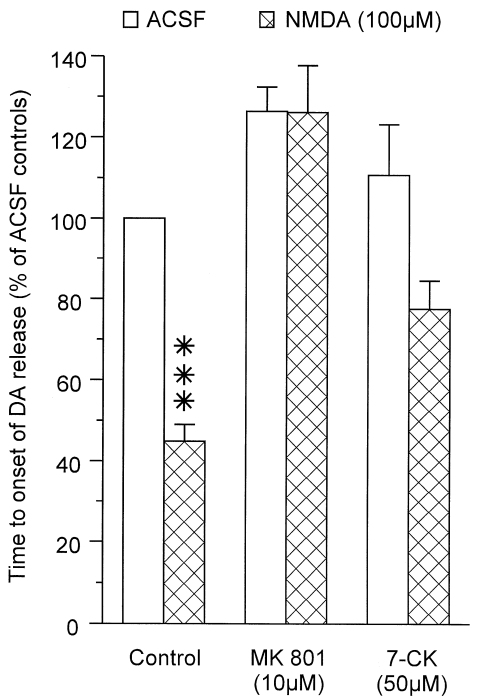

Fig. 4. Effects of MK 801 and 7-chlorokynurenic acid on the ability of NMDA to hasten hypoxia/hypoglycemia-induced dopamine release. Effects of MK 801 (10 μM) and 7-chlorokynurenic acid (7-CK, 50 μM) pretreatment on time to onset of DA release (T_{on}) in control slices (ACSF: open bars) and those treated with NMDA (100 μM: filled bars). All values (means±S.E.M. $n=4$–7). are expressed as a percentage of the control ACSF values. *** $P<0.001$ versus ACSF slices (t-test).

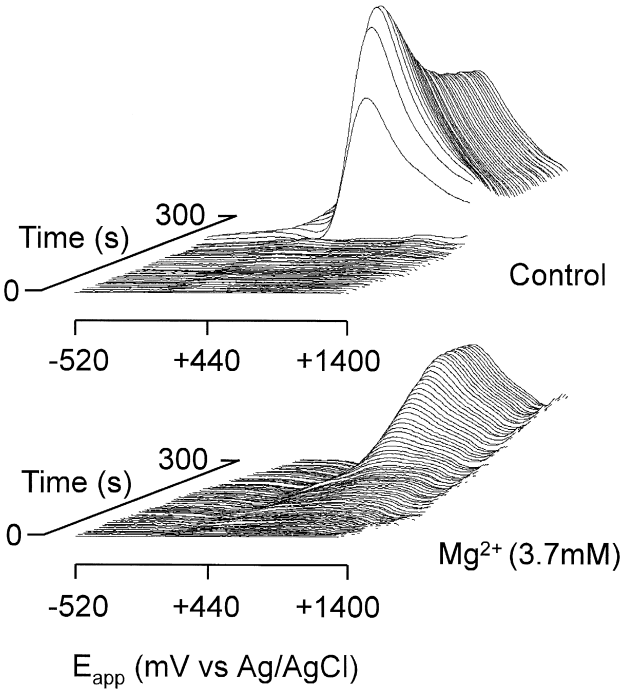

Fig. 5. Mg^{2+} delays and slows hypoxia/hypoglycemia-induced dopamine release in vitro. Cascades of sequential charging current-corrected voltammograms (oxidation scan shown from −520 to +1400 mV versus Ag/AgCl) obtained in a 'control' striatal slice (upper trace: 1.2 mM Mg^{2+}) and in one treated with Mg^{2+} (3.7 mM: lower signal). The vertical (y) axis is dopamine oxidation current, the x axis is the applied voltage (E_{app}) and the z axis, into the page, is time. The signals show the release of dopamine as the large peak. Mg^{2+} slowed dopamine release relative to controls ($P<0.05$, paired t-test).

Table 3
Effect of elevated Mg^{2+} on hypoxia/hypoglycemia-induced dopamine release

	T_{on} (s)	T_{pk} (s)	$\delta DA/\delta t$ (μM/s)	DA_{max} (μM)
Control (1.2 mM Mg^{2+})	161±7	17±2	4.2±0.7	84±11
Mg^{2+} (3.7 mM)	195±21	78±17[a]	1.8±0.6[a]	65±6
S.E.M. of difference	20.6	18.2	1.1	12.5

All data are means±S.E.M., $n=9$.
[a] $P<0.05$ versus matched controls (paired t-test).

Table 4
Effects of polyamine modulation on hypoxia/hypoglycemia-induced dopamine release

	T_{on} (s)	T_{pk} (s)	$\delta DA/\delta t$ (μM/s)	DA_{max} (μM)
Control	183±11	16±2	4.8±1.7	63±8
Spermidine (100 μM)	224±28	17±5	3.6±1.0	49±5
S.E.M. of difference	33.2	5.0	1.1	8.8
Control	177±2	19±6	5.0±1.4	69±8
Arcaine (100 μM)	198±23	9±1	3.9±1.1	49±10[a]
S.E.M. of difference	21.9	6.5	1.1	4.9

All data are means±S.E.M., $n=4$.
[a] $P<0.05$ versus matched controls (paired t-test).

Table 5
Effects of redox modulation on hypoxia/hypoglycemia-induced dopamine release

	T_{on} (s)	T_{pk} (s)	$\delta DA/\delta t$ ($\mu M/s$)	DA_{max} (μM)
Control	167 ± 8	19 ± 4	3.18 ± 0.72	70 ± 15
DTNB (1 mM)	198 ± 11[a]	37 ± 14	2.41 ± 0.91	70 ± 13
S.E.M. of difference	11.0	15.0	0.9	12.8
Control	166 ± 10	17 ± 4	6.06 ± 1.46	81 ± 8
Dithiothreitol (1 mM)	128 ± 10[a]	16 ± 5	7.26 ± 2.66	69 ± 15
S.E.M. of difference	13.5	4.7	1.9	10.1

All data are means ± S.E.M., $n = 6/7$.
[a] $P < 0.05$ versus matched controls (paired t-test).

The effect of NMDA (100 μM, added to the hypoxic ACSF) on T_{on} of dopamine release was prevented by MK 801 (10 μM). 7-Chlorokynurenic acid (50 μM) also caused a modest blockade of the effect of NMDA (Fig. 4).

3.4. Voltage-dependent Mg^{2+} block

Fig. 5 shows the effect of Mg^{2+} on cascades of sequential dopamine oxidation peaks recorded during hypoxia/hypoglycaemia-induced dopamine release. Increasing the Mg^{2+} concentration in the ACSF from 1.3 to 3.7 mM, significantly ($P < 0.05$) slowed dopamine release (decreased T_{pk} and $\delta DA/\delta t$: both $P < 0.05$) but did not affect T_{on}. Data for the group are shown in Table 3.

3.5. Polyamine modulation

Neither the polyamine, spermidine (100 μM) nor the polyamine antagonist arcaine (100 μM) affected T_{on}, T_{pk} or $\delta DA/\delta t$ although arcaine had a modest effect on DA_{max} (Table 4).

3.6. Redox modulation

Modulation of the NMDA receptor with the disulphide reducing agent dithiothreitol (1 mm) accelerated onset of dopamine release ($P < 0.05$) while oxidation with DTNB (1 mM) delayed onset ($P < 0.05$). The data are shown in Table 5. Neither drug affected T_{pk}, DA_{max} or $\delta DA/\delta t$.

4. Discussion

The NMDA receptor complex contains an ion channel which gates Na^+, K^+, and Ca^{2+} movement and is blocked in a voltage-dependent manner by Mg^{2+}. Membrane depolarisation relieves this block, allowing activation of the complex through the actions of glutamate (or NMDA) and glycine at their respective recognition sites. Other loci at which NMDA receptor function can be modulated are the polyamine site and the phencyclidine and Mg^{2+} binding sites within the ion channel pore. There are also modulatory sites for redox agents, protons and zinc. Many of these sites are interactive and there are notable differences in the relative sensitivity of individual NMDA receptor subtypes to agents acting at these different loci (Sucher et al., 1996).

The native NMDA receptor consists of at least one NR1 subunit with at least one of four different NR2 subunits, designated NR2A-D (Sucher et al., 1996). The NR1 subunit is essential for NMDA receptor activity (Ishii et al., 1993) and is held to contain the glutamate and glycine recognition sites (Corsi et al., 1996). The function of the NR1 subunit is modulated by the NR2 units to which it is attached and these show considerable regional variation (Monyer et al., 1992; Portera-Cailliau et al., 1996). Furthermore, different subtype combinations may modulate the release of different neurotransmitters (Nankai et al., 1995).

The neurotoxic effects of overstimulation of the NMDA receptor by excitatory amino acids (particularly glutamate) are central to the excitotoxin hypothesis of ischaemic neuronal injury (Benveniste, 1991). However, it is recognised that at sufficiently high concentrations, selective glutamate agonists evoke release of monoamines in the striatum (Werling et al., 1993; Ohta et al., 1994). There is ample evidence that monoamines exert neurotoxic effects in their own right and that drugs which modulate their release may have neuroprotective consequences. Since haloperidol can block quinolinic acid-induced excitotoxicity in the striatum (Garside et al., 1996), one might speculate that at least some of the neuropathological consequences of hypoxia/hypoglycaemia-induced glutamate release may be due to stimulation of monoamine release.

It has been well demonstrated that NMDA receptors can modulate *physiological* dopamine release in the striatum. The current study therefore explored the effects of agonists and antagonists at various sites on the NMDA receptor complex on *hypoxia/hypoglycaemia*-induced release of dopamine.

Much of our current understanding of NMDA receptors stems from molecular biology and the characterisation of cloned or recombinant receptors. We were keen to examine the effects of NMDA receptor manipulations at *native* NMDA receptors and examine their effects on a functional response — the release of dopamine.

4.1. The glutamate recognition site

NMDA is a selective agonist at the glutamate recognition site of the NMDA receptor complex whilst CGS 19755 is a potent, *competitive* antagonist at the same site (Lehmann et al., 1988). The acceleration of T_{on} in the presence of NMDA (100 μM) and the delay induced by CGS 19755 is thus consistent with the presence of presynaptic NMDA receptors on the dopaminergic terminals. Significantly, neither NMDA nor CGS 19755 affected T_{pk}.

The concentration of CGS 19755 used in the current study was similar to those previously shown to provide excellent neuroprotection in vitro and in vivo against NMDA-mediated toxicity and oxygen/glucose deprivation (Perez-Pinon et al., 1995). The delay in T_{on} in the presence of CGS 19755 indicates that NMDA receptor activation comes into play during hypoxia/hypoglycaemia-induced dopamine release.

4.2. The phencyclidine-binding site

Dextromethorphan is, in addition to being a clinically used antitussive, also a noncompetitive antagonist at the phencyclidine binding site within the ion channel of the NMDA receptor and has been shown to reduce ischaemia-induced neurodegeneration in vivo (Bokesch et al., 1994) and in vitro (Tortella et al., 1995). Dextromethorphan had no significant effect on T_{on} but delayed T_{pk} and reduced DA_{max}. Although this diminution of hypoxia/hypoglycaemia-induced dopamine release may have resulted from antagonism of the NMDA receptor, there is also evidence that dextromethorphan may act at presynaptic voltage-gated Ca^{2+} channels (Carpenter et al., 1988). We have previously shown that N- and P/Q-type Ca^{2+} channel blockade can influence hypoxia/hypoglycaemia-induced dopamine release (Toner and Stamford, 1997a) and thus it is possible that this action may underlie the observed response.

However, MK 801 is also a noncompetitive antagonist at the phencyclidine binding site but devoid of effects at voltage-gated channels (Wong et al., 1986). MK 801 is neuroprotective both in vitro and in vivo (Vornov et al., 1994; Hoffman and Boast, 1995) and has repeatedly been reported to inhibit NMDA- and glutamate-evoked dopamine release in striatal synaptosomes (Cheramy et al., 1994) and brain slices (Werling et al., 1993; Nankai et al., 1995). MK 801 has also been shown to reduce [^{3}H]-dopamine release in rat striatal slices, a finding consistent with the present study (Kim et al., 1995).

The pattern of effects observed with MK 801 treatment were almost identical to those seen with dextromethorphan, mainly a delayed T_{pk} and reduced DA_{max}. Interestingly, these effects differ functionally from those observed with CGS 19755 which only delayed T_{on}. One explanation of these differences lies in the nature of the block. Dextromethorphan and MK 801 are open channel blockers (i.e. their blockade only becomes manifest during activation of the receptor) whilst CGS 19755 exerts a competitive blockade that is present *before* NMDA receptor activation. Glutamate is released in ischaemia and it is plausible that this extracellular glutamate, above a threshold level, stimulates dopamine release via presynaptic NMDA receptors. Thus competitive blockade of the NMDA receptor by CGS 19755 would be expected to block the effects of early glutamate release, thereby delaying the onset of dopamine release. Conversely, MK 801 and dextromethorphan, being non-competitive open channel blockers, only *begin* to exert effective NMDA receptor blockade once glutamate release (and thus NMDA receptor stimulation) is already established.

4.3. The glycine site

Glycine is often described as an essential co-agonist for NMDA receptor activation (Kemp and Leeson, 1993). The glycine site of the NMDA receptor is an attractive therapeutic target since antagonists at this site produce less profound side effects than the channel blockers (Leeson and Iversen, 1994). Antagonists at the glycine recognition site reduce neuronal damage in slice cultures evoked by ischaemia (Newell et al., 1995) or NMDA (Boireau et al., 1996). Furthermore, under physiological conditions, NMDA-evoked dopamine release is *inhibited* by glycine site antagonists and *potentiated* by glycine (Werling et al., 1990; Cheramy et al., 1994; Nankai et al., 1995).

The finding that neither glycine nor glycine site antagonists had any intrinsic effect on hypoxia/hypoglycaemia-induced dopamine release in our model is, at first sight, puzzling. The failure of 100 μM glycine to have an effect is however relatively explicable. The glycine recognition site has a high affinity for glycine and is generally held to be fully saturated under physiological conditions. Under these conditions, further increases in glycine concentration would be unlikely to potentiate the effect.

More difficult to explain is the absence of effect with *antagonists* at the glycine site. However, such discrepancies are not without precedent. For example, spreading depression can also, like the dopamine release in this model, be delayed by antagonism of the NMDA receptor at the glutamate recognition site or phencyclidine binding site (Nellgard and Wieloch, 1992) and yet is also unaffected by potent glycine site antagonism with ACEA 1021 (Martin et al., 1994).

It has been shown that the several NMDA receptor subtypes have different affinities for glycine (Mori and Mishina, 1995). Furthermore one report demonstrated that cloned mouse NMDA receptors comprising $\varepsilon 1$ and $\zeta 2$ subunits (species homologues of the rat NR1 and NR2B subunits respectively) are able to generate modest currents upon exposure to glutamate (10 μM) in the nominal absence of glycine (Kutsuwada et al., 1992). Interestingly, despite being neuroprotective in the forebrain, glycine site antagonists do not appear to protect cerebellar granule cells against NMDA receptor-mediated toxicity (Widdowson et al., 1996).

It was with this in mind that experiments involving addition of NMDA during hypoxia/hypoglycaemia were performed. As already shown, NMDA hastened the onset of dopamine release (Fig. 1). We therefore sought to demonstrate that, in this model, the effect could be antagonized with the drugs used in this study at the concentrations used. MK 801 (10 μM) and 7-chlorokynurenic acid

(50 μM) both antagonised the effect of NMDA (100 μM), consistent with previous findings that 7-chlorokynurenic acid is an effective NMDA blocker at this concentration (Woodward, 1994).

The ability of 7-chlorokynurenic acid to block the effects of NMDA, despite its lack of effect on its own, may be explained in the light of recent patch clamp data: it has been shown that *plateau* currents mediated by continuing exposure to NMDA are often more sensitive to glycine site antagonism than the *peak* current on initial exposure (Molnar and Erdo, 1996).

In the present study, the durations of exposure of the NMDA receptor to agonist (NMDA or endogenous glutamate) are different in the two experimental situations. In the experiments involving addition of NMDA, stimulation of NMDA receptors by agonist is more prolonged, since it is present from the onset of hypoxia/hypoglycaemia. Conversely, hypoxia/hypoglycaemia-induced excitatory amino acid release is only likely to be present in high concentrations immediately following anoxic depolarisation (Scheller et al., 1989) at about the same time as monoamine release occurs (Toner and Stamford, 1997b). The former situation may be more analogous to a plateau type response while the latter may reflect a more dynamic form of NMDA receptor stimulation.

4.4. Voltage-dependent Mg^{2+} block

The NMDA receptor is blocked by Mg^{2+} in a voltage-dependent manner (Mayer et al., 1984). Under normal physiological (polarised) conditions, the NMDA receptor channel is blocked by Mg^{2+}. This block is relieved during transient ischaemia (Hori and Carpenter, 1994). Despite this apparent paradox, Mg^{2+} sulphate has reportedly been tried, with some success, in the treatment of cerebral ischaemia (Goldman and Finkbeiner, 1988).

As one might therefore expect, elevated Mg^{2+} had no effect on the dopamine release T_{on}, since anoxic depolarisation generally precedes neurotransmitter release (Toner and Stamford, 1997b). However, that the rate of hypoxia/hypoglycaemia-induced dopamine release was attenuated might mean that there is a *reduction*, rather than total loss, of the affinity of the channel binding site for Mg^{2+}. Thus, high Mg^{2+} concentrations such as, for example, those used in this study, might still be capable of partially blocking the channel (Cox et al., 1989).

4.5. Polyamine modulation

Polyamines (spermidine, spermine and putrescine) are present in the CNS in high concentrations and have a neuromodulatory role at the NMDA receptor (Williams et al., 1991; London and Mukhin, 1995), in all probability involving multiple sites (Ransom and Stec, 1988; Carter et al., 1990; McBain and Mayer, 1994). The NR2 subunit composition plays a major role in determining the polyamine pharmacology of the resultant NMDA receptor (Williams et al., 1991).

Spermidine enhances NMDA-induced whole cell currents in cultured neurones (Sprosen and Woodruff, 1990) while arcaine is a competitive polyamine antagonist (Reynolds, 1990) and has been shown to block NMDA-evoked release of noradrenaline (Sacaan and Johnson, 1990). The concentration of spermidine used in this study (100 μM) has been shown to enhance MK 801 binding tenfold (London and Mukhin, 1995). Given the significant effects of other NMDA receptor agonists and antagonists, it is initially surprising that arcaine and spermidine had little or no effect, respectively. This indicates that the polyamine site is not critically involved in hypoxia/hypoglycaemia induced striatal dopamine release in this model. In a recent patch clamp study, Wu and Johnson (1996) showed that spermine had no effect on NMDA-activated currents in midbrain dopamine neurones despite actions at other sites.

4.6. Redox modulation

It is known that the NMDA receptor may be modulated by the redox status of sulphydryl groups associated with the ion complex. For instance reduction with dithiothreitol increases the amplitude of currents through the NMDA ionophore whilst oxidation with DTNB has the opposite effect (Aizenman et al., 1989). The redox modulatory site also affects NMDA induced Ca^{2+} fluxes (Reynolds et al., 1990) and NMDA receptor mediated neurotoxicity (Levy et al., 1990).

In the present study, redox modulation of the NMDA receptor altered dopamine release T_{on}. Reduction of the NMDA receptor with the disulphide reducing agent dithiothreitol accelerated T_{on} while oxidation with DTNB delayed T_{on}. Interestingly, neither agent affected the rate or magnitude of dopamine release once initiated, suggesting that redox modulation may be inoperative or circumvented under conditions of strong NMDA receptor activation.

Dithiothreitol increases NMDA stimulated [^{3}H]NA release from cortical and hippocampal slices and more relevantly, tritiated and endogenous dopamine release from striatal slices (Woodward and Blair, 1991). The striatum is particularly sensitive to the effects of dithiothreitol (Woodward and Blair, 1991).

The redox site of the NMDA receptor presents a clinical paradox in the treatment of stroke. On the one hand, reducing agents are thought generally desirable to mop up the reactive oxygen species that prevail during reperfusion. On the other hand, increases in reducing equivalents in the brain following stroke (Levy et al., 1990) could increase the sensitivity of the NMDA receptor to released glutamate and contribute to neuronal death.

4.7. Conclusions

We have previously reported that hypoxia/hypoglycaemia-induced dopamine release is, in part, modulated

via N- and P/Q-type voltage sensitive Ca^{2+} channels (Toner and Stamford, 1997a). However, we concluded in that paper that, clearly, other mechanisms were involved. The involvement of presynaptic NMDA receptors on dopaminergic terminals suggests a likely alternative. In fact, we found that hypoxia/hypoglycaemia-induced dopamine release could be modulated via various sites within the NMDA receptor/ion complex, although most effectively at the phencyclidine binding site. Surprisingly, agonism and antagonism at the glycine recognition site was ineffective, as were agents acting at polyamine modulatory zones. These findings highlight differences between data obtained at recombinant and native NMDA receptors and suggest caution in the extrapolation of molecular biology data to functional studies.

The ability of NMDA receptor antagonists to modulate hypoxia/hypoglycaemia-induced dopamine release, coupled with the known neurotoxicity of dopamine and the finding that dopamine antagonists can block NMDA receptor-mediated excitotoxicity, may suggest that at least some striatal neuronal damage in hypoxia/hypoglycaemia may be mediated via glutamate-induced dopamine release. This conclusion may be of importance in determining neuroprotective strategies for the striatum.

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