

Chronic caffeine exposure decreases excitatory post-synaptic potentials in *Drosophila melanogaster* larvae

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ABSTRACT

Adenosine is a prevalent neurotransmitter that not only modulates brain activity, but also can have regulation effects throughout the body, such as modulation of locomotion within the mammalian spinal cord via different adenosine receptors, A1, A2A, A2B, A3. However, this modulation mechanism can be modified by adenosine receptor antagonists. The most consumed adenosine antagonist is caffeine. Caffeine is readily available to adults and children in drinks, such as coffee, tea, energy drinks, and soda. At the molecular level, caffeine binds to the adenosine receptors AdoR A1 and AdoA2A and prevents the activation of the subsequent pathways leading to a decrease in phosphorylation of target genes and proteins. Given a study conducted from 1999-2010 found that 73% of children in the United States drink caffeinated beverages, the implications of adenosine receptor antagonists on the development of neuron circuits is unclear. One study found caffeine has different stimulatory effects in adolescent rats versus adult rats. This contrast in locomotor responses points towards different circuitry and could have developmental impacts in adolescence. Therefore, this project seeks to understand the implication behind the effects of caffeine at the adenosine receptors by recording the changes in muscle fiber locomotor synapses in *Drosophila melanogaster* larvae. The *Drosophila* only has one adenosine receptor (DmAdoR) and it is homolog to mammalian A2A adenosine receptor. Thus, the *Drosophila* model system provides an excellent model to provide a fundamental understanding about the system. Similar to the previous studies in mice and rats, caffeine was also found to increase locomotor burst frequency as well as the amplitude of the burst in *Drosophila* adult flies. Given these findings, I expect the synapses in the larvae to excite at a high frequency under chronic exposure to high concentration of caffeine than those larvae not exposed to caffeine.

INTRODUCTION

Adenosine is a homeostatic neurotransmitter that not only modulates brain activity, but also can have regulation effects throughout the body, such as decreasing locomotion within the mammalian spinal cord via different adenosine receptors, A1, A2A, A2B, A3 (Witts, 2012). Adenosine impacts the presynapses and postsynapses of the CNS causing inhibitory or excitatory effects based on the type of AdoR that is activated. On the dendritic region, A2A activates Adenylate Cyclase (AC). A1 inhibits AC and the NMDA receptors while activating potassium channels. On the presynaptic terminal, A1 receptor inhibits calcium channels while A2A antagonize the A1 receptor inhibitory actions (Benarroch, 2008).

This modulation can be changed by adenosine receptor antagonists. The most consumed adenosine antagonist is caffeine. Adenosine receptors, A1 and A2A, are mainly responsible for the caffeine-induced locomotor effects. At the molecular level, caffeine binds to the adenosine receptors AdoR A1 and AdoA2A and prevents the activation of the subsequent pathways leading to a decrease in phosphorylation of target genes and proteins (Vaugeois, 2002). This can have effects in the developing nervous systems.

Caffeine is readily available to children in drinks, such as energy drinks and soda. A study conducted from 1999-2010 found that 73% of children in the United States drink caffeinated beverages (Branum et.al., 2013). In another similar study, ages 2-13 consume an average of 28.7 mg/day. For ages 14-21, males consumed an average 72.9 mg/ml while females consumed an average 62.0 mg/day (Somogyi, 2010). These statistics show caffeine is in significant consumption in children and adolescents. However, the implications of adenosine receptor antagonists, such as caffeine, on the development of neural circuits in these age groups is unclear. One study found caffeine has different stimulatory effects in adolescent rats versus adult rats (Martin et.al.,2010). This contrast in locomotor responses points towards different circuitry in children and adolescents, and towards developmental impacts..

Another study looked at the effects of maternal caffeine intake on perinatal development. Caffeine pre-treated (via maternal ingestion) female and male mice showed increased spontaneous locomotor activity in comparison to

the controls. This study suggests caffeine levels similar to human consumption of 3-4 cups of coffee influences the developing circuitry in offspring (Bjorklund et.al., 2008). Furthermore, another study analyzed the effects of caffeine on proteins involved in synapse development in embryonic mice. For instance, brain-derived neurotrophic factor (BDNF) is one of the main proteins involved in regulation of neuron synapses and enacts its effects through the TrkB receptor. At high levels of caffeine in embryonic day 18 hippocampus, TrkB expression decreased while BDNF decreased in the cortex. However, at embryonic day 20 BDNF increased in the cortex with no significant difference in the hippocampus (Mioranza et.al., 2014). With this study suggesting the neuron proteins are effected by caffeine, it is possible this could have altering effects on the structure of the brain and further analysis is needed to understand how these changes could potentially alter behavior in children.

Therefore, this project sought to understand the consequences of caffeine binding at the adenosine receptors by recording the changes in muscle fiber locomotor synapses in *Drosophila melanogaster* larvae. The *Drosophila* only has one adenosine receptor (DmAdoR) and homologous to mammalian A2A adenosine receptor. Thus, the *Drosophila* model system provides an excellent model to provide a fundamental understanding about the adenosine system modulating locomotion. Similar to the previous studies in mice and rats, caffeine was also found to increase locomotor burst frequency as well as the amplitude of the burst in *Drosophila* adult flies (Lin et. al., 2010). Given these findings, the synapses in the larvae that were chronically exposed to high concentration of caffeine were expected to exhibit a higher-than-usual frequency of excitatory post-synaptic potentials (EPSPs).

METHODS

Drugs

Caffeine was dissolved in ddH₂O for a final concentration of 2.5 mg/ml. 7ml of caffeine solution (7ml of ddH₂O for negative non-caffeinated control) was applied to 2.0g of potato flake media.

Drosophila melanogaster

Drosophila melanogaster wild-type flies were ordered from Carolina Biological Company. Flies for the experiment were collected as virgin flies in order for the caffeine to be present at the beginning of progeny development. Two batches of fly populations were made sequentially. Batch 1 Dm larvae were measured for behavioral locomotion and intercellular recordings. Then the same experiment was repeated with Batch 2 Dm larvae.

Behavioral Locomotion and Drosophila Larvae Dissections

The wandering third instar *Drosophila* larvae were removed from the vials, rinsed in *Drosophila* saline, and placed on 0.7% agarose gel. After 5 minutes of conditioning to new surroundings, the time of larval crawling locomotion was recorded for 2 centimeters squares. Crawling was recorded for a series of 4 times and then averaged.

Caffeine exposure

Since the caffeine was present in the media, the larvae were presumed to be constantly exposed to caffeine until they reached wandering third instar larvae stage. The larvae (n=8) were placed on a Sylgard petri dish and anesthetized with cold *Drosophila* saline. The dissection procedure revealed muscle fibers and the brain was left intact, since pattern generator neurons could be located within the larval brain (Cattaert and Birman, 2001). Recordings were made with a glass microelectrode, which was inserted between muscle fibers 6 and 13 in order to target the neuromuscular junction synapses. Both caffeinated and non-caffeinated larvae were initially recorded in *Drosophila* saline. After consistent recordings were made in the *Drosophila* saline, then 2.5ml/mg caffeinated saline solution was added to see if the added caffeine altered the excitatory state of the muscle fibers.

Intracellular Electrophysiology

Glass microelectrodes were made from 1.2mm diameter borosilicate capillary tubes with Sutter Instrument Company (Model P-87). Using a Powerlab/4sp amplifier and intracellular electrometer (IE-251A), passive intracellular recordings were made by inserting the tip of the microelectrode in-between muscles 6 and 13 where neuromuscular junctions are located. The LabChart software program from ADInstruments was used to record and analyze the data for excitatory post-synaptic potentials.

Statistical Analysis

Two batches of fly populations were made sequentially. Batch 1 Dm larvae were measured for behavioral locomotion and intercellular recordings. Then the same experiment was repeated with Batch 2 Dm flies. Data from both batches 1 and 2 of each growth condition were averaged together. Single ANOVA analysis was used to determine statistical significance with a threshold value of $p < 0.05$.

RESULTS

Behavioral Locomotion

After chronic 2.5mg/ml caffeine exposure, the caffeinated Dm larvae showed an average of 0.386mm/sec. For the non-caffeinated larvae, the average locomotion was 0.473 mm/sec. Statistically, the difference between caffeinated and non-caffeinated was insignificant ($p > 0.05$).

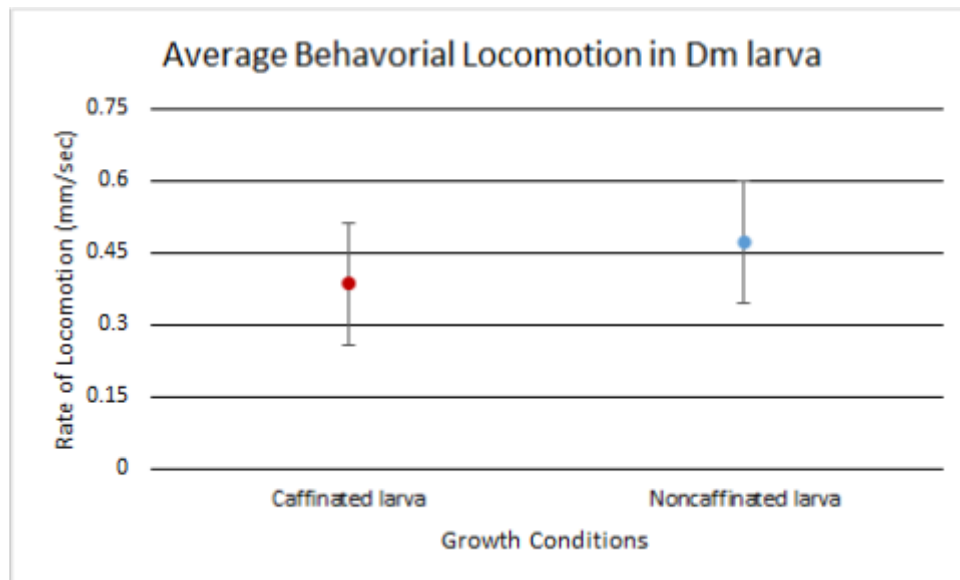


Figure 1. Average Behavioral locomotion rate in Dm larvae. Dm larvae were placed on agarose plates and crawling was timed for 2 cm for each type of growth condition. Caffeinated Dm larvae average locomotion was 0.386 mm/sec. Noncaffeinated Dm larvae average locomotion was 0.473.

Excitatory Post-Synaptic Potential in Drosophila larvae muscle fibers

In order to measure the potential changes caffeine had at the cellular level of the neurons, passive intracellular electrophysiology was used to record the number of EPSPs that disturb the muscle fiber. The non-caffeinated (negative control) larvae recordings in Dm saline had a higher EPSP frequency average in comparison to the caffeinated larvae recordings in Dm saline solution (See Figure 2). However, a single ANOVA analysis shows no significance between the two conditions ($p > 0.05$).

To see if the Dm saline solution caused any anomalies for the caffeinated larvae CNS, the non-caffeinated and caffeinated larvae preps were recorded in Dm saline + 2.5mg/ml caffeine. The non-caffeinated larvae showed slightly higher EPSP frequency average in comparison to caffeinated larvae. However, a single ANOVA analysis shows no significance between the two conditions ($p > 0.05$).

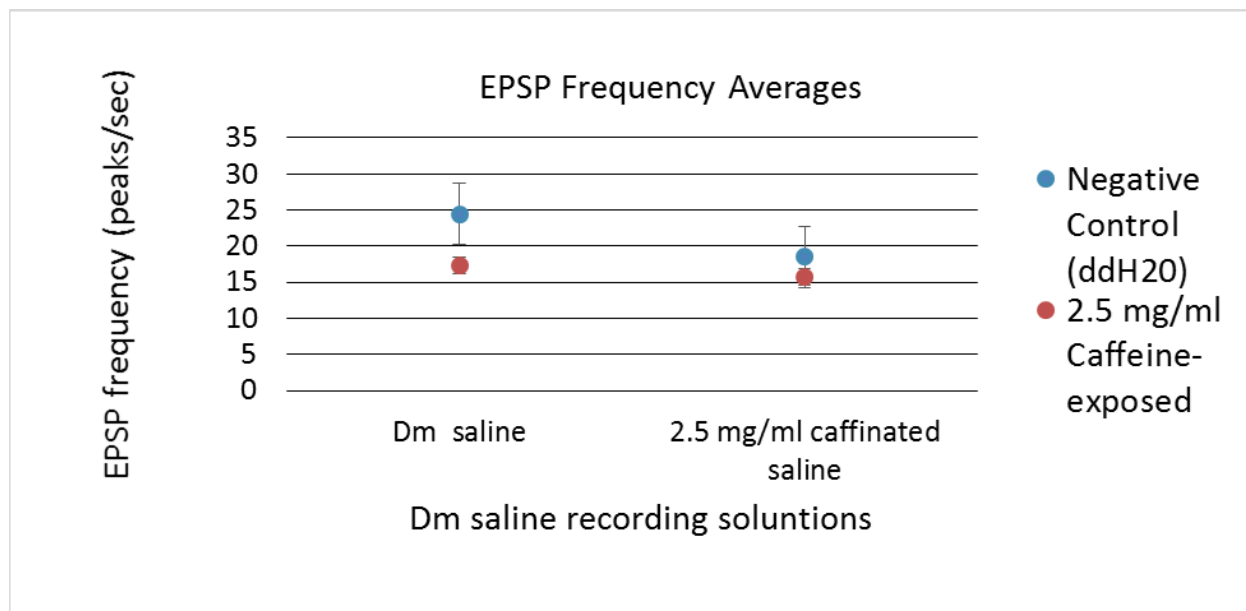


Figure 2. EPSP Frequency averages in caffeinated and negative control Dm larvae. This graph shows the average number of EPSPs for the different growth conditions (caffeine vs. non-caffeine) and different recording solutions (Dm saline vs. Dm saline + caffeine). In Saline recording solutions, the $p > 0.05$. In Caffeine recording solutions, the $p > 0.05$. Error bars represent the standard deviation within the negative control and caffeine-exposed groups.

EPSP Patterns (Observations)

In order to understand the patterns (certain neurons fire at alternate times) generated in *Drosophila* locomotion, EPSP burst patterns were analyzed in the recordings. One pattern noted frequently during passive intracellular recordings was a pattern with low-to-high burst of EPSP Frequencies with no-to-little EPSPs in between the low-to-high bursts. While analyzing these observations, a trend emerge within the some of the data recordings between caffeinated and non-caffeinated as shown in Table 1. In caffeinated larvae, the low-to-high burst frequencies were found in 5/11 preps in Dm saline + 2.5mg/ml caffeine and absent in Dm saline only. In non-caffeinated larvae, the low-to-high burst pattern was found in Dm saline only while absent in Dm saline + 2.5mg/ml caffeine solution.

Table 1. Number of observed EPSP Burst Patterns in Dm muscle fibers

Growth Conditions	Recording Solution	
	Saline (N=16)	Caffeine + saline (N=16)
2.5mg/ml Caffeine	2:10	5:10
ddH2O	5:11	1:10

Adult Dm (Observations)

There some notable observations in regards to the adult Dm flies. In both batches of flies, the male adult fly mortality rate reached 50% within 5 days, and there was no observed female mortality. Also, the caffeinated larvae required an additional 3 to 4 days to develop into pupae, as compared with the non-caffeinated larvae.

DISCUSSION

The purpose of this project was to see how caffeine would affect the developmental process in Dm larvae and how would this translated into effecting the neurophysiology of locomotion. Before electrophysiology recordings, the Dm larvae were measured for behavioral locomotion to see if caffeine had effect on the overall behavioral locomotor coordination of the larvae. The caffeinated larvae tended to have a slower rate of locomotion in

comparison to the non-caffeinated larvae. In addition to this behavioral response to the 2.5mg/ml of caffeine, the EPSP frequency also tended to be low in comparison to non-caffeinated larvae.

Even though the difference between caffeinated and non-caffeinated larvae is not statically significant, the trend in this data is similar to what is found in published literature. Caffeine has been shown to have biphasic effects on locomotion possibly due to the different caffeine concentrations. Low concentrations of caffeine have been shown to have stimulatory effects where as high concentrations of caffeine have inhibitory effects on locomotion (Marin et.al., 2011). The caffeine concentration of 2.5mg/ml was assumed to be of a high concentration with respect to Dm organisms. In previous preliminary tests (data not shown here), 5 mg/ml had lethal effects on male Dm flies a day or two after drug administration and no progeny resulted. Thus, assuming 2.5 mg/ml of caffeine is a high amount for Dm flies, then it is possible that the high concentration would result in attenuating Dm locomotion as opposed to stimulating locomotion.

The EPSP frequency pattern in the passive intracellular recordings also had a trend that differed in respect to caffeine larvae exposure and caffeinated Dm saline recording solution. Given this trend, it is possible that the caffeine had some influence on modifying the CNS locomotor neurons innervating the muscle fibers. Dm flies and larvae only have one adenosine receptor and it was assumed for these experiments that caffeine only inhibited this adenosine receptor (Kucerova et.al., 2012). With the application of caffeine in the Dm recording saline solution, the response from caffeinated and non-caffeinated larvae was inversely related to each other. This converse reaction could point to altered neurophysiological mechanisms due to chronic exposure to caffeine and further analysis with different pharmacology and molecular agents are needed to investigate this relationship.

In regards to the adult Dm fly observations, the attenuated developmental rate of Dm larvae could be due to the caffeine acting as an inhibitory factor in mating. The inhibitory sex pheromone and bitter-tasting mechanisms stimulate the same gustatory neuron system that inhibit male courtship behavior. Thus caffeine intake significantly decreases male courting behavior, which could be a factor in the later development of caffeinated Dm larvae in comparison to non-caffeinated larvae (Lacallie et.al., 2007). Furthermore, male mortality rate could be due to less consumption of media. It has been noted for caffeine to reduce proboscis extension reflex in both male and female flies and could lead to reduced appetite in male flies (Lacallie et.al., 2007).

Overall, the Dm larvae behavior and EPSP frequency suggests high caffeine decreases the locomotion response at the neuromuscular junction leading to slower behavioral locomotion in the larvae. Given the significant level of consumption among children and adolescents, this data could aid in understanding the impacts caffeine could have on the developing nervous system and support the action of decreasing the amount of caffeine accessible to this age group in caffeinated beverages, such as energy drinks. In addition, this data also could support attenuating caffeine consumption during pregnancy since caffeine can cross the placenta membrane and fetal brain tissue (Arnaud, 1987).

LIMITATIONS

Some limitations of this experiment were the chronic caffeine exposure, confirmation of caffeine within Dm larvae, and binding of caffeine to adenosine receptors. During this experiment, it was assumed the 2.5mg/ml of caffeine was present within the media for the one and half weeks during Dm larvae development. Future experiments would need to use a gas chromatography column to determine the amount of caffeine present over time.

In addition to not knowing the specific caffeine concentration in the media over time, the precise amount of caffeine within the Dm larvae is also undetermined. Confirmation would help support the hypothesis of caffeine affecting the CNS development and behavioral locomotion. Furthermore, confirmation of caffeine binding to the adenosine receptors would provide quality control for the experiment and ensure caffeine was blocking the Ado receptors within the larvae.

To provide a more comprehensive approach, morphological studies on the neuromuscular junction (NMJ) would also be documented in order to see if caffeine altered the structure of the NMJ with the muscle fiber.

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