NMDA Receptor Activity *In Utero* Averts Respiratory Depression and Anomalous Long-Term Depression in Newborn Mice

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Mutant mice lacking NMDA receptor 1 subunit (NR1) showed marked depression of respiratory and suckling activities *in vivo* and overexpression of synaptic long-term depression (LTD) in a brainstem cardiorespiratory-related region (nucleus tractus solitarius) *in vitro*. Pharmacological blockade of NMDA receptors in normal newborn mice mimicked the depression in suckling activity but not respiratory depression *in vivo* or brainstem LTD *in vitro*. Results at the behavioral and cellular levels demonstrate that NMDA receptor deficiency during prenatal development may unleash an anomalous form of NMDA receptor-independent LTD along with life-threatening respiratory depression consequences in the newborn. These findings raise the specter of cardiorespiratory dysregulation with increased risks of morbidity and mortality in the infant as a result of premature births or genetic or drug-induced NMDA receptor antagonism during pregnancy.

Key words: NMDA receptor; NR1 knock-out; synaptic long-term depression; respiratory failure; nucleus tractus solitarius; prenatal neural development; newborn mice

NMDA receptor plays an important role in many cellular functions or dysfunctions of the mammalian brain, including synaptic plasticity (Collingridge and Bliss, 1995; Bear, 1996), neural development (Hahm et al., 1991; Scheetz and Constantine-Paton, 1994), excitotoxicity (Rothman and Olney, 1995), and antiapoptosis (Ikonomidou et al., 1999). It has also been implicated at the behavioral level as a key factor in certain cognitive functions such as learning and memory (Kleinschmidt et al., 1987; Feldman et al., 1996; Tsien et al., 1996; Manahan-Vaughan and Braunewell, 1999; Tang et al., 1999) as well as epileptic seizure (Loscher, 1998) and other neurological disorders (Kornhuber and Weller, 1997; Mohn et al., 1999). By contrast, the role of NMDA receptor activity in prenatal neural development is poorly understood because of the difficulties of blocking NMDA receptors specifically in the fetus and not the maternal milieu.

A genetic approach offers a unique opportunity to study the effects of NMDA receptor malfunctioning in the fetus on prenatal neural development. Recent advances with molecular cloning have identified two families of subunits in the NMDA receptor (NMDAR) complex: the NMDAR1 family, which has only one member, NR1, and the NMDAR2 family, which has four members (for review, see Kutsuwada et al., 1992; Monyer et al., 1992; Nakanishi, 1992). Of these, NR1 is essential for NMDA receptor activity and is ubiquitous in the brain, whereas the other subunits provide functional diversity of the receptor complex and are more topographic.

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In the present investigation, we provide evidence that links the absence of NMDA receptor activity in the prenatal period to the postnatal evolution of catastrophic respiratory failure and an accompanying anomalous long-term depression (LTD) in the nucleus tractus solitarius (NTS), a brainstem region important for cardiorespiratory regulation. Thus, NMDA receptor activity during prenatal development *in utero* is crucial for subsequent neonatal survival. These findings raise the specter of cardiorespiratory dysregulation with increased risk of infant morbidity and mortality (Shannon et al., 1977; Schwartz et al., 1988; Eichenwald et al., 1997) as a result of premature births or genetic defects and warrant some cautions on exposure of the fetus to NMDA-antagonistic anesthetic or analgesic drugs (Orser et al., 1997) and certain substances of abuse (Spuhler-Phillips et al., 1997; Deutsch et al., 1998) during pregnancy.

MATERIALS AND METHODS

Genotyping. NR1 $^{-/-}$ mutant mice were produced as previously described (Li et al., 1994). Heterozygous females in BALB/c background were mated overnight with heterozygous males in either C57BL/6 or 129/Sv background. The parents had been back-crossed to C57BL/6 or BALB/c for at least six generations to remove most of the 129 strain phenotype. The day after mating the females were checked for a vaginal plug, and, if present, this would be referred to as embryonic day 0 (E0). Plugged

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females were housed individually, and they usually gave birth between E18.5 and E19.5. Approximately 2–8 hr after birth the pups were transferred from their nests to an incubator in preparation for the experiment.

NR1 $^{-/-}$ mutant mice were initially identified based on characteristic symptoms such as the absence of milk in the stomach. After the experiment, all animals were anesthetized on ice, and a piece of tail was taken from each animal and stored in a freezer. Tail DNA was extracted and genotyped by PCR analysis with a set of neo1 primers (5′-GCTTGGGTGGAGAGGCTATTC and 5′-CAAGGTGAGATGACAGGAGATC, 280 bp PCR product) and a set of primers to the deleted region of the mutant NMDAR1 allele (5′-TGACCCTGTCCTCTGCCATG and 5′-GCTTCTCCATGTGCCGGTAC, 550 bp PCR product).

Some normal (wild-type and heterozygous) mice were inadvertently identified as *NR1* $^{-/-}$ mutants during the experiment because they apparently had not been fed by the mothers before the experiment began. The true genotypes of all animals were verified after the experiment using PCR analysis.

In vivo *studies.* Normal and *NR1* $^{-/-}$ mutant mice were incubated at 34°C in a moist and ventilated chamber before the experiment. Respiration was monitored by means of a miniature plethysmograph (Depledge, 1985). The animal was placed in a two-compartment chamber in a head-out position, with the head and body compartments being separated by a latex diaphragm sealed around the animal's neck. Pressure changes in the body compartment were detected by a sensitive pressure transducer, and the resulting signal was amplified, digitized at 700 Hz, and continuously displayed on a computer monitor. The pressure (equivalent to volume) signal was calibrated at the beginning and end of the experiment by momentarily injecting into the body compartment 0.1 ml of air from a microsyringe; a good seal between the two compartments was indicated by a sustained elevated pressure, which was relieved by opening to the atmosphere through a three-way stopcock. The effects of changes in temperature and humidity in the chamber during the relatively brief experimental period were neglected.

Respiratory chemoreflex was assessed by exposing the animal to air enriched with 5% CO_2 for ~10 min and recording the steady-state respiratory movements for 2 min. Pharmacological agents were injected subcutaneously by using a Hamilton syringe, and their effects on suckling and respiration were examined after a stabilization period of ~30 min. Suckling reflex was induced by a gentle mechanical stimulation of the oral cavity using a fine catheter with a blunt tip, which led to a sequence of repetitive jaw opening–closing reactions. Suckling rhythm was registered as the number of jaw openings in the sequence induced over a 30 sec stimulation period.

In vitro *studies.* Synaptic transmission in rodent NTS was studied by using a brainstem slice preparation as described previously (Zhou et al., 1997; Zhou and Poon, 2000). Newborn mice were killed within 10 hr after birth by decapitation under metofane anesthesia, and their brains were rapidly removed and placed in chilled artificial CSF (ACSF) saturated with carbogen (95% O_2 and 5% CO_2) and containing (in mM): NaCl, 130; KCl, 5.4; KH_2PO_4, 0.8; $NaHCO_3$, 26; glucose, 30; $MgCl_2$, 1; and $CaCl_2$, 2. Transverse brainstem slices (400 μm) were cut using a vibratome at a level around the area postrema. After stabilization at room temperature (22–25°C) in ACSF for at least 1 hr, the brainstem slice was transferred to a recording chamber, stabilized under a nylon mesh, and submerged and continuously superfused with ACSF at 32°C. In all brainstem slices, bicuculline (10–20 μM, dissolved in DMSO) was added to the perfusate to suppress possible inhibitory synaptic transmission through $GABA_A$ receptors. A monopolar tungsten electrode with an ultrafine tip (FHC, Bowdoinham, ME; 5 MΩ, 2–4 μm tip diameter) was positioned by means of a micromanipulator at the tractus solitarius for discrete electrical stimulation (pulse width, 0.1 msec; frequency, 5 Hz; intensity, 3–10 V) of the primary afferent fibers. Excitatory postsynaptic response was elicited by a single electrical impulse. Whole-cell patch recordings of activity in NTS cells were obtained using a low-noise amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) with a fluid-filled micropipette (resistance, 4–10 MΩ) containing (in mM): KCl, 130, $CaCl_2$, 0.4, EGTA, 1.1; $MgCl_2$, 1; NaCl, 5; potassium HEPES, 10; Mg^{2+}-ATP, 2; and Na^{2+}-GTP, 0.1, pH 7.2–7.3. Neurons were approached blind, and a gigaohm seal was formed by a gentle suction of the micropipette. Once in the whole-cell mode, a voltage clamp was applied (holding potential, −70 mV), and a stabilization period of ~10 min was allowed before recordings began.

RESULTS

Relapse of *NR1* $^{-/-}$ mutant mice after birth

The heterozygous mutants had no phenotypic abnormalities compared with the wild-type animals (Li et al., 1994). However, physical examination of the *NR1* $^{-/-}$ mutants revealed two distinct phases in the postnatal period: a latent phase lasting 10–18 hr after birth, during which the mutant animals appeared to be healthy with flushed skins as their normal (heterozygous and wild-type) littermates, and a subsequent morbid phase (0.5–3 hr duration) of acute respiratory failure characterized by increasing cyanosis and decreasing motility. All *NR1* $^{-/-}$ mutants died within 12–20 hr after birth, in contrast to their normal littermates, which survived under similar environments.

NR1 $^{-/-}$ mutant neonates suffer from severe respiratory depression

Plethysmographic measurements (Fig. 1*A*) during the latent phase revealed marked depression of the respiratory rhythm in the *NR1* $^{-/-}$ mice compared with normal, with increased incidence and duration of apnea (Table 1). Thus, the respiratory failure during the morbid phase was probably the culmination of a sustained respiratory depression that developed at or soon after birth. Exposure of the mutants to air enriched with 5% CO_2 restored a regular respiratory rhythm (Fig. 1*A*, Table 1). Both normal and mutant mice showed an increase in respiratory frequency as well as tidal volume in response to the CO_2 challenge. Despite this, total ventilation of the *NR1* $^{-/-}$ mutants remained depressed (compared with normal) after CO_2 stimulation (Fig. 1*B*). The hypopnea is unlikely a consequence of decreased body metabolism or hypothermia, because both normal and mutant animals had similar body weights at birth (Li et al., 1994) and were incubated in a similar manner. Indeed, the mutant animals appeared to be more motile than the normal animals during measurement, and their breathing pattern tended to be more irregular (Table 1). Although arterial CO_2 tension could not be measured directly in these small animals, the hypoventilation suggests a decreased CO_2 chemoreflex in the mutants, i.e., with decreased CO_2 sensitivity and/or threshold (Cunningham et al., 1986).

To examine whether the respiratory depression in the mutant mice was secondary to feeding disorders, we monitored respiratory activity in a group of *NR1* $^{-/-}$ mutants and some littermates that had been misclassified as *NR1* $^{-/-}$ mutants (because of the absence of milk in their stomachs) at the time of the experiment but finally proved otherwise by genotypic analysis. These unfed normal mice were able to survive beyond the 20 hr maximum life span of their mutant littermates (Fig. 1*C*). Moreover, respiratory frequency in these normal animals remained significantly higher than their mutant littermates throughout the latent phase. Therefore, the respiratory depression in the *NR1* $^{-/-}$ mutants was not caused by malnutrition alone but was probably exacerbated by it to precipitate the terminal respiratory failure. The significance of respiratory failure as a primary cause for the early deaths of the mutants is also supported by the recent finding that *NR1* $^{-/-}$ mutant mice with artificial respiration treatment survived significantly longer than those without (Kolandaivelu and Poon, 1998).

Anomalous LTD in brainstem of *NR1* $^{-/-}$ neonates

Recent *in vitro* studies in rat brainstem slices (Zhou et al., 1997) showed that approximately half of the neurons in the medial and commissural NTS (type II neurons) exhibited LTD after low-frequency afferent stimulation, whereas the remaining (type I)

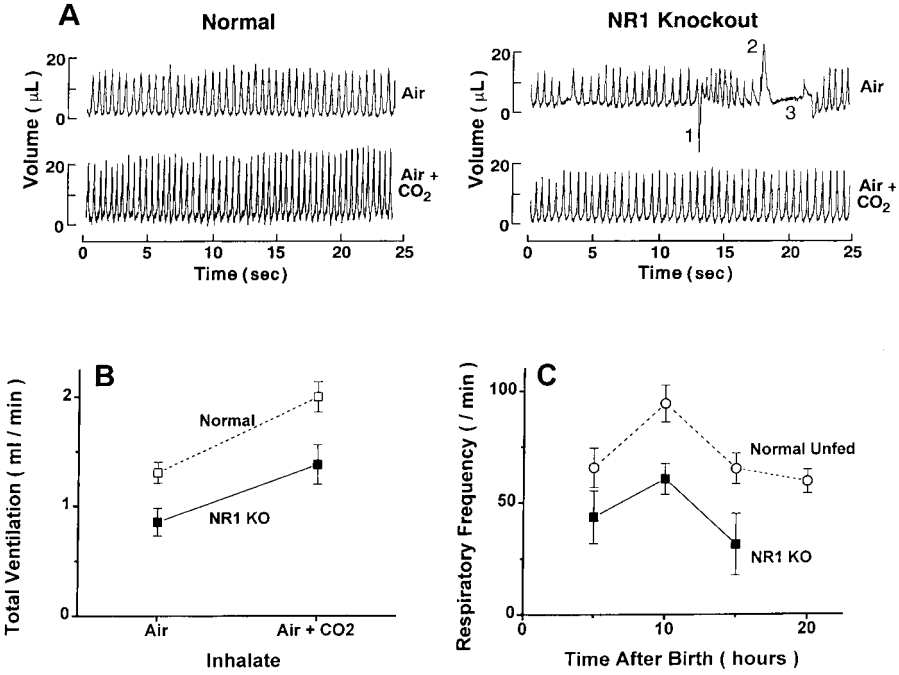

Figure 1. Respiratory depression in NMDAR1 gene knock-out ($NR1^{-/-}$) newborn mice on P0. *A*, Tracings of respiratory volume during quiet breathing with room air (*top*) and with 5% CO_2 in air (*bottom*) in normal and $NR1^{-/-}$ mutant mice during the latent phase after birth. Inspiration is *upward*. Note the slow and irregular breathing pattern of the mutant in room air characterized by frequent coughing (*1*), sighing (*2*), and apnea (*3*). Also note the restoration of regular breathing pattern with CO_2 stimulation. *B*, Total ventilation (respiratory frequency × tidal volume) was depressed in $NR1^{-/-}$ mutant mice (mean ± SE; n = 9) compared with normal littermates (n = 18) breathing room air or 5% CO_2 in air. Recordings were obtained in all animals within the first 10 hr after birth. *C*, Respiratory frequency was significantly decreased in mutant mice (n = 6) compared with normal littermates (n = 16) that were unfed. Measurements were made at 5 hr intervals until the animal died. All mutant mice died within 20 hr after birth, whereas the unfed normal littermates survived longer. Timing for the initial measurement (at the 5th hr) was estimated based on the elapsed time when the newborns were found and the time needed to prepare the animals for experimental studies. *KO*, Knock-out.

Table 1. Apneic and body movement episodes in normal and $NR1^{-/-}$ mutant newborn mice on PO breathing room air or air enriched with 5% CO_2

	Apnea		Body movement	
	Episodes/min	Duration (sec/min)	Episodes/min	Duration (sec/min)
Normal (n = 18)				
Air	1.2 ± 1.0	2.0 ± 1.0	0.6 ± 0.2	1.4 ± 0.6
CO_2	0.3 ± 0.5*	0.5 ± 0.7*	0.3 ± 0.3*	0.6 ± 0.5*
$NR1^{-/-}$ mutant (n = 9)				
Air	4.2 ± 1.0**	9.2 ± 2.5**	1.9 ± 0.3**	3.6 ± 1.0**
CO_2	0.9 ± 0.5*	1.2 ± 0.7*	0.5 ± 0.3*	0.8 ± 0.4*

Values are means ± SD. Data for each animal were obtained as the average values over a 15 min period. Apneic episode is defined as any end-expiratory pause >1 sec.

*Significantly different from air breathing.

**Significantly different from normal.

neurons did not. Figure 2*A* demonstrates such activity-dependent LTD of NTS neurons in brainstem slices from normal newborn mice, with similar proportions of type I and type II neurons. Surprisingly, LTD was found in NTS neurons of all $NR1^{-/-}$ mutants (Fig. 2*B*), in contrast to the abolition of hippocampal LTD in mutant animals with similar genetic defects (Kutsuwada et al., 1996). Indeed, the LTD was expressed even more robustly in NTS of the $NR1^{-/-}$ mutants than normal mice (100 vs ~50% of the cases; $p < 0.01$; n = 8, binomial distribution).

Abnormalities in $NR1^{-/-}$ neonates are absent in normal littermates with acute NMDA receptor blockade

To examine whether the brainstem LTD in the $NR1^{-/-}$ mutants resulted from the lack of NMDA receptor activity per se, we repeated the experiment in normal mouse brainstem slices perfused with the NMDA receptor antagonist D-AP-5 (50 μM). Unlike the NTS of the $NR1^{-/-}$ mutants, NMDA receptor-independent LTD was not induced in NTS neurons in any of these pretreated brainstem slices (Fig. 3*A*). This result is in agreement with previous studies (Zhou et al., 1997), which showed that acute pharmacological blockade of NMDA receptors abolished LTD in rat NTS. Taken together, these findings suggest that the absence of NMDA receptor activity in the $NR1^{-/-}$ mutants during the prenatal period may lead to an NMDA receptor-independent form of LTD in NTS of the neonate. Furthermore, the factors responsible for the expression of such NMDA receptor-independent LTD in NTS are unique to the $NR1^{-/-}$ mutant animals and are absent in the normal mice.

Because brainstem LTD in normal mice was abolished (instead of enhanced) by D-AP-5, it is unlikely that the respiratory depression in the $NR1^{-/-}$ mutants was caused by an absence of NMDA receptor activity per se in the postnatal period. To confirm this, we injected normal newborn mice with the noncompetitive NMDA receptor antagonist dizocilpine (MK-801; 3 mg/kg, s.c.) or an equal volume (~10 μl) of saline as control. Animals treated with MK-801 showed a marked depression of the suckling rhythm similar to that found in the $NR1^{-/-}$ mutants (Fig. 3*B*) and NMDAR2B mutants (Kutsuwada et al., 1996). By contrast, respiratory frequency was not significantly different between the MK-801 group and control group before and after drug administration

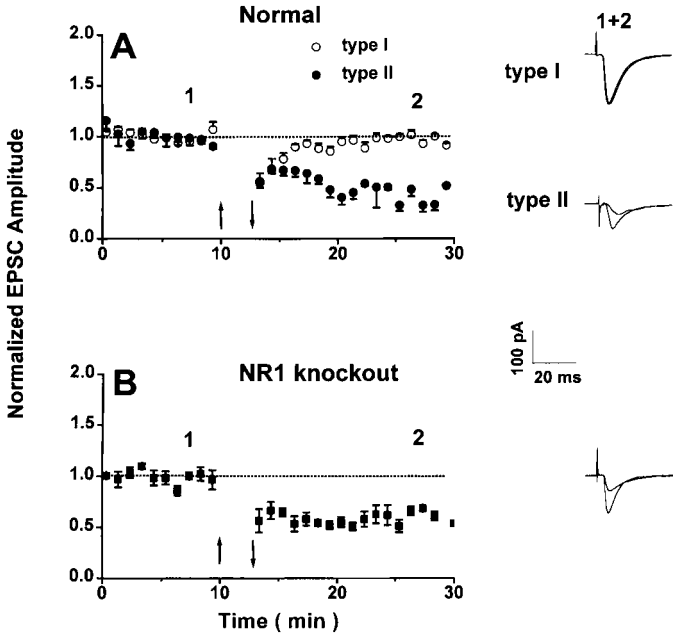

Figure 2. Long-term synaptic depression in NTS of normal and *NR1* $^{-/-}$ mice on P0. *A*, In brainstem slices (300–400 μm thick) from normal neonatal mice, low-frequency stimulation (LFS; 5 Hz, 3 min) of afferent fibers in the tractus solitarius elicited LTD in type II neurons (means ± SE; $n = 4$) but not in type I neurons ($n = 5$) in NTS. Synaptic strength is indicated by amplitude of EPSC evoked by an electrical impulse (0.1 msec, 3–10 V) delivered every 20 sec. *Arrows* indicate beginning and end of LFS [the phasic depression of synaptic transmission that normally occurred during LFS (Zhou et al., 1997; Zhou and Poon, 2000) is not shown]. Episodic data for each cell were averaged every minute and were normalized by the average response during the control period before LFS. *Insets*, Examples of averaged EPSCs (15 episodes) in the periods before (*1*) and after the induction of LTD (*2*) in each cell type. *B*, In *NR1* $^{-/-}$ mutant mice ($n = 8$), LFS elicited NMDA receptor-independent LTD robustly in all NTS cells tested. In *A* and *B*, each neuron was from a different animal.

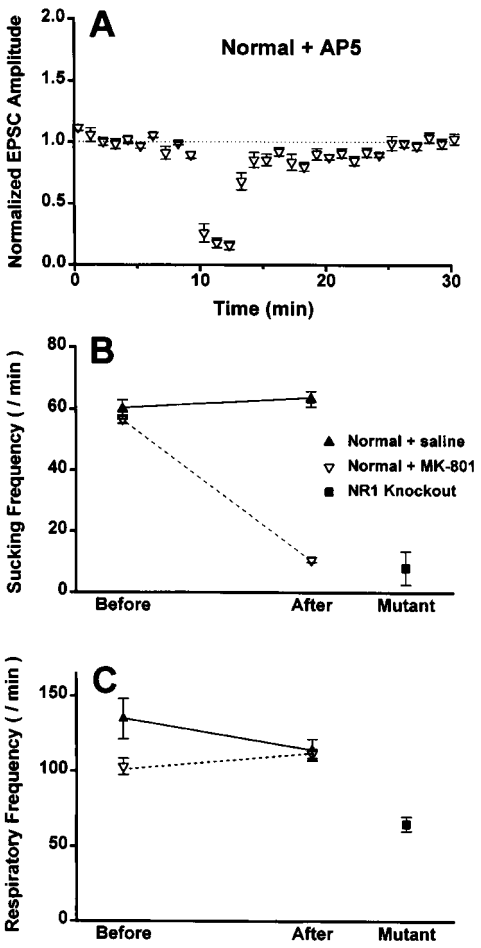

Figure 3. Effects of acute pharmacological blockade of NMDA receptor activity on physiological functions of newborn mice on P0 studied *in vitro* and *in vivo*. *A*, Brainstem slices ($n = 4$) treated with the NMDA receptor antagonist D-AP-5 did not demonstrate LTD in NTS when subjected to the same stimuli [low-frequency stimulation (LFS)] as shown in Figure 2, although the phasic synaptic accommodation during LFS (10th–13th min) remained intact. *B*, Suckling rhythm of normal mice ($n = 16$) was markedly depressed by the NMDA receptor antagonist MK-801 administered subcutaneously. The depressant effect of MK-801 is similar to that found in *NR1* $^{-/-}$ mutant mice ($n = 21$). Similar application of saline had no effect in the control group ($n = 12$). *C*, Respiratory rhythm of normal mice at P0 was not affected by MK-801 ($n = 7$) or saline ($n = 7$). This is in contrast to the depressed respiratory rhythm in the *NR1* $^{-/-}$ mutant mice ($n = 36$; also see Fig. 1).

(Fig. 3*C*). Thus, NMDA receptor activity in the postnatal period is integral to the sucking reflex but not requisite for respiratory rhythmogenesis in the newborn. The latter finding is in agreement with previous studies, which showed that MK-801 had little effect on respiratory rhythm in intact, unanesthetized mice on postnatal day 0 (P0) (Borday et al., 1998).

DISCUSSION

This study demonstrated that abolition of NMDA receptor activity by genetic disruption of NR1 resulted in overexpression of LTD in NTS and respiratory depression in the newborn mutant animal. Such abnormalities at the cellular and behavioral levels were not observed in normal newborn mice after acute pharmacological blockade of NMDA receptors, and thus they are most likely to develop prenatally. Because NMDA receptor–channel binding is not discernible in human fetal brainstem at midgestation (H. Kinney, personal communication), it appears that the critical periods for NMDA receptor-dependent development of normal respiratory rhythm and normal synaptic transmission in NTS occur late in fetal development. Thus, neonates of premature births may be particularly at risk of respiratory and suckling disorders similar to those found in the *NR1* $^{-/-}$ mutants (Eichenwald et al., 1997).

Respiratory depression in the *NR1* $^{-/-}$ mutants could be caused by multiple factors, potentially including abnormalities in respiratory tract, respiratory rhythmogenesis, and excitatory input to the rhythm generator. Abnormalities in respiratory tract can be ruled out, because functional NMDA receptors are requisite for excitotoxicity in the lungs (Said et al., 1995), and there is no evidence of pulmonary pathology in *NR1* $^{-/-}$ mutant mice until moments before they died (Forrest et al., 1994). Because respiratory rhythm is reportedly the same in the isolated respiratory pattern generators from normal and *NR1* $^{-/-}$ mutant mice (Funk et al., 1997), the depressed respiratory rhythm in the *NR1* $^{-/-}$ mutants is likely to reflect abnormality in chemoreflex (and possibly baroreflex) rather than in the pattern generator itself. This conclusion is consistent with our finding of profound anomalous LTD in NTS, a gateway for peripheral chemoafferent and other visceral inputs. Glutamatergic neurotransmission is essential for fetal breathing movements (Bissonnette et al., 1997), and NMDA receptor mediates peripheral chemoreceptor afferent input

(Ohtake et al., 1998) primarily via the NTS (Sapru, 1996). In contrast to the slow postnatal maturation of hypoxic chemosensitivity, peripheral chemoreceptor sensitivity to CO_2 is fully functional from birth (Canet et al., 1996). In addition, plasticity of afferent transmission plays an important role in respiratory control (Poon, 1996), and NMDA receptor has a profound influence on the short-term potentiation of peripheral chemoreflex (Poon et al., 1999). Thus, the respiratory depression in the *NR1*$^{-/-}$ mutant neonates may be ascribable at least in part to a blunting of peripheral chemoreflex (and/or baroreflex) or its short-term potentiation as a result of the anomalous LTD in NTS neurons. In the intact animal this deficit may add to other, as yet unidentified, cardiorespiratory abnormalities resulting from impairment of NMDA receptor-dependent activities such as pontine adaptation (Siniaia et al., 2000), neurotransmission in respiratory neurons (Dogas et al., 1995), or phrenic outflow (Sapru, 1996; McCrimmon et al., 1997). Nevertheless, because CO_2 chemoreflex was not totally abolished by the mutation (Fig. 1*A,B*), some chemosensitivity (of peripheral or central origin) remained in the *NR1*$^{-/-}$ mutant neonates.

Recently, it has been reported (Ikonomidou et al., 1999) that transient pharmacological blockade of NMDA receptors during the perinatal period may trigger widespread apoptotic neurodegeneration in newborn animals in certain brain regions with high NMDA receptor density such as dentate gyrus and hippocampal CA1 subfield. However, fetuses exposed to such pharmacological treatments as late as E17 during gestation did not show increased apoptosis in any of these brain regions after birth (Ikonomidou et al., 1999). Indeed, mutant mice with targeted deletion of the NR1 gene restricted to the CA1 pyramidal cells were able to survive to adulthood (Tsien et al., 1996). By contrast, the present study showed that disruption of NMDA receptor function in the fetus could result in the development of an anomalous form of LTD in a brain region (NTS) that controls vital functions, where such NMDA receptor-dependent neurodegenerative effects were not evident (Ikonomidou et al., 1999). Thus, NMDA receptor activity normally suppresses the expression of such anomalous LTD during prenatal development and helps avert postnatal respiratory depression that is detrimental to neonatal life. Such suppression of abnormal neural development by NMDA receptor during the prenatal period acts in concert with its postnatal facilitation of normal suckling activity to foster the viability of the newborn.

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