Systemic sodium selenate, a PP2A activator, attenuates conditioned taste aversion learning and phospho-MAP kinase induction

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Abstract

[REVISE, especially to include c-FOS]

CTA learning is constrained by phosphatase activity and is enhanced by, e.g.,okadaic acid (Oberbeck 2010) or calcineurin knockdown (Baumgartel 2008). Conversely, phosphatase activation should attenuate CTA. Recently, sodium selenate was found to be a specific activator of protein phosphatase 2A (PP2A; Corcoran 2010, van Eersel 2010). To characterize the effects of selenate on CTA, we determined 1) the dose of selenate which induces a CTA and 2) if a subthreshold dose of selenate would attenuate a LiCl-induced CTA. Water-restricted rats were given 10-min access to 0.125% saccharin, then injected with selenate (0, 0.5, 1, or 2 mg/kg ip, n=6 /dose). The next day, 2-bottle 24-h preference extinction tests of water vs. saccharin were begun and continued for 14 days. Rats that received saccharin paired with 1 or 2 mg/kg selenate acquired a CTA with reduced saccharin preference across extinction, but rats receiving 0 or 0.5 mg/kg showed a high preference and thus no CTA. A separate group of rats were injected on conditioning day with the low dose of selenate (0.5 mg/kg) or saline. 2h later, the rats were given 10min access to saccharin, then injected with either NaCl or LiCl (0.15 M, 6 ml/kg ip). Preference tests were run for 14 days. Vehicle-LiCl rats showed a persistent decrease in saccharin preference. While selenate-pretreated rats showed reduced saccharin preferences, their preferences were higher than vehicle-LiCl rats. This suggests that selenate interfered with the rats' ability to acquire a CTA, consistent with phosphatase activation.

1 Introduction

The reversible phosphorylation of specific intracellular substrates in several brain regions has been shown necessary for learning and memory formation in many learning paradigms (1) (2) (3). Protein kinases and phosphatases are responsible for the dynamic regulation of phosphorylation states of signaling protein substrates, and ultimately the cellular events that correlate with learning and memory, such as synaptogenesis and synaptic plasticity (4) (5). In the past, more research has focused on the role of kinases and positive regulation of substrate phosphorylation in learning and memory; however, recent interest has risen in the role of phosphatases and negative regulation of substrate phosphorylation.

Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that, in combination with PP1 and PP2B (calcineurin), is responsible for over 90% of the phosphatase activity in neurons (6). PP2A has a heterotrimeric arrangement in which the A subunit serves as a scaffolding subunit, the B subunit serves as a regulatory subunit, and the C subunit serves as a catalytic subunit. The pleiotropic effects of PP2A are regulated spatially and temporally by the attachment of the A subunit, various B subunits, and endogenous inhibitors. Although PP2A has several endogenous modulators, there are not many viable experimental tools for studying phosphatase dependent processes of learning and memory in vivo. As a result, it is important to find new chemicals with increased specificity for phosphatases to better define their activity (2) .

1.1 Sodium Selenate and PP2A Activation

Recently, Corcoran et al. found that sodium selenate (Na_2SeO_4) is a specific and potent activator of the PP2A heterotrimer (7). Several forms of selenium have been tested for possible pharmacological applications in models of Alzheimer's disease, in which enhancement of PP2A activity leads to a decrease in hyperphosphorylated tau protein (7)(8). A possible mechanism for selenate activation of PP2A is increased expression of the PR55 beta subunit, an obligate activator of PP2A for tau dephosphorylation (9).

1.2 [details of selenate and other forms of Learning Acquisition]

Traumatic brain injury in rats leads to increased phospho-tau and decreased PP2A activity; chronic selenate treatment (1 mg/kg/day for 12 weeks via osmotic mini-pump) improved behavioral deficits (such as water maze performance) increased PP2A activity , and decreased levels of tau phosphorylation(9).

Reduced tau phosphorylation in human neuroblastoma cells. In rats, acute sodium selenate (10-35 mg/kg, i.p) administered 2 h before stimulation reduced 6-Hz electrical stimulation seizures; chronic access to selenate in drinking water (0.12 or 1.2 mg/100 ml = 0.64 - 6.4 micromolar) reduced seizures induced by pentylenetetrazol (PTZ) or amygdalar stimulation (10).

In a brain slice preparation, selenate rescued deficits in long-term depression in THY-Tau22 mice which display tau hyperphosphorylation and impaired memory, as well as reversing the effects of okadaic acid (11).

1.3 Selenium Toxicity

Although selenium is an essential micronutrient, and sodium selenate appears to have superior characteristics for in vivo applications related to learning and memory, selenium is also toxic at doses not much higher than the dietary requirement (12)(13). The mammalian selenoproteome consists of some 2 dozen selenocysteine-containing proteins (24 in rodents, 25 in human) including the glutathione peroxidase, thioredoxin reductase and iodothyronine deiodinase enzymes (14) . Selenium toxicity is likely due to the generation of superoxide and other reactive oxygen species, the oxidation of thiols, and by substitution for sulfur in methionine to form selenomethione which then may be incorporated into many sulfur-containing proteins (15) .

Aversive effects of selenium compounds, especially at low sublethal doses, is by observing reduced intake, due to acute toxicity, and by learned food or fluid avoidance after conditioned taste aversion (CTA) acquisition. In CTA learning, animals will reduce subsequent intake of a diet if

consumption was previously paired with adverse consequences, even if the toxic effects were delayed for hours after consumption.

Reduced intake and avoidance of toxic diets containing selenium compounds has been demonstrated in many species. Because selenium is concentrated by certain species of plants in selenium-rich geographies, selenosis is a potential hazard for livestock and native herbivores. Waste from mining or agricultural run-off can also introduce selenium into the environment and hence into the food-chain. It has therefore been of interest to determine whether species can avoid selenium-rich food sources due to an aversive taste or odor of selenium compounds, or acute toxic effects, or learned food aversions. Aphids (16) (17), crickets and grasshoppers (18) , southern armyworms (19) , mallard ducks (20) , owls (21), prairie dogs (22) , sheep (12) (23), pigs (24), and cattle (12) have been shown to reduce intake of selenium-containing foods, or show reduced preference for selenium-containing foods over control diets. However, it has also been observed that honey bee foragers do not reduce their reflexive ingestive responses nor their consumption of selenium-adulterated sucrose solutions, even at toxic concentrations(25). Similarily, Argentine ants do not show decreased preference for sucrose containing selenium compounds over plain sucrose in short-term choice tests (26). Thus, the ability to detect or tolerate dietary selenium varies across species.

In laboratory studies, rats show a preference for control or low selenium-diets over high selenium diets (27) and [Franke and Potter 1935]. Consistent with the effects of other selenium compounds, selenate adulteration also causes a reduction of food (28)(29) (30) or water intake (31) (32) Acute injection of selenate can also reduce food intake and body weight gain (33)

Only a few of these studies (e.g. (12)(23) (20)) have determined if animals were responding to an acute perception of selenium compounds, or if the animals used food-associated cues, to avoid consumption, after acquiring a selenium-induced conditioned food aversion. Selenium absorbing plants may themselves have salient taste or odor: for example, Provenza et al. have demonstrated that the odor of Astragalus bisulcatus, a sulfur-containing selenium-absorbing plant, can serve as a CS that reduced preference for CS+barley-straw after pairing with oral-intubation of LiCl (34). Selenium compounds may also present distinctive cues. In particular, dimethyldiselenide, a major and volatile metabolite of selenite and selenate, has a pungent garlic odor to humans, that may be innately aversive or serve as an indicative CS in learned selenium aversions.

1.4 Conditioned Taste Aversion

Human phase 1, dose escalation study from 5 to 90 mg / day (tds) in patients with prostate cancer: dose limiting toxicity at 90 mg from fatigue, or diarrhea and muscle spasms (35). also reports of nausea and vomiting.

12 ug/ml for 12 weeks -> no effect on fluid intake in mice; also showed that selenate treated mice show preference for saccharin same as untreated mice (but not a CTA test, just unconditioned taste detection test). (8) .

Previous studies have found that when selenate (2.5-3.8 ug/ml) is added to their drinking water, rats reduce their fluid intake by 20-25% (31). One of the more recent studies found that administering

1.5 Goals

However, Na_2SeO_4 has not been utilized in CTA experiments, nor has a dose-response curve been characterized for its toxicity. The following experiments were designed to elucidate the dose-response relationship between systemic administration of Na_2SeO_4 and its potential aversive effects as measured by CTA induction and c-Fos induction in the visceral neuraxis. , and to determine the effect of Na_2SeO_4 administration on LiCl-induced CTA.

The goals of this study were

i. to determine a range of doses at which systemic sodium selenate paired with saccharin caused in CTA acquistion and activated the visceral neuraxis as measured by c-Fos induction, and

ii. to assess the ability of a low dose of sodium selenate to attenuate acquisition of a LiCl-induced CTA, and to attenuate neural activation as measured by reductions in c-Fos and

phospho-MAP kinase levels.

2 Methods

2.1 Animals

Adult male Sprague-Dawley rats (Charles River) were housed individually in polycarbonate cages in a temperature-controlled (22 ± 2 °C, 30-40% humidity) colony room. The rats were maintained on a 12 h light/dark cycle with lights-on at 7:00 A.M. All procedures were conducted during the light cycle. The rats had ad libitum access to Purina Rodent Chow and deionized-distilled water except as noted. All procedures were approved by the Florida State University animal care and use committee.

2.2 Experiment 1. To Investigate the Dose-Response Relationship for Sodium Selenate as the Unconditional Stimulus (US) in CTA Learning.

Na_2SeO_4 is a known toxin at excessive doses. In order to determine the toxicity of Na_2SeO_4, I measured rats' CTA acquisition after a variable dose of Na_2SeO_4 was paired with saccharin. Rats received access to the CS (saccharin) for 10 minutes and then received 1 of 4 doses of the US (0, 0.5, 1 or 2 mg/kg Na_2SeO_4 i.p.). A 2 bottle 24-hour preference test was used to measure CTA. It was predicted that rats given a higher dose (e.g.1and 2 mg/kg) of Na_2SeO_4 would acquire a CTA to saccharin; while those that received a low dose (0.5 mg/kg) and the vehicle would not.

Adult male Sprague-Dawley rats (n=24) were water restricted for 8 days. During this restriction, the rats received access to water for a period of time that was reduced every other day until rats had only 10 min water access a day. This ensured that the rats would sample the CS provided to them on the conditioning day. The next day, rats were given 10-min access to the CS (0.125% saccharin). Saccharin bottles were then measured in order to ensure that every rat sampled the CS. If any rat had not sampled the US, the bottle was placed on the cage for an additional 5 min. Twenty minutes later, the rats were injected with Na_2SeO_4 at 4 doses (0, 0.5, 1, or 2 mg/kg,

n = 6/dose); which served as the US. The next day rats underwent 2-bottle 24-h preference tests for 14 days. This preference test is comprised of giving the rats access to 2 bottles, one filled with water and one filled with the CS (0.125% saccharin), and weighing each bottle daily. Preference was calculated as (saccharin intake) / (total intake). Bottle location was switched daily in order to observe any place preference that could have confounded the association.

2.3 Experiment 2. To Investigate the Level of c-Fos Expression in the Visceral Neuraxis after Administration of Various Doses of Sodium Selenate.

In order to determine if Na_2SeO_4 elicits the activation of brain areas associated with the visceral neuraxis in a dose dependent manner, I injected rats with variable doses of Na_2SeO_4 and measured brain activation via c-Fos immunoreactivity. Rats were injected with 0, 0.5, 1, 2 mg/kg Na_2SeO_4 or 12 ml/kg of 0.15 M LiCl, euthanized, and then perfused. Region specific brain activation was accessed by measuring levels of c-Fos expression via DAB immunohistochemistry. It was predicted that levels of c-Fos expression in the visceral neuraxis would be higher in rats injected with high doses of Na_2SeO_4 (1and 2 mg/kg) and c-Fos expression would be lower in rats injected with 0.5 mg/kg Na_2SeO_4 or the vehicle.

Adult male Sprague-Dawley rats (n=30) were injected with doses of Na_2SeO_4 comparable to those used in experiment 1 (0, 0.5, 1, or 2 mg/kg Na_2SeO_4) or a 12 ml/kg dose of LiCl (0.15 M) to serve as a positive control. 2 hours later, rats were euthanized via i.p. injection of Nembutal and then perfused transcardially with 100 mL preperfusion solution and then 400mL paraformaldehyde. Brains were left to postfix for 2 hours and then stored in 30% sucrose. Forebrain and hindbrain sections of 40 micrometers were cut via microtome. Sections were then processed with goat anti-c-Fos antisera (Santa Cruz) using DAB chromogenic immunohistochemistry. For c-Fos quantification, the cells expressing darkly positive, nuclear staining were quantified with custom software (MindsEye, T. Houpt; Kwon et al., 2008). c-Fos was quantified in regions of the brain associated with CTA and the visceral neuraxis (i.e. central amygdala (CeA), paraventricular nucleus (PVN), nucleus of the solitary tract (NTS), parabrachial nucleus (PBN), and supraoptic nucleus (SON).

2.4 Experiment 3. To Investigate whether Activation of PP2A via a Low Dose of Sodium Selenate will Attenuate a LiCl-Induced CTA.

In order to determine whether PP2A interferes with CTA acquisition, I pretreated rats with a nontoxic dose of Na_2SeO_4 (a selective PP2A agonist) or vehicle and then measured CTA acquisition. Rats were initially separated into two groups and then received an i.p. injection of Na_2SeO_4 or vehicle. In order to elicit a CTA, rats in each group then received access to a CS (saccharin) which was paired with a US (6 ml/kg, 0.15 M LiCl) or vehicle. In order to test Na_2SeO_4's effects on a learned CTA, rats were then subjected to a 14 day 2-bottle 24-h preference test. It was predicted that the administration of an appropriate dose of sodium selenate would result in an attenuation of CTA learning via PP2A activation.

Adult male Sprague-Dawley rats (n=24) were water restricted for 8 days. The duration rats had access to water was reduced throughout the 8 days until rats were limited to 10 min water access a day. This ensured that the rats would sample the CS presented on the conditioning day. On the day of conditioning, rats were injected with either the low dose of Na_2SeO_4 (0.5 mg/kg) or a saline vehicle. This dose of Na_2SeO_4 was used because it did not elicit a CTA in experiment 1; therefore it was concluded to be a safe dose to study Na_2SeO_4's effects on learning and memory processes without the risk of resulting in an exaggerated US. Two hours later, the rats were given 10-min access to the CS (0.125% saccharin). Saccharin bottles were then measured in order to ensure every rat sampled the CS. If any rat did not sample the US, the bottle was placed on the cage for an additional 5 min and weighed again. Twenty minutes later, the rats received i.p. injections of either the US (6 ml/kg, 0.15 M LiCl) or vehicle (NaCl). This separated the rats into 4 groups: vehicle-NaCl, vehicle-LiCl, Na_2SeO_4-NaCl, and Na_2SeO_4-LiCl (n=6/group). The next day rats underwent 2 bottle 24-h preference tests that continued for 14 days. The preference test is comprised of allowing the rats access to 2 bottles, one filled with water and one filled with the CS (0.125% saccharin), and then recording the weight of each bottle daily. Preference was calculated as (saccharin intake) / (total intake). The bottle location of each tastant was switched daily in order to observe any place preference that may have confounded the association.

2.5 Experiment 4. Sodium Selenate Attenuation of c-Fos and phospho-MAP kinase induction

To assess the effects of Na_2SeO_4 pretreatment on neuronal activation in the visceral neuraxis, c-Fos and phospho-MAP kinase (pMAPK) induced by LiCl was examined with and without Na_2SeO_4 pretreatment

Adult male Sprague-Dawley rats (n=???) were injected with either Na_2SeO_4 (0.5 mg/kg) or vehicle (1 ml/kg 0.15 M NaCl). One hour later, rats were injected with either LiCl or NaCl (0.15M, 12 mk/kg), counterbalanced across selenate and vehicle groups. At 1 hour after the second injection, rats were euthanized via i.p. injection of Nembutal and then perfused transcardially with 100 mL preperfusion solution and then 400mL paraformaldehyde.

Brains were left to postfix for 2 hours and then stored in 30% sucrose. Forebrain and hindbrain sections of 40 micrometers were cut via microtome. Alternate sections were processed with goat anti-c- Fos antisera (Ab#, Santa Cruz) and anti-pMAPK (Ab#, Cell Signaling) using DAB chromogenic immunohistochemistry. c-Fos positive nuclei and pMAPK positive soma were quantified as in Experiment 2.

2.6 Statistical Analysis

Data are presented as the mean ± standard error of the mean. Preference scores were analyzed using Statistica software (Statsoft, Tulsa, OK), using either one-way or two-way, repeated measures analysis of variance (ANOVAs). For analysis of CTA magnitude, Treatment group (0.0, 0.5, 1, or 2 mg/kg Na_2SeO_4) served as the independent variable and saccharin preference and the first day of 2-bottle preference testing, served as the dependent variable for the one-way ANOVA. For analysis of CTA Extinction in Experiments 1 and 3, Treatment group (Experiment 1: 0.0, 0.5, 1, or 2 mg/kg Na_2SeO_4; Experiment 3: vehicle-vehicle, vehicle-LiCl, selenate-vehicle, and selenate-LiCl) served as the first independent variable, day of testing served as the second independent variable, and saccharin preference served as the dependent variable for the two-way repeated measures ANOVAs.

For analysis of c-Fos induction in Experiment 2, Treatment group (0.0, 0.5, 1, 2 mg/kg Na_2SeO_4, or 76 mg/kg LiCl) served as the independent variable and c-Fos counts in each brain region served as the dependent variable for a one-way ANOVA. Tukey-Kramer Highly Significant Difference pairwise comparisons were used for all post-hoc analyses.

3 Results

3.1 Experiment 1

A one-way, ANOVA revealed a significant main effect of treatment on the first day of 2-bottle preference tests ($F(3, 20) = 13.28$, $p = 0.000054$) (see figure 1). Post hoc analysis revealed that the mean saccharin preference of the 1 mg/kg selenate treatment group and the 2 mg/kg selenate treatment group were significantly lower than the mean of the 0.0 mg/kg selenate treatment group (negative control). The mean saccharin preference of the 2 mg/kg selenate treatment group was significantly lower than the 0.5 mg/kg selenate treatment group.

A two-way, repeated measures ANOVA across the 14 days of extinction testing revealed a significant main effect of the treatment ($F(3,39) = 4.45$, $p = 0.015$) and for day of testing ($F(13, 39) = 4.82$, $p = 0.0000$), but no interaction between the two factors (see figure 2). Post hoc analysis showed that the mean saccharin preference for the 0.5 mg/kg selenate treatment group was not significantly different from 0.0 mg/kg selenate treatment group (negative control group) across all 14 days of the 2-bottle preference tests. However, the mean saccharin preference for the 1 mg/kg selenate treatment group was significantly lower than the 0 mg/kg selenate control group on days 1-5 and 7-9 during the 14 days of 2-bottle preference testing. The mean saccharin preference for the 2 mg/kg selenate treatment group was significantly lower than the control group on days 1-13 of the 2-bottle preference test, but was not lower on day 14. Therefore, it was concluded that the high doses of sodium selenate (1mg/kg and 2 mg/kg doses) can serve as a CS if paired with saccharin and induce a conditioned taste aversion without the addition of another toxin; however, the low dose of sodium selenate (0.5 mg/kg dose) does not serve as a US when paired with saccharin and is not sufficient to induce a CTA.

3.2 Experiment 2

One-way ANOVAs revealed that injections of sodium selenate and/or LiCl significantly induced c-Fos in the NTS ($F(4,20) = 2.90$, $p < 0.05$), PBN ($F(4,22) = 3.67$, $p < 0.05$), CeA ($F(4,25) = 3.63$, $p < 0.05$) , PVN ($F(4,25) = 6.54$, $p < 0.001$), and SON ($F(4,24) = 4.55$, $p < 0.01$) (see figures 3, 4, 5, 6 & 7).

In the NTS, despite a significant overall effect of treatment, post hoc analyses showed no significant differences between any of the treatment groups and either of the control groups (negative or positive; see figure 3). Furthermore, in the NTS, none of the groups were significantly different from each other.

In the PBN, both the LiCl treatment group (positive control group) and the 2 mg/kg selenate treatment group induced significantly higher c-Fos counts than the vehicle (negative control group) (see figure 4). No other treatment group was significantly different from either control group.

In the CeA, only the 1mg/kg selenate treatment group showed a significantly higher c-Fos count than the vehicle; although elevated, LiCl-induced c-Fos counts failed to reach statistical significance (see figure 5). In the PVN, the LiCl injection induced significantly higher c-Fos than the vehicle; however, all other doses of selenate failed to induce significantly more c-Fos than the vehicle (see figure 6).

In the SON, selenate injections failed to induce any c-Fos and only the LiCl injections induced significantly more c-Fos than the vehicle (see figure 7). Therefore, it was concluded that, despite sodium selenate's lack of a uniform effect on c-Fos induction across all of the brain areas that were observed, sodium selenate induced c-Fos in patterns specific to neuroanatomical functional groups. In the brainstem (NTS and PBN), sodium selenate induced c-Fos in a dose dependent manner and this pattern possibly occurred in the CeA as well. However, in the hypothalamus (SON and PVN), none of the doses of sodium selenate induced c-Fos.

3.3 Experiment 3

A one-way ANOVA revealed a significant main effect of treatment on the first day of the 2-bottle preference test ($F(1, 20) = 20.83$, $p = 0.0002$) (see figure 8). Post hoc analysis showed that the mean saccharin preference of the NaCl-LiCl treatment group (positive control) was significantly lower than the mean saccharin preference of the NaCl-NaCl treatment group (negative control) on the first day of 2-bottle preference tests. The mean saccharin preference of the NaCl-LiCl treatment group (positive control) was significantly lower than the mean saccharin preference of the selenate-NaCl treatment group on the first day of 2-bottle preference tests. The selenate-LiCl group was not significantly different from the NaCl-NaCl treatment group (negative control) on the first day of 2-bottle preference tests, but had a p-value ($p = 0.0628$) that was very close to significance.

Two-way, repeated measures ANOVA across the 14 day extinction testing revealed a significant main effect of the treatment ($F(3,39) = 4.56$, $p = 0.014$) and of day of testing ($F(13, 39) = 6.80$, $p = 0.0000$), but no interaction between the two factors (see figure 9). Post hoc analysis revealed that the mean preference of the NaCl-LiCl treatment group (positive control group) was significantly lower than the NaCl-NaCl treatment group (negative control group) on all 14 days of the 2-bottle preference test. The mean preference for selenate-NaCl treatment group did not show a significant difference from the negative control group; however the mean preference of the selenate-NaCl treatment group was significantly higher than the positive controls for the full 14 days of the 2-bottle preference test. The mean preference for the selenate-LiCl treatment group was significantly lower than the NaCl-NaCl and selenate-NaCl groups on the first day, but failed to show a statistical difference on days 2-14 of the 2-bottle preference test. Furthermore, the mean preference of the selenate-LiCl group was significantly higher than the NaCl-LiCl group on days 5-9 of the 2-bottle preference test. Therefore, it was concluded that a low dose of sodium selenate (0.5 mg/kg) interferes with the learning and memory processes in conditioned taste aversion and increases the rate of memory extinction for the conditioned taste aversion.

3.4 Experiment 4

4 Discussion

Though it has been known for some time that phosphorylation of signaling protein substrates plays an important role in learning and memory, much of the research delineating the role of this particular intracellular signaling mechanism has focused specifically on the role of kinases. However, recent interest has risen in the role of phosphatases and negative regulation of signaling protein substrate phosphorylation in learning and memory. In 2010, Oberbeck et al. found that direct administration of okadaic acid, a known inhibitor of PP1 and PP2A, to the amygdala enhances learning of conditioned taste aversions and makes the memory of these aversions resistant to extinction. While studies looking at the inhibition of phosphatases are useful, they are incomplete alone. It is also important to look at the behavioral and intracellular effects of phosphatase activation and to increase the specificity of the pharmacological agents modulating phosphatase activities.

Corcoran et. al (2010) proposed that sodium selenate is a potent and specific activator of the PP2A heterotrimer, which makes Na_2SeO_4 an excellent candidate drug to study the role of PP2A on learning and memory. Selenium is an essential micronutrient that is incorporated in selenoproteins required for antioxidant defense, thyroid hormone production, and immune responses. However, Na_2SeO_4 is known to have toxic effects at doses that are only marginally higher than what is required by diet (Gasmi , 1997; Stowe, 1992). The proximal cause of selenium toxicity is unknown; however selenosis can result in cirrhosis of the liver, pulmonary edema, and death (Agency for Toxic Substances and Disease Registry, 2003). Nevertheless, Na_2SeO_4 is the best drug candidate out of all of the forms of selenium salt because it is the least toxic form of selenium, it shows no neurotoxicity, and it is the only form that activates PP2A (Corcoran e.t al, 2010). In this thesis, I showed that 0.5 mg/kg Na_2SeO_4 does not have toxic effects that can induce a CTA, that Na_2SeO_4 activates the visceral neuraxis and the CeA, and that Na_2SeO_4 weakens memory formation of CTAs presumably through activation of PP2A.

4.1 CTA Induction

Due to the fact that the learning and memory paradigm of conditioned taste aversion is sensitive to the effects of toxic substances, it was imperative to understand the dose-response relationships of Na_2SeO_4 toxicity before studying its effects on learning and memory. Since there is no literature regarding what constitutes a dose of Na_2SeO_4 that will modulate PP2A activity without showing toxic effects; experimentation was required to determine what constitutes such a dose. Previous studies addressing selenium salt dose toxicity have used both in vivo and in vitro methods (Xiao et al., 2006; Nogueira and Rocha, 2011; Lee and Jeong, 2012).

We are interested in finding a dose that will not confound the behavioral data of a CTA paradigm rather than characterizing the toxic effects of Na_2SeO_4 at the molecular and anatomical level. Therefore, determining which doses of Na_2SeO_4 can serve as a US when paired with a tastant is arguably the most valid method of investigating Na_2SeO_4 toxicity, translationally speaking. After pairing variable doses of Na_2SeO_4 with saccharin (the CS), we determined that a systemically delivered low dose (0.5mg/kg dose) of Na_2SeO_4 is not recognized as a toxin by the test rats. This was concluded with a behavioral assay. A physiological assay of the effects of Na_2SeO_4 could provide useful data as well. That was the objective of our second experiment.

When testing the toxicity of Na_2SeO_4, we used an acute dose, rather than a chronic treatment of the drug. Like acute doses of Na_2SeO_4, it has been found that chronic overexposure to selenium salts has additive effects that can enhance the potential risk of toxicity in a dose that would otherwise exhibit benign effects if delivered acutely. Chronic overexposure has been shown to increase the risk of diabetes type II, several types of cancer, and amyotropic lateral sclerosis (Vinceti et al. 1996, 1998, 2001, 2009, 2010; Stranges et al. 2010). It was concluded that the test animals' exposure to any dose of selenium exceeding their dietary requirement should be as brief as possible to reduce toxic side-effects.

Though the method used to discover toxic doses of Na_2SeO_4 in experiment 1 was translationally valid, this method may lack the specificity that other assays of toxicity have. Physiological assays have the benefit of showing specifically what doses result in damage to tissues and polymers

inside of the cells of affected organisms and the extent of this damage. Additionally, the treatment doses used in experiment 1 were fairly arbitrary. It may be beneficial to characterize the toxic effects of various doses of Na_2SeO_4 closer to the 0.5 mg/kg dose used in experiment 3 in order to see if there are more appropriate doses for various experimental purposes. Furthermore, it may be possible to bypass the toxic signaling of the visceral neuraxis altogether via intracranial cannulation and administration of site specific injections of Na_2SeO_4 to various nuclei (i.e. the amygdala), and thereby reduce the need to characterize the toxicity of various systemic doses. Despite these limitations, the results of experiment 1 show that a systemic dose of 0.5 mg/kg Na_2SeO_4 does not induce a CTA when paired with the CS; therefore it was concluded to be a suitable dose to test the effects of Na_2SeO_4 injections on the learning and memory processes of CTA in experiment 3.

4.2 c-Fos Induction

In experiment 2, we looked at the effects that systemic injections of Na_2SeO_4 had on the activation of a series of brain regions, including the visceral neuraxis. These regions included the parabrachial nucleus (PBN) and the nucleus of the solitary tract (NTS) of the hindbrain and the central amygdala (CeA), the paraventricular nucleus (PVN), and the supraoptic nucleus (SON) of the forebrain. Neural activation was determined by quantifying the immunoreactivity of the inducible transcription factor c-Fos, an antigen commonly used as marker of neural activity. Though there was not a consistent overall pattern of post treatment c-Fos induction, some patterns were apparent when the nuclei were grouped by functional circuitry. The c-Fos induction in the brainstem (PBN and NTS) showed a pattern of neural activation that most resembled a dose-response effect. A graded response in these brain areas would not be surprising considering that the NTS and PBN serve as the first and second sensory relays, respectively, for both taste and visceral signals coming into the brainstem and likely involve relatively little signal processing. Specifically, this dose dependent neural activation in the visceral neuraxis shows that the low dose of Na_2SeO_4 activates these nuclei similarly to that of vehicle injections; while the higher doses treatments results in activity comparable to that of LiCl. This is consistent with the behavioral evidence of experiment 1 showing that a low dose of Na_2SeO_4 does not cause a CTA, but higher doses do in a graded fashion. It is unknown whether Na_2SeO_4 is transduced as a toxin via the vagal pathway or the area postrema because c-Fos induction is not a reliable marker of neural activation in the area postrema.

The c-Fos induction patterns in the CeA showed more variability than any other brain region observed. There was an unusually high count of c-Fos in the negative control group as well as a lack of a significant difference between the negative control group and the positive control group in this experiment. This led to conflicting interpretations of the results in this brain region. There may be a dose-response effect, like that in the hindbrain, or possibly an all or nothing effect in which the 1 mg/kg of Na_2SeO_4 is the only dose that induced significantly higher c-Fos than the negative controls. Evidence shows that the CeA plays an important role in associative processes important for the learning and memory of a CTA. It is possible that the low dose does not generate a strong enough toxic signal to the CeA to result in significant neural activation in this brain region. Alternatively, activation of PP2A in the CeA, via Na_2SeO_4 administration, may have blocked learning and memory processes via some molecular pathway that also induces c-Fos expression.

Hypothalamic areas (the SON and the PVN) were sampled due to their involvement in stress responses. These areas show activation in response to LiCl injections as well as many other stimuli that can serve as an US. We observed little or no c-Fos induction when the rats were injected with Na_2SeO_4. These results were unusual and could mean that Na_2SeO_4 does not induce a stress response in animals after treatment, even at doses that induce conditioned taste aversions. Another possibility is that Na_2SeO_4 (i.e. activated PP2A) is interfering with a metabolic pathway which is necessary for c-Fos expression. These two possibilities may not be mutually exclusive, given that the interference of some aspect of the c-Fos pathway in the PVN and NTS could hypothetically be responsible for the diminished stress response that we observed. It would be interesting to see if sodium selenate's failure to activate the PVN and NTS was due to an insufficient dose size. It is possible that a much higher dose of Na_2SeO_4 will result in an increase in c-Fos expression in the PVN and NTS. c-Fos was used because it is a known marker of neural activity; but there are several caveats to c-Fos studies. c-Fos is not a marker for neural activity in all types of neurons (Morgan et al., 1987). Other methods of marking neural activity may be appropriate as well, if not more appropriate; such as quantifying pERK and zif-268 mRNA (Yong-Jing and Ru-Rong, 2009; Zhang et al. 1995). The interval of time between administration of each treatment and the euthanization of the test animals was chosen because it was previously shown that, 2 hours post Na_2SeO_4 administration, certain serine/threonine substrates will be dephosphorylated (Corcoran et al., 2010). Though it has been shown that Na_2SeO_4 crosses the blood brain barrier, we used a systemic administrative route in this experiment, so it is not conclusive whether neural activation was due to peripheral activation of the

visceral neuraxis or due to direct intracellular effects on central neurons. It would be interesting to see how other routes of administration affect c-Fos induction.

The results of experiment 2 were consistent with the results of experiment 1, in that the visceral neuraxis was activated in a dose dependent manner similar to the induction of a CTA. Additionally, we observed no activation of the visceral neuraxis or stress response associated hypothalamic areas by the 0.5 mg/kg dose of Na_2SeO_4. This confirms that the low dose of Na_2SeO_4 is likely a non-toxic dose that did not exacerbate the effects of the US in experiment 3.

4.3 CTA Attenuation

The ultimate goal of this study was to explore the presumed role PP2A plays in the processes involved in learning and memory via systemic injections of sodium selenate. After a non-toxic dose of Na_2SeO_4 was determined, we used this dose to look at the effects of Na_2SeO_4 on conditioned taste aversion learning and memory retention. We used the low dose (0.5 mg/kg) of Na_2SeO_4 as a pretreatment before pairing saccharin (the CS) with LiCl (the US). The results showed that the pretreatment of Na_2SeO_4 reduced the magnitude of the conditioned taste aversion and also caused the CTA to extinguish very rapidly. Due to sodium selenate's known specificity and agonist effects on PP2A, we hypothesized that this was likely due to the activation of PP2A and ultimately an increased removal of phosphate groups from signaling protein substrates. Due to evidence that the manipulation of certain kinases and phosphatases modulates the strength of learned associations and retention of conditioned taste aversions (there is evidence that this occurs in other learning and memory paradigms as well) we concluded that the results of experiment 3 were likely due to effects on associative processes or consolidation via alterations in intracellular signaling and gene expression. However, we cannot rule out the possibility that these experimental effects may have been due to manipulations of the taste and toxic transduction pathways.

The dose of sodium selenate used in experiment 3 was chosen because it did not induce a CTA in experiment 1 and was deemed non-toxic. The timing of the pre-treatment administration was chosen in order to further reduce the chance of strengthening the toxic signal of the US (CTAs are resistant to backwards conditioning) and because past literature has shown that Na_2SeO_4 has

effects on substrate phosphorylation 2 hours after an injection (Corcoran et al. 2010). Systemic injections were deemed appropriate for this study due to sodium selenate's ability to penetrate the blood brain barrier.

It would be interesting to give direct injections of Na_2SeO_4 to the CeA, or other nuclei of interest, via a cannula (rather than a systemic injection). This would help determine if there are non-specific effects of Na_2SeO_4 on peripheral organs that are confounding the data.

Additionally, it is possible that sodium selenate's effects were not due to activation of PP2A. Though other evidence would suggest that this is likely the mechanism through which Na_2SeO_4 caused our results, a phosphatase assay should be conducted to verify the efficacy of Na_2SeO_4.

Our results have important contributions to basic research and practical implications as well. Research regarding processes involved in the negative regulation of learning and memory has been neglected in the past; yet negative regulation is likely to be just as critical as positive regulation for the normal function of learning and memory processes. To date, little is known about the role of PP2A in learning and memory processes and even less is known about its involvement in CTA. For practical purposes, knowledge of the effects of Na_2SeO_4 on learning and memory could be important information due to certain possible medical applications. Na_2SeO_4 has been implicated as a possible treatment for Alzheimer's disease and cancer. If it is ever deemed appropriate for clinical use, it would be important to know how this treatment effects memory as a side effect. Likewise, selenium is essential in a normal diet. An understanding of how non-toxic levels of it effect cognitive processes is of some value. Additionally, due to the fact that Na_2SeO_4 can be used supplementally (if the dose is carefully monitored), it may be worthwhile to test it as a possible treatment for maladaptive forms of memory such as post-traumatic stress disorder.

4.4 selenate rescue of CTA in tau mice

4.5 selenate inhibition of phospho-MAP kinase, but not c-Fos

Chen et al PLOSone 2014 Phosphoproteomic profiling – check to see if anything interesting about MAPK

5 Acknowledgements

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5.2 Disclosures

The authors have no conflicts of interest.

6 Figure Legends

Figure 1. Initial magnitude of a conditioned taste aversion induced by sodium selenate. Expression of CTA following injections of variable doses of sodium selenate shown by quantification of first day 2-bottle preference test. Rats received an injection of one of four doses of sodium selenate 20 minutes after receiving 10 minutes access to saccharin. The vehicle injected group (0.0 mg/kg sodium selenate dose) showed a high preference for saccharin after the pairing. The 1.0 and 2.0 mg/kg sodium selenate dose group show a significantly lower saccharin preference than the vehicle control group, * $p<0.05$. The 0.5 mg/kg sodium selenate dose group showed a significantly higher saccharin preference than the 2.0 mg/kg sodium selenate dose group, ? $p<0.05$.

Figure 2. Extinction of a conditioned taste aversion induced by sodium selenate. Expression of CTA following injections of variable doses of sodium selenate shown by quantification of 14 days of 2-bottle preference test. Rats received an injection of one of four doses of sodium selenate 20 minutes after receiving 10 minutes access to saccharin. The vehicle injected group (0.0 mg/kg sodium selenate dose) showed a high preference for saccharin after the pairing. The 1.0 and 2.0 mg/kg sodium selenate dose group show a significantly lower saccharin preference than the vehicle control group that extinguishes before the end of the 14 days of preference testing, * $p<0.05$. The 0.5 mg/kg sodium selenate dose group did not show a significantly lower saccharin preference than the vehicle control group.

Figure 3. Example photomicrographs of c-Fos induction by sodium selenate. in the nucleus of the solitary tract (A), parabrachial (B), central amydala (C), paraventricular nucleus (D), and supraoptic nucleus (E).

[Insert example photomicrographs here]

Figure 3A NTS.

Figure 3B PBN.

Figure 3C CEA.

Figure 3D PVN.

Figure 3E SON.

Figure 4. Quantification of c-Fos induction. c-Fos positive immunoreactivity 2 hours after an injection of variable doses of sodium selenate or LiCl in the nucleus of the solitary tract (A), parabrachial (B), central amydala (C), paraventricular nucleus (D), and supraoptic nucleus (E). * $p<0.05$ vs. 0 mg/kg; ? $p < 0.05$ vs. LiCl-induced c-Fos.

Figure 5. Conditioned taste aversion after sodium selenate pretreatment. A. Initial magnitude of conditioned taste aversion with sodium selenate pretreatment. Saccharin preference on the first day of 2-bottle extinction testing. NaCl or selenate pre-treatment alone did not induce CTA. LiCl induced a significant CTA in NaCl-pretreated rats but not in selenate pretreated rats. * $p < 0.05$ vs. NaCl-NaCl group

B. Extinction of conditioned taste aversion with sodium selenate pretreatment. Expression of CTA following pretreatment of either vehicle or sodium selenate paired with vehicle injections or the US shown by quantification of 14 days of 2-bottle preference test. The vehicle-vehicle treatment group showed a high preference for saccharin after the pairing. The selenate pretreatment group that received a vehicle injection showed no significant difference from the vehicle-vehicle treatment group. The vehicle-LiCl treatment group showed a CTA throughout all 14 days of the 2-bottle preference test, * $p<0.05$. The selenate-LiCl treatment group only showed a CTA on day 1 of the 14 days of 2-bottle preference test that immediately extinguished, * $p<0.05$.

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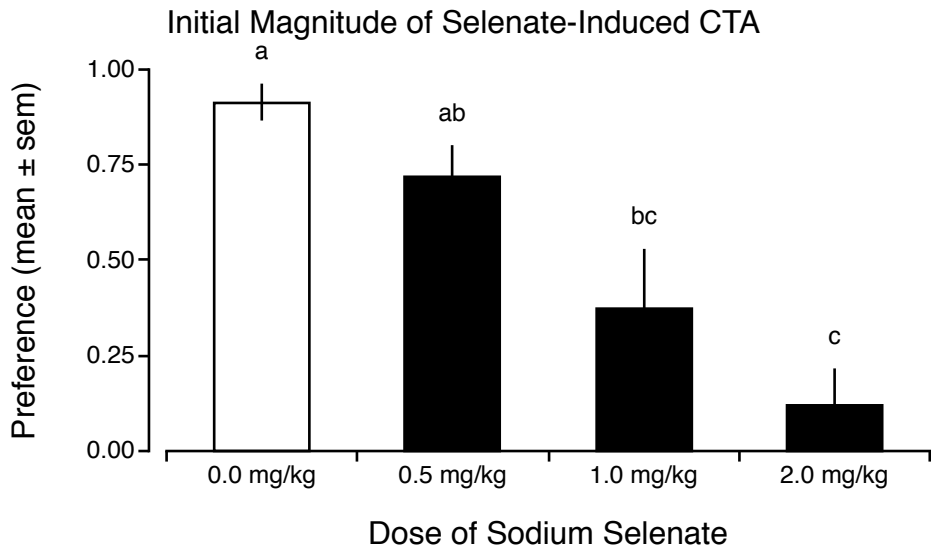
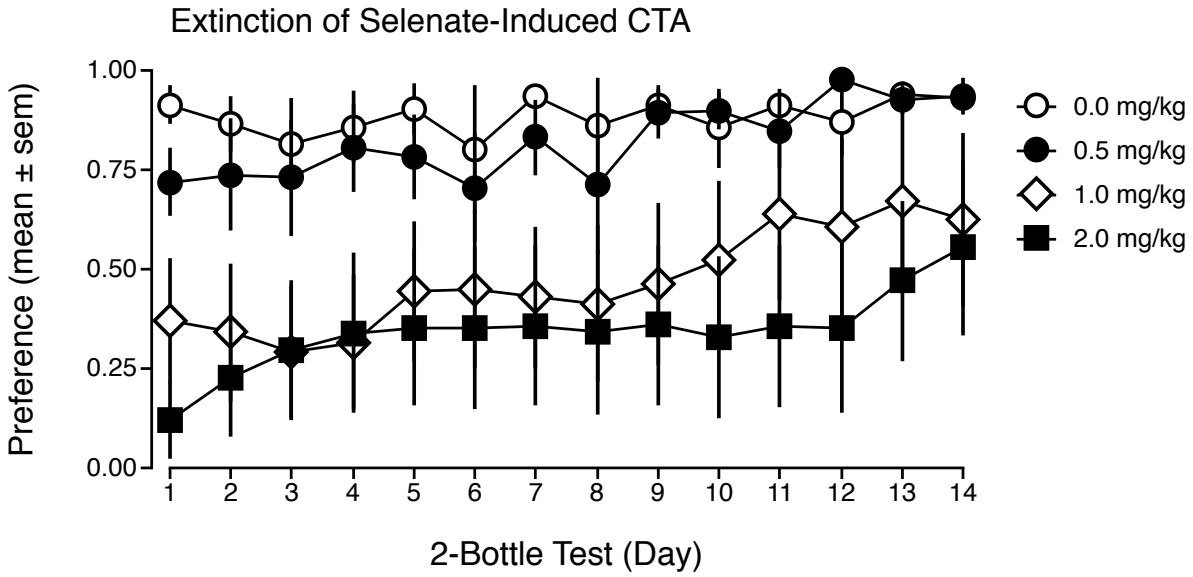

Initial Magnitude of Selenate-Induced CTA
a
ab
bc
c
1.00
0.75
0.50
0.25
0.00
Preference (mean ± sem)
0.0 mg/kg
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg
Dose of Sodium Selenate


Extinction of Selenate-Induced CTA
1.00
0.75
0.50
0.25
0.00
Preference (mean ± sem)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
2-Bottle Test (Day)
0.0 mg/kg
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg

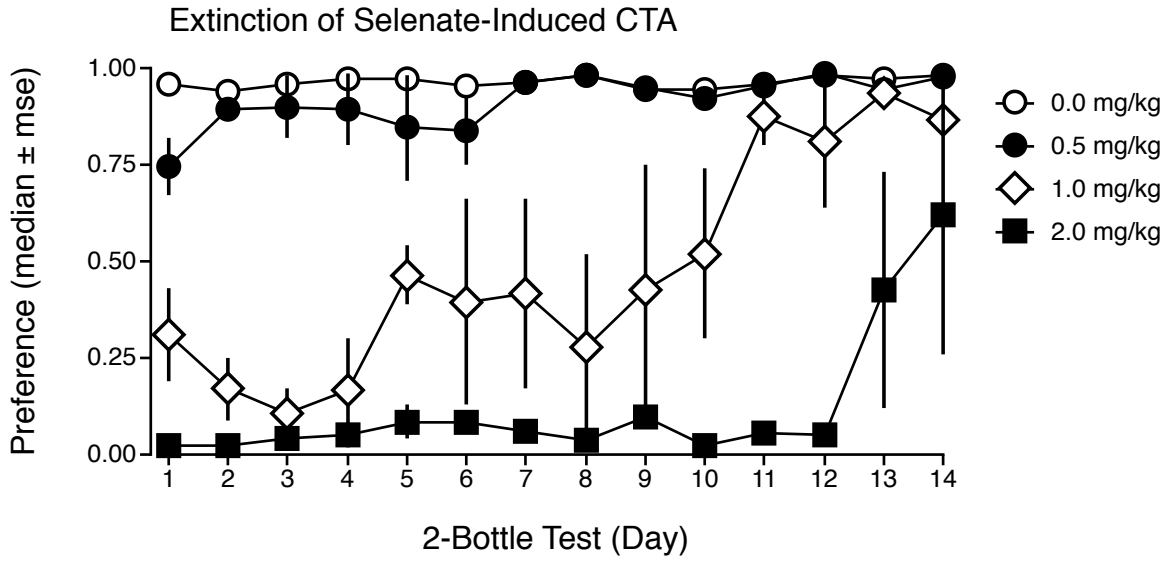
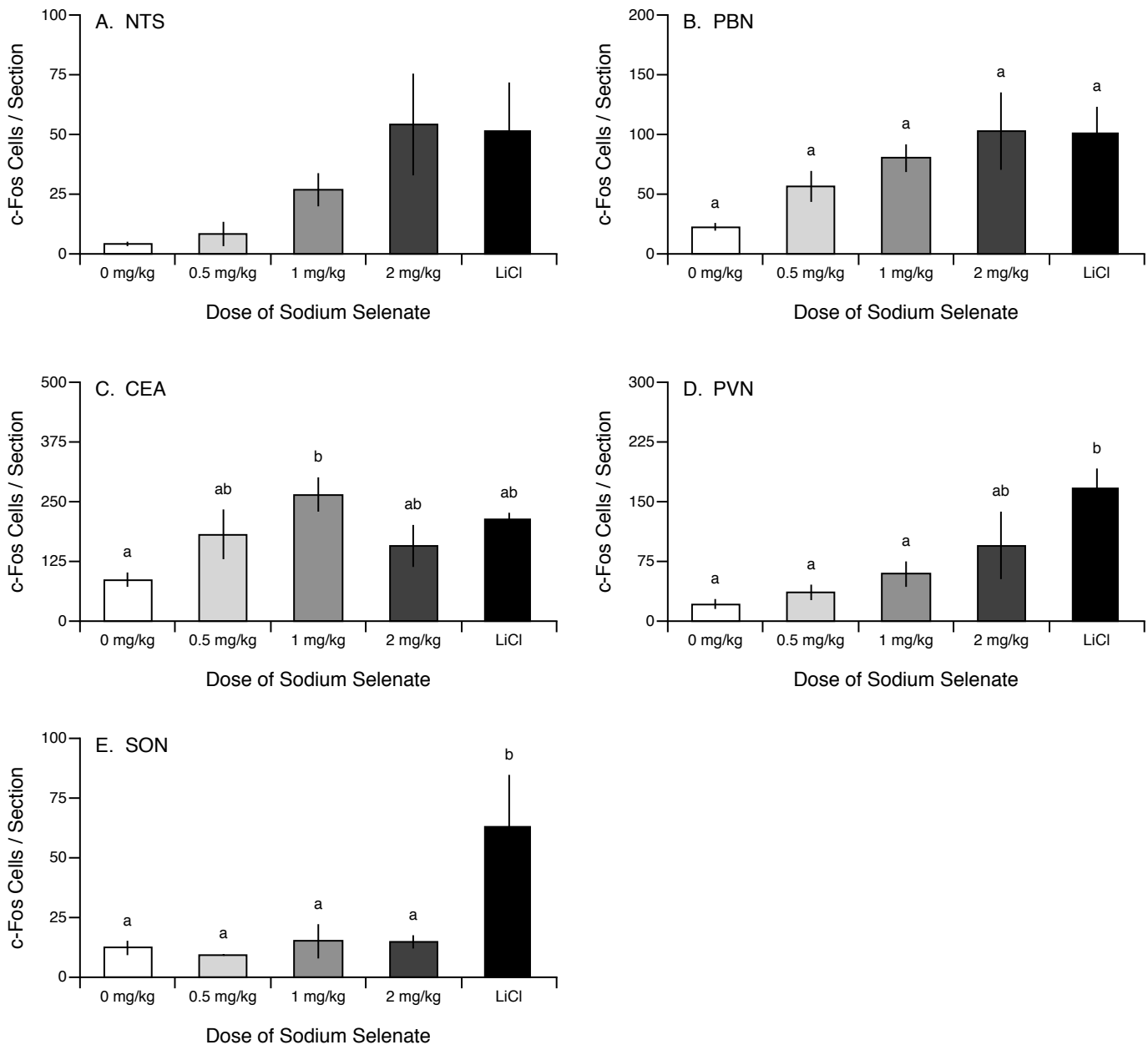

Extinction of Selenate-Induced CTA
1.00
0.75
0.50
0.25
0.00
Preference (median ± mse)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
2-Bottle Test (Day)
0.0 mg/kg
0.5 mg/kg
1.0 mg/kg
2.0 mg/kg

Figure 1


A. NTS
B. PBN
C. CEA
D. PVN
E. SON
c-Fos Cells / Section
Dose of Sodium Selenate
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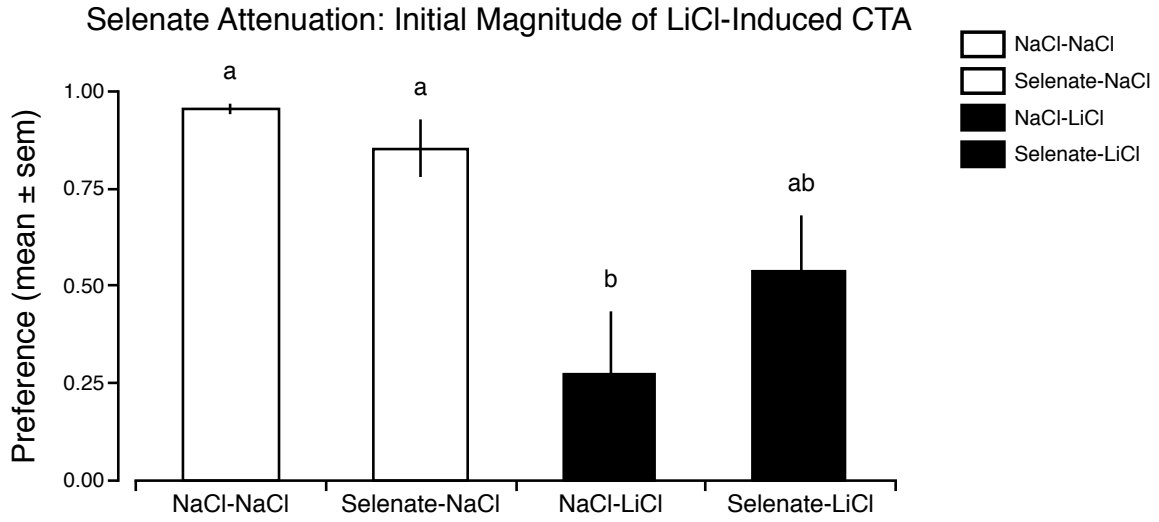
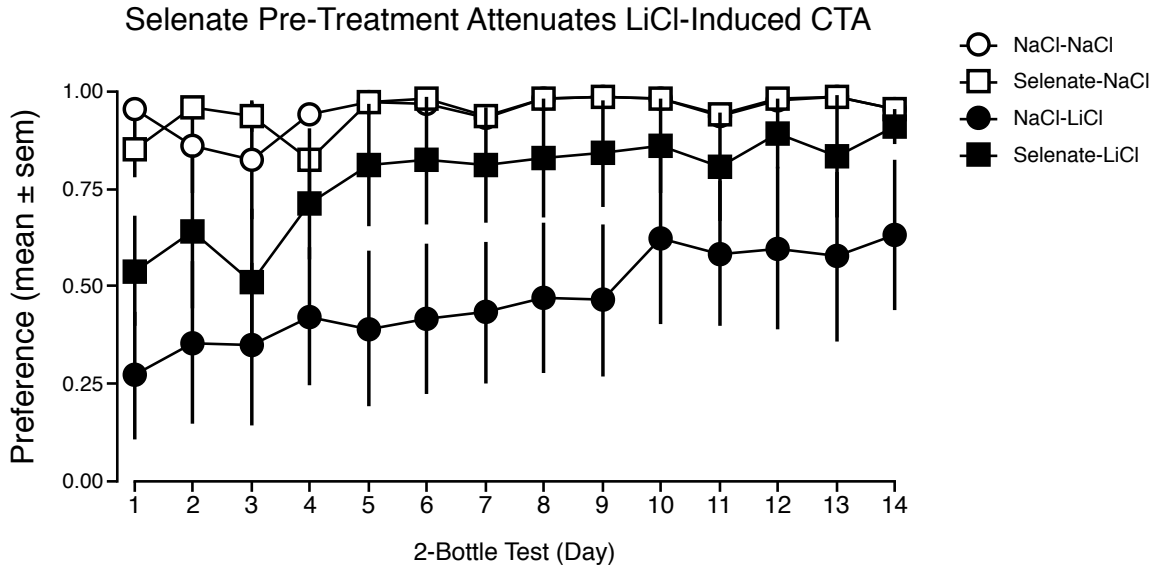
0.5 mg/kg
1 mg/kg
2 mg/kg
LiCl

Figure 2


Selenate Attenuation: Initial Magnitude of LiCl-Induced CTA
NaCl-NaCl
Selenate-NaCl
NaCl-LiCl
Selenate-LiCl
Preference (mean ± sem)
1.00
0.75
0.50
0.25
0.00
a
a
b
ab
NaCl-NaCl
Selenate-NaCl
NaCl-LiCl
Selenate-LiCl


Selenate Pre-Treatment Attenuates LiCl-Induced CTA
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Selenate-NaCl
NaCl-LiCl
Selenate-LiCl
Preference (mean ± sem)
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0.75
0.50
0.25
0.00
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2
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12
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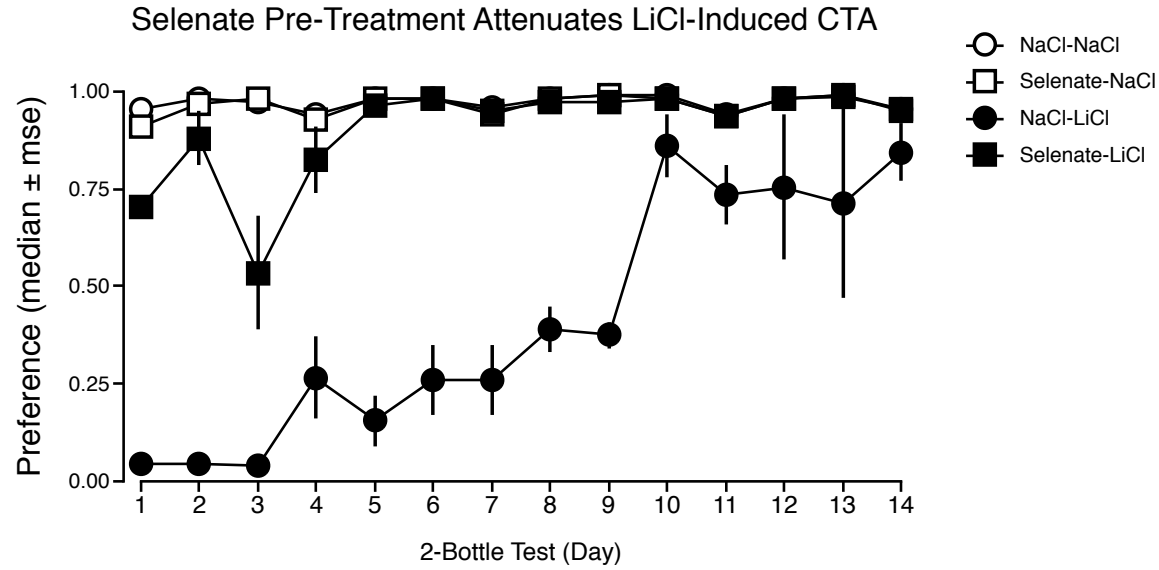
14
2-Bottle Test (Day)


Selenate Pre-Treatment Attenuates LiCl-Induced CTA
NaCl-NaCl
Selenate-NaCl
NaCl-LiCl
Selenate-LiCl
Preference (median ± mse)
1.00
0.75
0.50
0.25
0.00
1
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7
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9
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11
12
13
14
2-Bottle Test (Day)

Figure 3