Reduced N-Acetylaspartate in Prefrontal Cortex of Adult Rats with Neonatal Hippocampal Damage

Previous studies in animals suggested that neonatal lesions of the ventral hippocampus disrupt development of prefrontal cortex and its regulation of dopaminergic activity. In the present study, we assayed an in vivo chemical marker of neuronal integrity (proton magnetic resonance spectroscopy signal of N-acetylaspartate, NAA) in prefrontal cortex and striatum of rats with neonatal excitotoxic lesions of the ventral hippocampus. We also measured in post-mortem tissue expression of EAAC1 mRNA, a molecular marker of intrinsic neurons. In the cohort studied at juvenile age and again at young adulthood (postnatal day (PD) 37 and 71), we found selective reductions of NAA in the prefrontal cortex only at PD 71. Emergence of neuronal pathology was temporally associated with emergence of amphetamine-induced hyperlocomotion. Reduced prefrontal NAA was confirmed in the second cohort studied at an older age (PD 120). Expression of EAAC1 mRNA was significantly reduced in prefrontal cortex of the lesioned rats. No changes in NAA were found in the striatum in either cohort and cortical area size was not changed. These results suggest that early ventral hippocampal lesions produce developmental neuronal pathology in prefrontal cortex that is temporally associated with dysregulation of dopamine behaviors and is reminiscent of the temporal profile of the onset of schizophrenia.

Introduction
Animal models represent an important tool for investigating mechanisms underlying the neurobiology of human disorders. Lipska and Weinberger (Lipska and Weinberger, 2000) have elaborated an animal model of schizophrenia that involves neonatal excitotoxic injury to the ventral hippocampus. While at a juvenile age the neonatal ventral hippocampal lesion does not evoke obvious behavioral abnormalities, in early adulthood these rats exhibit a constellation of abnormalities reminiscent of some aspects of schizophrenia, including altered responsiveness to environmental stresses, amphetamine, and NMDA antagonists as well as abnormalities in prepulse inhibition of acoustic startle (PPI), latent inhibition and working memory (Weinberger and Laruelle, 1999; Callicott et al., 1997; Nelson et al., 1998; Solomen and Goldman-Rakic, 1999; Weinberger, 1999; Callicott et al., 2000). Moreover, one of the remarkable aspects of this animal model is that many of the abnormal behaviors do not appear before early adulthood, which is analogous to the typical age of onset of schizophrenia.

The current study was designed to identify in the PFC of rats with the neonatal lesion a subcellular correlate of the delayed emergence of dopamine-related behavioral phenomena. We used in vivo proton magnetic resonance spectroscopy (1H-MRS) to measure N-acetylaspartate (NAA) in rats with the neonatal lesion of the ventral hippocampus. NAA is an intracellular amino acid, found almost exclusively in mature neurons and their processes (Urenjak et al., 1993), with highest concentrations in pyramidal glutamate neurons (Moffett and Namboodiri, 1995). We chose to investigate NAA in PFC of these rats for two reasons. The first is that NAA is an in vivo measure of neuronal integrity and thus allows repeated measurements in the same animals; NAA measurements can also be used to detect trans-synaptic changes in vivo (Roffman et al., 2000). The second reason is that we and other groups have found the levels of the same molecule to be reduced in the hippocampal area and in PFC of patients with schizophrenia (Renshaw et al., 1995; Bertolino et al., 1996, 1998; Deicken et al., 1997; Cecil et al., 1998) with some exceptions (Bartha et al., 1997, 1999). Moreover, NAA reductions in PFC of patients with schizophrenia are associated with dysregulation of steady-state and amphetamine-evoked release of dopamine in the striatum as measured with radioreceptor neuroimaging (Bertolino et al., 1999, 2000) suggesting that NAA deficits may predict changes in dopaminergic function. Furthermore, in a previous experiment using 1H-MRS in non-human primates who had undergone neonatal removals of mesial temporal limbic structures (including the hippocampus, amygdala and entorhinal cortex), we found that in adulthood NAA measures are selectively reduced in the dorsolateral PFC (Bertolino et al., 1997). Similar reductions were not found in animals lesioned as adults. NAA reductions in dorsolateral PFC of these neonatally lesioned monkeys also predicted steady-state as
well as amphetamine-induced striatal release of dopamine as measured by in vivo microdialysis in a pattern very similar to that observed in patients with schizophrenia (Bertolino et al., 1999).

Thus, at least in these monkeys, NAA signals in PFC represent a marker of neuronal biology that is affected by abnormal development and that predicts trans-synaptic effects of prefrontal cortical projections on mesolimbic dopamine neurons. These studies in monkeys, however, were limited in some respects: the lesion was much more extensive and not specific to the hippocampal proper; NAA measures in dorso-lateral PFC were assessed only in adulthood, and there was no post-mortem assessment of neuronal integrity.

The objectives of the present study in rats with neonatal lesions of the ventral hippocampus were: (i) to assess whether the neonatal ventral hippocampal lesions produce changes of NAA in vivo in PFC; (ii) to determine the temporal pattern to the changes in NAA; (iii) to explore the temporal association between the emergence of PFC deficits and changes in dopaminergic behaviors; and (iv) to assay an independent subcellular marker of change in PFC neurons in post-mortem tissue.

Materials and Methods

Animal Care and Surgery

Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1986). Pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were obtained at 12–15 days of gestation. On postnatal day 7 (PD 7), the pups (weight 15–18 g) were anesthetized by hypothermia and placed in a stereotaxic frame (Kopf Instruments). Rats in the lesion group (n = 11) were infused with ibotenic acid (Sigma, 3 µg/0.3 µl over 2 min) bilaterally into the ventral hippocampus (coordinates AP 3.0 mm, ML ±3.5 mm, DV -5.0 mm, relative to bregma). Rats in the sham group (n = 10) received infusions of artificial cerebrospinal fluid (0.3 µl). Rats were weaned at PD 24, separated by lesion status and housed two or three to a cage. All rats in this group were scanned at PD 36 and PD 71.

To confirm the spectroscopy findings and their putative stability over time, we also scanned a second cohort of male Sprague-Dawley rats. Handling and lesion procedures were identical to those in the first cohort. This second group included seven male rats with a neonatal (PD 7) lesion of the ventral hippocampus, and nine male rats with a sham lesion. All rats in this group were scanned at PD 120.

Behavioral Testing

At PD 35 and PD 70, locomotor activity was assessed in Plexiglas cages equipped with photocell monitors (Omnitech, model RXYZCM, Columbus, OH). After a habituation period of 60 min, rats were injected with saline (1 ml/kg, i.p.) and monitored for an additional 60 min. Rats were then given injections of amphetamine sulfate (RBI, 1.5 mg/kg, i.p., a moderate dose not causing any lasting adverse side effects), and monitored for an additional 90 min. Locomotor activity was measured as total distance traveled (cm). One of the lesioned animals died before PD 70 and therefore was only tested once.

1H-MRS Procedure

Rats underwent in vivo NMR spectroscopy 1 day after each behavioral testing session (PD 36 and PD 71). Rats were anesthetized with 10:1 ketamine:xylazine (100 mg/ml, i.p.) via a 22 gauge i.p. catheter. Following insertion into a 1 m long plastic tray assembly and placement of a 20 mm diameter circular surface coil centered above the cranium, rats were positioned in a 4.7 T Varian INOVA horizontal bore magnet with a 20 mm diameter circular surface coil centered above the cranium. Following insertion into a 1 m long plastic tray assembly and placement in vivo Rats underwent localization of the prefrontal and striatal voxels (using the atlas of Paxinos and Watson) (Paxinos and Watson, 1986) and the riboprobe corresponding to a 0.5 µl of 35S-labeled riboprobe was synthesized using a clone containing 2284 base pairs of the rat EAAC1 cDNA sequence (kindly provided by Dr Marco Riva). To produce an antisense ribonucleotide probe, the Bluescript SK-vector was linearized with XbaI restriction enzyme; 200 ng (1 µl) of linearized plasmid was labeled using 150 µCi of 35S]dUTP (NEN, Boston, MA) and 1 µl (5 units) T3 RNA polymerase (for a sense probe Peul enzyme and T7 polymerase were used). Labeled RNA was digested with DNase (3 µl, 10 U/µl), precipitated with ethanol in the presence of yeast tRNA and ammonium acetate. After precipitation, the probe was washed with 70% ethanol (sp. act. 1.5 × 106 d.p.m./µg), resuspended in diethyl pyrocarbonate water, and added to the hybridization cocktail containing 5 µl of hybridization buffer (1200 mM NaCl, 20 mM Tris–HCl, 0.04% Ficoll, 0.04% BSA, 0.04% polyvinyl pyrrolidone, 2 mM EDTA, pH 8, 0.02% salmon sperm, 0.1% total yeast RNA, 0.01% yeast tRNA, 20% dextran sulfate), 5 ml formamide, 20 µl 50% sodium thiosulfate, 200 µl 5 M DTT and 100 µl 10% SDS to yield the final concentration of 5 ng/µl of the riboprobe corresponding to 4 × 1010 d.p.m./per section (25 µl of hybridization cocktail). In situ hybridization was carried out overnight in 55°C. The sections were treated with RNase A washed in 2× SSC for 15 min at room temperature, twice in 2× SSC at 50°C for 1 h, once in 0.2× SSC for 1 h in 55°C, once in 0.2× SSC at 60°C for 30 min and rinsed with
0.2× SSC at room temperature. The sections were then dehydrated and apposed with the 14C standards to BioMax Kodak film. Analysis of autoradiographic images was performed using NIH Image software. Optical density was converted to d.p.m./mm² using 14C standards (Miller, 1991). Four measurements were obtained per rat from two slides containing two sections each, and averaged. The sense riboprobe did not yield any hybridization signal (not shown).

Statistical Analysis
Analysis was conducted using Statistica, Version 5 (StatSoft, Inc.). Group means for lesioned and sham animals were compared using unpaired t-tests assuming unequal variance for all dependent measures. Correlations were assessed with Spearman's test. The level of significance was set at P < 0.05.

Results

Verification of the Lesion
Microscopic evaluation of Nissl-stained coronal sections of both cohorts of rats showed that neuronal loss was confined to the intended area in the ventral hippocampus (Fig. 1) as reported previously (Lipska et al., 1993). There was no discernible damage defined as neuronal loss or gliosis outside the hippocampus. However, even though ibotenic acid has axon-sparing properties, atrophy and some cavitation was observed in the ventral hippocampus and, thus, some damage of fibers of passage cannot be excluded.

Behavioral Testing
No significant difference in motor behaviors between lesion and sham rats was detected at PD 35 and 70 in the habituation period or after saline injection (all P > 0.3). After amphetamine at PD 35, sham and lesioned rats did not differ significantly in terms of hyperlocomotion (t = 1.5, d.f. 19, P > 0.1, Fig. 2). However, at PD 70, rats with neonatal lesions of the hippocampus displayed significantly greater amphetamine-induced hyperlocomotion (t = 2.7, d.f. 17, P < 0.01), consistent with earlier studies (Lipska et al., 1993; Wan et al., 1996).

1H-MRS Procedure
Figure 3 shows the structural MRI (with the dimensions of the acquired spectroscopy voxel) and spectra (PD 36 and PD 71) acquired from the PFC of a rat with the neonatal lesion of the hippocampus. No significant difference was found for the unsuppressed water signal between the two groups at both PD 37 and PD 71 (all P > 0.3). At PD 36, no significant difference for any metabolite ratio was found in the PFC (Fig. 4) or in the striatum of the two groups. In contrast, at PD 71, rats with the neonatal lesion of the hippocampus exhibited significantly lower NAA/H₂O in PFC (t = 2.5, d.f. 17, P < 0.02, Fig. 4). None of the remaining metabolite ratios in PFC as well as none of the metabolite ratios in the striatum were significantly different between the two groups (all P > 0.3, Fig. 4 for NAA). The results in the second cohort (PD 120) of rats were very similar. In the PFC, NAA/H₂O was significantly reduced in the lesioned group (t = 2.7, d.f. 14, P < 0.01). No other significant difference for other metabolite ratios in PFC or in the striatum was found (all P > 0.3). No correlation was found between NAA levels in PFC and locomotor activity.

PFC Area Measurements
In order to investigate if prefrontal NAA reductions were simply due to tissue shrinkage, we measured the area of the PFC. No significant difference was found between sham and lesioned rats (means ± SD: 0.12 ± 0.005 and 0.13 ± 0.01 for sham and lesioned rats, respectively, t = 1.03, P > 0.3).

EAAC1 mRNA In Situ Hybridization
The hybridization signal was present throughout all layers of the PFC but was most intense in deep layers V/VI (Fig. 5). White

Figure 1. A neonatal excitotoxic lesion of the ventral hippocampus shown in a diagram adapted from the atlas of Paxinos and Watson (Paxinos and Watson 1986) (A) and in photomicrographs of Nissl-stained coronal brain sections through the hippocampus of an adult rat with a neonatal sham (B) and ibotenic acid lesion (C). A gray area in a diagram indicates the boundaries of a lesion. Arrows point to the area showing loss of neurons in the ventral hippocampus in the lesioned brain (C).

Figure 2. Locomotor activity (total distance traveled, mean ± SD) of rats with the neonatal sham and ibotenic acid lesion of the ventral hippocampus at rats. The same rats were tested at PD35 and PD70 (*P < 0.01).
matter was devoid of hybridization signal. No signal was observed in PFC with a sense probe (data not shown). The lesioned animals had reduced EAAC1 mRNA expression in the PFC as compared with sham rats ($t = 2.6$, d.f. 15, $P < 0.02$, Fig. 5).

No correlation was found between EAAC1 mRNA expression and NAA levels in PFC.

Discussion

The results of the present study indicate that NAA measures in PFC of rats with neonatal lesions of the ventral hippocampus are reduced at a young adult age, analogous to our previous findings in monkeys (Bertolino et al., 1997). The longitudinal design of this study allowed us to establish that these changes emerge sometime around early adulthood. It is important to note that NAA measures in the striatum were not affected either before or after puberty, despite the fact that the ventral striatum receives dense hippocampal projections (Groenewegen et al., 1997). The prefrontal reduction in NAA also does not seem to be a reflection of reduced cortical size because changes in cortical cross-sectional area were not detected in the lesioned brains.

These results suggest that changes in PFC are regionally specific and not due to simple disconnection from the hippocampus, because they do not manifest at a juvenile age and are not seen in other target areas (i.e. striatum). Therefore, we propose that PFC NAA deficits may reflect developmental adaptations triggered by early hippocampal damage.

The data in the second cohort also provide support for the developmental nature of NAA reductions in PFC. This group was studied at an older age (PD 120) than the first cohort. If the process responsible for NAA reduction were degenerative in its nature, one might have expected to find more profound neuronal damage in the older cohort. Instead, the effect sizes of the two NAA reductions were almost identical (0.65 for the first cohort, 0.67 for the second cohort), thus suggesting lack of progressive neuronal damage. The results in the second cohort of rats also suggest that these phenomena are reproducible and enduring.

The temporal profile of NAA reductions parallels changes in putative dopaminergic behaviors elicited by amphetamine, suggesting a relationship between these phenomena. These abnormal behaviors may reflect abnormal cortical regulation of dopamine neuronal activity in brain stem and striatum as cortical glutamatergic neurons are involved in regulating steady-state and evoked dopamine activity (Grace, 1991). The reduced expression of EAAC1 mRNA, a marker of primarily glutamatergic neurons (Conti et al., 1998), suggests that this neuronal population may be involved in the developmental pathology related to the neonatal ventral hippocampal lesion. Moreover, a recent study reported that mRNA levels of AMPA receptor subtype GluR3 (lop) were significantly decreased at PD 60 in the PFC of neonatally lesioned rats (Stine et al., 2001). Because AMPA receptors incorporating flop variants exhibit faster desensitization, a shift towards a flip form in the lesioned rats might lead to increased neuronal excitability in the PFC, and thus inappropriate activation of subcortical dopamine systems regulated by PFC inputs. This possibility is further supported by the data of O’Donnell et al. showing enhanced excitability of prefrontal neurons to ventral tegmental area stimulation in these animals (O’Donnell et al., 1999). However, we cannot exclude the possibility that reductions in both NAA and EAAC1 (also found in GABAergic cells and glia) (Conti et al., 1998; Kugler...
and Schmitt, 1999) are consequential to pathology in GABAergic neurons. We have previously reported (Lipska and Weinberger, 2000) decreased expression of GAD-67 mRNA, a GABA synthetic enzyme, in PFC of rats with an early ventral hippocampal lesion.

The physiological role of NAA in neurons has yet to be fully elucidated. Several $^1$H-MRS studies have reported increases in NAA ratios or concentrations from infancy to adulthood (Kreis et al., 1993; Toft et al., 1994; Hashimoto et al., 1995; Huppi et al., 1995). Since the number of neurons does not increase significantly after birth, it might be assumed that the increase of NAA can be related to maturational processes. In the mature brain (when glial and neuronal cells are differentiated), NAA is found exclusively in neurons and in highest concentrations in pyramidal glutamatergic neurons (Urenjak et al., 1993). NAA

Figure 5. Expression of neuronal glutamate transporter EAAC1 mRNA in PFC of rats with the sham (A) and ibotenic acid lesion (B) of the ventral hippocampus. Expression of EAAC1 mRNA (d.p.m./mm$^2$) was measured by in situ hybridization in adult rats with the neonatal lesion, and found to be significantly reduced ($^{*}P < 0.02$) in the PFC of the lesioned rats as compared with the sham controls (C).
synthesis takes place in the mitochondria, and is ADP dependent. The synthetic reaction is a transamination catalyzed by L-aspartate-N-acetyl transferase that uses glutamate (source for aspartate) and either pyruvate or 3-hydroxybutyrate (source of acetyl CoA) as substrates (Clark, 1998). Moreover, NAA acts via the glutamatergic NMDA receptor to elevate intracellular calcium (Rubin et al., 1995); its concentrations are reduced by pharmacological inhibition of mitochondrial energy metabolism; it correlates highly with the relative reduction of ATP and O2 consumption (Bates et al., 1996). Moreover, a number of studies have demonstrated that NAA reductions are reversible, suggesting that NAA is sensitive to pathological processes affecting the functioning of neurons (De Stefano et al., 1995; Vion-Dury et al., 1995; Hugg et al., 1996; Cendes et al., 1997). Therefore, changes in the levels of these molecules may affect NAA synthesis and, consequently, concentrations.

The speculation that NAA levels may change along with the functional state of glutamatergic cells is also consistent with the well known role that glutamate plays in neuronal development. Early in development, there is an excessive number of neurons and synapses in the brain (Rakic, 1988). Some of these exuberant connections are stabilized by being incorporated into functioning systems. More labile contacts not incorporated into these systems regress, especially excitatory synapses. A series of factors seem to be essential in determining which axons, dendrites and synapses will form and survive, including axon growth enhancing factors, target feedback in the form of humoral growth factors (Changeux and Danchin, 1976), and neuronal electrical activity (Nowakowski, 1987) as well as the putative trophic effects of classical neurotransmitters such as glutamate (Mattson, 1988; Lipton and Kater, 1989). These developmental processes also make it likely that pathology localized to one of the nodes of a circuit will influence other areas in the circuit, especially if it occurs early in development. These speculations are consistent with a recent body of literature underlining the anatomical and functional connectivity of hippocampus, PFC and the dopaminergic system. It has been demonstrated that although hippocampal and dopamine afferents to the PFC do not converge onto common postsynaptic targets, these afferents are in close proximity to one another and thus may interact presynaptically (Carr and Sesack, 1996).

Moreover, integrity of the mesocortical dopaminergic system is thus may interact presynaptically (Carr and Sesack, 1996). These developmental processes also make it likely that pathology localized to one of the nodes of a circuit will influence other areas in the circuit, especially if it occurs early in development. These speculations are consistent with a recent body of literature underlining the anatomical and functional connectivity of hippocampus, PFC and the dopaminergic system. It has been demonstrated that although hippocampal and dopamine afferents to the PFC do not converge onto common postsynaptic targets, these afferents are in close proximity to one another and thus may interact presynaptically (Carr and Sesack, 1996). Moreover, integrity of the mesocortical dopaminergic system is necessary for complete expression of hippocampal–PFC NMDA receptor-dependent long-term potentiation (Gurden et al., 2000) and working memory tasks mediated through D1 receptors (Williams and Goldman-Rakic, 1995; Granon et al., 2000). Major developmental changes also take place in excitatory and dopaminergic inputs to prefrontal pyramidal neurons during adolescence (Lambe et al., 2000). Thus, it is conceivable that in our rats loss of early limbic cortical inputs to PFC has led to rewiring of intrinsic prefrontal neuronal circuitry and changes in the function of projection neurons that may lead to dopamine dysregulation.

Some limitations of the present study should be noted. The use of ketamine (a glutamate antagonist) for anesthesia might be a potential confound. However, rats in each group received the same sedative agent during scanning. Moreover, in previous studies we have not observed an effect of ketamine on NAA measures (Bertolino et al., 1997). Another potential confound is the relatively large size of the voxel (3.6 mm3) used for spectroscopy. Therefore, we cannot definitely exclude partial volume effects. However, we tried to minimize partial volume effects by positioning the voxel so that it covered the PFC bilaterally, thus covering the greatest extent of gray matter possible. The fact that the prefrontal cortical area does not appear to be reduced in rats with the lesion does not support the possibility of a differential gross partial volume effect.

Another potential limitation of the study involves the specificity of the NAA peak. Other signals such as NAAG, Glu, Gln, GABA and other macromolecules contribute to the NAA resonance at 2.01 p.p.m. Therefore, when measuring the peak at 2.01 p.p.m., there is a theoretical possibility that these other compounds contribute to measured changes. However, the relative contributions of these other signals to the NAA peak is rather small for NAAG (~7%, Stanley et al., 1995) and probably smaller for the other compounds. NAA accounts for ~85–90% of the N-acetyl group in the proton spectrum at 2.01 p.p.m. (Stanley et al., 2000). Moreover, both NAA and NAAG in the cortex are found almost exclusively in neurons and their processes while not in glia (Urenjak et al., 1993). On the other hand, all other metabolites measured with proton MRS such as creatine + phosphocreatine, choline-containing compounds, glutamate, glutamine, GABA are also found in glia. As a matter of fact, some of these other compounds such as choline-containing compounds and glutamine are found in higher concentration in glia than in neurons. In this respect, the selective reduction of the NAA peak is consistent with the assumption of neuronal pathology and with the EAAC-1 mRNA data. Moreover, if one metabolic process (the one causing reduction of the NAA peak) is abnormal in neurons, this does not imply that all metabolic processes (all other metabolites measured) in neurons have to be affected. This seems even more likely if the other metabolites are measured in higher concentrations from glia (i.e. the neuronal contribution is lesser).

It is also important to point out that the results of the present study are consistent with our previous study in monkeys in which we used a long TE imaging 1H-MRS technique (Bertolino et al., 1997). In that study, we reported specific prefrontal reductions of NAA ratios in monkeys with neonatal lesions of mesial–temporal structures. We did not find any change in choline-containing compounds or creatine + phosphocreatine. When using long TE, the contribution of other signals to the NAA peak is even less because of the shorter TE of these other metabolites. Therefore, the results in the monkeys lend further support to our interpretation of the present results in the rats. In light of these various considerations, it seems possible but unlikely that the NAA reduction we have found is attributable to any metabolite other than NAA.

**Implications for the Pathophysiology of Schizophrenia**

Altered development of connectivity between the hippocampus and the PFC has been proposed to play an important role in the pathophysiology of schizophrenia (Weinberger, 1991, 1996; Friston, 1998). Many studies have implicated the PFC and the hippocampal formation in schizophrenia. Post-mortem neuropathological studies have shown abnormalities in the hippocampal region and in the PFC of the brains of patients with schizophrenia (Arnold et al., 1991; Selemon et al., 1998; Pierri et al., 2001). The combined results of these studies suggest that in patients with schizophrenia failure of the normally developed communication between the hippocampus and PFC might be decisive for some aspects of the disease. Our current 1H-MRS data in rats support the possibility that the NAA reduction in PFC in schizophrenia may be a marker of a developmental defect of such connectivity.

In conclusion, the neonatal lesion of the ventral hippocampus leads to pathological changes in prefrontal neurons in rats. The findings may have implications for understanding developmental
aspects of the connectivity between the hippocampus and the PFC.

Notes
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