

PAR-1 activation by SFLLRNP decreases myelin deposition on lumbar motor neuron axons as assessed with cupric silver staining

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Working through protease-activated receptors (PARs), serine proteases have been shown to play important roles in neuronal and glial cell survival during development and in neurodegeneration. Past studies in chick embryos have shown that PAR-1 activation during the period of lumbar motor neuron programmed cell death (PCD) leads to a decreased number of surviving motor neurons by embryonic day 10 (E10). Retrospective analysis of these tissues revealed increased thionin staining in the white matter of these spinal cords, interesting because thionin does not normally stain myelinated areas. We hypothesized that PAR-1 activation decreases myelin deposition in developing spinal cords. To activate PAR-1, embryos were treated with the amino acid sequence SFLLRNP for five consecutive days beginning on E5. Embryos were sacrificed on E10, prepared for histology, and stained with cupric silver. The ventral hemispheres of lumbar sections were examined for the degree of demyelination as characterized by punctate or linear silver markings in the white matter. Experimental embryos were found to exhibit statistically more punctate markings and linear markings. This study shows that in addition to decreasing motor neuron survival, PAR-1 activation potentially decreases the conducting ability and viability of the surviving motor neurons.

Abbreviations: PAR – protease-activated receptor; E – embryonic day; SFLLRNP – serine-phenylalanine-leucine-leucine-arginine-asparagine-proline; PCD – programmed cell death; ALS – Amyotrophic Lateral Sclerosis

Keywords: programmed cell death; ALS; neurodegeneration

Introduction

Protease activated receptors (PARs), are transmembrane cell surface receptors activated by proteolytic cleavage of the extracellular domain. Extracellular serine proteases remove a portion of the N-terminus. While the removed portion is digested by circulating macrophages, the remaining amino acid sequence on the N-terminus undergoes a conformational change allowing it to bind to its own transmembrane segment. This sequence of events results in the receptor's activation (Steinhoff et al., 2005). In PAR-1, the protease thrombin activates the receptor by

exposing the amino acid sequence serine-phenylalanine-leucine-leucine-arginine-asparagine-proline, or SFLLRNP (Vu et al., 1991; reviewed in Salman et al., 2003). By exposing the receptor to SFLLRNP (Turgeon et al., 1998; Salman et al., 2003), or other similar truncated peptides (such as SFLLRN) from the N-terminus, thrombin's effect on PAR-1 is replicated (Smirnova et al., 2001).

The role of PAR-1 activation during coagulation and immune responses is well established (Steinhoff et al., 2005), but PAR-1's role in the nervous system is less

understood. Following the discovery of PAR-1 mRNA localization in motor neurons, dorsal root ganglia (Niclou et al., 1998), and oligodendrocytes (Wang et al., 2004), to name a few, several studies have emerged showing that PAR-1 activation affects neuronal and glial cells in various ways depending on the cell type, period of development, and other factors such as trauma (reviewed in Turgeon and Houenou, 1997). Stunted neurite growth and increased apoptosis in motor neurons is seen following PAR-1 activation both *in vitro* (Turgeon et al., 1998) and *in vivo* (Turgeon et al., 1999). However, PAR-1 activation can be neuroprotective depending on the concentration of thrombin (Donovan and Cunningham, 1998), and the specific physiological condition of the system (reviewed in Turgeon et al., 2000). For example, Vaughan et al., (1995) documents that treatment with SFLLRN elicits neuroprotection for hippocampal neurons and astrocytes during periods of hypoglycemia and oxidative stress, while under normal circumstances these concentrations of thrombin are toxic. Therefore, PAR-1 and thrombin may be involved in the balance between neuroprotection and neurodegeneration throughout the nervous system (Rohatgi et al., 2004).

While neurodegeneration is often associated with disease, it is not always detrimental to the organism; in fact, it is normal for developing organisms to undergo deliberate modification of neural elements, including the axon (Luo and O'Leary, 2005). These modifications, made through retraction and/or degeneration, are responsible for establishing meaningful connections and are involved in neural plasticity (Luo and O'Leary, 2005). In developing systems, neurodegeneration is hypothesized to be accomplished through cell-intrinsic programs, including receptor

activation and axon pruning that results in synapse elimination (Luo and O'Leary, 2005). Such plasticity is normally observed during nervous system development and maintenance (reviewed in Oppenheim, 1991), but neurodegeneration that occurs outside of development could lead to diseases, such as Amyotrophic Lateral Sclerosis (ALS) or exacerbate damage from a spinal cord injury.

While PAR-1 activation has been shown to decrease motor neuron survival in development (Turgeon et al., 1998; Smirnova et al., 1998; Turgeon et al., 1999) and decrease neurite length *in vitro* (Jalink and Moolenaar, 1992; Turgeon et al., 1998), we do not know how the remaining neurons have been affected. However, the use of previous slides from our lab provided a clue as to how the remaining cells of the spinal cord may be affected by SFLLRNP treatment. Slides that had previously been used to identify and quantify motor neurons were being used to train new research students in histology when the authors noticed increased thionin staining in the white matter of those embryos that were treated with SFLLRNP. Thionin is a typical hydrophilic stain used in nervous system histology, but it does not have a high affinity for myelin, which is hydrophobic. The presence of thionin staining in the white matter suggests that myelin is missing from the white matter of these tissues.

To replicate the conditions under which the aforementioned slides were obtained and to determine if PAR-1 activation was responsible for myelin loss and thus, the abnormal staining in the white matter, chicken embryos were treated with SFLLRNP or PBS (vehicle control). However, instead of staining with thionin, cupric silver histochemistry was employed. Cupric silver was used since it has been shown to depict accurately myelin loss (De Olmos et al., 1994; Festoff et al., 2004;

Grant et al., 2004), as it not only binds to the unmyelinated areas, but also because the deposition of the individual silver grains allows for the quantification of punctate (spots of demyelination) and linear (tracts of demyelination) marking. Axons in the white matter of the spinal cord should be completely encased in myelin with regularly spaced breaks or nodes of Ranvier. The range of the silver grain diameter used in this staining procedure is between 5-15 μm , which is much larger than the nodes of Ranvier at 1-2 μm , so that any silver markings seen represent spaces created by missing myelin (Switzer, 1991). Based on our previous observations that SFLLRNP treatment increased thionin deposition, presumably due to decreased myelin, we expected to see increased cupric silver staining in the white matter, supporting our hypothesis that PAR-1 activation leads to decreased myelin deposition during development.

Material and Methods

Fertilized *Gallus gallus* eggs were obtained from Charles E. Morgan Poultry Center (Clemson, SC). On E3, the eggs were windowed to reveal the chorioallantoic membrane containing the external vascular supply connecting the embryo to the extra-embryonic yolk and surrounding fluids. The resultant holes in the shells were resealed with transparent tape before returning them to the incubator (Percival Scientific; Atlanta, GA; 37°C, 95% relative humidity). Experimental embryos (n=20) were treated daily (E5-E9) by pipetting 200 μL of 100 μM SFLLRNP (Anaspec, San Jose, CA) onto the chorioallantoic membrane, whereby the solution was absorbed and delivered throughout the embryo. Control embryos (n=20) were treated in a similar manner with 200 μL of 1X phosphate buffered saline

(PBS; Sigma, St. Louis, MO). Before treating the embryos, the allotted volumes were incubated at 37°C for a period no longer than 5 minutes to minimize the shock to the embryos. On E10 the embryos were sacrificed by decapitation and fixed in 4% paraformaldehyde (Fisher Scientific, Atlanta, GA) overnight and transferred to 70% ethanol for another overnight period. Embryos were then post-fixed in a 30% sucrose solution (Fisher) made up in 4% paraformaldehyde and were kept in the post-fixative until they sank below the surface. Cryostat (2800 Frigocut N, Reichert Jung; Depew, NY) sections were cut at 25 μm (-27°C) and every tenth section was mounted on gel-coated slides (Lab Scientific Inc., Livingston, NJ).

Cupric Silver Staining

Cupric silver staining was carried out using the protocol by De Olmos et al. (1994). As directed, all glassware was washed with neutralized nitric acid (Fisher). The pre-impregnation solution [198.71 mg AgNO_3 (Sigma, St. Louis, MO), 198.71 mL dH_2O , 1.20 mL n-butyric acid (Fisher), 91.40 mg dl- α -alanine (Sigma), 0.5% $\text{Cu}(\text{NO}_3)_2$ (Fisher), 0.5% $\text{Cd}(\text{NO}_3)_2$ (Alfa Aesar, Ward Hill, MA), 0.5% $\text{La}(\text{NO}_3)_3$ (Sigma), 0.5% Neutral Red (Fisher), 1.987 mL pyridine (JT Baker, Phillipsburg, NJ), 1.987 mL triethanolamine (Sigma), 3.97 mL isopropanol (Carolina Biological Supply, Burlington, NC)] was microwaved at 60% power to reach 50°C and cooled for 1 hour. Slides were dipped in dH_2O and then transferred to the pre-impregnation solution at 50°C for 45-50 minutes. After this period, slides were cooled to room temperature. The diamine solution [6.975 g AgNO_3 (Sigma), 84.6456 mL dH_2O , 67.717 mL 100% ethanol, 0.846 mL acetone (Fisher), 0.4% LiOH (Sigma), 11.004 mL NH_4OH (Sigma)] was made up during this cooling period. Slides went through two

washes of acetone for 30-60 seconds before being moved into the diamine solution for 45-50 minutes under constant agitation (Orbitron Rotator II 260250; Fisher). The reducer solution [10% formalin (Sigma), 1% citric acid anhydrous (Sigma), 21.32 mL 100% ethanol, 189.53 mL dH₂O) was made up during this time. Slides were then moved to the reducer solution for 25 minutes at 32°C. While in the reducer solution for the 25 minutes several additional steps were taken. The protocol required that the reducer solution be constantly stirred for the first 2 minutes. After an additional 3 minutes in the reducer solution, 0.65 mL of the diamine solution was added and again at 5 minute intervals until the 20 minute mark. Slides were placed in 0.5% glacial acetic acid (Fisher) for 1-2 minutes, and then moved to dH₂O for 15 minutes. The slides were left overnight in another dH₂O wash. Bleaching solutions were made up before starting the second part of the stain. Slides were washed in the first bleaching solution [6% potassium ferricyanide (Sigma) in 4% potassium chloride (Acros Organics, Morris Plains, NJ), 4.216 mL undiluted lactic acid (Fisher)] for 20-60 seconds, being careful not to completely remove the color. The slides were moved to a 2 minute dH₂O wash before exposing them for 15-20 seconds to the second bleaching solution [0.06% potassium permanganate (JT Baker), 5% sulfuric acid (Fisher)]. The slides were placed in dH₂O and remained there while 2% sodium thiosulfate (Sigma) and Kodak Rapid Fixer (Eastman Kodak Company, Rochester, NY) were made up. Slides were put in 2% sodium thiosulfate (Fisher) for 1-3 minutes under constant agitation, then

moved to dH₂O for 2-5 minutes before being put in Rapid Fixer for 1-5 minutes. Slides were placed in dH₂O before being serially dehydrated (70%-95%-100% EtOH) ending in SafeClear (Fisher). Slides were then coverslipped.

Microscopy

Slides were observed at 400X total magnification using a light microscope (Meiji; Martin Microscope, Easley, SC) and photographed with a Nikon Coolpix 990 camera. The photographs were imported and analyzed with PhotoImpression, version 5.1.8.83 (ArcSoft Inc., Fremont, CA). All of the white matter from the lowest point of the central canal encompassing both ventral horns of the spinal cord was examined for the number of punctate and linear markings (n=25 cross sections per embryo) (Fig 1). The average number of punctate and linear markings were then determined per embryo.

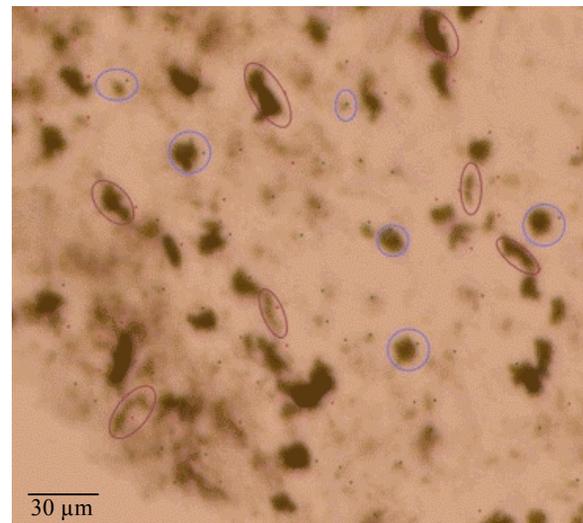


Figure 1. Characterization of demyelination as shown by cupric silver staining of lumbar spinal cord sections in *Gallus gallus* embryos following PAR-1 activation by treatment with SFLLRNP. Isolated circular markings were classified as punctate and markings of extended length were classified as linear. The white matter of the spinal cord pictured here displays two distinct markings: punctate and linear. Punctate markings are surrounded by circles and linear markings surrounded by ovals.

Statistical Analysis

Statistical analyses were computed using JMP IN, version 5.1.2 (SAS Institute, 2001). After the data was determined to be normally distributed (Goodness of Fit test), a Student’s t-test ($\alpha=0.05$) was used to analyze the differences in the number of punctuate markings between control and SFLLRNP-treated sections and then in linear markings between the two groups.

Results

Using a Goodness of Fit test all data was found to be normally distributed. Increased deposition of cupric silver was observed in the embryos treated with SFLLRNP (Fig. 2A-D). Experimental embryos were found to have a significantly higher number (mean \pm SEM) of linear markings (264.5 ± 67.90 ; Student’s t-test: $df=6$; $p=0.0280$) as well as a significantly higher number of punctate markings (180.25 ± 44.53 ; Student’s t-test: $df=6$; $p=0.0312$) in comparison to the control embryos (4.3 ± 2.3 punctate and 2.3 ± 1.45 linear; Fig. 3).

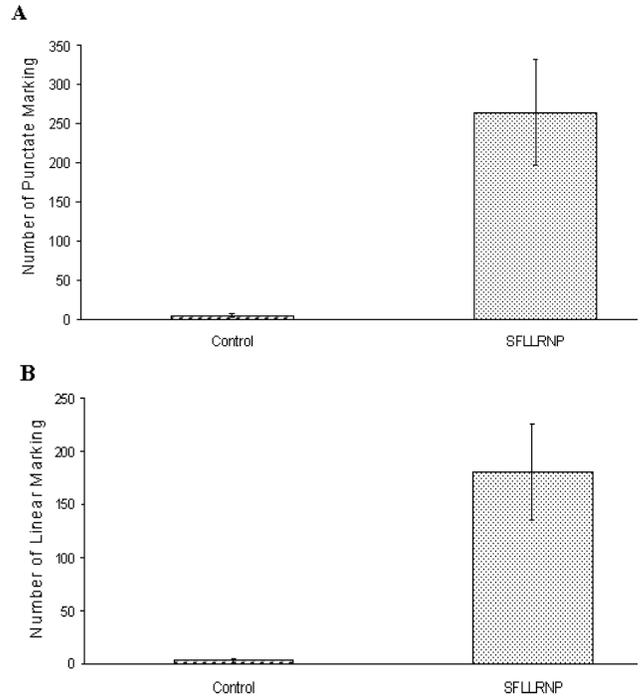


Figure 3. Mean silver markings (\pm SE) observed in the ventral half of spinal cord white matter following treatment with PBS (control) or 100 μ M SFLLRNP. Punctate (A) and linear markings (B) were significantly higher in SFLLRNP-treated embryos ($p=0.0312$ and 0.0280 , respectively; $df=6$ for both groups).

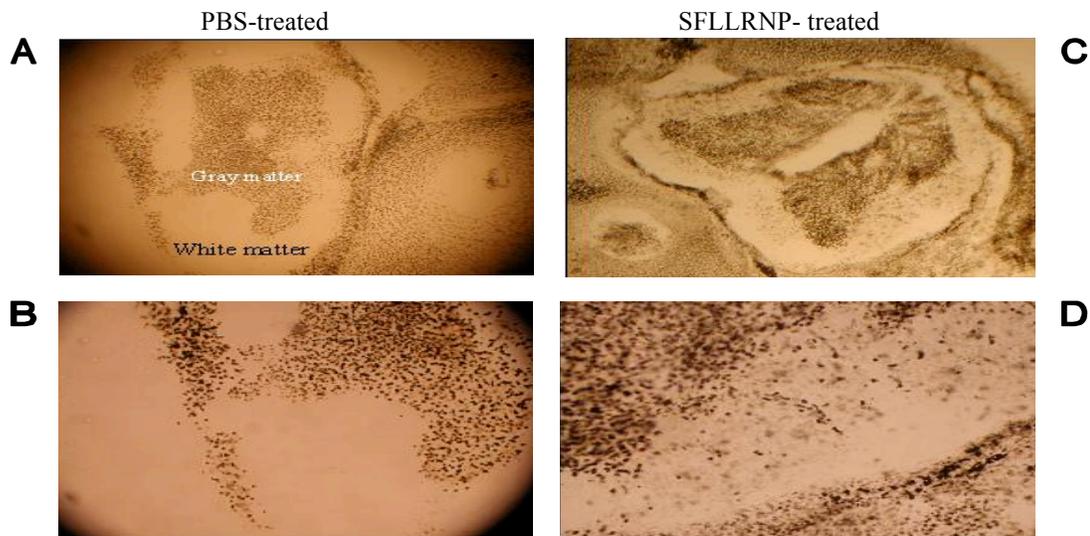


Figure 2. Representative spinal cord cross-sections taken from the lumbar regions of control and experimental chick embryos. From E5-E9 embryos were treated with PBS (control) or SFLLRNP and stained with cupric silver to assess axon degeneration. Panels A and C show an overview of sample sections (40X total magnification slight variation in cord level) while panels B and D (400X total magnification) show the white matter around the ventral motor horn. Panels A and B represent controls with little to no staining and C and D represent the experimental with markedly increased staining.

Discussion

Since the diameter of the silver grains make them too large to adhere to nodes of Ranvier, the silver grains observed in the slides represent unmyelinated areas in the spinal cord. Therefore, the fact that embryos treated with SFLLRNP had significantly more silver markings suggest that PAR-1 activation by SFLLRNP during the naturally occurring PCD for this population of neurons (E5-E10) decreases the deposition of myelin along the axons of *Gallus gallus* embryos. It can be concluded that markings observed are not due to normal developmental pruning because a significant loss of myelin occurred only in the SFLLRNP-treated embryos (Fig 3). Instead, the degeneration observed is pathological.

The punctate and linear markings quantified in this study could be the result of PAR-1 activation on the oligodendrocytes, since these cells do possess PAR-1 (Wang et al., 2004). This activation could result in a loss of oligodendrocytes, thereby decreasing the number of myelinating cells in the CNS, or it could result in decreased myelin production through process retraction. Although the length of the avian oligodendrocyte sheath contacting an axon can be quite variable (Anderson, 2003) at E10, the punctate markings depicted by single silver grains (5-15 μm) were interpreted to represent places where a single myelinating process from an oligodendrocyte was lost. Linear markings were interpreted to represent places where multiple processes were affected.

Whether specific markings were generated by the retraction of several oligodendrocyte processes from one cell, from multiple cells, or were the result of cell death is not yet known. However, studies examining oligodendrocyte numbers and myelin production following PAR-1

activation are currently underway in the laboratory. These studies will examine whether PAR-1 activation on the oligodendrocytes is the underlying cause of the decreased myelin deposition.

It is also possible that the decreased myelin is the result of an inflammatory response as PAR-1 activation has been shown to be involved in the cellular process of inflammation (Steinhoff et al., 2005). In a study regarding spinal cord injury, Festoff et al., (2004) supports thrombin's proinflammatory actions in producing secondary cell injury. Secondary cell injury occurs after the original disturbance and results from the damage of surrounding vasculature as well as the initiation of inflammatory responses (Mautes et al., 2000). Inflammatory cells are able to infiltrate the area after a breach in the blood-spinal cord barrier (Schlosshauer, 1993) and their presence has been shown to correlate with belated neuronal death and demyelination (reviewed in Mautes et al., 2000).

Similarly, when the blood-brain or blood-nerve barrier is compromised, fibrinogen is able to enter neural tissue where it can activate intercellular signaling pathways, causing extracellular matrix rearrangement (Akassoglou and Strickland, 2002). Under this disruption of the blood-brain barrier active thrombin can be generated (reviewed in Turgeon and Houenou, 1997). Thrombin can then convert this fibrinogen into fibrin (Xi et al., 2003) and subsequently cause damage to neural cells or changes in the myelin sheath. Kwon and Prineas (1994) showed that inflammatory demyelination can be linked to fibrinogen accumulations in the plaques formed in multiple sclerosis. Although it is possible that the vasculature was compromised by SFLLRNP treatment leading to a secondary inflammatory response or changes in the extracellular

matrix, no apparent vascular changes were observed. To examine this possibility further, transmission electron microscopy techniques would need to be employed to more closely examine the ependymal lining of the blood-spinal cord barrier.

It is clear that from this study and others that PAR-1 has a deleterious effect on developing motor neurons, but it is not known whether this is a direct or indirect effect. Astrocytes (Donovan et al., 1997) and motor neurons (Smirnova et al., 1998; Turgeon et al., 1998) are induced to undergo caspase-mediated apoptosis immediately following PAR-1 activation implying a direct effect. However, in other events related to PAR-1 activation, such as neurite rounding (Jalink and Moolenaar, 1992) and retraction (Jalink and Moolenaar, 1992; Turgeon et al., 1998), it is unclear as to how these events are stimulated. It is highly possible that PAR-1 activation affects motor neuron cell survival through both a direct and indirect pathway. Initial activation of the PAR-1 receptor on motor neurons could lead to cell death through caspase activation (Turgeon et al., 1998), but subsequent motor neuron cell death could ensue following demyelination of the surviving motor neuron axons. Circulating serine proteases are present during PCD, but up until now the focus has been solely on their direct impact on the neurons. The fact that many motor neurons survive PCD suggests that at some point in development either PAR-1 or the serine proteases are down-regulated; whereas, abnormal down-regulation could result in fewer surviving motor neurons.

Regardless of the specific components and steps involved in myelin formation, myelin plays a direct role in signal transduction and an indirect role in neuronal survival. Action potentials are propagated down the length of the axon through the opening of voltage-gated Na⁺ channels. Myelin effectively reduces the

time it takes for an action potential to reach the distal end of the axon by increasing capacitance along the length of the axon and reducing the number of Na⁺ channels that must be activated. Therefore any decrease in the myelin components may impair conduction signals of the affected motor neurons. Decreased transmission from the neuron to the target, not only slows stimulation of the target cell, but may also have indirect effects on continued viability of the neuron. In the case of the motor neuron, its target is a muscle cell, which upon stimulation contracts, and also produces growth/neurotrophic factors that are retrogradely transported from the axon terminal to the cell body (Deshmukh and Johnson 1997). The neurotrophic hypothesis contends that access to target-derived growth factors is essential to neuronal survival and that any condition limiting their access or uptake would affect neuronal cell viability (Oppenheim et al., 1988). Clarifying the mechanisms involved in cell survival following PAR-1 activation is important to not only understanding these processes that regulate spinal cord development, but also for those processes that mitigate cell death when PAR-1 is improperly activated as has been suggested in spinal cord injuries and in certain neurodegenerative diseases such as ALS. While further research is needed in this area, we have provided evidence that PAR-1 activation has both short- and long-term effects in spinal cord.

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