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Targeted gene delivery in the cricket brain, using *in vivo* electroporation

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ABSTRACT

The cricket (*Gryllus bimaculatus*) is a hemimetabolous insect that is emerging as a model organism for the study of neural and molecular mechanisms of behavioral traits. However, research strategies have been limited by a lack of genetic manipulation techniques that target the nervous system of the cricket. The development of a new method for efficient gene delivery into cricket brains, using *in vivo* electroporation, is described here. Plasmid DNA, which contained an enhanced green fluorescent protein (eGFP) gene, under the control of a *G. bimaculatus* actin (*Gb'*-act) promoter, was injected into adult cricket brains. Injection was followed by electroporation at a sufficient voltage. Expression of eGFP was observed within the brain tissue. Localized gene expression, targeted to specific regions of the brain, was also achieved using a combination of local DNA injection and fine arrangement of the electroporation electrodes. Further studies using this technique will lead to a better understanding of the neural and molecular mechanisms that underlie cricket behaviors.

1. Introduction

The cricket, *Gryllus bimaculatus*, is one of the classic model organisms used for studying neural mechanisms that underlie the behavioral traits of insects. Most studies, for example, those on escape behavior, phonotaxis, aggression and associative learning, have relied on electrophysiological, pharmacological and neuroimaging methods (Hedwig, 2006; Matsumoto and Mizunami, 2002; Ogawa et al., 2006, 2008; Stevenson and Rillich, 2012). In spite of the many studies into a broad repertoire of behavior, the molecular basis that underlies these traits is still unclear. This may be attributed to the existence of very few established techniques for the genetic manipulation of neurons in the cricket. The availability of a simple and effective method to introduce exogenous genes into the brains of living adult crickets will accelerate biological studies in a variety of fields.

There have been several successful attempts to introduce exogenous genes and other constructs into crickets (Zhang et al., 2002; Shinmyo et al., 2004; Nakamura et al., 2010; Mito et al., 2010; Watanabe et al., 2012). In these studies, which were performed to clarify development and regeneration mechanisms, eggs or nymphs were the main targets of gene transfer. In order to study the effects of genetic manipulation in adult crickets, the eggs and nymphs need to be reared to adulthood. The establishment of a simple and efficient method of gene delivery into post-mitotic cells of adult crickets would bypass the rearing of genetically manipulated eggs and nymphs, to allow the reduction of experimental time and resources.

Electroporation has been established as a powerful means of gene transfer and is applicable to post-mitotic cells. The electroporation method, which was initially invented for gene transfer in *in vitro* cultured cells (Neumann et al., 1982), has been successfully adapted to *in vivo* gene transfer in various species. It has been used in *Drosophila* (Kamdar et al., 1995), honeybee (Kunieda and Kubo, 2004), zebrafish (Buono and Linser, 1992), *Xenopus* (Eide et al., 2000), chick (Yasuda et al., 2000; Yasugi and Nakamura, 2000; Swartz et al., 2001) and mouse (Akamatsu et al., 1999; Miyasaka et al., 1999). Gene delivery by electroporation has been tested on a wide range of targets, from single cells (Haas et al., 2001) to tissues. However, the honeybee is currently the only insect in which gene transfer by electroporation

Abbreviations: eGFP, enhanced green fluorescent protein; PP, poring pulse; TP, transfer pulse; SR, success rate.

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has been carried out on the adult brain of a living insect (Kunieda and Kubo, 2004). Here, we describe a method for simple *in vivo* electroporation, which led to the expression of enhanced green fluorescent protein (eGFP) within the brains of living adult crickets. This study is the first report of successful transfer of a foreign gene into neural tissue of adult hemimetabolous insects. We also developed a novel method to target the delivery to specific regions within the brain, without the use of a cell-specific promoter. This was achieved by combining local microinjection of DNA with a fine arrangement of the electroporation electrodes. These techniques have the potential to extend the functional analysis of cricket behaviors.

2. Materials and methods

2.1. Animals

Adult male crickets (*G. bimaculatus*) from colonies maintained at Hokkaido University were used within 2 weeks from their imaginal molt. The animals were fed on a compounded diet for insects (Oriental Yeast, Tokyo, Japan) and were kept in a 12/12 h light/dark cycle at a constant temperature of 28 °C. Crickets were transferred from the colony to the laboratory environment during the week before the experiment. To avoid cannibalization, they were isolated in individual plastic cups with lids. To reduce body fluid, crickets were deprived of water for at least 3 days before the experiment. This allows easier fluid control and improves the success rate of plasmid injection and electroporation. During isolation, crickets were fed on insect pellets, *ad libitum*, as in the rearing environment.

2.2. Plasmids

To construct the plasmid DNA used for electroporation, the promoter of the *G. bimaculatus* cytoplasmic actin (*Gb′-act*) gene, the eGFP gene (derived from pEGFP-N1; Clontech Laboratories Inc., Mountain View, USA) and 3′ untranslated region (UTR), which contained the SV40 polyadenylation signal, were PCR amplified from the plasmid pGact-eGFP (Zhang et al., 2002). The PCR was performed with a sense primer that contained an *Xba*I site (5′-GC**TCTAGA**GCGGCCGCCACCGC-3′) and an antisense primer that contained an *Eco*RI site (5′-G**GAATTC**AGGGGGGGCCCGGTACCCAA-3′). The *Gb'-act* promoter is an 897 base pair (bp) genomic DNA fragment that is found upstream of the putative start codon of the *Gb′-act* gene (Accession Number: AB087882.1). Its strong activity in *G. bimaculatus* embryonic cells has been reported previously (Zhang et al., 2002; Shinmyo et al., 2004; Nakamura et al., 2010). A *Gb′-act* promoter-eGFP fragment was subcloned into a vector derived from a *C. elegans* plasmid, p*ttx-3::GFP* (pPD95.75 vector; Tsalik and Hobert, 2003), using the *Xba*I and *Eco*RI sites. The total length of the plasmid was 5606 bp and is shown in Fig. 1A. The plasmid was extracted and purified in distilled water using the QIAGEN Plasmid Plus Maxi Kit (QIAGEN, Hamburg, Germany). For the expression of Yellow Cameleon 3.60 (YC3.60, Nagai et al., 2004) that is a genetically encoded Ca^{2+} indicator (GECI) and Channel Rhodopsin 2 (ChR2, Nagel et al., 2003) that is a light-activated cation channel, plasmids *Gb'-act*-YC3.60-SV40 and *Gb'-act*-ChR2-DsRed2-SV40 were constructed by replacing the eGFP sequence with either YC3.60 or ChR2-DsRed2. The ChR2 gene and DsRed2 gene were used for observation of ChR2 expression (pDsRed2-C1 vector, Clontech 6974–1). The *Gb'-act*-YC3.60-SV40 and *Gb'-act*-ChR2-DsRed2-SV40 plasmids were 6388 bp and 6503 bp, respectively.

2.3. Preparation

For plasmid injection and electroporation, crickets were anesthetized by cooling on crushed ice. They were individually fixed with a harness set that consisted of a neck holder and an abdomen holder. The neck holder was made from the lid of small acrylic petri dish with a keyhole-shaped hole. Two pieces of plastic tape of ~18 mm by 20 mm and a third piece of ~18 mm by 25 mm, with a square hole in the center (4 mm by 4 mm), were used to harness a cricket to the neck holder. A 1.5 ml microtube was used as the abdomen holder, together with a piece of dental utility wax (GC Corp., Tokyo, Japan). The head of the anesthetized cricket was pushed through the hole in the neck holder, with the neck set at the narrow part of the keyhole. The rest of the hole was covered with a piece of plastic tape behind the neck of the cricket. The other piece of tape was used to immobilize the antennae. The piece of tape with the square hole was used to cover and stabilize the head, with the necessary part of the head exposed for access to the brain (Fig. 1B). The abdomen was placed in the abdomen holder, which was attached to the neck holder by the piece of dental utility wax. This prevented the body from twisting at the neck, which could cause fatal damage. For dissection, injection and electroporation, the neck holder was set on a tubular platform.

2.4. Plasmid injection

The plasmid DNA was manually pressure-injected using a glass needle connected to a disposable syringe. Glass needles were prepared from borosilicate glass capillary tubes (1B100–4, World Precision Instruments, Sarasota, USA) using a micropipette puller (PN-3, Narishige, Tokyo, Japan). The tips were broken to obtain a point diameter of approximately 10–15 μm. To expose the frontal surface of the brain, part of the head cuticle was carefully removed and the tissues covering the brain were pushed aside. The neural sheath that covers the brain was not removed. To deliver the DNA into the whole brain, a glass needle was filled with 200 nl of plasmid solution (250 ng/μl DNA, 0.01% Fast Green FCF in distilled water), which had been measured out with a micropipette (NPX-2, NICHIRYO, Koshigaya, Japan). Micromanipulators (MMN-1 and MMO203, Narishige, Tokyo, Japan) were used to insert the glass needle into the brain at the precise injection site. Injection of the entire volume within the needle was visually checked under a stereomicroscope (SMZ645, Nikon, Tokyo, Japan). For local microinjection, a glass capillary (ø = 1.0 mm) was filled with 100 nl of solution, which was measured out with a micropipette. Half of the volume (50 nl) was sucked from the capillary into a glass needle using a disposable syringe. Slightly less than half the volume (~20 nl) was injected into the targeted region to avoid injecting air into the brain. Three regions of the protocerebrum were targeted; the medial site close to the central–body complex, the antero-lateral site close to the mushroom-body calyx and the postero-lateral site close to the antennal lobe.

A pair of platinum wires (ø = 0.1 mm) was placed on either side of the targeted brain region in advance of the plasmid injection. Immediately after the plasmid injection, one poring pulse (PP; duration: 0.1 ms, voltage: 25–100 V), followed by five transfer pulses (TPs; pulse duration: 50 ms, frequency: 1 Hz, voltage: 10–50 V), were delivered at defined voltages using an electroporator (NEPA21, NEPA GENE, Ichikawa, Japan; Fig. 1B). After electroporation, the head cuticle was replaced into the original position. In some cases a small volume of cricket saline was placed onto the brain to prevent drying out. Crickets were left in the harness until the head cuticles stabilized. They were then released and left in individual plastic cups overnight. The following day, the whole brain was dissected and observed using stereoscopic fluorescence microscopy (MZ16F, Leica Microsystems, Wetzlar, Germany), with