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New insights into the acute actions from a high dosage of fluoxetine on neuronal and cardiac function: *Drosophila*, crayfish and rodent models

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ABSTRACT

The commonly used mood altering drug fluoxetine (Prozac) in humans has a low occurrence in reports of harmful effects from overdose; however, individuals with altered metabolism of the drug and accidental overdose have led to critical conditions and even death. We addressed direct actions of high concentrations on synaptic transmission at neuromuscular junctions (NMJs), neural properties, and cardiac function unrelated to fluoxetine's action as a selective 5-HT reuptake inhibitor. There appears to be action in blocking action potentials in crayfish axons, enhanced occurrences of spontaneous synaptic vesicle fusion events in the presynaptic terminals at NMJs of both *Drosophila* and crayfish. In rodent neurons, cytoplasmic Ca^{2+} rises by fluoxetine and is thapsigargin dependent. The *Drosophila* larval heart showed a dose dependent effect in cardiac arrest. Acute paralytic behavior in crayfish occurred at a systemic concentration of 2 mM. A high percentage of death as well as slowed development occurred in *Drosophila* larvae consuming food containing 100 μM fluoxetine. The release of Ca^{2+} from the endoplasmic reticulum in neurons and the cardiac tissue as well as blockage of voltage-gated Na^{+} channels in neurons could explain the effects on the whole animal as well as the isolated tissues. The use of various animal models in demonstrating the potential mechanisms for the toxic effects with high doses of fluoxetine maybe beneficial for acute treatments in humans. Future studies in determining how fluoxetine is internalized in cells and if there are subtle effects of these mentioned mechanisms presented with chronic therapeutic doses are of general interest.

1. Introduction

The serotonergic system is involved in many physiological and behavioral aspects of animals; therefore any dysregulation may lead to a wide range of disorders. Various medications, such as fluoxetine, have been developed that target the serotonergic system to ameliorate psychopathological symptoms (e.g. depression, obsessive–compulsive disorders, appetite, learning, and cognition). Fluoxetine (Prozac) is a commonly used medication for altering mood and the therapeutic action is mediated by blocking the reuptake of serotonin (5-hydroxytryptamine or 5-HT) into the presynaptic nerve terminals. Thus, fluoxetine is classified as a selective 5-HT reuptake inhibitor (SSRI); although, there are other mechanisms which contribute to the therapeutic effects of fluoxetine such as changes in microRNA expression which can result in the down regulation of SERT (Baudry et al., 2010). Moreover, it has been shown that fluoxetine can exert effects not related to the reuptake process (Donard et al., 2014). Usually the administration of fluoxetine does not result in severe side effects which explains its high use in prescriptions by physicians; however, high levels of fluoxetine in the body attained either by accumulation or augmented initial uptake can lead to serious health problems to even mortality. A case study documented that a child with cytochrome-P 450 2D6 genetic deficiency died because of an acute intoxication to fluoxetine (Sallee et al., 2000). The reduced metabolism caused the accumulation of the drug in the body resulting in death of the patient. In 1999 5% of acute intoxications lead to a fatal outcome; moreover, the treatment of ill patients (such as pulmonary disease or atrial arrhythmia) was even more severe resulting in death within ten days after the beginning of the treatment (Harris and Heil, 2013).

Further study is needed to obtain a better understanding of mechanisms associated with the acute effects of fluoxetine; this might change

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the effectiveness of treatments to counteract the resulting pathological conditions of an overdose. There are various reports that fluoxetine can block ion channels in neurons within a range of 10–100 μM (voltage-gated Na^+, Pancrazio et al., 1998; K^+ channels, Tytgat et al., 1997; Cl^- channels, Maertens et al., 1999) and K^+ channels in smooth muscle cells but this was varied depending on concentration (Farrugia, 1996). Deák et al. (2000) provided evidence of inhibition of T-, N- and L-type voltage-gated calcium channels by fluoxetine at a range of 1–10 μM which is within the therapeutic dosages. In examining the potential actions of fluoxetine in altering neuronal circuitry, during development within the larval *Drosophila* CNS, we noted acute actions on neural responses with high doses. In this current study, we pursued this observation to determine why a model neuronal circuit being used was altered rapidly with a high (100 μM) exposure.

Drosophila melanogaster is not only *a* genetically tractable organisms but many of the physiological functions at a cellular level are conserved and have been shown to be similar to those found in mammals (Strausfeld and Hirth, 2013). In addition, the serotonergic system is known to alter the CNS activity in larval *Drosophila* (Dasari and Cooper, 2004) which likely accounts for the altered functions in sleep and circadian cycles (Yuan et al., 2006; Nichols, 2007), mating (Becnel et al., 2011) and eating (Gasque et al., 2013) behaviors as well as forms of memory (Johnson et al., 2011). We did expect to observe some changes in developmental timing with feeding fluoxetine to larvae since 5-HT does have an effect on exposed larvae (Dasari and Cooper, 2004). In order to examine if the cellular mechanisms of action by fluoxetine are common in another invertebrate model, the crayfish animal model was used. The crayfish is commonly used as a model for synaptic transmission and neurophysiology (Katz and Kuffler, 1946; Wiese, 2005; Cooper and Cooper, 2009). The motor unit associated with opening the chelae on the first walking legs is well characterized (Cooper and Cooper, 2009). In this preparation the axon close to the terminals is large enough in diameter to obtain intracellular recordings; this allows one to assess actions of compounds on ion channels that shape the action potential (He et al., 1999; Sparks et al., 2003). The motor axons in *Drosophila* and in mammalian preparations are too narrow for readily obtaining intracellular recordings.

As with neurons, the regulation associated for developmental genes and ionic channels of the cardiac pacemaker as well as cardiac function is similar in some regards from *Drosophila* to humans (Bodmer, 1995; Bodmer and Venkatesh, 1998; Choma et al., 2011; Titlow et al., 2013; Majeed et al., 2014). During this investigation into the mechanisms of fluoxetine altering synaptic transmission it became apparent there might be an action on the release of Ca^{2+} from internal stores within the nerve terminal. So we used the *Drosophila* larval heart as another assay for perturbations in Ca^{2+} dynamics since previously it was demonstrated that this preparation is very sensitive to alterations in internal Ca^{2+} which is regulated by the plasma membrane pump (PMCA), the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Na^+/Ca^{2+} exchanger (NCX) (Desai-Shah et al., 2010).

Since this preliminary study focused on the fundamental mechanisms in the actions of high doses of fluoxetine, as noted with an overdose, we used mammalian glutamatergic neurons in culture for drawing parallels to commonalities in the acute effects noted for the *Drosophila* and crayfish preparations. Imaging of calcium flux within neurons was critical in differentiating extracellular and intracellular calcium dynamics in response to exposure of fluoxetine.

2. Methods

Canton S (CS) *D. melanogaster,* crayfish (*Procambarus clarkii*) and isolated brainstem neurons from mice were used in these experiments. The *Drosophila* were maintained on a 12/12 light dark cycle except when exposed to fluoxetine due to the light sensitivity of the compound. Controls for the experimental conditions were treated in the same manner except the food was not tainted with fluoxetine. *Drosophila* were raised on a mixture of cornmeal-agar-dextrose. The crayfish were obtained from Atchafalaya Biological Supply (Raceland, LA, USA). Crayfish were housed in an aquatic facility and fed dried fish food weekly. The crayfish and *Drosophila* saline is defined in previous publications (Cooper and Cooper, 2009; Desai-Shah et al., 2010). All compounds for the salines were obtained from Sigma-Aldrich as well as fluoxetine-HCl.

3. Behavioral studies—Drosophila

The developmental and survival assays were carried out by selecting late 1st instars and placing them in food containing fluoxetine and allowing them to develop at 21 °C. The vials were checked every 12 h and each individual pupa was marked on the side of the vials. The time to pupation and the time spent as a pupa were indexed for developmental time and survival rate (Li et al., 2002).

4. Behavior—crayfish

Tail flip and behavioral responses were performed as previously described (Kellie et al., 2001; Sparks et al., 2003; Pagé et al., 2007). Concentrated fluoxetine solution was injected into the animal (Listerman et al., 2000). The amount of concentrated fluoxetine to inject was determined by calculating the systemic dilution which would occur after injection. The volume of hemolymph is estimated to constitute 30% of the animal's gross weight (Gleeson and Zubkoff, 1977; Pagé et al., 2007). Saline injections of equal volume per weight of animal were used as sham controls.

5. Synaptic responses

5.1. Crayfish-evoked EPSPs at NMJ

The recording of evoked EPSPs was performed in the distal fibers of the opener muscle from the first or second walking legs. The dissection, stimulation and recording techniques for this preparation are described in Cooper and Cooper (2009), Chung et al. (2012) and Crider and Cooper (2000).

5.2. Drosophila-evoked EPSP at NMJ

Evoked EPSPs were recorded in 3rd instar larval body wall muscle fibers (m6 or m7) in abdominal segment 3. The dissection, stimulation and recording techniques for this preparation are previously described (Desai-Shah and Cooper, 2009; Lee et al., 2009) with the exception of using a modified physiological saline (De Castro et al., 2014). Briefly, a third instar larvae was dissected in a dorsal side-up position. The internal organs were removed and the segmental nerve was severed at the base of ventral nerve cord. A segmental nerve, from the third segment, was pulled into the suction electrode, which was filled with fly saline. Microelectrodes of 40 megaOhm were used for intracellular recordings. Muscle fiber 6 or 7 was impaled by microelectrode and the segmental nerve was stimulated at 40 Hz for 4 pulses in each train.

5.3. Spontaneous quantal events at NMJs of crayfish and Drosophila

In order to observe the spontaneous quantal responses, the same recording techniques were used as for obtaining the evoked responses in these preparations but in the absence of stimulating the motor nerves. The gain in the recording was increased from the recordings made for evoked responses to observe the small amplitude of the single quantal events (Wu and Cooper, 2012, 2013). In order to examine if fluoxetine was enhancing a Ca^{2+} influx through calcium channels, cadmium (1 mM) was used prior to exposure and during exposure to fluoxetine (100 μM). Nerve stimulated EPSPs are completely blocked with $CdCl_2$

at 1 mM in saline at *Drosophila* and crayfish NMJs (Kuromi et al., 2004; Bierbower and Cooper, 2013).

5.4. Action potential recording within crayfish motor neurons

Evoked action potentials were recorded within the excitatory motor neuron that innervates the opener muscle in crayfish with 3 M KCl filled microelectrodes. The nerve was stimulated at 1 Hz while monitoring the amplitude of the action potential prior to and during exposure to fluoxetine (10 μM, 100 μM 500 μM). The technique to record intracellular action potentials in this preparation is described in detail (He et al., 1999).

5.5. Drosophila heart rate

The *Drosophila* larval heart rate was monitored while directly exposing the heart to saline containing 100 nM, 1 μM, 10 μM or 100 μM fluoxetine. The larval dissection and importance of carefully monitoring of the saline pH for heart rate measures are described in video format as well as in text (Cooper et al., 2009; De Castro et al., 2014). Briefly, a third instar larvae was dissected in ventral side up position. The internal organs were removed carefully without damaging the cardiac tube. The dissected larvae was left for 1 min, and the heart beats were counted for 1 min inside saline. Then the saline was exchanged with saline containing various concentrations of fluoxetine. The heart beats were counted for 1 min. A percent change in rate was determined from saline exposure to fluoxetine.

5.6. Neuronal culture of mice neurons

Brainstem neurons were dissociated and cultured, as previously described (Brailoiu et al., 2013). Briefly, the mice were euthanized by cervical dislocation, the brains were quickly removed and immersed in ice-cold Hanks balanced salt solution (Mediatech, Manassas, VA). The cerebral cortex was dissected, minced, and the cells were dissociated by enzymatic digestion with papain, followed by mechanical trituration. After centrifugation at 1000 *g*, fractions enriched in neurons were collected and re-suspended in culture medium containing Neurobasal-A (Invitrogen), which promotes the survival of postnatal neurons, 1% GlutaMax (Invitrogen), 2% penicillin–streptomycin–amphotericin B solution (Invitrogen) and 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cells were plated on round, 25 mm glass coverslips previously coated with poly-L-lysine (Sigma-Aldrich) in 6-well plates. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. After 24 h, the cultured neurons were transferred in complete growth medium containing 10% fetal bovine serum. The mitotic inhibitor cytosine β-D-arabino furanoside (1 μM) (Sigma-Aldrich) was added to the culture to inhibit glial cell proliferation (Schoniger et al., 2001). Cells were used for imaging after 2–4 days in culture.

5.7. Identification of glutamatergic neurons in mice CNS

Glutamate levels were detected using an enzymatic assay (Duarte et al., 1996; Ayoub and Dorst, 1998; Innocenti et al., 2000). In the presence of glutamate, L-glutamic dehydrogenase (GDH) reduces 13-nicotinamide adenine dinucleotide (NAD+) to NADH, a product that fluoresces when excited with UV light. Provided that GDH and NAD+ are added to the saline in which neurons were bathed, any glutamate released in the medium can be detected as an increase in NADH fluorescence. Neurons on coverslips were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc, Melville, NY). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera. During the experiments, the Perfect Focus System was activated. The neurons were pre-incubated with 1 mM NADP+ and 50 U of glutamate dehydrogenase for 5 min. All the experiments were performed at room temperature (20–23 °C). The fluorescence (excitation—340 nm and emission—460 nm) was acquired and analyzed using NIS-Elements AR software (Nikon).

5.8. Calcium imaging of neurons from mice

Measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were performed as previously described (9, 10, 13). Briefly, cells were incubated with 5 μM Fura-2 AM (Invitrogen) in HBSS at room temperature for 45 min, and washed with dye-free HBSS. Coverslips were mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc., Melville, NY), equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). During the experiments the Perfect Focus System was activated. Fura-2 AM fluorescence (emission 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired/analyzed using NIS-Elements AR 3.1 software (Nikon). After calibration, the ratio of the fluorescence signals (340/380 nm) was converted to $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985).

6. Results

6.1. Evoked and spontaneous EPSPs at NMJ response to fluoxetine

Evoked transmission at the *Drosophila* larval NMJ (Fig. 1A1–2) and the crayfish opener muscle (Fig. 1B1–2) was rapidly and completely silenced upon exposure of saline containing fluoxetine (100 μM). The spontaneous EPSP responses could still be observed while evoked EPSPs were blocked. With repetitive exchanging the bath with fresh saline, the evoked responses did not recover in either preparation. The resting membrane potential did not depolarize upon exposure to fluoxetine.

With increased amplification of the recordings the spontaneous events at the NMJs are more readily observed. In normal saline, the spontaneous events usually occur as discrete events and are of relatively low frequency in occurrence. The crayfish muscle fibers are larger and have a lower membrane resistance than the small *Drosophila* larval muscles, resulting in a better signal to noise ratio for the *Drosophila* than crayfish muscle fibers to detect spontaneous events. In both preparations, the occurrence of spontaneous events increases upon exposure to fluoxetine. In order to examine if the increase in spontaneous events occurred due to Ca^{2+} leaking across the plasma membrane from the bath, a saline containing Cd^{2+} (1 mM) was exchanged with the normal saline before exposure to saline containing fluoxetine and Cd^{2+}. The 1 mM $[Cd^{2+}]$ is well established to block evoked transmission in both preparations (Bierbower and Cooper, 2013). The exposure to saline containing Cd^{2+} reduces the frequency of spontaneous events in all preparations ($P < 0.05$, rank sum). In five out of five preparations of the larval *Drosophila* as well as the crayfish NMJ exposure to fluoxetine (100 μM) in the presences of Cd^{2+} substantially increased the frequency of spontaneous events ($P < 0.05$, rank sum).To examine if a lower concentration of fluoxetine showed the same effect, a 10 μM was also used with a 1 mM Cd^{2+} combination followed by 100 μM. Representative traces of spontaneous quantal events recorded in the muscle fibers of *Drosophila* third instar larva for this experimental paradigm are shown in Fig. 2A–D. Some preparations had such an increase in the rate of spontaneous events with fluoxetine at 100 μM the events were not able to be counted. In preparations in which the rate of spontaneous events was practical for counting, the number of occurrences for a 10 s window for 10 μM and 100 μM exposure are shown in Fig. 3A. The 10 μM does not appear to have any effect on the resting membrane potential or the occurrences of spontaneous events. The resting membrane potential of the muscle is more negative for the 100 μM exposure (Fig. 3B). The crayfish opener preparation depicted similar effects with increase in spontaneous events for 100 μM exposure (Fig. 4).

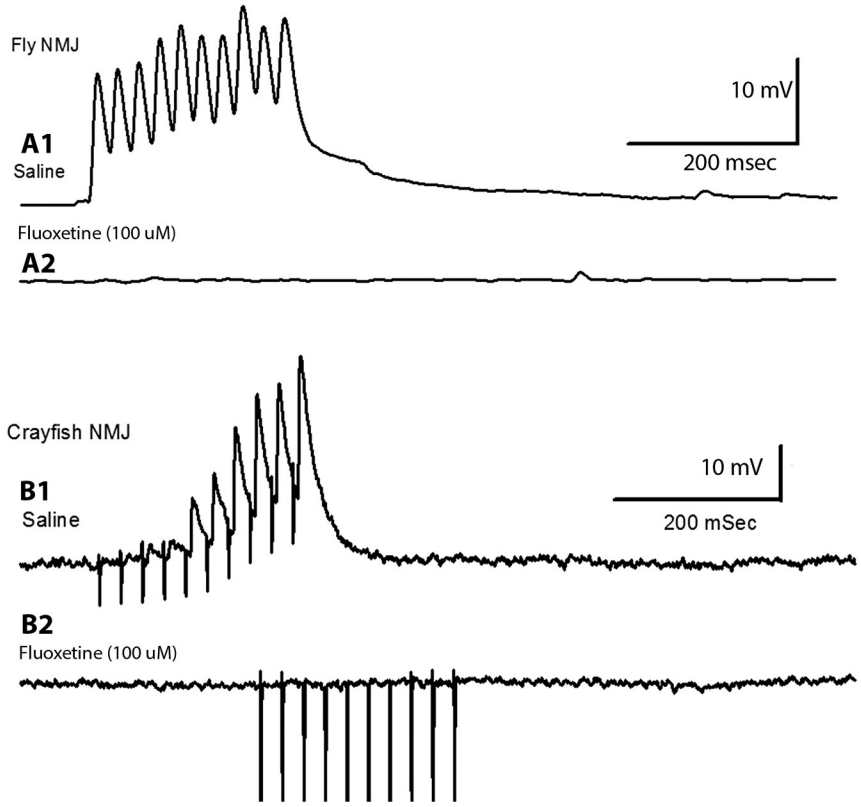

Fig. 1. Fluoxetine blocks neurotransmission at neuromuscular junctions (NMJ). Effect of fluoxetine on evoked excitatory postsynaptic potentials (EPSPs). The *Drosophila* larval NMJ (A1) as well as the crayfish (B1) NMJ had prominent amplitudes of EPSPs with the stimulation trains but were rapidly attenuated upon exposure to saline containing fluoxetine (A2 & B2). In the recordings shown for the *Drosophila* NMJ (A1 & A2) one can also observe the spontaneous events which occur before and after exposure with fluoxetine.

6.2. Fluoxetine dampens axon action potentials

Since evoked EPSP could not be induced after exposure to fluoxetine at 100 μM, or higher concentration if a prior exposure to 10 μM occurred, and the EPSPs did not show a gradual decrease in amplitude but a drastic cessation, we examined if the action potential in motor neurons might decrease below the threshold of the voltage gated presynaptic calcium channels. The axons are too small for intracellular recording in the *Drosophila* larval preparations; however, the crayfish axon to the opener muscle is large enough to obtain intracellular recordings.

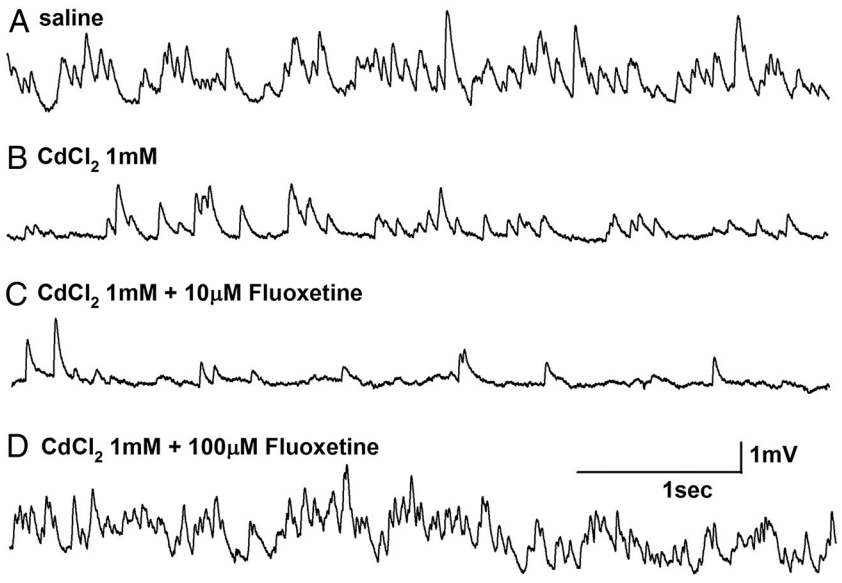

Fig. 2. Spontaneous quantal events and effect of fluoxetine. (A) The spontaneous events in muscle 6 of the larval *Drosophila* preparation exposed to normal saline. (B) Upon exposure to saline containing 1 mM Cd^{2+} the rate of spontaneous event decreased; however, the quantal events are still prominent in amplitude. Evoked transmission is completely blocked at this concentration of Cd^{2+} due to blocking presynaptic voltage-gated Ca^{2+} channels. (C) The low exposure to fluoxetine 10 μM with Cd^{2+} did not show any increase in spontaneous events. (D) Exposure to Cd^{2+} and fluoxetine at 100 μM promotes a high frequency of spontaneous events.

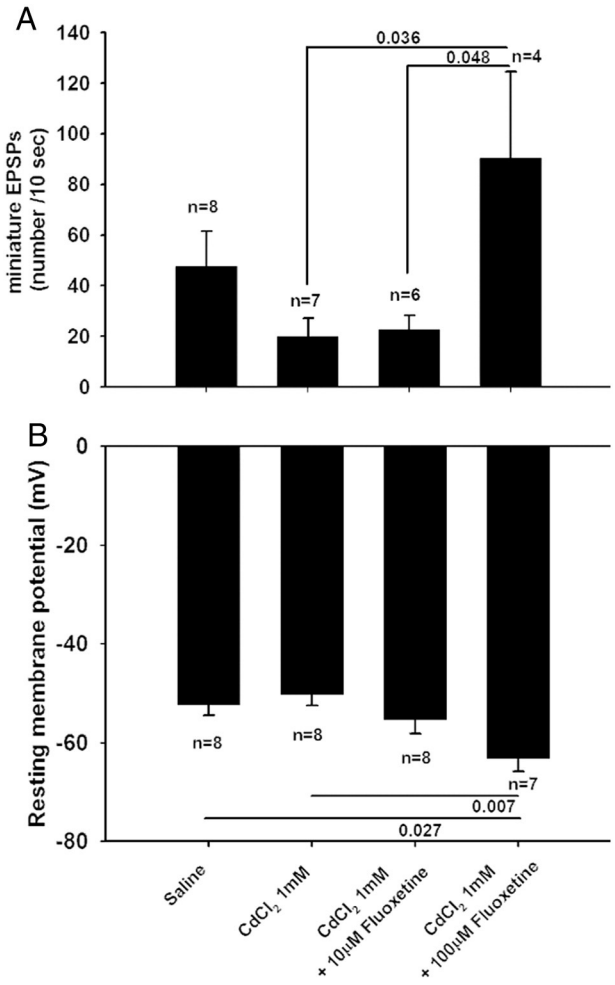

Fig. 3. Spontaneous quantal events and resting membrane potential for the *Drosophila* larval muscle preparation. (A) Four of the preparations in which the rate of spontaneous events for 100 μM fluoxetine was not too high to count, the number of occurrences in a 10 second period is shown in comparison to 10 μM fluoxetine, Cd^{2+} alone and saline exposures. The rate greatly increases for 100 μM exposure. (B) The resting membrane potential of the muscle significantly becomes more negative for the 100 μM fluoxetine exposure.

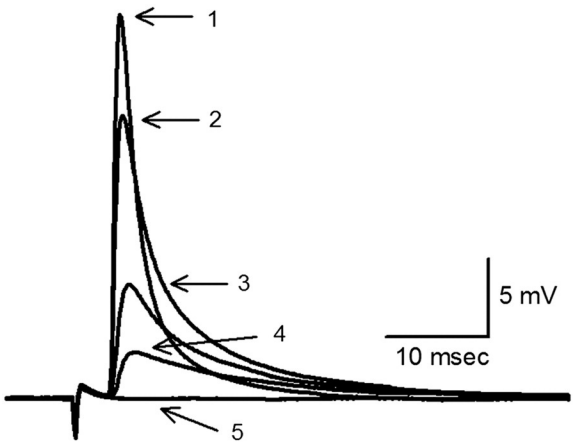

Fig. 5. A. Intracellular recordings in an opener motor neuron of action potentials while exposed to 100 μM fluoxetine over time. The initial saline (1) exposure and sequential (2 to 5) representative traces over the next few minutes of exposure to fluoxetine.

The action potential showed a decrease in amplitude relatively quickly (~1 min) to baseline (Fig. 5; 1 to 5 in series). When exposing the axon to 10 μM prior to 100 μM the effect of 100 μM was not always as prominent as exposure to 100 μM directly (Fig. 6). Increasing fluoxetine 500 μM in such cases did fully block the action potential from occurring.

6.3. Fluoxetine causes behavioral and heart rate alterations

In conducting behavioral assays with larval *Drosophila* being fed fluoxetine, we noticed that the hearts were decreasing in rate. Thus, we conducted a heart assay, exposing it to various concentrations of fluoxetine. At 100 nM no significant changes were noted; however, at 1 μM 2 out of the 5 preparations completed stopped beating. At 10 μM and 100 μM all preparations rapidly stopped beating when exposed to saline containing fluoxetine (Fig. 7; $P < 0.05$, rank sum).

In order to obtain insights into the long term effect of fluoxetine during development of larvae, early 2nd instar were placed in food containing various concentrations of fluoxetine. At concentrations less than 500 μM only slight delays in time to pupation with high concentrations (non-significant) and almost all the larvae survived to pupation. However, at 500 μM there was a very substantial delay in development and a large number of deaths. The larvae showed a slowed development upon exposure to fluoxetine and deaths occurred throughout the time to pupation (Fig. 8). Only 1/5 of the larvae survived to pupation.

As a comparison for the behavioral effects, we injected crayfish with fluoxetine and observed their responses to taps on the telson every 15 min (Fig. 8C). Various ranges in behavioral activity were assessed to index the responses. Controls were injected with saline of the same volume to determine if handling and injecting the crayfish altered responses. Injections, which were estimated to 100 μM in the circulating hemolymph, did not result in any noticeable acute effects. However, when the circulating concentration was at 2 mM the crayfish started to show altered responses to tail flip behavior. Seven out of the nine

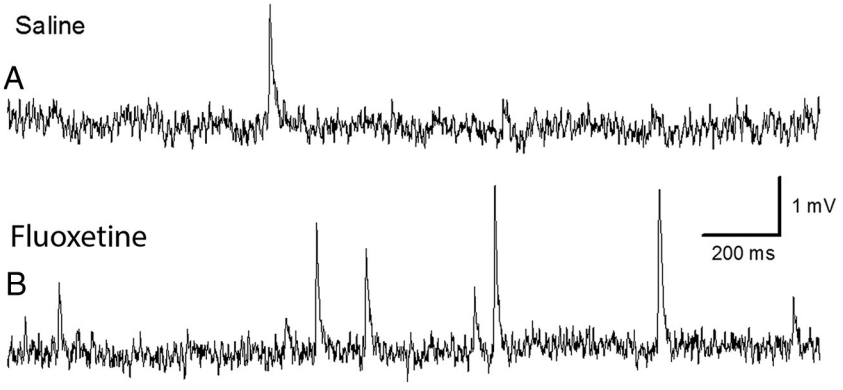

Fig. 4. The crayfish opener neuromuscular junction shows a similar phenomenon for exposure to a 100 μM fluoxetine in increasing the occurrences of spontaneous quantal events. Saline alone has few events normally (A) and a marked increasing when exposed to fluoxetine at 100 μM (B).

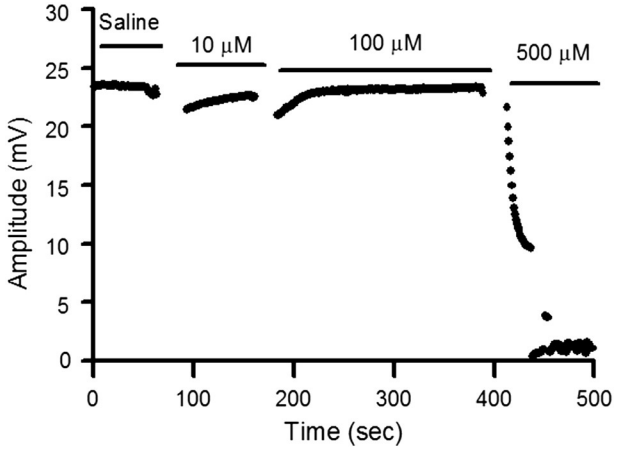

Fig. 6. The action potential amplitudes did not decrease as much when the preparations were previously exposed to 10 μM and then exposed to 100 μM. However higher concentrations 500 μM would result in a complete cessation of the action potential.

crayfish rapidly decreased responses to tail taps. One crayfish quickly became non-responsive and we almost assumed it was dead; however, after some time it regained its ability to move and respond to tail touches. Whereas another crayfish appeared to be recovering fully from the injection and then died. The black line on the graph (Fig. 8) for the controls consists of 5 crayfish which all showed full responsiveness each time tested.

Since the NMJs exposed to Cd^{2+} and fluoxetine still showed a substantial increase in spontaneous EPSPs it was assumed that Ca^{2+} must be released from internal stores of Ca^{2+}. We examined if glutamatergic neurons in the vertebrate brain stem would substantiate the possibility since these neurons loaded well with calcium indicators. The response was rapid in the intracellular calcium response in preparations bathed in a saline containing Ca^{2+} as well as a bathing saline devoid of Ca^{2+} (Fig. 9). Treating the preparations with thapsigargin, which blocks the SERCA uptake of calcium into the ER, demonstrated that fluoxetine's mechanism of action in increasing the internal calcium signal is due to dumping of Ca^{2+} from the internal ER store.

7. Discussion

Fluoxetine is commonly used as an anti-depressant agent. However, the detailed actions of fluoxetine on various cellular functions have not been fully studied. In this study, it was demonstrated that a high concentration of fluoxetine, which could occur with an overdose, delivered as a systemic exposure or in direct application to cardiac tissue or neurons results in side effects associated with ion channel blockage and intracellular release of Ca^{2+}. Even though in the current study, the concentrations of fluoxetine used were greater than the therapeutic range, the observed side effects may occur at varying degrees for individuals with altered metabolism and excretion of fluoxetine. A recent meta-analysis on pregnancy outcomes in patients taking SSRIs within a therapeutic range showed an increased risk of spontaneous abortion and major malformations during pregnancy (Nikfar et al., 2012). In addition, the use of fluoxetine to treat anxiety in patients may open a wider range therapeutic applications (Altieri et al., 2015) and potential for overdose and manifestation of subtle side effects. To treat an over dose and potential subtle side effects it is of interest to know what acute high dosages may have on physiological process.

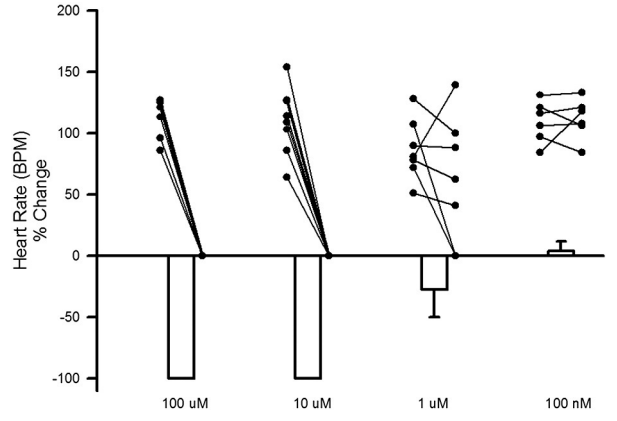

Fig. 7. Effect of fluoxetine on heart rate in dissected larval *Drosophila*. Fluoxetine at 10 μM and 100 μM stops the heart rate within 1 min; however, a low concentration of 100 nM did not show any significant effect. The 1 μM exposure showed a general trend of decreasing heart rate in all except one preparation.

Fluoxetine is water soluble and therefore it is therapeutically administrated as an oral medication and it is known to be readily taken up in the gastrointestinal tract of mammals (Hiemke and Härtter, 2000) as well as able to gain access to intracellular organelles (Caccia et al., 1990; Donard et al., 2014). Medications have effects on other organisms such as invertebrates (Fong and Ford, 2014) and many of the cellular process are conserved (Reaume and Sokolowski, 2011). It has been shown that fluoxetine has affinity to the *Drosophila* serotonin transporter (dSERT) (Corey et al., 1994; Demchyshyn et al., 1994). Also, a previous study has demonstrated that oral administration of fluoxetine is effective in *Drosophila* larvae (Neckameyer et al., 2007). Thus, delivering fluoxetine in the food for *Drosophila* is appropriate for administration in this model system; however, the pharmacokinetics, pharmacodynamics in *Drosophila* has not yet been elucidated. Furthermore, fluoxetine has been used to study the effect of 5-HT on behavior in crustaceans such as crayfish (Huber et al., 1997; Huber and Delago, 1998). Since it is not feasible to feed fluoxetine to crayfish we used injections into the hemolymph directly and used sham saline injections as controls. We do not know the free circulating concentration of fluoxetine from the injection paradigm as the drug could rapidly bind to proteins and tissues once in the hemolymph. The direct application to the dissected crayfish and *Drosophila* neuromuscular junctions, the *Drosophila* heart and the rodent brain stem neurons were very likely exposed to the dissolved concentrations in the saline applied as the tissue is minimal in comparison to the volume of bathing saline. Considering cellular metabolism and potential excretion within the fed or injected animals, the longer term effects might be due to metabolites of the fluoxetine. However, the rapid paralysis or lethargic effects of the crayfish upon injection into the hemolymph as well as the immediate responses in the heart rate, synaptic responses and calcium signaling with bath application would suggest direct actions of fluoxetine itself.

The immediate cessation of heart rate for all 12 larvae at the higher concentrations and for a few larvae at the lower concentrations would suggest a direct action on ion channels on the plasma membrane surface, potentially fluoxetine being internalized to have rapid action on either calcium dynamics or altering coupling of the electrical-mechanical action in cardiac contraction. It was shown that fluoxetine reduces calcium ion current and modifies other electrophysiological properties in mammalian cardiac tissue (Pacher et al., 2000). Therefore, we suggest that fluoxetine might block calcium channels on the plasma membrane of the *Drosophila* heart as well which causes cardiac arrest. Likewise, the rapid effect of stopping evoked synaptic transmission might be due to a reduction of the action potential amplitude. This would also suggest an effect on blocking ion channels. Given that the larval *Drosophila* heart has not been shown to express a voltage-gated sodium channel one might expect an action on another channels related to the pacemaker potential or one for contraction such as a voltage dependent calcium channel as a site of action. The effect of blocking voltage-gated calcium channels on the crayfish axon would not explain the rapid decrease in action potential amplitude; however, a blocking of the voltage-gated sodium channel would (Sparks et al., 2003). Considering that the resting membrane potential did not demonstrate rapid depolarization upon

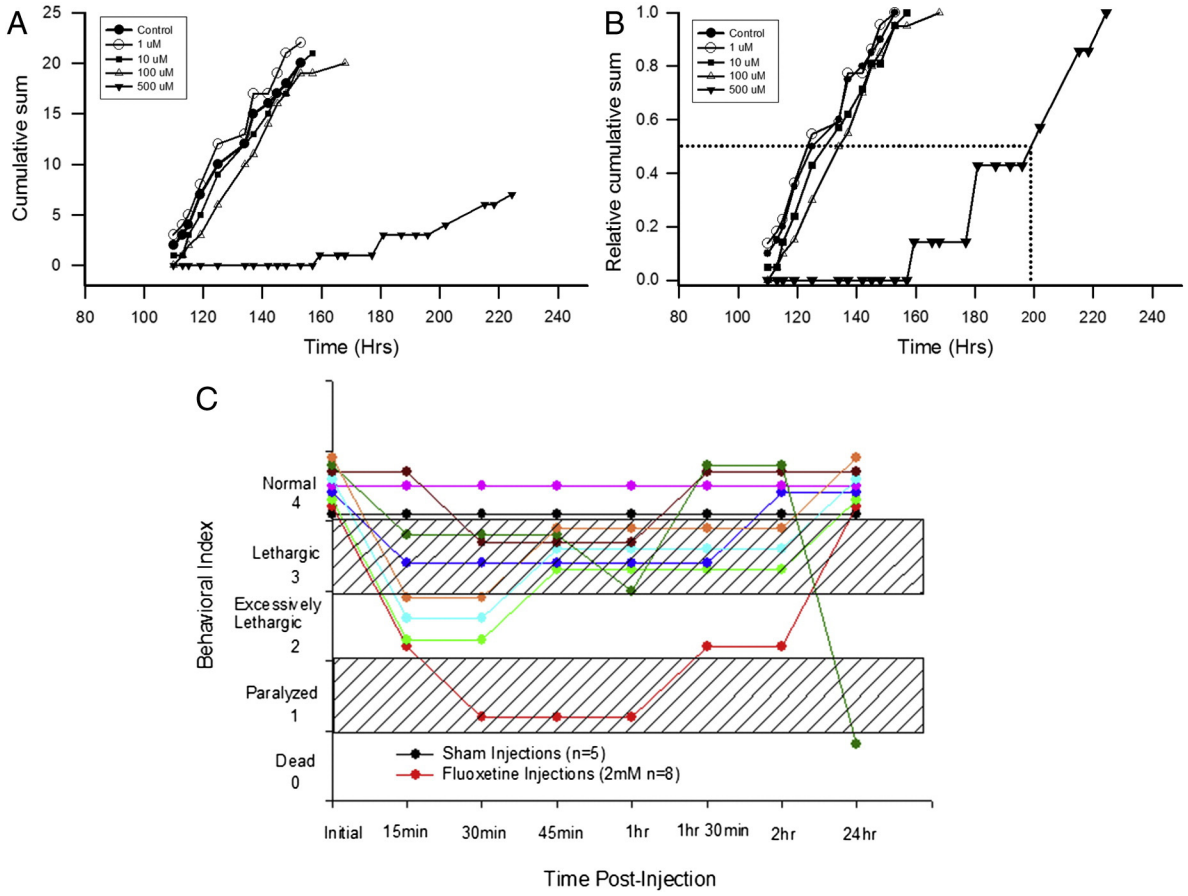

Fig. 8. Effect of fluoxetine on development in larval *Drosophila* and behavior in crayfish. (A1) Fluoxetine at various concentrations was fed to *Drosophila* larvae from first to third instar stage. The time to pupation from eggs were calculated for each larva. Cumulative sum for time to pupation from eggs at different concentrations was converted to relative cumulative sum for easier comparison with groups containing different numbers of samples (A2). Larvae fed the highest concentration took the longest time to pupation. Note 25 larvae were used to start and that many had died at the highest concentration of fluoxetine (A1). (B) Crayfish injected with fluoxetine to a circulating concentration estimated to be 2 mM generally decreased their movements and became less responsive to taps on their telson as indexed.

exposure to fluoxetine while other effects were prevalent would be indicative of the plasma membrane being stable in the presence of fluoxetine. There was no alteration in pH of the saline by fluoxetine as compared to normal saline. This was directly examined with sensitive pH paper (±0.2 units; Fluka Analytical pH strips 6.0–8.1). If the fluoxetine-HCl reduced the pH from 7.4 to 7.2 in the crayfish saline or decreased the pH from 7.2 to 7.0 in *Drosophila* saline this would still not explain the results observed. The *Drosophila* heart rate increases with a decrease in pH and will continue to beat for prolonged periods of time even at pH of 5.0 (Badre et al., 2005). Also, a low pH of crayfish saline of 5.0 results in a slight depolarization of the muscle as well as in the axon but both action potentials and EPSP are still able to be

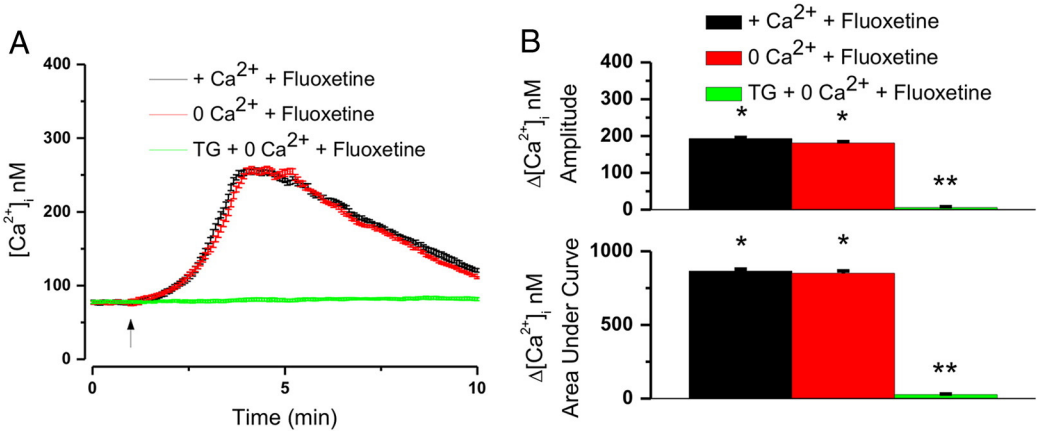

Fig. 9. The effect of fluoxetine on intracellular Ca^{2+}. (A) Exposure of fluoxetine to the rodent brain stem neurons in culture revealed an increase in internal Ca^{2+} only when the endoplasmic reticulum (ER) is able to unload the stored Ca^{2+}. With zero Ca^{2+} in the bath and exposure to fluoxetine a Ca^{2+} signal is present. When the ER is blocked from taken up Ca^{2+} by application with thapsigargin (TG) then the Ca^{2+} response to fluoxetine exposure is not present. (B) The representative responses of the Ca^{2+} signal over time in the same conditions shown in (A). There is a significant decrease in the response when TG is applied and a significant increase of intracellular Ca^{2+} during the fluoxetine exposure which indicates Ca^{2+} released from the ER by fluoxetine.

measured (Bierbower and Cooper, 2010) which is not the case for fluoxetine exposure.

The increase in spontaneous quantal events in the presence of cadmium, which blocks voltage gated calcium channels, is convincing evidence that the increase in spontaneous fusion is not due to an influx of Ca^{2+} through presynaptic calcium channels. The rapid rise in the frequency of spontaneous events upon exposure to fluoxetine is striking. The degree in increased number of occurrences for the given concentration of fluoxetine was always more dramatic for the *Drosophila* larval NMJs than for the crayfish opener NMJ. This may be due to the fact the motor nerve terminals are not as embedded in the subsynaptic reticulum as for the crayfish preparation (Atwood and Cooper, 1995, 1996a, 1996b). The pronounced effect lasted for a 2 to 5 min before there appeared to be run down in the high frequency of events. We did not quantify the depression in the rate of spontaneous events as it was not a focus for this study. The decrease in the resting membrane potential with fluoxetine at 100 μM for the skeletal muscle may be indicative of blocking cationic leak channels which would then allow the membrane potential to remain closer to the lower E_K potential.

Exchanging the bathing solution several times, very vigorously, did not wash out the effect of fluoxetine at the NMJs for either the *Drosophila* larval or crayfish preparations. Also, the action potentials did not recover in the crayfish motor axons where the intracellular recordings would have detected a slight recovery even if it were below the threshold of activating the voltage-gated calcium channels in the presynaptic terminal. Whatever the mechanism of action might be on the ionic channels it must be a strong interaction. Possible in vivo experiments with fluoxetine might have a strong association with circulating substances (proteins and lipids) to decrease the long term interaction with the ionic channels. Furthermore, fluoxetine might metabolize very quickly which could reduce its effect on ion channels. This may explain where in the one case in which the injected crayfish was paralyzed and then recovered.

The ability of fluoxetine to dump Ca^{2+} from the ER was substantiated in the imaging studies with and without thapsigargin treatment. In addition, the effect was rapid upon exposure to fluoxetine indicating that penetration and/or uptake across the membrane is rapid. Since it was shown that fluoxetine has an effect in disrupting mitochondrial function (Caccia et al., 1990; Donard et al., 2014) there is a possibility to also disrupt proteins in other cytoplasmic organelles. The mechanism remains to be determined how fluoxetine causes the ER to dump calcium (Desai-Shah and Cooper, 2009), but it might be selective to interacting with ryanodine-like proteins and not passively causing the ER membrane to become leaky as the resting membrane of the skeletal muscle and the axons did not show depolarization upon exposure to fluoxetine. It has been shown in human oral cancer (OC2) cells that fluoxetine increases the intracellular calcium concentration through PLC-PKC pathway. The calcium-free medium reduces the fluoxetine action on $[Ca^{2+}]_i$; however, it was shown that application of SERCA blockers in calcium-free medium blocks fluoxetine action on $[Ca^{2+}]_i$. In this study, the removal of extracellular calcium did not effect on fluoxetine-induced rise in $[Ca^{2+}]_i$. The inhibition of

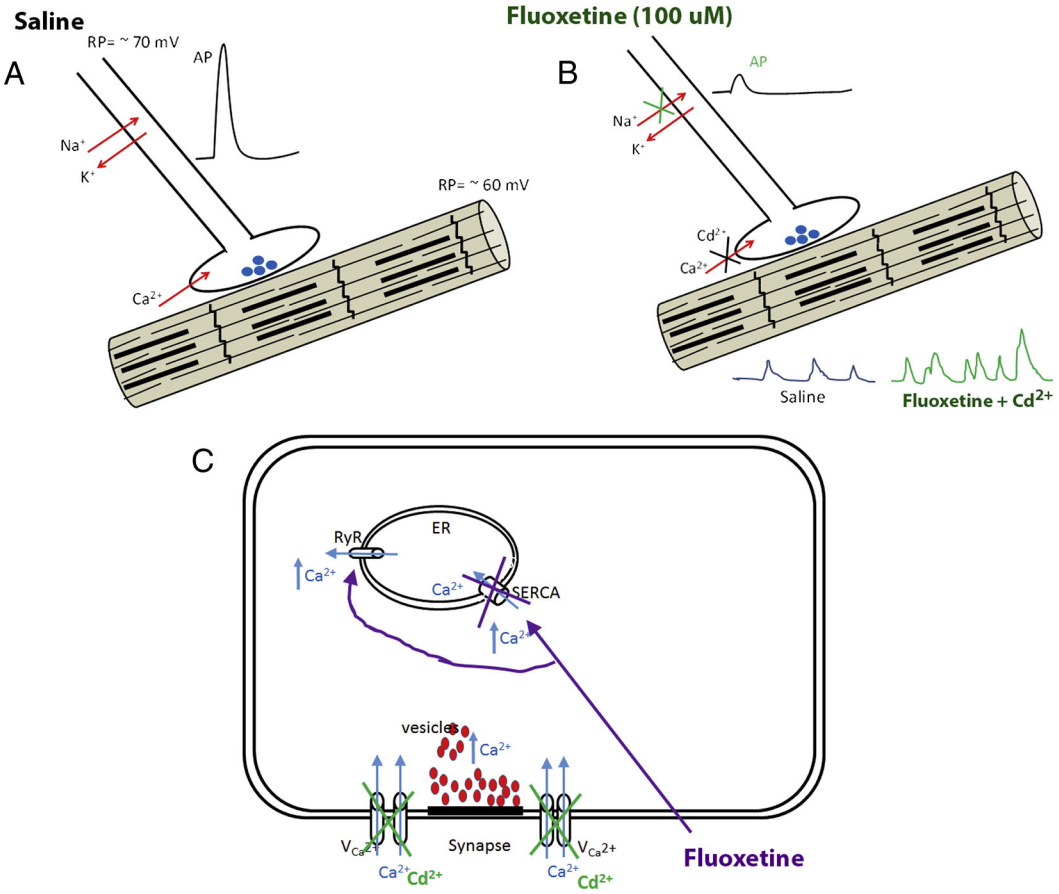

Fig. 10. A model representing the potential mechanisms of action by fluoxetine and experimental paradigms used in this study. A neuromuscular junction (NMJ) before (A) and after (B) exposure to fluoxetine. Fluoxetine reduces the amplitude of the action potential in the axon. Thus, there is a rapid block of evoked transmission. In the presence of Cd^{2+}, to block the presynaptic voltage-gated Ca^{2+} channels, there is a high rate of spontaneous quantal events with exposure to fluoxetine. (C) The ability of fluoxetine to raise intracellular Ca^{2+} likely occurs through ryanodine receptors (RyR) on the ER. If the SERCA is blocked with thapsigargin the ER will be unloaded of Ca^{2+} then no rise in intracellular Ca^{2+} occurs with treatment by fluoxetine. In modeling the NMJ in this regard, potentially fluoxetine maybe even blocks the SERCA as the occurrences of spontaneous events (vesicle fusion events) are prolonged even in the presence of Cd^{2+} blocking the voltage-gated presynaptic Ca^{2+} channels (V_{Ca}^{2+}). The rapid cessation of the larval *Drosophila* heart may also be explained by the dysregulation of the intracellular Ca^{2+} dynamics since it known to be very sensitive to intracellular Ca^{2+} regulation.

SERCA by thapsigargin blocked fluoxetine action on $[Ca^{2+}]_i$. In our preparations, PLC–PKC pathway might be activated to enhance ER Ca^{2+} release. A model representing these mechanisms is depicted in Fig. 10.

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