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The Role of Cl(-) Channel-inhibition in the Brain on the Phonotactic Selectivity of Female Crickets

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Abstract

The goal of this project was to determine if picrotoxin influences phonotactic selectivity of female crickets when nanoinjected into the supraesophageal ganglion. Adult females of *Acheta domesticus* were pre-tested for phonotaxis on a treadmill to a range of calling songs with different syllable periods. Following dissection to expose the brain, 9.2 nL of either saline (control group) or 10^{-5} M picrotoxin (experimental group) was nanoinjected into the supraesophageal ganglion. Post-tests following saline injection (10 min later) were similar to the pre-tests. Picrotoxin-treated females became more phonotactically selective displaying a preference for calling songs with the shorter syllable periods.

Keywords

Picrotoxin, recognition, modulation, plasticity

Introduction

Phonotaxis is the movement of an organism towards or away from a sound stimulus. Female crickets use phonotaxis to respond to the male's calling song during mating (Gerhardt & Huber, 2002). This behavioral response has been studied for several reasons. First, crickets possess a simple nervous system compared to vertebrates. Second, the natural phonotactic response of crickets is regulated not only by identified neurons within the cricket but also by neuromodulators. Models are being used to describe the neural control and modulation of these behavioral responses (Markovic, 2010).

Atkins *et al.* (1992) discovered that inactivation of the L1 and L3 neurons in the prothoracic ganglion of *Acheta domesticus* caused angular errors in the phonotactic response. This demonstrated that neurons in the prothoracic ganglion were involved in the phonotactic responses of female crickets. Nanoinjection of Juvenile Hormone III (JHIII), Picrotoxin (PTX), Benzodiazepine, and Histamine into the prothoracic ganglion all caused changes in phonotactic selectivity to the syllable period (SP) of the males call, which demonstrated that these compounds influenced phonotactic circuits (Atkins *et al.,* 2008; Yoon *et al.,* 2011). From this research, a model was developed to explain how the plasticity of neurons and hormones in the prothoracic ganglion modulate phonotaxis in a SP-selective manner (Markovic, 2010). The model describes how control of the inhibitory inputs of the omega neuron (ON1) are up-regulated by Histamine and down-regulated by both JHIII and PTX. These inhibitory inputs of the ON1 directly affect the responses of the delayed inhibition and selective excitation neurons that synapse with the L3 neuron. When JHIII (natural hormone) and PTX (an unselective Cl^- channel blocker) are nanoinjected into the prothoracic ganglion the inhibitory inputs of ON1 are

blocked which allows the delayed inhibitory and selective excitatory neurons to directly affect the L3 resulting in a selective phonotactic response. In contrast, when Histamine (the putative inhibitory transmitter of ON1) is nanoinjected into the prothoracic ganglion the inhibitory input of ON1 is increased resulting in the L3 and phonotaxis being less selective to SP.

The supraesophageal ganglion has also been shown to be involved in phonotactic selectivity. Selective heating experiments conducted by Pires and Hoy (1992) found that phonotactic responses of female crickets were altered the most when both the head and thorax were heated concurrently, indicating that both the prothoracic and supraesophageal ganglion were involved in phonotactic regulation. In addition, Schildberger (1984) used intracellular recording in the brain of females to identify SP-selective auditory neurons in the supraesophageal ganglion.

Because auditory neurons located in the prothoracic ganglion are modulated by JHIII, Markovic (2010) asked if nanoinjection of JHIII into the supraesophageal ganglion would have a similar effect as it did in the prothoracic ganglion. She found that unselective females became more selective for SP post-injection (Markovic, 2010). In addition she discovered that the increased selectivity to SPs was stronger than it had been in experiments involving JHIII in the prothoracic ganglion. Since the effect of JHIII was mimicked by PTX application in the prothoracic ganglion, indicating the role of Cl^- channel inhibition in the regulation of JHIII, my research focused on the effect nanoinjectioning PTX into the supraesophageal ganglion had on the SP-selective phonotaxis of female crickets.

Materials and methods

Cricket care

Four-week-old nymphs of *Acheta domesticus* were purchased from Fluker's Cricket Farm in Baton Rouge, Louisiana. The crickets were raised in 100-L containers. Cricket chow (Fluker's Cricket Farm, Baton Rouge, LA), water (in stoppered tubes), and egg cartons (for shelter) were provided for the crickets continuously. After reaching the adult stage, female crickets were transferred to smaller 16-L containers and provided with cricket chow, water, and egg cartons and kept separate from males. The temperature was maintained at 21-23 °C and lighting was under a LD 12:12 h photoperiod (Atkins *et al.* 2008). Females were kept in the 16-L containers until they were between 20-32 days old.

Phonotaxis testing

Warm wax was used to attach a tether to the cricket's pronotum. This tether connects to the arm of a metal rod extending above the free-floating ball of a spherical non-compensating treadmill (Atkins *et al.* 2008). Following a three-minute adaptation period a custom made computer program (Optical Kugel) was used to play model-calling songs (SoundStudio 3.0) of male crickets for 3 minutes each from a single speaker located at the level of the cricket beside the treadmill. These calling songs were standardized (chirp period of 666 ms; 3 syllables with a duration of 25 ms; 85dB). The syllable periods of the male calling songs ranged from 30-90 milliseconds (ms) and were played in a standard non-sequential order (50, 90, 60, 30, 70, 40, and 80 ms). Between each calling song the cricket experienced a 1-minute period of silence.

A positive phonotactic response of the cricket on the treadmill was determined by two measurements. The first was path orientation, which was determined by the ratio of the distance the cricket went towards the speaker vs. the distance it went away from it. The second was angular orientation. If the path orientation had a 2:1 ratio of towards to away and the angular orientation was +/- 60° the cricket was classified as performing positive phonotaxis towards the sound source (Atkins *et al.,* 2008).

Nanoinjection

Following the pre-test, dissection of the cricket was performed. This involved placing the cricket on a wax block ventral side down. Two U-shaped pins were used to secure the cricket, one just behind the head and the other over the abdomen and back legs. In addition, a thin strip of wax (Nakiplast) was placed over the top of the first pin just behind the head to prevent the cricket from moving forward or backward. Finally, two small balls of wax were placed on either side of the head in order to fully immobilize it. Microscissors were used to carefully puncture 4 holes in the exoskeleton on the top of the head. Two of the holes were slightly above the antennae and the others were in line with the ocelli (Fig. 1). The microscissors were then used to cut out a rectangular piece of the exoskeleton, which allowed access to the supraesophageal ganglion. The exoskeleton piece was set aside for later. For the controls, 9.2 nL of saline (Fielden, 1960) was nanoinjected into the supraesophageal ganglion using a nanoinjector (Drummond Nanoject II). The experimental group was dissected in the same way and nanoinjected with 9.2 nL of 10^{-5} M of PTX in saline. Following naninjection the exoskeleton piece was replaced and the cricket hemolymph was allowed to coagulate along the incision lines. The cricket was allowed to rest for approximately 10 minutes. The cricket was then post-tested,

which was identical to the pre-test described above. Pre and post-test phonotaxis were compared using a two-tailed paired *t*-test.

Results

The individual phonotactic responses of the control crickets varied somewhat between the pre and post-tests (Fig. 2). Some crickets broadened their responses to include more SPs. Others narrowed their responses somewhat by responding to fewer SPs in the post-tests. Some responded to the same number of SPs between the pre and post-tests. The overall effect of nanoinjecting saline into the 16 control crickets was not significant (t=1.54, p=0.145, d.f.=15).

In contrast, phonotactic selectivity between the pre and post-tests (Fig. 3) of the PTX-injected group (n=16) showed an overall narrowing of response to the male calling songs (Fig. 4) with a preference for the shorter syllable periods (t=3.95, p=0.001, d.f.=15). While the individual responses of the females varied between the pre and post-tests the 6 females which responded to the range of 1-3 SP's in the pre-tests (Fig. 5a) did not show an overall change in phonotactic response following nanoinjection (t=0791, p=0.465, d.f.=5). Five crickets responded to 4-5 SPs in the pre-tests (Fig 5b). Though responses varied between the pre and post-tests the overall shift in phonotactic response was found to be significant with females preferring the shorter (30-60 ms) SPs (t=2.95, p=0.042, d.f.=4). Five crickets responded to 6-7 SPs in the pre-tests (Fig. 5c). A similar overall shift in phonotactic selectivity occurred to the shorter SPs (t=4.24, p=0.013, d.f.=4).

Discussion

The results of nanoinjecting PTX into the supraesophageal ganglion causing female crickets to become more phonotactically selective (Fig. 3, 4), demonstrates that auditory neurons in the supraesophageal ganglion are involved in regulating phonotactic selectivity (Schildberger, 1984). Because PTX is an unselective Cl^- channel blocker the experimental results also indicate that inhibitory inputs in the brain dependent on Cl^- channel-inhibition are involved in the modulation of the SP-selectivity of phonotaxis.

Nanoinjection of JHIII into the brain caused unselective females to become more phonotactically selective with the preferred SPs centering between 50-70 ms (Markovic, 2010). The results of nanoinjection of PTX into the brain also result in females becoming more selective. However, the shift in the range of response is toward the shorter SPs, which is different from what was found for both JHIII nanoinjection into the supraesopageal ganglion (Markovic, 2010) and prothoracic ganglion and PTX nanoinjection into the prothoracic ganglion (Atkins *et al.,* 2008).

Schildberger's model may help explain this shift in selectivity (Carew, 2000). His model (Fig. 6) shows that auditory inputs entering the neuronal circuits of the brain are controlled by a high-pass and low-pass filter. Together these filters combine to create a bandpass-filter, which caused females to prefer the 50-70 ms SP range of the male calling song. The results from PTX nanoinjection in the brain are consistent with the possibility that the low-pass filter was effectively blocked by PTX, allowing only the higher frequency or shorter SPs through. This would result in the crickets preferring the shorter SPs.

To evaluate this hypothesis, future work could include trying to block the high-pass filter causing crickets to prefer the longer SPs. This could include nanoinjection of Amiodarone, an unselective K+ channel blocker (the other possible form of inhibition that could lead to selective changes), into the supraesophageal ganglion (Kiehn *et al.,* 1998). If Amiodarone had this effect then the hypothesis could be tested further by nanoinjecting PTX and Amiodarone to see if the resulting phonotaxis became centered around the midrange of SPs (50-70 ms) typical of selective untreated crickets.

Acknowledgements

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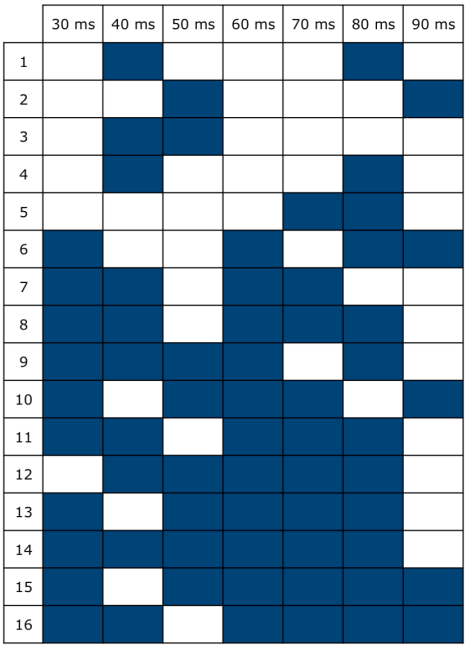
Fig. 1. Picture showing the position of the four holes (indicated by the circles) in the early dissection.

Saline pre-test



Saline post-test

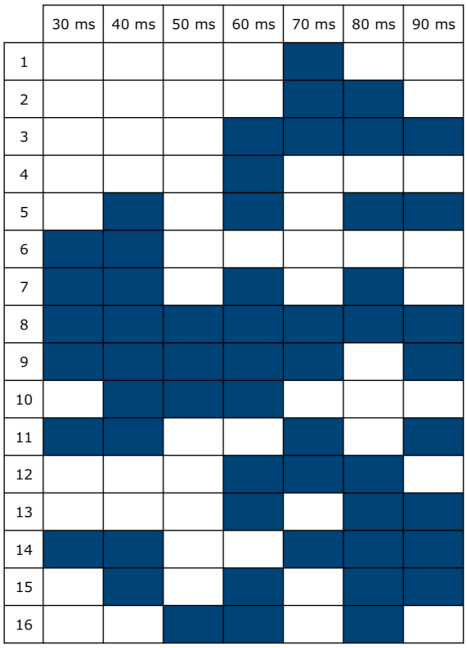
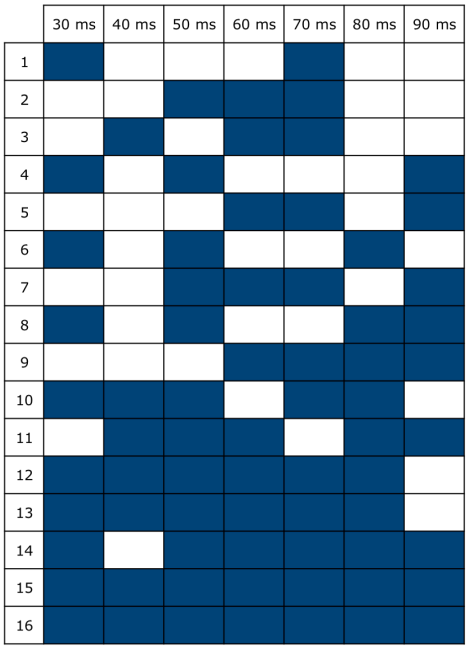


Fig. 2. Effects of saline nanoinjection on phonotactic selectivity. Tables show the pre and post-test phonotactic responses of female crickets. Blue boxes indicate positive phonotaxis and unshaded boxes indicate no phonotaxis for that SP. Sixteen crickets were used (each row represents one female's response) and the post-test table indicates the response after nanoinjection of 9.2 nL of saline into the supraesophageal ganglion of the same crickets.

PTX pre-test



PTX post-test

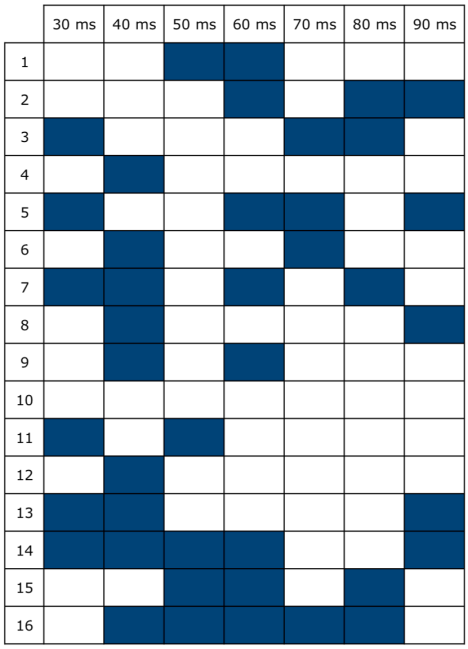


Fig. 3. Effects of PTX nanoinjection on phonotactic selectivity. Tables show the pre and post-test phonotactic responses of female crickets. Blue boxes indicate positive phonotaxis and unshaded boxes indicate no phonotaxis for that SP. Sixteen crickets were used (each row represents one female's response) and the post-test table indicates the response after nanoinjection of 9.2 nL of 10^{-5} M PTX (dissolved in saline) into the supraesophageal ganglion of the same crickets.

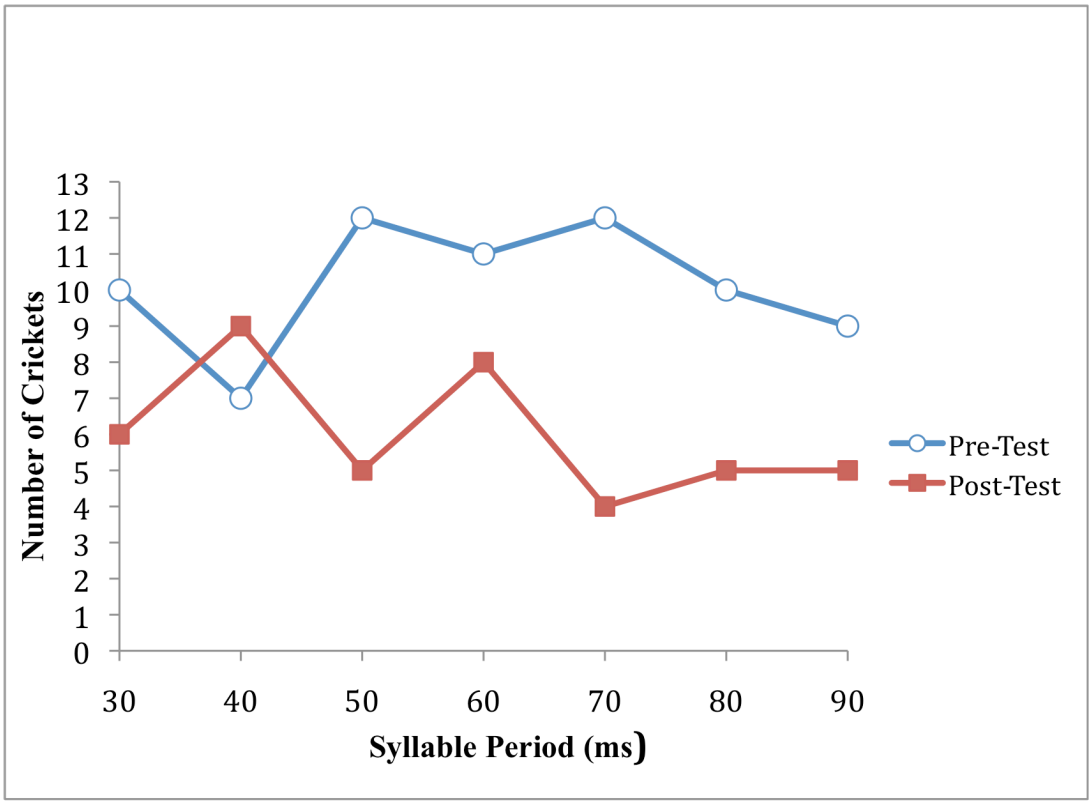


Fig. 4. Graph showing the overall variation in the number of crickets (n=16) that responded to SPs between 30-90 ms before and after nanoinjection of 9.2 nL of 10^{-5} M PTX (dissolved in saline).

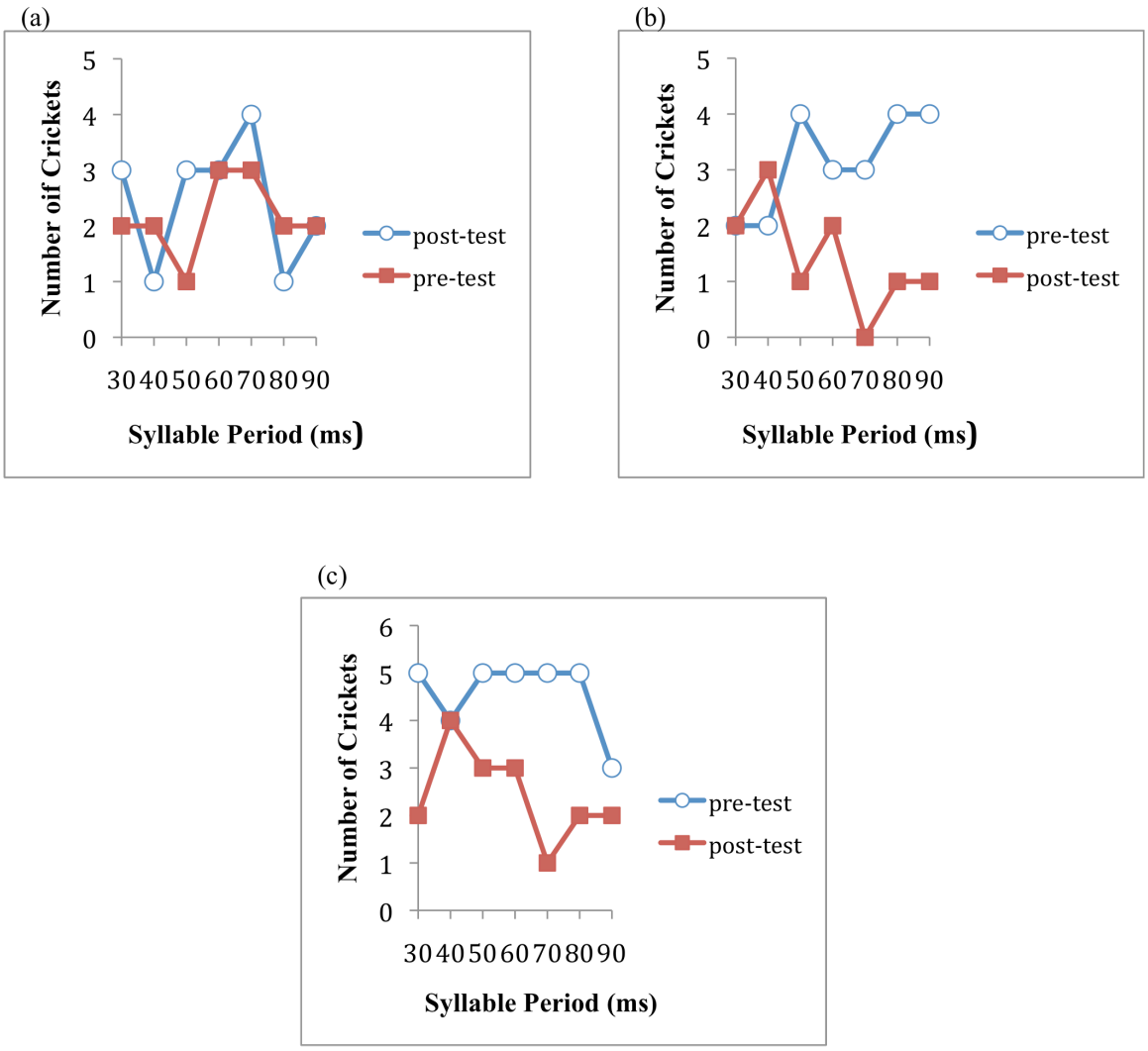


Fig. 5. Graphs showing the phonotactic responses of females to nanoinjection of 9.2 nL of 10^{-5} M PTX into the supraesophageal ganglion. Females were put into different groupings based on their pre-test selectivity. (a) shows the 6 female crickets who responded to 1-3 SPs. (b) shows the 5 female crickets who responded to 4-5 SPs. (c) shows the 5 female crickets who responded to 6-7 SPs.

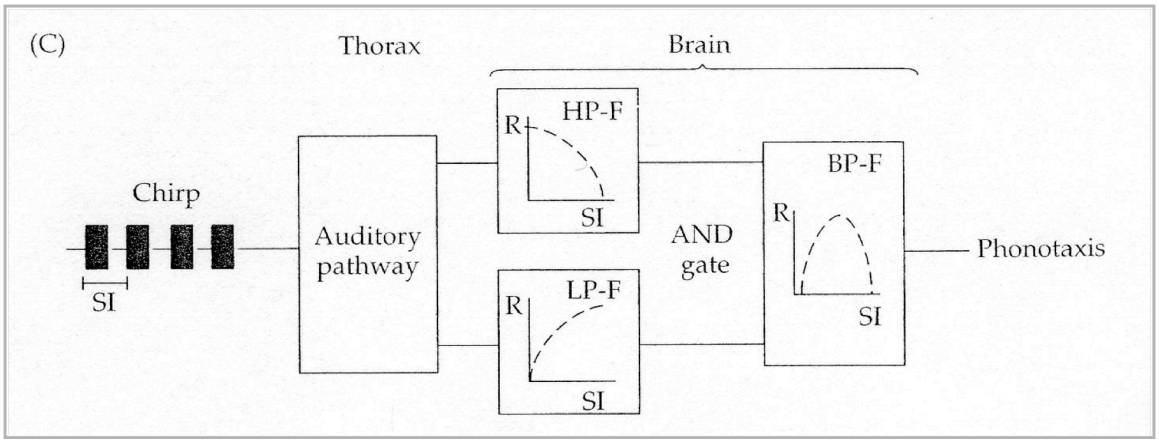


Fig. 6. Diagram (modified from Carew, 2000) showing Schildberger's model of how the high-pass filter (HP-F) and low-pass filter (LP-F) modulate auditory input in the brain and combine together to form the bandpass-filter (BP-F) which causes an optimal phonotactic response to occur to SP's of 50-70 ms.