Impact of neonatal NOS-1 inhibitor exposure on neurobehavioural measures and prefrontal-temporolimbic integration in the rat nucleus accumbens

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Abstract

Nitric oxide (NO) is a gaseous neurotransmitter that plays a significant role in the establishment and refinement of functional neural circuits. Genetic and post-mortem studies have suggested that neuronal NO synthase (NOS-1) activity may be compromised in frontal and temporal lobes, and related structures, in schizophrenia. The goal of this study was to determine if there is a link between neonatal disruptions in NO signalling and disturbances in the development and function of prefrontal–temporolimbic circuits. Neonatal rats were injected on postnatal days PD3–5 with the selective NOS-1 inhibitor Nω-propyl-L-arginine (NPA) and tested in adulthood (≥PD60) or as juveniles (PD30). Adult rats treated with NPA as neonates exhibited increased amphetamine-induced locomotion compared to animals receiving vehicle as neonates, whereas this was not observed in juvenile rats treated with NPA as neonates. Adult rats exposed to NPA as neonates also exhibited deficits in social interaction and short-term recognition memory, as well as reduced brain weight, compared to vehicle-treated controls. Finally, neonatal NPA exposure increased the responsiveness of nucleus accumbens neurons to prefrontal cortical input and disrupted the modulation of cortico-accumbens circuits by hippocampal afferents that is normally observed in adult animals. These results show for the first time that neonatal inhibition of NOS-1 during a critical neurodevelopmental period leads to aberrant behaviours that manifest in adulthood, as well as electrophysiological abnormalities in prefrontal–temporolimbic circuits. Greater understanding of the role of NOS-1 in the development of these circuits will shed light on how developmental insults translate to pathophysiology associated with schizophrenia.

Received 18 June 2013; Reviewed 12 July 2013; Revised 27 July 2013; Accepted 7 August 2013; First published online 12 September 2013

Key words: Hippocampus, nucleus accumbens, prefrontal cortex, schizophrenia.

Introduction

Nitric oxide (NO) is a gaseous neurotransmitter implicated in the etiopathophysiology of schizophrenia. NO is produced by NO synthase (NOS) (Garthwaite, 2008). The neuronal isoform of NOS (NOS-1) and the postsynaptic target of NO, soluble guanylyl cyclase (sGC), are ubiquitously expressed in the adult brain. NOS-1 and sGC are also prevalent in a number of brain regions during early development (Breit and Snyder, 1994; Giulii et al., 1994), including cortical and limbic structures that are involved in motivated and mood-related behaviours. Moreover, there is now substantial evidence that NO plays a significant role in all major histogenetic events involved in the establishment and refinement of functional neural circuits, including proliferation, differentiation, axon outgrowth, synaptogenesis, synaptic pruning, and programmed cell death (Contestabile, 2000; Sunico et al., 2005; Nikonenko et al., 2008).

Neurodevelopmental disruption of NO signalling results in a wide range of behavioural and anatomical abnormalities which are associated with pathological function in fronto-temporolimbic circuits. For example, male NOS-1 knockout mice and animals exposed to NOS-1 inhibitors exhibit robustly increased impulsive aggression and abnormal social and sexual behaviour (Nelson et al., 1995; Demas et al., 1997). More recent studies have reported that NOS-1 knockout mice also exhibit hyperactivity, impaired spatial memory, and increased dopamine (DA) D1 receptor signalling (Tanda et al., 2009). Furthermore, studies by Black and colleagues showed that transient, neonatal exposure to the non-selective NOS...
inhibitor L-nitroarginine induces behavioural changes in male and female rats suggestive of disturbances in DA and glutamate transmission (Black et al., 1999, 2002). Specifically, male rats exhibited hypersensitivity to amphetamine (AMPH) and deficits in social interaction and pre-pulse inhibition in adulthood, but not juvenile animals (Black et al., 1999, 2002). Subsequent studies replicated and extended several of these observations and provided further evidence for behavioural abnormalities expressed primarily in adulthood as a result of neonatal NOS inhibition, including hyperlocomotion in response to stress, latent inhibition deficits, and hypersensitivity to DA agonists (Morales-Medina et al., 2008; Black et al., 2009). Neuroanatomical abnormalities of the prefrontal cortex (PFC) and hippocampus have also been reported following neonatal disruption of NOS activity. These include decreased dendritic spine density and dendritic length in pyramidal neurons in the hippocampus (Morales-Medina et al., 2007) and lower expression of synapsin 1 in the PFC (Sánchez-Islas and Léon-Olea, 2004).

Interestingly, abnormal distributions and morphology of NOS-1 positive interneurons have been reported in prefrontal–temporolimbic circuits in post-mortem studies of patients with schizophrenia (Akbarian et al., 1993a,b; Lauer et al., 2005; Fritzen et al., 2007). Regulatory polymorphisms in the NOS-1 gene which lead to decreased NOS-1 activity have also been shown to contribute to the genetic risk for developing schizophrenia (Shinkai et al., 2002; Reif et al., 2006; Tang et al., 2008; Silberberg et al., 2010). The presence of regulatory polymorphisms in the NOS-1 gene was also shown to be negatively correlated with performance on cognitive tasks associated with PFC activation (Reif et al., 2006; Rose et al., 2012). Additionally, the expression of the NOS-1 adaptor protein NOS-1AP, which reduces N-methyl-D-aspartate receptor (NMDAR)-NOS-1 coupling and NOS-1 activation, has been found to be abnormally elevated in patients with schizophrenia (Xu et al., 2005; Wrattn et al., 2009). Taken together, these strong links between aberrant NO transmission and schizophrenia suggest that NO signalling cascades may represent promising targets for antipsychotic treatments (Pálsson et al., 2010; for review see Bernstein et al., 2011). Abnormalities in NO signalling have also been linked to other neuropsychiatric disorders affecting motivational processes, including affective disorders, impulsive aggression, and suicidal behaviour (Reif et al., 2009; Cui et al., 2010). The nucleus accumbens (NAc) plays a central role in these motivational processes, and prefrontal–temporolimbic inputs to the NAc can drive motivated behaviours (Mogenson et al., 1980). However, little is known about the role of NO in the maturation of limbic circuits and neural processing in the NAc.

Studies in the developing rat brain have shown that high concentrations of NO-stimulated cGMP are present in the CNS immediately after birth and decline during the first 3 wk postnatal (DeVente and Steinbusch, 1992). Additionally, NOS-1 activity in the rat neocortex has been shown to peak within the first week postnatal (Ogilvie et al., 1995). Thus, the present study used a sub-chronic NOS-1 inhibitor treatment regimen delivered on PD3–5 in order to determine how compromised NO signalling during early stages of neonatal development leads to aberrant function within prefrontal–temporolimbic-NAc circuits in adulthood.

Methods

Chemicals

Urethane and amphetamine sulfate (AMPH) were purchased from Sigma Chemical (USA). The selective NOS-1 inhibitor N<sup>C</sup>-propyl-L-arginine (NPA; Zhang et al., 1997) was purchased from Tocris (USA). All other reagents were of the highest grade commercially available.

Subjects

All experimental procedures were conducted under protocols approved by either Rosalind Franklin University of Medicine and Science or Abbott Laboratories Institutional Animal Care and Use Committee. All animals were housed under conditions of constant temperature (21–23°C) and maintained on a 12:12 light/dark cycle with food and water available ad libitum. Male Sprague–Dawley pups born to timed-pregnant dams were used for all experiments (Harlan, USA or Charles River Laboratories, USA). Male pups were injected (s.c.) with either saline or NPA (10 mg/kg) on PD3–5 and returned to their mother and litter mates. NPA was employed in these studies because of its superior potency and selectivity as a NOS-1 inhibitor (Zhang et al., 1997). Moreover, we have previously demonstrated the effectiveness of systemic NPA administration for decreasing NOS activity in multiple studies/brain regions using several different parallel and complementary amperometric (Sammut et al., 2007) and histochemical approaches (Hoque et al., 2010; Hoque and West, 2012). Upon reaching PD25 rats were weaned and housed in pairs with another rat that received the same injection regimen (i.e. saline control or NPA). Prior to experimentation, male rats were allowed to mature until either PD30 (juvenile) or ≥PD60 (adult) as indicated.

Amphetamine locomotion test

Rats were allowed to habituate in the test room for a period of 3 h on the day prior to testing. On both the habituation and the test day, each rat was placed into a clear test chamber. Three photobeam devices were used to monitor the horizontal locomotor activity of each animal for 3 h (data were collected in 10 min bins). On the test day, vehicle and NPA-treated rats were divided...
into two groups and received either saline or AMPH (0.5 mg/kg s.c.). Animals were allowed to acclimate to the testing environment for 20 min prior to drug or vehicle treatment. No evidence of stereotypies (e.g. abnormal tremors, oral movements, or dyskinesias) was observed in any of the treatment groups during testing. Animals that were administered AMPH were not used in any other experiments.

Social interaction and short-term recognition memory
Male rats were tested in the light phase of a 12 h light:12 h dark schedule (lights on at 06:00 hours). Animals were allowed free access to food and water except for during the test period. Adult (350–450 g) and juvenile (50–80 g) animals were allowed to acclimate to the test room for 90–120 min. Adult rats were then placed alone in their respective test cages and, after a brief habituation period (30 min), allowed to interact for 5 min with a juvenile rat (trial one; T1). During the interactive trial, the adults exhibit investigative behaviours that include close following, grooming and/or sniffing of the juvenile for as much as 40–50% of the trial duration. The juvenile rat was then removed and the adult rats were left in the test cage. A second identical 5 min interactive trial (trial two; T2) was conducted 40 min later in the same test cage. Interaction times (T1) and recognition ratios of time spent investing the familiar juvenile (T2/T1) were calculated.

Surgery
Rats were deeply anaesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic apparatus as previously described (Sammut et al., 2010). Burr holes (α 2–3 mm in diameter) were drilled in the skull overlying the PFC (coordinates from bregma: 3.2 mm anterior, 0.8 mm lateral), NAc (coordinates from bregma: 1.2–1.8 mm anterior, 0.6–1.4 mm lateral), and hippocampal fimbria (coordinates from bregma: −1.3 mm anterior, 1.4 mm lateral). The dura mater was resected and concentric bipolar stimulating electrodes were lowered into the PFC (4 mm ventral from dura) and hippocampal fimbria (3.8 mm ventral from dura). All experiments were initiated ca 2 h post-surgery.

Electrical stimulation
Concentric bipolar electrodes were used to deliver stimuli to the PFC and fimbria and stimulation currents delivered to the PFC were titrated to an intensity (200–1400 μA) which reliably evoked spike activity approximately 50% of the time (Ondracek et al., 2008). This intensity was then used for all subsequent stimulation trials. Fimbria train stimulation (250 μA, 30 Hz, 500 ms train duration) was delivered intermittently (2 s ITI) during a single trial lasting 50 s (25 individual trains) with each train followed by a single PFC test pulse (500 ms delay). Stimulation trials were performed in the following order: PFC stimulation alone (pre-train), fimbria train and PFC stimulation, PFC stimulation alone (post-train trials 1, 2 and 3).

Extracellular recordings
Recordings of PFC- and fimbria-evoked extracellular single-unit activity were made in the NAc (see Fig. 3). Single-unit microelectrodes were manufactured as previously described (Ondracek et al., 2008; Sammut et al., 2010). In order to isolate single-units, microelectrodes were lowered incrementally through the NAc using a micromanipulator (MO-8, Narashige) while single-pulse electrical stimuli were applied to the cortex and fimbria. Neurons in the NAc were recorded only if they received excitatory inputs from both the PFC and fimbria as determined by evoked spike activity. Cells exhibiting relatively high spontaneous firing (>4.0 Hz) or spike characteristics resembling striatal interneurons (Ondracek et al., 2008) were not recorded in this study. Electrode potentials isolated from putative NAc medium-sized spiny neurons (MSNs) were filtered (highpass, 500 Hz; lowpass 10 kHz) and then amplified and digitized using a Multiclamp 700B amplifier and Digidata 1322A interface (Axon Instruments/Molecular Devices, USA) or Xcell 3+ micro-electrode amplifier (FHC, USA). Data were acquired at a sampling rate of 20 kHz using Axoscope 10.1 software (Axon Instruments/Molecular Devices) and analysed using Clampfit 10 (Axon Instruments/Molecular Devices). Cortical local field potentials were monitored as previously described (Tseng et al., 2011). After completion of each experiment, rats were deeply anaesthetized and perfused transcardially as previously described (Sammut et al., 2010). Brains were then removed and processed for histological assessment of stimulating and recording electrode sites (Fig. 7).

Data analysis
Locomotor activity was quantified as the total number of beam breaks occurring within 10 min epochs. The statistical significance of AMPH-induced changes in locomotor activity in rats exposed to neonatal saline or NPA was determined using two-way repeated measures (RM) analysis of variance (ANOVA) (Sigma Stat, Jandel). The two factors examined were perinatal drug treatment and juvenile/adult drug treatment. Social interaction and recognition were defined as the total amount of time(s) spent in active social interaction as described above. Significant differences between group means were assessed using an unpaired Student’s t-test. The statistical significance of changes in these gross anatomical measures induced by perinatal NPA treatment was determined using an unpaired Student’s t-test. Electrophysiological data was analysed in Clampfit as previously described (Sammut et al., 2010). Probability, latency, and coefficient of variation of evoked spikes were determined using a two-way RM ANOVA. The two factors
examined were perinatal drug treatment and stimulation induced changes in spike activity occurring across stimulation trials. In all studies using ANOVA, Tukey post-hoc tests were used to conduct pair-wise comparisons to determine which factors contributed to overall differences.

**Results**

**Effect of neonatal NPA administration on the responsiveness of juvenile and adult rats to amphetamine**

In rats treated with vehicle or NPA as neonates and tested as juveniles (Fig. 1(a), (c)), significant main effects of AMPH \((F_{3,288}=17.689, p<0.001)\) and time \((F_{8,288}=44.469, p<0.001)\) on locomotion were observed. Additionally, significant interactions were observed between AMPH treatment and time \((F_{24,288}=6.786, p<0.001)\). However, rats treated with NPA as neonates and tested as juveniles exhibited no difference in responsiveness to AMPH (0.5 mg/kg s.c.) as compared to rats treated with vehicle as neonates (two-way ANOVA/Tukey test, \(n=10\) animals per group). (b) Rats treated with NPA as neonates and tested in adulthood (≥PD60) exhibited increased responsiveness to AMPH (0.5 mg/kg s.c.) as compared to rats treated with vehicle as neonates \((**p<0.001,\) two-way ANOVA/Tukey test, \(n=13-15\) animals per group). Note: this finding was replicated in a separate cohort of animals (data not shown). (c) Juvenile (PD30) test: locomotion averaged over the first 20 min of testing was significantly elevated in both AMPH-treated groups \((**p<0.001\) or \(**p<0.005)\), as compared to respective vehicle-treated control groups, two-way ANOVA/Tukey test, \(n=10\) animals per group), however no significant differences were observed between the vehicle and NPA-treated animals \((p>0.05)\). (d) Adult (≥PD60) test: locomotion averaged over the first 20 min of testing was significantly elevated in both AMPH-treated groups \((**p<0.001,\) two-way ANOVA/Tukey test, \(n=13-15\) animals per group). A significant increase in the response of NPA-treated animals to AMPH was also observed, as compared to the vehicle-treated controls \((###p<0.001)\).

**Fig. 1.** Effect of neonatal NPA administration on amphetamine induced locomotion measured in juvenile and adult rats. (a) Rats treated with NPA as neonates and tested as juveniles (PD30) exhibited no difference in responsiveness to AMPH (0.5 mg/kg s.c.) as compared to rats treated with vehicle as neonates (two-way ANOVA/Tukey test, \(n=10\) animals per group). (b) Rats treated with NPA as neonates and tested in adulthood (≥PD60) exhibited increased responsiveness to AMPH (0.5 mg/kg s.c.) as compared to rats treated with vehicle as neonates (**\(p<0.001\), two-way ANOVA/Tukey test, \(n=13-15\) animals per group). Note: this finding was replicated in a separate cohort of animals (data not shown). (c) Juvenile (PD30) test: locomotion averaged over the first 20 min of testing was significantly elevated in both AMPH-treated groups (**\(p<0.001\) or **\(p<0.005\)), as compared to respective vehicle-treated control groups, two-way ANOVA/Tukey test, \(n=10\) animals per group), however no significant differences were observed between the vehicle and NPA-treated animals (\(p>0.05\)). (d) Adult (≥PD60) test: locomotion averaged over the first 20 min of testing was significantly elevated in both AMPH-treated groups (**\(p<0.001\), two-way ANOVA/Tukey test, \(n=13-15\) animals per group). A significant increase in the response of NPA-treated animals to AMPH was also observed, as compared to the vehicle-treated controls (###\(p<0.001\)).
increased responsiveness to AMPH as compared to rats treated with vehicle as neonates (Fig. 1(b); \( p<0.001 \)). Locomotor counts summed over the first 20 min were examined further as this was the period of maximal response to AMPH. Neonatal NPA exposure did not significantly alter AMPH-induced locomotion in juveniles (Fig. 1(c), \( p>0.05 \)). However, AMPH administration significantly increased locomotion in the NPA-treated, adult animals above that observed in vehicle-treated controls (Fig. 1(d), \( F_{3,36}=29.748, \ p<0.001 \)). Given that we did not observe significant differences in AMPH-induced locomotion in the juvenile group, all further experiments were performed in adult rats.

**Effect of neonatal NPA administration on social interaction and recognition in adult rats**

Male rats treated with NPA (10 mg/kg, s.c.) as neonates and then exposed to a strange juvenile as adults displayed significant decreases in the investigative duration of trial 1 (T1), showing an impairment in normal social interaction when compared to vehicle-treated controls (Fig. 2(a); \( p<0.001, \ t\text{-test} \)). During a subsequent exposure to the same juvenile 40 min later (trial 2; T2), the NPA-treated rats investigated the familiar juveniles for a similar duration (\( T2/T1 \approx 1.0 \)), which is an indication of short-term memory impairment. Conversely, vehicle-treated rats exhibited a \( T2/T1 \) ratio \( \approx 0.5 \), indicating memory retention (Fig. 2(b); \( p<0.001, \ t\text{-test} \)).

**Effect of neonatal NPA treatment on PFC-evoked activity recorded in the NAc of adult rats**

All electrophysiological recordings were performed once rats reached adulthood. The recording protocol and representative traces of evoked responses are depicted in Fig. 3. Animals treated with NPA as neonates required smaller current amplitudes to evoke spike activity (suggesting increased PFC-NAc circuit excitability) as compared to vehicle-treated controls (Fig. 4(a); \( t\text{-test}, \ p<0.05 \)). However, no significant difference in spike probability or the mean number of spikes evoked in the pretrain stimulation trials were observed between the NPA and vehicle-treated animals (Fig. 4(b); \( t\text{-test}, \ p>0.05 \)). Additionally, no significant differences in spike latency or coefficient of variance of spike latency were observed between groups (data not shown).

**Modulation of PFC-evoked activity in the NAc during train stimulation of the hippocampal fimbria**

In the majority of adult rats from both NPA- and vehicle-treated groups, PFC-evoked spike activity in the NAc was modified when preceded by high frequency train stimulation of the hippocampal fimbria (Fig. 5). As described in our previous studies on the dorsal striatum (Ondracek et al., 2008; Sammut et al., 2010), three response types were observed: excitatory (Fig. 5(a), left), inhibitory (Fig. 5(a), right), and no change. The magnitude of the excitatory and inhibitory responses induced during the fimbria stimulation trial was not affected by neonatal treatment (Fig. 5(b), (c); \( p>0.05 \)). However, significant main effects of time on spike probability were observed in excitatory response types (\( F_{4,36}=9.056, \ p<0.001; \) Two-way RM ANOVA and Tukey post-hoc test), with NPA-treated animals exhibiting a more transient facilitation of
Thus, stimulation in cells exhibiting synaptic suppression.

100 pulses per trial. (Fisher Exact test, p < 0.05; t-test). No significant differences in body weight or brain volume were observed between groups (Fig. 8(a), (c); p > 0.05).

Discussion

The present study found that adult rats treated with the selective NOS-1 inhibitor NPA (Zhang et al., 1997) as neonates exhibit increased AMPH-induced locomotion and deficits in social interaction and short-term recognition memory as compared to animals receiving vehicle as neonates. Animals exposed to NPA as neonates exhibited decreased brain weight in the absence of changes in brain volume or overall body weight. Recordings in adulthood also showed that neonatal NPA exposure increased the responsiveness of NAC neurons to PFC stimulation. Most interestingly, neonatal NPA exposure decreased the incidence and duration of excitatory effects of hippocampal drive on PFC-evoked activity. Following neonatal NOS-1 disruption, this fimbria-induced excitation was replaced with a prominent and enduring inhibition of PFC-evoked activity. These findings suggest a vital role for NO signalling in normal neurodevelopment. Indeed, early disruptions in NO signalling may lead to a reorganization of prefrontal–temporolimbic-NAC circuits and pathophysiological function in adulthood.

Neuroimaging studies have shown that patients with schizophrenia are more responsive to AMPH as indicated by greater displacement of radioligands.

PFC-evoked spiking (Fig. 5(b)). Interestingly, neonatal exposure to NPA significantly decreased the recovery of PFC-evoked spike activity observed following fimbria stimulation in cells exhibiting synaptic suppression. Thus, significant main effects of time on spike probability were observed in inhibitory response types (F(4, 65) = 27.098, p < 0.001). Moreover, significant interactions were observed between neonatal drug treatment and time in inhibitory response types (F(4, 65) = 3.456, p < 0.05). Pairwise comparisons revealed that neonatal NPA administration induced a robust decrease in cortically-evoked spike activity, following high frequency fimbria train stimulation, in NPA-treated animals as compared to vehicle-treated controls (Fig. 5(c); p < 0.01). No significant changes (p > 0.05) in spike latency or coefficient of variance were observed in NPA-treated animals (data not shown). Although all three response types were observed in both NPA and vehicle-treated animals, the relative proportion of these responses varied significantly depending on neonatal treatment (Fig. 6). Thus, neonatal NPA treatment resulted in a significant decrease in the incidence of excitatory responses (Fisher Exact test, p < 0.05), as well as a significant increase in the proportion of inhibitory responses observed during train stimulation of the fimbria (Fig. 6; Fisher Exact test, p < 0.05). None of the above effects of neonatal NPA treatment were related to electrode placement in the PFC, fimbria, or NAC as histological assessments were similar across groups (Fig. 7(a)–(c)).

**Effect of neonatal NPA treatment on overall brain mass**

To assess the potential impact of anatomical changes induced by neonatal exposure to NPA on the above electrophysiological observations, brain weight and volume and body weight were compared across groups. The wet brain weight of adult rats treated with NPA as neonates was significantly decreased as compared to vehicle-treated controls (Fig. 8(a); p < 0.05, t-test). No significant differences in body weight or brain volume were observed between groups (Fig. 8(b), (c); p > 0.05).

Fig. 3. *In vivo* extracellular recordings of NAC MSNs and afferent stimulation. (a) Areas that projected to the NAC were stimulated in consecutive stimulation trials (25–100 pulses each). Stimulation was either applied to the PFC alone or paired with fimbria stimulation. PFC stimulation applied in absence of fimbria stimulation (0.5 Hz, 0.5 ms, 200–1400 μA) was delivered for a total of 100 pulses per trial. (b) Representative traces of spike activity of a single unit in the NAC evoked via low frequency electrical stimulation (0.5 Hz, 500–1000 μA) of the PFC (10 superimposed traces). Arrow indicates the location of the stimulus artifact. (c) Fimbria train stimulation (250 μA, 30 Hz, 500 ms train duration, 2 s ITI) was followed by (500 ms delay) PFC stimulation as described above. This dual stimulation was repeated 25 times within a single train stimulation trial.
binding to striatal D2 receptors as compared to control subjects (Laruelle et al., 1996; Breier et al., 1997). Given these observations, measures of AMPH-induced hyperactivity in rodents have been used extensively to assess the presence of positive symptoms analogous to psychotic behaviour observed in schizophrenia (for reviews see Lipska, 2004; Tseng et al., 2009). Given that AMPH-induced hyperlocomotion can be blocked at the level of the NAc (Creese and Iversen, 1974; Kelly et al., 1975), the increased AMPH-induced locomotion observed in adult NPA-treated animals in the current study may be largely a result of hyperactive mesolimbic DAergic transmission induced by this neonatal insult. Discrepancies between studies that found hyposensitivity (Semba et al., 2000) and hypersensitivity (Black et al., 1999) to amphetamine were likely due to the age of the animals that were tested, with full symptoms manifesting in adulthood as observed in our current study.

One influential hypothesis on the etiology of schizophrenia asserts that this disease is a neurodevelopmental disorder in which full manifestation of symptoms does not develop until adolescence or early adulthood (for review see Keshavan and Hogarty, 1999). This phenomenon may be linked to neural systems that normally reach physiological maturity in late adolescence and early adulthood. In humans, the PFC and hippocampus are not fully myelinated until early adulthood (Yakovlev and LeCours, 1964; Benes, 1989). The onset of adolescence is also accompanied by a significant increase in synaptic pruning of glutamatergic inputs in the human PFC (Huttenlocher, 1984). Increased DAergic innervation of the PFC is also observed to occur during adolescence in primates and rat (Kalsbeek et al., 1988; Zecevic et al., 1989). Additionally, a study in rats found that the modulatory effect of DA on the membrane excitability of PFC interneurons did not develop until after puberty (Tseng and O'Donnell, 2007). The findings of the current study showing that AMPH-induced behavioural differences between NPA and vehicle-treated animals did not appear until after puberty, further strengthen the face validity of the NOS-1 inhibitor model.

The deficits in social interaction and short-term recognition memory observed in our experiments also provide evidence that neonatal NOS-1 inhibitor exposure affects prefrontal-temporolimbic-NAc circuits and behaviours dependent on these systems. Social withdrawal is a common behaviour associated with the negative symptoms of schizophrenia. Social interaction has been utilized as a measure of negative symptom expression in animal models of schizophrenia (Sams-Dodd, 1998; Black et al., 2002; Tanda et al., 2009). Although the precise neural substrates for deficits in social interaction and other negative symptoms are still poorly understood, there is some evidence that implicates disturbances in PFC DA transmission and D1 receptor activation in this behaviour (Brozoski et al., 1979; Arnsten et al., 1994). Additionally, cognitive deficits, including abnormal memory function, are prevalent in patients with schizophrenia and often detected through psychological memory tasks (Langraff et al., 2011; Zandbelt et al., 2011).

In apparent contrast to the current study, other investigators have reported that chronic postnatal L-nitroarginine administration (PD0–24) did not affect the performance of adult rats on cognitive tests such as the Morris water escape and two-way active avoidance tasks (Prickaerts et al., 1998). This prolonged treatment regimen did result, however, in detrimental side effects such as weight loss, increased mortality rate, and transient, but severe, stomach hypertrophy. It is difficult to directly compare the current findings with the work...
of Prickaerts and colleagues as several key differences in methodology exist across studies. For example, in addition to using a broad spectrum NOS inhibitor and different behavioural measures, the Prickaerts study focused on much older rats (i.e. 4 months of age vs. ca 2 months in the current study). Nonetheless, outcomes from these studies indicate that additional time course studies are needed to determine the precise impact of NOS-1 disruption on maturing neural systems and neurobehaviour at critical stages of brain development and adulthood.

In addition to behavioural abnormalities, a decrease in overall brain weight was observed in adult animals that received neonatal NPA-treatment. No significant difference in body weight or brain volume was observed between groups in our study, suggesting that these factors did not contribute significantly to overall changes in brain weight observed following NPA exposure. These observations are consistent with the findings of previous studies which induced neurodevelopmental insults by chemically-disrupting embryonic brain development (Moore et al., 2006). Interestingly, decreased brain size is also reported in post-mortem studies of human patients with schizophrenia (Harrison et al., 2003).

The present electrophysiological data showed that NAc neurons recorded in animals treated with NPA as...
neonates exhibited increased responsiveness to PFC stimulation compared to vehicle-treated animals. At this time, it is not known whether this apparent increase in PFC-NAc pathway excitability is a result of changes in cortical excitability and/or alterations in NAc MSN excitability. Although speculative, these changes may be due to decreased GABAergic inhibition of projection neurons in the PFC, a phenomenon that has been postulated to occur in patients with schizophrenia (reviewed in Lewis et al., 2005). Furthermore, stimulation of NOS activity has been shown to play a key role in inducing long-term activity-dependent changes (e.g. long term potentiation) in synaptic efficacy of glutamatergic transmission in the PFC (Nowicky and Bindman, 1993). Therefore, it is possible that transient disruption of NO signalling during neonatal neurodevelopment results in dysfunctional glutamatergic drive in mature cortico-accumbens projection neurons. It should also be noted that previous studies using chronic postnatal treatment with the NOS inhibitor N\textsuperscript{\textnd} -nitro-L-arginine methyl ester (PD3-23) did not find evidence of altered markers of cortical cholinergic, glutamatergic, or GABAergic neurons (Virgili et al., 1999). However, these studies did not examine functional measures of synaptic activity or neuroplasticity. Thus, future studies examining the membrane properties of cortico-accumbens neurons and NAc MSNs in NPA treated animals are needed to clarify this issue.

Previous studies have shown that hippocampal afferents drive shifts in the steady-state membrane potential of NAc MSNs, causing them to oscillate between depolarized ‘up states’ and hyperpolarized ‘down states’ (reviewed in O’Donnell, 2003; Goto and Grace, 2008). Stimulation of hippocampal afferents was found to induce the depolarizing phase (i.e. up state) of these oscillations, thus increasing the probability that subsequent PFC stimulation would evoke spike activity in the NAc MSNs. Therefore, temporally coordinated excitatory drive to the NAc increases PFC throughput through a hippocampal-mediated gating mechanism. Our observations that fimbria stimulation primarily facilitates PFC-evoked spiking in control animals are in agreement with this gating model. Also consistent with our current work, more recent studies have shown that PFC stimulation alone can also facilitate transitions into ‘up states’ and can drive NAc MSN spike activity independent of hippocampal stimulation (Gruber and O’Donnell, 2009). Furthermore, it is also becoming clear that paired fimbria-PFC stimulation can result in a suppression of PFC-evoked NAc MSN activity (Wolf et al., 2009). Our findings that ca 25% of MSNs recorded in control rats exhibited suppression of PFC-evoked responses following fimbria stimulation are consistent with these studies.

The current electrophysiological studies also found that NAc neurons recorded in animals treated with NPA as neonates exhibited a decrease in the incidence and duration of excitatory effects of hippocampal drive on PFC-evoked activity. Moreover, the predominant response to fimbria stimulation in NPA-treated animals was the opposite (an enduring suppression of PFC-evoked spiking) of the facilitation most frequently observed in control animals. Thus, neonatal NPA exposure was found to essentially reverse the ‘sign’ of hippocampal input to the NAc from predominantly excitatory to one of enduring inhibition. These profound effects of neonatal NPA exposure may be due to a disruption of the aforementioned gating mechanism of the hippocampal fimbria inputs. However, it is also possible that overactive mesolimbic DA transmission may play a role during and, more likely, after termination of fimbria stimulation in mediating the increased incidence of synaptic suppression. Prior studies have shown that DAergic transmission is modulated by hippocampal afferents that project from the ventral subiculum to the NAc, and then ultimately to the ventral tegmental area (Floresco et al., 2001). DA release in the NAc has also been shown to selectively attenuate PFC inputs through presynaptic D\textsubscript{2} receptor stimulation (for review see O’Donnell, 2003; Goto and Grace, 2008).

Given the above, it is plausible that the abnormal behavioural responses observed following neonatal NPA exposure (i.e. hyper-responsiveness to AMPH; decreased social interaction and short-term recognition memory) arise in part as a result of excessive DA D\textsubscript{2} receptor-mediated suppression of PFC input to the NAc and abnormal processing of hippocampal drive (i.e. the normal hippocampal facilitation of PFC input is replaced...
by inhibition in NPA-treated rats). Elevated DA release and over-activation of D2 receptors in the NAc may be driven by AMPH in the case of the locomotion studies, and hippocampal fimbria stimulation in the electrophysiological studies (see above). Furthermore, in normal subjects the hippocampus has been proposed to convey contextually relevant information through hippocampal afferents which, by working in concert with PFC and DAergic afferents at the level of the NAc, facilitate goal-directed behaviour (Goto and Grace, 2008). Thus, in addition to increasing AMPH-induced locomotion, an over-responsive mesolimbic DA system induced by neonatal NPA exposure could attenuate motivated behaviours such as social interaction and executive functions involved in short-term memory tasks by disrupting the normal hippocampal gating of PFC input to the NAc. Future studies will need to address the precise mechanism(s) associated with the NPA-induced changes in mesolimbic DA transmission and afferent regulation in the NAc.

In conclusion, the current studies indicate that transient inhibition of NOS-1 during a critical neonatal

Fig. 7. Histological assessment of stimulating and recording electrode placements. The termination site of each stimulating electrode tip is mapped in the (a) PFC and (b) hippocampal fimbria. Each stimulation site is color coded to indicate the response type associated with each electrode placement (diagrams are derived from the atlas of Paxinos and Watson, 1986). (c) The termination site of each recording electrode was mapped in the NAc of both vehicle and NPA treated rats. No obvious relationship was noted between cell location and prevalence of excitatory (E) or inhibitory (I) response types (see Fig. 6 for description of response types).
developmental period results in abnormal prefrontal–temporolimbic circuit interactions at the level of the NAc, and point to a vital role for NO in the normal neurodevelopment of these circuits. Outcomes from this study also provide further evidence that aberrant NOS-1 expression may contribute to the complex etiology of schizophrenia. Indeed, disruption of prefrontal–temporolimbic integration in the NAc following neonatal NOS-1 inhibition accurately reproduces some of the behavioural endophenotypes associated with schizophrenia. Thus, further characterization of the role(s) of NO signalling in the maturation of limbic circuitry may provide important insights into synaptic dysfunction associated with this devastating disease and reveal novel drug targets and better treatment strategies for psychiatric disorders.

Acknowledgments
This work was supported by Abbott Laboratories (KLK) and NARSAD (MEW and ARW).

Statement of Interest
Dr Wolf owns shares in Grace Laboratories LLC and CIS Biotech Inc., but these companies are unrelated to the present work. All other authors report no biomedical financial interests or potential conflicts of interest related to the present work.

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