2012

The Role of Protein Kinase C in the Supraesophageal Ganglion of Female Acheta Domesticus

Sunny Kim
Andrews University, kisunny@andrews.edu

John Nevins Andrews Scholars
Andrews University Honors Program

Honors Thesis

The role of protein kinase C in the supraesophageal ganglion of female *Acheta domesticus*

Sunny Kim

2 April, 2012

Advisor: Dr. Gordon Atkins

Primary Advisor Signature:_____

Department: _____

ABSTRACT

The phonotactic response of female *Acheta domesticus* to the male's calling song is modulated by hormones. The presence of Juvenile Hormone III in the supraseophageal ganglion causes crickets to be more selective. This project evaluates the possibility of a second messenger system involving protein kinase C as the molecular mechanism of Juvenile Hormone III control. A solution of Juvenile Hormone III and a protein kinase C blocker was nanoinjected into the supraesophageal ganglion of unselective female crickets, causing them to remain unselective. This suggests that Juvenile Hormone III utilizes a protein kinase C-mediated second messenger system.

INTRODUCTION

Crickets are used as a simple model to study how small networks of neurons control biologically relevant behavior through hormonal control. This model is particularly useful for evaluating the plasticity of neural networks, such as the auditory system, through hormonal control (Stout *et al.* 2002). Crickets have a convenient behavior called phonotaxis that can be easily tested and modulated. In order to mate, female crickets respond to the calling songs of male crickets by exhibiting phonotaxis, the movement towards or away from the sound stimulus. Positive phonotaxis is movement of the female cricket towards the direction of the male calling song, and negative phonotaxis is the movement away from the calling song (Gerhardt & Huber, 2002). Positive phonotaxis allows females to locate and approach the male for reproduction (Stout *et al.* 2010). The phonotactic behavior of female crickets ranges from being unselective to selective. Unselective crickets respond to a wide range of calling songs; in the lab, they respond to 5-7 syllable period calling songs (out of 7) between 30-90 ms syllable periods in 10 ms increments. Selective females respond to 1-3 syllable period calling songs, often in the range of 50-60 ms (Stout *et al.* 2002*;* Stout *et al.* 2010).

There has been a large focus on the prothoracic ganglion of female *Acheta domesticus* to describe the neural control and modulation of cricket phonotaxis and selectivity. In 1992, Atkins *et al.* discovered that the inactivation of the L1 and L3 neurons in the prothracic ganglion caused angular errors in the phonotactic response. This demonstrated that neurons in the prothoracic ganglion were involved in the phonotactic responses of female crickets. Many different hormones, such as juvenile hormone III (JHIII), picrotoxin (PTX), and histamine, have been experimented on the prothoracic ganglion in order to develop a fuller picture of the neural circuitry of female crickets (Atkins et al. 2008, Yoon et al. 2011). A model was developed to explain how the plasticity of neurons and hormones in the prothoracic ganglion modulates phonotaxis in a syllable period-

selective manner. The proposed neuronal model shows that JHIII down-regulates the synapse between the omega neuron (ON1) and L3, neurons in the prothoracic ganglion. This effect is mirrored in the cricket's behavior, as nanoinjections of JHIII into the prothraocic ganglion shows changes in the female's overall selectivity (Atkins *et. al* 2008). In young females, high levels of JHIII are produced. During this time, the female crickets are highly selective in their response to the male's calling songs by responding to only certain syllable periods of calling song. In older females, the concentration of JHIII decreases, making them less selective (Stout *et al.* 2002).

Recent studies show that the supraesophageal ganglion of the brain is also involved in phonotactic selectivity (Markovic 2010, Sherwin 2011). Selective heating experiments conducted by Pires and Hoy in 1992 showed that the phonotactic responses of female crickets were altered the most when both the head and thorax were heated at the same time, rather than when it was heated separately. These results indicate that both the prothoracic and supraesophageal ganglia are involved in phonotactic selectivity of female crickets. Since the auditory neurons located in the prothoracic ganglion are modulated by JHIII, Markovic examined if the supraesophageal ganglion was under the same control. The effects of nanoinjecting JHIII into the supraesophageal ganglion of the brain were similar to the prothoracic ganglion, making old, unselective female crickets significantly more selective (Markovic 2010). Picrotoxin, which mimics the effect of JHIII, in the supraesophageal ganglion was also shown to increase the selectivity of females (Sherwin 2011).

Research has suggested that some actions of certain juvenile hormones in insects work through a protein kinase C (PKC) signaling pathway within the cells (Wheeler *et al.* 2003). This project seeks to evaluate the possibility of a second messenger system involving PKC as the molecular mechanism of JHIII control in the supraesophageal ganglion of crickets.

METHODOLOGY

Cricket care

4-week-old nymphs of *A. domesticus* were purchased from Fluker's Cricket Farm in Baton Rouge, LA. Themale and female crickets were raised together in 100-L containers at 22-24°C in a walk-in climate-controlled chamber. Cricket chow (Fluker's Cricket Farm, Baton Rouge, LA) and water in stoppered tubes were provided for the crickets continuously. Egg cartons were also provided for shelter. Artificial lighting was maintained on an LD 12:12 hour photoperiod (Atkins *et al.* 2008). After reaching the adult stage, female crickets were separated and transferred to smaller 16-L containers that were also supplied with food, water and shelter. There was a maximum of 30 female crickets per container, and they were housed in the same incubator as the nymphs. Adult males were discarded.

Phonotaxis testing

Warm wax was used to attach a tether to each cricket's pronotum on its dorsal surface. The cricket was then placed on a free-floating ball of a spherical non-compensating treadmill using the tether attachment (Atkins *et al.* 2008). A three-minute adaptation period preceded the data collection for each cricket. Optical Kugel, a custom made computer program, was used to play computer-generated male calling songs (Sound Studio 3.0) from a single speaker located at the level of the cricket beside the treadmill. The calling songs were standardized (chirp period of 666 ms; 3 syllables; 85 dB) and consisted of seven different chirps with syllable periods ranging from 30 to 90 ms, in 10 ms increments. The calling songs were played in a standard non-sequential order (50, 90, 60, 30, 70, 40, and 80 ms) for 3 minutes each, with a 1-minute period of silence between each syllable period test. The parameters used to determine positive or negative phonotaxis were the path orientation and angular orientation. Path orientation was determined by the ratio of the distance the cricket

went towards the speaker over the distance it went away from it. If the path orientation had a 2:1 ratio of towards to away and the angular orientation was $\pm 60^{o}$ toward the speaker, the cricket performed positive phonotaxis (Atkins *et al.* 2008). If positive phonotaxis was demonstrated to more than 5 of the tested calling songs, the cricket was classified as unselective (Stout *et al.* 2010).

Nanoinjection

Following the pre-test, the dissection of the cricket was performed. The cricket was placed on a wax block ventral side down, and secured with a strip of wax (Nakiplast) over its abdomen. To fully immobilize the cricket head, a thin strip of wax was used to surround the head and secure it onto the wax block. Microscissors (BSB International, b43) were used to puncture 4 holes in the exoskeleton on the top of the head, with two holes slightly above the antennae and the other two in line with the ocelli (Sherwin 2011). Using the 4 holes as the framework, microscissors were used to cut out a rectangular piece of the exoskeleton to expose the supresophageal ganglion. The exoskeleton piece was set aside. For the control group, 9.2 nL of acetone was injected directly into the suprasesophageal ganglion of twenty crickets using a nanoinjector (Drummond Nanoinject II). The first experimental group of 20 crickets was nanoinjected with 9.2 nL of 10^{-5}M JHIII dissolved in acetone. The second experimental group, also consisting of 20 crickets, was nanoinjected with 9.2 nL of 10^{-5}M JHIII and Chelerythrine Chloride (CC), a protein kinase C blocker, dissolved in acetone. Following the nanoinjection, the exoskeleton piece was placed back onto the cricket's head and the cricket hymolymph was used as a seal, as it was allowed to coagulate along the incision lines. The cricket was allowed to rest for approximately 10 minutes, and was post-tested using the same procedure as the pre-tests. Pre- and post-test phonotaxis results were compared using a two-tailed paired *t*-test.

RESULTS

Individual control crickets showed varying phonotactic responses between the pre- and post-tests (Fig. 1). After the dissection and nanoinjection of acetone into the supraesophageal ganglion, the crickets responded differently in the post-test from what was shown in the pre-test: some crickets responded to more syllable periods, some narrowed their responses, and some responded to the same number of syllable periods but to different syllable periods. However, the overall effect of nanoinjecting acetone into the 20 control crickets was not significant (paired *t*-test, $p=0.428$).

The 20 crickets injected with JHIII showed an overall significant difference between the phonotactic responses of the pre- and post-tests (paired *t*-test, $p=0.002$). Although there were slight variances in individual crickets, the crickets showed an overall narrowing of response to the male calling songs with a preference for the 50 ms syllable period calling song (Fig. 2).

For the 20 crickets treated with the solution of JHIII and CC, the overall phonotactic responses between the pre- and post-tests were not significant (paired *t*-test, $p=0.382$). As shown in the previous groups, there were individual differences of response to the number of calling songs the cricket responded to, and which syllable period it responded to. However, on average, the whole group did not show significant difference (Fig. 3).

DISCUSSION

The results confirm that the supraesophageal ganglion of female A. domesticus controls the plasticity of the neuronal mechanism underlying modulation. The control group did not show significant difference, confirming that the dissection and nanoinjection process did not significantly affect the phonotactic behavior of the crickets. The first experimental group showed that the nanoinjection of JHIII into the supraesophageal ganglion caused unselective female crickets to become more selective, which further validated Markovic's work done in 2010.

The results of the second experimental group showed whether PKC was involved in the possible mechanism of the brain. The nanoinjection of JHIII and CC simultaneously into the supraesophageal ganglion showed that unselective crickets remained unselective. CC blocked the natural effect of JHIII of making crickets more selective, which was shown by Markovic in 2010 and again in the first experimental group of this project. Because CC is a PKC blocker, it can be concluded that CC nullified the effect of JHIII by blocking a PKC pathway. This indicates that PKC is part of the JHIII machinery that affects the plasticity of the responsiveness of female crickets to male calling songs.

While the results showed a definite trend, the data for the pre-tests of all three groups were not exactly as expected in regards to the crickets' age group. Crickets of older age, including crickets that are 4-weeks old, should be very unselective as they have very little to no amount of endogenous JHIII. However, some of the crickets were behaving more selectively than expected. This may be due to the mechanics of the treadmill, or the difference in seasonal crickets. Although it may be worth re-testing, the trends were still clearly significant enough to interpret the data.

Since CC, a PKC blocker, nullifies the action of JHIII in the supraesophageal ganglion, JHIII may work through a PKC-mediated 2nd messenger system. To evaluate this further, future research should try to validate and solidify this claim. A possible method would be to test young

crickets typically produce high levels of natural JHIII (Walikonis *et. al* 1991), and inject CC into its supraesophageal ganglion. If CC, the PKC blocker, successfully blocks the action of the endogenous JHIII by making the selective young crickets less selective, this would confirm that JHIII uses a PKC pathway. Additional probing for a PKC pathway involvement for JHIII control would be to inject calcium-release blockers and/or enzymes that breakdown diacylglycerol, which would block the release of calcium and diacylycerol in the cell. Both calcium and diacylglycerol are known to play a role in PKC-mediated 2nd messenger systems, so this would test if their release affects the phonotactic selectivity of crickets.

LITERATURE CITED

Atkins, G., Kilmer, J., Scalfani, M., Navia, B., Stout, J. (2008). Modulation of syllable period-selective phonotaxis by prothoracic neurones in crickets (*Acheta domesticus*): juvenile hormone, picrotoxin and photoinactivation of the ON1 neruones. *Physiological Entomology*, 33, 322-333.

Gerhardt, H. & Huber, F. (2002). Acoustic communication in insects and anurans. University of Chicago Press, Chicago, IL.

Markovic, C. & Atkins, G. (2010). The effect on phonotaxis of naninjecting juvenile hormone III into the supraesophageal ganglion of female crickets. *Senior Honors Thesis*, Andrews University, MI.

Sherwin, B. & Atkins, G. (2011). The role of Cl- channel-inhibition in the brain on the phonotactic selectivity of female crickets. *Senior Honors Thesis*, Andrews University, MI.

Stout, J., Atkins, G., Bronsert, M., Hao, J., Walikonis, R. (2002). Influence of juvenile hormone III on the development and plasticity of the resopnsiveness of female crickets to calling males through control of the response properties of identified auditory neurons. *Hormones, Brain and Behavior*, 3, 167-193.

Stout, J., Navia, B., Jeffery, J., Samuel, L., Hartwig, L., Butlin, A., Chung, M., Wilson, J., Dashner, E., Atkins, G. (2010). Plasticity of the phonotactic selectivness of four species of chirping crickets (*Gryllidae*): Implications for call recognition. *Physiological Entomology*, 10, 1-7.

Wheeler, D.E., Nijhout, H.F. (2003). A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. *Bioessays*, 25, 994-1001.

Yoon, J., Atkins, G. (2011). Effects of nanoinjecting inhibitory neurotransmitters into the prothoracic ganglion on the selectivity of phonotaxis in female crickets. *Senior Honors Thesis*, Andrews University, MI.

Acetone Pre-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1	■			■			
2			■		■		
3			■	■	■		
4			■	■		■	
5	■	■	■	■			
6	■	■			■		■
7		■	■	■		■	
8		■	■		■	■	
9	■	■		■	■		■
10	■	■	■	■			■
11	■	■		■	■	■	
12	■			■	■	■	■
13		■	■	■	■	■	
14		■	■	■	■	■	
15		■	■	■	■	■	
16	■	■	■	■	■		
17		■	■	■	■	■	■
18	■		■	■	■	■	■
19	■	■	■	■	■		■
20	■	■	■	■	■	■	■

Acetone Post-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1		■			■	■	■
2			■				
3			■		■		
4	■		■		■	■	
5	■		■	■	■		
6		■	■	■	■	■	■
7			■	■	■	■	
8	■	■	■	■		■	
9		■		■	■	■	■
10			■	■	■	■	■
11		■	■	■	■	■	
12	■	■	■			■	
13	■	■		■	■		
14	■	■	■	■		■	
15	■			■			■
16	■		■	■	■	■	
17	■	■		■	■	■	■
18	■	■	■	■			■
19			■	■	■		■
20	■	■	■	■	■	■	

Fig. 1: The effects of acetone nanoinjection on phonotactic selectivity. Tables show the pre- and post-test phonotactic responses of female crickets. Each row represents the response of one female cricket. The shaded boxes represent positive phonotaxis, and the unshaded boxes represent no phonotaxis for the indicated syllable periods. Twenty female crickets were used, and the post-test table depicts the response after the nanoinjection of 9.2 nL of acetone into the supraesophageal ganglion of the same crickets.

JHIII Pre-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1			■		■		
2		■		■			
3			■	■			■
4	■		■			■	
5		■		■	■		
6		■		■	■		
7	■	■	■				
8			■	■		■	
9	■		■	■			
10	■		■	■	■		
11		■	■	■			■
12		■		■	■	■	
13	■	■			■	■	
14		■		■	■		■
15			■		■	■	■
16		■	■		■	■	
17			■	■		■	■
18	■	■	■	■		■	
19	■	■	■	■		■	
20		■	■	■	■	■	■

JHIII Post-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1				■			
2		■	■				
3			■		■		
4	■	■	■	■	■	■	■
5							
6	■		■				
7		■	■		■	■	
8		■	■				
9			■			■	
10							
11				■			
12				■			■
13	■	■					
14			■		■		
15			■	■		■	
16				■			■
17			■	■		■	■
18						■	
19	■						
20		■	■	■			

Fig. 2: The effects of JHIII nanoinjection on phonotactic selectivity. Tables show the pre- and post-test phonotactic responses of female crickets. Each row represents the response of one female cricket. The shaded boxes represent positive phonotaxis, and the unshaded boxes represent no phonotaxis for the indicated syllable periods. Twenty female crickets were used, and the post-test table depicts the response after the nanoinjection of 9.2 nL of 10^{-5}M JHIII dissolved in acetone into the supraesophageal ganglion of the same crickets.

JHIII + CC Pre-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1				■		■	
2	■		■				
3	■					■	
4	■	■					
5		■			■		
6		■		■		■	
7	■		■			■	
8		■	■		■		
9			■		■	■	■
10		■	■	■		■	
11				■	■	■	■
12		■		■	■		■
13		■	■	■	■		
14	■	■		■	■		
15		■	■		■	■	■
16	■	■			■	■	■
17	■		■	■	■	■	
18	■		■	■			■
19	■	■	■	■	■	■	■
20	■	■	■	■	■	■	■

JHIII + CC Post-test

Syllable Period (ms)

	30	40	50	60	70	80	90
1	■		■	■			■
2						■	
3					■	■	
4		■	■				
5	■		■			■	
6	■		■	■		■	■
7	■	■		■			■
8		■		■			■
9			■	■	■	■	
10	■	■		■	■	■	
11			■	■	■		■
12				■	■		
13		■	■			■	
14		■	■		■	■	
15		■	■		■		
16		■	■	■		■	■
17	■	■	■		■	■	
18	■	■	■				
19		■	■	■		■	■
20	■	■	■	■	■	■	■

Fig. 3: The effects of JHIII + CC nanoinjection on phonotactic selectivity. Tables show the pre- and post-test phonotactic responses of female crickets. Each row represents the response of one female cricket. The shaded boxes represent positive phonotaxis, and the unshaded boxes represent no phonotaxis for the indicated syllable periods. Twenty female crickets were used, and the post-test table depicts the response after the nanoinjection of 9.2 nL of 10^{-5}M JHIII + CC dissolved in acetone into the supraesophageal ganglion of the same crickets.