Functional Respiratory Rhythm Generating Networks in Neonatal Mice Lacking NMDAR1 Gene


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Funk, G. D., S. M. Johnson, J. C. Smith, X.-W. Dong, J. Lai, and J. L. Feldman. Functional respiratory rhythm generating networks in neonatal mice lacking NMDAR1 gene. J. Neurophysiol. 78: 1414–1420, 1997. N-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission is implicated in activity-dependent developmental reorganization in mammalian brain, including sensory systems and spinal motoneuron circuits. During normal development, synaptic interactions important in activity-dependent modification of neuronal circuits may be driven spontaneously (Schatz 1990b). The respiratory system exhibits substantial spontaneous activity in utero; this activity may be critical in assuring essential and appropriate breathing movements from birth. We tested the hypothesis that NMDA receptors are necessary for prenatal development of central neural circuits underlying respiratory rhythm generation by comparing the responsiveness of control mice and mutant mice lacking the NMDA receptor R1 subunit (NMDAR1) gene to glutamate receptor agonists and antagonists and comparing endogenous respiratory-related oscillations generated in vitro by brain stem-spinal cord and medullary slice preparations from control and mutant mice. In control mice, local application of NMDA and the non-NMDA receptor agonist, (R,S)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid hydrobromide (AMPA), over the pre-Bötzinger Complex, the C4 cervical motor neuron pool, and the hypoglossal motor nucleus produced profound increases in inspiratory frequency, tonic discharge on C4 ventral nerve roots, and inward currents in inspiratory hypoglossal motoneurons, respectively. Responses of mutant mice to AMPA were similar. However, mutant mice were completely unresponsive to NMDA applications. Preparations from mutant mice generated a respiratory rhythm virtually identical to control. Results demonstrate that NMDA receptors are not essential for respiratory rhythm generation or drive transmission in the neonate. More importantly, they suggest that NMDA receptors are not obligatory for the prenatal development of circuits producing respiratory rhythm.

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors could contribute to developmental tuning of neural circuits as suggested by their role in several systems, including formation and refinement of topographic maps in frog tectum (Cline et al. 1987), ferret lateral geniculate nucleus (Hahm et al. 1991), cat cortex (Bear et al. 1990; Kleinschmidt et al. 1987), and rat trigeminal nuclei (Li et al. 1994); selective stabilization of synapses of climbing fiber/Purkinje cells in cerebellum (Rabacchi et al. 1992); expression of Cat-301 proteoglycan, a proposed marker for experience-dependent development, in hamster spinal motoneurons (Kalb and Hockfield 1990); and activity-dependent modification of mouse dorsal root ganglion-ventral horn synapses in vitro (Fields et al. 1991; Nelson et al. 1990).

The respiratory system must be fully functional from birth. Thus a critical period for prenatal tuning may exist for respiratory circuits. Moreover, fetal breathing movements may be important for development of lung and respiratory muscle (Jansen and Chernick 1991) and/or for tuning central respiratory circuits. NMDA receptors are present in respiratory circuits in neonatal cat (Sàles et al. 1993) and rat (Morin et al. 1989). Exogenous application of NMDA to brain stem-respiratory networks of fetal (unpublished observations) and neonatal rat in vitro (Greer et al. 1991), like adult rat (Connelly et al. 1992) and cat (Feldman et al. 1992; Pierrefiche et al. 1991) in vivo, potently stimulates breathing. Thus NMDA receptors could play an obligatory role to ensure functional neural circuits for breathing at birth. If this is true, mice lacking the NMDA receptor R1 subunit (NMDAR1) gene are likely to have dysfunctions in these circuits.

In vivo comparison of the breathing pattern between mice lacking the NMDAR1 gene and their normal siblings would not test of this hypothesis, however, because afferent input could mask deficits. For example, cats breathe normally after global blockade of NMDA receptors; subsequent removal of afferent signals from the lung reveals a profound disruption in central circuits that manifests behaviorally as apneusis (Feldman et al. 1992; see DISCUSSION). In control cats, deafferentation alone has only minor effects on respiratory pattern (Feldman and Gautier 1976). Thus normal ventilation in NMDAR1 knockout mice would not assure normal central neural circuits. Moreover, preliminary data indicate that these mutant mice have decreased respiratory frequency, have increased incidence of apnea (Poon 1996; Poon et al. 1994, 1996), and die of apparent respiratory failure within 24–48 h of birth (Forrest et al. 1994). The deficiencies could result from multiple developmental deficits, including central neural circuits generating respiratory rhythm or pattern; receptors or central circuits processing sensory signals related to blood gases, pH, or lung volume; or other regulatory systems (e.g., Kutsuwada et al. 1996) that impact on respiratory function. Therefore a preparation eliminating such complications is desirable for examining the functional role of NMDA receptors in central respiratory circuits.

Central mechanisms underlying respiratory rhythm and pattern formation can be studied in isolation of confounding afferent signals using in vitro preparations of brain stem slice or en bloc brain stem spinal cord that maintain respiratory rhythm (Feldman and Smith 1994; Funk and Feldman 1995). Although these preparations do not include all re-
regions of the central respiratory network (e.g., pontine respiratory group), they do contain the basic elements essential for rhythm and pattern formation (Smith et al. 1991; for reviews, see also Duffin et al. 1995; Feldman and Smith 1995; Funk and Feldman 1995). We therefore used these preparations to determine if the development or function of brain stem neural circuits generating respiratory rhythm were affected in NMDAR1-deficient mice at and shortly after (<8 h of) birth. Preliminary results have appeared in abstract form (Feldman et al. 1993).

METHODS

Production and identification of NMDAR1 mutant mice

NMDAR1 mutant mice generously were supplied by Dr. Y. Li and Dr. S. Tonegawa [Howard Hughes Medical Institute at the Center for Learning and Memory and the Department of Biology, Massachusetts Institute of Technology (MIT), Cambridge, MA]. NMDAR1 mutant mice were produced as previously described (Li et al. 1994). Heterozygous females were mated overnight with heterozygous males (both in C57BL6 × 129/Sv background). Females were checked for a vaginal plug the next day, and if present, this day was referred to as embryonic day zero (E0). Plugged females were housed individually and gave birth between E18.5 and E19.5. For determination of mouse genotype, a piece of tail was taken from each animal after induction of anesthesia, immediately before isolation of the neuraxis (see next section). Tail DNA was extracted, and its genotype confirmed as homozygous wild type (+/+), heterozygous (±) or homozygous for the NMDAR1 deletion (mutant; −/−) by PCR analysis with a set of neo1 primers (5'-GCTTGGGTTGGAGGCTATTC and 5'-CAAAGTGAGATGACAGGAGTCC, 280-bp product) and a set of primers to the deleted region of the mutant NMDAR1 allele (5'-TGACCTGTCTCTGCATG and 5'-GCTTCTCCATGTGCGGATG, 550-bp product) (Li et al. 1994).

Brain stem-spinal cord and medullary slice preparations

Experiments were performed on brain stem-spinal cord (en bloc) and medullary slice preparations from mutant and control mice at postnatal day zero (P0). Although performing experiments blind to genotype would have been ideal, screening was essential due to the limited availability and viability of the transgenic mice. Shipment of heterozygous pregnant female mice from MIT to the University of California occasionally resulted in a delay in parturition. Thus two of the six mutant mice were delivered by cesarean section from anesthetized females (2% halothane in 100% O2) 1 or 2 days after their expected birth. All experiments performed on mice delivered by cesarean section were performed blind to genotype. Normal term mutant mice typically were used between 2 and 8 h of birth because absence of milk in the stomach at this time tentatively distinguished mutant from control mice. Genotyping was later performed via analysis of tail DNA (see preceding paragraph). In vitro behavior of preparations from normal term neonates and from mice delivered by cesarean section were not different, thus all data were pooled according to genotype.

Details of the procedures for mice were similar to those described previously (en bloc, Smith and Feldman 1987; slice, Funk et al. 1993, 1994; Smith et al. 1991). Briefly, neonatal mice were anesthetized with ether, decerebrated, and the neuraxis (spinal cord and medulla) isolated by dissection in a bath containing control artificial cerebrospinal fluid (ACSF) [which contained (in mM) 128 NaCl, 3.0 KCl, 1.5 CaCl2, 1.0 MgSO4, 21 NaHCO3, 0.5 NaH2PO4, and 30 d-glucose] equilibrated with 95% O2-5% CO2 at 27–28°C. The neuraxis was either pinned down on silicone elastomer (Sylgard) in the recording chamber or transferred to a vibratome bath for sectioning. Slice preparations were made using a vibratome (Technical Products International, VT 1000) to section the neuraxis serially in the transverse plane starting from the rostral medulla to within 150 μm of the rostral boundary of pre-Bötzinger Complex (pre-BöC). A single transverse slice extending caudally to obex then was cut (400–500 μm thick) and pinned down in the recording chamber. Slices contained the pre-BöC and most of the rostral ventral respiratory group. After initial experimentation, some brain stem spinal cord preparations from mutant mice were subject to the slicing and further experimentation.

En bloc and slice preparations were superfused continuously with ACSF. In slices, but not en bloc preparations, extracellular K+ concentration was raised to 9 mM in the ACSF, perfusing the slices (ACSF) to maintain respiratory network activity (quodd vide Funk et al. 1993). Population respiratory activity was recorded with suction electrodes applied to cut ends of cranial (IX, X, and XII) and spinal nerve roots (C1, C2, C3, C5, T1, and T2) en bloc to cranial nerve XII in slice preparations.

Drug application

Drugs were applied directly to the perfusate bathing the en bloc and slice preparations or applied locally via pressure injection (5–40 psi) from triple-barreled pipettes (8-μm tip diam per barrel) (Funk et al. 1993). Effects of bath-applied 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Research Biochemicals, 0.5–5 μM), a non-NMDA receptor antagonist, and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminemaleate (MK801, Research Biochemicals, 100 μM), an NMDA receptor antagonist, were assessed after a 10-min equilibration period by measuring respiratory frequency and respiratory burst amplitude [amplitude represents the peak of rectified, filtered (Paynter filter, τ = 15 ms) signals of respiratory nerve discharge].

Drugs were applied locally to the ventral surface of the brain stem overlying the ventrolateral medulla (VLM) at the rostrocaudal level of the pre-BöC where rhythm generating circuits are located (Smith et al. 1991), to the ventral surface of the spinal cord at C4 in the en bloc preparations, and over the hypoglossal nucleus in slice preparations. Duration and timing of pressure pulses was controlled by a digital stimulator (MASTER-8, AMPI). Effects of (R,S)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid hydrobromide (AMPA, Research Biochemicals, 200 μM) and NMDA (Sigma, 1 mM) applied over the VLM and AMPA, NMDA, and 2-amino-4-phosphonobutyric acid (AP-4, Sigma, 1–2 mM) applied over C4 were assessed by monitoring respiratory frequency and respiratory burst amplitude.

Whole cell recordings from XII motoneurons

Inspiratory synaptic activity in hypoglossal (XII) motoneurons and neuronal responses to locally applied AMPA and NMDA were monitored in slices using “blind” whole cell recording techniques (Blanton et al. 1989). CURRENTS were recorded from XII motoneurons 180–200 μm below the slice surface with an Axopatch 1-D amplifier (Axon Instruments; 2–5 kHz low-pass filter). Electrodes (3.5–4.5 MΩ, 1.5- to 2-μm tip size) contained K+-glutamate solution consisting of (in mM) 120 K+-glutamate, 5 NaCl, 1 CaCl2, 1 MgCl2, 10 N-2-hydroxyethylpiperazone-N’-2-ethanesulfonic acid (Sigma), 10.1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, tetra K+ salt (Sigma), 2 ATP (Mg2+ salt), Lucifer yellow (dipotassium salt, 1%; Sigma), pH adjusted to 7.3 using KOH, and coated with Sigmacote to reduce electrode capacitance. Seals ranged from 1.5 to 2.5 GΩ.

Series resistance and whole cell capacitance were estimated using brief voltage pulses (100 Hz, −10 mV, 3.0 ms). Maximum possible series resistance compensation was used (≥80%).
input resistance \( (R_{\text{in}}) \) was calculated at \(-60\, \text{mV} \) holding potential from the current responses to 10-mV hyperpolarizing voltage steps (300-ms duration). Postsynaptic responses of XII motoneurons to NMDA (1.0 mM) and AMPA (200 \( \mu \)M) were examined in control animals after motoneurons were isolated synthetically by blocking Na\(^+\)-dependent action potentials with tetrodotoxin (TTX, Sigma, 0.5–1.0 \( \mu \)M). Mutant motoneurons were not synaptically isolated before NMDA and AMPA application to maximize the number of recordings from identified mutant respiratory motoneurons. TTX abolishes network activity and precludes functional identification of respiratory neurons. In addition, TTX was not applied to mutant preparations to increase the sensitivity of the cellular tests because sensitive presynaptic neurons could contribute to a response.

Data analysis

Intracellular signals were recorded on videocassette via pulse code modulation \((\text{Vetter Instruments})\). Segments of data were digitized (2–10 kHz/channel) with a VAX 3200 computer, and signal amplitudes (peak synaptic current), integral (total charge transfer), and time course were analyzed with a customized version of a commercial time signal analysis software package \((\text{RS/PROBE, BBN Laboratories})\). Details of the analysis are given in RESULTS. Statistical values are reported as means \( \pm \) SD. Differences between means were tested with analysis of variance \((\text{ANOVA})\) and Bonferroni simultaneous confidence intervals for multiple comparisons \((\text{RS/PROBE, BBN Laboratories})\). Values of \( P < 0.05 \) were assumed significant.

RESULTS

We compared rhythmic activity generated by neuraxes isolated from 15 control (+/+) and 6 mutant animals homozygous for the NMDARI gene deletion \((-/-)\). Mutant mice were identified by the presence of a 280-bp band \((\text{neomycin-resistant gene})\) and absence of the 500-bp band \((\text{NMDARI gene})\) on an agarose gel of polymerase chain reaction \((\text{PCR})\). CNQX \((\text{control, n} = 4; \text{mutant, n} = 4; \text{data not shown})\), whereas AP-4 markedly increased the frequency of respiratory oscillations in control preparations \((n = 7)\). A sixfold increase in the duration of NMDA application to 30 s had no effect on preparations from mutant mice \((n = 5; \text{Fig. 2} A)\). Respiratory frequency averaged 16.5 \( \pm \) 9.5 cycles per minute for the 10 cycles before NMDA application and 14.9 \( \pm \) 8.3 cycles per minute during drug application. As described previously for control brain stem spinal cord \((\text{Figs. 2 and 7 of Greer et al. 1991})\) and medullary slice preparations \((\text{Figs. 3, 8, 9, and 12 of Funk et al. 1993})\) from neonatal rat, bath application of MK801 blocks the effects of exogenously applied NMDA in control mice. In this study, MK-801 did not affect ongoing rhythm in control \((n = 7)\) or mutant mice \((n = 2)\).

We examined the effects of the non-NMDA receptor agonist, AMPA, on rhythm generating networks to confirm that the failure of mutant mice to respond to NMDA was due to disruption of NMDA receptors rather than a more general disruption of glutamate-mediated synaptic transmission or an inability of mutant respiratory networks to increase activity. Five-s application of 200 \( \mu \)M AMPA over the VLM in the same site where NMDA had been applied previously produced a reversible 7.0 \( \pm \) 2.0-fold increase in respiratory frequency in control \((n = 2)\) and a 7.6 \( \pm \) 4.8-fold increase in mutant mice \((n = 4; \text{Fig. 2} A)\). AMPA-mediated responses were blocked in a dose-dependent manner by bath-applied CNQX \((\text{control, n} = 2; \text{mutant, n} = 2)\). CNQX also blocked respiratory oscillations at concentration \(>2.0\, \mu\text{M} \) \((\text{control, n} = 2; \text{mutant, n} = 2)\).

Motor output from respiratory motor neuron pools in mutant mice also was unaffected by NMDA. NMDA applied locally over \(C_4\) for \(<1\, \text{s}\) produced sustained tonic discharge in \(C_4\) of preparations from control mice \((n = 9; \text{Fig. 2} B)\). NMDA application for 20–60 s had no effect on \(C_4\) population activity of mutant mice \((n = 5; \text{Fig. 2} B)\). In contrast, effects of AMPA and AP-4 on population activity were similar in control and mutant mice. AMPA produced sustained tonic discharge in cervical nerves \(C_1-C_4\) \((\text{control, n} = 2; \text{mutant, n} = 4; \text{data not shown})\), whereas AP-4 markedly reduced the amplitude of \(C_4\) population inspiratory output \((\text{control, n} = 2; \text{mutant, n} = 2; \text{data not shown})\).

To address the possibility that NMDA effects were sub-threshold for action potential generation, we applied AMPA \((200\, \mu\text{M})\) and NMDA \((1.0\, \mu\text{M})\) to inspiratory-modulated XII motoneurons under whole cell recording conditions. AMPA, applied for 0.5–1.0 s, induced strong inward currents in control \((n = 5)\) and mutant motoneurons \((n = 4; \text{Fig. 2} B)\). NMDA, applied for 0.5–1.0 s, induced current only in motoneurons from control mice \((n = 4; \text{Fig. 2} B)\). Forty-fold increases in duration of NMDA application \((20\, \text{s})\) induced no current or change in potential in motoneurons from mutant mice. In spite of the difference in glutamate receptor complement, inspiratory synaptic inputs to XII motoneurons were similar in control \((n = 7)\) and mutant mice.
**Discussion**

We sought to determine the obligatory role of genomic NMDA receptors on the in utero development and postnatal function of central circuits underlying respiratory rhythm. We conclude that respiratory rhythm generating circuits can develop in the absence of genomic NMDA receptors. Before elaborating on this conclusion, we need to address whether our results in vitro are obvious based on the observations that intact mutant mice (Forrest et al. 1994; Li et al. 1994) breathe at birth.

Mutant mice initially were reported to have “no obvious” distinguishing features at birth or for the first 1–2 postnatal hours (Forrest et al. 1994) and skin that “is flushed and has a reddish coloration much like their wild-type littermates” (Li et al. 1994). Thus while “cardiovascular and respiratory systems are functional” at birth (Li et al. 1994), the inference that central circuits are normal is incorrect. Central deficits in respiratory circuits may be masked by normal afferent signals such that breathing patterns of control animals and those with central deficits are indistinguishable (Feldman and Gautier 1976; Fig. 2 in Feldman et al. 1992) (see Introduction). Moreover, preliminary data suggest that within 7 h after birth, mutant mice have respiratory deficits observable as decreased breathing frequency and increased incidence of apnea (Poon 1996; Poon et al. 1994, 1996).

In vitro brainstem-spinal cord and medullary slice preparations, although not without limitations (for discussion, quod vide Berger 1990; Feldman and Smith 1995; Funk et al. 1993), facilitate analysis of mechanisms underlying endogenous generation of respiratory rhythm. In addition, they isolate the medullary respiratory rhythm generating network from potentially confounding signals that may contribute to deficits in respiratory behavior of mutant mice in vivo, for example, pontine and peripheral NMDA-mediated inputs (quod vide McCrimmon et al. 1994). These inputs may underlie respiratory perturbations during acute NMDA receptor block in kitten (Schweitzer et al. 1990; Sica et al. 1992), cat (Feldman et al. 1992; Foutz et al. 1988, 1989), and rat (Connelly et al. 1992; Monteau et al. 1990).

We found that the medullary respiratory circuits of mutant mice produce a rhythm in vitro virtually indistinguishable from that of control mice. Only one notable difference in the rhythmic respiratory activity of control and mutant preparations in vitro, that related to NMDA, was apparent. Thus medullary rhythm generating networks, motoneuron population activity, and respiratory motoneuron currents in prepara-
ity-dependent reorganization of central topographic maps not indicate that malformation of central circuits underlying NMDA-mediated synaptic transmission is required during is consistent with respiratory failure as the cause of death 1 et al. 1990) and medullary slice preparations (Funk et al. irregular during the first postnatal day, with duration and cellular and system levels the absence of functional NMDA death in the NMDAR1 mutant mice because hand-feeding mutation of either class of ionotropic glutamate receptors 1996) may contribute to the early morbidity in the NMDAR1 METHODS). 1 ± 2 days of birth. Increased viability after hand feeding of applied to block the inspiratory currents in control preparations only (see respiratory motor circuits because mutant mice die within 10.220.33.3 on November 2, 2016 http://jn.physiology.org/ Downloaded from

![Diagram](https://example.com/diagram.png)

**FIG. 2.** Local application of glutamate receptor agonists over the ventrolateral medulla and spinal cord of brain stem-spinal cord preparations and XII motor nucleus of medullary slice preparations. A: application of 200 µM (R,S)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid hydrobromide (AMPA) and 1.0 mM N-methyl-D-aspartate (NMDA) to the ventrolateral medulla at the level of the pre-Bötz in control and mutant mice. Note that sixfold increases in duration of NMDA application were without effect on mutant preparations. B: local application of 1.0 mM NMDA over the ventral spinal cord at C4 for 2 s evoked tonic discharge from the C4 nerve in control preparations, but 20 s of NMDA was without effect on C4 discharge in mutant preparations. C: local application of 200 µM AMPA (0.5 s) and 1.0 mM NMDA (0.5 s) over the hypoglossal motor nucleus induced an inward current of ~100 pA in a control XII motoneuron recorded under whole cell voltage-clamp conditions. AMPA produced a similar current in a XII motoneuron from a mutant mouse, but a 20-fold increase in duration of NMDA application had no effect on membrane current. AMPA and NMDA responses are from the same cell. Periodic, 500- to 700-ms, ~100-pA currents superimposed on the current traces from the mutant neuron represent inspiratory synaptic currents. Tetrodotoxin was bath-applied to block the inspiratory currents in control preparations only (see METHODS).

... from control mice were modulated strongly by activation of either class of ionotropic glutamate receptors (NMDA and non-NMDA). In contrast, preparations from mutant mice were modulated by non-NMDA receptors only; they were unaffected completely by NMDA, confirming at cellular and system levels the absence of functional NMDA receptors. Thus in early life, NMDA receptor-mediated synaptic transmission is not required for rhythm generation in the neonatal brain stem spinal cord (Greer et al. 1991; Liu et al. 1990) and medullary slice preparations (Funk et al. 1994; Smith et al. 1991), in vitro, or transmission of respiratory drive to (pre-) motoneurons.

We had considered that NMDA receptors were essential for development of respiratory rhythm generating and motor circuits prior to birth based on several observations. 1) NMDA-mediated synaptic transmission is required during critical developmental periods (pre- and postnatal) for activity-dependent reorganization of central topographic maps (Bear et al. 1990; Clne et al. 1987; Hahn et al. 1991; Kleinschmidt et al. 1987; Li et al. 1994). 2) Normal development of spinal motoneurons in rats depends critically on NMDA receptors between P7 and P21 (Kalb and Hockfield 1988, 1990, 1992). 3) Brain stem neurons of neonatal rat (Morin et al. 1989) and putative respiratory neurons and motoneurons in neonatal cat (Salès et al. 1993) and adult rat (Petralia et al. 1994) express high levels of NMDA receptors. 4) NMDA is a powerful modulator of respiratory rhythm in embryonic (unpublished observations) and neonatal rodents (Greer et al. 1991) as well as adult rats (Connelly et al. 1992). 5) Central respiratory circuits produce high levels of spontaneous activity before birth (Jansen and Chernick 1991). 6) The respiratory system must be fully and continuously functional from birth. Thus a critical period for prenatal tuning, as seen in the lateral geniculate nucleus (Shatz 1990a), may exist for respiratory circuits.

Our results suggest that NMDA receptors are not required for the prenatal development of the basic circuits underlying respiratory rhythm generation and drive transmission to motoneurons innervating respiratory muscles. It is important to point out, however, that the plasticity of the neonatal nervous system is an important factor in interpreting the development of knockout mice. Thus the possibility that undefined mechanisms compensated for the deletion of NMDAR1 (Conquet et al. 1994; Forrest et al. 1994) and that NMDAR1 normally plays a role in activity-dependent development of respiratory rhythm and pattern forming circuits must be considered but were beyond the scope of the present study to explore. We note that this concern is not unique to this study and confounds the interpretation of genomic deletion experiments in general, whether or not there is a phenotype.

Critical periods for NMDA-mediated activity-dependent development occur postnatally in many systems (e.g., visual cortex, Shatz 1990b; trigeminal system, Li et al. 1994; spinal cord motoneurons, Kalb and Hockfield 1990). Respiratory networks continue to develop after birth (Haddad et al. 1994). Thus there may be postnatal tuning in the respiratory system that requires NMDA receptors. We did not address the role of NMDA receptors in postnatal development of respiratory motor circuits because mutant mice die within 1–2 days of birth. Increased viability after hand feeding of mutant mice lacking the NMDA receptor ε2 subunit suggests that a deficit in the suckling response (Kutsuwada et al. 1996) may contribute to the early morbidity in the NMDAR1 mutant mice. Unlike ε2 mutant mice, however, NMDAR1 mutant mice lack all functional NMDA receptors. Furthermore, malnutrition does not appear to be the main cause of death in the NMDAR1 mutant mice because hand-feeding produces only minor increases in longevity (Y. Li, personal communication).

Respiration in mutant mice in vivo becomes increasingly irregular during the first postnatal day, with duration and frequency of apneas increasing (Poon et al. 1994, 1996). As yet, whether these respiratory irregularities result directly from the NMDAR1 deletion on respiratory-related structures or secondary to dysfunctions in other physiological systems has not been established. Lung pathology of the mutant mice is consistent with respiratory failure as the cause of death (Forrest et al. 1994). However, lung pathology per se does not indicate that malformation of central circuits underlying...
Rhythm generation contributes to the early death of mutant mice. Histological and histochemical analysis of brain stem regions important in respiratory regulation does not reveal structural differences between control and mutant mice (Forrest et al. 1994). Similarly, our results suggest that primary deficits of the basic central respiratory rhythm generating networks do not contribute to early death because the neuraxes from mutant preparations, when isolated from afferent inputs, generate a rhythmic output similar to controls. 

Even though rhythm generating circuits function properly, the respiratory system as a whole may be dysfunctional. NMDA receptors may be necessary for proper development or function of afferent inputs from mechanosensory, chemosensory, or pontine respiratory areas (quod vide McCormon et al. 1994) important in regulation but not generation of respiratory rhythm. Absence of NMDA-mediated inputs may compromise the ability of the mutant respiratory system to maintain homeostasis and therefore could result in hypoxemia, hypercapnia, and/or acidosis precipitating death. This suggestion is consistent with the findings that the respiratory frequency of mutant mice in vivo is depressed relative to control mice (Poon et al. 1994), that block of NMDA receptors with MK801 in vivo disrupts respiratory rhythm in kitten (Schweitzer et al. 1990; Sica et al. 1992), but MK801 has no effect on respiratory activity in vitro, i.e., deafferented, rat preparations (Funk et al. 1993; Greer et al. 1991), and that synaptic transmission within the nucleus of the solitary tract, a region important in processing respiratory-related afferent information, is disrupted in NMDAR1 knockout mice (Poon et al. 1996). Alternatively, NMDA receptors may be important in initiation of fetal breathing rhythm. If fetal breathing is important for lung and respiratory muscle development (Jansen and Chernick 1991), a reduced incidence of fetal breathing in mutant mice could contribute to early postnatal death.

In summary, the respiratory system must develop in utero into a fully functional motor system by birth. In spite of the profound effects on breathing of NMDA receptor blockade and exogenous application of NMDA to brain stem respiratory circuits and the critical role of NMDA-mediated synaptic transmission in development of many neural systems, NMDA receptors are not essential for the development of basic respiratory rhythm and motor circuits before birth. Thus genetic cues direct prenatal respiratory circuit formation, or NMDA-independent activity is responsible for refinement of respiratory circuits during the prenatal period (at least in mutant mice). This does not preclude a role of NMDA receptors in such refinement in normal mice. NMDA-independent forms of synaptic plasticity occur in the hippocampus (quod vide, Nicoll et al. 1988) and cerebellum (Conquet et al. 1994). Further application of the combined transgenic/in vitro approach described in this study should prove useful in distinguishing between the above possibilities.

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