# Localization and quantification of acetylcholine receptors in the brains of knockout and wild-type mice

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## Introduction

Nerve agents such as soman (GD) are organophosphorus compounds that inhibit acetylcholinesterase and cause acetylcholine (ACh) to accumulate in the synaptic cleft. This accumulation results in lethal repeated firing of neurons called status epilepticus (Johnson & Kan, 2010). In response to status epilepticus and neuronal damage, proinflammatory pathways are activated that can exacerbate these outcomes after exposure. A potential strategy to reduce damage after exposure is to inhibit the pro-inflammatory pathways Interleukin (IL)-1 and tumor necrosis factor α (TNFα) (Pavlov et al., 2009). The US Army Medical Research Institute of Chemical Defense (USAMRICD) has developed an animal model that mimics nerve agent exposure using knockout mice to identify pathways that may be used in neuroprotective treatments. One of these strains, the IL-1 receptor 1 (IL1R1) /TNF receptor 1A (TNFR1A) knockout mouse, has been observed to be particularly resistant to nerve agent poisoning with reduced mortality, seizure initiation and brain damage. A potential explanation for this resistance is an increased amount of inhibitory ACh receptors in the brain. There are two main types of ACh receptors, muscarinic receptors, which indirectly affect the cell through internal reactions, and nicotinic receptors, which let ions pass into the cell to cause immediate changes. Muscarinic receptors break down further into five subtypes, with the M2 receptor being an inhibitory receptor. (Albuquerque, Pereira, Alkondon, & Rogers, 2009) Therefore, this study focused on potential changes in M2 receptor density and localization in three strains of mice through immunohistochemistry (IHC) to determine if an increase in initial M2 receptor density might account for the beneficial effects seen in the TNFR1A/IL1R1 double knockout mice.

## Materials and Methods

Brain slides from tumor necrosis factor receptor 1A/interleukin-1 receptor 1 (TNFR1A/IL1R1) knockout mice, C57/BL6J wild-type mice, and tumor necrosis factor receptor 1A/tumor necrosis factor 1B (TNFR1A/B) knockout mice were deparaffinized in EZ DeWax™ solution, then rinsed in distilled $H_2O$, and lastly immersed in $H_2O_2$ to halt peroxidase activity. These slides were rinsed again and then microwaved in citric acid. After microwaving, the slides rinsed in phosphate buffered solution (PBS) between each step. Tissue was treated for thirty minutes with a 2% blocking solution then treated with a muscarinic acetylcholine receptor 2 (mAChR2) antibody for one hour at room temperature and then 48 hours at 4 °C. The slides were then prepared for either fluorescent or 3,3'-diaminobenzidine tetra-hydrochloride (DAB) secondary labeling. Double stained fluorescence slides were treated with a fluorescent 594 secondary antibody in blocking serum for 30 minutes. An additional primary antibody was added that targeted Glial fibrillary acidic protein (GFAP) for one hour. Tissues were then treated with a fluorescent 488 tertiary antibody for 30 minutes. Afterwards, slides were cover slipped with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) and sealed with nail polish. DAB slides were treated with a secondary antibody solution for 30 minutes. After being rinsed, the slides were incubated with an avidin-biotin-peroxidase solution for thirty minutes. The final step for the densitometry slides was to rinse with PBS and treat the slides with a DAB solution for about two minutes to achieve desired stain intensity. The densitometry slides were dehydrated to xylene and cover slips were mounted using Permount™. Tissue sections were examined with an Olympus BX61 equipped with a DSU spinning disk confocal system and DP-70 CCD camera. Fluorescent pictures were taken for localization and DAB pictures were taken for densitometry. Fluorescent photos were zoomed to 10x and taken through the 488 nm channel, 594 nm channel, and 461 nm channel and then combined in cellSens, a program used for microscopy by USAMRICD. DAB photos were taken at 40x across the entire slide through an automated macro. Densitometry was performed on the whole slide of each strain by importing the 40x pictures into ImageJ and running a macro. This macro was set to count all pixels within a particular range. The counts represent the amount of receptors stained by the primary antibody, so when recorded, the counts indicated the number of mAChR2 that are present in the entire brain slide.

## Materials and Methods (cont.)

## Results

The 10x fluorescence photos were qualitatively analyzed in reference to GFAP and DAPI. As seen in Figure 1, the mAChR2 were observed to cluster about the axons and cell bodies of neurons in the hippocampus. Staining was also seen in the same manner in the cortex, thalamus, piriform cortex, and amygdala, but was not shown here.

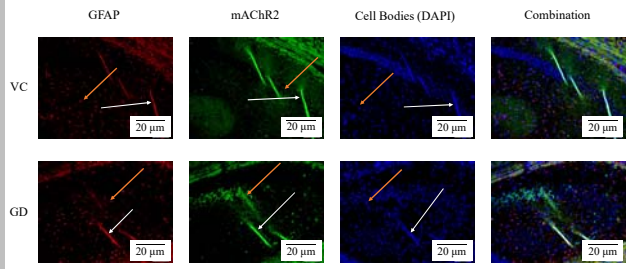
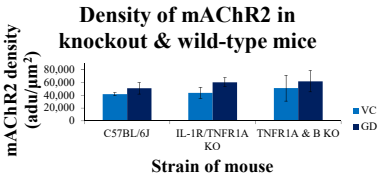



Figure 1: CA1-CA2 hippocampal region with individual 10x channels shown. Artifacts (white arrows) are present in each channel and cancel each other out. Positive staining for each channel, shown by the orange arrow, shows how antibodies in each channel stain.

## Results (cont.)

A one-way ANOVA analysis with a post-Bonferroni analysis was performed with an *n* of 24, in which a *p*-value of 0.7843 was obtained. There was no significant difference in mAChR2 concentrations between each strain of mice. As seen in Graph 1, mAChR2 density increased in GD exposed mice compared to vehicle mice.




Graph 1: DAB stained mAChR2 area for each strain of knockout and wild-type mouse. Mice exposed to GD that expressed a pathology response such as seizing had a higher area of receptors in all strains compared to vehicle mice with no pathology. Error was calculated as the standard error from the mean.

## Conclusion

The purpose of this project was to determine if a different density of the mAChR2 in the brain was responsible for the increased resilience to nerve agent poisoning in TNFR1A/IL1R1 double knockout mice. The null hypothesis is that there would be no significant difference in mAChR2 amounts in the brain between the TNFR1A/ILR1 knockout mouse and the C57/BL6J wild-type mouse. IHC was performed on slides from TNFR1A/IL1R1 knockout mice, C57/BL6J wild-type mice, and TNFR1A/B knockout mice. Double staining and DAB IHC were used to localize and determine density of mAChR2 in mice brains, and found that there was no significant difference in mAChR2 density between TNFR1A/IL1R1 knockout mice, C57/BL6J wild-type mice, and TNFR1A&B knockout mice. The null hypothesis was not rejected, and the evidence supported the hypothesis that there is another factor responsible for the resilience to nerve agent poisoning seen in TNFR1A/IL1R1 knockout mice. Further research into neuronal receptor density is needed to verify that other factors are responsible for nerve agent resilience, but this project has yielded important information for the development of neuroprotective therapies.

## References

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