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Plasminogen Activator Promotes Recovery Following Spinal Cord Injury

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Abstract

Plasminogen activators play an important role in synaptic plasticity associated with the crossed phrenic phenomenon (CPP) and recovery of respiratory function after spinal cord injury. A genetic approach using knockout mice lacking various genes in the plasminogen activator/plasmin system has shown that induction of urokinase plasminogen activator (uPA) is required during the first hour after a C2-hemisection for the acquisition of the CPP response. The uPA knockout mice do not show the structural remodeling of phrenic motor neuron synapses characteristic of the CPP response. As shown here uPA acts in a cell signaling manner via binding to its receptor uPAR rather than as a protease, since uPAR knockout mice or knock-in mice possessing a modified uPA that is unable to bind to uPAR both fail to generate a CPP and recover respiratory function. Microarray data and real-time PCR analysis of mRNAs induced in the phrenic motor nucleus after C2-hemisection in C57Bl/6 mice as compared to uPA knockout mice indicate a potential cell signaling cascade downstream possibly involving β-integrin and Src, and other pathways. Identification of these uPA-mediated signaling pathways may provide the opportunity to pharmacologically upregulate the synaptic plasticity necessary for recovery of phrenic motoneuron activity following cervical spinal cord injury.

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This research contribution is dedicated to the memory of a dear friend and mentor Marshall W. Nirenberg, who always said don't be afraid to explore the big questions in science.

Keywords

Plasminogen activator; uPA; uPAR; Crossed phrenic phenomenon; Spinal cord plasticity

Introduction

The crossed phrenic phenomenon (CPP) is one of the most striking 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 numerous mammalian species including the mouse (Minor et al. 2006) following a cervical C2 spinal cord hemisection, which paralyzes the ipsilateral hemidiaphragm by interrupting the descending flow of respiratory impulses from the medulla to phrenic motor neurons, which in turn innervate the diaphragm (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. These axons from the premotor rVRG descend in the lateral and ventral funiculi of the spinal cord and enter the gray matter between C3 and C6 to innervate phrenic motor neurons (Watson and Harvey 2009; Qui et al. 2010). A small population of these rVRG axons cross-over the spinal cord midline during development and contact ipsilateral phrenic motor neurons (Zimmer and Goshgarian 2005). Both the loss of ipsilateral rVRG input to the PMN as a result of the hemisection coupled with the increased activity from crossed contralateral rVRG fibers is thought to convert some of their presumptive synaptic contacts on phrenic motor neurons on the paralyzed side of the spinal cord from a “functionally ineffective” state pre-hemisection to a “functionally latent” state; i.e., 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; i.e., “the crossed phrenic phenomenon” (Fig. 2). Activation of these synapses and the crossed pathway requires an inter-operative 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 motor neurons (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. We propose that plasminogen activator is a likely candidate for playing an active role in these early cellular remodeling events promoting the CPP.

Plasminogen activators are best known for their proteolytic action, 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 (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 motor neuron 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). Here we show that the urokinase plasminogen activator (uPA) acting through its cell surface receptor uPAR is required for the CPP response in mice.

Materials and Methods

Male mice all on a C57Bl/6J genetic background 3–5 months of age were used in the following studies. Wild-type mice and those deficient for the expression of tPA or uPA (tPA −/−, uPA−/−) were originally obtained as breeding stock from The Jackson Laboratory (Bar Harbor, ME). Plasminogen deficient mice (plgn−/−) were the gift of Dr. Jay Degen, while mice lacking the uPA receptor (uPAR−/−) (Dewerchin et al. 1996) and the uPA mutant mice unable to bind to uPAR [*Plau*$^{(\mathbf{GFDhu/GFDhu})}$] (Connolly et al. 2010) were the generous gift of Dr. Thomas Bugge. All mice were bred locally and were cared for at the UCHSC Center for Laboratory Animal Care. All surgical and experimental procedures were approved by our Institutional Animal Care and Use Committee. Left C2-hemisection and right-side phrenicotomy were performed as previously described (Minor et al. 2006). In brief all animals were pre-medicated with an intramuscular injection of atropine sulfate (1 μg/25 g mouse) to reduce respiratory mucus secretions and anesthetized by an intra-peritoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). A dorsal C2 laminectomy was performed and the spinal dura cut. A left spinal cord lesion extending from the midline laterally through the lateral funiculus was completed caudal to the C2 dorsal roots. Following spinal cord lesion, paravertebral muscles and skin were closed with 4-0 silk sutures and animals allowed to recover. Contralateral phrenicotomies were carried out by a midline incision on the ventral surface of the throat followed by rostral retraction of the submaxillary glands to expose the trachea. Using a blunt probe, the right sternomastoideus muscle and clavicle were moved laterally and caudally to expose the phrenic nerve. Finepoint #5 forceps were used to isolate and sever the nerve. The caudal end of the cut nerve was pulled gently to remove any possible respiratory input from the accessory phrenic nerve.

Following an abdominal laparotomy to expose the diaphragm just prior to induction of CPP by contralateral phrenicotomy, silver bipolar electrodes were placed on the left and right hemidiaphragm and electromyography (EMG) recordings were made (as described, Minor et al. 2006). While recording, animals were observed to ensure that the EMG signal was a respiratory-related activity and all recordings were performed while the animals were breathing spontaneously. Spinal cord C2 hemisections were considered functionally complete only when there was a complete absence of respiratory activity on the left ipsilateral hemidiaphragm.

EMG recordings were taken at various time points from 1 to 30 min following phrenicotomy in each animal. Each recording was obtained over a 5-second interval. A “good” CPP response is one that built with time, and the peak response had at least one respiratory burst during the 5 s recording period with 10 or more spikes per burst and at least one respiratory burst with an amplitude of at least 0.5 mV. A “weak” CPP response is characterized by a small amplitude (less than 0.5 mV) or less than 10 spikes/respiratory burst during the 5 s

recording period. “Non-responders” showed no respiratory signal in the ipsilateral hemidiaphragm at all.

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions from the C4-5 spinal cord ventral gray matter containing the phrenic motor nucleus on the hemisected or the contralateral side of the mouse spinal cord in wildtype or uPA−/− mice. mRNAs in these phrenic motor nucleus preparations were compared for components of JAK/STAT or MAPK pathways using real-time PCR with SABiosciences pre-coated 96-well plates following the procedure described by the manufacturer. Relative differences between mRNA expression among the samples was determined by normalizing the ΔCt to 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. Microarray analysis of these same mRNA samples was assayed on the Affymetrix Mouse Gene 1.0ST chip (Affymetrix, Santa Clara, CA). Generation of double-stranded cDNA preparations and labeling of cRNA hybridization to the arrays, washing, and scanning were performed according to the standard Affymetrix protocol, and performed by the Molecular Genomics Core of the University of Colorado Cancer Center. Their Bioinformatics Group compared each of the four RNA samples in pairs one-to-the-other, and calculated the fold-difference between samples for each probe sequence on the chip. The top 10% of genes showing the greatest fold-differences in expression ratios between hemisected wildtype and hemisected uPA−/− animals was compared. For the data in Table 1 the values were corrected for those differences between the un-operated uPA−/− and wildtype mice.

Results and Discussion

In earlier studies (Minor and Seeds 2008) we showed that uPA mRNA is rapidly induced in ipsilateral phrenic motor neurons following a C2-hemisection. This uPA mRNA expression is transient and seen from 0.3 h to 8 h but absent by 20 h post-hemisection, as is the induced uPA protein. The importance and relationship of PA induction to the synaptic changes and activation of phrenic motor neurons during the CPP was assessed using knockout mice. Mice deficient in uPA, tPA or plasminogen (Plgn) genes, were compared to their heterozygous littermates or wild-type C57Bl/6 mice for recovery of diaphragm function via the CPP at 6 h post-hemisection, when about 90% of wildtype mice show a good CPP response (as defined by Minor et al. 2006). All the uPA ± heterozygotes showed a CPP response at 6 h, 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. 3). However, there is a striking failure by most uPA−/− mice to generate a good CPP response at 6 h. 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 20 h 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. Furthermore, the uPA−/− mice failed to undergo the characteristic synaptic remodeling events of an increase in multiple axo-dendritic synapses and a lengthening of the synaptic active zone on phrenic motor neuron dendrites, coincident with the acquisition of a CPP response as elicited in wildtype mice (Minor and Seeds 2008).

Since most Plgn−/− mice show a good functional CPP response, suggesting that plasmin formation by uPA may only play a minor role, we explored how uPA might act to bring about the structural changes and functional recovery that occur during the CPP. Although, uPA is capable of proteolytically cleaving substrates other than plasminogen, including the

extracellular matrix molecule fibronectin (McGuire and Seeds 1990), activating extracellular pro-matrix metalloproteases (Keski-Oja et al. 1992; Baramova et al. 1997; Siconolfi and Seeds 2003), and activating the pro-neurotrophic factor HGF (Naldini et al. 1992; Mars et al. 1993; Thewke and Seeds 1996), it may act 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 participate in cell signaling cascade and are dramatically up-regulated in the ipsilateral PMN immediately following a C2-hemisection (Seeds et al. 2009). Therefore, we next explored whether uPA is promoting the CPP response via its receptor uPAR.

Two different mouse mutants deficient in the binding of uPA to uPAR have been used to assess the importance of uPA signaling via uPAR. The uPAR−/− knockout mouse (Dewerchin et al. 1996) and a uPA mutant mouse (*Plau*$^{\mathbf{GFDhu/GFDhu}}$ knockin) (Connolly et al. 2010) that retains all uPA activities except that it fails to bind to uPAR, were compared to wildtype mice for their ability to generate a good CPP response (as defined in “Materials and methods” section) and demonstrate functional diaphragm recovery at 6 h post-C2 hemisection. As seen in Fig. 4, neither the uPAR−/− knockout nor the *Plau*$^{\mathbf{GFDhu/GFDhu}}$ knockin mouse are capable of generating a good CPP response. These mutant mice usually expire following some intercostal-mediated gasping shortly after phrenicotomy. These studies indicate that uPA binding to uPAR possibly activating cell signaling cascades is required for the CPP response.

uPA binding to its glycosyl phosphatidylinositol (GPI)-linked receptor uPAR leads to interaction with several different cell surface molecules in dynamic signaling complexes that produce a variety of activities favoring structural remodeling events in different tissues including cell migration and invasiveness (for review see Blasi and 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 (Degryse et al. 1999; Mazzieri et al. 2006). Furthermore, uPAR interaction with β1-integrin leads to activation of a Src pathway (Monaghan-Benson and 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. uPAR may interact with these proteins and the pathways they mediate either directly or indirectly.

Changes in gene expression and activation of specific signaling pathways downstream from uPAR activation were assessed with mRNA isolated from the ipsilateral ventral horn gray matter of the C4-5 spinal cord of un-operated or 4 h following a C2-hemisection of wildtype or uPA−/− mice. Real-time PCR was performed on 96-well microarray plates containing probe sequences for genes of the JAK/STAT or MAPK pathways (SABiosciences) using these four different mRNA samples. Comparison of un-operated wildtype to uPA−/− mice controlled for gene expression differences due to knocking-out uPA, and mRNA levels were similar except for major decreases in Src, SH2B adaptor 1, EGF-R, Socs2, and YY1 transcription factor in the un-operated uPA−/− mice. A comparison, as reflected in $2^{-\Delta\Delta Ct}$ fold differences of RNAs, of C2-hemisected wildtype and uPA−/− mice showed even greater differences in these same genes, as well as uPA−/− decreases in Stat 5b, Growth hormone receptor, nuclear receptor 3C1, MAP2k6, cyclin-dep kinase inhibitor 2C and Bcl2-like 1 gene expression in response to hemisection (Seeds et al. 2009).

A complete analysis of mouse gene expression was assessed in these same RNA samples taken from C4-5 ventral spinal cord of un-operated wildtype and uPA−/− mice, and of C2-hemisected wildtype and uPA−/− mice and carried out on the Affymetrix Mouse Gene 1.0ST chip. C2-hemisected mice as compared to the un-operated wildtype mice led to an increased expression in several of the same kinases and transcription factors as detected by

the real-time PCR, as well as cell surface receptors including uPAR. A comparison of un-operated wildtype and uPA−/− mice showed that the gene knockout led to similar changes as seen in the real-time PCR. More importantly a comparison of mRNA from uPA−/− hemisected mice to that from hemisected wildtype mice, when corrected for those gene differences seen in the un-operated uPA−/− mouse, showed trends of difference in molecules related to axonal pathfinding, synaptic activity, transcription factors, growth factors, kinases and receptors including IGF, FGF, patched, notch, Eph (B4, B2, A2), proto-cadherins, and vitronectin (Table 1). Interestingly the axon midline crossing factor Robo 3, which shows minimal expression in the adult spinal cord, also stood out in the RNA from C2-hemisected wildtype mice. Thus, these differences reflect primarily gene expression events resulting from uPA action that are required for the successful CPP response.

Current studies, using western blotting and immunohistochemistry, are assessing the relative amounts of the protein gene product for many of these up-regulated mRNAs, that may indicate downstream signals from uPA binding to uPAR. In this regard, both vitronectin and Src, known to play important roles in uPAR signaling, show noticeable differences between the wildtype and uPA−/−mRNAs following hemisection in both real-time PCR and microarray analysis; thus suggesting that uPA/uPAR cell signaling may involve interaction with *β*-integrin and subsequent up-regulation or activation of Src.

In summary, uPA plays a critical role in the acquisition of the crossed phrenic phenomenon via its binding to uPAR and subsequent cell signaling, possibly with vitronectin/*β*-integrin leading to Src activation and synaptic remodeling of phenic motor neuron dendrites.

Phrenic motor neuron remodeling increases both the number of axo-dendritic contacts and their area of active zone contact with those rVRG axons that cross the midline (Minor and Seeds 2008). These findings suggest the potential for using plasminogen activators, or their agonists, or drugs mimicking their action in a therapeutic regenerative model for spinal cord injury.

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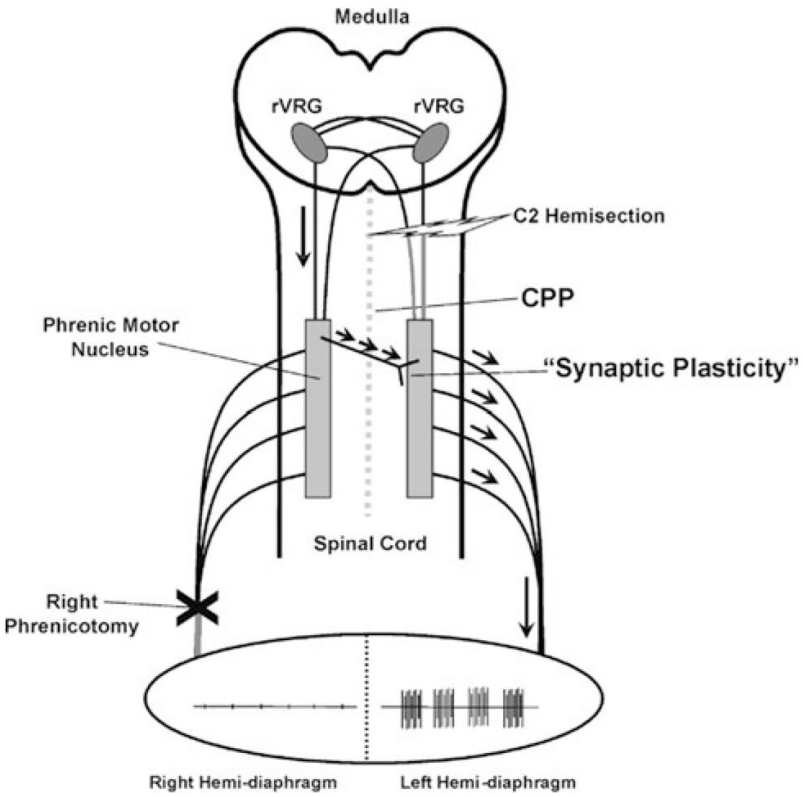

Fig. 1.

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 motor neurons extending from C3 to C6. Thus, blocking phrenic motor neuron 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)

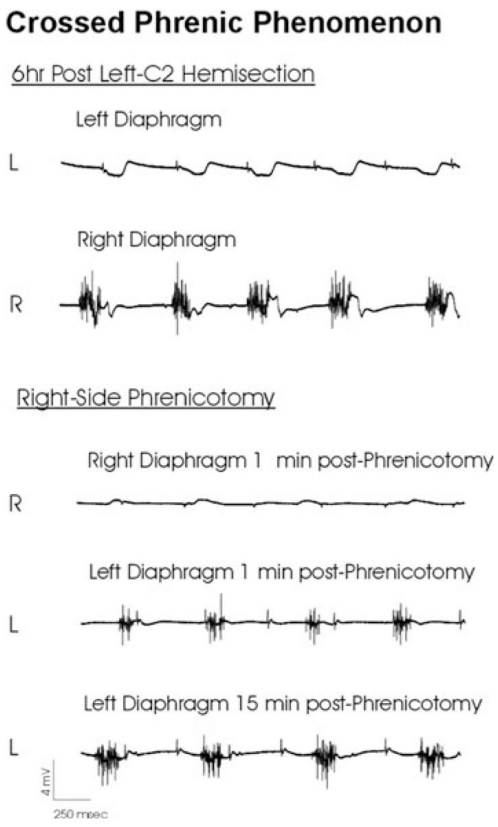

Fig. 2.

Crossed phrenic phenomenon response. Post-C2-hemisection silencing of the left hemidiaphragm of a mouse, as compared to the active right hemidiaphragm at 6 h post-hemisection. Immediately prior to a transection of the contralateral right-phrenic nerve, which silences the right hemidiaphragm and leads to immediate recovery (at 1 min) of respiratory bursts that build with time (15 min) in the previously quiet left hemidiaphragm; demonstrating the crossed phrenic phenomenon

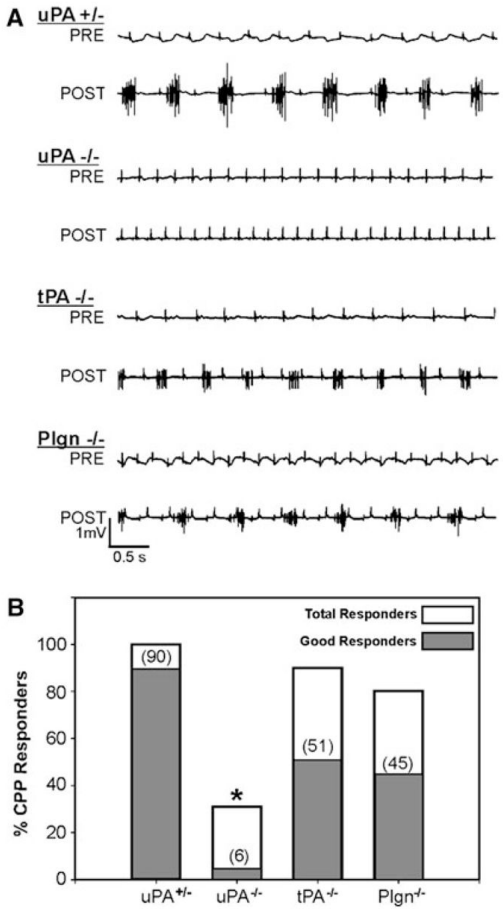

Fig. 3.

uPA knockout mice are impaired in the CPP response. **a** Typical electrophysiological recording of ipsilateral hemidiaphragm activity both pre- and post-phrenicotomy done 6 h post-hemisection in C57Bl/6 uPA ± mice ($n = 20$) as compared to uPA−/− ($n = 32$), or tPA −/− ($n = 16$) or plgn−/− ($n = 18$) knockout mice. A dramatic absence of a CPP response is seen in uPA−/− mice as compared to others. **b** Graph compares the percentage of mice giving a Good (as defined in “Materials and methods” section) CPP response, or Total (good + weak) CPP response. Most of the uPA−/− mice ($n = 32$) fail to generate a Good CPP response and their response is significantly different (*$P < 0.001$) from the heterozygote or wildtype mice. They also fail to show a CPP at 20 h post-hemisection (reprinted from Minor and Seeds 2008)

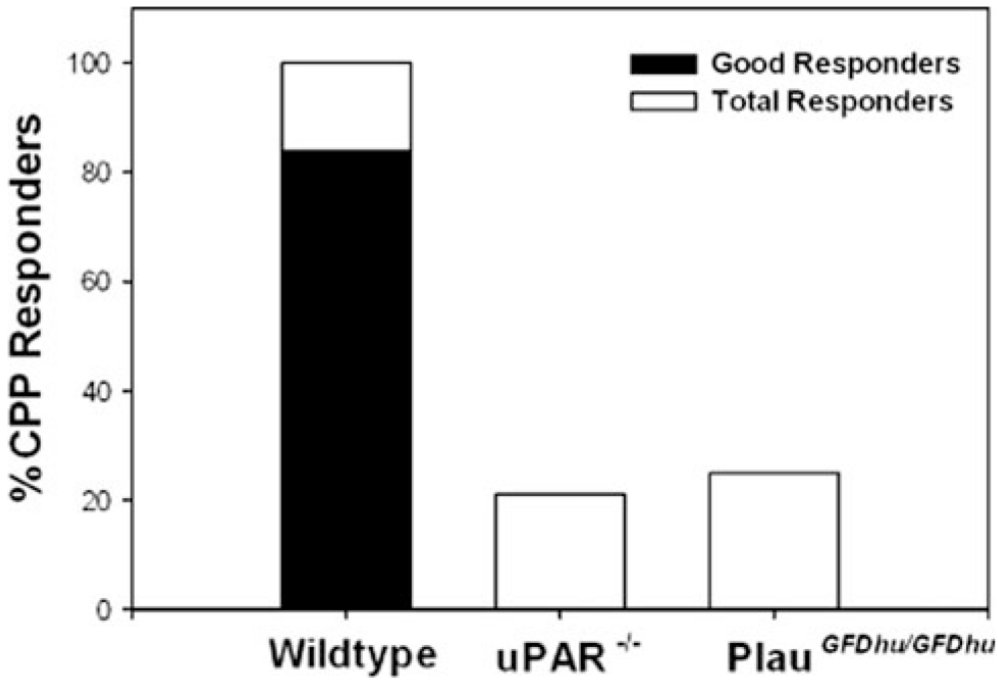

Fig. 4.

uPA binding to uPAR is required for the CPP response. The graph compares the percentage of mice giving a Good CPP response or Total (good + weak) CPP response, when assayed at 6 h post-hemisection. uPAR−/− knockout mice (n = 14) fail to generate a Good CPP response as compared to wildtype C57Bl/6 mice. Similarly, the uPA knock-in ($Plau^{GFDhu/GFDhu}$) mice (n = 8), whose growth factor domain of uPA is modified and unable to bind to uPAR, also fail to generate a Good CPP response. These studies suggest that uPA binding to uPAR leading to cell signaling events is required for the CPP response

Table 1

Microarray analysis of potential genes related to the CPP response. Increased mRNAs in wildtype as compared to uPA−/− mice 4 h post- C2-hemisection

Protein type
Matrix/Adhesion: collagen 8; vitronectin; P-CAM; fibronectin
Cytoskeleton: γ-actin; β-tubulin
Growth factor: IGF binding proteins 2,4,7; connective tissue growth factor; BMP-8; PDGF-R; FGF 5,11; somatostatin-R; neuron-derived neurotrophic factor; CNTF-R; BMP-4; NGF-R; Src
Axonal growth and pathfinding: Eph B4; MAPKAPK 3; Rho-GEF; Rho; Rho-GAP; Eph A2; Robo-3; Sema 6c; Eph B2;
Ion channels: K + inwardly rectifying channel 2.4; gap junction membrane channel 5; voltage-gated K + channel; Na + channel Nav1.8
Receptors: Patched; Notch 3; neuropeptide Y receptor; LRP 3; thrombin-R
Synapses: protocadherins 12 and 18; synaptic Ras GTPase GAP; synaptopodin; neuroligin; rapsyn; nicotinic ACh-R; synaptoporin; CAMkII inhibitor
Protease and inhibitors: uPA; serpina1c; calpain 10; MMP-8; MMP-25
Neural protein: α-synuclein; proteolipoprotein 2; S100; presenilin 2; LIM; β-synuclein
Transcription factors: Hoxb8; Hoxa4; Sox1; Snip 1; neurogenin; engrailed; PAX-1; Sox4
Cell division: cyclin A1; cdc42; cyclin-dep ki2C; cyclin B3; Cdk 5
Apoptosis: caspase 7; Bax

Genes were selected from among the top 10% of genes showing the greatest difference in mRNA expression following C2-hemisection in wildtype mice relative to uPA−/− mice. The genes are listed in each category by decreasing difference in expression between genotypes. The listing has been corrected for those genes that are simply down-regulated in the un-operated uPA knockout mouse as compared to gene expression in wildtype mice