

Cyclosporin A Enhances Cell Survival in Neural Precursor Populations in the Adult Central Nervous System

Jessica Hunt and Cindi Morshead

Department of Surgery, University of Toronto, Donnelly CCBR, 160 College Street, Toronto, Ontario, Canada

PharmSight on Hunt J et al., Cyclosporin A has direct effects on adult neural precursor cells. *J Neurosci* 2010;30:2888-96.

Abstract

Cyclosporin A is a widely used immunosuppressive drug that selectively inhibits cell-mediated immune reactions. The discovery of Cyclosporin A has been critical to the success of transplantation therapies and distinguishing cellular mechanisms of the immune response. This PharmSight will focus on a novel application of Cyclosporin A in modulating the fate and behavior of Neural Precursor Cells (NPCs), a cell population consisting of stem cells and their progeny, which provide the basis for adult neurogenesis. Our recently published data indicate that Cyclosporin A, at therapeutically relevant concentrations, acts directly on NPCs to enhance their survival both *in vitro* and *in vivo*. The action of Cyclosporin A on NPC survival is promising for the development of regenerative strategies which aim to utilize NPCs to repair and regenerate damaged or diseased Central Nervous System (CNS) tissue. Herein, we present an overview of our recent findings, and further demonstrate the ability of Cyclosporin A to enhance the survival of regionally distinct NPC populations, as well as consider the potential mechanisms by which Cyclosporin A may mediate this effect. Finally we conclude by speculating on the potential therapeutic benefits of Cyclosporin A in the treatment of CNS injury and disease.

Keywords: Cyclosporin A; Neural precursor cells; Survival; Adhesion

Introduction

The Pharmacology and Therapeutic Uses of Cyclosporin A

Cyclosporin A is well-known for its potent immunosuppressive properties, and it is routinely used to treat autoimmune disorders and prevent graft rejection following organ transplant (1, 2). The drug is a small, lipophilic, cyclic polypeptide that freely crosses the plasma membrane and binds to several receptors from a family of peptidyl-prolyl cis-trans isomerases known as cyclophilins (3-5). Cyclophilins are distributed in various regions of a cell, from the cytosol to specific organelles, where they facilitate protein folding, act as chaperones, and play a role in cell signaling (6). Although Cyclosporin A can alter multiple cellular pathways by binding different cyclophilins, the most well established pathway is that which was first identified: the immunosuppressive effect of Cyclosporin A on T-lymphocytes. Cyclosporin A prevents antigen stimulated T-lymphocyte activation and proliferation following binding to cyclophilin A, creating a drug-receptor complex which binds and inhibits calcineurin, a Ca^{2+} /calmodulin activated phosphatase (7, 8). Blocking calcineurin inhibits the translocation of Nuclear Factor of Activated T-cells (NFAT) from the cytosol into the nucleus, thus preventing transcription of Interleukin 2 (IL-2), a cytokine responsible for T-lymphocyte propagation (9-11) (Figure. 1). As a result, cell-mediated immune responses to antigens are repressed in the presence of Cyclosporin A.

The ability of Cyclosporin A to selectively inhibit T-lymphocyte mediate immune responses was recognized as a major breakthrough in immunopharmacology; however, since its discovery and approval for clinical use, various cellular

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Correspondence: Cindi Morshead, Ph.D., Department of Surgery, University of Toronto, Donnelly CCBR, 160 College Street, Toronto, ON, Canada, M5S 3E1. Tel. 416-946-5575, Fax. 416-978-3844. email: cindi.morshead@utoronto.ca

pathways and interactions of Cyclosporin A have been documented (6). Cyclosporin A binds and blocks intracellular and/or extracellular cyclophilins which alters cell behavior and function. For example, Cyclosporin A is neuroprotective in CNS and spinal cord models of injury (12, 13). Although the exact mechanism of action is not well established, several theories exist as to how Cyclosporin A protects neuronal cells following injury. Theories suggest Cyclosporin A may lessen cell death by reducing inflammation associated with injury, decreasing lipid peroxidation, preventing neurotransmitter excitotoxicity, and acting directly on cells to down regulate apoptotic factors and up regulate pro-survival factors and pathways (14). Hence, Cyclosporin A may act through many pathways to directly or indirectly alter cell behavior and fate.

The Therapeutic Potential of Neural Precursor Cells

Multipotent, self-renewing neural stem cells and their progeny (collectively referred to as Neural Precursor Cells (NPCs)) may well provide a foundation for regenerative strategies that aim to replace lost and damaged neural cell types in an injured CNS (15). The adult mammalian forebrain contains a population of neural stem cells that reside in the subependyma, a 3-4 cell layer thick region adjacent to the ependymal lining of the lateral ventricles (16). In vivo, subependyma neural stem cells proliferate slowly to generate progeny which expand the precursor pool by producing neuroblasts that either die or migrate to the olfactory bulb where they differentiate into olfactory interneurons (17, 16). Neural stem cells can be isolated in vitro in the presence of growth factors whereby single cells proliferate to form free-floating colonies termed neurospheres (18). Neurospheres contain a mixed population of a small number (<1%) of neural stem cells and a large number (>99%) of progenitor cells. The inherent capacity of NPCs to proliferate, migrate, and differentiate in vivo, along with the capacity for in vitro expansion, makes them excellent targets for neural repair strategies and much interest has surrounded the identification of extrinsic cues and intrinsic signals that regulate NPC kinetics and fate (for review see 19). Indeed, one area of current research actively pursues the identification of novel therapeutics which target cellular pathways to augment the innate ability of endogenous NPCs to repair or replace lost or damaged cells and thereby restore neurological function.

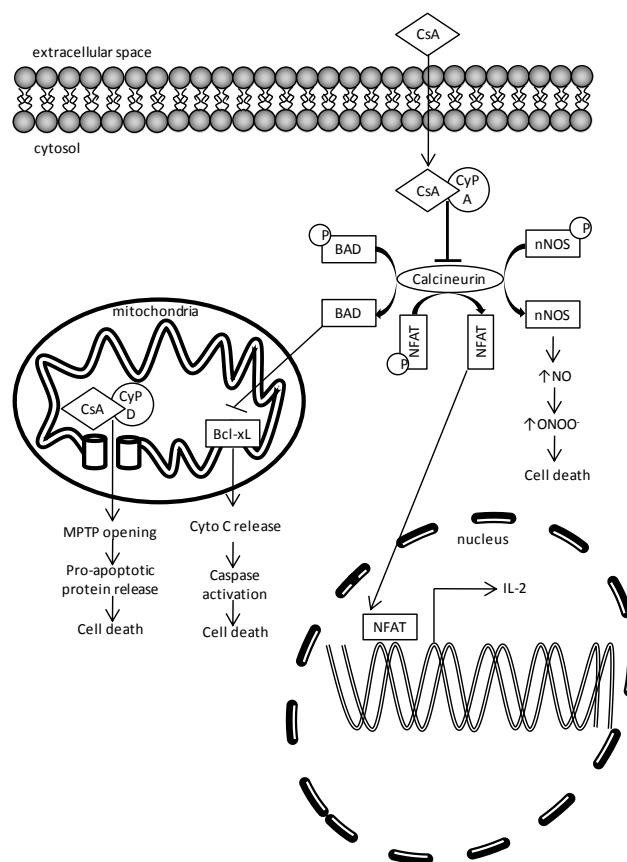


Figure 1. Cyclosporin A may potential affect cell survival through calcineurin-dependent and calcineurin-independent pathways. Cyclosporin A blocks the phosphatase activity of calcineurin preventing: a) the transcription of NFAT regulated genes, including IL-2; b) production of free radicals by nNOS, and; c) BAD inhibition of bcl-xL, a pro-survival protein found in the mitochondria. Along with binding Cyclophilin A, Cyclosporin A blocks the *cis-trans* isomerase activity of all other cyclophilins. In the mitochondria, Cyclosporin A blocks the opening of mitochondrial permeability transition pores by binding to Cyclophilin D, and therefore preventing the release of pro-apoptotic proteins and enhancing cell survival. (BAD = Bcl-2 Associated Death promoter; CsA = Cyclosporin A; Cyp = Cyclophilin; CytoC = Cytochrome C; MPTP = Mitochondrial Permeability Transition Pore; NFAT = Nuclear Factor of Activated T-cells; NO = Nitric Oxide; nNOS = neuronal Nitric Oxide Synthase; ONOO- = peroxynitrate).

Immune-relevant molecules are of particular interest as targets as recent studies have demonstrated changes in NPC proliferation, migration, and differentiation in response to CNS inflammation and degenerative damage (20-23). For example, inflammatory components, such as mononuclear cells, reactive astrocytes, microglia, and endothelial cells are hypothesized to release cytokines and chemokines that overwhelm the response of NPCs to chronic inflammation, thus resulting in failure of neural stem cells to contribute to neural repair following injury to the CNS (23).

While numerous studies reveal a neural-immune interaction, the exact mechanisms by which immune-relevant factors and NPCs communicate and importantly, whether interactions between immune cells and/or immune signals and NPCs are direct or indirect, was not clear. Our recent work was the first to demonstrate a direct effect of the immunosuppressive drug Cyclosporin A on NPCs in the adult CNS.

Methods and Materials

Neurosphere Assay

Adult male Cd1 mice (6-8 wks, 25-30 gm; Charles River, QC) were housed in the University of Toronto animal facilities, and maintained in accordance with the Institutional guidelines. Neural stem cells were isolated from the spinal cord as previously described (24). Briefly, tissue was digested with enzyme (0.01% papain, 0.1% protease, 0.01% DNase I; all from Sigma-Aldrich, St. Louis, MO) for 40 min at 37°C. Enzyme activity was inhibited with ovomucoid protease inhibitor (Sigma-Aldrich, St. Louis, MO), and the tissue was mechanically dissociated into a single cell suspension. Cells were plated at clonal density (<5 cells/ μ L) (25) in 24-well polystyrene plates (VWR, Mississauga, ON) with serum free media supplemented with EGF (20 ng/ml), bFGF (10ng/ml), and heparin (7.35 ng/ml) (all from Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen, Eugene, OR). Neurospheres were counted on day 7 of culture. Stock solution of Cyclosporin A (0.2 mg/ml; Bioshop Canada Inc., Burlington, ON) was made by dissolving solid Cyclosporin A in a 1:1 anhydrous ethyl alcohol:serum free media solution, and subsequently added to the cultures at various concentrations.

In Vivo Studies

Adult male Long Evans rats (6-8 wks, 250-300 gm; Charles River) were housed in the University of Toronto animal facilities, and maintained in accordance with the Institutional guidelines. Animals were immunosuppressed with a daily dose of Cyclosporin A (15 mg/kg/day) delivered subcutaneously via an osmotic mini-pump (Alzet Osmotic Pumps, Cupertino, CA). The pump delivered Cyclosporin A or saline at a rate of 0.5 μ L per hour for 14 days. Neural stem cells were isolated by dissection of the central canal of the spinal cord as described above. The cells were plated at a density of 5 cells/ μ L in growth factor supplemented

serum free media, and the neurospheres that formed were counted on day 7 of culture.

Statistical Methods

Statistical analysis was performed using the Student's t test for two-group comparisons and ANOVA for multiple group comparisons (SigmaStat statistical package). All data are reported as means $\pm$ SEM.

Results and Discussion

Cyclosporin A has Direct Effects on Neural Precursor Cells

We examined the effects of Cyclosporin A on NPC proliferation kinetics, survival, and fate using in vitro assays at the population level and at the single-cell level (26). The use of pure populations of subependymal-derived NPCs (i.e. in cultures devoid of other neural, endothelial or blood derived cells), revealed a direct effect of Cyclosporin A on cell survival, resulting in increased numbers and larger colonies, with no effect on proliferation kinetics. These effects are independent of the T-lymphocyte cytokine, IL-2, establishing that non-immunosuppressive mechanisms are mediating the observed effects of Cyclosporin A on NPC behavior. Cyclosporin A did not alter the differentiation profile of NPC colonies, indicating that it did not promote selective survival of a particular neural lineage. Consistent with the in vitro observations, in vivo administration of Cyclosporin A to adult animals via systemic administration or infusion directly into the brain, resulted in a significant increase in the numbers of NPCs within the neurogenic niche lining the lateral ventricles. Together, our findings establish that Cyclosporin A has direct effects on NPCs both in vitro and in vivo, making it a promising candidate molecule for developing clinically relevant strategies to stimulate NPCs for CNS repair. We were further interested in asking whether the enhanced NPC survival was specific for brain derived NPCs or whether Cyclosporin A had similar effects on regionally distinct NPC populations in the adult CNS.

Cyclosporin A Enhances Spinal Cord-derived NPC Survival

Neural stem cells have been isolated from along the entire neuroaxis of the developing and adult CNS including: olfactory bulb (27); retina (28); and, the central canal of the spinal cord (24, 29-31). Regional differences in proliferation (24), gene

expression (32-34), the ability to generate specific cell types (35, 36) and migration patterns (37) occur within these distinct neural precursor pools. We questioned whether the effect of Cyclosporin A on cell survival translated across the different neural stem cell populations, and specifically examined spinal cord-derived NPCs. The general application of promoting cell survival and thereby increasing the pool of NPCs within the adult CNS has broad implications for the treatment of CNS injury or disease. In the case of spinal cord-derived NPCs, their potential to contribute to neural tissue repair following spinal cord injury and inflammation is an exciting prospect. Similar to brain derived (subependymal) neural stem cells, spinal cord neural stem cells proliferate in response to exogenously applied growth factors to form clonally derived, self-renewing and multipotent neurospheres (30). Different from the NPCs in the brain is the fact that spinal cord NPCs do not contribute to active neurogenesis in the adult mammalian nervous system. Hence, general conclusions about the effects of Cyclosporin A on brain-derived NPCs may not translate to regionally distinct populations and must be examined independently.

In a series of *in vitro* experiments using pure populations of spinal cord NPCs, we observed a significant 2-fold increase in the numbers of spinal cord-derived neurospheres cultured in the presence of Cyclosporin A relative to control (no Cyclosporin A) cultures (72 ± 7 vs. 35 ± 7 neurospheres, 100 ng/ml Cyclosporin A vs. 0 ng/ml Cyclosporin A; $p < 0.01$) and comparable to the results using subependymal-derived NPCs where we observed a 1.7-fold increase in the presence of Cyclosporin A (Figure. 2). Moreover, a similar effect on neurosphere size was seen as that observed with brain-derived neurospheres, whereby Cyclosporin A exposure resulted in significantly larger neurospheres ($80 \pm 2 \mu\text{m}$ vs. $59 \pm 5 \mu\text{m}$, 100 ng/ml Cyclosporin A vs. 0 ng/ml Cyclosporin A; $p < 0.01$). These results, increased numbers and size of neurospheres, indicate Cyclosporin A acts on spinal cord stem and progenitor cells, respectively to enhance their survival.

We next asked whether Cyclosporin A administration can enhance the numbers of spinal cord NPCs *in vivo*. Systemic infusion of Cyclosporin A for 7 or 14 days results in increased numbers of proliferating NPCs in the subependyma, as well as increased numbers of neurospheres isolated *in vitro*.

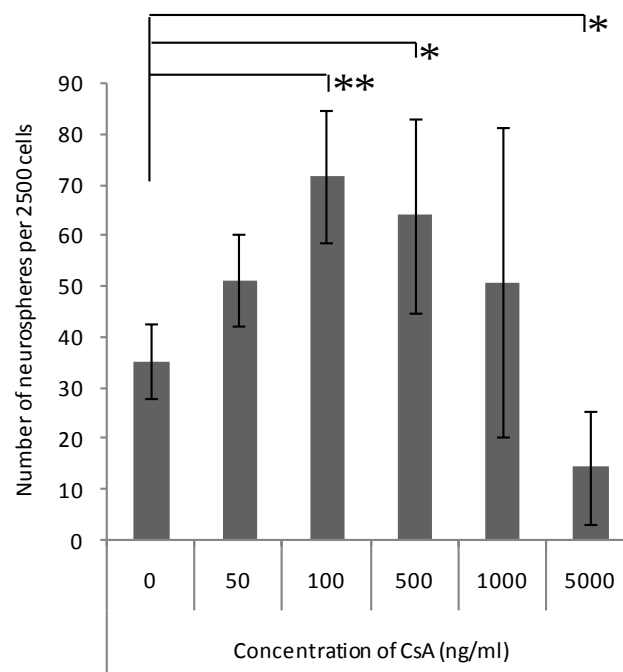


Figure 2. Cyclosporin A increased the numbers of spinal cord-derived neurospheres *in vitro*. Spinal cord NPCs were exposed to various concentrations of Cyclosporin A and the numbers of neurospheres that formed was assayed after 7 days *in vitro*. A significant increase in the numbers of spinal cord derived neurospheres was observed at similar concentrations of Cyclosporin A (100 ng/ml and 500 ng/ml). The magnitude of the increase and the effective Cyclosporin A concentration are similar to what was observed with brain-derived neurospheres. (* = $p < 0.05$, ** = $p < 0.01$; $n = 3$).

Uninjured animals (rats) received systemic infusion of Cyclosporin A (15 mg/kg/day) for 14 days by intraperitoneal implanted osmotic mini-pumps. The spinal cord was dissected, dissociated into single cells and cultured in the absence of Cyclosporin A. We observed a significant increase in the number of neurospheres derived from Cyclosporin A infused animals compared to control (saline infused) animals (30 ± 1 vs. 10 ± 5 neurospheres, treatment vs. control; $p < 0.05$) (Figure 3). Hence, Cyclosporin A increases the number of brain and spinal cord NPCs *in vivo*, suggesting potential implications for regenerative strategies that aim to increase NPC numbers to replace neural cells lost or damaged following spinal cord injury.

Investigating the Mechanism of Action: Calcineurin-dependent Pathways Regulating Survival

We demonstrated that cyclophilin A and calcineurin are abundantly expressed in pure

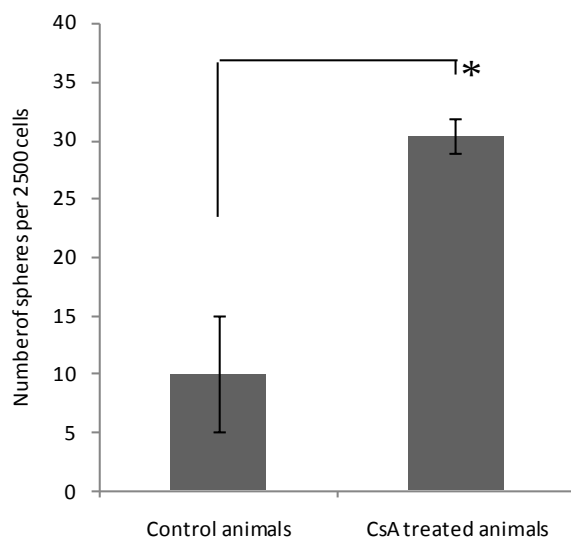


Figure 3. Cyclosporin A increased the numbers of spinal cord-derived NPCs *in vivo*. Cyclosporin A infused rats show increased numbers of neural stem cells *in vivo* following infusion of Cyclosporin A for 14 days. ($p < 0.05$; $n = 3$ animals per group).

populations of NPCs, and given that cyclophilin A has the highest affinity for Cyclosporin A compared to other cyclophilins, we asked whether the effects on cell survival were mediated through inhibition of IL-2 expression in NPCs. We were unable to detect the production or release of IL-2 in NPC cultures, and demonstrated that the addition of IL-2 to NPC cultures did not affect neurosphere numbers or size, thus, we concluded that IL-2 inhibition by Cyclosporin A does not mediate the enhanced survival on NPC populations. So the question remains as to how Cyclosporin A promotes cell survival. Based on what is known about the intracellular signaling pathways, there are several mechanisms by which Cyclosporin A may promote cell survival which include both calcineurin-dependent (genetic and non-genetic) as well as calcineurin-independent pathways.

Calcineurin inhibition by Cyclosporin A may enhance cell survival through two separate pathways: neuronal Nitric Oxide Synthase (nNOS) and/or expression of the pro-apoptotic protein, Bcl-2 Associated Death promoter (BAD). Both nNOS and BAD signaling pathways play a role in cell death and are activated by the phosphatase activity of calcineurin (Figure 1). Hence, blocking calcineurin would effectively enhance cell survival. Indeed, Cyclosporin A and the calcineurin blocking immunosuppressant, FK506, have been shown to be neuroprotective supporting the idea that inhibition

of calcineurin specifically, as opposed to other mechanisms, enhances cell survival (38-40). In the case of nNOS activity, calcineurin up-regulates the activity of nNOS augmenting the levels of nitric oxide (NO) (41). Overproduction of NO is toxic due to the production of free radicals such as peroxynitrite (ONOO^-), a reaction product of NO, and superoxide anion (O_2^-) and by altering conformational changes in proteins that result in misfolding and protein aggregation (42). Inhibition of calcineurin by Cyclosporin A would keep nNOS in its inactive, phosphorylated state and thereby reduce nitric oxide radical production. This would lessen formation of the nitric oxide-derivative reactive oxygen species, peroxynitrite, which is known to be rupture cell membranes leading to cell death and posttraumatic injury (43). It has recently been shown that Cyclosporin A attenuates peroxynitrate-induced oxidative damage to mitochondrial proteins following traumatic brain injury (44).

Calcineurin also influences the viability of cells through activation of the pro-apoptotic protein, BAD. An increase in cytosolic calcium leads to calcineurin activation and dephosphorylation of BAD, which in turn triggers its translocation to the mitochondria where BAD binds and blocks Bcl-xL protein impeding Bcl-xL's pro-survival actions. Pores form in the inner mitochondrial membrane leading to disrupted membrane potential and release of pro-apoptotic proteins such as cytochrome c which activates caspases and cell death ensues. Thus, the inhibition of calcineurin by Cyclosporin A may enhance cell survival by maintaining phosphorylated nNOS or BAD in the cytoplasm.

FK506, also known as tacrolimus, is structurally unrelated to Cyclosporin A; however, it has the same immunosuppressive effects at the cellular and molecular level. FK506 binds to members of an immunophilin family known as FKBP (FK506 binding proteins) which catalyze cis-trans conformation of prolyl bonds in a similar manner as cyclophilins. Immunosuppression is associated with the smallest FKBP isoform, FKBP12, which under normal conditions is part of the IP_3R /ryanodine receptor in the endoplasmic reticulum (45). Similar to Cyclosporin A-cyclophilin A, the FKBP12-FK506 complex specifically binds to and inhibits the phosphatase activity of calcineurin. Currently, we are investigating the possibility of calcineurin-dependent pathways of enhanced survival using FK506. We predict that if Cyclosporin A enhances survival through nNOS or BAD by calcineurin

inhibition then FK506 will similarly increase the numbers and size of NPC colonies.

Since calcineurin inhibition can mediate pro-survival effects through two pathways, it will also be interesting to test whether nNOS or BAD alone mediate NPC survival effects. Our preliminary works using nNOS knockout mice which effectively have a complete lack of nNOS, NO, and ONOO⁻, suggest that nNOS activation does not mediate the pro-survival effects. We would predict that if nNOS mediates the pro-survival effects of Cyclosporin A on NPCs, we will observe no increase in neurosphere numbers or size in cultures derived from nNOS^{-/-} mice treated with Cyclosporin A. However, our studies to date show similar, significant increases in both neurosphere numbers and size in Cyclosporin A treated cultures from nNOS^{-/-} and nNOS^{+/+} (littermates) cultures. While this work is preliminary, it suggests that calcineurin mediated nNOS activation is not playing a role in the enhanced cell survival hence it will be interesting to investigate the role of BAD as we continue to tease apart the various pathways that promote cell survival.

Investigating the Mechanism of Action: Cyclophilin D: A Calcineurin-independent Pathway Regulating Survival

In conjunction with Cyclosporin A's ability to inhibit the phosphatase activity of calcineurin, the drug can also influence multiple cellular pathways by binding to other members of the cyclophilin family. Cyclophilins are a highly conserved family of proteins that are ubiquitously expressed in all organisms from prokaryotes to mammals, and they possess peptidyl-prolyl isomerase (or rotamase) activity, which facilitates cis-trans isomerization of peptide (amide) bonds (46, 47). Cyclosporin A binds and blocks the peptidyl-prolyl isomerase active site of all cyclophilins. One of the best characterized mechanisms by which Cyclosporin A enhances cell survival is through inhibition of the Mitochondrial Permeability Transition (MPT) pore which is a pore the forms in the inner mitochondrial membrane. The MPT pore is composed of the Voltage-Dependent Anion Channel (VDAC), the Adenine Nucleotide Translocase (ANT), and cyclophilin D. It is located between the outer and inner mitochondrial membrane. The pore can open in response to oxidative stress, high Ca²⁺, and low ATP releasing pro-apoptotic protein including cytochrome c, Apoptosis-Inducing Factor (AIF) and Second mitochondria-derived activator of caspase/Direct

IAP Binding protein with Low pl (Smac/DIABLO), and pro-caspases which are involved in downstream programmed cell death pathways (48-50). By binding and blocking Cyclophilin D, Cyclosporin A prevents pore formation and thus impedes cell death (51-53). We are currently employing small molecules to manipulate cyclophilin D to determine its role (if any) in the pro-survival effects of Cyclosporin A on NPCs.

Concluding Remarks

The findings that Cyclosporin A enhances NPC survival have enormous implications for the development of regenerative medicine strategies that target endogenous precursor cells and potentially promote self-repair mechanisms in the CNS. Towards this end, studies investigating the effect of Cyclosporin A on NPCs following injury are underway. Albeit in its infancy, the ability of Cyclosporin A, a clinically relevant, FDA approved drug, to enhance cell survival without causing changes in the cell cycle time, will undoubtedly facilitate the translation of neural repair strategies to the clinic.

Acknowledgements

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Conflicts of Interest

No potential conflicts of interest to disclose.

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