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Expression of AMPA Receptor Flip and Flop mRNAs in the Nucleus Accumbens and Prefrontal Cortex after Neonatal Ventral Hippocampal Lesions

Christy D. Stine, Ph.D., Wenxiao Lu, M.D., and Marina E. Wolf, Ph.D.

Many lines of evidence implicate dysfunctional excitatory amino acid (EAA) transmission in schizophrenia. The present study examined α-amino-3-hydroxy-5-methylisoxazole-4-priopionate (AMPA) receptor expression in the prefrontal cortex (PFC) and nucleus accumbens (NAc) using a rat model of schizophrenia in which excitotoxic lesions of ventral hippocampus (VH) on postnatal day (PD) 7 lead to the postpubertal emergence of behavioral abnormalities that resemble schizophrenic symptoms. In situ hybridization histochemistry with ^{35}S-labeled oligonucleotide probes was used to quantify mRNA levels for GluR1-3 flip and flop variants at prepubertal and postpubertal time-points (PD 35 and 60, respectively). Comparisons of PD 35 and PD 60 groups suggested that there may be changes in the relative expression of flip and flop isoforms during normal development. The most pronounced change was an apparent increase in the flip/flop ratio for GluR1 from PD 35 to PD 60 in both the PFC and the NAc. Neonatal VH lesions did not significantly alter GluR1-3 flip and flop mRNA levels in the NAc at either time-point. However, in the PFC, GluR3 flop mRNA levels were significantly decreased in lesioned rats at PD 60. AMPA receptors incorporating flop variants exhibit faster desensitization, so a shift towards flip incorporation might lead to increased neuronal excitability in the PFC, thereby influencing subcortical DA systems regulated by PFC efferents. In summary, an early developmental injury can predispose rats to abnormal development of EAA transmission in the PFC. This may contribute to the postpubertal onset of symptoms after neonatal VH lesions.
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KEY WORDS: *Alternative splicing; AMPA receptors; In situ hybridization; Nucleus accumbens; Prefrontal cortex; Schizophrenia*

Recent hypotheses about the pathophysiology of schizophrenia have posited defects in excitatory amino acid (EAA) transmission (e.g., Carlsson and Carlsson 1990; Coyle 1996; Krystal et al. 1999; Olney and Farber 1995). A number of different lines of evidence support the involvement of EAAs. First, N-methyl-D-aspartate (NMDA) receptor antagonists produce effects in normal human subjects that resemble those associated with schizophrenia (Krystal et al. 1999). Second, schizophrenic brains exhibit abnormalities in glutamate metabolism (Bartha et al. 1997; Do et al. 1995; Faustman et al. 1999; Tsai et al. 1995) and glutamate receptor expression (reviewed by Meador-Woodruff and Healy 2000). Finally, drugs that alter glutamate transmission can ameliorate schizophrenic symptoms (Heresco-Levy et al. 1996; Javitt et al. 1994; Tsai et al. 1998). Conversely,

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antipsychotic drug treatment alters EAA levels in the CSF and serum of schizophrenic patients (Evins et al. 1997; Labarca et al. 1995).

Preclinical research also supports a role for EAAs in the pathogenesis of schizophrenia. When tested in rats, NMDA receptor antagonists produce behavioral responses that model schizophrenic symptoms and are blocked by antipsychotic drugs, including clozapine (e.g., Hauber 1993). Chronic administration of antipsychotic drugs alters glutamate receptor expression in rat brain (Chen et al. 1998; Eastwood et al. 1994, 1996; Fitzgerald et al. 1995; Healy and Meador-Woodruff 1997; Riva et al. 1997; Tascedda et al. 1999) and alters EAA levels as measured by microdialysis (Daly and Moghaddam 1993; See and Chapman 1994; See and Lynch 1996; Yamamoto and Cooperman 1994).

The α-amino-3-hydroxy-5-methylisoxazole-4-priopionate (AMPA) subtype of glutamate receptor mediates the majority of excitatory transmission in brain. Different combinations of four AMPA receptor subunits (GluR1-4) give rise to heteromeric receptors with distinct properties (Hollman and Heinemann, 1994). The purpose of the present study was to examine the possible role of alterations in AMPA receptor expression in schizophrenia, using a rat model for schizophrenia in which excitotoxic lesions of ventral hippocampus (VH) are produced on postnatal day (PD) 7 (Lipska et al. 1993). By interrupting connections between the limbic system and cortex during the vulnerable neonatal period, neonatal VH lesions may produce developmental changes related to those that are believed to underlie schizophrenia (Benes 1995; Lewis 1997; Weinberger and Lipska 1995). Just as schizophrenia often emerges during late adolescence or early adulthood, rats with neonatal VH lesions appear normal until puberty (PD 56) but then develop a constellation of abnormalities that is reminiscent of schizophrenic symptoms. These include behavioral and neurochemical changes in subcortical dopamine (DA) transmission, enhanced behavioral responsiveness to stress, social interaction deficits, disruption of latent inhibition, decreased prepulse inhibition (PPI), and an exaggerated reduction in PPI after treatment with apomorphine (Brake et al. 1999; Grecksch et al. 1999; Lillrank et al. 1999; Lipska et al. 1993, 1995a,b; Lipska and Weinberger 1993, 1994; Sams-Dodd et al. 1997; Wan and Corbett 1997; Wan et al. 1996, 1998).

We focused on AMPA receptor expression in the prefrontal cortex (PFC) and the nucleus accumbens (NAc). Both regions are implicated in schizophrenia (Csernansky and Bardgett 1998; Goldman-Rakic 1991; Grace 2000; Lewis and Anderson 1995; Weinberger and Lipska 1995) and receive EAA projections from the hippocampus (PFC: Carr and Sesack 1996; Jay et al. 1989; Jay and Witter 1991; Swanson 1981) (NAc: Brog et al. 1993; Groenewegen et al. 1987; Kelley and Domesick 1982; Sesack and Pickel 1990). In both regions, the response to EAA inputs is mediated primarily by AMPA receptors (e.g., Hu and White 1996; Jay et al. 1992; Pennartz et al. 1990). Thus, we hypothesized that loss of hippocampal EAA inputs as a result of neonatal VH lesions could lead to compensatory changes in AMPA receptor subunit expression in PFC and NAc.

Several prior studies have examined AMPA receptors in the basal ganglia and cortex of schizophrenic brains. Healy et al. (1998) found no change in AMPA receptor binding or GluR1-4 mRNA levels in either prefrontal cortical or striatal regions (NAc, caudate, putamen) in a population that included neuroleptic-treated and drug-free patients. Other studies have reported similar results (Breese et al. 1995; Freed et al. 1993; Kurumaji et al. 1992), although Sokolov (1998) found that mRNA levels for GluR1 (as well as NMDAR1, GluR7, and KA1 subunits) in frontal cortical regions were significantly lower than controls in neuroleptic-free (>26 weeks) schizophrenics but not in schizophrenics receiving neuroleptics at the time of their death. Noga et al. (1997) found an increase in AMPA binding in caudate (and a trend in the NAc).

However, AMPA receptor function can be regulated in many ways that would not be reflected by measurements of AMPA binding or subunit levels. For example, changes in AMPA receptor subunit phosphorylation or trafficking contribute to activity-dependent synaptic plasticity (Malenka and Nicoll 1999). RNA editing, which regulates the Ca^{2+} permeability of GluRs, is altered in a number of disease states, including schizophrenia (Akbarian et al. 1995a). Finally, AMPA receptor subunits exist in two isoforms, flip and flop, which arise from alternative splicing (Sommer et al. 1990). AMPA receptors containing flop isoforms of the GluR1-4 subunits exhibit a faster rate of desensitization than receptors containing flip isoforms (Lambolez et al. 1996; Mosbacher et al. 1994; Sommer et al. 1990). Increased incorporation of flip variants into synaptic AMPA receptors might enable synapses to switch from low gain to high gain (Sommer et al. 1990).

Changes in the flip/flop ratio for GluRs in the hippocampus have been demonstrated in various seizure models (Kamphuis et al. 1994; Lason et al. 1997, 1998; Pollard et al. 1993), including a neurodevelopmental model (Rafiki et al. 1998). Most relevant to the present study, 2 weeks of haloperidol treatment increased the flip/flop ratio for GluR2 in the rat hippocampus and dorsolateral striatum, while 16 weeks of haloperidol increased the ratio in the striatum and frontoparietal cortex (Eastwood et al. 1994). An increased flip/flop ratio for GluR2 was also observed in the hippocampal formation of schizophrenics (Eastwood et al. 1997). Based on these findings, we examined flip and flop mRNA levels for GluR1-3 in the rat PFC and NAc after neonatal VH lesions. Pre- and postpubertal time points were exam-

ined to identify developmentally regulated abnormalities in AMPA receptor expression.

METHODS

Animals

All animal use procedures were in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. Rats were housed in a colony room maintained on a 12-hr light/dark cycle with constant temperature (21–23°C) and humidity (40–50%). Food and water were freely available. Pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) obtained at 14 days gestation were housed individually in breeding cages. Male offspring were used for all studies. On PD 25, animals were weaned, separated based on lesion status, and housed 3 per cage.

Neonatal Lesions of the Ventral Hippocampus

VH lesions were performed exactly as described by Lipska et al. (1993). On PD 7, rats were randomized to sham or lesion status and anesthetized by hypothermia. After immobilizing each rat on a stereotaxic device, an incision was made in the skin overlying the skull. Either ibotenic acid (0.3 μl at 10 μg/μl) or artificial cerebrospinal fluid (aCSF) was infused bilaterally using an infusion pump and a Hamilton syringe at 0.15 μl/min. Coordinates for the VH were: A −3.0 mm, L ± 3.5 mm, V −5.0 mm, relative to bregma (Paxinos and Watson 1997). The needle was withdrawn 4 min after infusion. After recovery from surgery, rats were returned to the colony, housed 3 per cage, and killed either on PD 35 or PD 60. Thus, 4 groups of rats were generated: PD 35 sham rats, PD 35 lesioned rats, PD 60 sham rats, and PD 60 lesioned rats (N = 10 rats per group). The same rats were used for all of the GluR1-3 flip and flop mRNA expression studies. Upon the completion of experiments, lesion boundaries were determined by high-power microscopic analysis of Nissl-stained sections (see next section).

Perfusion and Histological Procedures

Rats were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and perfused transcardially first with ice-cold saline containing 0.02% diethyl pyrocarbonate (an RNase inhibitor) and then with fixative solution containing 4% paraformaldehyde, 1.5% sucrose and 0.1 M phosphate buffer (pH 7.2). Brains were removed, immersed in fixative solution for an additional hour, and then immersed in sequential solutions containing 0.1 M phosphate buffer, 0.1 % sodium azide, and either 10, 20, or 30% sucrose. Forty μm sections were cut frozen on a sliding microtome and placed sequentially into the wells of a cell culture plate such that each well contained a group of coronal sections that had been spaced 480 μm apart in the intact brain. Sections were then stored in cryoprotectant solution (30% sucrose, 30% ethylene glycol, and 0.1 M phosphate buffer) at −20° C. Sections through the hippocampal formation were used for lesion verification by high-power microscopic analysis of Nissl-stained sections. Based on criteria used by Lipska et al. (1993), lesions were considered acceptable if there was cell loss in CA1 and CA2 regions from bregma −4.8 to −5.3. Rats were excluded from analysis if lesions were smaller than this or if histological examination revealed extensive damage outside the intended lesion site (e.g., neuronal loss and/or cavitation in the septum, cortex, or thalamus). Figure 1 depicts an acceptable lesion. There is sparing of the most dorsal aspects of the hippocampal formation, while the VH shows neuronal loss and disorganization, atrophy, and some cavitation.

In Situ Hybridization Histochemistry (ISHH)

The previously stored sections were transferred from cryoprotectant solution into a net in a glass dish. The brain sections were rinsed in 50% formamide and 4X SSC (1X SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.2) twice for at least 30 min each time with gentle agitation. Hybridization buffer consisted of ^{35}S-labeled probe (10×10^6 cpm/ml), 50% formamide, 4X SSC, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 100 μg/ml denatured salmon DNA, 250 μg/ml yeast RNA, 50 mM dithiothreitol (DTT), and 10% dextran sulfate. Oligonucleotide

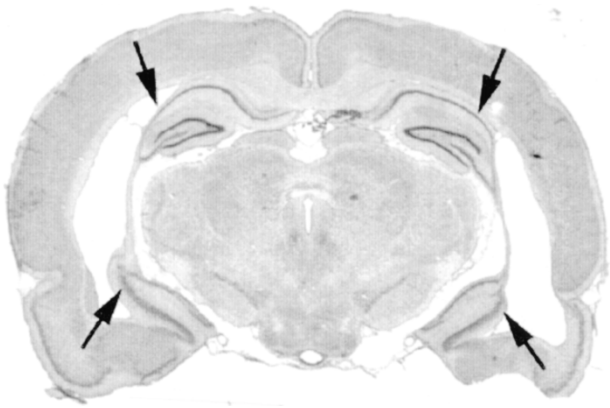


Figure 1. Bilateral VH lesions in a PD 60 rat. This Nissl-stained section shows sparing of the most dorsal aspects of the hippocampal formation, while the VH shows neuronal loss and disorganization, atrophy, and some cavitation. The arrows bracket the dorsal and ventral extent of the lesioned area. The location of this coronal section is approximately 5.0 mm posterior to bregma.

probes for GluR subunits were obtained commercially (DuPont, Wilmington, DE) and labeled at the 3′-terminal with terminal transferase (Boehringer Mannheim, Indianapolis, IN) and ^{35}S-dATP (DuPont, Wilmington, DE). The probe labeling procedure was performed under nuclease-free conditions. Sections were transferred into 0.5–1 ml of hybridization buffer in 2 ml tubes. They were then placed in an incubator at 37° C for 20 hr, with continuous gentle agitation by a mixer. After hybridization, sections were transferred to a net in a glass dish and rinsed sequentially in 2X SSC, 1X SSC, and 0.5X SSC (two times in each buffer, 10 min each rinse) at room temperature. For the high stringency post-hybridization wash, the sections were rinsed in 0.5X SSC at high temperature (54–60° C), that is, 10–15° C lower than the melting temperature of each probe. The sections were mounted onto gelatin-coated slides and dried overnight.

This method was modified from a previously described method for free-floating sections (Lu and Haber 1992) in that RNase inhibitors were included in steps previously vulnerable to RNase digestion (first perfusion step, pre-hybridization rinses). As described in Lu et al. (1996), this standardizes mRNA preservation and hybridization throughout the procedure, resulting in higher levels of specific hybridization and greater reproducibility between rats, enabling between-group comparisons. The new method has been characterized for preproenkephalin and GluR 1-4 mRNAs. Using this method, we have been able to detect and quantify modest changes (10–20%) in mRNA levels for AMPA and NMDA receptor subunits in the NAc and the PFC resulting from repeated amphetamine administration (Lu et al. 1997, 1999).

Autoradiography and Image Analysis

Sections were exposed to BioMax-MR film (Kodak, Rochester, NY) with ^{14}C-standard microscale strips (Amersham, Arlington Heights, IL) for approximately 2–4 days. Sections from lesioned and shams in each group were exposed to the same film, to avoid possible differences between films. Films were developed with GBX developer (Kodak) for 4 min and fixed with rapid fixer (Kodak). Autoradiographs were scanned by a computer system (Power Macintosh G3, Cupertino, CA). For each of the 10 rats in each experimental group, four to five sections between bregma +3.7mm and +2.2mm were scanned for the PFC, and 4 to 5 sections between bregma +1.7mm and +0.7mm were scanned for the NAc. Both left and right sides of each section were scanned. Figure 2 shows regions scanned for the PFC and the NAc, based on Paxinos and Watson (1997). The boundary of the PFC was defined to include prelimbic, infralimbic, and dorsal anterior cingulate regions. All layers of the PFC were scanned. For NAc, we scanned the entire NAc as well as core and shell subregions, carefully excluding surrounding areas with high levels of signal, such as the islands of Calleja and olfactory tubercle. Because of the lack of a clear boundary between the core and shell subregions, a transitional zone was avoided in scanning the autoradiographs. Thus, the total area for the core and shell subregions is smaller than the area scanned for the entire NAc (see Figure 2).

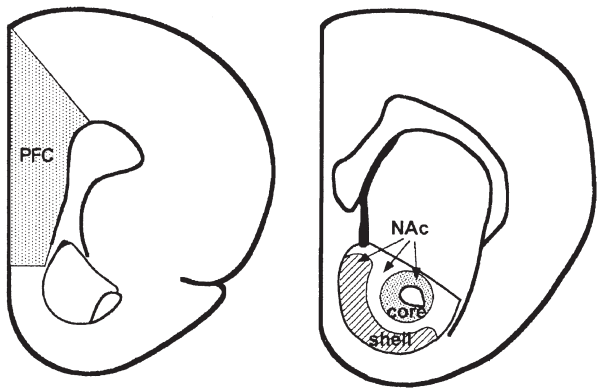


Figure 2. Scanned regions in the PFC and NAc. The boundary of the PFC was defined to include prelimbic, infralimbic, and dorsal anterior cingulate regions. All layers of the PFC were scanned. Core and shell regions were scanned in coronal sections between bregma +1.7mm and +0.7mm according to indicated boundaries, excluding a transitional zone between these subregions. (Adapted from Paxinos and Watson 1997.)

NIH Image software was used for quantitative analysis based on optical density (O.D.). Within the PFC and the NAc, there are areas that should not be included in the analysis of specific signals, such as white matter areas located within these structures (i.e., the anterior commissure), blood vessels, and areas where the section was damaged. To separate such areas from those with specific signals, the threshold function of the NIH Image program was used with a cut-off value. The cut-off value was determined by making background measurements in surrounding white matter regions and was defined as the mean of these background measurements + 2 standard deviations (to set the cut-off level at a point that would be greater than 95% of all background measurements). Areas within the PFC or the NAc that exhibited values lower than this cut-off were defined as background. In regions with values greater than the cut-off, the specific signal was defined as the total signal minus the mean background signal. Data were expressed as nano-curies (nCi) per gram of dry tissue weight, determined using ^{14}C-standard microscales.

Data Analysis

At PD 35 or PD 60, sham and lesioned groups were compared using unpaired, two-tailed Student's *t*-tests with significance set at $p < .05$.

RESULTS

Methodological Considerations

To reduce differences from experimental procedure between groups, all manipulations (including lesioning, brain perfusion, sectioning, mounting, and ISHH) were performed at the same time or under similar conditions. However, it was physically impossible to perform all of the ISHH experiments on all the groups with all of the GluR probes at the same time. Thus, each of six oligoprobes (GluR1-3 flip and GluR1-3 flop) was ^{35}S-labeled once and used for all of the rats in all four groups (PD 35 sham, PD 35 lesioned, PD 60 sham, PD 60 lesioned). Because this still represented too many sections to be processed simultaneously, the PD 35 rats (sham and lesioned) and the PD 60 rats (sham and lesioned) were analyzed in separate ISHH experiments. Therefore, the only truly valid quantitative comparisons that can be made are between sham and lesioned animals at PD 35, and between sham and lesioned animals at PD 60. For this reason, statistical analyses comparing PD 35 and PD 60 are not reported. However, these comparisons are interesting to consider and will be addressed in the Discussion. Similarly, ratios of flip and flop isoforms are presented but statistical analyses comparing these ratios in different groups are not reported. Comparison of the signals obtained with flip and flop probes is further complicated by possible differences in hybridization efficiency and specific activity.

Developmental Changes in GluR1-3 Flip and Flop mRNA Levels in the PFC

Developmental changes in GluR expression are suggested by comparisons of PD 35 and PD 60 groups. GluR1 flip mRNA levels increased while GluR1 flop mRNA levels decreased as a function of age, both for sham and lesioned groups (Figure 3). The ratio of GluR1 flip/flop reflects this developmental change (Figure 3). No dramatic differences in GluR2 flip or flop mRNA levels were apparent between PD 35 and PD 60. This is reflected in similar flip/flop ratios at both time-points (Figure 4). For GluR3, flip appeared to decrease more than flop with age, resulting in a modest decrease in the flip/flop ratio on PD 60 (Figure 5).

Effect of Neonatal VH Lesions on GluR1-3 Flip and Flop mRNA Levels in the PFC

For GluR1-3 in the PFC, we were able to make valid comparisons between sham and neonatal VH-lesioned groups at PD 35, and between these groups at PD 60 (see above). There were no statistically significant differences in levels of GluR1 flip and flop mRNA between the groups at either time-point (Figure 3), although

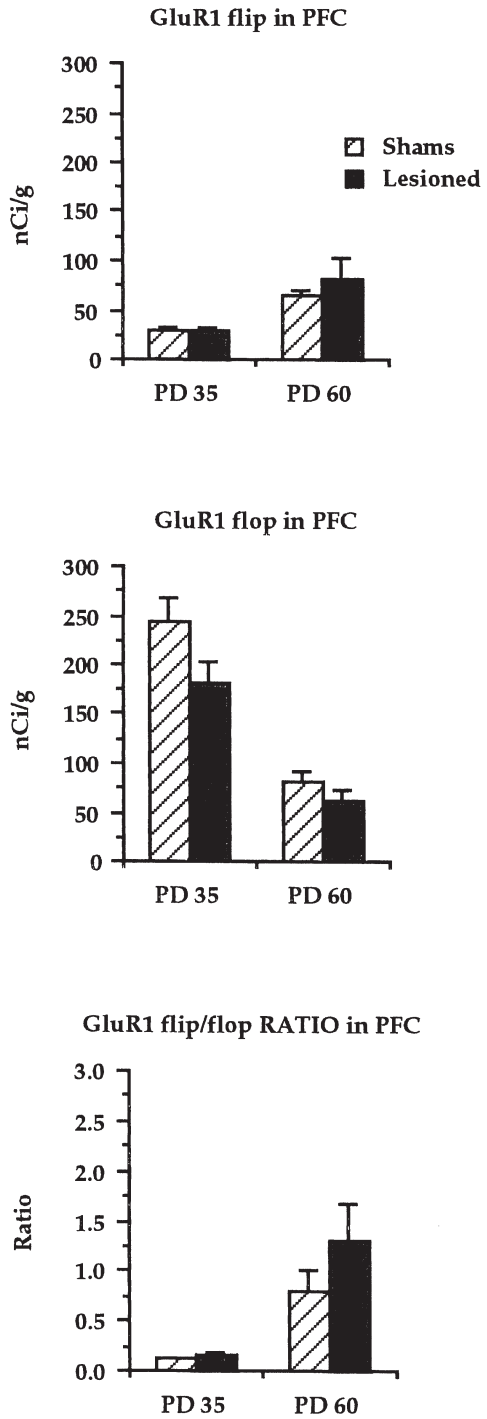


Figure 3. GluR1 flip, GluR1 flop, and GluR1 flip/flop ratio of mRNA expression in the PFC. Autoradiographs were analyzed quantitatively using NIH Image software and ^{14}C-standard microscales. For each rat in each treatment group, the average mRNA level in the PFC (or NAc; see below) was determined by scanning both right and left sides of 4–5 coronal sections at various rostral-caudal levels (see Methods). Bars represent the mean of such determinations for 10 rats in each treatment group. Sham and lesioned groups at each time-point (PD 35 or PD 60) were compared using a two-tailed Student's *t*-test. No significant differences were found between sham and lesioned groups.

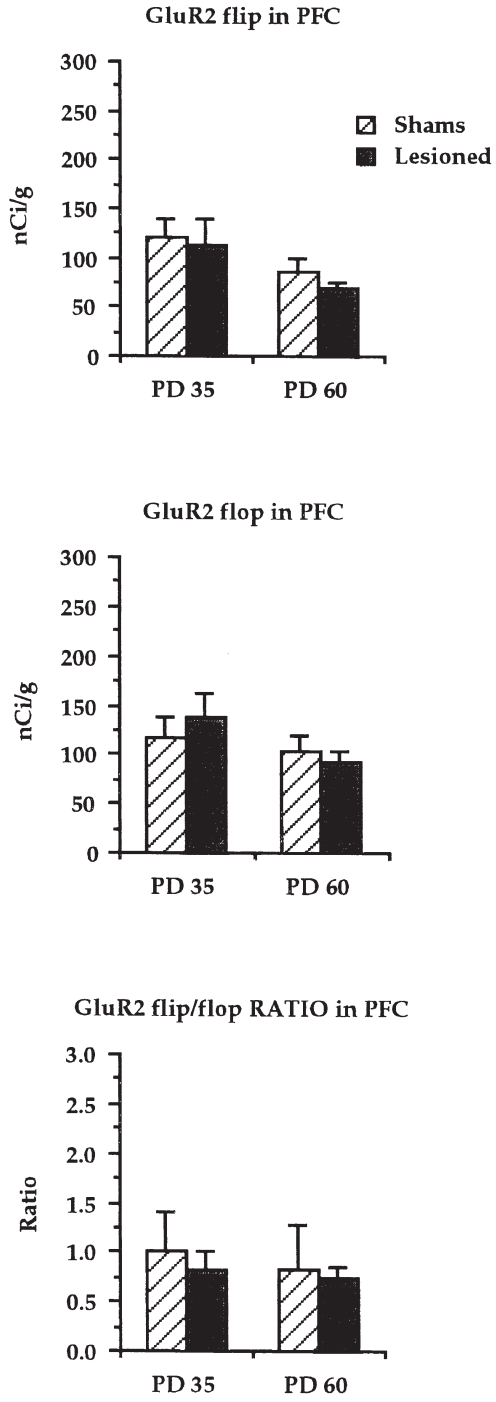


Figure 4. GluR2 flip, GluR2 flop, and GluR2 flip/flop ratio of mRNA expression in the PFC. No significant differences were found between sham and lesioned groups. N = 10 rats/group. See legend to Figure 3 for details of analysis.

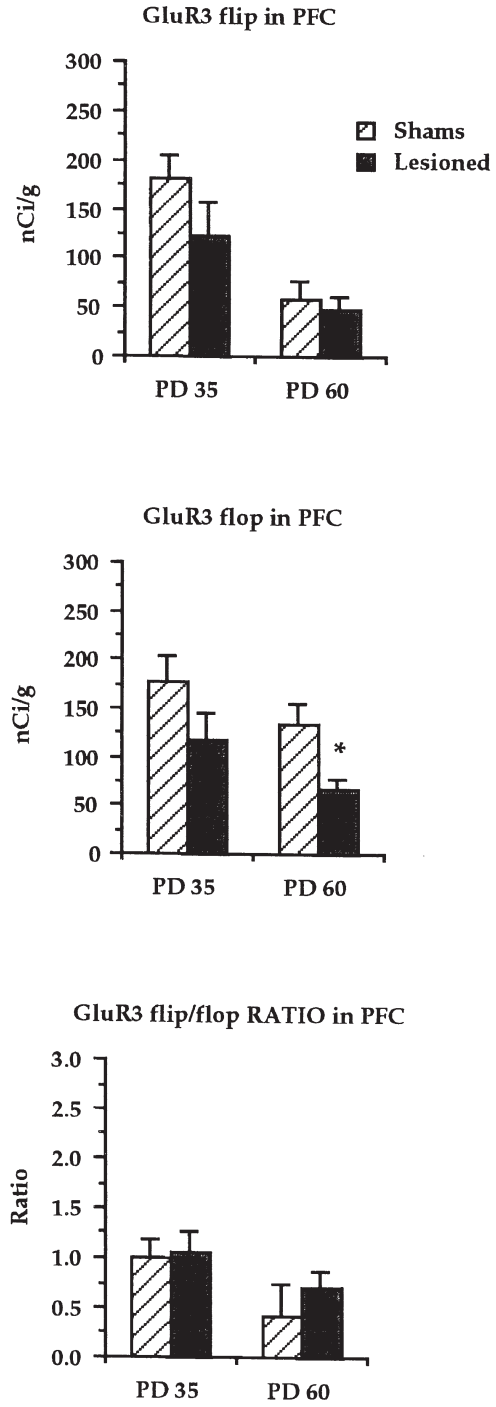


Figure 5. GluR3 flip, GluR3 flop, and GluR3 flip/flop ratio of mRNA expression in the PFC. GluR3 flop mRNA levels were significantly decreased in lesioned rats compared to sham rats on PD 60 (*$p < .05$). N = 10 rats/group. See legend to Figure 3 for details of analysis.

there was a strong trend towards decreased GluR1 flop mRNA levels in lesioned rats on PD 35 compared to sham operated controls ($p = .07$; two-tailed Student's *t*-test). Levels of GluR2 flip and GluR2 flop mRNA in the PFC did not differ between sham and lesioned groups at either PD 35 or PD 60 (Figure 4). For GluR3, there were trends towards decreases in both flip and flop levels in lesioned rats compared to shams on PD 35. On PD 60, there was a statistically significant decrease in GluR3 flop in the lesioned rats (*$p < .05$; Figure 5).

Developmental Changes in GluR1-3 Flip and Flop mRNA Levels in the NAc

Similar to results for GluR1 in the PFC, mRNA levels for GluR1 flip increased while GluR1 flop decreased from PD 35 to PD 60 in the NAc (Figure 6). This apparent developmental change is reflected in an increase in the ratio of GluR1 flip/flop on PD 60 (Figure 6). Changes in other subunits between PD 35 and PD 60 were less pronounced. There was a trend towards a small decrease in the GluR2 flip/flop ratio with age (Figure 7), while both flip and flop variants of GluR3 decreased slightly with age (Figure 8). To determine if core and shell subregions of the NAc were differentially affected by neonatal VH lesions, these subregions were analyzed separately using the boundaries defined in Figure 1. Developmental profiles within each subregion were very similar to those found for the NAc as a whole (data not shown).

Effect of Neonatal VH Lesions on GluR1-3 Flip and Flop mRNA Levels in the NAc

When the entire NAc was scanned, levels of GluR1 flip and GluR1 flop mRNA did not differ significantly between sham and lesioned groups at PD 35 or PD 60 (Figure 6). Similarly, no significant differences between sham and lesioned groups were found for GluR2 (Figure 7) or GluR3 (Figure 8) at either PD 35 or PD 60. To determine whether core and shell subregions of the NAc were differentially affected by neonatal VH lesions, these subregions were analyzed separately using the boundaries defined in Figure 1. Results for each subregion scanned alone were extremely similar to those obtained for the entire NAc (data not shown).

DISCUSSION

Expression of GluR1-3 flip and flop isoforms in rats with neonatal VH lesions and sham-operated rats was examined on PD 35 (before puberty) and on PD 60 (after puberty). There were two major findings. First, GluR3 flop mRNA levels in the PFC were decreased significantly in the lesioned group at PD 60 compared to the sham group. Second, our results suggest that there may be changes in the relative expression of flip and flop isoforms during normal development. The most pronounced change was an apparent increase in the flip/flop ratio for GluR1 from PD 35 to PD 60 in both the PFC and the NAc.

Relationship between mRNA and Protein Levels for AMPA Receptor Subunits

We studied GluR1-3 flip and flop variants at the mRNA level because there has been no way to distinguish be-

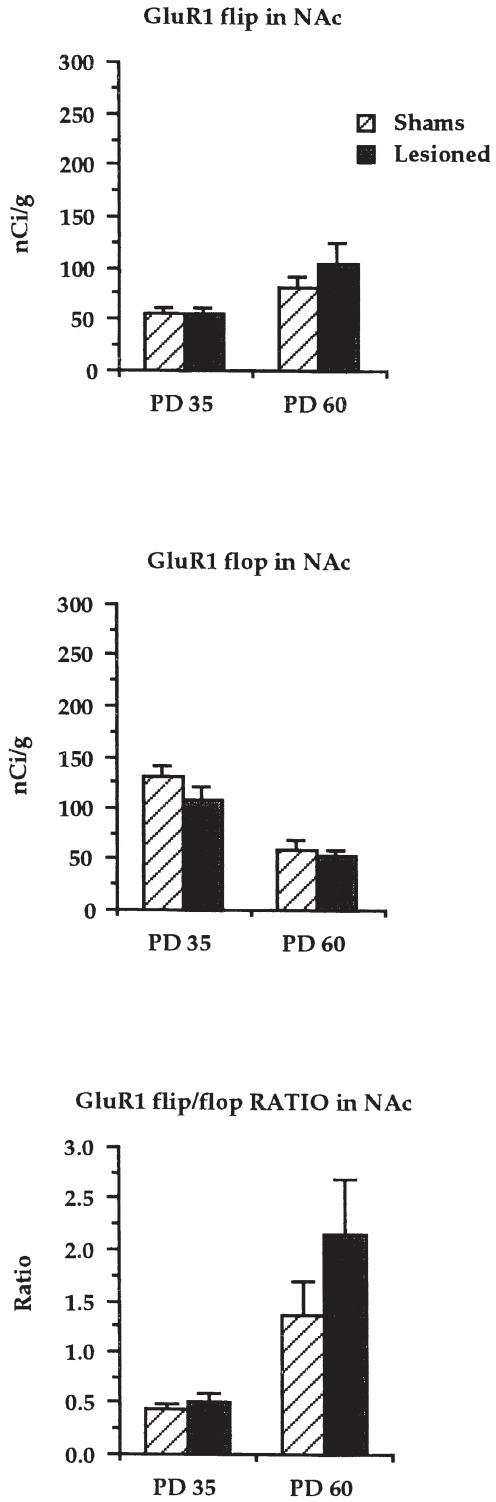
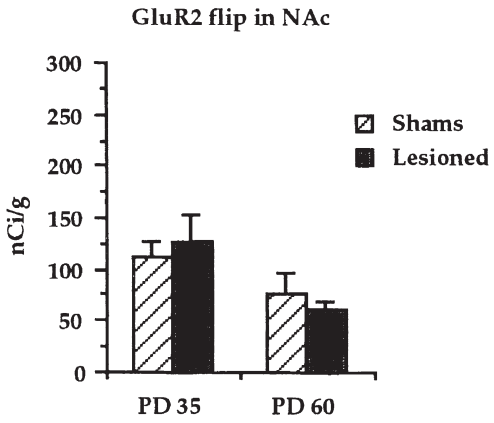
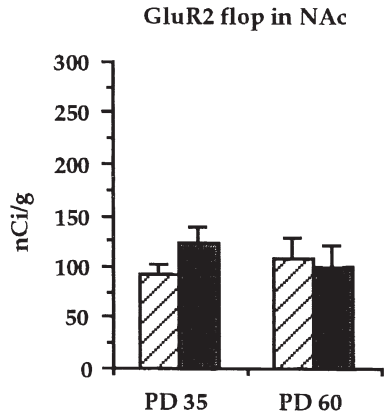


Figure 6. GluR1 flip, GluR1 flop, and GluR1 flip/flop ratio of mRNA expression in the entire NAc. No significant differences were found between sham and lesioned groups. N = 10 rats/group. See legend to Figure 3 for details of analysis.





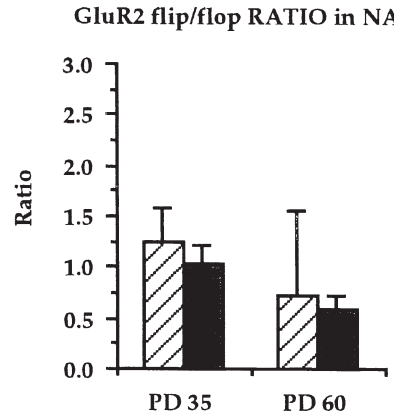
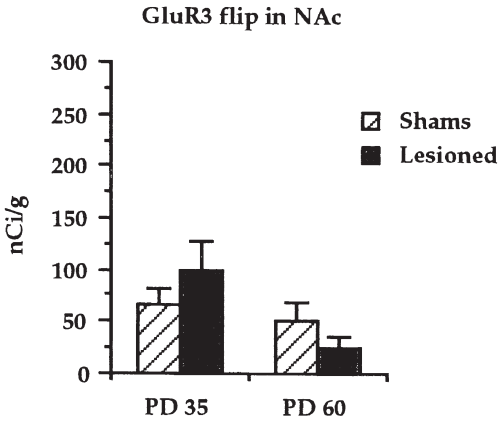
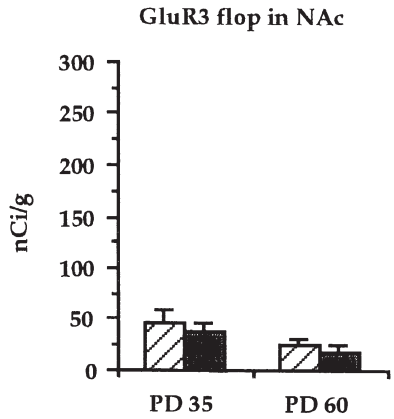


Figure 7. GluR2 flip, GluR2 flop, and GluR2 flip/flop ratio of mRNA expression in the entire NAc. No significant differences were found between sham and lesioned groups. N = 10 rats/group. See legend to Figure 3 for details of analysis.





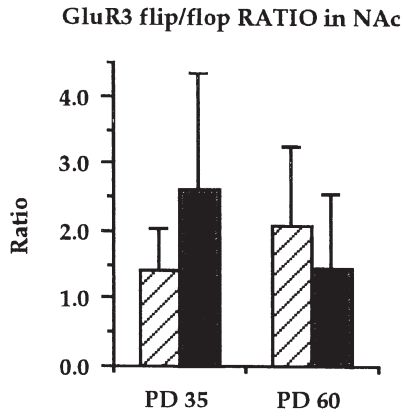


Figure 8. GluR3 flip, GluR3 flop, and GluR3 flip/flop ratio of mRNA expression in the entire NAc. No significant differences were found between sham and lesioned groups. N = 10 rats/group. See legend to Figure 3 for details of analysis.

tween them at the protein level, although a monoclonal antibody selective for GluR1 flop has been developed very recently (Tönnes et al., 1999). However, many studies suggest that regulation of mRNA levels for individual AMPA receptor subunits represents an important mechanism for determining the subunit composition of heteromeric AMPA receptors. Most convincingly, a very good correlation has been found between mRNA levels for AMPA receptor subunits and functional properties of AMPA receptor channels in the same cell (Angulo et al. 1997; Bochet et al. 1994; Geiger et al. 1995; Jonas et al. 1994; Lambolez et al. 1992). Moreover, the regional and developmental pattern of expression for GluR1 flop as determined using a newly generated monoclonal antibody (above) is generally similar to that revealed by prior studies of GluR1 flop mRNA (Tönnes et al. 1999). Finally, we have found that chronic amphetamine-induced changes in AMPA receptor subunit expression at the mRNA level, assessed with the same quantitative ISHH procedures used herein, are nearly always accompanied by corresponding changes at the protein level (Lu et al. 1997; Lu and Wolf 1999).

Developmental Expression of GluR Flip and Flop mRNAs During Postnatal Development

There has been one prior detailed study of GluR1-4 flip and flop expression during rat brain development (Monyer et al. 1991). In the embryonic brain, GluR mRNA was primarily of the flip form. Distribution patterns for the flip form remained largely invariant during postnatal development. While there was regional variation, GluR flop mRNA was almost absent in embryonic brain, was expressed at very low levels relative to flip at PD 8, underwent a general increase in expression after PD 8, and reached adult levels by PD 15. This study did not compare later postnatal time-points and focused on hippocampus and cerebellum; no results are reported for the NAc or the PFC (Monyer et al. 1991). A second study examined flip and flop but only compared expression at PD 7 and in adulthood (Jakowec et al. 1998). Flip predominated over flop for all subunits, while both flip and flop isoforms of GluR1-3 showed reduced expression in the adult compared to the neonate. Again, specific information about NAc and PFC was not presented (Jakowec et al. 1998). Two studies of adult striatum reported a predominance of flop over flip for GluR1-3 (Wüllner et al. 1994; Tallaksen-Greene and Albin, 1996), although data presented in another study suggested the opposite for GluR1 and GluR3 (Sommer et al. 1990).

The present study extends these findings by showing that changes in the flip/flop ratio continue to occur during later stages of postnatal development. In the PFC, GluR1 flop expression dominated at PD 35 in both sham and lesioned groups. After puberty, GluR1 flop mRNA levels in both groups decreased considerably, leading to a flip/flop ratio close to 1 in the sham group and greater than 1 in the lesioned group. GluR2 flip and flop variants showed nearly equivalent expression from PD 35 to PD 60 in both sham and lesioned groups. For GluR3, flip expression was roughly equivalent to flop expression at PD35 in both sham and lesioned groups, but flip mRNA levels declined in both groups after puberty, resulting in a flip/flop ratio less than 1 on PD 60. For technical reasons discussed in Results, comparison of our flip and flop signals can only provide an estimate of the relative abundance of these mRNA species. However, our results suggest that there are changes in the flip/flop ratio for GluR1 and perhaps for GluR3 in the PFC during the period surrounding the onset of puberty. This may correlate with late development of other neurotransmitter systems in the PFC (see below).

In the NAc, GluR1 flop expression predominated at PD 35 in both the sham and lesioned groups, while flip predominated on PD 60. Thus, the flip/flop ratio for GluR1 appears to increase from PD 35 to PD 60, similar to results obtained in the PFC. Changes in other subunits were more modest. For GluR2, the flip/flop ratio was $\sim$1 on PD 35. However, flip levels decreased slightly from PD 35 to PD 60 while flop remained constant, resulting in a modest decrease in the flip/flop ratio. For GluR3, flip forms predominated at both timepoints, although both flip and flop expression appeared to decrease with age. The predominance of GluR1 and GluR3 flip variants in the NAc at PD 60 (flip/flop ratios $>$ 1) may be related to the fact that medium spiny NAc neurons have very hyperpolarized resting membrane potentials (Pennartz et al. 1994). Greater relative expression of flip variants, which are associated with slower desensitization and larger current amplitude (see Introduction and next section), may be a mechanism designed to enhance the ability of excitatory inputs to activate the normally quiescent NAc neurons.

Effect of Neonatal VH Lesions on GluR Flip and Flop mRNAs in the PFC and the NAc

Neonatal VH lesions did not significantly affect GluR1 flip or flop mRNA levels at either PD 35 or PD 60 in the PFC or in the NAc. In the NAc, it is likely that GluR1 is a constituent of most heteromeric AMPA receptors in medium spiny neurons (see Lu and Wolf 1999 for discussion). These medium spiny neurons represent 90–95% of total neurons in adult striatum (Gerfen 1992; Smith and Bolam 1990) and in primary cultures of the NAc (Shi and Rayport 1994). Thus, a lack of change in GluR1 expression suggests that the lesions do not affect the absolute number of AMPA receptors in the NAc. The situation is more complicated for the PFC, where AMPA receptor subunit expression by particular cell types has not been characterized (see below). Sham and lesioned rats did

not differ at either developmental time point with respect to expression of GluR2 flip or flop mRNAs in the PFC or the NAc. Since GluR2 controls calcium permeability of AMPA receptors (Geiger et al. 1995; Hollman et al. 1991; Jonas et al. 1994), our results suggest that this property is not altered by neonatal VH lesions.

In the NAc, GluR3 flip and flop mRNAs were not altered by neonatal VH lesions. However, GluR3 flop mRNA levels in the PFC were significantly decreased in lesioned rats compared to shams at PD 60. The postpubertal emergence of this effect suggests that changes resulting from loss of VH inputs interact with other changes in rat PFC that are occurring during puberty, such as continued maturation of DA and GABA systems (e.g., Benes et al. 1996; Kalsbeek et al. 1988; Vincent et al. 1995). Continued refinement of neuronal circuitry within the human PFC during adolescence may account for the tendency for symptoms of schizophrenia to emerge during late adolescence or early adulthood (Lewis 1997).

In what cell types of the PFC are lesion-induced decreases in GluR3 flop mRNA levels occurring? Compensatory changes in GluRs on pyramidal neurons might be expected based on the observation that excitatory inputs from hippocampus to PFC form at least 95% of their synaptic contacts onto pyramidal neurons (Carr and Sesack 1996). On the other hand, a single cell reverse transcriptase PCR study found that 90% of GluR1-4 mRNAs in nonpyramidal neurons of rat neocortex are flop variants, whereas 92% in pyramidal neurons are flip variants (Lambolez et al. 1996). Thus, decreased GluR3 flop expression may indicate a preferential effect of the lesion on nonpyramidal cells, that is, GABA local circuit neurons. Most studies have not found decreases in subclasses of GABA neurons in the PFC of schizophrenics (Daviss and Lewis 1995; Woo et al. 1997; but see Benes et al. 1991). However, these neurons do exhibit changes in gene expression, such as decreased mRNA levels for glutamic acid decarboxylase (Akbarian et al. 1995b).

Although neocortical regions studied by Lambolez et al. (1996) showed segregation of flop and flip variants in nonpyramidal and pyramidal neurons, respectively, this may not hold true in the PFC. Thus, it remains possible that decreased GluR3 flop mRNA is occurring in a population of neurons, either pyramidal or nonpyramidal, that also expresses GluR3 flip variants. In this case, decreased flop expression might result in the formation of heteromeric AMPA receptors that include proportionately more flip variants. Flop isoforms are associated with less current entry than flip isoforms due to faster desensitization (see Introduction). For example, homomeric GluR3 flop receptor channels desensitize four times faster than GluR3 flip channels (Mosbacher et al. 1994). Thus, the observed decrease in GluR3 flop expression in the PFC could increase neuronal excitability.

It is difficult to speculate further about the identity of cell populations that experience lesion-induced changes in GluR3 flop, because GluR3 is widely distributed. Thus, a study that examined GluR1-4 expression in rat somatic sensory cortex estimated that 64% of neurons contained GluR3 mRNA, with labeling in all layers except layer I, although labeling was higher in layers II-III and V-VI than in layer IV (Conti et al., 1994). Parallel immunocytochemical studies using antibody recognizing GluR2/3 showed that the majority of labeled cells were pyramidal, although nonpyramidal cells were also labeled and represented the majority of GluR2/3-containing neurons in layer IV (Conti et al. 1994). These findings are generally in agreement with other studies that examined various cortical regions in the rat (e.g., Boulter et al. 1990; Gutierrez-Ibarluzea et al. 1997; Keinanen et al. 1990; Martin et al. 1993; Petralia and Wenthold 1992; Sato et al. 1993). The only study in the PFC itself was conducted in the Macaque monkey. Patterns of labeling were very similar to those obtained in rat neocortex, but specific information about GluR3 was not obtained because the study used an antibody recognizing GluR2 and GluR3 (Vickers et al. 1993). No previous studies have examined GluR flip and flop expression in the rat PFC, although a study in parietal cortex found preferential expression of flip versions of GluR1-3 mRNAs in some layers (II, II and VI) while flop expression was more uniform (Sommer et al. 1990).

The functional consequences of decreased GluR3 flop expression remain to be determined. If it is associated with increased neuronal excitability in the PFC, and if this occurs in pyramidal neurons, an increase in the activity of PFC projections might be expected. Since PFC projections regulate the activity of midbrain DA neurons (see Sesack and Pickel 1992), this could contribute to dysregulation of subcortical DA systems after neonatal VH lesions (see Introduction). In addition, because these PFC projections use EAAs as their transmitter substances, the lesions might alter EAA transmission in projection areas of the PFC, such as the NAc. In other studies of neonatal VH lesioned rats, we have found that depolarization-induced glutamate and asparate efflux in the NAc, measured by microdialysis, is enhanced on PD 60 in lesioned rats compared to sham-operated rats [Stine CD, Xue C-J, Wolf ME (submitted): Excitatory amino acid transmission in the nucleus accumbens and prefrontal cortex: Effect of neonatal ventral hippocampal lesions]. Thus, alterations in EAA receptor expression in the PFC have the potential to produce widespread changes in subcortical regions implicated both in behavioral abnormalities produced by neonatal VH lesions and in the pathophysiology of schizophrenia.

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