



Research article

Examining the effect of manganese on physiological processes: Invertebrate models



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ABSTRACT

Manganese (Mn^{2+} as $MnSO_4$ &/or $MnCl_2$) is a common and essential element for maintaining life in plants and animals and is found in soil, fresh waters and marine waters; however, over exposure is toxic to organisms. $MnSO_4$ is added to soil for agricultural purposes and people are exposed to Mn^{2+} in the mining industry. Hypermanganesemia in mammals is associated with neurological issues mimicking Parkinson's disease (PD) and appears to target dopaminergic neural circuits. However, it also seems that hypermanganesemia can affect many aspects of health besides dopaminergic synapses. We examined the effect on development, behavior, survival, cardiac function, and glutamatergic synaptic transmission in the *Drosophila melanogaster*. In addition, we examined the effect of Mn^{2+} on a sensory proprioceptive organ and nerve conduction in a marine crustacean and synaptic transmission at glutamatergic neuromuscular junctions of freshwater crayfish. A dose-response effect of higher Mn^{2+} retards development, survival and cardiac function in larval *Drosophila* and survival in larvae and adults. $MnSO_4$ as well as $MnCl_2$ blocks stretch activated responses in primary proprioceptive neurons in a dose-response manner. Mn^{2+} blocks glutamatergic synaptic transmission in *Drosophila* as well as crayfish via pre-synaptic action. This study is relevant in demonstrating the effects of Mn^{2+} on various physiological functions in order to learn more about acute and long-term consequences Mn^{2+} exposure.

1. Introduction

Given that manganese (Mn^{2+}) is found in many plants and animals that are consumed by humans as food, it is essential to understand the effects of consuming high levels of Mn. It is well established as an essential element required for photosynthetic abilities in plants as well as cellular function in mammals. For example, Mn acts as a cofactor for aiding in the function of various essential enzymes (i.e., superoxide dismutase) in organisms. However, Mn is also toxic at high levels. Thus, it is of interest to understand the threshold at which subtle effects in physiological processes become compromised. It is difficult to determine what physiological symptoms may manifest when chronically exposed to low levels of manganese but examining acute actions at high levels may provide some insight to what the potential effects might be. Mn is found in seaweed (130 to 735 mg/kg dry weight), formula milk

consumed by developing infants (above recommended daily limits; Frisbie et al., 2019), marine and freshwater fish (<0.2 to 19 mg/kg dry weight) and can bioaccumulate in blueberries as well is found on plants from application of agricultural sprays in the range of 2000–4000 mg/kg (Howe et al., 2004).

Hypermanganesemia in mammals can be toxic and is associated with neurological issues mimicking Parkinson's disease (PD). In fact, the neural injury from Mn affects the same locations within the central nervous system known to be dysfunctional in PD. Manganese has been shown to accumulate in these areas (Calne et al., 1994; Eriksson et al., 1987; Erikson et al., 2004a, 2004b; Brennehan et al., 1999; Nagatomo et al., 1999; Newland, 1999; Pal et al., 1999; Baek et al., 2003; Nelson et al., 2018). Such findings have led, in part, to suggesting that Mn selectively targets dopaminergic neurons. However, it is not known what the effect(s) are of Mn on neurons which use other types of

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neurotransmitters or exactly how Mn^{2+} is offsetting the dopaminergic neural circuit in the mammalian brain. Bonilla-Ramirez et al. (2011) addressed whether Mn was accumulating in the dopaminergic regions of the adult brain of *D. melanogaster* and found that there indeed was an accumulation and that there was a dose-dependent effect on the adults being able to locomote in a climbing assay. Additionally, acute and long-term feeding of $MnSO_4$ decreased survival of the adult *Drosophila* likely due to damage in the dopaminergic regions within the CNS.

Nelson et al. (2018) reported on studies conducted with bivalve mollusks where the cilia beating is controlled by dopaminergic and serotonergic innervation. Low concentration of Mn^{2+} at 50 μM reduced the dopaminergic effects on the cilia beating but a higher concentration of Mn^{2+} blocked the effect with application of DA. This implied that low concentrations reduced the neuron's function, potentially by a decrease in DA release but that high concentrations blocked the postsynaptic DA response (Catapane et al., 1978; Aiello, 1990; Carroll and Catapane, 2007; Martin et al., 2008; Nelson et al., 2010, 2018). Nelson et al. (2018) investigated the mechanism in which Mn^{2+} altered the postsynaptic response. This previous study focused to address if Mn^{2+} blocked the DA receptors as well as the downstream effects of adenylyl cyclase for the G protein coupled metabotropic receptors (GPCR) of DA. It appeared that Mn^{2+} resulted in a reduced sensitivity to DA by likely desensitizing the DA receptors and not acutely altering adenylyl cyclase (Nelson et al., 2018). The notion in the effect of Mn exposure is that oxidative stress and altered mitochondrial function occurs (Bowman et al., 2011; Nelson et al., 2018), resulting in the neurons becoming dysfunctional. Such cellular mechanisms would likely be affected in all neurons and other cell types as well. There are many potential actions on neurons by affecting channels and screening electrical potential on membranes. To gain an overview in the effects of Mn^{2+} on various physiological functions, we conducted experiments in a few model systems in different animals on development, survival, cardiac, sensory, motor, and glutamate synaptic responses.

Larval *D. melanogaster* have a relatively simple neural circuit to analyze metal toxicity effects on locomotive behaviors. Body wall crawling, and mouth hook movements are readily assessed behaviors to examine the dietary actions of consuming Mn^{2+} (Badre and Cooper, 2008; Dasari et al., 2007). Thus, to gain some insight into the effects of dietary Mn^{2+} , these larval behaviors were examined in this study. As expected, if the neural circuits for eating and locomotion are severely affected in adults and larvae, one would expect manganese to also affect development and survival. Thus, the general effects on the pupation rate as well as survival of larvae and adults with manganese-tainted food was surveyed herein.

The direct effect of Mn^{2+} on sensory neurons is lacking in literature. Since Mn^{2+} can block voltage-gated calcium channels, then it may be possible that Mn^{2+} can block stretch-activated channels (SACs). We examined the effects of Mn^{2+} exposure on primary sensory neurons activated by SACs in proprioceptive neurons of a model crab chordotonal organ (Alexandrowicz, 1972; Cooper, 2008; Hartman and Boettiger, 1967; Whitear, 1938).

To address whether Mn^{2+} has an effect beyond that on dopaminergic synapses, evoked and quantal responses of glutamatergic synapses were also observed when acutely exposed to Mn^{2+} . The spontaneous quantal events of larval *D. melanogaster* and crayfish neuromuscular junctions (NMJs) were used to assess the acute actions on the model glutamatergic synapse (Titlow and Cooper, 2018). If there remains the presence of spontaneous quantal events of the same general magnitude before and during exposure to Mn^{2+} while evoked responses are retarded, then these effects would indicate a presynaptic action; however, if both evoked and spontaneous quantal events were gradually declining in amplitude with exposure, then it would be suggestive that the glutamate receptors are affected by Mn^{2+} .

In rodent models, Mn^{2+} exposure (1–8 mM) was found to inhibit myocardial contraction and electrical function (Jiang and Zheng, 2005). Thus, we examined the acute effect on the heart rate in larval

D. melanogaster within in situ preparations. When the in situ larval hearts are bathed in a large amount of saline, leaving them isolated from any endogenous hormonal actions, then the direct effects of exogenously applied compounds can be assessed independently of endogenous compounds of the hemolymph. The heart rate is rapidly altered with release of dopamine, serotonin or octopamine by the larval CNS, (Malloy et al., 2017) which is not an issue with saline flushed in situ preparation of the larval heart. Since the *D. melanogaster* model is increasingly becoming a model to address cardiac function and pacemaker activity, it serves as a rapid approach to screen the effect of Mn^{2+} on cardiac function. Since the larval heart tube is not innervated until the transition from larvae to pupa, the direct action on the heart is able to be addressed independently of neural innervation (Johnstone and Cooper, 2006). The heart rate in larval *D. melanogaster* is very sensitive to extracellular Ca^{2+} levels, pH, and other modulators, so the change in heart rate can be used as a sensitive index for the effects of compounds that alter these functions (de Castro et al., 2014; Desai-Shah et al., 2010; Majeed et al., 2013, 2014; Titlow et al., 2013). Thus, it was assumed that there would likely be an acute effect by Mn^{2+} on the heart rate in larval *Drosophila* due to the responsiveness to Ca^{2+} in bathing media.

The common forms of Mn in nature are found in the oxidation state of +2 (e.g., manganese chloride [$MnCl_2$]), +4 (e.g., manganese dioxide [MnO_2]), and +7 (e.g., potassium permanganate [$KMnO_4$]) (Howe et al., 2004). Both $MnSO_4$ and $MnCl_2$ are both readily soluble in water and readily dissociate in water. Since cytotoxicity varies between $MnSO_4$ and $MnCl_2$ in some studies (Marreilha dos Santos et al., 2008), we examined the two compounds in some studies herein. The differences in the physiological responses between the compounds appear to be due to differences in the rate of transport into different tissues (Erikson et al., 2004a, 2004b). In the current study, some of the physiological assays were performed with $MnSO_4$ and $MnCl_2$ either being dissolved in food or in the saline applied to exposed tissues.

Surveying several physiological systems in various animals will provide a general understanding of the effect of Mn^{2+} . This knowledge can then be applied to understand the effects on organisms potentially exposed to Mn^{2+} through industrial mining or agricultural use of $MnSO_4$ in foliar sprays intended to benefit plant health. Mn^{2+} is noted to be found in various tissues of the edible blue crab in marine environments (*Callinectes sapidus*) (Zotti et al., 2016) and edible crayfish of freshwater systems. Given that $MnSO_4$ is used in agriculture to aid plant health, it is also found on food sources for insects not only in the soil but also in and on plants (Chen et al., 2020; Jhanji et al., 2014). Increasing manganese concentrations also seems to be linked to idiopathic blindness in the American lobster, a condition affecting roughly half of the species population (Ochs et al., 2020). Additionally, commonly eaten crabs in the wetlands of China were found to contain high amounts of manganese that deemed them to be inedible (Zhang et al., 2019). Manganese can be taken up across the gills of crustaceans and is found in the hemolymph (7.35 $\mu g/g$ wet weight) as well as the midgut of hermit crab (1596 $\mu g/g$ dw) and in crayfish (374 $\mu g/g$ dw). In skeletal muscle of crayfish it has been found to be as high as 87 $\mu g/g$ and in the central nervous system in lobster as high as 193 $\mu g/g$. Baden and Eriksson (2006) and Zhang et al. (2019) reviewed the bioaccumulation in various tissues of crustaceans under different conditions. A detailed review of accumulation of Mn^{2+} in tissues of eatable lobster was reviewed by Ochs et al. (2020). Certain edible termites have also been found to contain astonishingly high amounts of Mn^{2+} , some species containing enough that minimal consumption could exceed the upper limits for intake of Mn^{2+} in humans (Verspoor et al., 2020).

Because of the Mn^{2+} exposure on various populations in the ecosystem examining the effects on *D. melanogaster* can provide an index (Mohandas et al., 2017) of the potential effects on other insect species in the environment. As it is well established, the effects of agents on non-humans are relevant to understanding the potential actions of that agent in humans. To uncover the potential effects of compounds on physiological functions, a higher concentration than that which a system

is normally exposed to can unmask the more subtle effects of the compound present at a lower level over longer period of time. Since Mn is of higher concentration in some infant formula products, there is some interest in understanding the potential effects of Mn^{2+} on neuronal function of human infants fed formula milk in comparison to those fed human breast milk (Frisbie et al., 2019). Thus, with all of these potential avenues for Mn exposure, it is crucial to understand more about the effects of manganese in various other organisms in order to better predict the potential effects on humans.

2. Methods

2.1. Animals

Drosophila melanogaster, Canton S (CS) flies were used in all behavioral and physiological assays. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington Drosophila Stock Center (BDSC). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. Blue crabs (*C. sapidus*) were obtained from a local supermarket in Lexington, KY, USA which were delivered from a distribution center in Atlanta, GA, USA. They were bought and maintained in a seawater aquarium for several days prior to use in order to assess their health. The crabs were adults and in the range of 10–15 cm in carapace width (from point to point). All crabs used were alive and were very active upon autotomizing a leg for experimentation. Red Swamp Crayfish (*Procambarus clarkii*) were obtained from a distribution center in Atlanta, GA, USA and delivered to, and bought from, a local supermarket in Lexington, KY, USA. Throughout the study, mid-sized crayfish measuring 6–10 cm in body length and 12.5–25 g in body weight were used. Each animal was housed in individual standardized plastic containers with dry fish food exchanged weekly and aerated water (20–21 °C).

2.2. Survival and developmental studies of *Drosophila melanogaster*

To address the effect of Mn^{2+} on life cycle alterations, the time to pupation from 1st instar larva was measured when exposed to varying dosages of $MnCl_2$ and $MnSO_4$ treatments in tainted food. Ten 1st instars were placed on the food located at the bottom of the glass vials. The vials were used to allow for an aerated and moist environment at 21 °C. The larvae were then left to develop into pupa and each day, the number of pupae were counted for each condition. After 7 days, the food was removed and the vial was examined for dead larvae. Each concentration (2.5 mM, 30 mM and 100 mM) of $MnCl_2$ and $MnSO_4$ was performed in triplicate.

Assessing the survival of adults involved the collection of adults within 3 days after encasing from pupa. The adults were placed in plastic vials containing the tainted food with $MnCl_2$ or $MnSO_4$. The survival of the adults was monitored daily for 21 days. The conditions were replicated in 6 trials for each concentration of $MnCl_2$ and $MnSO_4$ (5 mM, 15 mM and 30 mM). Each trial started with 20 adults. Each experimental group and control group, also referred to as a saline group, had 120 adults in total.

2.3. Behavioral assays

Locomotion of larvae was assessed by recording body wall contractions per minute with the use of a dissecting microscope. The 2nd instar larvae were placed in food containing a concentration of either 15 mM or 30 mM $MnCl_2$ or $MnSO_4$. This was made by combining 1 g of corn meal food, estimated as 1 mL in volume, and 1 mL of water to produce 2 mL. The $MnCl_2$ and $MnSO_4$ was dissolved in the 1 mL of water at double the concentration and then was mixed with the food to provide the final concentrations. After 24 h, the 3rd instars were placed on apple-juice agar (1% agar) Petri dishes (about 8.5–9 cm diameter). The larvae were left inside the dish for 1 min to acclimate to the environment. Then

body wall movements (BWMs) were counted for 1 min via a microscope in a lightly illuminated environment at room temperature (21–22 °C).

Next, the larvae used for body wall movements were used to assess larval feeding behavior after being transferred to a small Petri dish (5.5 cm diameter) that contained a yeast solution (a few dried yeast granules were mixed with water). The larvae were left for 1 min, and then the mouth hook movements (MHMs) were counted for 1 min.

2.4. Heart rate measures in larval *Drosophila*

A detailed description, in video format, of exposing the larval heart in early 3rd instars is provided in Cooper et al. (2009). In brief, the larvae were dissected ventrally and pinned on four corners to expose the heart tube. The preparation was then bathed in physiological saline (de Castro et al., 2014). A modified HL3 saline was used to maintain the in situ hearts and body wall muscles ($NaCl$ 70 mM, KCl 5 mM, $MgCl_2 \cdot 6H_2O$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 25 mM, and $CaCl_2 \cdot 2H_2O$ 1 mM, pH 7.1; Stewart et al., 1994). In the experimental groups, varying concentrations of $MnCl_2$ or $MnSO_4$ was dissolved in this saline. The pH of the saline was maintained at 7.1. Each concentration of $MnCl_2$ and $MnSO_4$ (2.5 mM, 15 mM and 30 mM) was examined for 8 to 10 larvae. The heart rate was measured in a 30 second period and was converted to beats per minute for graphical analysis. The rate was taken in the caudal region of the heart tube (Fig. 1). The heart rate was first obtained during the initial saline exposure before the bathing solution was exchanged to one containing a compound. Two minutes after the new solution containing an experimental compound was added, the heart rate was obtained again. This was followed by flushing out the compounds with fresh saline three times and then waiting 2 min before recording data of the heart rate after the Mn^{2+} was removed.

2.5. Crab proprioceptive neurons

Similar dissection procedures and electrophysiological measures as those thoroughly described in text and video format by Majeed et al. (2013) were used. In brief, the animal was induced to autotomize the first or second walking leg by lightly pinching with pliers at the base of the leg. The propodite-dactylopodite (Pd) chordotonal organ spans the last segment of the leg and was exposed by cutting a window of the cuticle on both sides of the leg (in the propodite segment). The leg was then pinned in a Sylgard-lined dish and covered with crab saline (Fig. 2). The Pd nerve was then exposed and pulled into a suction electrode for recording. During the experiment, the dactyl was moved from a flexed position to an open position in a 1 s time frame, held for 10 s, and then moved back to the starting position (see Fig. 1A in McCubbin et al., 2020). An insect dissecting pin was used as a reference for the maximum displacement range, and each displacement was marked on the

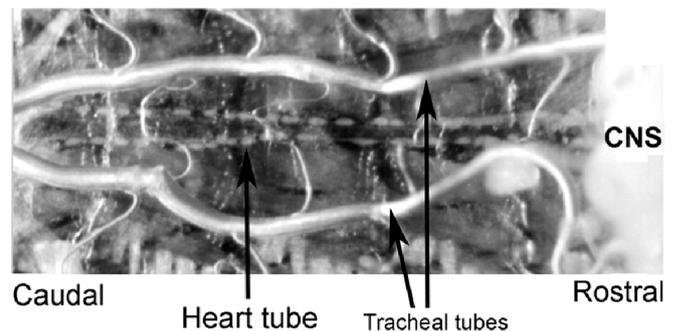


Fig. 1. The filleted larva preparation used for measurement of heart rate when exposed to $MnSO_4$ and $MnCl_2$. Heartbeats were counted by manual inspection through a dissecting microscope before and after switching to the compound of interest. Heart rates were measured from the caudal end of the preparation near the point where the two tracheal tubes bifurcate.

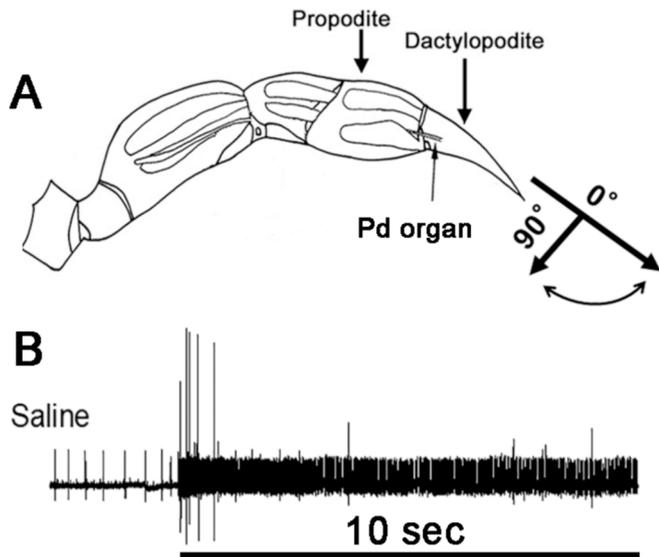


Fig. 2. The first or second walking leg of the crab was used to expose the Pd organ and associated nerve. (A) The joint was initially bent at 90 degrees and then extended out to 180 degrees within 1 s and held for at least another 9 s. (B) The entire 10 s was then used for analysis in the number of spikes that occurred while being bathed in different solutions.

computer recording file. The crab saline used during recordings of the sensory nerves consisted of (in mM): 470 NaCl, 7.9 KCl, 15.0 CaCl₂·2H₂O, 6.98 MgCl₂·6H₂O, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4. Each concentration of MnCl₂ and MnSO₄ (1 mM, 15 mM, 30 mM and 100 mM) was examined for 5 to 6 preparations. As a control for osmolarity differences, preparations exposed to 1 M sucrose made in standard crab saline were also studied. The numbers of spikes recorded over the first 10 s from the start of the joint displacement were used as an index in the neural activity. Preparations were also examined with 1 M mannitol to examine the effect on the neural activity of the Pd organ.

To measure the compound action potential, the Pd nerve was dissected away from the main leg nerve and from the Pd organ to the autotomy plane in the proximal region of the leg (Fig. 3A). The proximal half of the shell of the leg was removed after the nerve was pulled to the side of the leg. The proximal end of the nerve was pulled into a suction electrode to record the neural activity from the proprioceptive sensory cells as well as to evoke a compound action potential (CAP) when the stimulating electrode induced the neurons to produce action potentials (Fig. 3B). A region of the distal part of the nerve was pulled into a suction electrode to evoke stimulation in an *en passant* manner of the nerve. The voltage of the stimulating electrode was increased to produce a maximum amplitude and duration and then the voltage was slightly reduced so the CAP amplitude would be below the maximum response. Thus, any increase in the CAP would be able to be detected upon exposure to Mn²⁺. Both ends of the nerve, which were placed in the suction electrodes, were packed with clear petroleum jelly to seal the nerve in the electrode and increase resistance around the nerve to the electrode to obtain the largest extracellular signals. A ground wire was placed in the bathing media.

2.6. Neuromuscular junctions of larval *Drosophila* and crayfish

The effect of Mn²⁺ on the membrane potential, evoked excitatory junction potentials (EJPs), and spontaneous quantal events (mEJPs) were examined for brief exposures of saline containing Mn²⁺. This was followed by flushing of the preparation with standard fly saline to determine if the preparations were still viable after exposure to Mn²⁺.

Third instar larval *D. melanogaster* were dissected in physiological

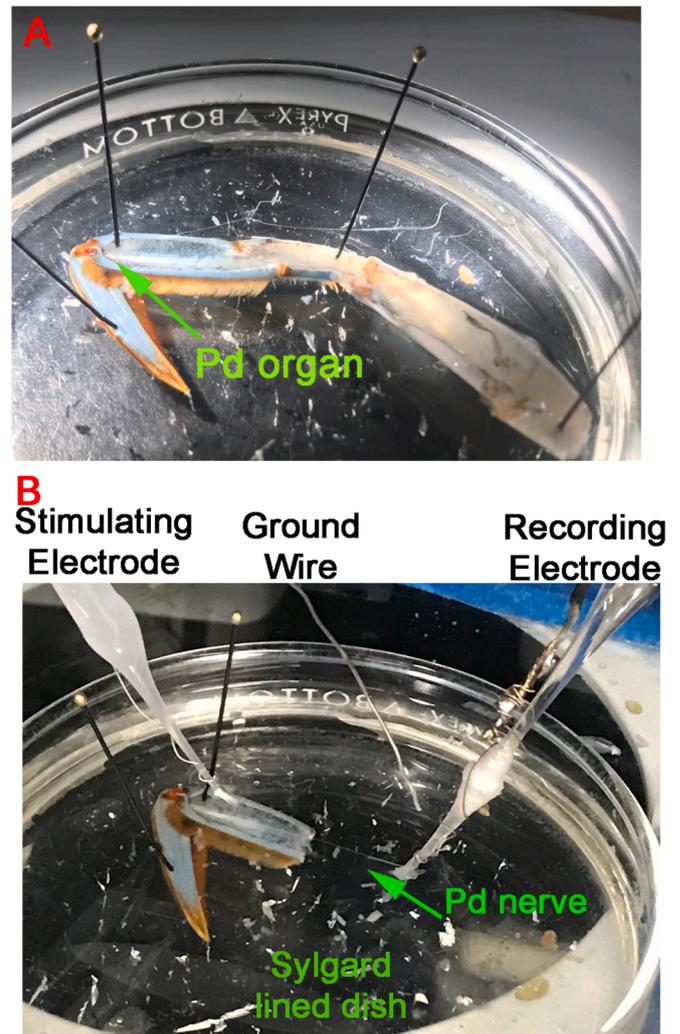


Fig. 3. Recording the evoked compound action potential (CAP) of the Pd nerve in a crab first walking leg. The Pd nerve is dissected out of the leg from the Pd organ to the autotomy plane of the leg. The proximal end is used to record the Pd activity and induced CAP in different bathing media. The distal end of the nerve close to the Pd organ was pulled into a suction electrode to induce CAPs in the Pd nerve. The preparation is pinned to a Sylgard lined dish to hold the dissected leg in place. A ground wire is placed in the bathing media.

saline. The segmental nerves were cut and sucked into a suction electrode, which was filled with saline and stimulated. The segmental nerves were stimulated at 0.5 Hz (S88 Stimulator, Astro-Med, Inc., Grass Co., West Warwick, RI, USA). To monitor the transmembrane potentials of the body wall muscle (m6) of 3rd instar larvae, a sharp intracellular electrode (30 to 40 M resistance) filled with 3 M KCl impaled the fiber (Fig. 4). An Axoclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage was used. Fly saline used was the same as detailed above for the heart rate monitoring.

The opener muscle of the crayfish has been historically reviewed for studying synaptic transmission (Cooper and Cooper, 2009). The use of the most distal muscle fibers in the preparation provides a reference for consistency among preparations (Cooper et al., 1995). The dissection to expose and selectively stimulate the excitatory motor neuron of the opener muscle is described in textual and video format (Cooper and Cooper, 2009). In brief, the excitatory neuron was isolated from the inhibitor neuron and was then stimulated in the meropodite segment. The stimulation paradigm consisted of providing a train of pulses at 60 Hz with 10 s between trains (Fig. 5). Responses were recorded with an Axoclamp 2B (Axon Instruments, USA), converted with a PowerLab,

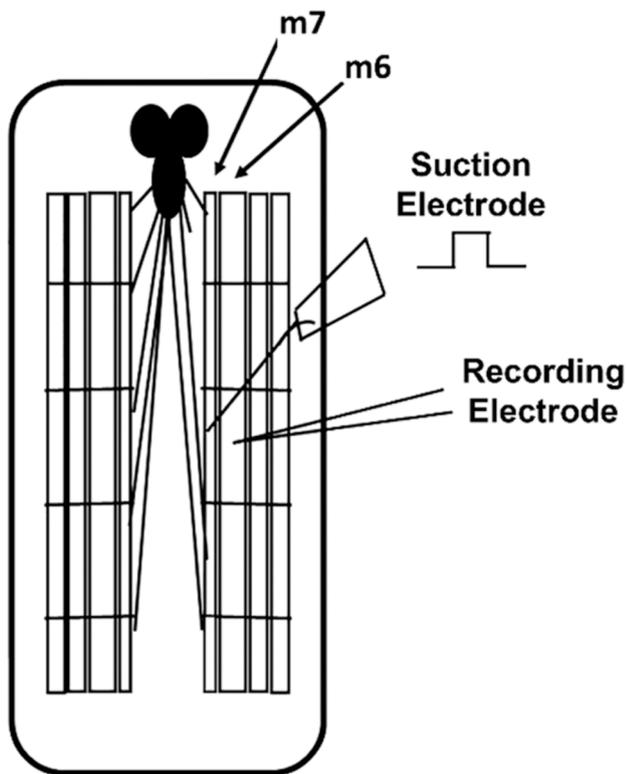


Fig. 4. A schematic diagram of the recording arrangement of a 3rd instar larva for obtaining evoked and spontaneous synaptic excitatory junction potentials from m6 muscle fiber.

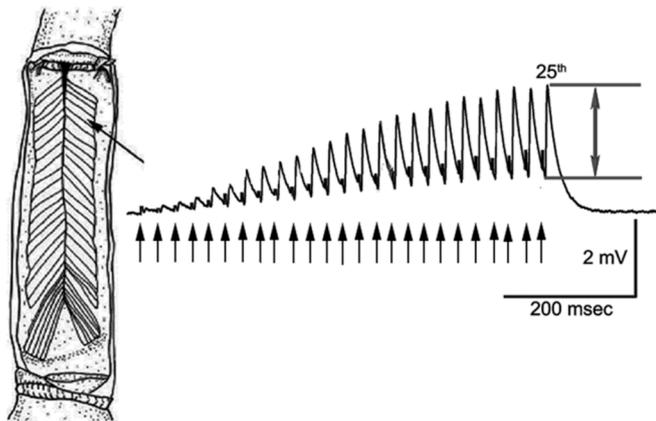


Fig. 5. The opener muscle of the crayfish walking leg with facilitated synaptic responses in the excitatory junction potentials from the distal muscle fibers. The synaptic responses depicted show the facilitation which occurs over 25 stimuli. The arrows indicate the stimuli given to the nerve.

4SP (ADInstruments, USA), and analyzed with LabChart 7.0 (ADInstruments, Colorado Springs, CO, USA) on a computer at a 10 or 20 kHz sampling rate. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mmol/L: 205 NaCl; 5.3 KCl; 13.5CaCl₂·2H₂O; 2.45 MgCl₂·6H₂O; 10 glucose; 0.5 HEPES adjusted to pH 7.4).

2.7. Statistical methods

In general, when normality was to be assumed, Shapiro-Wilks tests were used to validate the assumptions in order to determine the use of a *t*-test, a paired *t*-test or a Wilcoxon Signed Rank Test. An ANOVA was

used when necessary, along with Bonferroni post-hoc *t*-test.

For the survival analysis in *D. melanogaster*, Weibull regression was used to model the survival curves with the factors being solution type (Control, MnCl₂, MnSO₄) and strength of solution (0, 5 mM, 15 mM and 30 mM). ANOVA was then used to determine if a significant difference existed between the factors. If a factor was considered statistically significant, $p < 0.05$, a Bonferroni correction was used to determine the pairwise post-hoc differences in the factors.

For the behavioral studies, ANOVA was used to determine the difference between the 5 treatments (Control, 15 mM and 30 mM of both MnCl₂ and MnSO₄). If a significant difference was detected, a Bonferroni post-hoc *t*-test was conducted to determine which treatments were statistically different.

For the heart rate experiment, the data consisted of an initial saline treatment followed by application of either a saline, MnSO₄ or MnCl₂ treatment and then another saline application. The original data was not normally distributed (Shapiro-Wilks, $p < 0.05$). Therefore, new variables were created with a pre and a post variable creation. Pre is defined as the initial saline treatment minus the application of either saline or Mn solution and post was defined as the initial saline treatment minus the terminal saline treatment. The transformed data did pass the Shapiro-Wilks test ($p > 0.05$). With the two new variables, a 3-way repeated measures ANOVA evaluating the effects of compound (MnSO₄ vs MnCl₂), dose level (2.5 mM, 15 mM and 30 mM) along with time (pre and post) was conducted using the difference in heart rate. Since we found a significant two-way interaction, we followed up the initial repeated measures ANOVA with a one-way ANOVA with application, treatment, and time held constant to determine the difference in the doses. This *p*-value was also adjusted using the Bonferroni paradigm. Once a significant result was found, all post-hoc analysis was done with the Bonferroni test.

For the crab neuron and NMJ studies, either a paired *t*-test (if Shapiro-Wilks test passed) or a Wilcoxon Signed Rank test was used to compare various sensitivities.

A significance level of 0.05 was used in all studies unless the Bonferroni *t*-test was used, in which case the significance level was adjusted accordingly.

3. Results

3.1. Survival and developmental studies of *Drosophila melanogaster*

Larval development and survival study were conducted in triplicate with ten 1st instars, providing a total of 30 individuals for each condition. Within a 6 h window from hatching, the 1st instars were placed in food tainted with NaCl, MnSO₄ and MnCl₂ at various concentrations (0.0025, 0.015, 0.03 and 0.1 M). The most significant effect was for the higher concentrations of 0.03 and 0.01 M MnSO₄ and MnCl₂ as this was toxic for the 1st instar. In these conditions, there were no 2nd instars observed. When exposed to a 0.015 M concentration of both MnSO₄ and MnCl₂, there was a high percent of death. The 0.015 M MnCl₂ was toxic at 1st instar stage since only 1 out of 30 larvae produced a pupa and went on to emerge as an adult. The 0.015 M MnSO₄ was not conducive for full survival of all 30 larvae as 15 out of the 30 survived to pupa and adults. In these conditions, there were a total of eight 2nd instars present in three vials and the rest of the dead larva (seven) appeared to be 1st instar but degraded as seen for the vials at 0.03 and 0.01 M concentrations (Table 1).

The survival of a different set of adults were examined for survival when exposed to food also tainted with MnSO₄ and MnCl₂. The young adults did not show a dose-dependent effect with the low dose (5 mM) of either compound when compared to controls (Fig. 6); however, the 15 mM and 30 mM showed a greater percentage of deaths over time (Fig. 6). The 30 mM of MnSO₄ showed a steep decline in survival after 5 days and by 8 days the adults had died. In the same time period, there were a few still surviving in 30 mM MnCl₂ but the majority were

Table 1

Larval development and survival study for NaCl, MnSO₄ and MnCl₂ at 0.0025, 0.015, 0.03 and 0.1 M. Larvae were placed in food with the compounds from early 1st instar. The number of pupae formed are recorded. The number of adults which emerged from the pupae are also recorded over the days from placing 1st instars in the food.

Food: NaCl (mM)				MnSO ₄				MnCl ₂				Days	
2.5	15	30	100	2.5	15	30	100	2.5	15	30	100		
0	0	0	0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	0	0	0	3
18	17	15	17	18	6	0	0	18	1	0	0	0	4
30	30	30	29	30	15	0	0	30	1	0	0	0	5
30	30	30	29	30	15	0	0	30	1	0	0	0	6
30	30	30	29	30	15	0	0	30	1	0	0	0	7
30	30	30	29	30	15	0	0	30	1	0	0	0	8
30	30	30	29	30	15	0	0	30	1	0	0	0	9
No adults	No adults	No adults	No adults	No adults	No adults	No pupa	No pupa	No adults	No adults	No pupa	No pupa	No pupa	10
1	2	6	0	4	0	-	-	0	0	-	-	-	11
17	20	19	15	20	15	-	-	16	0	-	-	-	12
30	30	30	30	30	15	-	-	30	1	-	-	-	13

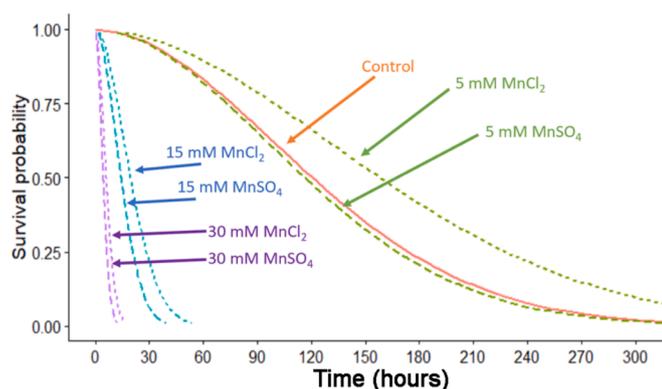


Fig. 6. Survival of adults exposed to food tainted with MnSO₄ or MnCl₂ as compared to controls. Controls were fed standard cornmeal food while others had food tainted with 5, 15 or 30 mM MnSO₄ or MnCl₂. Twenty adults were placed into each condition and observed daily.

deceased. By 14 days, all the MnCl₂ at 30 mM were dead and >90% of the 15 mM MnSO₄ had died. We found a significant difference between at least two of the solutions (ANOVA, $p < 0.001$). Two sets of curves were statistically different from each other, the Control and MnCl₂ curves (Bonferroni t -test, $p < 0.001$) and the Control and MnSO₄ curves (Bonferroni t -test, $p < 0.001$). However, MnCl₂ and MnSO₄ were not significantly different (Bonferroni post-hoc, $p > 0.05$). Similarly, we found at least 2 of the strengths of the solutions were different from each other (ANOVA $p < 0.001$). In fact, the 15 mM and 30 mM solutions were significantly different from the control as well as different from 5 mM (all 4 Bonferroni t -tests, $p < 0.001$). 15 mM and 30 mM were also different from each other (Bonferroni t -test, $p < 0.001$). Lastly, the control and 5 mM solutions were not significantly different (Bonferroni t -test, $p > 0.05$).

3.2. Behavioral assays

After only 24 h of consuming the food tainted with Mn²⁺, there were obvious differences in the behaviors associated with consuming 30 mM MnSO₄ or MnCl₂ (Fig. 7). The lower concentration of 15 mM did not show as large of differences. Specifically, the mouth hook movements were significantly different from controls for both MnSO₄ and MnCl₂ at both concentrations of 15 mM and 30 mM. Also, the mouth hook movements from the 15 mM and 30 mM exposure were significantly different from each other (ANOVA, $N > 20$; $p < 0.05$, Bonferroni t -test).

The statistical differences in the body wall movements were not as pronounced as the mouth hook movements (Fig. 7). The 15 mM MnCl₂

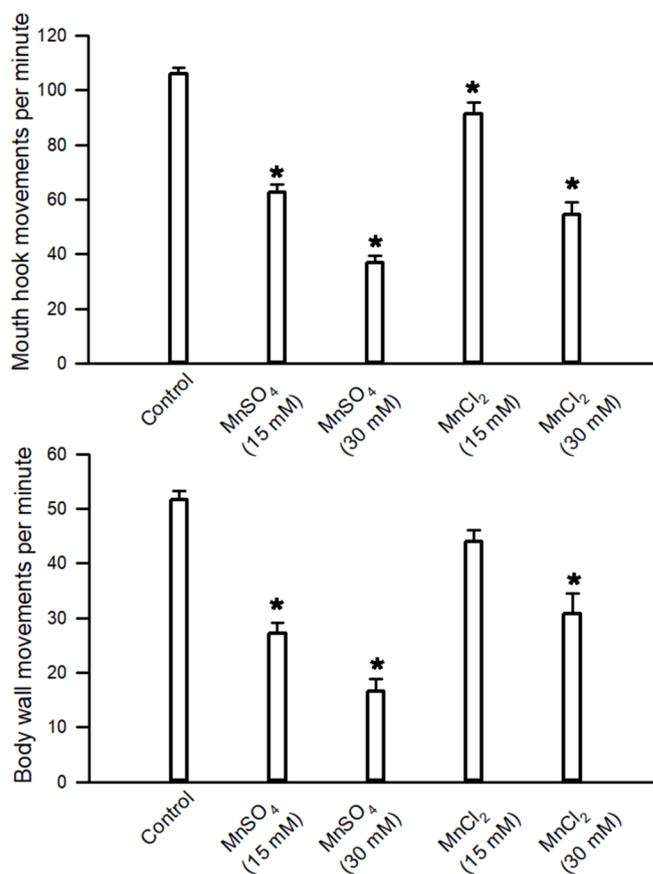


Fig. 7. Larval behaviors after 24 h of exposure to tainted food of 15 or 30 mM MnSO₄ or MnCl₂ as compared to standard media. The mouth hook movements per minute were significantly reduced with both 15 and 30 mM of MnSO₄ or MnCl₂. The larvae were transferred to a dilute solution of yeast mixed with distilled water for the assay. Crawling behavior, as indexed in body wall contractions per minute, indicated that being exposed to food of 30 mM for MnSO₄ or MnCl₂ reduced the rate. However only the 15 mM MnSO₄ resulted in a significantly lower rate than controls. The rates are expressed as a mean \pm SEM and significance is $p < 0.05$ ANOVA, $N > 20$ larvae for each condition.

did not produce a significant difference from the controls but did when compared to 30 mM MnCl₂. The effect of 15 mM MnSO₄ was statistical different from controls and both MnSO₄ and MnCl₂ at 30 mM. The effects of 15 mM and 30 mM MnCl₂ were not statistically different from each other. Likewise, the effects of 30 mM of MnSO₄ or MnCl₂ were not

statistically different from each other (ANOVA, $N > 20$; $p > 0.05$).

3.3. Heart rate measures in larval *Drosophila*

The hearts of the larvae were very sensitive to the exposure of Mn^{2+} from either $MnSO_4$ or $MnCl_2$ as even the lowest concentration of 2.5 mM influenced the heart rate (Fig. 8). We found a significant two-way interaction between dosage and time (pre vs post) (3-way repeated measures ANOVA, $p < 0.002$). Therefore, we needed to focus on the treatment applications separately.

For the $MnCl_2$ treatment, there was a statistical difference between post measurements (initial – terminal saline) between the 3 dose levels (ANOVA, $p = 0.024$). There was a statistical difference between the 2.5 mM and 30 mM doses (Bonferroni *t*-test, $p = 0.0011$) and a lesser significant difference between the 2.5 mM and 15 mM doses (Bonferroni *t*-test, $p = 0.0358$). However, there was not statistical difference between pre -measurements, initial saline to treatment application, (ANOVA, $p > 0.05$) between the 3 dose levels.

For the $MnSO_4$ treatment, there were statistical differences between both the pre- and post- measurements between the 3 dose levels (ANOVA, pre $p = 0.004$, ANOVA post < 0.0001). For the post measurements, the lowest dose and highest dose were significantly different from each other (Bonferroni *t*-test, $p < 0.001$) and the lowest dose and middle dose were also significantly different (Bonferroni *t*-test, $p < 0.001$). Finally, for the pre-measurements, there was only a marginal

significance with a Bonferroni *t*-test with 2.5 mM versus 15 mM doses ($p = 0.079$).

A percent difference in the heart rate provides a normalized view from the initial differences that can be used for comparisons (Fig. 9). The paradigm in exposing the in situ hearts to the compounds allowed comparisons in the heart rate from the initial saline exposure to the compound (Saline to Mn) and the rate in the initial saline to after

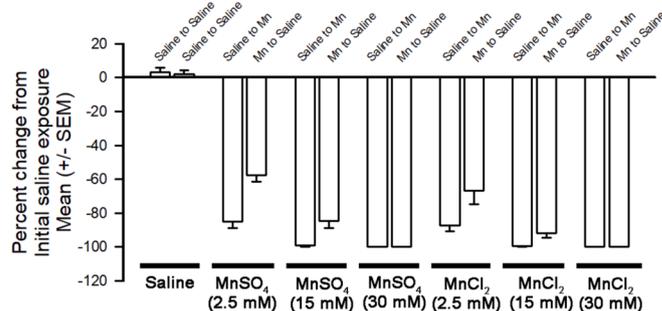


Fig. 9. Percent differences in heart rate from saline to exposure of $MnSO_4$ or $MnCl_2$. The percent differences are compared for each condition to the initial rate in saline for each individual larva. The “Mn to saline” is the condition of flushing away of Mn to fresh saline and the percent difference is from the initial saline condition.

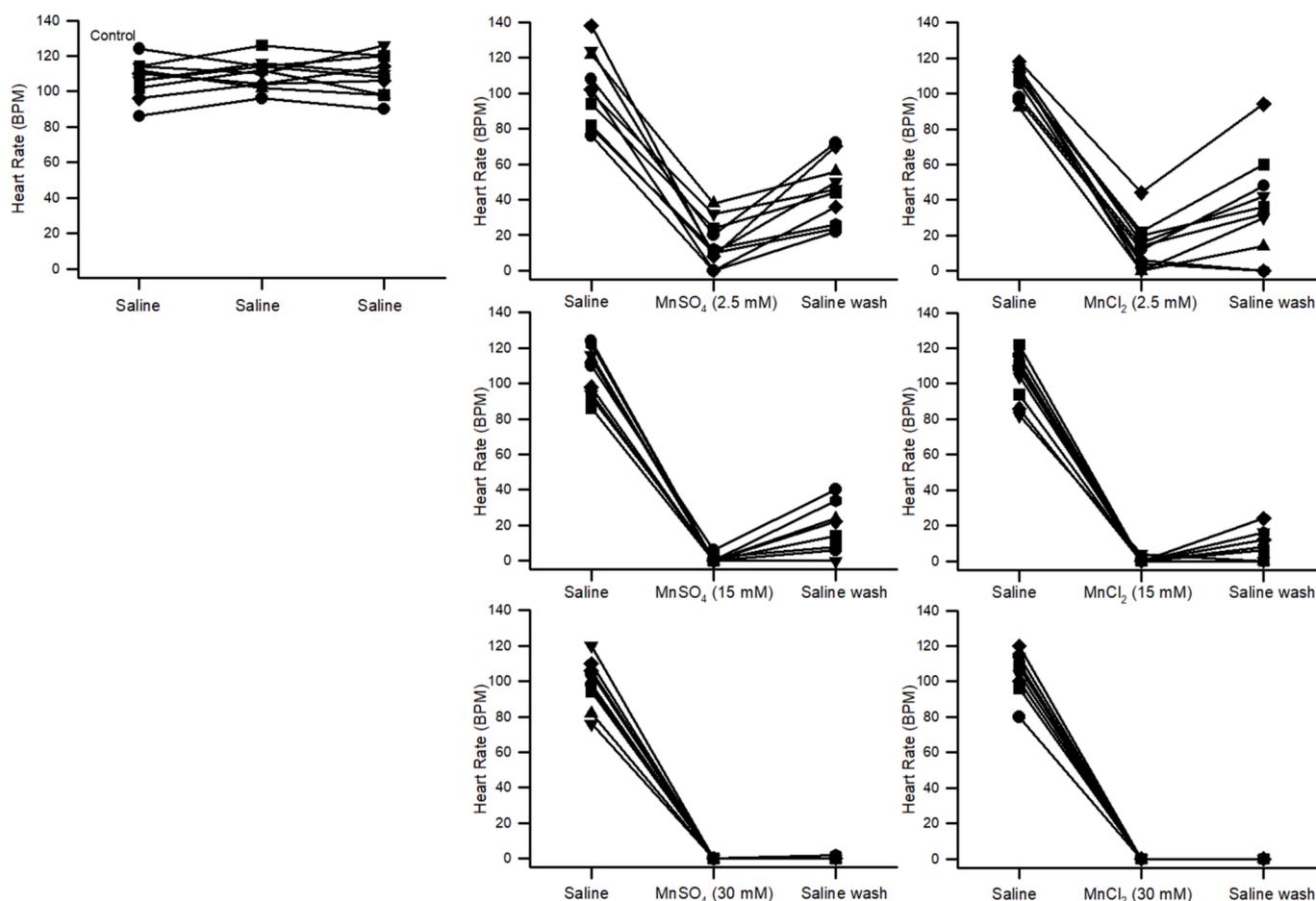


Fig. 8. The effect of $MnSO_4$ or $MnCl_2$ exposure on heart rate of in situ hearts in 3rd instar larvae. The early 3rd instar larvae is dissected and filleted in order to expose the heart tube to the bathing saline and flush away any endogenous compounds. The heart rate is counted in saline and then the media is changed to saline or to a concentration of either $MnSO_4$ or $MnCl_2$, and this is followed by flushing the preparation with fresh saline without any Mn^{2+} and the heart rate is counted again. At all concentrations of $MnSO_4$ or $MnCl_2$, the rate dropped significantly upon exposure and the extent in the decreased rate is concentration-dependent. The heart rate did not recover even after 2 min following flushing with fresh saline for the 30 mM concentrations for either $MnSO_4$ or $MnCl_2$. There was no significant effect for saline exchanged with saline only. Each line represents an individual larva, and each larva was only examined once.

flushing the Mn^{2+} bath and returning to the saline without Mn^{2+} (Mn to Saline) (see Fig. 8). Upon flushing the Mn^{2+} removing saline from the preparations, the heart rate did not return to the initial values, but a semi-recovery was best at 2.5 mM in comparison to the higher concentrations. The 30 mM exposure, for either $MnSO_4$ or $MnCl_2$, affected the heart so substantially that the heart rate was not able to recover in the time observed (2 min after flushing away the Mn^{2+} containing bath).

3.4. Crab proprioceptive neurons

The sensory neurons associated with the crab Pd chordotonal organ showed sensitivity to Mn^{2+} presented as either $MnSO_4$ or $MnCl_2$ at the high concentration of 100 mM with the depression of neural activity (Wilcoxon Signed Rank test, $p < 0.05$). The 30 mM $MnSO_4$ significantly depressed activity (Wilcoxon Signed Rank test, $p < 0.05$), but the 30 mM $MnCl_2$ did not show significant differences as only 3 of the 6 preparations decreased in average in the number of spikes during the limb displacements while exposed. The 15 mM exposure for either the $MnSO_4$ or $MnCl_2$ did not show significant differences in the number of spikes (Wilcoxon Signed Rank test $p > 0.05$, Fig. 10). A high concentration of 1 M for $MnSO_4$ or $MnCl_2$ drastically decreased the activity of the Pd nerve

and the activity did not return with exchanging the bath back to fresh saline.

Since there was a large variability among preparations for exchanging the bath to low concentrations of Mn^{2+} , it was of interest to know if this also occurred with only exchanging the bath with saline as a sham control (Fig. 11A). On average, the percent change in activity with the initial saline to the following saline media exchange increased but was not statistically significant (Fig. 11B; Shapiro-Wilks $p > 0.05$, paired t -test, $N = 6$).

To examine if the 1 M Mn^{2+} blocked the electrical condition of action potentials in the Pd nerve, evoked nerve activity was induced in order to monitor CAPs. To address the effect of Mn^{2+} , two concentrations were examined (100 mM and 1 M Mn^{2+}). The 100 mM $MnSO_4$ shut down the Pd organ activity, but it was still able to conduct experimentally stimulus-induced nerve impulses (CAPs) along the nerve (Fig. 12). In this procedure, the leg is pinned in a static position and the SACs are stimulated in the Pd strand in order to give rise to the spikes seen in Fig. 13A. Every 2 s, the nerve was electrically stimulated to generate a compound action potential (CAP) as noted by stimulations (i.e. Stim) in Fig. 12A1, B1 and C1. Note the CAPs induced are shown in Fig. 12A2, B2 and C2 for the same conditions. The CAPs still can be induced while the

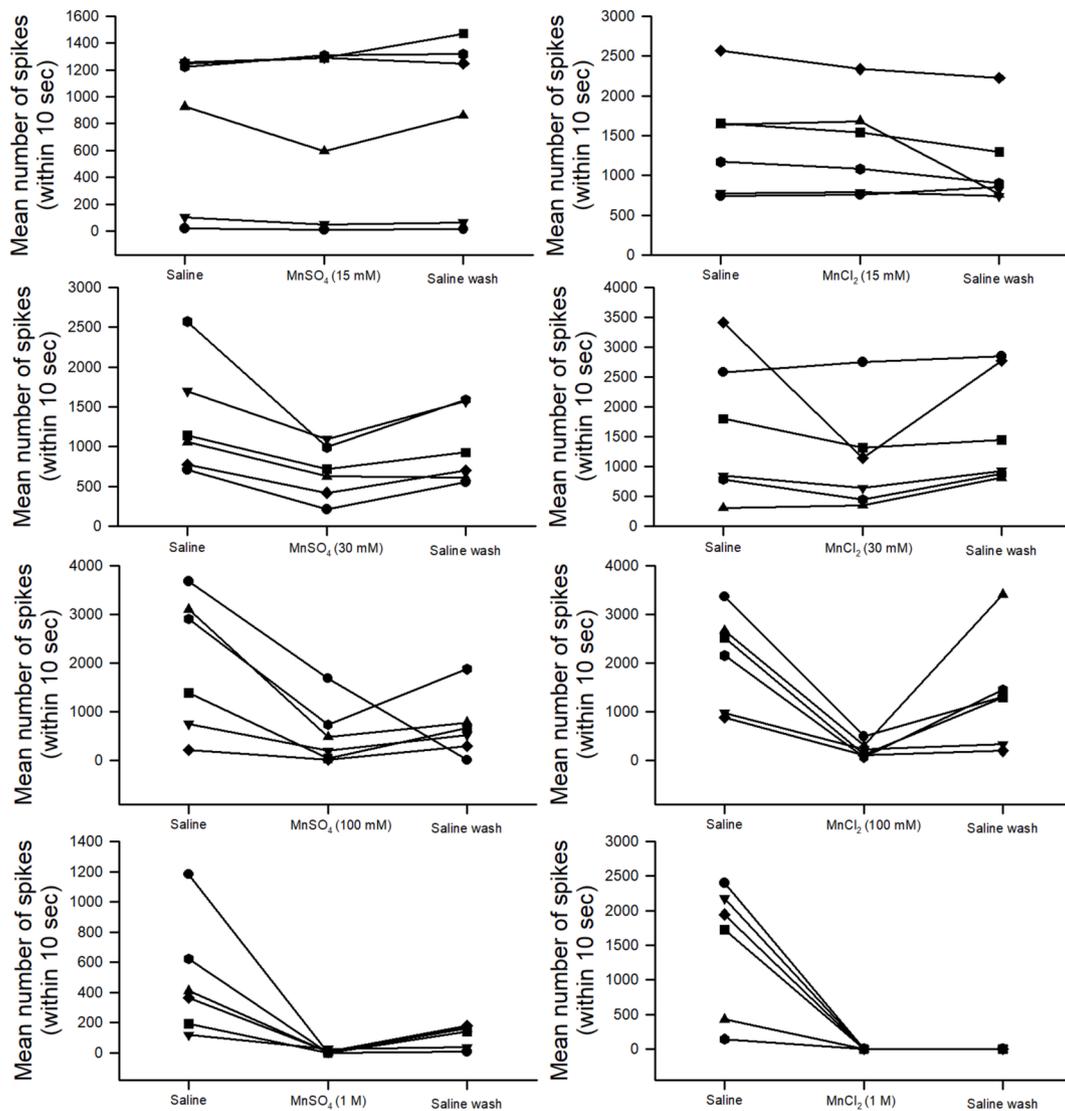


Fig. 10. Activity of the Pd nerve with displacement of the Pd joint from the flexed position to an extended position. The mean number of spikes (averaged of three displacements in each bathing media) for 10 s with the 1st second moving the joint from a flex position to an extended position for the remaining 9 s (static position). Six preparations were used for each concentration (15, 30, 100 mM and 1 M) of $MnSO_4$ or $MnCl_2$. Each line is an individual preparation which was used only once.

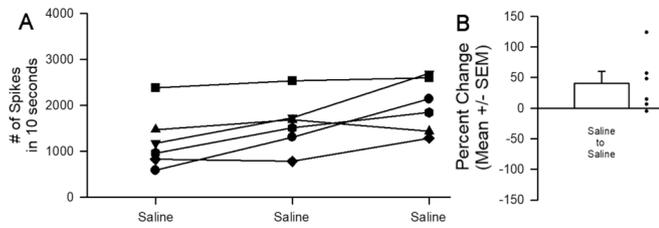


Fig. 11. Activity of the Pd nerve with displacement of the Pd joint from the flexed position to an extended position with repeated saline-only bath exchanges. (A) Six preparations were used while measuring the mean number of spikes (average of three displacements for each time the bathing media was exchanged) over 10 s with the 1st second being the movement of the joint from a flexed position to an extended position, and the remaining 9 s consisting of the extended static position. Each line is an individual preparation which was used only once. (B) The mean percent change from the initial saline exposure to the following bath exchange is depicted along with each individual preparation as dots.

static position sensitive neurons have stopped firing. This is most likely due to the Mn^{2+} blocking the SAC within the sensory endings.

The activity of the Pd organ rapidly decreased when the joint was

held in a static position or during joint displacement when exposed to 1 M Mn^{2+} (Fig. 13A). The CAP was not able to be induced in the nerve during 1 M $MnCl_2$ exposure. Considering that a high concentration of 1 M could alter the nerve's biophysical properties, an equally high concentration of 1 M mannitol was used to examine the activity of the nerve and the evoked CAPs during the joint displacement regime. The activity of the Pd organ during joint movement and while held in an extended position was still robust while exposed to 1 M mannitol (Fig. 13B1 and B2). The shape of the CAP was also very similar before and after exposure to 1 M mannitol (Fig. 13C1 and C2). Thus, the high concentration of 1 M $MnCl_2$ is not just due to the concentration. In addition, nerve activity with the same displacement regime was used before and during exposure to 1 M sucrose for six preparations. The percent change in the number of spikes in saline and exposure to 1 M sucrose did not produce a significant change (Fig. 14; Shapiro-Wilks, $p > 0.05$, paired t -test, $N = 6$, $p = 0.216$).

3.5. Neuromuscular junctions of larval *Drosophila* and crayfish

For assessing synaptic transmission at the neuromuscular junction of the larval *D. melanogaster* and crayfish, only $MnSO_4$ at 2.5 mM was used because low concentrations of manganese still had a drastic effect in

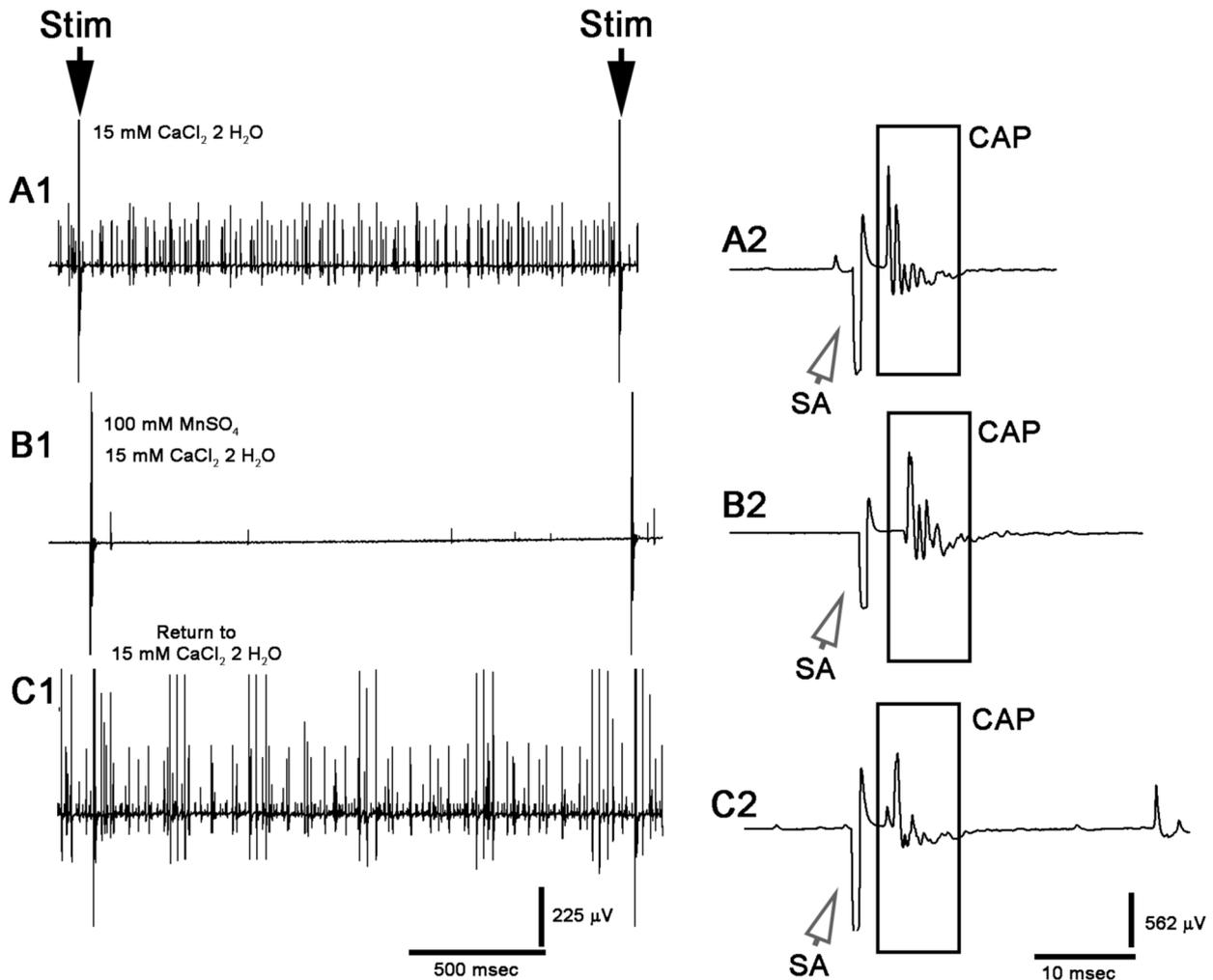


Fig. 12. The activity of the stretch-activated channels in the Pd organ and electrical conduction along the Pd nerve. (A1) The activity of the Pd nerve is induced by static-position-sensitive neurons firing while the joint is pinned in a given position (flexed). While recording the activity in the Pd nerve, a stimulation (Stim) is given every 2 s by the second suction electrode to induce compound action potentials (CAPs). The CAP from such stimulation is shown in A2. The stimulus artifact (SA) is seen followed by the CAP in the rectangular box. (B1) Exposure to 100 mM $MnSO_4$ decreased the stretch-activated channels (SACs) induced activity in the Pd nerve, but not the ability of the nerve to conduct experimentally induced CAPs (B2). (C1) Return of the sensory response from the SACs appears with the flushing away of Mn^{2+} , but the CAPs are still present (C2).

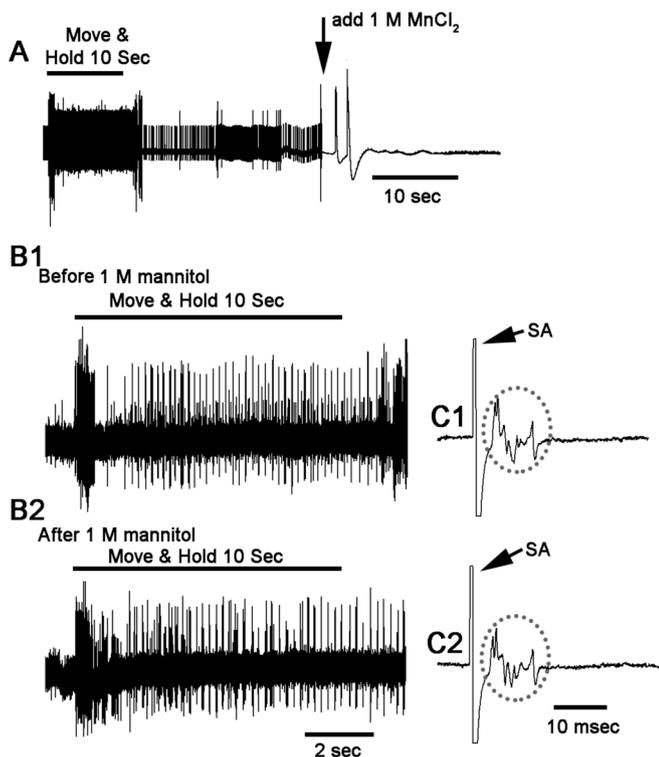


Fig. 13. The effects of high concentration of $MnCl_2$ or mannitol on the activity of the Pd organ during joint movement and induction of compound action potential (CAP). (A) The activity before and during exposure to 1 M $MnCl_2$. The recording was continuous while adding the $MnCl_2$, which resulted in the activity of the Pd organ to stop immediately. The two large deflections in the trace are due to swishing the bathing media over the nerve. (B) The activity during the movement and hold paradigm before (B1) and during 1 M mannitol exposure (B2). In separate experiments, the CAP was induced before (C1) and during 1 M mannitol exposure (C2). The dotted circle outlines the CAP while the stimulus artifact (SA) is the electrical event in the media from the stimulation.

blocking the evoked responses. The intracellular recordings of the body wall muscle (m6) in the larval *D. melanogaster* have many observable spontaneous quantal events. Note that the evoked excitatory junction potentials (EJPs) rapidly depressed upon exposure and slowly regained some of the amplitude upon removal of the Mn^{2+} (Fig. 15A). However, the full amplitude of the EJPs did not return with flushing fresh saline over the preparation repeatedly. Since the quantal events are present while the evoked responses were blocked, this would indicate that the postsynaptic glutamate receptors on the muscle were not blocked by Mn^{2+} (Fig. 15B). This same trend, as depicted in Fig. 15, was observed in 6 out of 6 preparations (Wilcoxon Signed Rank test $p < 0.05$).

The NMJs on the opener muscle of the crayfish, like the NMJs of larval *D. melanogaster*, were significantly depressed when eliciting evoked EJPs. Additionally, the removal of the Mn^{2+} partially recovered the amplitude of the EJPs. The occurrence of spontaneous quantal events on the crayfish muscle of the opener muscle was not as frequently observed as for the larval *D. melanogaster* preparation. However, in the presence of Mn^{2+} , the quantal responses were still present while evoked responses were depressed. This also indicates that the postsynaptic glutamate receptors on the crayfish muscle were not blocked by Mn^{2+} . This same trend, as depicted in Fig. 16, was observed in 6 out of 6 preparations for the crayfish NMJ (Wilcoxon Signed Rank test $p < 0.05$).

4. Discussion

In this study, we have shown that Mn^{2+} from $MnCl_2$ or $MnSO_4$ can have profound effects in a dose-dependent manner on development,

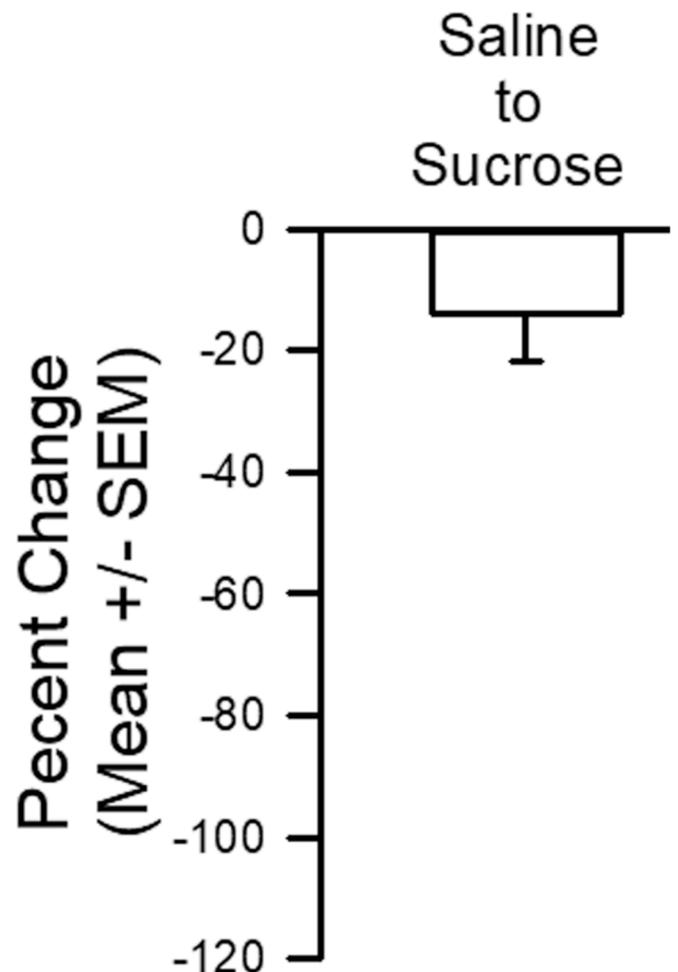


Fig. 14. The mean percent change in the number of spikes before and during exposure to 1 M sucrose with displacement of the Pd joint for 10 s. There was no significant difference in the activity due to exposure of sucrose.

survival of larvae and adults, behaviors, and cardiac function of *D. melanogaster*. In addition, Mn^{2+} depresses synaptic transmission at the *D. melanogaster* and crayfish NMJs with the proposed mechanism being the blockage of evoked presynaptic transmission. The presence of spontaneous quantal events indicates that the sensitivity of the postsynaptic glutamate receptors is not altered while evoked EJPs are blocked. The proprioceptive sensory input in the crab leg is reduced by Mn^{2+} . The most sensitive physiological disturbances by Mn^{2+} are on cardiac function and synaptic transmission which are possibly due to blocking voltage-gated Ca^{2+} channels. The acute 24-hour dietary supplement of Mn^{2+} depressed body wall and mouth hook movements, which may well be due to effects within the CNS as well as at the NMJs producing the movements.

It would be expected that development and survival would be impacted with acute high concentration of exposure, since low concentrations depressed cardiac function and synaptic transmission at NMJs. Lower concentrations over longer periods of time could potentially allow for compensatory mechanisms to rebuff the effects of a more subtle actions. It is of interest to learn the long-term effects of sublethal doses on neural development and other physiological functions in larvae. *D. melanogaster* adults were examined by Bonilla-Ramirez et al. (2011) at 0.5 and 1.0 mM for longer term effects. Adults showed declining climbing abilities over time at these lower doses.

Toxicity studies by feeding $MnCl_2$ or $MnSO_4$ have been used to investigate the effects of exposure to Mn; however, the concentration directed on tissues is more precisely addressed by direct exposure. In this

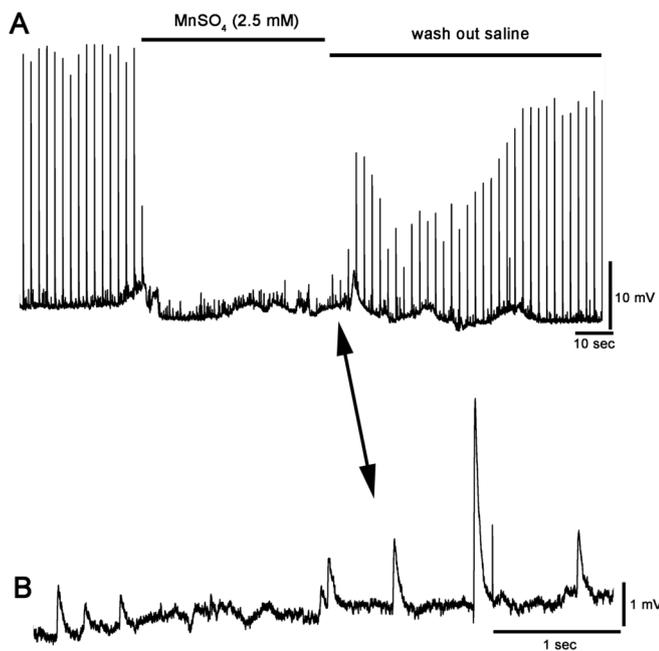


Fig. 15. The effect of exposure to MnSO_4 (2.5 mM) to synaptic transmission at the larval *D. melanogaster* neuromuscular junction. (A) Evoked excitatory junction potentials (EJPs) (0.5 Hz stimulation rate) reduced rapidly upon exposure to MnSO_4 and slowly recovers upon washing away the MnSO_4 . (B) The spontaneous quantal events are still present while evoked EJPs are depressed.

study, larvae were dissected to directly expose the heart to the bathing saline with known concentrations of Mn^{2+} . There were not any large notable differences between delivering Mn^{2+} via MnCl_2 or MnSO_4 . This is expected as both are salts, which would readily dissociate in water. Thus, if there were differences in the effects, it would be due to the Cl^- or SO_4^{2-} anions. One consideration is the osmolarity changes due to adding MnCl_2 or MnSO_4 to saline or to the food. Small changes in osmolarity in the saline used for the *D. melanogaster* heart and NMJs of only 2.5 mM do not cause alterations in function. The BES (a pH buffer) compound as high as 25 mM has been added to the *D. melanogaster* standard saline for long term studies of hours without decreasing function in neural activity or cardiac function (de Castro et al., 2014, 2019). Synaptic transmission at the crayfish NMJ will generally increase with higher osmolarity (Delaney et al., 1991). If the synaptic vesicle pools would be depleted by prolonged 300 mOSM treatment, then there would be synaptic depression, but one would first observe a large burst of synaptic activity. The effect of osmolarity is also consistent for frog NMJs (Kita and Van der Kloot, 1977; Tanabe and Kijima, 1988; Van der Kloot and Molgo, 1994). Thus, the depression in evoked synaptic transmission at the

D. melanogaster and crayfish NMJs is not due to the slight increase osmolarity from the 2.5 mM of MnSO_4 . The crab saline is very high in osmolarity (~1041 osmolarity), which is common for saline used with marine invertebrates. Increasing osmolarity with a 100 mM MnCl_2 or MnSO_4 solution may have some impact on the SACs and would most likely result in the activity declining, since it would raise osmolarity outside the neurons and result in shrinkage. However, we have exposed the Pd organ to 1 M sucrose and 1 M mannitol in normal crab saline without any significant changes to neural activity within the time frame used for these studies. We have noted in another on-going study that if Ca^{2+} and Mg^{2+} is removed from the saline and then sucrose is added to account for the osmolarity change, there is a depression in overall neural activity of the Pd nerve. This depression does not occur if Ca^{2+} and Mg^{2+} is present in the saline along with sucrose as in this study (Parker et al., 2021).

For the developing larvae, consuming MnCl_2 or MnSO_4 in the diet may have additional effects in relation to increasing osmolarity or even the Cl^- or SO_4^{2-} anions. As a control, NaCl at the same concentrations as MnCl_2 or MnSO_4 were used; however, using the same concentration is not equivalent to the osmolarity in solution. The increased Na^+ could also have an impact itself with transport mechanics within the gastrointestinal tract due to co-transporters with Na^+ ions. The effects of NaCl in the diet did not cause any alteration in time to pupation or survival of the larvae from 2.5 mM to 100 mM.

The effects at the glutamatergic NMJs of the larval *D. melanogaster* and crayfish at a low concentration (2.5 mM) illustrate that Mn^{2+} is not specific to dopaminergic synapses. Oddly, it appears Mn^{2+} exposure enhances dopamine release from dopaminergic neurons in rodent models (Lin et al., 2020) where it depressed synaptic transmission at the glutamatergic NMJs. Blocking the Ca^{2+} channels with pharmacological agents prevents the Mn^{2+} -induced transmitter release in the dopaminergic neurons in which Lin et al. (2020) suggests is due to Mn^{2+} fluxing through Ca^{2+} channels. Mn^{2+} has been noted to drastically depress evoked synaptic transmission in frog cholinergic NMJs (70 μM ; Meiri and Rahamimoff, 1972) and block central glutamatergic synapses in the frog at 1 mM (Hackett, 1976).

Since MnSO_4 is used at high levels in agricultural sprays, (2000–4000 mg/kg in blueberries; Korcak, 1988) and as a nutrient in soil, it is of interest to know what levels might run off into streams, lakes, marine environments, as well as bioaccumulated in the food chain (Eriksson, 2000a). If Mn^{2+} can be accumulated in a mother's diet as well as formula milk, the subtle effects on long-term exposure during fetal and infant develop need to be addressed (Howe et al., 2004; Eriksson, 2000b). Taking a comparative approach among different animals and systems has provided an overview of the potential effects of Mn^{2+} in the environment on invertebrates in general.

In summary, this study demonstrated that Mn^{2+} obtained as either MnSO_4 or MnCl_2 has pronounced effects on *D. melanogaster* larval

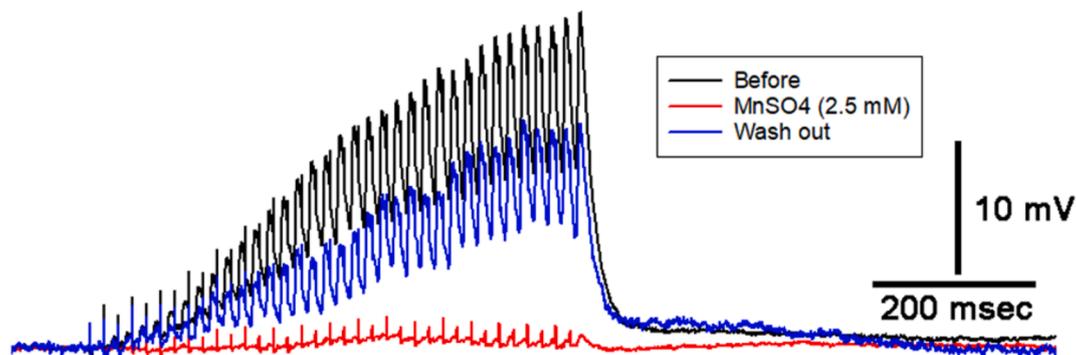


Fig. 16. The effect of exposure to MnSO_4 (2.5 mM) on synaptic transmission at the neuromuscular junction of the opener muscle in the walking leg of crayfish. Evoked excitatory junction potentials (EJPs) (60 Hz stimulation rate for 30 pulses) are reduced rapidly upon exposure to MnSO_4 and partially recover after washing away the MnSO_4 .

survival. Concentrations higher than 10 mM in the diet were toxic for the 1st instar of larval *D. melanogaster*. For adult *D. melanogaster*, a 15 mM concentration in tainted food resulted in low survival rates and 30 mM was toxic. Additionally, the crawling and eating behaviors of larval *D. melanogaster* indicated that they are not healthy after 24 h of consuming 15 mM tainted food. Direct exposure of the larval heart to saline containing 2.5 mM rapidly decreases the heart rate and can even stop the heart from beating. Even after flushing the Mn^{2+} -containing saline away, the heart rate did not recover. Proprioceptive sensory neurons in the marine crab decreased in function with acute exposure at 15 mM while nerves were still able to conduct electrical signals. This indicated that the stretch-activated channels in the sensory ending were likely targeted by Mn^{2+} . Synaptic transmission at NMJ was rapidly blocked in both larval *D. melanogaster* and the freshwater crayfish with saline at 2.5 mM Mn^{2+} while spontaneous quantal responses were still observed. This indicated a presynaptic block of evoked transmission which is likely due to the blocking of voltage gated Ca^{2+} channels.

In future studies, the sensitivity to exogenously applied Mn^{2+} directly on in situ exposed sensory–central nervous system–motor circuit can be compared to the behavior of animals fed Mn^{2+} . It would be of interest to know if fetal and embryonic tissue also accumulate Mn^{2+} in various animal models from insects to humans.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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