The Effects of Rolipram, a Selective Phosphodiesterase Inhibitor, on Immortalized Schwann Cell Proliferation, AKAP95 and Cyclin D3 Expression

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Introduction: Schwann cells are a vital component of the peripheral nervous system (PNS) due to their ability to protect and myelinate axons in addition to aiding in neural repair. Schwann cell differentiation, proliferation, and migration at the site of injury depends on the levels of intracellular cyclic adenosine monophosphate (cAMP).[1] When intracellular cAMP levels are low, Schwann cells exhibit a myelinating phenotype while high concentrations result in a proliferating phenotype.[2] Due to this capability, Schwann cell transplants have been attempted to treat damaged nervous tissue found in both the PNS and the central nervous system (CNS). In the CNS, due to inadequate growth and cell signaling factors, there is little success with these transplants.[3] The regulation of Schwann cell growth *in vitro* is facilitated by heregulin, a neuron-secreted growth factor, and an unknown mitogen that activates the cAMP pathway.[3,4] In recent years, researchers have used phosphodiesterase inhibitors as an alternative form of treatment for spinal cord damage, multiple sclerosis, Alzheimer's Disease, and other neurodegenerative diseases. Phosphodiesterase inhibitors increase the abundance of intracellular cAMP in Schwann cells by targeting and hydrolyzing phosphodiesterases (PDEs) a family of enzymes responsible for the regulation of the universal secondary messenger.[5,6] Besides inducing increased rates of cell proliferation, intracellular cAMP binds to the regulatory subunit of Protein Kinase-A (PKA) which releases the catalytic subunits to phosphorylate protein substrates. A-Kinase anchoring proteins (AKAPs) are a family of scaffolding proteins that anchor PKA and bind to PDEs and regulate cAMP concentration in cardiac myocytes. Previous studies have shown that AKAP95, a nuclear AKAP, known for scaffolding cyclins, is essential for Schwann cell growth.[7,8] Currently there is no literature that explores the effects of phosphodiesterase inhibitors such as rolipram on Schwann cells proliferation, AKAP95, and cyclin D3 expression. Based on these reports, it was hypothesized that increasing the concentration of rolipram would elicit a dose-dependent increase in Schwann cell proliferation by augmenting the expression of AKAP95 and cyclin D3.

Experimental Design:

- **Schwann cell culturing:** Schwann cell line CRL-2941 (ATCC) was purchased and cultured in Dulbecco's Modified Eagle Media (DMEM), 10% Fetal Bovine Serum (FBS), and 1% 1/1 penicillium:streptomycin at 37 °C and 5% CO_2 until 80% confluency and were continuously sub-cultured.
- **MTT Assay:** For 12 and 24-hour MTT assays, 50,000 cells were seeded per well in a 96 well plate with colorless DMEM until 85% confluent. Cells were then treated with no mitogen (N2) media for 24-hours and received the following treatments: control media (N2), 12.5 ng/mL heregulin (Hrg), 1 µM forskolin (Fsk), or heregulin + forskolin (Hrg + Fsk) with various doses of rolipram (0, 0.5, 1, 5, 10, 25, or 50 µM). Prior to the Vybrant MTT assay, Schwann cell morphology was visualized at 10X magnification using a Zeiss Primovert microscope and Axiocam ER c5S camera attachment. After incubation, cell viability was determined by reading the optical absorbance at 570 nm.
- **AKAP95 and cyclin D3 exxpression:** S16 Schwann cells (ATCC) were treated with no mitogens (N2), heregulin (Hrg) at 12.5 ng/mL, forskolin (Fsk) at 1 µM, heregulin + forskolin(Hrg + Fsk), and various concentrations of the phosphodiesterase inhibitor rolipram, ranging from 0 µM to 50 µM for 12-hours at 37 °C and 5% CO_2. Cell extracts were prepared, processed, ran in polyacrylamide gels, and transferred onto nitrocellulose membranes. The expression of AKAP95 and cyclin D3 were then analyzed by performing western blots and normalizing with actin.

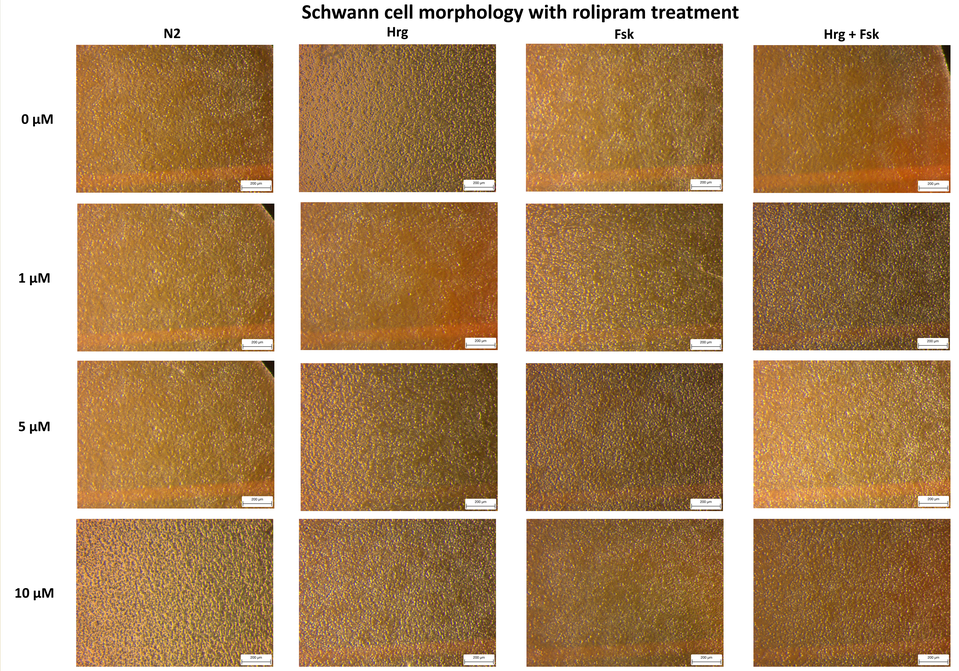

Figure 2 – S 16 Schwann cell were cultured in control media (N2), 12.5 ng/mL heregulin (Hrg), 1 µM forskolin (Fsk), heregulin + forskolin (Hrg + Fsk) at 37 °C and 5% CO_2. Cells were also incubated with various dosages of rolipram as specified above for cell proliferation assay. Cells treated with 5 µM of rolipram at 12 hours and 1 µM of rolipram at 24 hours expressed the greatest amount of proliferation while cells treated with more than 5 µM of rolipram revealed a decrease in cell number and proliferation. The above result is one representative set of images from a 12-hour incubation of rolipram. The experiment contained three independent trails for 12-hours and four for 24-hours.

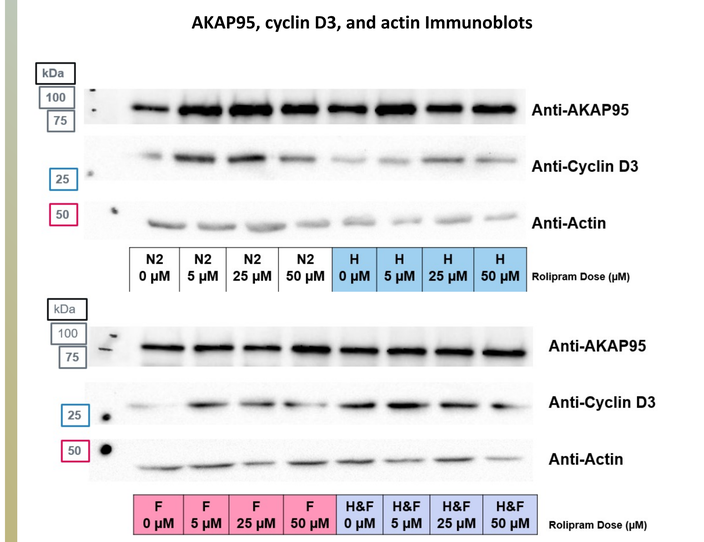

Figure 4 - S16 Schwann cells (ATCC) were treated with no mitogens (N2), heregulin (Hrg) at 12.5 ng/mL, forskolin (Fsk) at 1 µM, heregulin + forskolin(Hrg + Fsk), and various concentrations of the phosphodiesterase inhibitor rolipram, ranging from 0 µM to 50 µM for 12-hours. The expression of AKAP95 and cyclin D3 were analyzed by immunoblotting and normalizing with actin. The above result is one representative blot from three independent experiments.

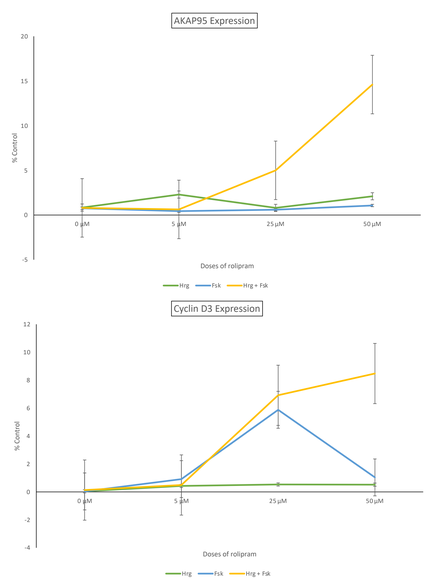

Figure 5 – Quantitative analysis of AKAP95 and cyclin D3 expression as analyzed by densitometry is shown above. Protein expression was normalized with signal intensities of actin protein and expressed as percent control over basal levels of unstimulated cells and displayed as the mean + SEM. Results from all three experiments were examined using one-way ANOVA and tested with post hoc Tukey test. The cells were treated for 12 hours. The cells cultured with heregulin plus forskolin and 50 µM rolipram exhibited an upregulation of AKAP95. Cells cultured with forskolin 25 µM rolipram, and heregulin plus forskolin and 50 µM rolipram exhibited an upregulation of cyclin D3 (n=3, p<0.05).

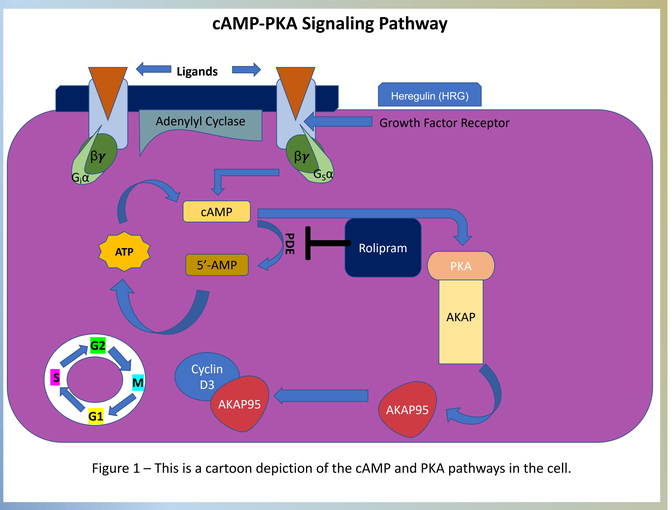

Figure 1 – This is a cartoon depiction of the cAMP and PKA pathways in the cell.

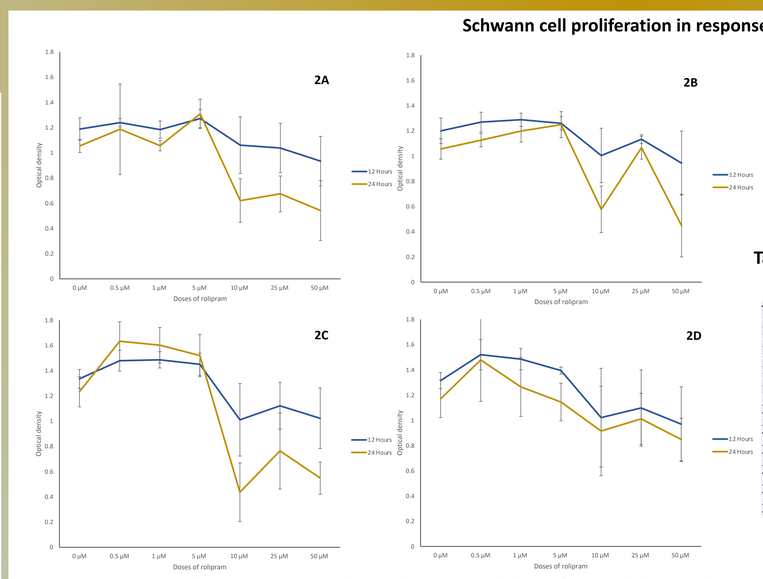

Figure 3 – S16 Schwann cells were seeded 50,000 cells per well using a 96 well plate until they reached 80% confluency. Cells were incubated in colorless control (N2) media for 24 hours then cultured with no mitogens (figure 2A), 12.5 ng/mL heregulin (figure 2B), 1 µM forskolin (figure 2C), heregulin + forskolin (figure 2D), and various concentrations of rolipram ranging from 0 µM to 50 µM for either 12 hours or 24 hours. Cell proliferation and survival was measured using Vybrant™ MTT cell proliferation assays with the means + SEM displayed. Statistical analysis using one-way ANOVA revealed that overall, there was no significantly different effects between timepoints. However, using a post-hoc Tukey test, the following groups were statistically significant at $P < 0.05$ (Table 1). Replicates for the 12-hour MTT assay were repeated three times and 24-hour MTT assay were repeated four times.

Table 1 - Table of P-Values of rolipram treatment groups that elicited statistical significance

Time	Treatments	P-Value
12 Hrs	Hrg + Fsk at 50 µM R was significant to Fsk at 0.5 µM R	0.03
12 Hrs	Hrg + Fsk at 50 µM R was significant to Fsk at 1 µM R	0.033
12 Hrs	Hrg + Fsk at 50 µM R was significant to Hrg + Fsk at 0.5 µM R	0.042
12 Hrs	Hrg + Fsk at 50 µM R was significant to Hrg + Fsk at 1 µM R	0.022
24 Hrs	N2 at 0.5 µM R was significant to Fsk at 50 µM R	0.021
24 Hrs	N2 at 0.5 µM R was significant to Hrg + Fsk at 10 µM R	0.025
24 Hrs	N2 at 0.5 µM R was significant to Hrg + Fsk at 50 µM R	0.038
24 Hrs	Hrg + Fsk at 0.5 µM R was significant to Fsk at 50 µM R	0.025
24 Hrs	Hrg + Fsk at 0.5 µM R was significant to Hrg + Fsk at 10 µM R	0.021
24 Hrs	Hrg + Fsk at 0.5 µM R was significant to Hrg + Fsk at 50 µM R	0.024

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Conclusions:

- Cells treated with 5 µM of rolipram at 12 hours and 1 µM of rolipram at 24 hours expressed the greatest amount of proliferation.
- Cells cultured with heregulin and forskolin and 50 µM rolipram exhibited an upregulation of AKAP95 at 12 hours ($p<0.05$).
- Cells cultured with forskolin 25 µM rolipram, and heregulin and forskolin and 50 µM rolipram exhibited an upregulation of cyclin D3 at 12 hours ($p<0.05$).

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