INVESTIGATING THE ROLE OF GLYMPHATIC CLEARANCE OF AMYLOID BETA THROUGH EXERCISE IN C57BL6/J MICE

By

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ABSTRACT

Alzheimer's disease (AD) is a very prevalent neurodegenerative disorder characterized by widely distributed amyloid plaques and neurofibrillary tangles. AD is clinically associated with a progressive decline in memory and other cognitive functions. Several pieces of evidence have indicated that amyloid beta accumulates to form oligomeric states in the AD brain and cause the cognitive dysfunction commonly seen in patients. While a decrease in cognitive function is considered a hallmark of the disease, AD patients also exhibit decreased motor abilities and difficulties learning new motor tasks. Our lab's previous investigations found voluntary running to decreased amyloid beta burden in C57/BL6 mice. The present experiment seeks to further explore the mechanism through which exercise induced amyloid beta clearance occurs. Previous studies have pointed to the function of the glymphatic system in the clearance of amyloid beta. The level and distribution of aquaporin 4 (AQP4) is crucial to the normal function of the glymphatic system. We hypothesize that mice receiving intraperitoneal TGN injections, a selective AQP4 antagonist, thus blocking the function of AQP4, will experience decreased glymphatic clearance of amyloid beta. To test this hypothesis mice were given intraperitoneal injections of saline every morning for one week. The following two weeks, doses of either TGN or saline injections were given twice daily morning and night. During this period, mice were moved into individual cages with running wheels at 6:00 PM daily and returned to group housing cages the following morning at 7:00 AM. 24 hours following treatment mice were trained and tested in hippocampus dependent contextual fear conditioning (CFC) to explore possible cognitive differences between treatment groups.

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INTRODUCTION

Alzheimer's disease (AD) is a very prevalent neurodegenerative disorder and is the 6th leading cause of death in the United States. AD is characterized by widely distributed amyloid plaques and neurofibrillary tangles, and is clinically associated with a progressive decline in memory and other cognitive functions (Gargia-Alloza and Monica, 2006). The severe cognitive dysfunction and memory impairment continues to worsen over time and ultimately leads to complete loss of independent living. Given increasing life expectancy, the prevalence of AD is expected to continue to rise, afflicting 50% of the population over 85. It is an increasingly serious problem throughout society and there is no treatment or cure available at this time. Early and accurate diagnosis of AD could save up to $7.9 trillion in medical and care costs. There is currently no cure for Alzheimer's and an urgent need for better understanding (Alzheimer's Association 2018).

The neuronal degradation within the hippocampus of the brain associated with AD leads to observable behavioral changes in those afflicted. Memory loss is one of the earliest signs and changes observed. Initially the memory loss is mild, effecting short term memory, resulting in forgetfulness of minor events, recalling names, or recent conversations. As the disease progresses memory impairments worsen and a profound dementia develops affecting behavioral and cognitive activity (Heneka and O'Banion, 2007). AD causes difficulty concentrating and thinking about abstract concept such as numbers, making paying bills or managing finances an increased hassle. Changes in personality and mood are seen and patients often experience social withdrawal, depression, irritability, delusions and mood swings (Mayo Clinic 2018). In severe stages, motor skills decline resulting in slow uncoordinated movements, an inability to control normal physiological functions, and speak clearly. Once routine activities become a struggle,

including cooking or dressing. Patients may be unaware of time and unable to recognize their environment or even their family members surrounding them. At this stage, patients require assistance with almost all daily activities (Heneka and O'Banion, 2007). These changes leave an enormous emotional burden on surrounding family and loved ones. In addition to the emotional toll, AD becomes a large financial burden as well due to hospital visits, prescriptions, and at home care.

The neuropathological trademarks of AD patients are characterized by the presence of extracellular neuritic plaques of the β-amyloid peptide (Aβ) and intracellular neurofibrillary tangles (NFT) in the hippocampus. The major protein component of neuritic plaques is amyloid beta peptide (Galimberti et al., 2013). Several pieces of evidence have indicated that amyloid beta accumulates to form oligomeric states in the AD brain and cause the cognitive dysfunction commonly seen in patients. In forming these plaques, amyloid beta precursor protein (APP) is cleaved by two proteins, β- and γ-secretase. β- secretase first cleaves APP to release a large peptide derivative, sAPPβ. The remaining fragment of APP remains membrane bound and is then cleaved by γ-secretase to generate Aβ. Cleavage by γ-secretase is somewhat imprecise and results in numerous different Aβ species of varying length and hydrophobicity. The most abundant peptides cleaved are those ending at position 40 (Aβ40, ~80-90%) and 42 (Aβ42, ~5-10%). The longer form of Aβ, particularly Aβ42, is more hydrophobic and clusters together to form oligomers. These oligomers accumulate into larger fibrils and then into plaques deposited in the brain (Murphy and Levine, 2010). Amyloid beta plaques bind to neurons causing synaptic dysfunction and inaccurate neuronal communication, blocking key processes involving memory and learning. This leads to neuronal and cognitive dysfunction and ultimately neurotoxicity and cell death (Qin et al., 2018). This damage in addition to those incurred due to NFT are

considered the root cause of brain degeneration associated with AD. Amyloid beta oligomers are considered a reliable biomarker for the diagnosis of AD and can be detected through enzyme linked immunosorbent assay (ELISA) and evaluated by Western blot analysis (Yang et al., 2013).

In addition to the neuronal dysfunction caused by the buildup NFT and amyloid beta plaques, amyloid beta may promote neurodegeneration by activating microglial cells and astrocytes. Activated microglial cells induce an inflammatory response releasing various inflammatory mediators and neurotoxic pro-inflammatory cytokines. Aβ in the brain leads to an excess of inflammatory cytokines that induces the production of even more amyloid beta. Several lines of evidence have indicated all of these factors contribute to eventual neuronal dysfunction and cell death in AD (Heneka and O'Banion, 2007).

While a decrease in cognitive function is considered a hallmark of the disease, AD patients also exhibit decreased motor abilities and difficulties learning new motor tasks. Motor dysfunction seems to occur sooner than and is predictive of cognitive declines (Buchman and Bennett, 2011). Lifestyle modifications such as physical activity and exercise help manage AD as well as reduce the risk of developing AD, delaying onset, and improving AD symptoms (Buchman and Boyle, 2011). Our lab's previous investigations found voluntary running to decreased amyloid beta burden in C57/BL6 mice. To show this, we used intraperitoneal injection of lipopolysaccharide (LPS) administered once daily for seven days in non-transgenic mice to initiate AD-like pathology. Our lab has previously demonstrated that LPS leads to increases in proinflammatory cytokines in addition to central accumulation of Aβ. The mice were allowed access to voluntary running wheels for 12 hours at night in individual housing and their wheel activity was measured by number of wheel turns. The results showed exercise animals

demonstrated increased clearance of amyloid beta as shown by less amyloid beta in our ELISA analysis.

The present experiment seeks to investigate the mechanism through which exercise induced amyloid beta clearance occurs. Nedergaard and colleagues previously discovered the function of the glymphatic system in the clearance of amyloid beta (Iliff et al., 2012). The glymphatic system is responsible for the clearance of neuronal extracellular protein waste mainly via the cerebrospinal fluid (CSF)- intersitital fluid (ISF) exchange (Iliff et al., 2012). The level and distribution of aquaporin 4 (AQP4) in the vascular endfeet of astrocytes is crucial to the normal function of the glymphatic system. AQP4 influences synaptic plasticity and mediates Aβ clearance, whereas AQP4 deficiency impairs learning and memory (Lan et al., 2016). There has been indication that messenger RNA transcripts involved in AQP4 expression are down-regulated in major depressive disorder subjects compared to control. Major depressive disorders are highly linked to the development of Alzheimer's and depression is considered a risk factor for AD (Medina et al., 2016). In opposition to a healthy brain that is able to clear amyloid beta via glymphatic drainage, in AD there is a gradual accumulation in the brain parenchyma and vascular structures. However, the exact mechanisms and importance of the glymphatic system continues to be studied. Our experiment sought to determine a more detailed analysis of the role of AQP4 in the glymphatic clearance of amyloid beta. Using TGN-020, a selective AQP4 antagonist, the project aimed to further determine the role of glymphatic clearance of amyloid beta in C57BL6/J mice through exercise. We hypothesize that mice receiving intraperitoneal TGN injections, thus blocking the function of AQP4, will experience decreased glymphatic clearance of amyloid beta. It is hypothesized that these mice will therefore have greater levels of Aβ following voluntary exercise due to decreased clearance.

MATERIALS AND METHODS:

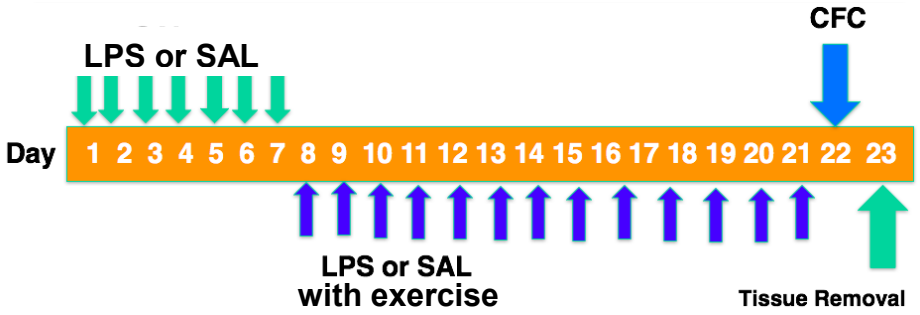
Subjects and housing

Ranging from 4-6 month old male C57BL/6J mice were used in this experiment. The mice were bred in the TCU vivarium, however originated from the Jackson Laboratory breeding colony located in Bar Harbor, Maine. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) and in congruency with the procedures outlined by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. The animals were housed in standard cages measuring 12.5 cm x 15 cm x 25cm. Each cage held a group of three to four animals. Mice were moved into individual cages, all of which had running wheels, at 6:00 PM daily. The mice were then returned back to their group housing cage without running wheels at 7:00 AM daily. Cage conditions remained the same throughout the experiment. Likewise, control groups were subjected to the same living conditions as the experimental group. Daily rhythms were kept with the lights turning on at 7:00 AM and turning off at 7:00 PM.

Treatment conditions

Prior to testing, mice were given intraperitoneal injections of LPS of 250 μg/kg dissolved in 0.2 ml saline every morning for one week. The following two weeks, doses of either 13.3 mg/kg TGN dissolved in 0.4ml saline or the same equivalent of saline injections were given 2 hours prior to placement in running cages. Saline injections were used as the control group to make certain the differences seen were not elevated due to stress of consecutive injections. The mice received intraperitoneal injections and were weighed daily. Running wheel activity was

measured during the time in which the mice were housed in their individual cages from 6:00 PM to 7:00 AM.



Aβ ELISA procedure

The LEGEND MAX β-Amyloid x-42 ELISA Kit (Biolegend) was performed according to manufacturer's instructions. After coating each well with antibody, the wells were filled with either the sample or standards of known concentration. The incubation buffer and the standard intermediates were made before beginning the assay. The Standard Diluent was used in order to make the standard curve as well as the Aβ standard. Samples were subsequently diluted in the buffer, which included the HRP labeled detection antibody at a ratio of 2 to 1. The plates were then incubated overnight at 2-8 degrees Celsius. The proceeding day, each well was washed 5 times using 1X wash buffer. After the washes, 200 µL of TMB was added. TMB is the HRP enzyme substrate. The plate then incubated for 45 minutes at room temperature within a darkroom. Following this, an optical density of 620 nm was used to read the plates.

Statistical Analyses

Using an analysis of variance (ANOVA) statistics, we were able to quantify significant differences between experimental and control groups. If a significant omnibus F was found, then the use of Fisher's PLSD post hoc test was implemented to conclude which groups were significantly different.

Behavioral Paradigms

Contextual fear conditioning

CFC (contextual fear conditioning) is a behavioral paradigm utilized to assess the cognitive function of mice. The first day is the training session, and the testing session takes place 24 hours later. Training took place the day after the last night of wheel running. During the training session, there was a 120 second acclimation period preceding a 2 second 0.5 mA mild aversive stimulus, followed by a 60 second phase where they remained within the unit. The total training time is 182 seconds. The following day is the testing period, in which the mice were once again placed in the unit. During testing day, no shock was administered. Instead, the animals' movements were recorded for 120 seconds continuously in order to observe freezing behaviors. The total time spent freezing indicates a stronger association between the context and the aversive stimulus, hippocampus dependent contextual learning. Freeze Monitor System and software was used to measure freezing behaviors. The CFC unit (Coulbourn Instruments, Whitehall, PA) has a floor containing an electric grid which emits the negative stimulus to the mouse. The ceiling of the unit houses both a small light bulb as well as a camera to track and monitor the movements of mouse.

RESULTS

TGN does not affect the ability to exercise in mice

In order to properly assess the effect of TGN on exercise mediated amyloid beta clearance, it was essential to show that TGN itself does not affect the ability to exercise in mice. It has been previously shown that exercise mobilizes AQP4 in the muscles so we needed to assure that blocking AQP4 did not have an adverse effect on exercising muscles or impact the activity level. If TGN had effected the activity level it would be inaccurate to make conclusions

regarding the role of TGN on amyloid beta clearance since the exercise level would be a compounding factor in the clearance. To this end, the running wheel rotations were measured daily and the treatment groups were compared to assure all the groups had the same level of exercise. Our results (Figure 1) showed there was no significant differences in daily activity regardless if the mice received LPS or saline, or TGN or saline ($p<0.05$). These findings were imperative because they allow us to compare the exercise mediated clearance between each treatment group, since the exercise levels were the same between all groups.

TGN treatment did not decrease exercise mediated clearance of Aβ

To illustrate that exercise increased the clearance of Aβ through the glymphatic system and the use of AQP4, it was necessary to measure the levels of Aβ. We used an x-42 ELISA kit to measure these levels. The results demonstrated that TGN did not decrease the clearance of Aβ (Figure 2). Our data illustrated a significant increase in Aβ burden between animals that received intraperitoneal injections of LPS versus animals who received injections of saline. However, no effect of TGN was shown as there was no significant difference between animals who received TGN or saline ($p<0.05$). This data indicates that TGN treatment did not affect exercise mediated clearance of amyloid beta.

TGN treatment did not affect cognitive function

In addition to discerning if TGN inhibits the increased amyloid beta clearance mediated by exercise, we were looking at the effect of TGN on hippocampus dependent contextual learning (Figure 3). By measuring freezing behavior in CFC on testing day, we were able to determine there was no significant difference in percent freeze time between animals who received LPS or saline, or TGN or saline ($p<0.05$). TGN did not further decrease cognitive function which correlates with its inability to decrease the clearance.

DISCUSSION

In the present study, we sought to determine whether or not previously observed exercise mediated clearance of Aβ were due to clearance mediated through AQP4 within the glymphatic system. Our lab has previously shown that intraperitoneal injections of LPS for 7 days results in elevated levels of Aβ in the hippocampus as well as cognitive deficits visible through CFC testing. From a prior study conducted by Lan et al (2016), we know that AQP4 is implicated in the clearance of Aβ. Cerebrospinal fluid functions as a sink for brain extracellular solutes and it has been demonstrated that Aβ is transported along this route. Animals that lacked AQP4 in astrocytes exhibited ~70% decrease in solute clearance suggesting AQP4 to be involved in the drainage pathway of removing Aβ from the CNS. Additionally, it has been shown that Aβ might alter the expression of AQP4 as AQP4 downregulation appears to occur in later stages of Aβ plaque formation. (Illif et al., 2016). Since Aβ has been proven to have deleterious effects on cognition and contribute to AD behaviors, targeting AQP4 as a potential mechanism of Aβ clearance is a very attractive area of research. TGN-020 has been shown to be a selective AQP4 inhibitor according to various previous studies (M Xia et al, 2016). Therefore, we hypothesized that using a known AQP4 inhibitor such as TGN-020 in concert with intraperitoneal LPS injections would allow us to see if the observable Aβ clearance mediated through exercise were attributed to the glymphatic system and function of AQP4.

To test our hypothesis, we used four experimental groups that received injections once daily (saline-saline, saline-TGN, LPS-saline, LPS-TGN) in which the first 7 days the mice received either LPS or Saline and for the following 2 weeks received either TGN or Saline. We predicted mice that received injections of LPS along with TGN would show significantly elevated levels of Aβ when compared to mice that received LPS and saline due to exercise

mediated clearance being reduced through inhibition of AQP4. Since previous investigations have shown elevated levels of amyloid beta to correlate with hippocampus dependent behavioral deficits, we reasoned that mice receiving LPS-TGN would exhibit increased cognitive deficits as shown by CFC. To prevent any confounding results, it was imperative to show that TGN-020 itself does not prevent or hinder the ability of mice to exercise. This was especially critical to examine because AQP4 is stabilized in exercising muscles. To do so, we measured the daily wheel rotations of each experimental group. There were no significant differences in average activity level between any group indicating TGN-020 did not implicate the ability to run.

After completions of injections we needed to assess behavior utilizing CFC. By measuring freezing behavior, we could determine if any of the treatment groups were experiencing cognitive deficits in learning and memory. In order to make a conclusion there needed to be a significant difference in freezing behavior during testing day. Our results indicated that there were actually no significant differences between any treatment group animals. We had hypothesized that animals receiving LPS-TGN injections should exhibit cognitive deficits shown by a decreased time freezing which would indicate an inability to associated the context of the foot shock with the environment. In addition, our results did not confirm previously seen results that LPS-saline animals have a decreased freezing behavior compared to saline-saline. The explanation for our lack of significant results is likely due to the timing of CFC compared to the injections as well as the added component of exercise. We performed CFC 2 weeks after LPS injections whereas if we had performed them directly following the 7 days of initial injections we would have seen significant cognitive deficits in mice receiving LPS. Likewise, within the 2 week span of injections following 7 days of LPS injections, the mice had access to running wheels. It's likely that enough Aβ had been cleared

through exercise mediated functions during these 2 weeks that there wasn't a high enough concentration of Aβ left accumulated in LPS animals to show significant cognitive deficits in our LPS group versus our saline group. However, we would still expect to have seen an adverse effect of TGN-020 on cognitive function and this was not noted through CFC testing.

Lastly, it was important to directly examine the concentration of Aβ in the hippocampus of the various experimental groups to determine the effect of TGN-020 on exercise mediated Aβ clearance. Our results showed a significant elevation of Aβ in animals that received LPS versus saline, however no effect of TGN-020 was noted. This result suggests TGN-020 was unsuccessful in blocking exercise mediated clearance of Aβ through AQP4. Although we did not specifically test the ability of TGN-020 to bind to AQP4 within the brain previous literature confirms it as a successful AQP4 antagonist. It is likely that our concentration of TGN-020 was not high enough to have a significant effect. Considering exercise stabilizes AQP4 in the muscles as well, its reasonable to conclude that the exercising mice had an increased concentration of AQP4 in the muscles that our TGN-020 was inhibiting. This increase of AQP4 in the muscles became saturated with TGN-020 such that there was not enough TGN-020 to fully inactive the AQP4 channels in the brain and show an effect on Aβ clearance. In addition, its plausible that the TGN was too quickly metabolized in the body of the mice. This could be a possibility for further exploration in which we examine the results with an increase of TGN-020 dosage. Diffusing TGN-020 directly into the brain may also ensure binding to AQP4 channels in the blood brain barrier preferentially as opposed to in the muscles. Examining the role of the glymphatic system and AQP4 is a possible target for Alzheimer's Disease therapy that warrants further exploration.

Figure 1

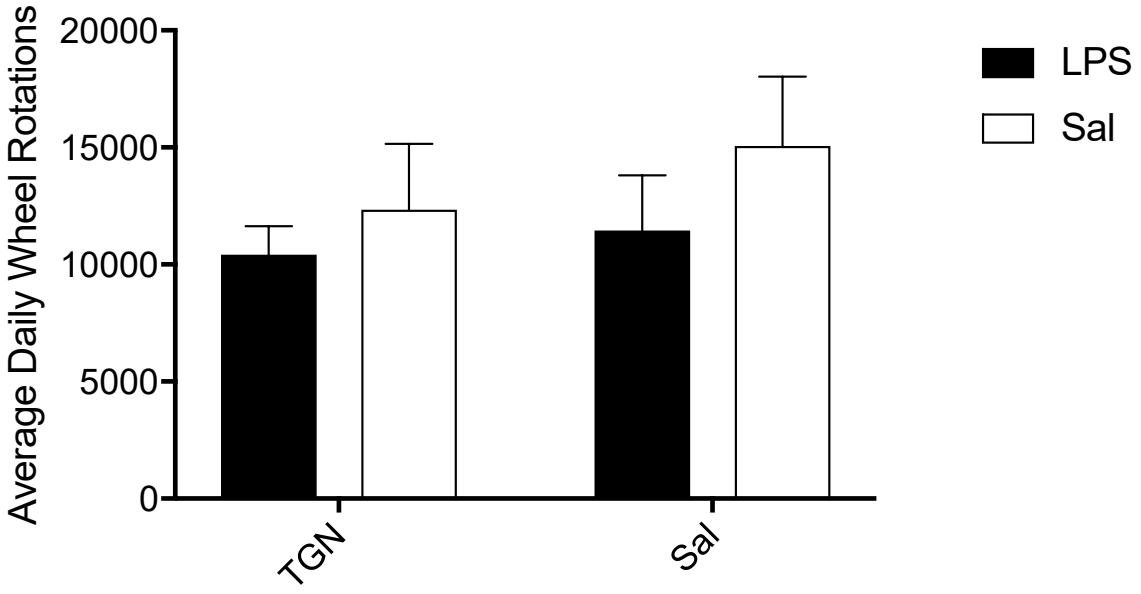


Figure 1. Average Daily Wheel Rotations. To determine the activity level of each experimental group, the wheel rotations were recorded each night for each animal. After the two-week period of exercise and injections, the average daily wheel rotations were calculated according to which injections the mice received. There were no significant differences in average daily wheel rotations between any experimental group. This suggests there was no effect of TGN on exercise level.

Figure 2

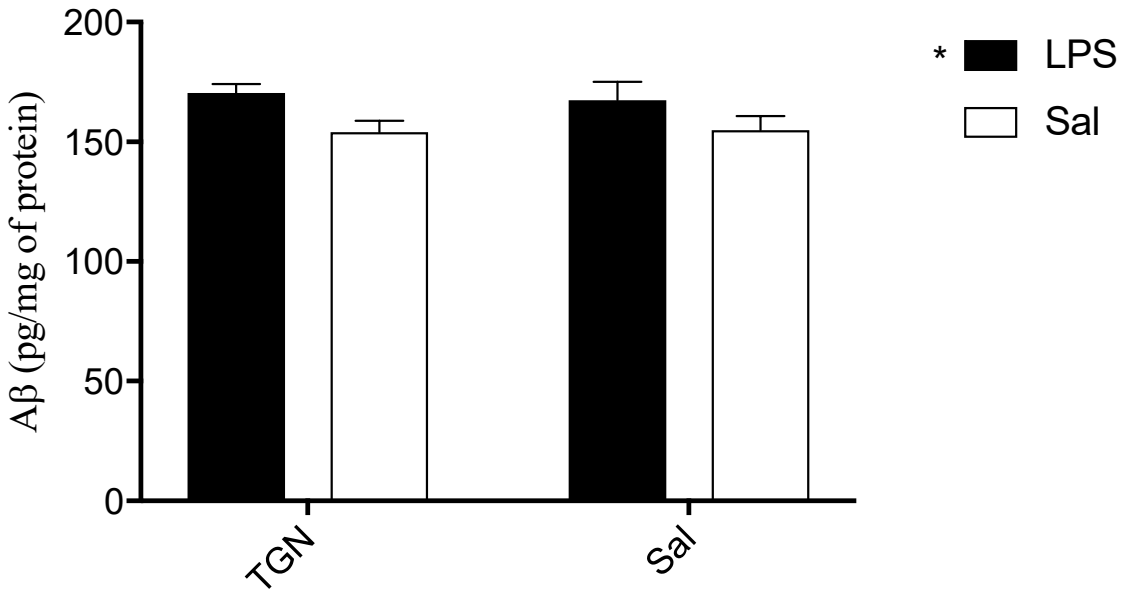


Figure 2. Amyloid beta levels following a two-week period of injections and access to running wheels. There was a significant difference ($p<0.05$) of Aβ levels in the hippocampus of mice in LPS groups compared to saline, however no effect of TGN was shown.

Figure 3

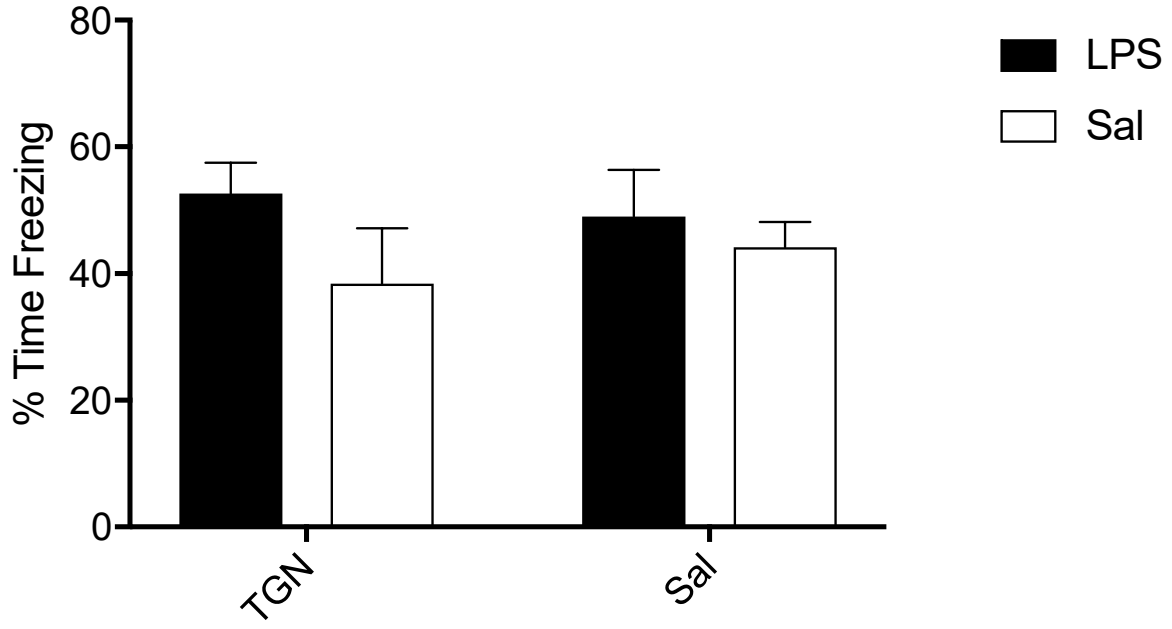


Figure 3. Cognitive deficits as observed in CFC. LPS-induced deficits in cognition are not observed as there is no significant difference in percent time freezing between any treatment group. Likewise, TGN show no adverse effect on cognition. Bars represent +/- SEM.

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