Journal of Insect Physiology 56 (2010) 21–27

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Functional consequences of mutations in the *Drosophila* histamine receptor HCLB

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ARTICLE INFO

Article history:
Received 25 March 2009
Received in revised form 17 June 2009
Accepted 19 August 2009

Keywords:
Histamine receptor
Anaesthesia
Avermectin
Visual signalling

ABSTRACT

The gene *hclB* encodes a histamine-gated chloride channel subunit in *Drosophila melanogaster*. Mutations in *hclB* lead to defects in the visual system and altered sensitivity to the action of ivermectin. To investigate whether this member of the Cys-loop receptors is common across the Insecta, we analysed the genomes of seven other insect species (Diptera, Hymenoptera, Coleoptera) and revealed orthologues of *hclB* in all of them. Sequence comparisons showed high identity levels between the orthologues, indicating similar constraints and conserved function between the species. Two *D. melanogaster* mutants, $hclB^{T1}$ (P293S) and $hclB^{T2}$ (W111*, a null mutation) were tested for the lapse into, and recovery from, paralysis induced by high temperature or the anaesthetic action of halothane. At 41 °C, the $hclB^{T2}$ flies lapsed into coma faster than wild-type or the $hclB^{T1}$ flies, while both mutants recovered more slowly. A substantially impaired recovery rate was also observed in $hclB^{T1}$ after anaesthesia with halothane. Enhanced synaptic signalling at low-intensity light stimuli was registered on electroretinograms recorded from the two mutant strains. Our results suggest that HCLB may play an essential and conserved role in insect neurophysiology.

1. Introduction

Fast neurotransmission mediated by histamine (Hardie, 1987, 1989a) is a common signalling mechanism in arthropod visual pathways (Hardie, 1989b; Nassel, 1999; Stuart, 1999; Stuart et al., 2007). Two genes, *hclA* and *hclB* (the genetic nomenclature is according to Geng et al., 2002), each encoding a distinct ionotropic histamine receptor subunit, were recently identified in *Drosophila* (Geng et al., 2002; Gisselmann et al., 2002; Witte et al., 2002; Zheng et al., 2002). *In vitro* expression studies demonstrated the formation of homomeric HCLA and HCLB chloride channels (Gisselmann et al., 2002; Zheng et al., 2002), or heteromeric HCLA/HCLB channels (Zheng et al., 2002), all of which responded to histamine. Heterologously expressed HCLB and HCLA/HCLB channels are also been activated by the macrocyclic lactone, ivermectin (Zheng et al., 2002), whose mode of action is the specific and essentially irreversible activation of ligand-gated chloride channels. The two subunits have the highest sequence identity (30–40%) to mammalian glycine and GABA receptors.

In the visual system, HCLA is expressed in the large monopolar cells (LMCs), while HCLB is exclusively localized to the glial cells in the lamina (Pantazis et al., 2008). *hclA* (initially known as *ort*) mutations lead to defective vision, documented as electroretinogram (ERG) records lacking the on- and off-transient components (Koenig and Merriam, 1977; O'Tousa et al., 1989). The studies of Heisenberg (1971) and Coombe (1986) demonstrated that the lack of these transient components is the result of impaired synaptic transmission between the photoreceptors and their targets, the large monopolar cells in the lamina, which is consistent with the expression data. By contrast, ERG records from *hclB* mutants have been reported to contain both components; the on-transients only (Pantazis et al., 2008) or both on- and off-transients (Yusein et al., 2008) having higher amplitudes than those from control flies. At the same time, the LMCs response to low-intensity brief flashes, where the on- and off-components are not separated, were shown to be less sensitive than the corresponding responses of the controls (Pantazis et al., 2008). It was suggested that the HCLB channels participate in the modulation of the visual responses. However, it remains unknown if the sensitivity of on- and off-transient responses in all *hclB* mutants is affected in a similar way.

The use of the reporter gene strategy (Hong et al., 2006) allowed the mapping the expression of these proteins in many other cells of adult brain and the thoracic ganglia. It is well-known that mutations in a single synaptic protein may result in diverse neurological effects, as reported for the *Drosophila* voltage-sensitive sodium channel *paralytic* (Loughney et al., 1989), where mutations result in hypersensitivity to increased temperature and defects in olfaction, circadian rhythms and courtship (reviewed in: Wu and Ganetzky, 1992; Smith, 1996; Young, 1998; Greenspan

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0022-1910
doi:10.1016/j.jinsphys.2009.08.016

and Ferveur, 2000). Known non-visual phenotypes of the *ort* mutant flies include abnormal responses to mechanical shock (bang sensitivity) or diethyl ether (Iovchev et al., 2002), and mutant-specific temperature preferences (Hong et al., 2006), all implying that HCLA has functions outside the visual system. Hong et al. (2006) also described the first *hclB* null mutant (*HisCl1*^{134}) and demonstrated that it not only prefers a higher temperature than normal flies, but also has a reduced tolerance for high temperatures. Recently, we identified two new *Drosophila* mutants, *hclB*^{T1}(P293S) and *hclB*^{T2}(W111*, a null mutation), and showed that they have allele-specific visual system phenotypes and altered susceptibility to ivermectin (Yusein et al., 2008). Since the proline residue at position 293 affected in *hclB*^{T1} is highly conserved across the ligand-gated chloride channel family, we explore here the effect of its substitution on the tolerance of flies to high temperature. As it is known that the HCLA subunit is involved in the response to anaesthesia (Iovchev et al., 2002), we also explored the influence of HCLB-containing channels on sensitivity to the anaesthetic agent, halothane.

We show that mutations in *hclB* lead to allele-specific responses of mutant flies to both high temperature and anaesthesia. We also demonstrate that the absolute sensitivity of both on- and off-transient responses in the ERG is increased to almost the same degree in the *hclB* mutants. The high degree of identity of HCLB orthologues from various insect species allows us to conclude that the gene has an important role in nervous systems across the Insecta.

2. Materials and methods

2.1. Drosophila stocks

Two strains with mutant *hclB* alleles having the genotype: *st hclB*^{T1}*/TM3*, *Sb* and *st hclB*^{T2}*/TM3*, *Sb* were described in Yusein et al. (2008). The flies were kept on yeast-molasses medium at 25 °C. For all experiments we used 3–7-day-old hemizygous females produced by crossing of mutant strains with flies *Df(3R)E79/MRS*, *Sb* where the deficiency (86F1–87B9) eliminates the *hclB* chromosomal region. Hemizygous control flies were obtained in a similar way from Oregon R (OR).

2.2. Genetic nomenclature

Here we follow the nomenclature proposed by Geng et al. (2002): *hclA* (*ort*) and *hclB*. The two genes are also referred to as *HisCl2* and *HisCl1* respectively by Zheng et al. (2002) and *HisCl-α1* and *HisCl-*α2 by Gisselmann et al. (2002).

2.3. Bioinformatics analysis

The *hclB* orthologues from *Apis mellifera* (honeybee, Hymenoptera), *Anopheles gambiae* (malaria mosquito, Diptera), *Aedes aegypti* (the yellow fever mosquito, Diptera), *Tribolium castaneum* (red flour beetle, Coleoptera), *Drosophila ananassae*, *Drosophila pseudoobscura*, and *Drosophila virilis* were identified under the analysis of alignments of the *Dm-hclB* (NM_169429) sequence to the corresponding whole genome sequences from NCBI trace archives at (http://www.ncbi.nlm.nih.gov/BLAST/tracemb.shtml). Alignments of the predicted amino-acid sequences to known protein database were performed by the use of the Predict Protein software (http://www.cubic.bioc.columbia.edu/predictprotein/). The HCLBs from *Drosophilidae* species were aligned and compared by whole protein sequences (1–426aa of Dm-HCLB), whereas other *Insecta* species were aligned as following Aeae: 35–426aa; Anga: 29–426aa; Amel: 51–371aa; Tric: 51–426aa because of incomplete genome sequences.

2.4. Behavioral assays

2.4.1. Knock-down analysis

Flies were placed in groups of ten in the vials, 2 cm in diameter, with cotton plugs by brief anaesthesia with carbon dioxide. After 1 h recovery they were put into an incubator with a transparent front, set at 41 °C. The number of flies that were unable to stand and fell to the bottom of vials was recorded at 1 min intervals. After 10 min all flies were knocked down.

2.4.2. Measurement of arousal time after heat shock

The assays were modified from Hong et al. (2006). After knock-down analysis, flies were immediately removed from the thermostat and placed in plastic Petri dishes at 20 °C. The number of flies that could stand and walk was counted; for the first 30 min the number of aroused flies was recorded at 5 min intervals. All recovered animals were removed from the Petri dish by aspiration with a pipette to avoid any influence on the rest. After measurements at 45 and 60 min flies were replaced in vials containing a minimal medium covered with living yeasts and their recovery was measured after 3 and 24 h.

2.4.3. Measurement of time for anaesthesia with halothane

All experiments were performed with halothane ('Narcotan', Zentiva International, Czech Republic) in a temperature controlled room at 22 °C. Adults from the three genotypes were anaesthetized in a desiccator of 5400 cm^3 volume. The surfaces of parts that can detach were coated with silicone. An oval-shaped easel with places for vials was placed into the desiccator. A glass dish was placed into the middle of the easel and liquid anaesthetic was poured from the hole of the lid. Ten flies were placed in each vial by brief anaesthesia with carbon dioxide. Flies were allowed to recover for 1 h. Then they were placed into the desiccator. The recording started immediately after closing of the lid and addition of 5 ml halothane. During the experiment a saturated atmosphere of halothane was gradually reached. Every minute flies that were unable to stand and fell to the bottom of vials were counted. All flies were paralyzed after 15 min.

2.4.4. Time of recovery after anaesthesia with halothane

The flies anaesthetized as described above were replaced into plastic Petri dishes at 25 °C. The recording of fly recovery was performed in the same way as the arousal time after heat shock. The viability of flies was counted after 24 h.

2.5. ERG recording

Recordings were carried out as described by Yusein et al. (2008). The electroretinograms were recorded using glass pipette microelectrodes with a tip diameter of 15–20 μm. The microelectrodes were filled with Ringer solution (in mmol/l: NaCl 130, KCl 4.7, $CaCl_2$ 1.9, $MgCl_2$ 4, HEPES 1.3; pH 7.14). The ERG responses were amplified at a bandpass of 0–1000 Hz using low noise WPI ISO-DAM preamplifier. They were digitized at 5 kHz and analysed using WPI LAB-Trax4 Data acquisition system (Data-Trax software). Diffuse light from green LUXEON®V LED (LXHL-PMo2; Lumileds Future Electronics) with a dominant wavelength of 530 nm was used for light stimulation. The stimulus intensity was changed at 0.5 log unit steps within a range of 5 log units. The maximal intensity used (denoted by 0) was 5.39×10^6 quanta s^{-1} μm^{-2} at the plane of the eye. After 2 min dark adaptation, intermittent stimuli with 2 s ON and 8 s OFF periods were given.

2.6. Data analysis

Student's *t*-test was used for statistical evaluation of all data.