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# Activated Protein C Promotes Neuroprotection: Mechanisms and Translation to the Clinic

**John H. Griffin**[a,b], **José A. Fernández**[a], **Patrick D. Lyden**[c], and **Berislav V. Zlokovic**[d,e]

[a]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA

[b]Department of Medicine, Division of Hematology/Oncology, University of California San Diego, San Diego, CA, USA

[c]Department of Neurology, Cedars-Sinai Medical Center, Los Angeles, CA, USA

[d]Department of Neurosurgery, University of Southern California, Keck School of Medicine, Los Angeles, CA

[e]Department of Neurosurgery, Zilkha Neurogenetic Institute, University of Southern California, Keck School of Medicine, Los Angeles, CA, USA

## SUMMARY

Activated protein C (APC) is a plasma serine protease that is capable of antithrombotic, anti-inflammatory, anti-apoptotic, and cell-signaling activities. Animal injury studies show that recombinant APC and some of its mutants are remarkably therapeutic for a wide range of injuries. In particular, for neurologic injuries, APC reduces damage caused by ischemia/reperfusion in the brain, acute brain trauma, and by chronic neurodegenerative conditions. For these neuroprotective effects, APC requires endothelial cell protein C receptor. APC activates cell signaling networks with alterations in gene expression profiles by activating protease activated receptors 1 and 3. To minimize APC-induced bleeding risk, APC variants were engineered to lack > 90 % anticoagulant activity but retain normal cell signaling. The neuroprotective APC mutant, 3K3A-APC which has Lys191-193 mutated to Ala191-193, is very neuroprotective and it is currently in clinical trials for ischemic stroke.

### Keywords

Activated Protein C (APC); Stroke; Neuroprotection; Neurogenesis; Traumatic Brain Injury

Corresponding author: John H. Griffin, jgriffin@scripps.edu; phone 858-784-8220.

**Conflict of interest statement**

John H. Griffin is a consultant for ZZ Biotech LLC and inventor for patents involving 3K3A-APC. Berislav V. Zlokovic is a founder and the Chief Scientific Officer of ZZ Biotech LLC, a biotechnology company with a mission to develop APC and its functional mutants for the treatment of stroke and other neurological disorders.

## 1. Introduction

The protein C system is a key component of the body's host-defense systems which evolved to maintain tissue homeostasis and minimize damage from both foreign bodies and from host [1–7]. Initial key findings about the functions of protein C in man came from the discoveries of human hereditary deficiencies; in particular, severe protein C deficiency causes neonatal purpura fulminans which is fatal unless treated aggressively [8, 9]. Moreover, knockout of the murine protein C gene causes pathologic lesions in the brain of embryos and also causes perinatal lethality [10, 11]. In short, results from clinical and murine studies document protein C's essential physiologic roles as an antithrombotic and anti-inflammatory factor.

Clinical data indicate that the brain has particular needs for protein C. Very often, children born with severe protein C deficiency are blind and may also have neurologic deficits [12, 13]. Endogenous APC circulates in an active state at ~ 40 pM in plasma. In the brain, APC is increased during the short ischemic phase of routine carotid endarterectomy in the human brain [14], implying that it might play some neuroprotective role during ischemia. Moreover, circulating APC levels are decreased in stroke patients with antecedent infection/inflammation compared to controls [15]. Finally, it was shown in prospective epidemiologic studies of stroke that plasma protein C levels were inversely associated with the incidence of stroke [16]. Hence, clinical data are consistent with a neuroprotective function for APC. This brief review is focused on the neuroprotective activities of APC [17–22].

## 2. APC provides neuroprotection for ischemic stroke

APC and its variants have been extensively studied in rodent models of ischemic stroke. Our initial study of murine focal ischemia after middle cerebral artery occlusion (MCAO) (hereafter simplified as murine "stroke") showed that human APC dose-dependently reduced infarct volume and other markers of brain damage [19]. This success led us to clone murine protein C and then show that recombinant murine wild type (wt)-APC as well as human wt-APC was neuroprotective for murine stroke [20]. Subsequent mechanistic studies showed that wt-APC provided anti-apoptotic activity for ischemic brain microvascular endothelial cells by reducing p53, normalizing the proapoptotic Bax/Bcl-2 ratio, and lowering caspase-3 signaling [21]. Those studies also provided the first *in vivo* proof of concept evidence for the APC-induced signaling paradigm requiring protease activated receptor (PAR)-1 and endothelial protein C receptor (EPCR) [21].

## 3. APC is neuronal protective and promotes neurogenesis

Neuronal damage in stroke is greatly reduced by APC. Both *in vivo* and *in vitro* studies indicate that APC's neuronal protection involves signaling and requires PAR-1, PAR-3 and EPCR [18, 22–27]. Current therapy for human stroke patients who meet certain restrictive criteria can involve recombinant tissue plasminogen activator (tPA) therapy which, although beneficial, can cause hemorrhagic transformation; moreover, tPA may possibly be toxic for neurons [28]. Surprisingly, wt-APC, in spite of its potent anticoagulant activity, was vasculoprotective in murine stroke studies and in various test systems it was neuronal-

protective both *in vivo* and *in vitro* as it reduced tPA-induced hemorrhage and neuronal damage caused by tPA or by NMDA [22, 29, 30]. Thus, we suggested that APC is attractive for adjunctive therapy with tPA. The relationships between APC and neurons became even more fascinating when we discovered that APC promotes neurogenesis *in vivo* following murine stroke [31] and *in vitro* in cultures of human embryo-derived neuroprogenitor cells (NPCs) [32]. The ability of 3K3A-APC to promote differentiation and growth of human NPCs requires PAR-1, PAR-3 and sphingosine phosphate receptor-1. Thus, APC and its 3K3A-APC variant are able not only to reduce damage to neurons during and following ischemia reperfusion but also to promote growth and regeneration of neurons [31, 32].

## 4. APC reduces brain damage following traumatic brain injury

Because wt-APC and 3K3A-APC were found to provide neuroprotection in stroke, they were studied for their ability to reduce damage following traumatic brain injury. For this purpose, a controlled cortical impact injury to mice was used and the effects of APC administration at multiple time points after injury on neuroprotection, angiogenesis and neurogenesis were determined [33, 34]. First, therapy with high doses of 3K3A-APC was safe in the model. Second, APC-treated mice showed improved motor function in a variety of functional tests. Third, APC significantly reduced lesion volumes, increased new blood vessel formation as shown by CD105+/Ki-67+ double immunostaining, and promoted post-traumatic proliferation of neuroblasts in the subventricular zone. Thus, therapy with APC or its variants appears very promising for traumatic brain injury.

## 5. Cell signaling mechanisms mediate APC's neuroprotective activities

While the mechanisms for APC's anticoagulant effects were fairly known last century, the mechanisms for APC's cell signaling actions are today only partially clear and remain the subject of much ongoing research. In 2001, Joyce et al [35] demonstrated direct effects of APC on endothelial cells and showed that APC was antiapoptotic, anti-inflammatory and altered gene expression profiles. This led others to discover that PAR1 and EPCR were required for APC's alteration of gene expression [36] and APC's anti-apoptotic activity [37]. The first in vivo proof of concept for the APC-PAR1/EPCR paradigm for APC-initiated cell signaling came from studies of APC's neuroprotective actions [21, 22].

Based on many subsequent studies since 2003 of the multiple cytoprotective actions of APC, these activities generally require EPCR and involve APC's ability to activate PAR1 which can likely involve biased signaling by APC-specific unusual cleavages in PAR1 at Arg46 or in PAR3 [38, 39]. However, various reports for which there is not space here to review also variably implicate other receptors including S1P receptor$_1$, Mac-1 (CD11b/CD18), apolipoprotein E receptor 2, EGFR, Tie-2 and others.

For APC's neuroprotection and neurogenesis, the following receptors have been identified as contributory, if not strictly required: EPCR, PAR1, PAR3 and S1P receptor 1 [18, 21, 22, 23–27, 30–32]. The finding that signaling-selective 3K3A-APC is normally neuroprotective, like wt-APC, further supports the view that APC's cytoprotection is due to APC-induced cell signaling [34, 40–43].

## 6. Signaling-selective 3K3A-APC is in clinical trials for ischemic stroke

To dissect the importance of APC's anticoagulant activity from its cell signaling activities in cellular and preclinical studies, we and others have engineered APC with selective loss or gain of these two types of APC activities (see [1, 3, 7, 44–48]). For all *in vitro* and *in vivo* studies of APC's neuroprotection, signaling-selective APC was neuroprotective [34, 40–43]. In contrast, an anticoagulant-selective APC mutant lacking normal anti-apoptotic and cytoprotective activities was actually neurotoxic in murine stroke, possibly due to its anticoagulant activity that was 400% of wildtype APC [49]. Thus, clinical trial studies have been based on the principle that APC-initiated cell signaling underlies its neuroprotective actions.

All available studies of the signaling-selective 3K3A-APC, including knowledge for its mechanism of action, enabled moving forward with translation from bench to beside, i.e., undertaking clinical trials of 3K3A-APC that has < 10 % of APC's anticoagulant activity for ischemic stroke therapy [50, 51]. Preclinical studies of human 3K3A-APC in two animals for toxicity [50] and in two animal stroke models for efficacy [40–43, 52] enabled moving to a phase 1 study [51]. The phase I safety study in normal subjects showed that high dose bolus regimens which resulted in very high transient levels of 3K3A-APC (e.g., plasma levels of 4,500 ng/ml) are safe, including when such high dose boluses are administered every 12 hr over 2.5 days. Moderately severe headache, nausea and vomiting were reported in one of two subjects treated with the maximum dose studied of 0.720 mg/kg, so a 0.540 mg/kg dose was considered the maximum tolerated dose. Elevations in aPTT were minimal, implying bleeding risk is minimal. These very favorable results next need to be confirmed in stroke patients with relevant comorbidities.

The RHAPSODY trial is a Phase 1, dose-escalation and safety trial comparing 3K3A-APC versus placebo in acute stroke patients treated with intravenous recombinant t-PA, intraarterial mechanical thrombectomy [28], or both (https://clinicaltrials.gov/ct2/show/NCT02222714). The trial is being conducted within the NeuroNEXT clinical trials network at 15 sites funded by NINDS. Patients are randomly assigned to treatment group; clinical and imaging endpoints are blinded. A novel hypothesis in RHAPSODY is that the incidence of post-recanalization petechial hemorrhage could be reduced by treatment with 3k3A-APC. The trial is 50% completed as of January 2016.

## 7. Conclusions

Much has been learned about APC's neuroprotective activities. There is a remarkable variety of potential indications for APC's neuroprotective activities, including both acute injury and chronic neurodegenerative conditions [53, 54]. A successful phase 1 clinical study showed that high dose intravenous repeated doses of the signaling-selective APC variant, 3K3A-APC which has < 10 % anticoagulant activity, are safe, setting the stage for ongoing and future clinical trials using this variant for ischemic stroke or for other brain pathologies.

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## Highlights

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