N-methyl-D-aspartate modulation of nucleus accumbens dopamine release by metabotropic glutamate receptors: fast cyclic voltammetry studies in rat brain slices in vitro

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Abstract.
The N-methyl-D-aspartate (NMDA) receptor antagonist, phencyclidine, induces behavioural changes in rodents mimicking symptoms of schizophrenia, possibly mediated through dysregulation of glutamatergic control of mesolimbic dopamine release. We tested the hypothesis that NMDA receptor activation modulates accumbens dopamine release, and that phencyclidine pretreatment altered this modulation. NMDA caused a receptor-specific, dose-dependent decrease in electrically stimulated dopamine release in nucleus accumbens brain slices. This decrease was unaffected by picrotoxin, making it unlikely to be mediated through GABAergic neurones, but was decreased by the metabotropic glutamate receptor antagonist, (RS)-α-methyl-4-sulfonophenylglycine, indicating that NMDA activates mechanisms controlled by these receptors to decrease stimulated dopamine release. The effect of NMDA was unchanged by in vivo pretreatment with phencyclidine (twice daily for five days), with a washout period of at least seven days before experimentation, which supports the hypothesis that there is no enduring direct effect of PCP at NMDA receptors after this pretreatment procedure. We propose that NMDA depression of accumbal dopamine release is mediated by metabotropic glutamate receptors located pre-or peri-synaptically, and suggest that NMDA evoked increased extra-synaptic spill-over of glutamate is sufficient to activate these receptors that, in turn, inhibit dopamine release. Furthermore, we suggest that enduring functional changes brought about by sub-chronic phencyclidine pre-treatment, modelling deficits in schizophrenia, are downstream effects consequent on chronic blockade of NMDA receptors, rather than direct effects on NMDA receptors themselves.

Keywords.
Brain slices, dopamine, metabotropic glutamate receptors, n-methyl-D-aspartate, nucleus accumbens, phencyclidine
Introduction

The glutamate theory of schizophrenia posits that the core deficits leading to the behavioural dysfunctions are in glutamate systems \(^1,2\). In particular NMDA-type glutamate receptors (NMDA-R) are implicated, since drugs such as ketamine and phencyclidine (PCP), which are non-competitive antagonists at NMDA-Rs, both induce schizophrenia-like symptoms in normal people, and exacerbate symptoms in schizophrenia patients\(^1\). However, dopaminergic systems are also implicated in schizophrenia: drugs which increase dopamine function, such as amphetamine, induce schizophrenia-like symptoms, and antipsychotic drugs currently used clinically have in common an antagonist action at dopamine receptors\(^3,4\). A unifying theory proposes that the core deficit in glutamate function causes a dysregulating of glutamatergic modulation of dopamine release\(^1,4,5\). In particular changes in nucleus accumbens (NAc) dopamine function appear to be critical for the expression of positive symptoms\(^4,6\), whereas the mesocortical pathway terminating in frontal cortex, has been more implicated in negative and cognitive symptoms\(^7\). A central question in elucidating the underlying deficits in schizophrenia is to understand the mechanism of the interaction between glutamate systems, particularly those mediated through NMDA-Rs, and mesolimbic dopamine function.

Activity in the mesolimbic dopamine pathway is under direct excitatory control from glutamatergic neurones originating in frontal cortex, amygdala, thalamus and hippocampus \(^8\), and glutamatergic modulation of dopamine release, mediated through both ionotrophic (including NMDA-R) and metabotropic (mGluR) glutamate receptors has been described \(^8,9,10\). Whilst some of these effects are mediated through ‘long-loops’ involving actions on the cell body region in the ventral tegmental area (VTA), local effects have also been reported \(^5,10,11\). In NAc, it has been widely reported that NMDA-R activation evokes increased dopamine release, measured by brain microdialysis. However, using fast-scan cyclic voltammetry (FCV), which has much superior (e.g. sub-second) time resolution to microdialysis, NMDA has been seen to decrease both basal and stimulated dopamine release \(^12\), although the mechanisms of this action have yet to be elucidated. Although studies in synaptosomes have suggested the presence of NMDA-R on dopamine terminals \(^13\), and dual labelling studies found
tyrosine hydroxylase activity in terminals expressing NMDA-Rs, the evidence for localisation of NMDA-Rs on dopamine terminals in NAc is sparse, suggesting that a direct effect of NMDA on the dopamine terminals in NAc is unlikely. Therefore it is likely that NMDA-mediated modulation of DA release is through complex process involving interactions between multiple cellular types and/or chemical mediators.

The NAc is important in the control of motivation-related processes, including reward, stress and arousal. It is separated into two anatomically and functionally distinct subregions, the core and the shell: the core region connects predominantly with basal ganglia motor structures, whereas the shell connects with more limbic-related subcortical structures. Functionally, the shell subregion is predominantly involved in acquisition and expression of motivated behaviour, while the core region is more concerned with the motor components of such behaviour. Dopaminergic neurones comprising the mesolimbic pathway innervate both regions extensively, and exercise modulatory control of information throughput. In the context of schizophrenia, the shell region appears to be particularly implicated, with its key involvement in processes such as stimulus salience processing, attentional selection, and cognitive flexibility, which are dysfunctional in positively symptomatic schizophrenia sufferers: our studies therefore focussed on the shell region.

The first aim of the present study was to assess the action of NMDA on local dopamine release in NAc shell. Subsequently, the study aimed to identify mechanisms through which this modulation occurs: given the paucity of NMDA-R on dopamine terminals in NAc, this is unlikely to be mediated through a direct action of NMDA on dopaminergic terminals (see above). Rather, we hypothesised that the action of NMDA on dopamine release may be mediated indirectly through inhibitory links intrinsic to NAc. Given the central role of GABAergic neurones in NAc, and that activation of GABA-A receptors may depress accumbal dopamine release through a presynaptic action on dopamine terminals, we hypothesised that NMDA activated GABAergic neurones, which feedback on to dopamine terminals to decrease dopamine release. A second possibility is that the effect is mediated through cholinergic systems, since ionotropic glutamate receptor activation has been shown to modulate dopamine
release in dorsal striatum via cholinergic mechanisms. A third possibility is that mGluR may regulate this effect, since group 2 and 3 mGluR have been reported to be present on dopaminergic terminals in NAc, and activation has been shown to decrease accumbal dopamine release through presynaptic mechanisms. We further hypothesised that the psychomimetic effects of PCP are mediated, at least in part, through long-term changes in the NMDA-R-mediated modulation of accumbal dopamine release.

In the studies reported here, we used fast-scan cyclic voltammetry (FCV) in brain slices in vitro to measure the effects of drugs on electrically stimulated dopamine release from NAc shell. We aimed to characterise the effect of NMDA-R activation on NAc dopamine release, and to test the hypotheses (1) that NMDA-R activation modulates dopamine release in NAc shell via an indirect route, involving GABA, acetylcholine or mGluR receptors; and (2) that this modulation would be dysregulated in PCP pretreated animals, modelling schizophrenia.

Results and discussion

Repeated electrical stimulation (30 pulses; 60 Hz; 4 ms pulses; 500 µA; presented twelve times at 3 min intervals) of NAc shell in rat brain slices in vitro evoked a robust and reproducible increase in dopamine release, measured by FCV (Fig 1 a,b, and Fig 2 a,b; see also supplementary data, Figs S1 to S4), which corresponded to an increase of 730 ± 80 nM (mean ± SEM; n = 12), calculated by comparison to dopamine calibration recordings. Over 12 repeated stimulations at 3 min intervals there was no significant change in the magnitude of the stimulus-evoked dopamine release (n = 12; Fig 1 - 4: No drug condition). The NMDA-R agonist, NMDA (15 µM, 30 µM, and 60 µM) in the superfusate caused a dose-dependent decrease in electrically stimulated dopamine release (Fig 1 and Fig 2 c,d). Two-way ANOVA showed a main effect of drug (F[3,396] = 72.91; p < .0001) and of stimulus number (F[11,132] = 8.32; p < .0001) and a significant interaction (F[33,396] = 3.69; p < .0001). Post hoc analysis revealed no change over repeated stimulations in the control slices (No drug). Although there was an indication of a small decrease during the lowest dose of NMDA (15 µM), this was not significant, but significant
decreases were seen with the two higher doses of NMDA (30 μM and 60 μM). There was a highly significant linear trend in the effect of different doses of NMDA during the drug application (Fig 1b; $R^2 = .3573; p < .0001$) confirming the concentration-dependence of the effect. Following the end of NMDA superfusion, the stimulated release of dopamine showed limited, dose-dependent return towards baseline over the subsequent four stimulations: after 15 μM NMDA responses returned to 100% of baseline, while after 30 μM NMDA responses showed a partial return to baseline levels, but after 60 μM NMDA there was little sign of reinstatement of the response.

Figure 1. Dose-dependent effect of NMDA (15 μM, 30 μM, 60 μM), compared to control (No drug). (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods.

Data are mean ± SEM. * $p < .05$; ** $p < .01$; *** $p < .001$; Dunnett’s post hoc test, based on a significant interaction from ANOVA: n=12 per group.
Figure 2. Example FCV data from a single slice showing effect of NMDA (30 µM). (a) Colour plot and (b) current vs time plots of electrically stimulated (red bar) dopamine release during baseline period (S1), before the start of drug application. (c) Colour plot and (d) current vs time plots of electrically stimulated (red bar) dopamine release during superfusion of 30 µM NMDA (S8). In each case “S” indicates the stimulus artefact. Insets in (c) and (d) indicate cyclic voltammograms measured during the release, confirming the identity of dopamine (scale bar = 20 nA).

These data show a clear dose-dependency of the NMDA-evoked depression of stimulated dopamine release. Although there was an indication of a decrease at the lowest dose tested (15 µM) this was not statistically significant when compared with non-drug treated slices. However, the dose-dependency of the effect was confirmed across the three concentrations tested through the highly significant linear trend in response suppression with concentration. These data support previous findings that agonists at ionotropic glutamate receptors generally and specifically NMDA-R.
in dorsal striatum caused a decrease in stimulated dopamine, across a similar dose range, albeit that unlike those of Wu et al. \textsuperscript{12}, our studies did not require the removal of Mg\textsuperscript{2+} to observe NMDA-mediated changes (data not shown). At the end of the drug application, there was complete or partial return to baseline levels of response at the two lower concentrations, but at the high concentration the blockade was maintained over the full recording period. It is possible that these changes reflected excitotoxic damage. While we cannot rule this out in the highest concentration tested (100 µM), it is unlikely to account for the effects measured with a concentration of 30 µM, since (1) we saw at least a partial return to baseline levels and (2) staining of a representative sample (n = 4 for each concentration) of slices with the mitochondrial function marker 2,3,5-triphenyl tetrazolium chloride (TTC) after completion of recording, showed strong staining in all cases, indicating a healthy slice (good mitochondrial function): indeed the level of staining was similar to non-drug treated slices (see supplementary data, Fig S5). On the basis of these data, 30 µM NMDA was chosen as the most appropriate dose for subsequent antagonist studies as it showed a robust response, and at least a partial return to baseline during washout.

To ascertain that the effect of NMDA was specifically mediated through NMDA-Rs, rather than through other non-specific mechanisms, the selective NMDA-R antagonist, AP-5 (50 µM) was applied concomitantly with NMDA (30 µM) in the superfusate. This experiment replicated the previous depressant effect of NMDA (30 µM: Fig 1), which was entirely prevented by AP-5, while AP-5 alone had no effect (Fig 3). Statistical analysis using 2-way ANOVA confirmed a significant main effect of drug (F[3,240] = 166.0; p < .0001) and of stimulus number (F[11,240] = 11.48; p < .0001), and a significant interaction (F[33,240] = 9.844; p < .0001). Post hoc analysis (Dunnett’s test) showed a significant decrease in stimulated release during NMDA superfusion, which was abolished by AP-5.
Figure 3. Reversal of the effect of NMDA (30 μM), by NMDA receptor antagonism (AP-5, 50 μM) (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. Data are mean ± SEM. ** p < .01; ### p < .001; Dunnett’s post hoc test, based on a significant interaction from ANOVA: n=6 per group. # p < .01; #### p < .001 Significant Reversal of the NMDA effect by AP5 (Tukey’s HSD)

Therefore, the NMDA mediated depression of stimulated dopamine release was mediated specifically through NMDA-R, rather than by some non-specific route. Interestingly, and in contrast to previous reports of similar studies in dorsal striatum, the antagonist alone had no effect on stimulated dopamine release, indicating that any glutamate released by the electrical stimulation did not impact on the stimulated levels of dopamine.

It is unlikely that the depressant effect of NMDA is due to a direct action on dopamine axons, since (1) histological evidence shows a paucity of NMDA-Rs located on dopamine terminals, and (2) any direct effect of NMDA would be expected to be excitatory. Three possible indirect routes for this action were considered; via GABAergic systems, given the abundance of GABAergic medium spiny neurones and interneurons in NAc, via cholinergic mechanisms, on the basis of the evidence showing cholinergic modulation of dopamine release in the striatum or through mGluR-mediated mechanisms, since mGluR group 2 and 3 receptors are widely distributed in striatal areas, have been shown to be
inhibitory, and they decrease dopamine release, probably through presynaptic receptors located on dopamine terminals²⁹,³⁰.

To assess the role of GABAergic systems, the non-competitive GABA-A receptor channel blocker picrotoxin (100 µM) applied concomitantly with NMDA (30 µM) did not reverse the effect of NMDA: indeed there is an indication that picrotoxin may have slowed the reinstatement of the response. There was a main effect of stimulus number (F[11,324] = 9.31; p < .0001) and of drug (F[2,324] = 48.15; p < .0001) and a significant interaction (F[22,324] = 3.00; p < .0001). Post hoc analysis (Dunnett’s test) showed that NMDA (30 µM) caused a significant decrease in stimulated release either in the absence or the presence of picrotoxin (100 µM: n=6 for picrotoxin, n=7 for NMDA; Fig 4 a,b)

Given the abundance of GABAergic neurones in NAc (see above), and the observations that GABA-A receptors in NAc shell inhibit dopamine-mediated behaviours³¹, it seemed plausible that the inhibition of dopamine release might be mediated through NMDA-activation of GABA neurones, which in turn feed back on to the dopamine terminals. However, in our experiments, picrotoxin did not reverse the release inhibition induced by NMDA: indeed there is some evidence that it may have enhanced and prolonged it, in line with previous observations that picrotoxin decreases evoked dopamine release in dorsal striatum²⁸. However, due to floor effects this conclusion is ambiguous, and would require further investigation.

To test whether the NMDA-evoked suppression of stimulated dopamine release was mediated through nicotinic cholinergic systems, the α4β2 nicotinic receptor subunit antagonist, dihydro-β-erythroidine (DHβE), was applied with NMDA. Concomitant superfusion of DHβE (1 µM) with NMDA (30 µM) caused an attenuation of the NMDA-evoked depression of stimulated dopamine release. There was a main effect of stimulus number (F[11,252] = 4.276; p < .0001) and of drug (F[3,252] = 323.8; p < .0001) and a significant interaction (F[33,252] = 15.82; p < .0001). Post hoc analysis showed that the decrease in NMDA-evoked depression of dopamine was significantly attenuated, although not abolished, by DHβE. Notably, however, DHβE alone caused a significant increase in stimulated dopamine release (n = 5 to 6: Fig 4 c,d).
Figure 4. Effect of GABA-A receptor antagonism, (picrotoxin (PIC), 100 µM; a,b), α4β2 nicotinic receptor antagonism, (DHβE, 1 µM; c,d) or mGluR2/3 receptor antagonism, (MSPG, 200 µM; e,f) on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM). (a,c,e) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b,d,f) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. Data are mean ± SEM. * p < .05; ** p < .01; *** p < .001: significant difference from control (Dunnett’s post hoc test). # p < .05; ## p < .01; ### p < .001: significant difference between NMDA alone and (a) NMDA + picrotoxin, (c,d) NMDA + DHβE or (e,f) NMDA + MSPG (Tukey’s HSD test): n = 5 to 7.

Previous studies have shown that both muscarinic 24 and nicotinic 25 cholinergic mechanisms contribute to NMDA-mediated control of dopamine release in the striatum. We tested the effect of the nicotinic receptor blockade on the NMDA-mediated attenuation of stimulated dopamine by concomitant application of the α4β2 subunit antagonist DHβE. The antagonist did attenuate the NMDA-evoked depression of stimulated dopamine release, although it did not abolish it completely: the responses were still significantly below baseline. However, DHβE alone caused a substantial and significant augmentation of the stimulated dopamine release. It is therefore likely that the apparent attenuation of the effect of NMDA by DHβE is due to the co-occurrence of two independent processes, one depressant (NMDA) and one facilitatory (DHβE), rather than to the NMDA effect being mediated through cholinergic mechanisms, although further investigation would be required to confirm this.

Since mGluR activation has been reported to modulate accumbal dopamine release through presynaptic mechanisms 26, we tested whether the NMDA-evoked suppression of stimulated dopamine release was mediated through mGluR. Blocking group 2 and 3 mGluR using the relatively non-specific receptor antagonist (RS)-α-methyl-4-sulfonophenylglycine (MSPG: 200 µM) did attenuate
the effect of NMDA (30 μM) on stimulated dopamine release. There was a main effect of stimulus number \((F[11,228] = 14.45; p < .0001)\) and of drug \((F[3,228] = 149.3; p < .0001)\) and a significant interaction \((F[33,228] = 9.165; p < .0001)\). Post hoc analysis (Dunnett’s test) showed that NMDA caused a significant decrease in stimulated release which was attenuated in the presence of MSPG, while MSPG alone had no effect. Tukey’s HSD test on each time point showed a significant attenuation of the NMDA-evoked decrease during, and immediately after the drug application period \((n = 5 \text{ to } 6: \text{Fig 4 e,f})\).

The widespread distribution of mGluR in striatal areas, including NAc has been extensively reported. Eight different mGluR have been identified, which cluster into three main groups. While group 1 receptors are primarily excitatory, and found postsynaptically, groups 2 and 3 receptors (mGluR-2 and mGluR-3 respectively) are inhibitory and are located extra synaptically (mGluR-2) and presynaptically (mGluR-2, mGluR-3) as both autoreceptors and heteroreceptors. They are therefore exquisitely placed to modulate release of glutamate itself, and other neurotransmitters, including an inhibitory effect of mGluR-2/3 activation on dopamine release in NAc. The present experiments showed that concomitant application of the non-specific mGluR group2/3 receptor antagonist MSPG reversed the NMDA-evoked decrease in stimulated dopamine release, but alone had no effect. From this we may conclude that the NMDA effect is mediated via group 2 and/or 3 mGluR. The most likely location for these receptors would be on the axons or terminals of the afferent mesolimbic dopamine neurones, providing presynaptic inhibition of dopamine release. One possible mechanism for this may be through extrasynaptic ‘volume’ transmission by glutamate, which has previously been suggested as one mechanism of action of glutamate in NAc. Thus, NMDA-R activation would lead to increased spill-over of glutamate from the synaptic cleft, allowing activation of inhibitory mGluR located presynaptically and perisynaptically on dopamine terminals, leading to the observed decrease in dopamine release.

Sub-chronic pre-treatment with PCP leads to behavioural changes mimicking those seen in schizophrenia, which endure long after the end of the drug treatment, precluding this from being a
short term, acute action relying on the presence of the drug in the brain at the time of testing. Since this drug is an antagonist at NMDA-R, we proposed that the long term changes evoked by this pretreatment may cause dysregulation of NMDA-R mediated modulation of mesolimbic dopamine function.

Animals were pretreated in vivo with PCP (2 mg/kg, twice daily for five days), then remained drug-free for the remainder of the experiment (wash out period). One week after the end of pretreatment they were tested in a novel object recognition task to confirm the behavioural effect of the PCP pretreatment. Both saline pretreated and PCP pretreated animals showed similar exploration of both objects during the acquisition stage. During the test stage, animals pretreated with saline showed the expected preference to explore the novel object over the familiar one. However, animals pretreated with PCP did not show this preference, but instead spent similar amounts of time exploring each object. Discrimination index (DI) is a measure of how much animals discriminate between the two objects, with a value of zero indicating no discrimination. There was a significant difference in DI between saline-pretreated animals and PCP-pretreated animals (T[10] = 3.686; p = .004). More specifically, the saline pretreated group showed, as expected, a discrimination index significantly above zero (T[5] = 5.26; p = .003), indicating that they were able to discriminate between the familiar and the novel objects, as would be expected from control animals. However, the PCP pretreated animals showed no such discrimination, with the DI not significantly different from zero (T[5] = 2.334; p = .067), indicating a disruption in visual working memory in these animals.
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Table 1: Results from the NOR test. (a) time (s) time spent exploring the two familiar objects during the Acquisition stage (3 min); (b) time (s) time spent exploring the familiar and novel objects during the Test stage (3 min); (c) DI calculated from the time spent exploring familiar and novel objects during the Test stage. * p < .01; significantly different from zero; one-sample t-test; ## p < .01; significantly different from saline pretreated group; independent samples t-test).

Data are mean ± SEM; n = 6 per group.

The results from the behaviour testing showed the expected reduction of NOR after PCP pretreatment\(^{36,37}\), confirming that the pretreatment was effective. Although the ANOVA of the times spent exploring each object showed a main effect of object type (F[1,20] = 6.508, p = .02), but not of drug treatment (F[1,20] = .10; p = .75) nor any interaction (F[1,20] = 3.16; p = .09) this is most likely down to the low numbers of animals in each group giving a lack of power for this type of analysis. On the other hand, statistical analysis of the DIs, which take better account of the within-animal variability did confirm the disruption of the NOR effect statistically. Importantly the behavioural testing was carried out one week after the final drug pretreatment, ensuring that there was no drug present in the system at the time of testing. Therefore the impact on NOR is due to enduring changes in neural systems brought about by the sub-chronic PCP pretreatment, rather than by an acute effect of the drug at the time to testing\(^{37,38}\).
Three to eight days after behavioural testing, brain slices were taken from these animals for in vitro testing. In tissue taken from rats pretreated with PCP, NMDA produced a similar decrease in electrically stimulated dopamine release as in tissue taken from either saline pretreated animals (Fig 5) or non-pretreated animals (Fig 1,3,4). Three way ANOVA showed a main effect of stimulus number ($F[11,11] = 19.57; p < .0001$) and of NMDA ($F[1,11] = 531.7; p < .0001$), but no main effect of PCP ($F[1,11] = .518; p = .472$) and a significant stimulus number x NMDA interaction ($F[11,11] = 26.98; p < .0001$): no other interactions were significant. Post hoc analysis (Tukey’s HSD test) showed that PCP pretreatment had no effect on stimulated dopamine release in control (No drug) slices or NMDA treated slices. Specifically, NMDA caused a significant decrease in stimulated release in both PCP pretreated and saline (vehicle) pretreated animals ($n = 5$ to $6$: Fig 5). Importantly, before culling the animals to take the tissue for the in vitro experiments reported here, the animals were tested behaviourally on a novel object recognition task, known to be sensitive to PCP pretreatment $^{37,38}$. Whilst saline pretreated animals showed normal novel object recognition, in PCP pretreated animals this was disrupted, providing evidence that the PCP pretreatment was effective in the animals from which the tissue was taken for the in vitro experiments.

Figure 5. Effect of PCP (2 mg/kg, i.p., twice daily for 5 days) or saline vehicle (1 ml/kg, i.p., twice daily for five days) on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM). (a) Time course of the effects over twelve stimulations
at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. Data are mean ± SEM. ** p < .01; *** p < .001: significant difference from baseline period (Dunnett’s post hoc test), and from the appropriate saline pretreated groups (Tukey’s HSD test): n = 5 to 6.

From this experiments we conclude that it is unlikely that the effect of sub-chronic PCP is mediated through a direct change in NMDA-R regulated control of dopamine release in NAc shell. Importantly, the PCP pretreatment was completed seven days before behavioural testing, and 10 to 15 days before the in vitro experiments, indicating that the effects seen are due to enduring consequences of the drug having been present, rather than acute effects of the drug being present during testing 37. In a broader context, too, these data suggest that the long term effects of PCP pretreatment are not mediated through global changes in NMDA-R function. This leads to the possibility either that only localised changes to NMDA-R function occur in regions outside NAc, or that the enduring changes which occur due to PCP pretreatment are downstream effects consequent on chronic blockade of NMDA receptors over the period of five days, rather than direct effects on NMDA-R function.

In summary, using repeated electrical stimulation, we were able to measure the effect of NMDA receptor activation on stimulated dopamine release in slices of NAc shell, and to investigate the mechanism of the interaction. In the experiments reported here we demonstrated three replications of the depressant effect of NMDA on dopamine release: a very similar depressant effect of NMDA was seen in slices taken from untreated animals (two replicates: Fig 1 and Fig 2, 3), and from slices taken from saline pretreated animals (Fig 4). The effects were blocked by the NMDA-R antagonist AP-5, indicating a specific, NMDA-R mediated mechanism, but not by picrotoxin, indicating that an intermediary action via GABA-A receptors is unlikely. Cholinergic systems are known to modulate of striatal dopamine release, through both nicotinic and muscarinic mechanisms 13,23,24,25. In the present
study we found that the α4β2 nicotinic antagonist, DHβE, attenuated the NNDA-R evoked suppression of stimulated dopamine release. However, since DHβE alone produced a substantial enhancement of stimulated release on its own, this effect is difficult to interpret, and requires further investigation. The NMDA-evokes suppression of stimulated dopamine release was blocked by the mGluR group 2/3 receptor antagonist MSPG, which alone had no effect, indicating a role for these inhibitory mGluR in the depressant effect of NMDA. We suggest that the NMDA activation provokes increased spill-over of glutamate from the synaptic cleft, which then interacts with inhibitory mGluR located on dopamine terminals to decrease dopamine release. Finally we showed, that PCP pretreatment, sufficient to bring about behavioural deficits in novel object recognition, did not change the NMDA-mediated decrease in dopamine release, indicating, somewhat surprisingly, that the sub-chronic treatment with this drug, a non-competitive NMDA-R antagonist, does not cause long term changes in NMDA-R function at least in NAc shell, suggesting that the changes evoked involve mechanisms downstream of NMDA-R action.

**Methods**

**Animals:** For experiments using non-pretreated animals, female Wistar rats (Division of Biomedical Services, University of Leicester) were humanely killed 4 to 10 days after weaning (age 25 to 31 days), and the brains removed and placed in ice cold artificial cerebrospinal fluid (aCSF: (mM), NaCl (126.0), KCl (2.0), KH₂PO₄ (1.4), MgSO₄ (2.0), NaHCO₃ (26.0), CaCl₂ (2.4), Glucose (4.0)). For pretreated animals, drug treatment started 4 to 5 days after weaning (age 25 to 26 days). Animals were injected twice daily with PCP (2 mg/kg; i.p.) or saline vehicle (1 ml/kg; i.p.) for five days, after which they remained drug free for the remainder of the experiment. Seven days after the end of the drug pretreatment they underwent behavioural testing with novel object recognition (NOR) and at least three days after that they were humanely killed and tissue and the brain removed, as described above. All procedures using animals were carried out with appropriate personal and project licence (PPL 6004390) approval under the Animals (Scientific Procedures) Act, 1986, and with local ethical approval from the University of Leicester Animal Welfare and Ethical Review Body (AWERB).
Procedure: Seven days after the end of drug pretreatment, animals were tested behaviourally, using a novel object recognition task, as previously described. Briefly, they were habituated to the test arena (60 x 60 x 60 cm, black plexiglass) for 20 minutes on two consecutive days. The day after habituation, they were placed individually in the test arena for 3 min with no objects present, then removed and placed in a holding cage for 1 min. During this time, two identical objects (a filled metal cylinder: 12cm high x 10 cm diameter) were placed in the arena, in opposite corners, 10 cm from the edge of the arena. The rat was then returned to the arena and allowed to explore for a further 3 min acquisition period. They were then removed from the test arena and again placed in the holding cage for 1 min (inter-trial interval), while the objects were changed: one object (familiar) was identical to the ones exposed during acquisition, and the other (novel) object was a glass jar (9cm high x 10 cm diameter), placed in the same positions in the arena as in acquisition. The rat was again introduced to the arena and allowed to explore for the 3 min test period. The position of the familiar and novel objects was counterbalanced across animals. The sessions were videoed continuously, and scored off line by two independent scorers, blinded to the drug treatment condition. Using a digital stopwatch, the time spent exploring each object was recorded in both the acquisition and test periods. The DI was calculated for each animal as the \[
\frac{\text{Time spent exploring the novel object} - \text{Time spent exploring the familiar object}}{\text{Time spent exploring the novel object} + \text{Time spent exploring the familiar object}}
\] A DI of zero indicates no discrimination between objects, while a DI of 0.5 indicates spending twice as much time exploring the novel object as the familiar object.

After removal from the skull (see above) brains were transferred to a tissue slicer (NVSLM1 Vibroslice, World Precision Instruments) and consecutive 400 μm slices were cut. Each slice was cut in half along the mid-line, and placed in a tissue saver, comprising a wire mesh suspended in continuously oxygenated aCSF at room temperature, for at least one hour to recover from slicing. For recording a unilateral slice containing NAc shell was transferred to the tissue chamber of the recording apparatus, and superfused with continuously oxygenated aCSF at 33° ± 1°C, flow rate 2 ml/min, for 60 minutes, using a peristaltic pump (Gilson Minipuls3), before the start of recording. Each brain yielded 2 or 3 bilateral slices containing NAc shell, giving sufficient tissue for 4 to 6 treatments per brain. In each
case, different drug conditions were used on each slice from any one brain, with the order of testing randomised across brains, meaning that the n-values quoted are the number of slices, each from different brains.

For FCV recording of electrically stimulated dopamine release, a bipolar tungsten stimulating electrode, constructed in the lab, was lowered into NAc shell \(^{39}\), until the stimulating tips were approximately 50 μm below the surface of the slice. The stimulating electrode comprised two tungsten wires (0.075mm diameter) inserted into a glass capillary, including a glass septum to ensure electrical isolation (theta capillary; 1.5 mm o.d.; World Precision Instruments, UK), and pulled over a flame to achieve a tip, with electrodes separated by 700 ± 100 μm. FCV recordings were made at a carbon fibre working electrode, constructed in the lab as described by Clarke and co-workers \(^{40}\).

Briefly, a 2 cm length of 8 μm carbon fibre was inserted into a 1 cm length of vitreous silica tubing (OD 90 μm, ID 20 μm; CM Scientific, Cambridge, UK) under ethanol. After drying, the carbon fibre working tip was sealed with epoxy (Devcon, 5 minute epoxy, ITW Polymers, Danvers, USA) and a gold plated pin socket (MillMax, 0667; id 0.6mm; Farnell Electronics, Leeds, UK) was secured to the opposite end with silver, conducting epoxy (Circuitworks CW2400, Farnell Electronics, Leeds, UK), ensuring a contact between the carbon fibre and the socket. Finally the carbon fibre working tip was cut to a length of 120 μm (± 20 μm) with iris scissors. The FCV electrode was lowered into the tissue to a depth of approximately 120 μm, to lie approximately 500 μm from the stimulating electrode, and centrally located between the poles of the stimulating electrode.

For FCV recording, a triangular waveform (-0.4 to +1.3 to -0.4 v; 400 V/sec relative to Ag/AgCl reference electrode) was applied to the carbon fibre working electrode, at a frequency of 10 Hz, and the dopamine concentrations were assessed by measuring the oxidation current at +600 mV in the background subtracted signal, using Demon Voltammetry software \(^{41}\) (Wake Forest, USA).

For control experiments, twelve stimulus trains (30 pulses; 500 μA, 4 ms, 60 Hz; offset by 10 ms from the FCV waveform) were applied at 3 minute intervals from a constant current stimulus isolator (Iso-Flex; AMP Instruments), driven by the Demon software. Repeated 15 sec current recordings were made, such that the stimulation occurred 5 sec into each recording. In preliminary experiments (data
not shown), these stimulus parameters were found to produce consistent and reproducible TTX-dependent dopamine release over twelve consecutive stimulations required to characterise the drug effects (see supplementary data, Fig S1 and “No Drug” condition in Figs 1, 3, 4 and 5). Where drugs were applied to the tissue, the superfusion medium was switched to aCSF containing the drug(s) immediately after the completion of the fourth stimulus train: superfusion was then returned to aCSF immediately after the eighth stimulation. Taking into account the time taken for the drug to travel from the reservoir to the tissue chamber (80 sec), the tissue was exposed to the drug from 90 sec before the fifth stimulus train. Separate slices were used for each of the treatments, such that each slice received only twelve electrical stimulus trains, and a single drug treatment, and the order of testing of the different conditions was randomised. Experiment 1: NMDA (15 μM, 30 μM or 60 μM) was superfused during stimulation trains 5 to 8, and compared to the control condition where no drug was applied. In experiment 2, the effects of antagonists on the NMDA-evoked decrease in stimulated dopamine was assessed by comparing the control (no drug) condition with NMDA (30 μM) alone, NMDA (30 μM) + D-(-)-2-amino-5-phosphonopentanoic acid (AP-5: 50 μM), AP-5 alone (50 μM), NMDA + DHβE (1μM), DHβE alone (1 μM), NMDA (30 μM) + MSPG (200 μM), MSPG alone (200 μM), or NMDA (30 μM) + picrotoxin (100 μM). For experiment 3, animals were pretreated with PCP or saline (see above), and the slices were superfused with NMDA (30 μM) and compared to control slices receiving no drug. In each case, following the end of drug application, a further 4 electrical stimulations were applied at 3 min intervals (stimulations 9 to 12).

On completion of the recording, a representative sample of untreated slices and slices treated with NMDA (15 μM, 30 μM, 60 μM; n = 4 of each) were stained with TTC, to ascertain the viability of the slices. Slices were transferred to 1cm diameter dishes (3 ml) and 1 ml 2% TTC in aCSF was added and incubated at room temperature, and in darkness, for 30 min. After staining, TTC aspirated off and replaced with 1 ml formal saline (10% formalin in 0.9 % saline solution). The degree of staining (showing functional mitochondria, indicative of living tissue) was assessed qualitatively in each slice under low magnification.
Chemicals and Drugs: PCP hydrochloride, AP-5 and MSPG were purchased from Tocris, UK. All other drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK). Stock solutions of drugs (10 mM) were made up as follows, aliquoted, and stored at -80°C: MSPG was dissolved in 10 mM NaOH; picrotoxin was dissolved in 100 mM in DMSO; NMDA and DHβE dissolved in water. Working concentrations of the drugs were made freshly on the day of each experiment by appropriate dilutions of the stock solutions in aCSF. PCP for i.p. injection was dissolved in sterile physiologic saline.

Data Analysis: The current generated by dopamine oxidation (+600 mV) in the background subtracted signal was measured on line (Demon Voltammetry software), and the maximum concentration of dopamine release was calculated from the oxidation current, by comparison with a dopamine standard calibration (5 µM) carried out prior to each experiment session. For each experiment, the mean baseline increase was calculated from the first four stimulus presentations. The changes measured at all twelve stimulus presentations were then expressed as the percentage of this mean baseline value. Pooled data from repeated experiments are expressed as mean ± SEM percentages of the baseline stimulated release measured during the first four stimulations in each experiment. Statistical analysis was by mixed two- or three-way analysis of variance (ANOVA), with appropriate post hoc comparisons using either Dunnett’s test or Tukey’s HSD. Statistical analysis was performed by using GraphPad Prism 7.

Author Contributions

EY carried out all the experiments and analysis, and contributed to the preparation of the manuscript. AMJY supervised all experimental work and analysis, and took the lead in preparing the manuscript.

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Conflict of Interest

None

References


