

Early Appearance of Functional Deficits after Neonatal Excitotoxic and Hypoxic-Ischemic Injury: Fragile Recovery after Development and Role of the NMDA Receptor

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Key Words

Cerebral ischemia · Stroke · Cerebral hypoxia-ischemia · Neonatal brain injury · Sensorimotor deficit · Plasticity · Excitotoxicity · N-methyl-D-aspartate · MK-801

Abstract

We sought to determine whether neonatal rats that sustain unilateral cerebral hypoxic-ischemic or excitotoxic insults (1) manifest contralateral sensorimotor deficits during development or in adulthood and (2) recover from those deficits. Seven-day-old (P7) rats received a right intrastriatal injection of the glutamate analog N-methyl-D-aspartate (NMDA). Unilateral hypoxia-ischemia (HI) was induced by right carotid ligation followed by 1.5 h in 8% O₂. Both procedures produce neuronal loss in the striatum and sensorimotor cortex. Nonlesioned controls were included. We scored percent forepaw placement on the edge of a horizontal surface, with lateral vibrissa stimulation, from P9 to P19, and at P33 and P50. Then, on P50, rats were treated with the NMDA antagonist MK-801 to determine whether deficits could be reinstated. NMDA- and HI-lesioned rats exhibited a

deficit in contralateral vibrissa-stimulated forepaw placing that emerged during the second week of life. Yet, by P33 and P50, the lesioned groups and controls were indistinguishable. MK-801 injection on P50 resulted in transient reinstatement of the placing deficit. After unilateral neonatal excitotoxic or HI brain injury, contralateral sensorimotor deficits are detected, but in many animals, these deficits have resolved by adulthood. Thus, timing of sensorimotor tests may influence their sensitivity for detection of focal neuropathology originating in the neonatal period.

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Introduction

Human trials of promising neuroprotective therapies (e.g. hypothermia) utilize neurologic function as their primary outcome measure. In rodent trials of potential neuroprotective therapies, there is increasing use of functional outcomes to complement neuropathologic outcomes. Questions that still need to be answered regarding such testing in developing rodents include: 'what are the appro-

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priate tests?' and, 'when is the optimal time to test?' An additional question, closely related to the latter question, is 'do deficits change over time?'

In adult rodents, there is evidence, in several injury models, that sensorimotor deficits become less pronounced over time [Schallert et al., 2000]. Such deficits can, however, be reinstated pharmacologically using central nervous system (CNS) depressant drugs [Barth et al., 1990; Schallert et al., 1986]. A variety of cognitive and sensorimotor deficits have been reported in immature rats that undergo hypoxic-ischemic lesions [Almli et al., 2000; Altemus and Almli, 1997; Balduini et al., 2000; Bona et al., 1998; Bona et al., 1997; Chou et al., 2001; Ford et al., 1989; Ikeda et al., 2001; Jansen and Low, 1996a; Jansen and Low, 1996b; Liu et al., 2001; Tomimatsu et al., 2002; Wagner et al., 2002; Young et al., 1986]. Many reports of functional deficits after neonatal hypoxia-ischemia (HI) involve only a single time point; few incorporate serial testing. Rats with moderately severe hypoxic-ischemic lesions on day 7 of life (P7) had a deficit in Rota-Rod sensorimotor performance at 3 weeks that persisted in weekly testing until 9 weeks of life [Jansen and Low, 1996a]. The same group of rats also manifested asymmetry in apomorphine-stimulated rotation from 3 to 15 weeks of age [Jansen and Low, 1996a], consistent with severe striatal damage [Schallert and Whishaw, 1985]. In an immature (P17) rat model of traumatic brain injury, sensorimotor deficits resolved over time [Adelson et al., 1997]. Kolb [1999] has shown that after neonatal cortical aspiration lesions, the extent and time course of functional recovery parallels changes in dendritic morphology in the remaining intact cortex.

In a preliminary experiment to characterize the evolution of sensorimotor deficits after an episode of neonatal cerebral HI, we found evidence of recovery or compensation from a unilateral deficit. Specifically, in a test of paw placement while exploring a Plexiglas cylinder, rats that underwent unilateral (right) HI on P7 exhibited a right-sided paw weight-bearing preference, peaking on P35. Yet these same rats no longer had this ipsilateral preference as adults [Barks et al., 2002].

In the experiment reported here, we injected the glutamate analog N-methyl-D-aspartate (NMDA) into the right striatum. Our goal was to create lesions that were less heterogeneous than HI lesions and that were sufficiently localized to produce predominantly motor deficits. These animals were serially tested over the next 2 months to evaluate reflex ontogeny, forelimb placing and strength, and sensorimotor integration.

Methods

NMDA Lesioning

Isoflurane-anesthetized P7 Sprague-Dawley rats from two litters (n = 10) underwent a midline scalp incision followed by stereotaxic right intrastriatal injections of NMDA (Sigma) 10 nmol in 0.5 μ l (pH 7.4), using coordinates (relative to bregma) AP 0, ML 2.0, V 3.8 mm [Hagan et al., 1996]. Pups recovered in a 37 °C incubator and were returned to the dam when active. To begin to evaluate whether recovery of function was possible after other neonatal brain injuries, unilateral cerebral HI was elicited in a small number of littermates in one experiment. In 3 rats, double ligation and division of the right carotid artery was followed 1 h later by 1.5 h in 8% O₂ (balance nitrogen). Littermate controls (n = 12) did not undergo surgery. Both the NMDA lesioning and HI lesioning protocols were designed to produce lesions of moderate severity.

NMDA Antagonist Injection

On P50, after sensorimotor testing, all NMDA-injected and HI rats received an intraperitoneal injection of the noncompetitive NMDA receptor antagonist MK-801 (dizocilpine maleate, Sigma-RBI, 1 mg/kg in PBS). The purpose of these injections was to determine whether, as in adult-lesioned rats, a sensorimotor deficit could be reinstated after behavioral recovery had occurred [Barth et al., 1990].

Developmental Assessment

Measures of development that were assessed at P7 (pre-NMDA lesion), and P9, 11, 13, and 15, using a 0- to 5-point Likert scale, included: growth (fur, ear, eye opening); general development (auditory startle, bar hold, surface righting, negative geotaxis), and the sensorimotor skill of forelimb placement [Altman and Bayer, 1997; Lapointe and Nosal, 1979]. Forelimb placement was assessed at the above ages and at P17 and P19, by the number placed of 10 trials/limb in response to ipsilateral vibrissa or head-on (chin) stimulus on a horizontal surface.

Postdevelopmental Sensorimotor Assessment

At P33, P50 (pre-MK-801) and P51–P53 (post-MK-801), forelimb placement in response to ipsilateral vibrissae and head-on stimuli were assessed as above for both litters [Hua et al., 2002; Schallert et al., 2000; Schallert et al., 2002b; Wu et al., 2002]. Other sensorimotor assessments included: cylinder test [number of placements of each forelimb on rearing in a clear Plexiglas cylinder (R – L)/total]; sticker test (number of and latency to contact and removal of an adhesive dot placed on each upper extremity); cross-vibrissa placing (number placed of 10 trials/limb in response to contralateral vibrissa stimulus), and parachute test (posture in response to tail suspension; number of left-turn postures per 10 trials) (fig. 1) [Whishaw et al., 1981]. The cylinder and sticker tests were completed at P33, P50 and P53 in the first litter. The cross-vibrissa and parachute tests were completed at P50 and P51–P53 for the second litter.

Brain Pathology

After the completion of all testing, rats were weighed and euthanized. Brains from the first litter were weighed and then frozen under powdered dry ice, for coronal sectioning and cresyl violet staining. Brains from the second litter were fixed in situ with PBS followed by 4% paraformaldehyde under deep chloral hydrate anesthesia; in this litter, 1 NMDA-injected and 1 control rat were sacrificed on P21 to

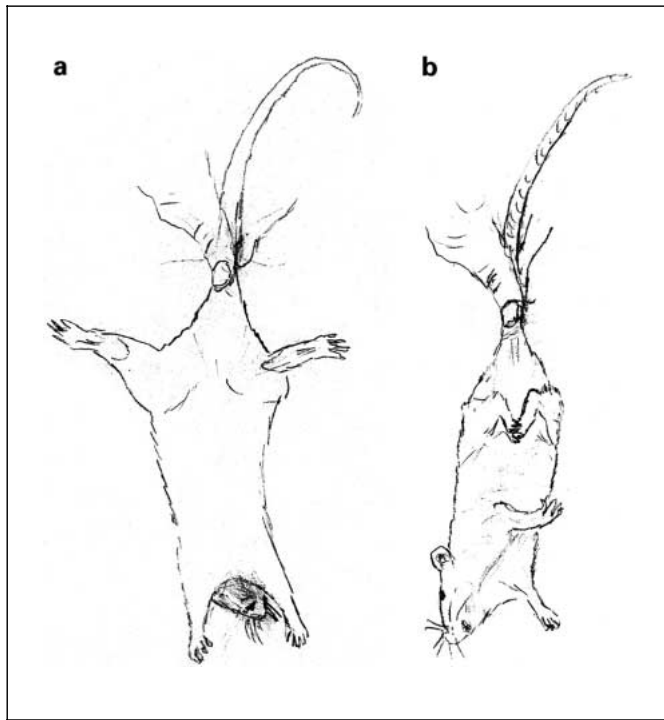


Fig. 1. Tail-suspension response in normal and NMDA-lesioned rats. At P50–P53, rats were suspended by the tail above a horizontal surface and lowered toward the surface. In the normal response, a nonlesioned animal reaches toward the surface below with both forelimbs extended and abducted; the hindlimbs are also extended and abducted (a). b Abnormal response frequently seen in the neonatally NMDA-lesioned rats. The contralateral forelimb (in this case, the left) is flexed, adducted and internally rotated, and both hindlimbs are adducted and internally rotated, with the hindpaws claspings each other.

assess the extent of injury and microgyria at this age. Cortical and striatal cross-sectional areas in 20- μ m coronal sections (5/brain through striatum and dorsal hippocampus) were measured by microcomputer-based image analysis (NIH image). As an index of atrophy, percent damage of each ipsilateral structure, compared with the nonlesioned side, was calculated using bilateral regional areas, by the formula $\% \text{ damage} = 100 \times (L - R)/L$. Sections from NMDA-lesioned rats in the second litter, at P54, were also stained by the von Kossa method with a neutral red counterstain to demonstrate calcium deposition [Pathology, 1992].

Data Analysis

Measures of development and sensorimotor skill emergence, and percent forelimb placement, were compared among NMDA-injected and control rats, and HI littermates, by ANOVA or t test for continuous and χ^2 for categorical variables. Repeated-measures ANOVA was also applied as appropriate. Significance was set at $p < 0.05$. Body and brain weights were compared between groups by t test or ANOVA. As an index of ipsilateral striatal and cortical damage

severity, the percent atrophy (i.e. percent damage) was calculated from the bilateral mean regional cross-sectional areas for each animal, using the formula $\% \text{ damage} = 100 \times (L - R)/L$. The relationship between the ultimate striatal and cortical percent damage and percent success in contralateral forelimb placing was evaluated by linear regression at P11, P13, P15, P17, P19, P33 and P50–P53.

Results

Growth and Physical Development

There were no differences in day of eye opening or ear opening, nor in fur development between the NMDA-injected, HI and control pups. There was no difference in mean body weight between groups, at the end of testing (P54).

Reflex Development

There were no differences among groups in auditory startle, surface righting, negative geotaxis or bar hold during development.

Forelimb Placing

In nonlesioned controls, vibrissa-stimulated lateral forepaw placement was not detected until P11, and was fully established (10/10 trials in all subjects) by P19 (fig. 2). In the NMDA-lesioned rats, the ontogeny of ipsilateral (right-sided) forepaw placement was indistinguishable from nonlesioned controls. In the NMDA-lesioned rats, there was no delay in onset of contralateral (left-sided) vibrissa-stimulated forepaw placement, but there was an overall performance deficit compared with controls during the preweaning period (fig. 2; $p < 0.05$, repeated-measures ANOVA). When evaluating results on individual testing days, between-group differences in contralateral placing were not detected on P11 or P13, but they were detected on P15, P17 and P19 (fig. 2; $p < 0.001$, Student t test). On P33 and P50, contralateral performance in the NMDA-lesioned rats was no longer different from controls (fig. 2). The 3 HI rats exhibited a transient contralateral deficit in forepaw placing, on P15 and P17, which had resolved by P19 (fig. 2; $p < 0.02$, Student t test). Twenty-four hours after MK-801 injection on P50, control rats exhibited a mild bilaterally symmetric reduction in forepaw placing that resolved at 72 h after MK-801. Among the NMDA-injected rats, treatment with MK-801 on P50 resulted in a total (0/10 trials) deficit in contralateral vibrissa-stimulated placing 24 h later in all animals that had resolved by 72 h after MK-801 injection (fig. 2). Among the HI comparison group, 2/3 exhibited marked deterioration (0–1/10 trials) in contralateral placing 24 h

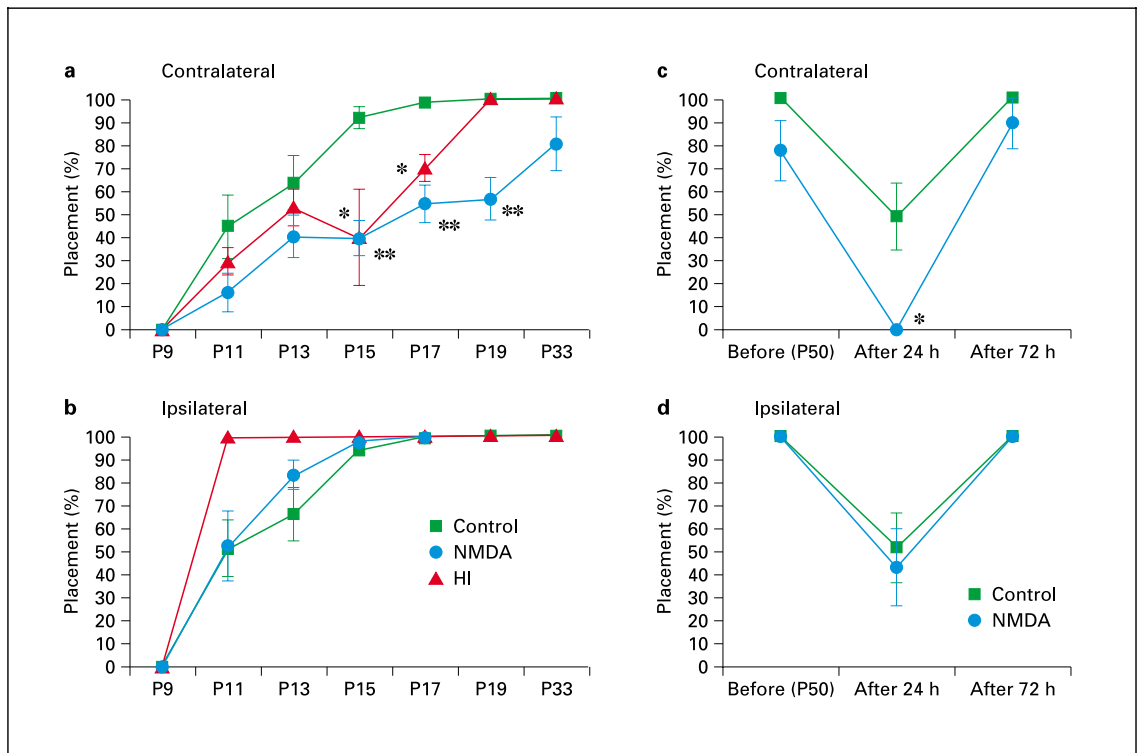


Fig. 2. Neonatal NMDA and HI lesioning induce deficits in forepaw placing ontogeny. a, b The mean \pm SE of vibrissa-stimulated lateral forelimb percent placement (of 10 trials/side), from P9 to P33 for both the contralateral (left, a) and ipsilateral (right, b) sides, in rats that received either intrastriatal NMDA injections (10 nmol, $n = 10$) on P7 (NMDA), noninjected littermate controls ($n = 12$), and a comparison group of 3 littermates that underwent unilateral right cerebral HI (HI; see Methods). As the lateral vibrissa placing response developed in controls, the NMDA-injected animals exhibited a contralateral placing deficit ($p < 0.01$, repeated measures ANOVA). When group data from individual days were compared, the NMDA group had a lateral placing deficit on P15, P17 and P19 (** $p < 0.001$, t test), and the HI group had a placing deficit on P15 and P17 (* $p < 0.02$, t test). c, d By P33 and P50, there was no significant difference

in vibrissa-stimulated contralateral forelimb placing among groups (mean \pm SE). No deficit in vibrissa-stimulated placing was detected ipsilaterally to the NMDA or HI lesions. After vibrissa-stimulated forepaw placing testing on P50, all rats received an injection of the NMDA antagonist and CNS depressant MK-801 (1 mg/kg, i.p.). Twenty-four hours later, controls had a modest bilateral deterioration in placing, which had resolved by 72 h. In contrast, the entire NMDA-lesioned group was unable to place contralaterally (c), 24 h after MK-801 (* $p < 0.02$, ANOVA with Fisher PLSD, NMDA vs. control). The ipsilateral placing performance of NMDA-lesioned rats 24 h after MK-801 was similar to controls (d). The MK-801-induced forepaw-placing deficit in the NMDA-lesioned animals had resolved by 72 h after MK-801 treatment. Similar trends were detected in the HI group (not shown).

after MK-801 injection. No lateralizing deficit in head-on (chin-stimulated) forepaw placing was detected either during development, prior to or following MK-801 treatment.

To attempt to determine whether placing deficits were attributable to sensory vs. motor dysfunction, we tested cross-midline vibrissa-stimulated placing in animals of the second litter. On P50, 1 each of the NMDA and HI animals had a residual contralateral vibrissa-stimulated placing deficit (2 left forepaw placings/10 trials after left vibrissa stimulation). When these animals were tested by crossed vibrissa placing, i.e. the left vibrissae were stimu-

lated by the edge of the bench, with the left forelimb restrained, the right forepaw was successfully placed in 10/10 trials. The latter finding implies that left vibrissa sensation was intact, and that the lesion-induced contralateral (left) forelimb dysfunction was primarily motoric.

The sensitivity of lateral vibrissa-stimulated forelimb placing to both striatal and cortical lesion severity during development was evaluated by linear regression, which included all subjects, both lesioned and controls. There was a linear relationship between striatal percent atrophy and contralateral percent placement on P15, P17 and P19 ($R^2 = 0.426, 0.254$ and 0.179 , respectively, $p < 0.05$), but

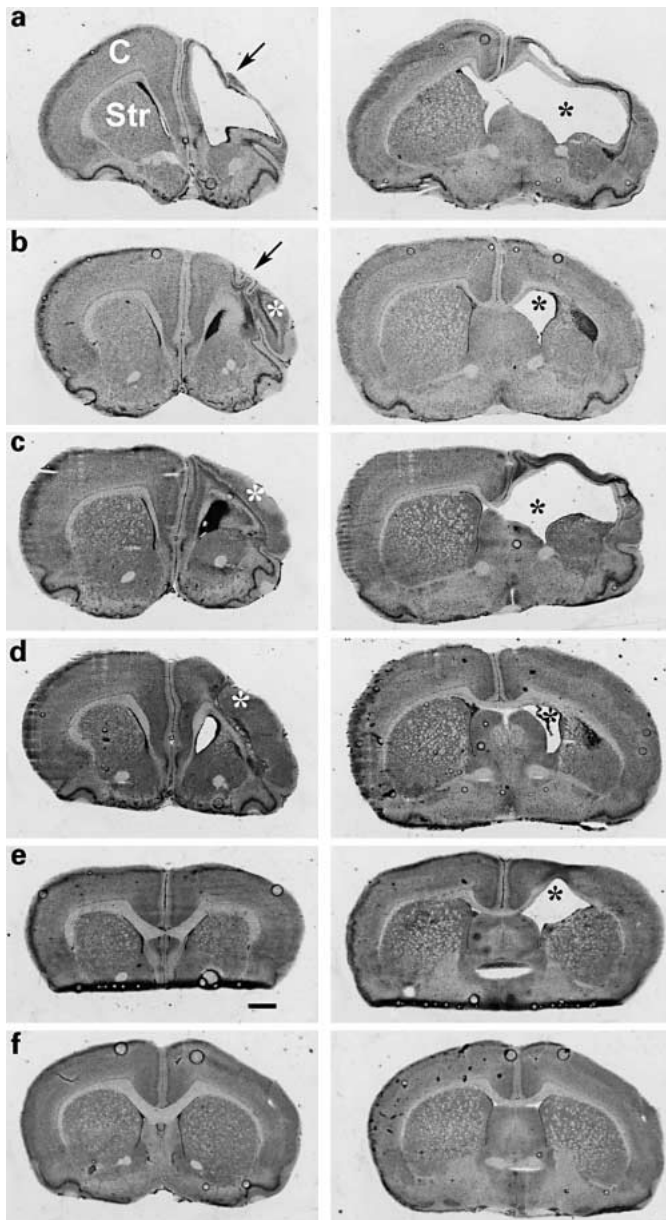


Fig. 3. Histopathology in NMDA-injected rats. Coronal cresyl violet-stained sections at the level of the rostral striatum (left column) and anterior commissure (right column) are presented for each of 6 rats from a single experiment that received right intrastriatal injections of NMDA (10 nmol) 7 week earlier. Note ipsilateral striatal atrophy in all cases, and dilatation of the ipsilateral ventricle (black asterisk) in 5/6 cases (a–e). Abnormal cortical morphology, including microgyria (black arrows) and abnormal layering (white asterisk) is evident in 4/6 (a–d). C = Cortex; Str = striatum. Scale bar = 1 mm.

not at earlier ages nor at P33. There was a linear relationship between cortical percent atrophy and contralateral percent placement only on P15 ($R^2 = 0.2790$, $p < 0.05$). On P50, prior to MK-801 treatment, and again on P53, 72 h after MK-801 treatment, there was a linear relationship between both striatal and cortical percent atrophy and percent contralateral placement ($R^2 = 0.32$ and 0.493 , respectively, on P50, and 0.194 and 0.36 , respectively, on P53, $p < 0.05$). Twenty-four hours after MK-801 treatment, when contralateral placing was at its nadir in lesioned animals, there was no relationship between damage severity and percent placement.

Tail Suspension

In all (4/4) controls in the second litter, the normal symmetric hindlimb response (fig. 1a) was elicited on P35. In all (3/3) NMDA-injected rats, and in 2/3 HI rats in the second litter, hindlimb adduction and clamping was elicited. The number of abnormal forelimb and hindlimb responses in the NMDA-lesioned rats was greater than in the controls (median, control vs. NMDA: forelimb 2.5 vs. 8, hindlimb 0 vs. 8, $p < 0.05$, Mann-Whitney).

Other Assessments

There was no significant difference between the experimental groups for latency to touch or remove the stickers at P33, before MK-801 (P50) or at 72 h after MK-801 (P53). Similarly, there was no significant group difference for upper extremity use in the cylinder task at P33, P50 and P51.

Neuropathology

In the first litter, the brains of the NMDA-injected rats were 7% smaller than those of controls (mean \pm SD: NMDA-injected 1.64 ± 0.08 g; control 1.77 ± 0.06 g, $p < 0.01$). Based on our previous experience evaluating NMDA-induced neuropathology 5 days after P7 lesioning, we expected to find ipsilateral striatal atrophy and thinning of the overlying neocortex, as a result of excitotoxic neuronal loss in the striatum and cortex, with ex vacuo dilatation of the right lateral cerebral ventricle [Hagan et al., 1996]. These expectations were confirmed on examination at 47 days after intrastriatal NMDA lesioning; there was a considerable degree of heterogeneity in damage severity (fig. 3). Two additional findings were noteworthy. First, areas of microgyria dorsolateral to the lesioned striatum, primarily seen rostral to the anterior genu of the corpus callosum, were noted in 8/10 NMDA-injected rats. Calcium deposition was assessed in the second litter at P54 and noted in the ipsilateral hemisphere

in the NMDA-injected rats. Foci of ipsilateral calcification included the dorsolateral striatum and deep layers of the overlying residual cortex (fig. 4). Of the 3 HI animals, all followed to P54, 2 had moderate ipsilateral striatal and cortical infarction with foci of microgyria and 1 had complete ipsilateral hemisphere infarction.

Discussion

Based on the location of the excitotoxic lesions in these experiments, with damage to the striatum and overlying cortex, a sensorimotor deficit, such as a contralateral deficit in vibrissa-stimulated lateral forepaw placing is not unexpected. Analogous functional deficits have been reported in adult rats with similarly located lesions [Schallert et al., 2000]. Similar deficits were detected in the small number of HI animals. Crossed vibrissa-stimulation testing suggested that these performance deficits reflected more motor than sensory dysfunction [Schallert et al., 2002a]. The deficits in lateral vibrissa-stimulated placement were dependent on lesion severity both during development and in adulthood. The functional deficits that we detected in the preweaning period were transient, consistent with previous reports of plasticity in the primate developing nervous system after injury [Goldman and Galkin, 1978]. The deficit in contralateral vibrissa-stimulated lateral forelimb placing demonstrated substantial recovery during the preweaning period, i.e. within 2 weeks of lesioning. Recovery from sensorimotor and cognitive deficits has been reported after traumatic brain injury in immature rats; the time course of functional recovery was 3–10 days, depending on lesion severity [Adelson et al., 1997]. Recovery of function has also been described after ischemic and ablation cortical injury in adult rats, but in mature animals, reports suggest less recovery of function when the lesion includes deeper structures [Schallert et al., 2000]. The placing deficit was not detected immediately after lesioning. The most likely explanation for the latter finding is that a deficit could not be detected until the age at which rats normally develop a reliable placing response during the second week of life. That is, the animals developed into the impairment [Goldman, 1978]. In clinical practice, similar situations arise; a hemiparesis may not become evident until the age at which an infant would be expected to have voluntary control of reaching and grasping.

Our inability to detect a deficit in head-on forepaw placement indicates that the motor program for contralateral forelimb placing per se is not impaired but rather the

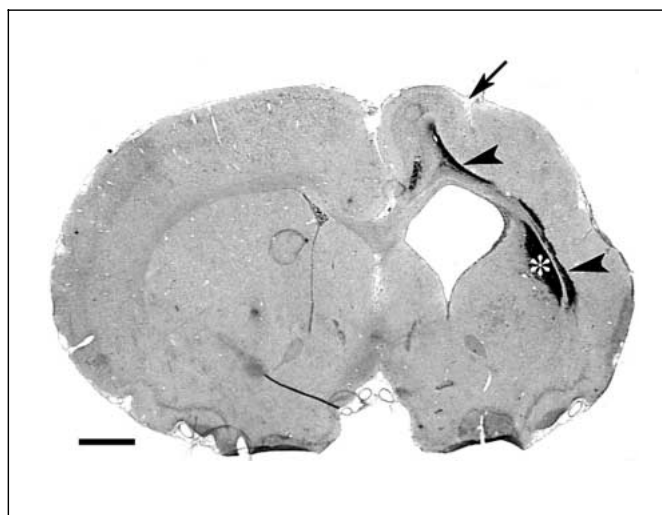


Fig. 4. Calcium deposition in NMDA-lesioned brain. This representative section from a P54 rat that received intrastriatal injections of NMDA (10 nmol) on P7 was stained by the von Kossa method (see Methods) to detect deposition of calcium in brain tissue. Note the black staining (silver grains), indicative of calcium deposition, in the lesioned dorsolateral striatum (white asterisk) and in the deep layer of the residual, damaged overlying cortex (arrowheads). There is no calcium deposition in the remaining upper cortical layers of a lesion-induced microgyrus (arrow). Scale bar = 1 mm.

capacity for the vibrissae on the contralateral side to initiate placing in that forelimb. The head-on procedure stimulates both ipsilateral and contralateral vibrissae. To the best of our knowledge, only when there is severe damage to striatal or nigrostriatal neurons is there a lasting deficit in the capacity of the nonimpaired vibrissae, when stimulated, to initiate placing in the impaired forelimb (as though sensory input to the intact hemisphere can activate motor programs in the injured hemisphere). Therefore, it is not unexpected that the head-on procedure would not detect impairment in the neonatal HI and NMDA-injected animals at time points when sensory input into the injured hemisphere is unable to consistently activate motor programs in that same injured hemisphere. It is also unlikely that there is sensory impairment in the contralateral vibrissae, based on our crossed stimulation studies. In every unilateral adult injury model examined, including severe striatal and cortical ischemic or neurotoxic injuries from which forelimb placing does not recover, we have found in cross-midline placing tests that the vibrissae on the contralateral side of the body can readily activate placing of the ipsilateral forelimb [Schallert et al., 2002a; Schallert et al., 2002b].

Microgyria has been described previously after focal cortical freeze or excitotoxic lesions on the first or second day of life in rats or mice [Humphreys et al., 1991; Marret et al., 1995; Redecker et al., 1998]. In our previous experience with striatal NMDA lesioning, we did not note microgyria, but did not evaluate neuropathology later than P12 [Hagan et al., 1996]. In younger animals, acquired microgyria takes between 5 and 10 days to develop after freeze-induced cortical necrosis [Rosen et al., 1992]. In our previous studies with striatal NMDA injections, we did not evaluate for calcium deposition at P12. Yet, the calcium deposits at P54 in NMDA-lesioned animals are not an unexpected finding. Calcium deposition is frequently seen in human infants after neonatal-perinatal hypoxic-ischemic brain injury [Ansari et al., 1990], and has been described in neonatal and adult cortical aspiration lesions [Braun et al., 1972; Whishaw et al., 1981] and in excitotoxic lesions of adult rats [Mahy et al., 1999].

Our findings suggest that in developing rodents, depending on the lesion and the test used, the time window for detection of functional deficits and for detection of behavioral evidence of plasticity may be quite narrow. Thus, the persistence of Rota-Rod sensorimotor performance deficits from 2–8 weeks after neonatal HI lesioning [Jansen and Low, 1996a] could represent a missed opportunity to detect earlier evidence of recovery rather than absence of plasticity. The phenomenon of recovery from functional deficits might also underlie some reported difficulties in detecting functional evidence of neuroprotection using a battery of sensorimotor tests 5 weeks after treatment of neonatal rats with post-HI hypothermia [Bona et al., 1998]. Our findings suggest that the time course of functional deficits in immature rats should be carefully characterized to facilitate optimal choice of time for testing.

The forelimb placing deficit in the neonatally NMDA-injected rats was transiently reinstated by treatment with the noncompetitive NMDA receptor antagonist MK-801.

Reinstatement of injury-induced functional deficits in adult rats after MK-801 or ethanol is associated with alteration of postinjury dendritic plasticity [Kozlowski et al., 1997; Kozlowski et al., 1994]. Our findings indicate that behavioral plasticity in response to neonatal brain injury is fragile, and NMDA receptor dependent. During normal CNS development, excitatory amino acid (EAA) receptors play a key role in use-dependent plasticity, and chronic treatment with EAA receptor antagonists can disrupt developmental plasticity [Gu et al., 1989; Ramoa et al., 2001; Schlaggar and O'Leary, 1993]. Thus, we speculate that EAA receptor-dependent neuroplasticity may also play a role in the recovery of function after neonatal brain injury. Regardless of the mechanism underlying the reinstatement of the functional deficit by MK-801, from a practical viewpoint, the injection of MK-801 in adult rats might be a useful adjunct to increase the sensitivity of testing for sensorimotor deficits related to neonatal lesioning.

In conclusion, we have demonstrated sensorimotor deficits and patterns of recovery using a battery of tests that assess distinct functional capabilities after neonatal brain injury. In our own experience and in published reports, adult animals with similar severity of damage would not be expected to demonstrate the degree of functional sparing or recovery seen in our developing animals. If we had only examined these animals behaviorally early after injury, we would have failed to appreciate the amount of functional recovery that could occur. Using multiple behavioral tests at multiple time points was important in detecting the degree of injury as well as the extent of recovery/compensation. Knowledge about the onset of a development-sensitive behavioral deficit and its temporal pattern of recovery, as well as of residual deficits and spared functions or compensations, would be useful for targeting studies of time-dependent molecular, cellular and structural events that might be involved in the mechanisms underlying recovery from early brain injury.

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