

# Muscarinic-1 Receptor Antagonists Upregulates ATF3 via ERK Phosphorylation

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## Background:

Peripheral Neuropathy is a serious condition that results in weakness and loss of feeling in the hands and feet due to damage of the peripheral nerves. Peripheral neuropathy is most commonly found in diabetic patients and people undergoing chemotherapy. As of right now there is no cure to peripheral neuropathy. Previous research has identified activating transcription factor 3 (ATF3) as a key factor in the regeneration of peripheral axons (Hunt et al, 2012). A key finding was that ATF3 is found in extremely low levels in normal neural cells but expressed at higher levels when our neural cells are injured (Hunt et al, 2012). While ATF3 is believed to play an important role in neural regeneration not much is known about the regulation of its activity and the signalling pathway that activates it in response to neural injury. Through previous research we have found that muscarinic antagonists that are specific or selective for muscarinic-1 receptors enhance neurite outgrowth (Calcutt et al, 2017). This enhancement is believed to occur due to the biased agonism between muscarinic-1 receptors and beta-arrestin2. A selective muscarinic antagonist binds to a muscarinic-1 receptor which results in the recruitment of beta-arrestin2 and the phosphorylation of ERK.

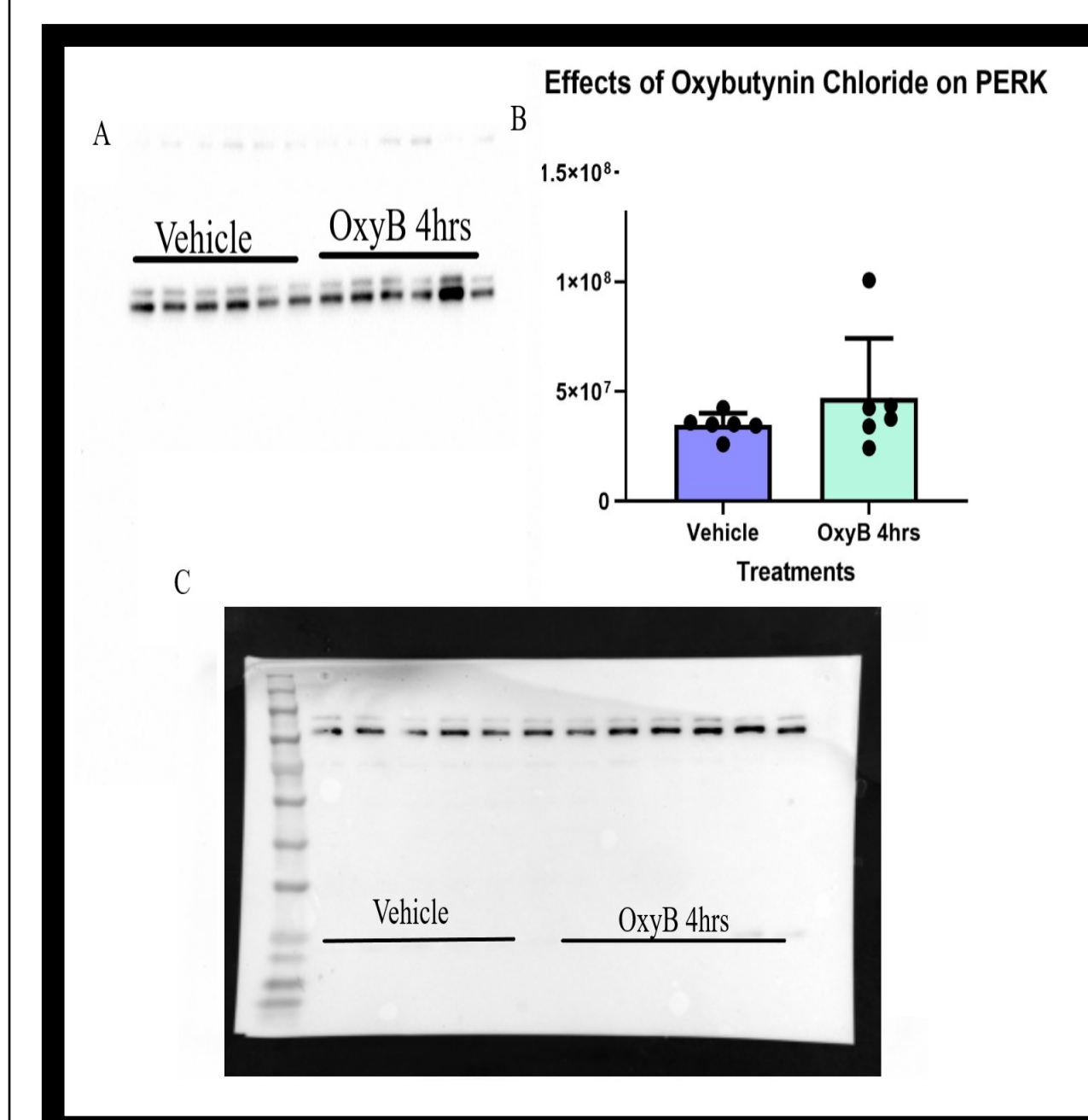
## Research Aims:

To discover the link between muscarinic-1 receptors, ERK, and ATF3 in the regeneration of peripheral neurons. Muscarinic-1 antagonists lead to the phosphorylation of ERK via biased agonism and beta-arrestin2. We hypothesized that as ERK phosphorylation increased due to muscarinic-1 receptors so would ATF3 levels.

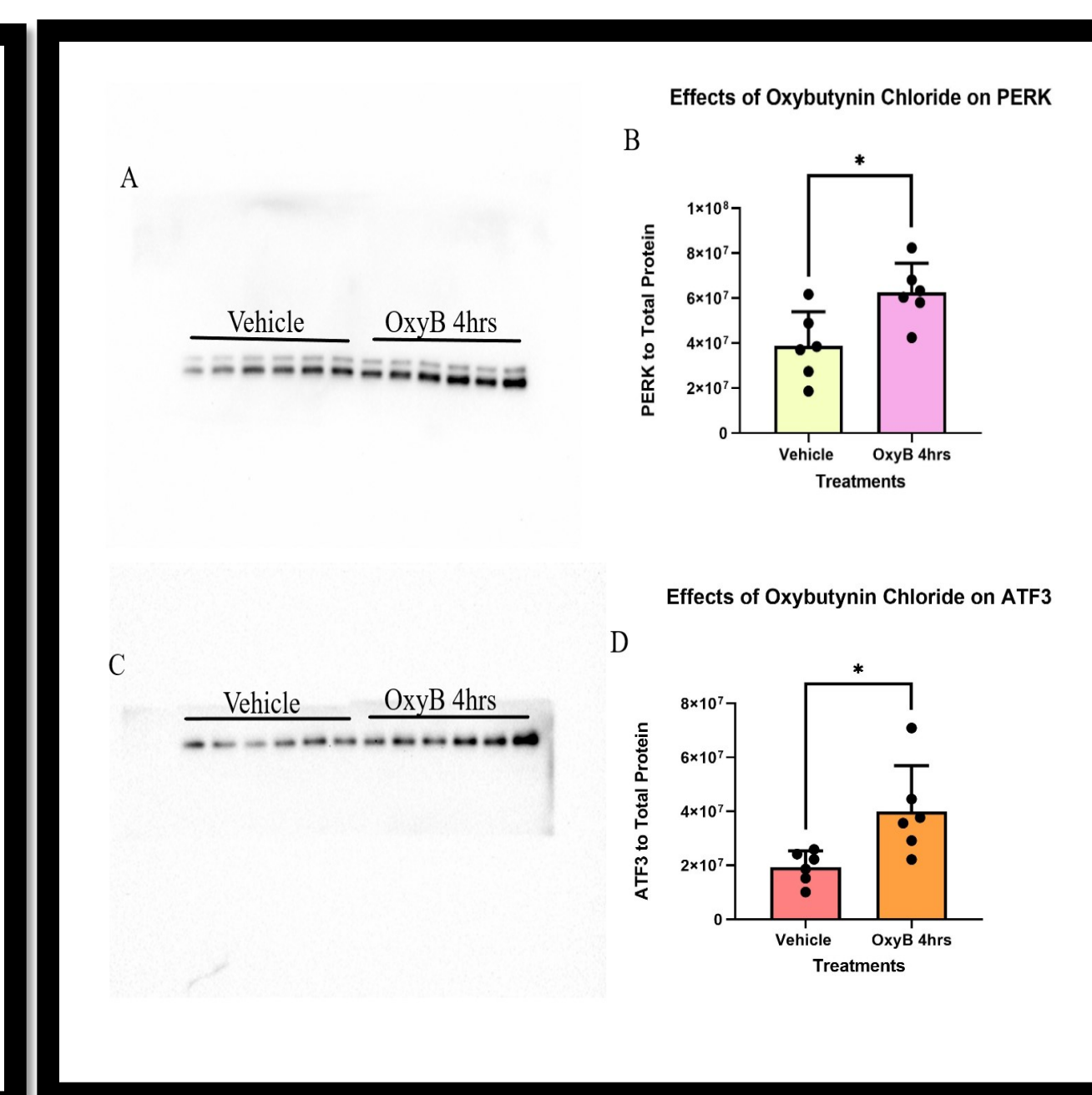
## Methods:

Human embryonic kidney cells (HEK293) were the cell lines used for all experiments. The cells were co-transfected with M1RNLUC (muscarinic-1 receptors tagged with NLUC) and  $\beta$ -ARR-HALO (Beta-arrestin2 tagged with HALO) to cause an overexpression of muscarinic-1 receptors. After transfection the cells were serum starved to synchronize all cells to the same cell cycle before starting drug treatments. The drug treatments used over the course of my experiments were 100nM oxybutynin chloride which is a non-selective muscarinic receptor antagonist and 1 $\mu$ M pirenzepine which is a selective muscarinic-1 receptor antagonist. After treatment with drugs, protein was harvested from the cells. My proteins of interest were ERK, P-ERK, and ATF3. To test for their presence I used western blotting.

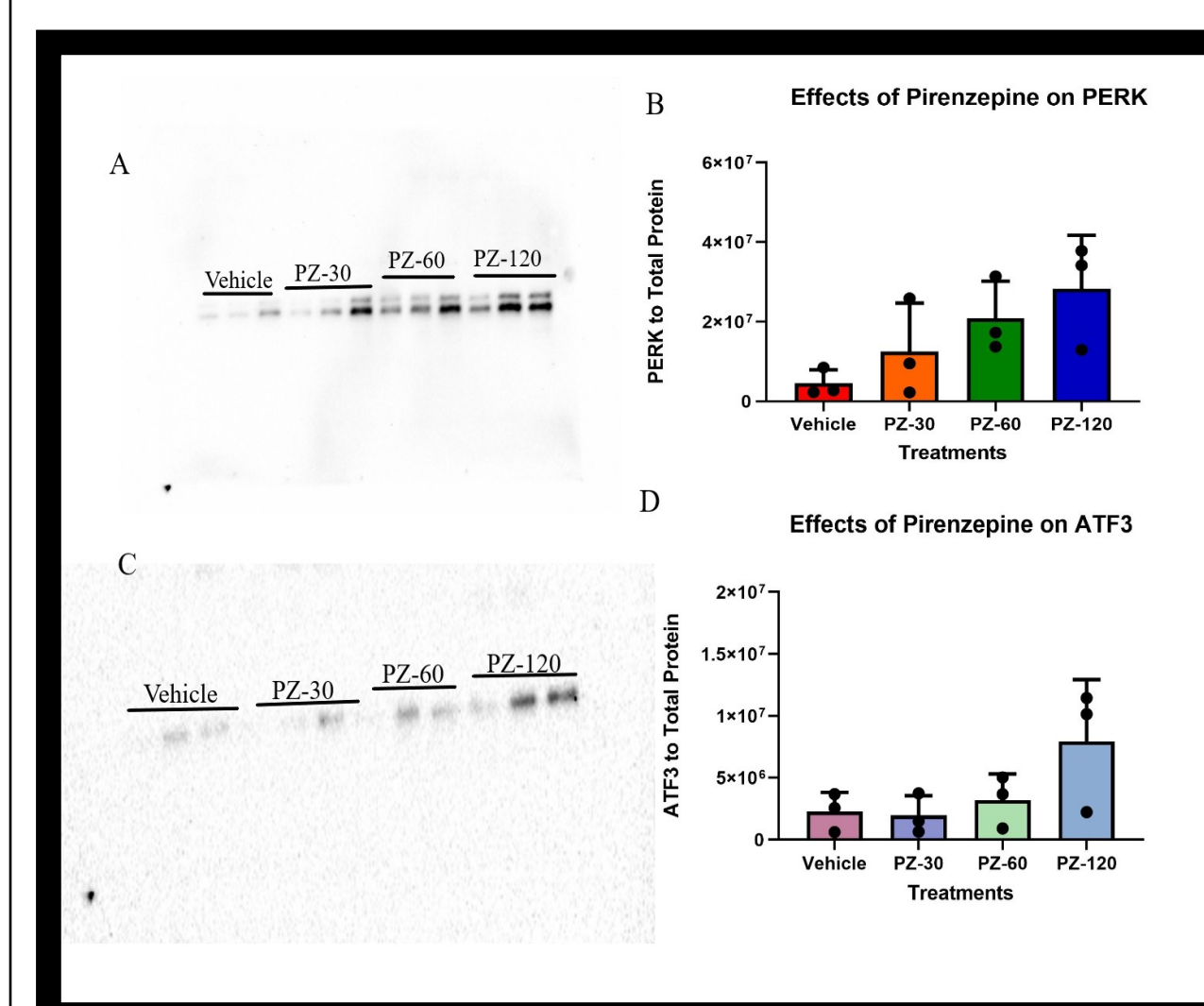
## Results:



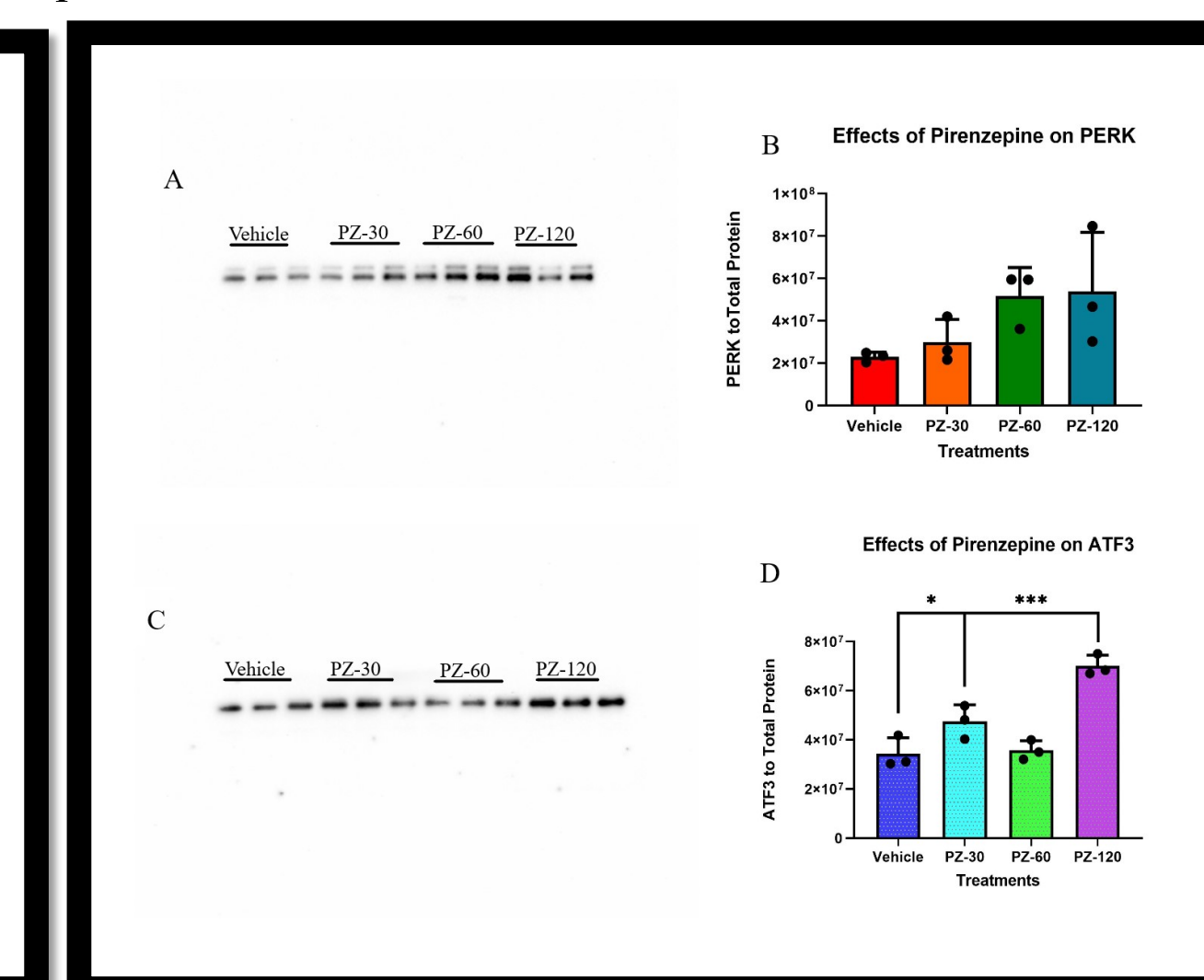
**Figure 1:** HEK293 cells exposed to a 5 hours starvation/serum withdrawal in DMEM-F12 media. The cells were then treated with 100nM Oxybutynin Chloride for 4 hours. **A:** Image of a western blot treated with P-ERK antibody. **B:** Analysis of results in figure 1A using a one way anova test. **C:** Image of a western blot treated with ATF3 antibody



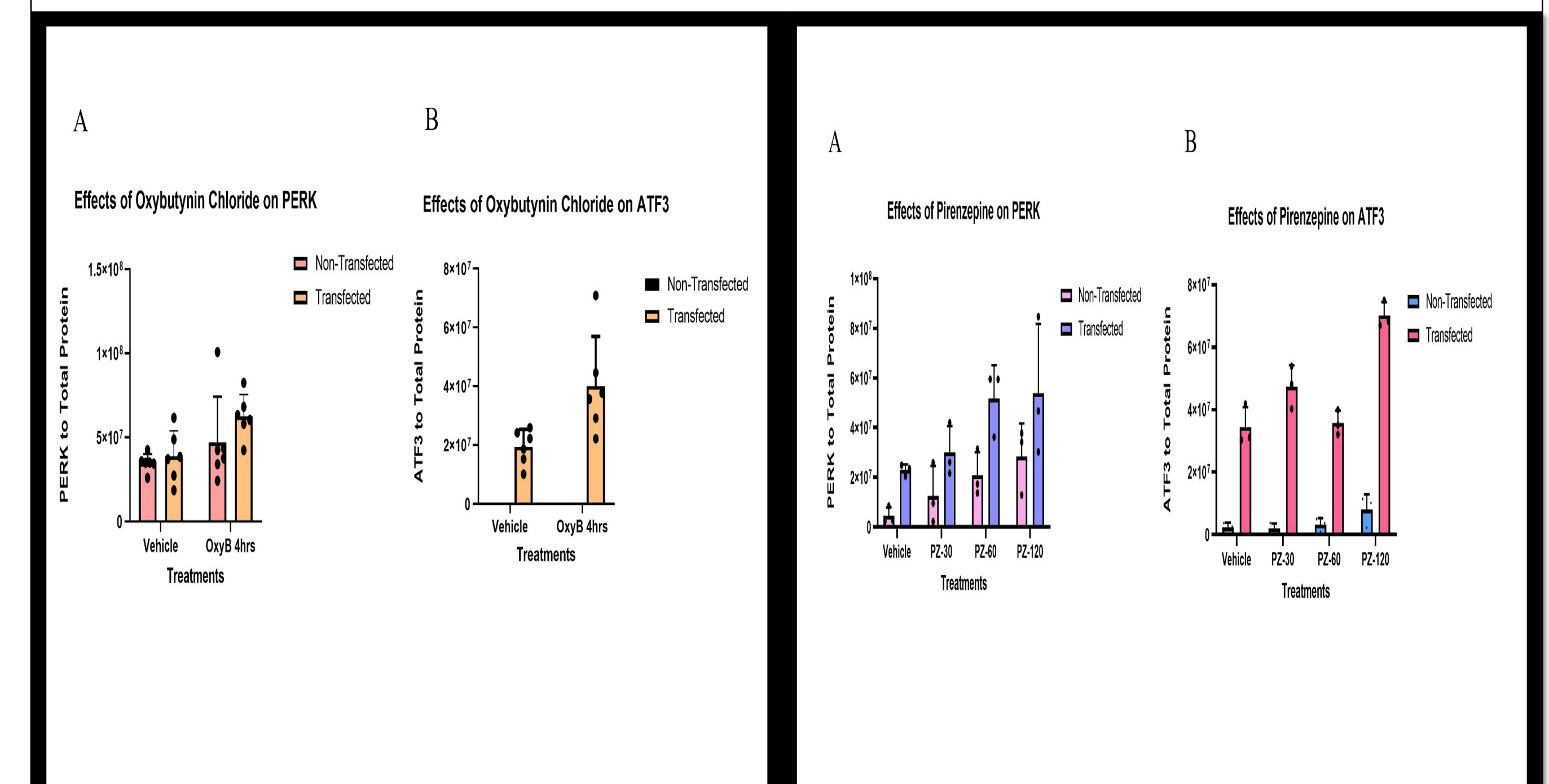
**Figure 2:** HEK293 cells co-transfected with M1RNLUC and  $\beta$ -ARR-HALO, then exposed to a 5 hours starvation/serum withdrawal in DMEM-F12 media. The cells were then treated with 100nM Oxybutynin Chloride for four hours. **A:** Image of a western blot treated with P-ERK antibody. **B:** Analysis of results in figure 2A using one way anova. \* indicates that  $p < 0.05$ . **C:** image of a western blot treated with ATF3 antibody. **D:** Analysis of results in figure 2C using one way anova. \* indicates that  $p < 0.05$ .



**Figure 3:** HEK293 cells exposed to a 5 hours starvation/serum withdrawal in DMEM-F12 media. The cells were then treated with 1 $\mu$ M Pirenzepine for varying time intervals. **A:** Image of a western blot treated with P-ERK antibody. **B:** Analysis of results in figure 3A using a one way anova test. **C:** Image of a western blot treated with ATF3 antibody. **D:** Analysis of results in figure 3C using a one way anova test.



**Figure 4:** HEK293 cells co-transfected with M1RNLUC and  $\beta$ -ARR-HALO, then exposed to a 5 hours starvation/serum withdrawal in DMEM-F12 media. The cells were then treated with 1 $\mu$ M Pirenzepine for varying time intervals. **A:** Image of a western blot treated with P-ERK antibody. **B:** Analysis of results in figure 4A using a one way anova test. \* indicates that  $p < 0.05$ . **C:** Image of a western blot treated with ATF3 antibody. **D:** Analysis of results in figure 4C using a one way anova test. \* indicates that  $p < 0.05$ .



**Figure 5:** Results from figure 1 and 2 comparing the effects of oxybutynin chloride on (A) P-ERK and (B) ATF3 in non-transfected and transfected HEK293 cells.

**Figure 6:** Results from figure 3 and 4 comparing the effects of pirenzepine on (A) P-ERK and (B) ATF3 in non-transfected and transfected HEK293 cells.

## Conclusion:

As you can see from the results in figure 1-6. Muscarinic receptor antagonists have a strong effect on the level of P-ERK and ATF3. With both oxybutynin chloride and pirenzepine we observe the same trend in results. In non-transfected HEK293 samples our P-ERK and ATF3 levels are lower than what is observed in the transfected HEK293 samples. We can also see an overall trend in my transfected samples that as P-ERK levels increase so do ATF3 levels. Figures 5-6 also show that pirenzepine which is a selective muscarinic-1 receptor has a stronger effect on P-ERK and ATF3 than oxybutynin chloride which is a non-selective muscarinic receptor. Based on my results we can see that overexpressing muscarinic-1 receptors and treating them with an antagonist results in an increase in P-ERK and ATF3 levels. To further support my hypothesis that muscarinic-1 receptor antagonists upregulates ATF3 via ERK phosphorylation, a future direction would be to run an experiment with an ERK inhibitor. An ERK inhibitor would decrease the levels of P-ERK and with that we would expect to see decreased ATF3 levels as well.

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