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Role of Plasminogen Activator in Spinal Cord Remodeling after Spinal Cord Injury

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Abstract

Plasminogen activators play an active role in synaptic plasticity associated with the crossed phrenic phenomenon (CPP) and recovery of respiratory function following spinal cord injury. A genetic approach has been used to identify molecular mechanisms underlying this synaptic plasticity. Knockout mice lacking different genes in the plasminogen activator/plasmin system demonstrate that expression of urokinase plasminogen activator (uPA) is required during the critical 1-2h delay period following C2-hemisection for acquisition of a good CPP response. uPA knockout mice fail to show the structural remodeling of phrenic motor neuron synapses that underlie the CPP response. Potential mechanisms by which uPA may promote phrenic motor neuron synaptic plasticity have been explored. Expression of uPA receptors, uPAR and LRP-1, are both up-regulated in the ipsilateral phrenic motor nucleus (PMN) following C2-hemisection. A comparison of microarray data and real-time PCR analysis of mRNAs induced in the PMN after hemisection indicate potential cell signaling pathways downstream of uPA's interaction with these cell surface receptors in the PMN. Knowledge of these uPA-mediated signaling pathways may identify potential means for pharmacological activation of the synaptic plasticity required for recovery of phrenic motor neuron activity.

1. Introduction

There are two plasminogen activators (PA) in mammals, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Plasminogen activators are best known for their proteolytic action as "clot busters", when they activate the proenzyme plasminogen to the broad-acting and fibrin-degrading protease plasmin in the vascular system (Collen, 1980). However, plasminogen activators play important roles in numerous tissues where they have been shown to promote cellular remodeling associated with a number of physiological events, including angiogenesis, ovulation and trophoblast implantation, bone growth, muscle differentiation, and tumor cell metastasis (for review see Dano et al., 1999); as well as, activating other proenzymes such as matrix metalloproteases (Keski-Oja et al., 1992; Baramova et al., 1997; Siconolfi and Seeds, 2003). In the nervous system PAs play an active role in neural development, where they are secreted by both CNS and PNS neurons to facilitate neuronal cell migration and axonal outgrowth (Krystosek and Seeds, 1981; Pittman, 1985; McGuire and Seeds, 1990; Verrall and Seeds, 1989; Friedman and Seeds, 1995; Seeds et al., 1999); as well as, being up-regulated to promote axonal regeneration following nerve injury

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(Hayden and Seeds, 1996; Nakajima et al., 1996: Siconolfi and Seeds, 2001a,b). In addition, PAs in the nervous system can directly activate pro-neurotrophic factors including the motorneuron survival factor hepatocyte growth factor (HGF) (Mars et al., 1993; Thewke and Seeds, 1996), or indirectly via plasmin formation activate pro-BDNF (brain-derived neurotrophic factor) and pro-NGF (nerve growth factor) to their active forms (Pang et al., 2004). Furthermore, PAs play an active role in dendritic spine formation (Oray et al., 2004) and have been implicated in synaptic remodeling associated with cerebellar motor learning, visual cortex ocular dominance columns, and both hippocampal and corticostriatal long-term potentiation (LTP) (Seeds et al., 1995; Seeds et al., 2003; Muller and Griesinger, 1998; Mataga et al., 2004; Huang et al., 1996; Baranes et al., 1998)

The crossed phrenic phenomenon (CPP) is one of the most dramatic examples of spinal cord plasticity resulting in the recovery of respiratory function following a high cervical spinal cord injury (for a review see Goshgarian, 2003). The CPP occurs in several mammalian species including the mouse (Minor et al., 2006) following a cervical C2 spinal cord hemisection (C2HS), which paralyzes the ipsilateral hemidiaphragm by interrupting the descending flow of respiratory impulses from the medulla to phrenic motorneurons (Fig. 1). The hemisection leads to a stronger respiratory drive to the contralateral phrenic motor nucleus (PMN) via the contralateral rostral ventral respiratory group (rVRG) fibers, a small population of which cross-over the spinal cord midline during development and contact phrenic motorneurons (Zimmer and Goshgarian, 2005). The loss of ipsilateral rVRG input to the PMN coupled with this increased activity from crossed contralateral rVRG fibers is thought to convert some of their presumptive synaptic contacts on phrenic motorneurons on the paralyzed side of the spinal cord from a pre-hemisection “functionally ineffective” state to a post-hemisection “functionally latent” state; ie. an anatomically/physiologically modified cell contact that requires a specific time interval for conversion in mice and rats, yet still does not restore hemidiaphragm functional activity under normal conditions (Goshgarian, 2003). Subjecting the animal to additional respiratory stress by transecting the contralateral phrenic nerve, “activates” these latent synapses and function is restored to the paralyzed hemidiaphragm. Activation of these synapses and the crossed pathway requires an interoperative delay of several hours between hemisection and phrenicotomy to elicit the CPP in rats and mice (O’Hara and Goshgarian, 1991; Minor et al., 2006). During this delay period, ultrastructural changes occur in the PMN including elongation of the synaptic active zones and increased numbers of multiple axo-dendritic synapses on phrenic motorneurons (Castro-Moure and Goshgarian, 1997; Liou and Goshgarian, 1997; Minor and Seeds, 2008). However, the molecular mechanisms underlying this cellular remodeling, synaptic plasticity and functional recovery associated with the CPP, and its inter-operative delay are not well defined. Plasminogen activator is a likely candidate for playing an active role in these early cellular remodeling events promoting the CPP.

2. CPP in the mouse

Recently has the CPP been shown to occur in the mouse, an animal model readily amenable to a molecular genetic approach to dissect mechanisms underlying the CPP (Minor et al., 2006; Minor and Seeds, 2008). Axons from the premotor rVRG descend in the lateral and ventral funiculi of the spinal cord and enter the grey matter between C3 and C6 to innervate phrenic motorneurons. Although careful anatomical mapping has not yet been done for the mouse, organization of the vestibulospinal tracts is consistent among amniote vertebrates (Watson and Harvey, 2009). A C2HS disrupts the descending input leading to paralysis of the ipsilateral hemidiaphragm, and an immediate increase in the duration of respiratory bursts and the amplitude of individual spikes within each burst by the contralateral diaphragm is seen in spontaneously breathing mice (Minor et al., 2006). Although this response is similar to that seen in other mammals (Golder et al., 2001), mice show no change in spike frequency within

a burst in marked contrast to the increase in spike frequency seen in spontaneously breathing rats 24h after a C2HS (El-Bohy and Goshgarian, 1999). If the C2-hemisected mice are subjected to additional respiratory stress by transection of the contralateral phrenic nerve immediately after hemisection, the mice must be ventilated or they will die; whereas, if the phrenicotomy is delayed until the next day there is an immediate and rapidly building recovery of function by the previously silent hemidiaphragm; i.e., the CPP response (Fig. 2). Although this immediate classic CPP response in mice and rats is similar, rats have been reported to undergo spontaneous recovery of the silent diaphragm after several weeks to a month (Nantwi et al., 1999; Fuller et al., 2006). In contrast, mice fail to show any spontaneous recovery even when examined three months after a C2HS; yet they give an immediate and strong CPP response upon contralateral phrenicotomy at three months post-surgery (Fig. 3). This finding suggests a potential difference in the long-term CPP recovery mechanisms between these species. In this regard, rat phrenic motorneuron dendrites have been shown to cross the midline and may form contacts with the contralateral rVRG fibers (Lindsay et al., 1991; Prakash et al., 2000; Boulenguez et al., 2007) facilitating spontaneous recovery; however, whether mouse phrenic motoneurons also display contralateral dendritic projections is not presently known.

As the inter-operative interval between the spinal cord lesion and the contralateral phrenicotomy was reduced, the strength of the crossed phrenic respiratory signal decreased. Although greater than 90% of the mice demonstrated a good CPP response at 6hr post-hemisection, at earlier times the number of mice showing a CPP response decreased as did the strength of the response (Minor et al., 2006). A critical latent period of 1–2h post-hemisection was required prior to any CPP response, similar to the 2–4h latency period seen in rats (O'Hara & Goshgarian, 1991). The important molecular events required for the CPP that occur during this 1–2hr window post-hemisection have been the focus of our attention.

3. Plasminogen activator and the critical period for the CPP

The premise that synaptic plasticity associated with the CPP may be similar to the synaptic remodeling associated with the different learning paradigms discussed above, where the extracellular protease PA plays an active role was explored. In situ hybridization with ^{35}S-cRNA antisense probes to uPA or tPA showed that mRNAs for both molecules were rapidly induced in C4–5 phrenic motorneurons immediately following a C2HS (Minor and Seeds, 2008). Induction of uPA mRNA was restricted to a limited number of large retrogradely WGA-labeled phrenic motorneurons in the ipsilateral PMN and was not seen at the C2–3 interface or the C6–7 interface ventral spinal cord above and below the PMN (Fig. 4). The limited number of motorneurons expressing uPA mRNA is not surprising since fewer than 10% of the phrenic motorneurons, which represent only 11% of the total motoneurons at the C4–5 level, appear to be activated during the CPP response (Zimmer and Goshgarian, 2005). Interestingly, uPA mRNA expression was transient and seen from 0.3h to 8h, but was absent at 20h post-hemisection. In contrast to uPA mRNA expression which is not commonly seen in the uninjured spinal cord, tPA mRNA is apparent at a low level throughout the cervical spinal cord. However, tPA mRNA expression also increased in the ipsilateral PMN about 2-fold greater than the contralateral PMN following C2HS, but in contrast to uPA mRNA it was not specific to the retrogradely WGA-label phrenic motorneurons (Minor and Seeds, 2008). Dramatic induction of uPA protein was seen within 1h in ipsilateral phrenic motorneurons, reaching maximal levels by 6h and disappearing by 20h post-hemisection. tPA protein showed similar increases in both phrenic motorneurons and Neu-N positive interneurons within the PMN; however, elevated tPA levels were still seen 20h post-hemisection (Minor and Seeds, 2008). Thus, PA induction was concomitant with the critical latent period in recovery of diaphragmatic function during the CPP.

The potential importance and relationship of PA induction to the presumptive synaptic changes and activation of phrenic motorneurons during the CPP was assessed using knockout mice. Mice deficient in uPA, tPA or plasminogen (Plgn) genes, were compared to their heterozygous littermates or wildtype C57Bl/6 mice for recovery of diaphragm function via the CPP at 6h post-hemisection, when >85% of wildtype mice show a good CPP response (as defined by Minor et al., 2006). All the uPA +/− heterozygotes showed a CPP response at 6h, and most tPA−/− and Plgn−/− mice give a CPP response, although the overall strength of the CPP response was not as strong as that seen in the heterozygotes or wildtype mice (Fig. 5) (Minor and Seeds, 2008). Interestingly, most of the uPA−/− mice fail to generate a good CPP response at 6h. Typical diaphragm electromyographic responses are shown for these knockout mice (Fig. 5). To assess whether acquisition of the CPP response was just delayed in these knockout mice, several mice of each genotype were not given a phrenicotomy until 20h post-hemisection. All the tPA−/− and Plgn−/− mice now gave a good CPP response; however, the uPA−/− mice still failed to respond, indicating that uPA gene expression was critical for the CPP.

Ultrastructural analysis at the electron microscope level of PMN synapses showed that uPA−/− mice failed to undergo the characteristic synaptic remodeling events, an increase in multiple axo-dendritic synapses and a lengthening of the synaptic active zone, coincident with the acquisition of a CPP response as elicited in wildtype mice. C2HS of wildtype mice showed an increase in the number of multiple axo-dendritic synapses on identified phrenic motorneuron dendrites from 3.76±0.6 per 100 dendrites in the uninjured mice to 8.73±1.3 per 100 dendrites by 4 h post-hemisection. Similarly, the average length of the synaptic active zone significantly increased from 0.28μm in the control mice to 0.39μm in the hemisected mice. Both of these synaptic changes are congruent with an increased synaptic input by the latent rVRG crossed pathway on the ipsilateral PMN, leading to enhanced phrenic nerve output and diaphragm function, and are similar to those seen in the rat during acquisition of the CPP (Castro-Moure & Goshgarian, 1997). tPA−/− mice showed changes similar to wildtype mice following hemisection. Strikingly, the hemisected uPA−/− mice showed no increases in multiple axo-dendritic synapses (3.35±0.9 per 100 dendrites) nor in active zone length (0.29μm) (Minor and Seeds, 2008). Thus, uPA gene expression promotes synaptic remodeling in the PMN coincident with the CPP response and recovery of diaphragm function.

4. Potential mechanisms for uPA action in the CPP response

How might uPA act to bring about the structural changes and functional recovery that occur during the CPP? Most Plgn−/− mice show a functional CPP response, suggesting that plasmin formation by uPA during the critical latent period may play a minor role in the CPP response. However, uPA is capable of proteolytically cleaving substrates other than plasminogen, including the extracellular matrix molecule fibronectin (McGuire and Seeds, 1990) and activating extracellular pro-matrix metalloproteases (Keski-Oja et al., 1992; Baramova et al., 1997; Siconolfi & Seeds, 2003) that may act in tissue remodeling events (Fig. 6). Also, uPA can proteolytically activate the pro-neurotrophic factor HGF (Naldini et al., 1992; Mars et al., 1993; Thewke and Seeds, 1996) that promotes motorneuron survival and axonal growth (Ebens et al., 1996).

Alternatively, uPA may be acting as a cytokine by binding to either of its two cell surface receptors uPAR or LRP-1 (the LDL-like receptor protein), both of which are capable of cell signaling events. Interestingly, both uPAR and LRP-1 expression are dramatically up-regulated in the ipsilateral PMN immediately following a C2HS (Fig. 7), and may be targets for uPA mediated cell signaling. Both uPA and tPA interact with LRP-1 to signal an up-regulation of PKA activity and cAMP levels within neurons (Zhuo et al., 2000;Herz & Bock, 2002). Furthermore, tPA interaction with LRP-1 has recently been shown to be coupled to phosphorylation of LRP-1's cytoplasmic NPxY-motif to promote interaction with neuronal

PSD95 that binds to and opens the NMDA-R for calcium influx leading to MAPK signaling (Martin et al., 2008). The possibility that uPA can also signal via LRP1 to open NMDA-R calcium channels is currently under investigation (Seeds, unpublished observations). The NMDA-R and other glutamate receptors are present on phrenic motorneurons; thus calcium influx may promote synaptic activity in the CPP.

uPA binding to its glycosyl phosphatidylinositol (GPI)-linked receptor uPAR leads to interaction with several different cell surface molecules with subsequent cell signaling events producing a variety of activities favoring structural remodeling events in different tissues including cell migration and invasiveness (for review see Blasi & Carmeliet, 2002). For example in non-neural tissue, uPAR is known to interact with EGF-R leading to stimulation of the Erk pathway, and uPAR interaction with the G-protein coupled receptor FPRL1 is required for cell migration (Mazzieri et al., 2006). Furthermore, uPAR interaction with β1 integrin leads to activation of a Src pathway (Monaghan-Benson & McKeown-Longo, 2006), and such interactions with integrins can organize both matrix molecules and the underlying cytoskeleton in the process of cell attachment/detachment, mechanisms that may favor structural remodeling events.

Other studies on a molecular level have shown increased levels of serotonin in ventral spinal cord following a C2HS (Hadley et al.,1999) and that blockers of 5-HT receptors inhibit CPP respiratory recovery (Zhou et al., 2001), antagonists of adenosine receptors enhance the CPP response (Nantwi and Goshgarian, 2002), while elevated cAMP-mediated PKA activation appears necessary for recovery of diaphragm function following a C2HS (Kajana and Goshgarian, 2008). Up-regulation of the NR2A subunit of the NMDA-R, as well as the GluR1 subunit of the AMPA-R have also been reported following C2 injury and have been implicated in spontaneous recovery of diaphragm function (Alilain and Goshgarian, 2007; Grossman et al., 1999). Using a different model of respiratory functional recovery, phrenic long-term facilitation (pLTF) Mitchell and coworkers (Baker-Herman et al., 2004; Golder & Mitchell, 2005) found that enhanced activation of 5-HT2A receptors leads to increased BDNF synthesis, TrkB activation and MAPK that may strengthen inputs on phrenic motorneurons. Similarly, adenosine A2a receptor agonists can elicit pLTF presumably by up-regulating the same MAP kinases (pERK1/2) via a different pathway (Golder et al., 2008). However, these molecular changes occur days to weeks after C2HS and would not appear to be changes directly associated with the 1–2h “critical latent period” necessary for acquisition of the uPA-dependent CPP as described above.

5. Potential uPA-mediated cell signaling pathway activation for the CPP

If uPA is acting as a cytokine or protease & cytokine in facilitating the CPP response it is important to identify molecules influenced by uPA mRNA induction. Knowledge of the signaling pathway(s) and downstream molecules activated by uPA signaling will provide a more thorough understanding of this novel form of synaptic plasticity. Such plasticity may well be able to occur at other sites further down the spinal cord and lead to a similar recovery of function. Furthermore, if uPA appears to signal via a unique or more limited cellular pathway it may then be a potential target for drug therapy via agonists and antagonists that may help promote synaptic plasticity leading to functional recovery following SCI.

If uPA is acting through its binding to uPAR then, based on other tissues, we may expect to see activation of Src and G-proteins, or Stat and ERK1/2 pathways, along with EGF-R and integrin-associated signaling. If uPA is interacting through LRP-1 then MEK-1 and ERK1/2 may be activated. As a first approach to addressing this question real-time PCR 96-well plate microarrays for gene profiling of signaling pathways (ie. Superarray Biosciences) were used. RNA isolated from the ipsilateral grey matter of C4–5 ventral spinal cord of control uninjured

wildtype mice or those 4h post-C2HS, and uninjured uPA−/− mice or those with C2HS 4h post-surgery, was compared for relative mRNA expression on 96-well microarray JAK/STAT or MAPK pathway genes using RT-PCR. Relative differences between RNA samples are determined by normalizing the ΔCt to the housekeeping genes and the results expressed as $2^{-\Delta\Delta Ct}$ fold differences up or down. A change was considered when it appeared in all paired experiments and the magnitude of the change (+ or −) had a minimum value of two; validation of differences used the paired *t* test.

5.1 Src and Jak/Stat pathways

When gene changes are compared 4hr following a C2HS (Table 1), both wildtype and uPA−/− mice show similar increases (blue) in Socs3, Cyclin-dep kinase inhibitor 1A, Protein tyrosine phosphatase non-receptor type1, SH2B adaptor2, Myc, IL-4 receptor, and decreases in IL-10 receptor. Uninjured uPA−/− mice show a reduced expression of a number of genes (yellow) when compared to uninjured wildtype mice, most notable are Smad1,2&5, Src, Stam, YY1, Myc, Jak2, Nuclear receptor 3C1, Growth hormone receptor. C2HS in uPA−/− mice (red) leads to a dramatic decrease in EGF-R, and decreases in Src, YY1, Socs2, SH2B-adaptor1 that are not seen in C2-hemisected wildtype mice. Furthermore, when C2-hemisected uPA−/− and C2-hemisected wildtype gene expression are directly compared there are major decreases (red) in Src, EGF-R, YY1 and Growth Hormone receptor. Two of the genes showing the greatest increase with C2HS are Socs-3 and protein tyrosine phosphatase non-receptor type 1, both negative regulators of the JAK/STAT pathway. Socs-3 is known to promote neuron survival in response to IGF-1 stimulation (Yadav et al., 2005). Also, negative regulation of signal transduction pathways is necessary for correct cellular response to cytokine stimulation. Reductions in Src and EGF-R are not unexpected since both play a role in uPA mediated signaling through uPAR. uPA signaling via uPAR and Src has been shown to promote cytoskeleton reorganization and cell migration in smooth muscle cells (Degryse et al.; 1999). Similar cytoskeletal changes may be important in the morphological re-structuring of phrenic motorneuron dendrites during the crossed phrenic phenomenon.

5.2 MAPK pathways

When compared to wildtype mice the uPA−/− mice showed (Table 2) notable reductions in expression of Cyclin B2, Cdki 1C, MAP3k1, MAP2k6, and modest reductions in several other genes (yellow). However, Cdki1A and 2C mRNAs show large increases at 4h post-hemisection in both wildtype and uPA−/− mice (blue); interestingly, both genes are regulated by Erk1/2. Cdki1A (Rho-kinase inhibitor) is known to enhance axonal regeneration and functional recovery after spinal cord injury (Tanaka et al., 2004), while Cdki2C shows highly specialized expression in only a few regions of the adult nervous system and at specific times (Zindy et al., 1997;Legrier et al., 2001). A third up-regulated protein, MAP2k6 is an upstream activator of the widely active p38 MAPK. Previous reports (Hu et al., 2002;Velardo et al., 2004) have shown a major decline in neural gene expression following spinal cord injury. CPP induction in wildtype mice led to a decline in many of the mRNAs characteristic of the MAP kinase pathway as shown (Cdki1c, Creb-1, Elk-1, Ets-1, Mapk14), while in C2HS uPA−/− mice several mRNAs (Cyclin B2, Creb-1, Fos, MAP2k5) show apparent increases, but in genes whose expression is decreased in the un-injured uPA−/− mouse compared to wildtype. These decreased mRNAs following C2HS in the wildtype mouse may be indicative of critical gene shutdown related to acquisition of the CPP. Also differences between uPA−/− and wildtype mice following C2HS indicate potential critical components in the CPP as it occurs in wildtype mice; the most dramatic effect is seen with decreases in MAP2k6, MAP3k1, and Cdki1C & 2C.

A pilot study with these same mRNAs assayed on the new Affymetrix Mouse Gene 1.0ST chip showed that C2HS led to an increased expression (the top 1–2% of genes by difference) in

several of these same kinases and transcription factors, as well as cell surface receptors, most interestingly uPAR when compared to uninjured C4–5 ventral spinal cord. Comparison of uPA −/− hemisected to wildtype hemisected gene expression showed major (top 1–2%) decreases in several kinases, transcription factors, growth factors and receptors including IGF, EGF, patched, notch, EphB4, cadherin, vitronectin, and interestingly the axon midline crossing factor Robo3. Current studies are assessing changes in the respective proteins, and monitoring mRNA differences at earlier time points following C2-hemisection.

6. Summary

These studies indicate that plasminogen activators play an active role in the acquisition of the crossed phrenic phenomenon and may be important players in spinal cord motor neuron synaptic plasticity; thereby, setting the stage for the potential use of plasminogen activators, or their agonists, or drugs mimicking their action in a therapeutic regenerative model for spinal cord injury.

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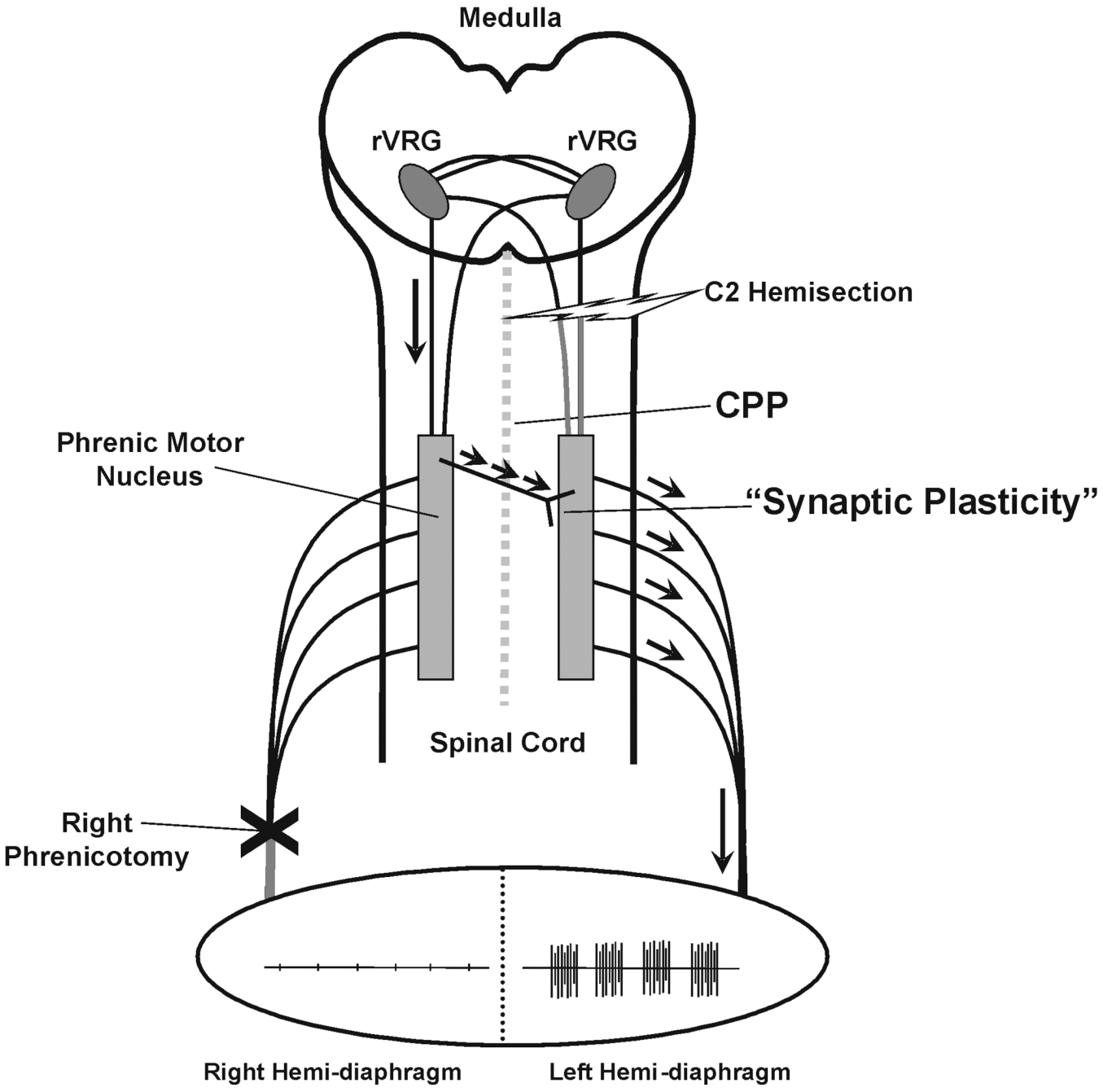

Figure 1. Schematic of the crossed phrenic pathway

A spinal hemisection was performed at C2 disrupting the rostral ventral respiratory group (rVRG) descending axons that innervate the PMN, a column of motorneurons extending from C3 to C6. Thus, blocking phrenic motorneuron axonal output and silencing the ipsilateral left hemidiaphragm. Subsequent transection of the contralateral phrenic nerve leads to asphyxia; however, if performed after a critical delay period of synaptic remodeling, the rVRG axons on the contralateral side of the spinal cord now convey respiratory impulses across the midline (arrows) via activated synaptic contacts on the silent phrenic motor nucleus (PMN) and recovery of hemidiaphragm function (lower recording) on the hemisected left side. (reprinted from Minor and Seeds, 2008).

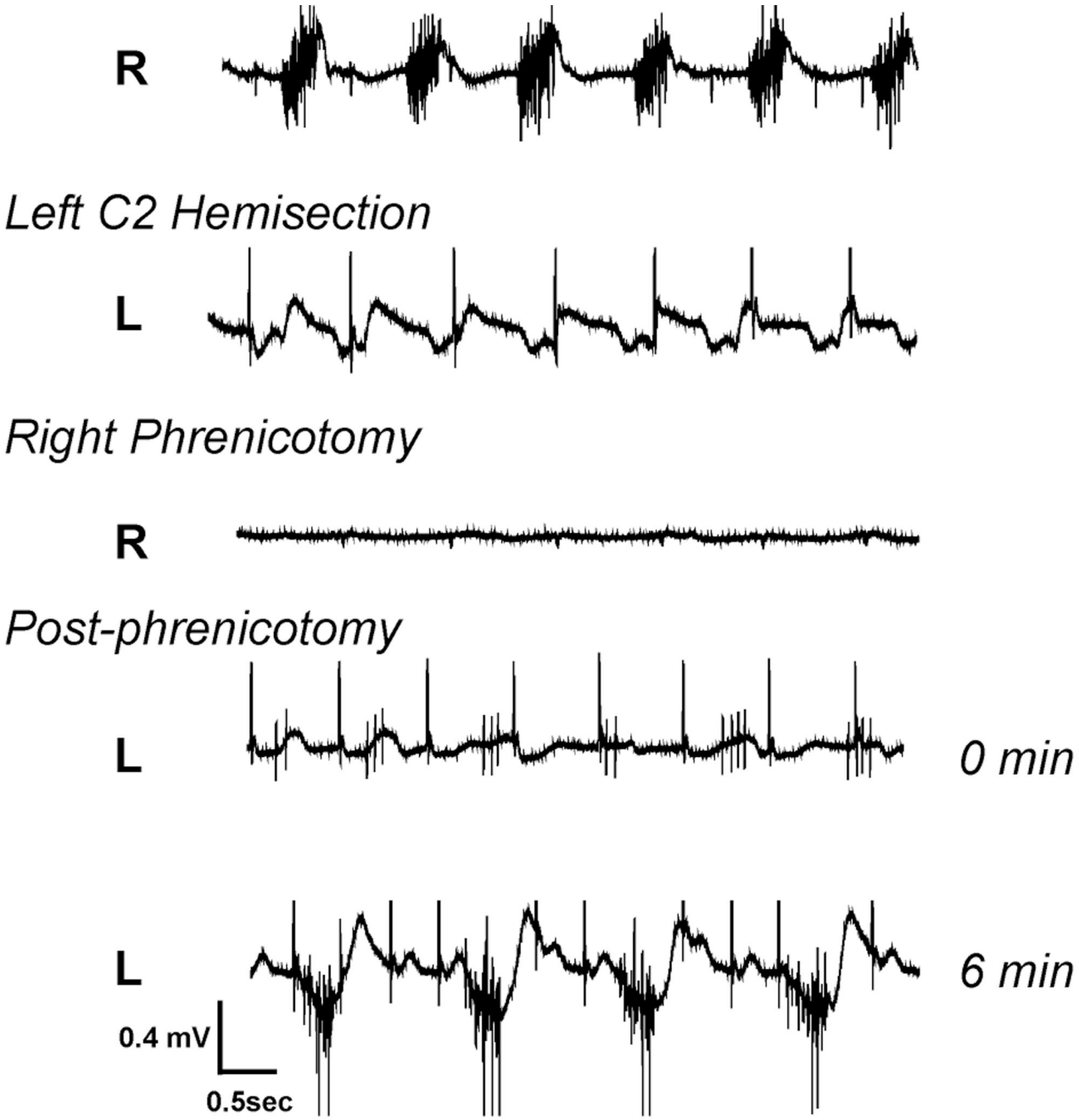

Figure 2. Crossed phrenic phenomenon in mice

Post-C2HS silencing of the left hemidiaphragm, a subsequent transection of the contralateral (right) phrenic nerve at 24h post-hemisection silences the right hemidiaphragm, and leads to immediate recovery (at 0min) of respiratory bursts that build with time (6min) in the previously quiet left hemidiaphragm; thus, demonstrating the crossed phrenic phenomenon. (reprinted from Minor and Seeds, 2008). Electrocardiographic artifacts are seen in these and subsequent electromyographic (EMG) traces.

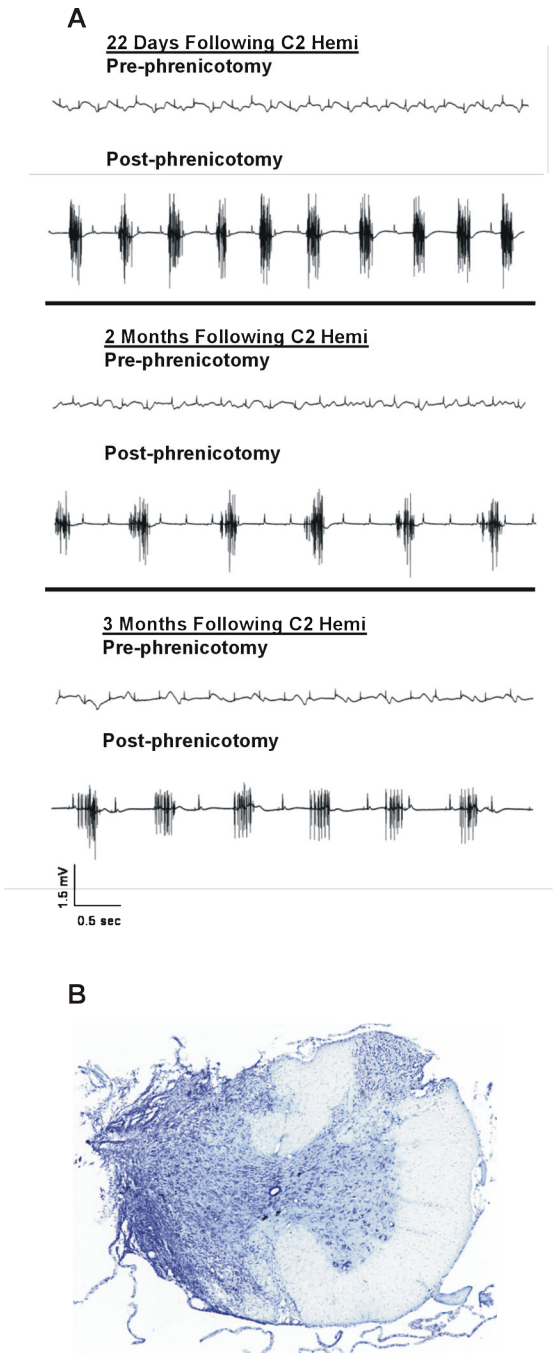

Figure 3. Mice fail to show spontaneous recovery following a C2-hemisection

Diaphragm activity and the CPP are only induced after phrenicotomy at one, two or three months post-C2HS (A). Extensive cellular changes are seen at C2 on the ipsilateral side of the spinal cord at three months post-C2HS, where extensive fill-in of the lesion site by astrocytes has changed the tissue morphology (B). In contrast to rats where a cavity forms at the SC lesion site, mice fill-in the lesion site with reactive astrocytes.

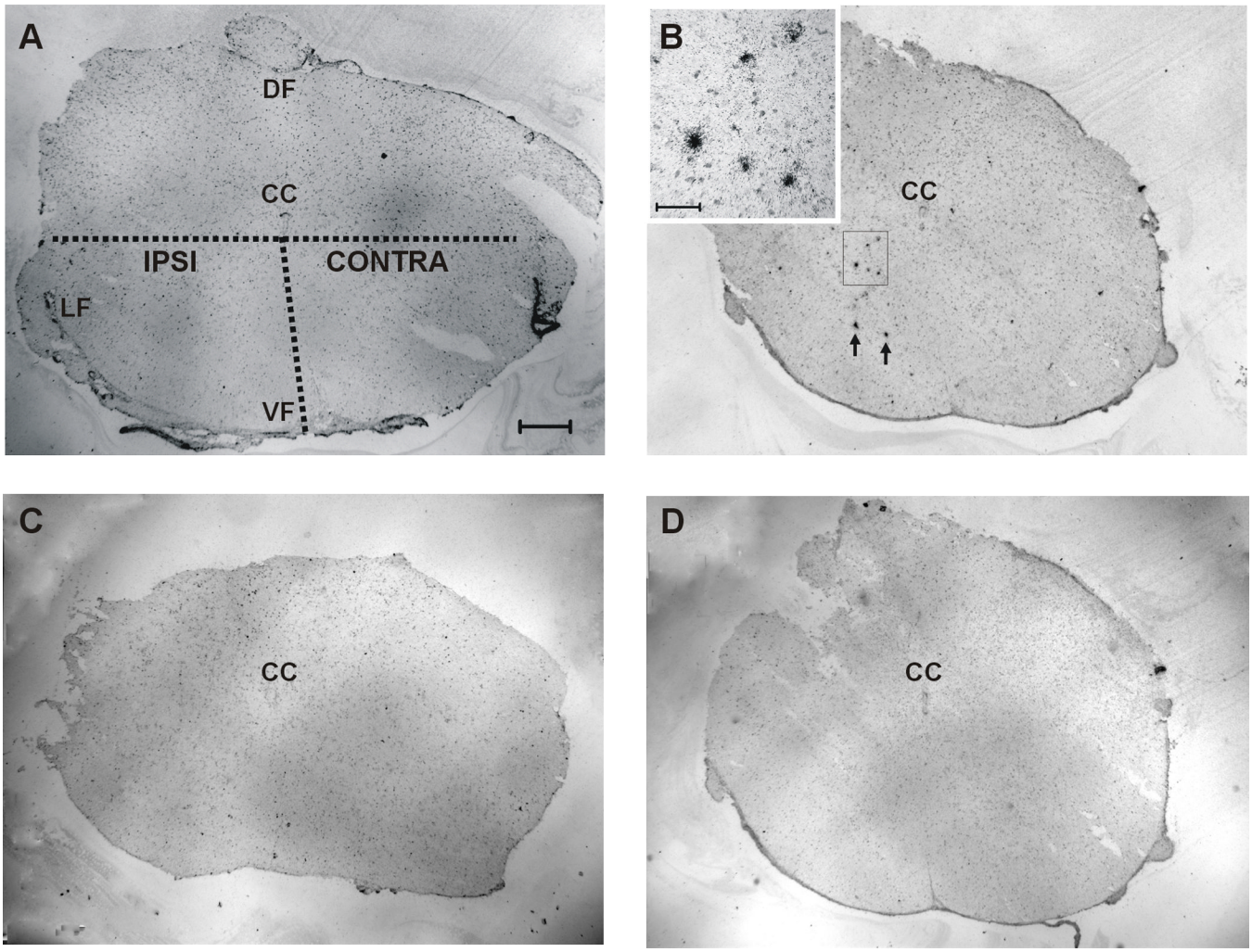

Figure 4. Induction of uPA mRNA in the PMN following C2-hemisection

In situ hybridization of uPA mRNA using a ^{35}S-cRNA probe in the C4 mouse spinal cord 1h following a left C2HS (B) compared to an uninjured mouse (A). Induced uPA mRNA expression is restricted to the ventral spinal cord in a limited number of large motorneurons in the ipsilateral PMN. The PMN induction is specific with no uPA mRNA expression in the contralateral spinal cord, or above the PMN at the C2-C3 interface (C), or below the PMN at the C6-C7 interface (D). The magnification bar in A is 250μm and the same for all four panels, while the bar in B inset is 50μm.

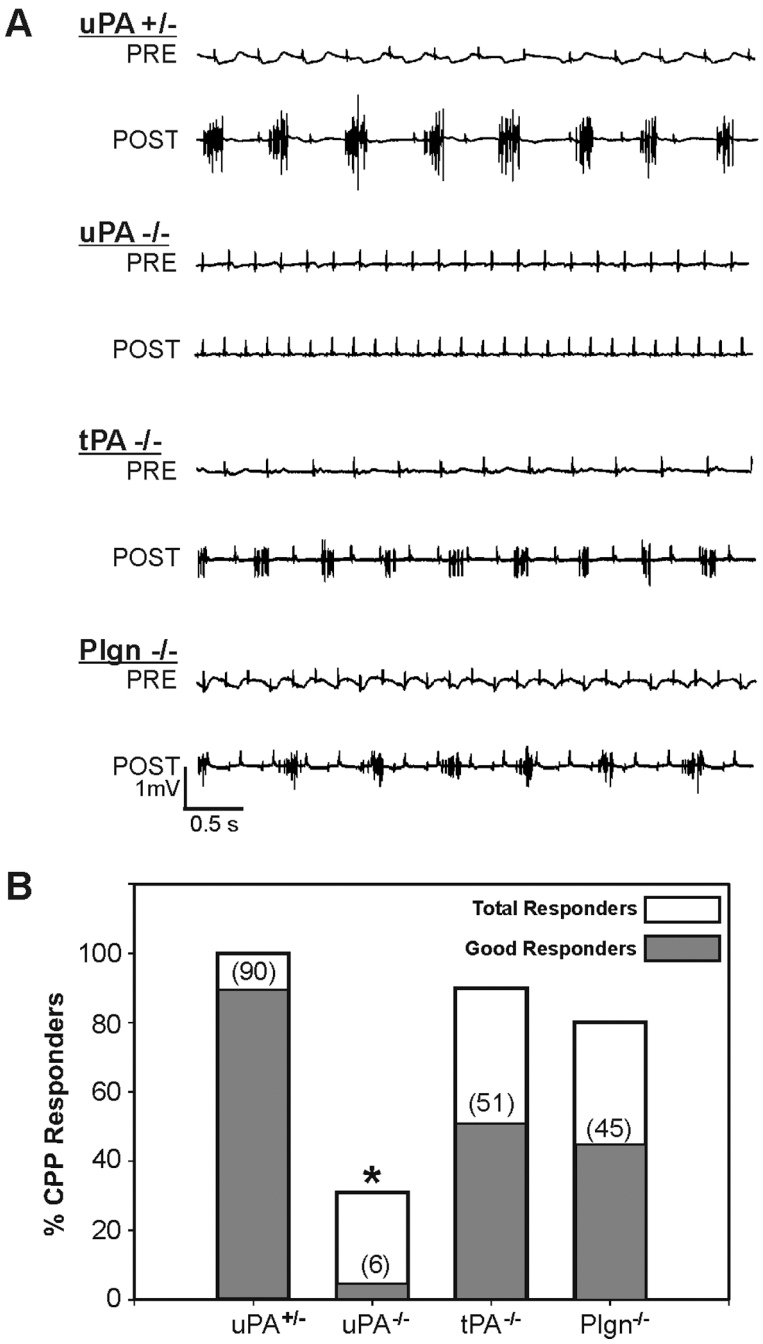

Figure 5. uPA knockout mice are impaired in the CPP response

(A) Typical electrophysiological recording of ipsilateral hemidiaphragm activity both pre- and post-phrenicotomy done 6h post-C2HS in uPA+/− mice as compared to uPA−/−, tPA−/− or plgn−/−knockout mice. Wildtype C57Bl/6 and uPA+/− mice showed a similar response. Most notable is a dramatic absence of a CPP response in uPA-knockout mice. (B) Graph compares the percentage of mice giving a good CPP response (as defined, Minor et al., 2006) or Total (good + weak) CPP response. Most of the uPA−/− mice fail to generate a good CPP response and their response is significantly different (**P*<0.001) from the heterozygote uPA+/− or wildtype C57Bl/6 mice. (reprinted from Minor and Seeds, 2008).

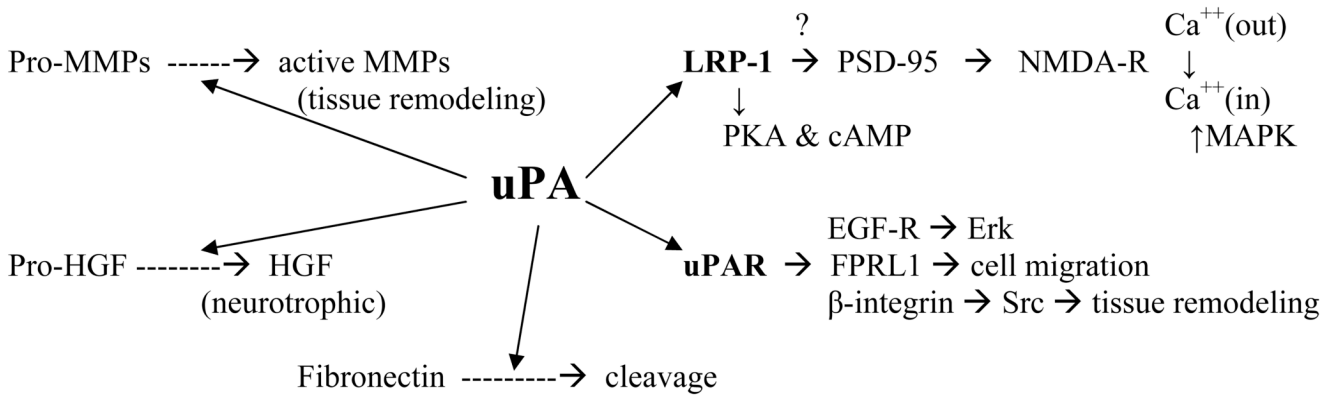

Figure 6. Potential actions of uPA during acquisition of the Crossed Phrenic Phenomenon

uPA proteolytic cleavage can activate both pro-matrix metalloproteases to their active MMPs, and can activate Pro-Hepatocyte Growth Factor to active HGF a potent neurotrophic factor for motor neurons. uPA can directly cleave fibronectin to influence integrin signaling. uPA binding to its cell surface receptor uPAR can set off several signaling cascades via uPAR interaction with EGF-R, FPRL1 or β-integrin. uPA also binds to the cell surface receptor LRP1 where it can up-regulate PKA and cAMP levels. Also uPA binding to LRP1 may act like tPA and lead to the phosphorylation of LRP1's cytoplasmic NPxY-motif to promote interaction with neuronal PSD95 that binds to and opens the NMDA-R for calcium influx leading to MAPK signaling and increased synaptic activity.

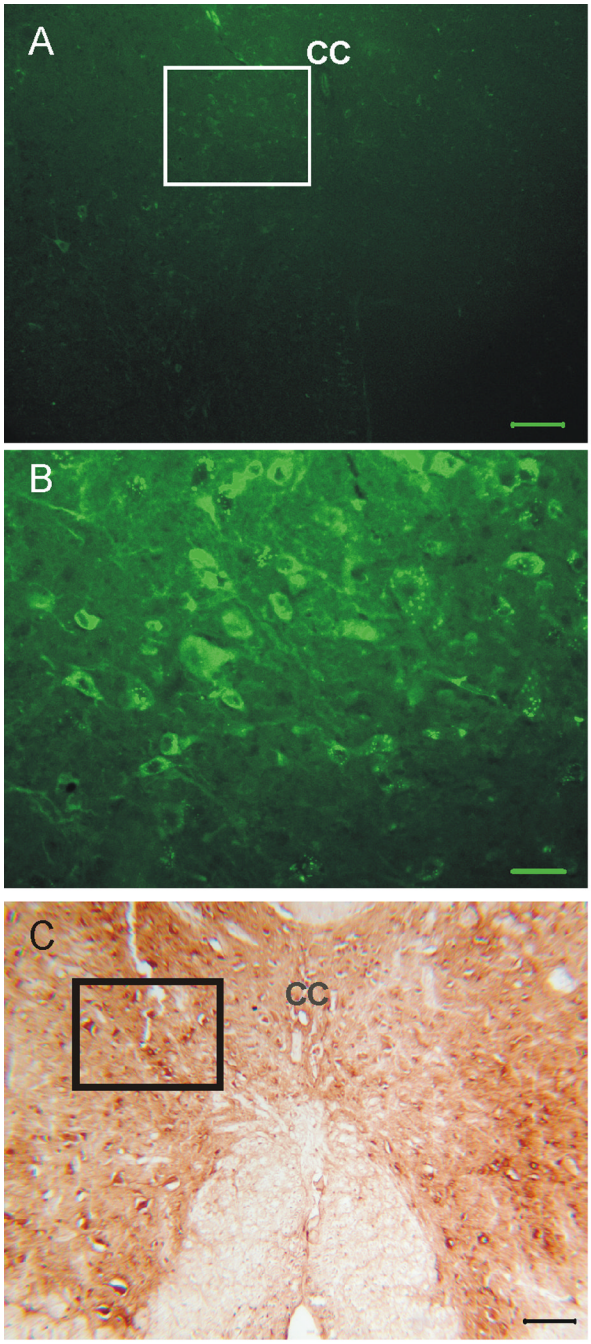

Figure 7. Up-regulation of LRP1 and uPAR in the PMN following C-2 hemisection

Antibodies to LRP1 (panels A & B) and uPAR (panel C) show ipsilateral induction of both uPA cell surface receptors in the C4-C6 ventral grey matter spinal cord 3h post-C2HS. Both receptors are up-regulated on neurons in the PMN (boxed areas in panels A and C). Panel B is the magnified boxed-area indicated in panel A. Bar in A & C = 100μm, and 25μm in B.

Table 1

Jak/Stat pathway gene expression following C2-hemisection (uPA−/− vs. wildtype)

Gene in JAK/STAT pathway	wt+/+ Hemi/Con	uPA−/− Hemi/Con	uPA−/− Con wt+/+ Con	uPA−/− Hemi wt+/+ Hemi
JAK 2	− 1.59	1.67	− 8.82	− 3.32
Stat 5b	1.95	− 1.34	− 1.34	− 3.49
EGF-R	− 1.36	− 34.54	− 2.28	− 57.76
Growth hormone receptor	1.07	− 2.89	− 8.41	− 26.03
Interleukin 10 receptor beta	− 2.48	− 2.17	− 1.79	− 1.57
Interleukin 4 receptor alpha	2.21	2.06	1.08	1.01
SH2B adaptor 1	1.28	− 2.68	1.01	− 3.39
SH2B adaptor 2	2.55	2.83	− 1.02	1.09
Src	1.91	− 4.76	− 24.93	− 226.29
Stam	1.71	1.85	− 22.78	− 21.01
Nuclear receptor 3C1	− 1.88	− 3.25	− 7.16	− 12.41
Myc	2.15	2.14	− 8.34	− 8.35
YY1 transcription factor	1.13	− 3.25	− 15.45	− 65.89
Smad 1	1.31	2.39	− 48.84	− 26.76
Smad 2	− 1.32	2.31	− 4.47	− 1.48
Smad 5	− 2.29	− 1.35	− 20.11	− 11.81
Oncostatin M	− 6.53	4.14	− 1.25	21.68
Protein Tyr-phosphatase non-recpt	3.71	5.17	− 1.96	− 1.41
Socs 2	− 1.07	− 3.01	− 1.84	− 5.18
Socs 3	22.51	23.75	− 1.27	1.35
Bcl2-like 1	2.57	1.64	− 3.14	− 4.93
Cyclin-dep kinase inhibitor 1A	5.04	5.24	− 1.16	− 1.12

Comparison of JAK/STAT pathway gene expression 4h post-C2HS analyzed using real time PCR and probes for specific mRNAs. Only differences in expression ($2^{-\Delta\Delta Ct}$) of two or greater are shown. Gene expression increased following hemisection relative to un-injured controls are blue, and those decreased are grey. Gene expression decreased in the uPA−/− mice compared to wildtype control mice are yellow. Those mRNAs that are decreased in the uPA−/− mice following hemisection as compared to un-injured uPA−/− mice or hemisected wildtype mice are red, while those that are increased are green.

Table 2

MAPK pathway gene expression following C2-hemisection (uPA−/− vs. wildtype)

Gene in MAPK pathway	wt+/+ Hemi/Con	uPA−/− Hemi/Con	uPA−/− Con wt+/+ Con	uPA−/− Hemi wt+/+ Hemi
MAP 3K 1	− 1.53	− 1.36	− 14.12	− 12.62
MAP 2K 5	1.21	4.09	− 4.32	− 1.27
MAP 2K 6	2.51	− 1.64	− 8.28	− 34.25
MAPK 14	− 3.21	1.04	− 1.21	2.75
Creb-1	− 3.56	4.18	− 4.66	3.19
Elk 1	− 3.39	1.09	− 2.38	1.31
Ets 1	− 2.97	− 1.79	− 1.48	1.12
Fos	− 1.03	3.59	− 2.38	1.55
Grb 2	1.16	1.92	− 3.32	− 2.01
Cyclin B2	− 2.66	9.86	− 11.24	2.23
Cyclin D1	− 1.61	1.06	− 4.92	− 2.91
Cyclin-dep kinase inhibitor 1A	5.35	7.79	− 1.45	1.01
Cyclin-dep kinase inhibitor 1C	− 4.14	1.53	− 20.97	− 3.31
Cyclin-dep kinase inhibitor 2C	122.79	12.57	1.95	− 5.02

Comparison of MAPK pathway gene expression 4h post-C2HS analyzed using real time PCR and probes for specific mRNAs. Only gene expression differences ($2^{-\Delta\Delta Ct}$) of two or greater are shown. Gene expression increases following hemisection are blue, and those decreases are grey. Gene expression decreases in the uPA−/− mice compared to wildtype control mice are yellow. Those mRNAs that are decreased in the uPA−/− mice following hemisection as compared to hemisected wildtype mice or their un-injured controls are red, while those that are increased are green.