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The potential role of phrenic nucleus glutamate receptor subunits in mediating spontaneous crossed phrenic activity in neonatal rat

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Abstract

Cervical spinal cord hemisection rostral to the phrenic nucleus leads to paralysis of the ipsilateral hemidiaphragm in adult rats. Respiratory function can be restored to the paralyzed hemidiaphragm by activating a latent respiratory motor pathway. The latent pathway is called the crossed phrenic pathway. In adult rats, the pathway can be activated by drug-induced upregulation of NMDA receptor NR2A subunit and AMPA receptor GluR1 subunit in the phrenic nucleus following hemisection. In neonatal rats, this pathway is not latent as shown by the spontaneous expression of activity in the ipsilateral hemidiaphragm following hemisection. We hypothesized that the NR2A and GluR1 subunits may be highly expressed naturally on phrenic motoneurons of neonatal rats and may play a potential role in mediating the spontaneous expression of activity in the ipsilateral hemidiaphragm after hemisection. To test this hypothesis, the protein levels of NR2A and GluR1 in different age rats were assessed via western blot analysis immediately following C2 hemisection and EMG recording of crossed phrenic activity. The protein levels of NR2A and GluR1 were transiently high in postnatal day 2 (P2) rats and then was significantly reduced in P7 and P35 animals. An immunofluorescence study qualitatively supported these findings. The present results indicate that the developmental downregulation of the phrenic nucleus glutamate receptor subunits correlates with the conversion of the crossed phrenic pathway in older postnatal animals from an active state to a latent state.

Keywords

NR2A; GluR1; respiration; crossed phrenic pathway; spinal cord injury

Introduction

Cervical spinal cord injury may impair normal respiratory function because the injury may interrupt the descending respiratory drive from the rostral ventral respiratory group (rVRG) of neurons in the medulla to phrenic motoneurons (Golder and Mitchell, 2005, Fuller et al., 2008). Cervical (C2) spinal cord hemisection leads to the complete paralysis of the ipsilateral hemidiaphragm in adult rats (Goshgarian, 1979; Moreno, et al., 1992). Respiratory function can be restored to the paralyzed hemidiaphragm following spinal cord hemisection in adult rats by the administration of various drugs (Nantwi and Goshgarian, 1998; Alilain and

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Goshgarian, 2007; Kajana and Goshgarian, 2008). The restored function to the paralyzed hemidiaphragm is mediated by activating a latent crossed respiratory pathway which has been referred to as “the crossed phrenic pathway” (Goshgarian, 2003; Moreno, et al., 1992, Fig. 1). In this paper, recovered activity recorded in the ipsilateral hemidiaphragm and mediated by the activation of the crossed phrenic pathway will be referred to as “crossed phrenic activity”.

The crossed phrenic pathway is initially active and functional during early postnatal development as shown by both an *in vitro* study as well as an *in vivo* study (Zimmer and Goshgarian, 2005; Huang and Goshgarian, 2009). In the *in vivo* study, crossed phrenic activity was recorded immediately following hemisection in ventral, lateral, and dorsal parts of the ipsilateral hemidiaphragm in the first postnatal week. After the first postnatal week, however, this activity was observed only in the ventral area of the ipsilateral hemidiaphragm and the extent of crossed phrenic activity was reduced quantitatively from P7 to P28 (Huang and Goshgarian, 2009). By postnatal day 35, the pathway converted from a functional to a latent state and the activity was no longer recorded in the hemidiaphragm ipsilateral to hemisection. Although the spontaneous expression of crossed phrenic activity has been observed in neonatal rats (Huang and Goshgarian, 2009), the mechanisms that mediate the spontaneous activity are still unknown.

In bulbospinal respiratory pathways, glutamate is the major excitatory neurotransmitter (McCrimmon, *et al.*, 1989; Liu, *et al.*, 1990; Ge and Feldman, 1998; Tai and Goshgarian, 1996). Glutamate receptors can be divided into two main classes, the metabotropic receptors and the ionotropic receptors. The ionotropic receptors are further classified into three subtypes, alpha-amino-3- hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors. During development, the NMDA and AMPA receptor subunits are expressed variably in spinal cord (Kalb et al., 1992; Jakowec et al., 1995). The protein levels of NMDA receptor subunits and AMPA receptor subunits in the spinal cord are transiently high during the first weeks after birth and then these levels gradually decrease over the next several weeks to adult levels (Brown et al., 2002). In addition, recent studies have demonstrated that the NR2A subunit is a mediator for synaptic strengthening during long term potentiation and helps the insertion of AMPA receptors to the post-synaptic membrane (Hayashi et al., 2000; Liu et al., 2004; Massey et al., 2004). There is evidence indicating that spontaneous upregulation of NR2A and GluR1 subunits in phrenic motoneurons occurring 12 to 16 weeks following hemisection may mediate recovery of the paralyzed hemidiaphragm in adult rats (Alilain and Goshgarian, 2008; Nantwi and Goshgarian, 1999). Moreover, it has been shown that the NMDA receptor antagonist, MK-801 can upregulate NR2A expression on the phrenic nucleus and induce the recovery of the paralyzed hemidiaphragm in acute C2 hemisected adult rats (Alilain and Goshgarian, 2007). Based on the above, we hypothesized that the spontaneous expression of crossed phrenic activity observed in neonatal rats is temporally related to the transiently high expression of NR2A and GluR1 subunits in the phrenic nucleus and downregulation of these receptor subunits during later postnatal development also correlates with the conversion of the active crossed phrenic pathway to a functionally latent state.

Based on above, these predictions were verified through western blot analysis and immunofluorescence in the present study. Normal and crossed phrenic activity was recorded by bilateral EMG recording in the diaphragm immediately following C2 hemisection.

Materials and methods

Animal surgery protocol

Timed pregnant female Sprague-Dawley rats were purchased from Harlan Rodent Laboratories and allowed to give birth in the animal care facilities at Wayne State University, School of

Medicine. Litters of rat pups were housed with mothers together and individual postnatal rats were brought to the laboratory before each experiment. Since a significant change of spontaneous crossed phrenic activity during postnatal development occurs at three different ages (Postnatal day 2 (P2), P7 and P35, Huang and Goshgarian, 2009), three groups of postnatal rats at P2, P7 and P35 was involved in the present study. Postnatal rats were prepared for aseptic hemisection surgery of the left C2 spinal cord. Rat pups were anesthetized with ketamine (30–40 mg/kg, ip) and xylazine (10 mg/kg, ip) and placed on a warming pad (37 °C) during surgery. A dorsal midline incision (1 cm) was made through the cervical skin and the paravertebral muscles above the first three cervical vertebrae to expose the second vertebra. A laminectomy of the C2 vertebral bone and durotomy were performed to expose the cervical spinal cord. A left C2 spinal cord hemisection was performed with microscissors just caudal to the C2 dorsal rootlets under magnification. Then, the paravertebral muscles were sutured at the midline with 5.0 absorbable sutures and the skin was closed with tissue glue.

Immediately after hemisection, the pups were placed in the supine position and a horizontal incision was made through the abdominal skin and muscle (1–2 cm) on the ventral surface caudal to the rib edge. By retracting the ventral cut edge of the abdominal muscle rostrally, the abdominal surface of the diaphragm was exposed. Respiratory activity on both sides of the diaphragm was recorded from ventral, lateral, and dorsal areas in that order by using bipolar Grass E2B platinum wire recording electrodes (see detailed protocol in Huang and Goshgarian, 2009). The EMG signals were filtered (0.1–3 Hz) and amplified (10 K) by using a Tektronix 502 amplifier. Animals were not ventilated and were allowed to breath spontaneously throughout the experiment.

Gel electrophoresis and Western blotting

Following EMG recordings, anesthetized rat pups at different postnatal days (P2, P7 and P35, N=6 for each group) were sacrificed and the spinal cord was harvested without fixative perfusion. The spinal cord tissue from non-hemisectioned animals from P2, P7 and P35 were also used as controls in the study (N=6 for each group). The C3–C6 spinal cord was removed and the dorsal half was separated from the ventral half. The ventral part of the left cervical spinal cord containing the left phrenic nucleus from both the hemisectioned and control animals was stored at –80°C until needed for electrophoresis. The total proteins of the tissues from different age groups were extracted through homogenization. The tissues were sonicated in a modified Glasgow protein extraction buffer containing 150 mM NaCl, 1.0 mM EDTA, 50 mM Tris, 1% NP40, 0.1% Triton X-100 and protease inhibitor. The tissue lysates were then centrifuged for 30 minutes to collect the supernatant. The total protein concentration was determined by Bradford assay (Sigma, St. Louis, MI).

A sample containing 50µg total protein was loaded, separated and transferred to a polyvinylidene difluoride (PVDF) membrane. After transfer, the PVDF membrane was blocked with 8% milk, 2% BSA, 2% normal goat serum (NGS) and then washed with a Tris/Tween/saline buffer (TBST). Following the blocking step, the PVDF membrane was probed overnight at 4°C with the primary antibody diluted in a 1% BSA and 1% NGS TBST solution. The primary antibodies were rabbit polyclonal anti-NR2A (1:1000) and rabbit polyclonal anti-GluR1 (1:400, Millipore, Billerica, MA). The membranes were then incubated in the anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) for 2 hr at room temperature. Following extensive washing, the membranes were incubated in an enhanced chemiluminescence peroxidase substrate (Millipore, Billerica, MA) for 1 minute and then developed. β-actin controls (1:5000, Sigma, St. Louis, MO) were used to confirm the equal loading.

The films for the relevant bands for NR2A and GluR1 along with β-actin were scanned with the image Scanner II. Bands corresponding to the NR2A and GluR1 subunits were relatively

quantified as optical density (OD) by using the ImageQuant TL program (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence staining

Additional postnatal rats from P2, P7 and P35 ages (N=6 for each group) were anesthetized with ketamine and Xylazine. Following exposure of the diaphragm, five 5 μ l injections of a 0.4% solution of Dextran Texas Red (invitrogen, Carlsbad, CA) were made into the left hemidiaphragm for retrograde labeling of the phrenic motoneurons. After 36 hours, three pups from each age group were hemisected at C2 under anesthetization and recorded bilateral hemidiaphragmic activity. Following EMG recording, all the animals were transcardially perfused with 20–100 μ l 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS) under anesthetization. The rest of the pups were used as non-hemisection controls and also were perfused following retrograde staining. The spinal cord (C3–C6) was dissected out and post-fixed in the perfusate. After two hours of post fixation and cryoprotection in 30% sucrose for 48 hours, the spinal cord containing the phrenic nucleus was transversely sectioned at a 50 μ m thickness on a cryostat. The sections were blocked with a 5% normal goat serum and 0.1% bovine serum albumin in PBS for 2hr at room temperature. Sections were then incubated in primary polyclonal anti-NR2A (1:1000) and anti-GluR1 (1:200–700) antibodies (Millipore, Billerica, MA) diluted in blocking buffer overnight at 4°C. Following extensive washing, sections were incubated in secondary antibodies conjugated to Alexa Fluor 488 (1:500, invitrogen, Carlsbad, CA) for 2 hr at room temperature. The sections were then washed and mounted with fluorescence protect solution. Sections were viewed and photographed by using a Zeiss Apotome Fluorescent microscope. The Dextran Texas Red produced a red fluorescence color while Alexa Fluor 488 produced a green fluorescent color under a fluorescence microscope.

Statistical analysis

Statistical significance was analyzed by using SPSS 11 software for the Western blot experiment. A one-way multiple analysis of variance (ANOVA) was used to compare the protein levels of NR2A and GluR1 among different age groups and the successive two group difference was tested by a Tukey test of Post-Hoc analysis (SPSS 11). Significance was set at $P < 0.05$.

Results

Crossed phrenic activity was recorded in P2, P7 and P35 animals as shown by EMG recordings immediately following complete C2 hemisection (Fig. 2). Although the activity ipsilateral to hemisection was markedly lower in amplitude qualitatively compared to the activity of the contralateral hemidiaphragm, crossed phrenic activity was spontaneously expressed in the ventral, lateral and dorsal parts of the left hemidiaphragm in P2 neonatal rats (Fig. 2A). The respiratory activity of the left hemidiaphragm was coincident with activity on the right non-hemisected side. In P7 rats, crossed phrenic activity was still observed spontaneously only in the ventral part of the hemidiaphragm ipsilateral to hemisection and qualitatively reduced as compared to the activity in the P2 animals (Fig. 2B). However, during further postnatal development, P35 animals did not display any crossed phrenic activity by EMG recording of the left hemidiaphragm following left C2 hemisection (Fig. 2C).

We next sought to investigate the protein level of NR2A and GluR1 subunits in the left ventral cervical spinal cord (containing the phrenic nucleus) during the period of changing crossed phrenic activity in the postnatal rats. The protein levels of NR2A in the C3 to C6 spinal cord from P2, P7 and P35 rats were quantified and compared by western blot technique (Fig. 3). There was a significant difference of NR2A subunit expression among the different age groups

as revealed by ANOVA analysis ($P < 0.01$, Fig. 3A). In P2 animals, the relative optical density (OD) of NR2A was 0.71 ± 0.11 which was the highest in the three age groups and significantly higher than that of P7 and P35 groups ($P < 0.05$, $P < 0.01$ individually, Fig. 3A). During postnatal development, the NR2A protein level of the P35 group was markedly lower than P7 group ($P < 0.01$, Fig. 3A). Similar changes were also observed in control animals (Fig. 3B). No significant differences were found in the NR2A protein levels between hemisected animals and non-hemisected controls at the same age (Fig. 3C).

Similar to NR2A, the receptor protein of the GluR1 subunit was expressed with the highest level in the P2 animals (1.03 ± 0.12) and the lowest level at P35 (0.31 ± 0.06 , Fig. 4A). ANOVA analysis revealed a significant difference in GluR1 protein expression among the P2, P7 and P35 groups ($P < 0.01$, Fig. 4A). Similar to NR2A, this glutamate receptor subunit was transiently expressed at a higher level in P2 as compared to the P7 ($P < 0.05$) and P35 animals ($P < 0.01$, Fig. 4A). With development, the GluR1 protein level was significantly reduced in P35 as compared to P7 ($P < 0.01$, Fig. 4A). Similar changes also occurred in control animals (Fig. 4B). In addition, there was no significant difference in the protein levels of the GluR1 receptor subunit between the hemisected animals and non-hemisected controls at the same age (Fig. 4C).

To localize the expression change of membrane bound NMDA receptor subunit 2A and AMPA receptor subunit 1 on phrenic motoneurons, NR2A and GluR1 immunofluorescence study with the omission of Triton X-100 combined with retrograde staining was carried out in the present study. The phrenic motoneurons which were labeled with a retrograde tracer, Texas red (red color, Fig. 5A, D, G and 6A, D, G). The expression of NR2A subunit was observed on phrenic motoneurons of the P2, P7 and P35 rats (green color, Fig 5B, E, H). After overlapping the green and red fluorescence, the phrenic motoneurons appeared as yellow or orange in color indicating dual labeling in the three age groups (Fig 5C, F, I). Qualitatively, the NR2A subunit was expressed at a much higher level on phrenic motoneurons at P2 compared to the P7 and P35 animals. In agreement with the above western blot results, the expression of the membrane bound NR2A subunit was conspicuously reduced in P35 rats as compared to P7 pups (Fig 5B, E, H).

The expression of the membrane bound GluR1 subunit, as evaluated by immunofluorescence, also appeared as a green color on the motoneuron surface (Fig. 6B, E, H). With the combination of Texas red, the different expression levels on phrenic motoneurons were revealed in different age groups (Fig. 6C, F, I). An apparently higher expression of GluR1 subunit was found in P2 as compared to P7 as well as P35 animals (Fig. 6B, E, H). During development, there appeared to be a downregulation of GluR1 expression in P35 as compared to P7 rats. In the non-hemisection control animals, the developmental downregulation of the GluR1 and NR2A subunits was also observed in P7 and P35 animals (data not shown).

Discussions

In the present study, we demonstrated that the protein levels of the NR2A and GluR1 subunits were significantly lower in the ventral C3-C6 level of the spinal cord in P7 and P35 animals as compared to that of P2 rats by using western blot analysis. Through the combination of immunofluorescence combined with retrograde staining, it was demonstrated qualitatively that the downregulation of NR2A and GluR1 also occurs specifically on phrenic motoneurons. P35 was chosen as the end time point of the postnatal developmental study because it has been suggested that the levels of glutamate receptor in the spinal cord decline to adult levels by the fifth week after birth (Brown et al., 2002; Kalb and Fox, 1997). Consistent with our present study, the reduced expression of NMDA receptor and AMPA receptor subunits has been demonstrated in non-injured brainstem and spinal cord during development (Jakowec et al.,

1995; Brown et al., 2002; Liu and Wong-Riley, 2005). For AMPA receptor subunits, the most conspicuous change is the downregulation of GluR1 expression in the rat spinal cord over the first three to four weeks of postnatal life (Jakowec et al., 1995). For NMDA receptor subunits, the most significant change happens to NR2A (Oshima et al., 2002).

In the respiratory system, the AMPA glutamate receptor is the primary mediator of the descending respiratory drive to the phrenic motoneurons (McMrimmon et al., 1989; Liu et al., 1999). The NMDA receptors can also drive respiratory rhythm independent of AMPA receptors (Morgado-Valle and Feldman, 2007). It has been observed that the administration of NMDA receptor antagonist (MK-801) to the C2 hemisectioned adult rats results in the upregulation of the NR2A expression in the cervical spinal cord from C3 to C6. NR2A is an essential component of synaptic strengthening in long term potentiation (Liu et al., 2004; Massey et al., 2004; Alilain and Goshgarian, 2007). One result of long term potentiation is the insertion of AMPA receptors subunit, specifically GluR1 to the postsynaptic membrane (Hayashi et al., 2000) and this results in an increase in phrenic motor output. We have previously shown that the spontaneous upregulation of the NR2A and GluR1 subunits in adult rats with chronic hemisection injury may contribute to the spontaneous functional recovery of the ipsilateral hemidiaphragm (Alilain and Goshgarian, 2008). Therefore, increased expression of both NR2A and GluR1 receptors is an important regulator of activation the latent crossed phrenic pathway in adult rats. In addition, NR2A and GluR1 play an important role in the regulation of developmental neuroplasticity (Yashiro and Philpot, 2008). The transiently high expression of the NR2A and GluR1 subunits on phrenic motoneurons in neonatal rats could result in synaptic strengthening between respiratory premotor neurons and motoneurons, mediating spontaneous crossed phrenic activity. During postnatal development, the decreased expressions of the two subunits on phrenic nucleus may contribute to the conversion of the crossed phrenic pathway from a functional to a latent state.

Studies have shown that significant decreases in the protein level of GluR1 and NR2A occur at 24 hrs after acute spinal cord injury (Brown et al., 2004). In chronic spinal cord injury and recovery (>28 days), the expression of GluR1 and NR2A significantly increased compared with uninjured animals (Alilain and Goshgarian, 2008; Brown et al., 2004). However, there were no significant changes of the protein level of NR2A and GluR1 subunits between the hemisection and non-hemisectioned animals in present study. This is likely because the spinal cord tissue in the hemisection rats was taken immediately following hemisection surgery and there was very little time for receptor plasticity to occur. In the western blot analyses, we limited our analysis to the ventral portion of C3 to C6 cervical spinal cord which contains the phrenic nucleus. However, this analysis is limited because the tissue contains other motor neurons. This limitation of our western blot results was partially corrected by our immunofluorescence study combined with phrenic nucleus fluorescent staining reflecting the expression changes of the NR2A and GluR1 subunits specifically in the phrenic nucleus.

Another factor that may contribute to spontaneous crossed phrenic activity in neonatal rats is the anatomy of the crossed phrenic pathway. There is a significant difference in the anatomy of the pathway between neonatal and adult rats (Huang and Goshgarian, 2009 submitted manuscript). The neonatal crossed phrenic pathway is comprised of not only spinal cord midline-crossing collaterals from the ipsilateral rVRG premotor axons, but also the midline-crossing dendrites of phrenic motoneurons. The medially projecting crossing phrenic dendrites may receive descending respiratory afferents from the contralateral side of the cervical spinal cord in neonates (Zimmer and Goshgarian, 2005; Zimmer, et al., 2008). However, in adult rat, the pathway is composed of the midline-crossing collaterals from bilateral rVRG premotor neurons and there are no midline-crossing dendrites. The developmental changes of the crossed phrenic pathway occur within the first four postnatal weeks (Huang and Goshgarian, 2009 submitted manuscript). Therefore, this developmental change of the crossed phrenic pathway

may also play an important role in mediating the change of crossed phrenic activity during postnatal development.

In summary, the present study only begins to uncover the underlying mechanisms of spontaneous crossed phrenic activity following hemisection in neonatal rats. Our results suggest a potential role of phrenic nucleus glutamate receptors in mediating the spontaneous expression of crossed phrenic activity in neonatal rats. Understanding these receptors in the respiratory system and their regulation in developmental neuroplasticity may prove to be beneficial in exploring new strategy following spinal cord injury.

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Abbreviations

NR2A

N-methyl-D-aspartate (NMDA) receptor subunit 2A

GluR1

alpha-amino-3- hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 1

P2

postnatal day 2

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Figure 1.

Diagram of the crossed phrenic pathway in the adult rat. The pathway involves bilateral rVRG respiratory premotor axons with axon collaterals that cross the midline of the spinal cord. Arrows indicate the direction of respiratory impulses to the paralyzed hemidiaphragm after hemisection.

Figure 2.

Electromyographic (EMG) recordings from the left and right ventral hemidiaphragm of postnatal rats before and after a complete left C2 spinal cord hemisection showing activity on both sides of the diaphragm. A, B) The spontaneous crossed phrenic activity which drives the left ventral hemidiaphragm after left C2 hemisection was expressed in a P2 and P7 neonatal rat. C) No spontaneous crossed activity was observed in a P35 hemisected rat. This reflects the adult rat pattern in which no crossed phrenic activity is expressed after ipsilateral spinal cord hemisection.

Figure 3.

Western blot analysis revealed significant decreases in NR2A receptor subunit during postnatal development. A) The left ventral spinal cord (C3 to C6) in P2 rats had a significantly higher relative optical density (OD) value corresponding to NR2A protein levels as compared to the other age groups. The OD in the P7 group was significantly higher than the P35 age group. B) The non-injured control group has a similar downregulation of NR2A receptor subunit. C) There is no significant difference in the protein level of NR2A subunit between control and hemisection groups at each individual age. ** indicates significance with a $p < 0.01$. * indicates significance with a $p < 0.001$.

A graph showing that there is downregulation of AMPA GluR1 subunit in left ventral spinal cord (C3 to C6) during postnatal development as revealed by western blot analysis. A) GluR1 protein level in the P2 group was significantly higher than the other age groups. The protein value in the P35 group was significantly lower compared to the P7 age group. B) Non-injured control groups have a similar downregulation of GluR1 receptor subunit. C) No significant difference of the protein level of GluR1 was found between the control and the hemisection group at each individual age. ** indicates significance with a $p < 0.01$. * indicates significance with a $p < 0.001$.

Figure 5.

Immunofluorescence images depicted the NR2A subunit on phrenic motoneurons at different ages. A, D, G show examples of phrenic motoneurons which were retrogradely labeled with dextran Texas red (red color). B, E, H show the expression of the NR2A subunit on motoneurons in the same ventral area of the spinal cord (green color). C, F, I merges the two fluorescence images and depicts the expression level of the NR2A subunit on the labeled phrenic motoneurons (yellow or orange color). A downregulation of the NR2A expression is demonstrated qualitatively in P7 (F) and P35 (I) animals as compared to P2 rats (C). PM-DTR means phrenic motoneurons labeled with dextran Texas red. Magnification of all immunofluorescence figures is the same and scale bar equals 50 μ m.

Figure 6.

Immunofluorescence images delineated the expression of the GluR1 subunit on phrenic motoneurons. A, D, G show phrenic motoneurons which were retrogradely labeled with dextran Texas red (red color). B, E, H show the expression of the GluR1 subunit on motoneurons of the ventral spinal cord (green color). C, F, I depicts the expression of the GluR1 subunit on the labeled phrenic motoneurons (yellow or orange color) by overlapping the two fluorescence images. There appeared to be a downregulation of GluR1 expression in P7 (F) and P35 (I) as compared to that of P2 (C). PM-DTR means phrenic motoneurons labeled with dextran Texas red. Magnification of all figures is the same and scale bar equals 50 μ m.