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

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**Abstract:** Schwann cells are a vital component of the Peripheral Nervous System and aid in the repair of axons following injury. The regulation of Schwann cell growth *in vitro* is facilitated by heregulin, a neuron-secreted growth factor, and an unknown mitogen that activates the cyclic adenosine monophosphate (cAMP) pathway. The abundance of intracellular cAMP is regulated by a family of enzymes called phosphodiesterases (PDEs). PDE inhibitors such as rolipram have therapeutic potential in various disorders and function by increasing the levels of intracellular cAMP. A-Kinase anchoring proteins (AKAPs), a family of scaffolding proteins that belong to the cAMP/Protein Kinase A (PKA) pathway are known to bind both PDE and PKA to 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. 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. Immortalized Schwann cells were cultured with no mitogens, 12.5 ng/mL heregulin, 1 µM of forskolin (a pharmacological activator of cAMP), heregulin + forskolin, and various doses of rolipram at 0, 0.5, 1, 5, 10, 25, and 50 µM for 12 or 24 hours. Using the MTT assay, preliminary results indicate that cells incubated for 12 hours and 24 hours exhibited the highest rate of proliferation at a dose of 5µM and 10 µM rolipram, respectively. Meanwhile, immunoblot analysis revealed that in cells treated with heregulin + forskolin, the expression of cyclin D3 and AKAP95 was highest when incubated with 25 µM and 50 µM of rolipram, respectively. These results suggest that increasing the concentration of cAMP by inhibiting phosphodiesterases augments Schwann cell proliferation by amplifying the expression of proteins regulating cell division.

**Introduction:** Schwann cells are a vital component of the peripheral nervous system (PNS) by protecting and myelinating 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 of intracellular cAMP levels yield a proliferating phenotype.[2] Due to this capability, Schwann cell transplants have been used in an attempt 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 been using 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 the family of enzymes called phosphodiesterases (PDEs) 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. 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.[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.

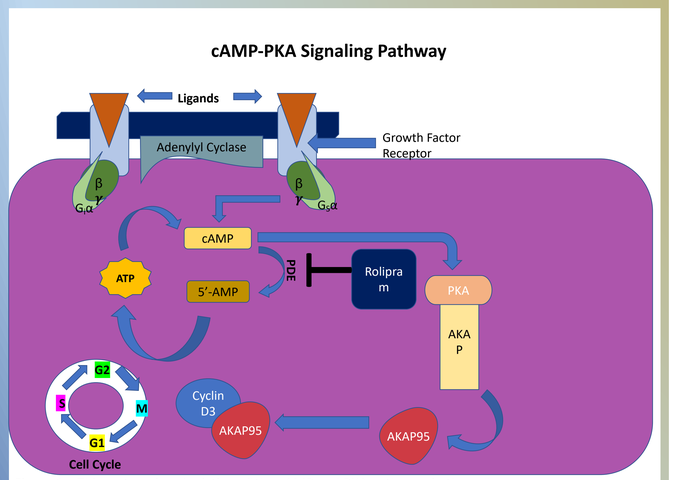


Figure 1 – This cartoon is a depiction of the cAMP and PKA pathways in the cell.

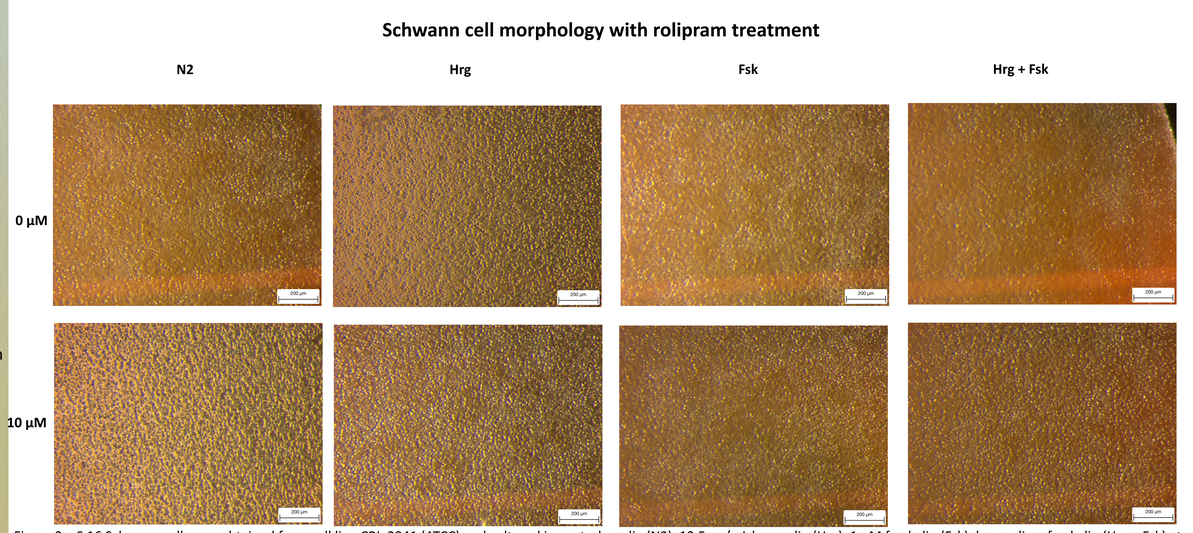


Figure 2 – S 16 Schwann cell were obtained from cell line CRL-2941 (ATCC) and cultured in control media (N2), 12.5 ng/mL heregulin (Hrg), 1 µM forskolin (Fsk), heregulin + forskolin (Hrg + Fsk) at 37 °C and 5% $CO_2$. Cells were also incubated with various dosages of rolipram as specified above for cell 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. Morphological features of the cells were visualized with a Zeiss Primovert microscope and Axiocam ER c5S camera attachment at 10X magnification and the scale bar indicates 200 µm. The above result is one representative set of images from three independent experiments for 12 hours and four trials for 24 hours.

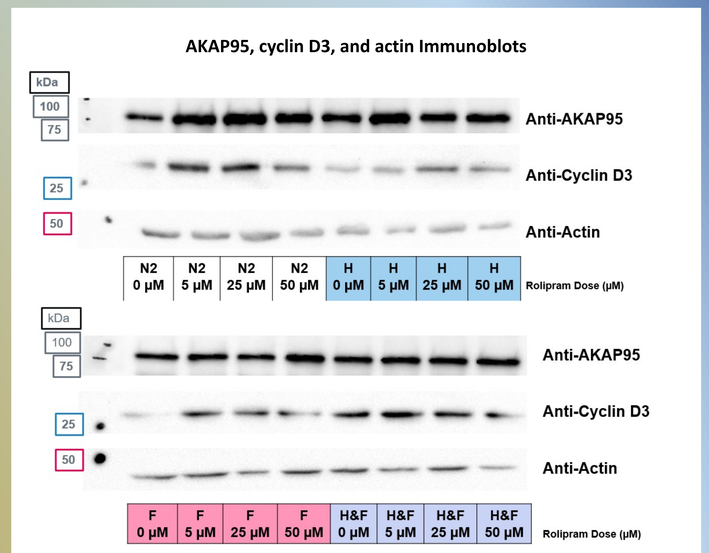


Figure 4 - S16 Schwann cells (ATCC) were treated with no mitogens (N2), heregulin (Hrg) at 12.5 ng/mL, forskolin (Fsk) at 1 µM, heregulin + forskolin(Hrg + Fsk), and various concentrations of the phosphodiesterase inhibitor rolipram, ranging from 0 µM to 50 µM for 12 hours. The expression of AKAP95 and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

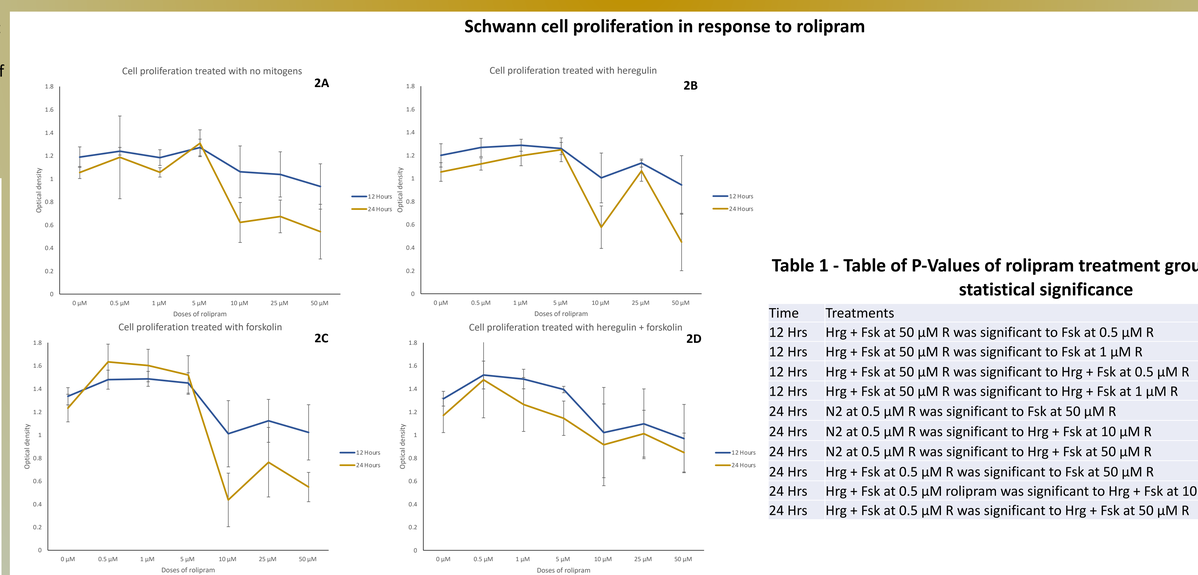


Figure 3 – S16 Schwann cells were seeded 50,000 cells per well using a 96 well plate until they reached 80% confluency. Cells were incubated in colorless control (N2) media for 24 hours then cultured with no mitogens (figure 2A), 12.5 ng/mL heregulin (figure 2B), 1 µM forskolin (figure 2C), heregulin + 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. Statistical analysis using one-way ANOVA revealed that overall effects were not significantly different. However, using the 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 the 24-hour MTT assay was 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 rolipram 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 |

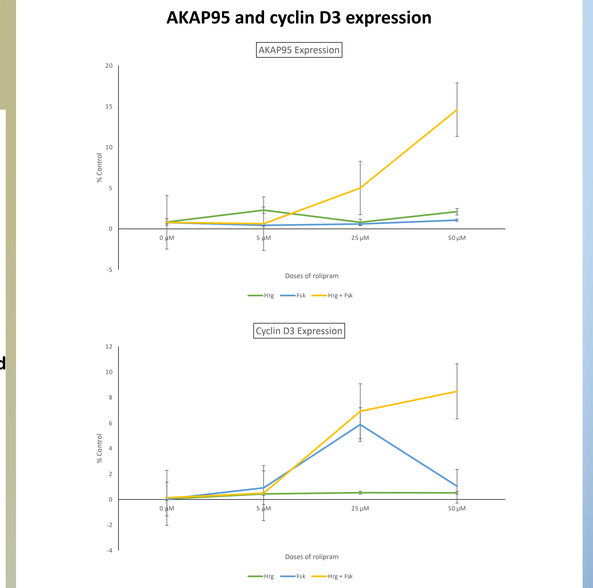


Figure 5 – Quantitative analysis of AKAP95 and cyclin D3 expression as analyzed by densitometry is shown above. Protein expression was normalized with signal intensities of actin protein and expressed as percent control over basal levels of unstimulated cells and displayed as the mean + SEM. Results 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, $p<0.05$).

**References:**

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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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