Whole-brain activity mapping for neuroethological study in the cricket

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1. Introduction

Two-spotted cricket *Gryllus bimaculatus* has been widely used in neuroethological studies to understand the neural basis of sensory processing and motor control, as well as their instinctive behaviors (e.g. agonistic behavior)[1-4]. Studies using the crickets largely rely on limited experimental techniques such as neurophysiological techniques (e.g. electrophysiological recordings, neuroimaging). Although neurophysiological techniques allow us to directly monitor electrical/physiological activities of neural circuits, theses techniques are vulnerable to mechanical disturbances caused by animal movements.

Histological detection of neuronal activity-regulated genes has been widely employed to visualize neuronal activation in recent behavioral neuroscience[5,6]. The advantage of this technique lies in its ability to visualize the key neural circuits related to a certain behavior. In the present study, we established a neuronal activity-regulated transgenic reporter system for whole-brain activity mapping in the cricket.

2. Neuronal immediate-early gene (IEG)

Neuronal IEGs are defined as genes that show rapid and transient expression in response to increased neuronal activity in the absence of *de novo* protein synthesis (Figure 1). Therefore, expression of neuronal IEGs is a first round of genomic response to increased neuronal activity. We identified neuronal IEGs expressed in the cricket brain, and constructed a transgenic reporting system that mimics the expression of neuronal IEGs.

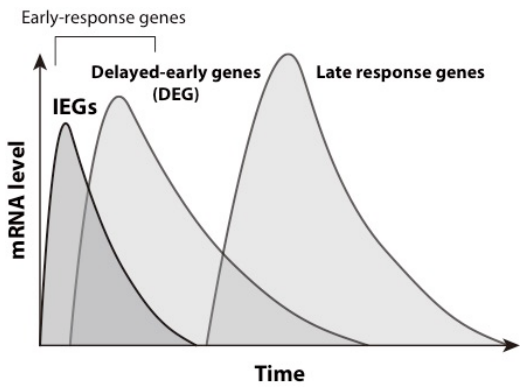

Figure 1: Stimulus-dependent gene expression.

3. Materials and methods

Neuronal IEGs in the cricket brain were selected according to the following criteria: genes that show significant increase 30 min after injection of picrotoxin (PTX; $GABA_AR$ antagonist) under the influence of protein synthesis inhibitor cycloheximide. The mRNA expression levels were determined by RT-qPCR. The promoter region of neuronal IEGs were isolated to construct a transgenic vector (Figure 2). The IEG reporter cassette contains a nuclear-targeted destabilized EYFP (*EYFPnls::PEST*) driven by the promoter region of a neuronal IEG. The transgene expression cassette was integrated into the cricket genome by using *piggyBac* transposon-based transgenic system. Successful transgenic crickets were selected to establish homozygous transgenic line (IEG reporter line). Expression of reporter gene and innate neuronal IEGs were compared in various conditions (e.g. PTX treatment, drug-induced activation of various intracellular signaling pathways) by using RT-qPCR. The distribution of reporter protein was detected by immunohistochemistry against EYFPnls::PEST protein.

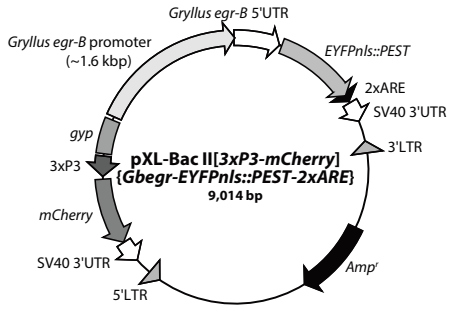

Figure 2: Transgenic vector for the IEG promoter-driven reporter system.

4. Results

4.1. Neuronal IEGs in the cricket brain

We examined activity-regulated expression of three candidate neuronal IEGs, *fos-related antigen* (*fra*), *jun-related antigen* (*jra*), and *early growth response* (*egr*). In the cricket brain, *fra* B isoform (*Gryllus fra-B*) and *egr* B isoform (*Gryllus egr-B*) were induced as neuronal IEGs after PTX injection. *Gryllus fra-B* showed a small magnitude of up-regulations (~2-fold up-regulation), whereas *Gryllus egr-B* showed an ~5-fold up-regulation 60 min after PTX injection.

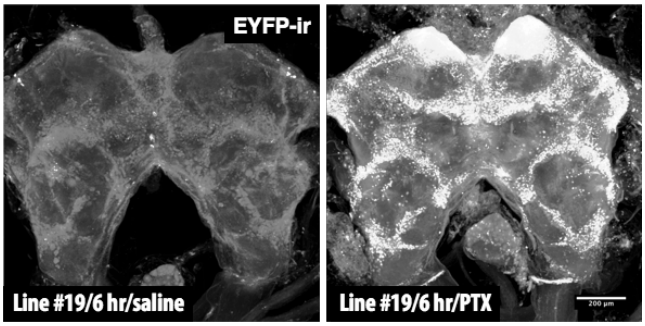

Figure 3: EYFP immunoreactivity (EYFP-ir) in the brain of the IEG reporter line induced by PTX injection.

4.2. Isolation of an IEG promoter

A ~ 2.2 kbp genomic fragment upstream to the translation initiation site of *Gryllus egr-B*, which contains the major promoter region of the gene, was isolated. This region contained potential binding sites for transcription factors regulated by various intracellular signaling pathways (e.g. CREB, AP-1, SRF), as well as core promoter elements conserved across insect/crustacean *egr-B* homologues.

4.3. Generation of the IEG reporter line

The transgene expression cassette was integrated into the cricket genome by using *piggyBac* transposon-based transgenic system. Successful transgenic crickets were selected according to the expression of visible marker gene (*3xP3-mCherry*), which resulted in red fluorescent eyes. Three out of 37 mCherry-expressing transgenic lines showed EYFP-immunoreactivity in the brain after PTX injection (Figure 3). One transgenic line with a low background EYFP expression was selected, and a homozygous transgenic line was established as an IEG reporter line for subsequent analyses.

4.4. Characterization of the IEG reporter line

In the brain of the IEG reporter line, mRNA expression of reporter gene reached a maximum (~1.8-fold up-regulation) 60 min after PTX injection, and sustained at near peak level by 120 min after injection. On the other hand, mRNA expression of *Gryllus egr-B* reached a maximum (5-fold up-regulation) 60 min after PTX injection, and decreased to near baseline level by 120 min after injection. The expression levels of *EYFPnls::PEST* and *Gryllus egr-B* were strongly correlated (Pearson's r=0.738; Figure 4), indicating the expression of IEG reporter mimics innate *egr-B* expression.

Six hours after PTX injection, the reporter protein was distributed throughout the brain of the IEG reporter line (Figure 3). In the brain of naïve animals, the reporter protein was detected in the clusters of neurons located in the lateral parts of the supraoesophageal ganglion. In both preparations, the receptor protein was restrictedly localized in the nuclei of the neurons.

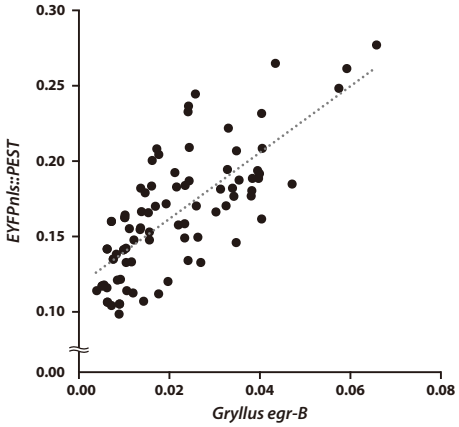

Figure 4: Correlation plot between *EYFPnls::PEST* and *Gryllus egr-B* in the brain of the IEG reporter line.

5. Discussion

Our IEG promoter-driven transgenic reporter system was capable to visualize strong, prolonged stimulation of neuronal activity by blocking GABAergic inhibition. It is necessary to test whether the IEG reporter system is sensitive enough to visualize sensory-evoked or behaviorally-evoked neuronal activity.

6. Conclusion

- *Gryllus fra-B* and *Gryllus egr-B* were inducibly expressed as neuronal IEGs in the cricket brain.
- The promoter region of *Gryllus egr-B* was enriched with sequence elements important for activity-regulated inducible gene expression.
- The promoter region of *Gryllus egr-B* was used to construct neuronal activity-regulated reporter system in the cricket.
- In the brain of the IEG reporter line, reporter gene mimicked the expression of innate neuronal IEGs.
- The reporter system was capable to visualize neuronal activation caused by pharmacological inhibition of GABergic system.

References

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