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RNAi reveals the key role of Nervana 1 in cockroach oogenesis and embryo development

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ABSTRACT

Na^+, K^+-ATPases is a heterodimer protein consisting of α- and β-subunits that control the ion transport through cell membranes. In insects the β-subunit of the Na^+, K^+-ATPase, known as Nervana, was characterized as a nervous system-specific glycoprotein antigen from adult *Drosophila melanogaster* heads. Nervana is expressed ubiquitously in all insect tissues, and in epithelial cells appeared located in a basolateral position as part of the septate junctions. Herein we study two Nervana isoforms from *Blattella germanica*, a cockroach species with panoistic ovaries. The sequencing and the phylogenetic analysis results suggest that these two isoforms are orthologs of *D. melanogaster* Nervana 1 and Nervana 2, respectively. Nervana 1 is highly expressed in the ovary of *B. germanica*, and depleting its expression results in changes in oocyte shape that do not impair oviposition. However, the resulting embryos show different defects and never hatch. These findings highlight the importance of this type of membrane pump in insect oogenesis as well as in embryo development, and its possible regulation by juvenile hormone.

1. Introduction

The flow of nutrients and metabolites through cell membranes is facilitated by ion gradients that change the cell membrane potential and contribute to the resting membrane potential, thereby serving as a driving force for the transport of materials. An important part of this ion gradient is established by Na^+ pumps which maintain the low internal Na^+ and high internal K^+ concentrations characteristic of most animal cells (Grindstaff et al., 1996). These pumps are sodium- and potassium-dependent adenosine triphosphatase (Na^+, K^+-ATPase), a plasma membrane enzyme that couples ATP hydrolysis to Na^+ and K^+ exchange against their respective chemical gradients (Kaplan, 2002).

Na^+, K^+-ATPase is a heterodimer protein consisting of α- and β-subunits present in equimolar ratios (Kaplan, 2002; Lingrel and Kuntzweiler, 1994). Multiple isoforms of both α- and β-subunits have been identified, and their number can vary among species. Thus, three isoforms of the β-subunit and two of the α-subunit have been described in the fruit fly *Drosophila melanogaster*, (Paul et al., 2007; Sun and Salvaterra, 1995b) whereas four isoforms have been reported for each subunit in humans (Kaplan, 2002; Lingrel et al., 1990). These subunits may associate into αβ-dimers in different combinations displaying distinct distribution patterns between and within tissues during ontogenesis (Lopina, 2001; Mobasheri et al., 2000). The α-subunit, which has a molecular mass of 113 kDa, shows 10 transmembrane domains containing short extracellular loops and larger cytoplasmic regions (Kaplan, 2002). A phosphorylation site in this subunit plays a major role in the catalytic function of the enzyme (Lingrel et al., 1994). The β-subunit of Na^+, K^+-ATPase, known as Nervana in *D. melanogaster*, is a glycoprotein containing a single transmembrane domain with a short amino-terminal cytoplasmic domain (around 35 residues) and a larger carboxy-terminal extracellular region that usually contains a number of glycosylation sites and three disulfide bonds (Kaplan, 2002). This β-subunit corresponds to the small subunit of the enzyme (55 kDa) and is involved in the maturation of the holoenzyme (Ackermann and Geering, 1990; Geering et al., 1989) and subsequent transport of the α-subunit to the plasma membrane (Fambrough, 1988; Noguchi et al., 1987; Takeyasu and Kawakami, 1989). In an evolutionary context, both α- and β-subunits exhibit high similarity in the conserved amino acid domains, across the entire animal kingdom (Sun et al., 1998), where gene duplication of both α- and β-subunit genes has independently led to the

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occurrence of isoform diversity within various animal phyla (Okamura et al., 2003).

Cell volume regulation associated with potassium exchange has been observed in many cell types. In the cockroaches *Blattella germanica* and *Periplaneta americana*, for example, Kunkel (1991) and Kunkel and Faszewski (1995) highlighted the importance of ion fluxes for vitellogenin uptake and oocyte growth, and established that these fluxes may be mediated by Na^+, K^+ pumps. Similarly, in *D. melanogaster*, Bohrmann (1991) and Bohrmann and Braun (1999) used biochemical and cytochemical methods to show that changes in the volume of follicular cells are associated with variations in the extrafollicular currents pattern. Moreover, Sun and Salvaterra (1995a) purified and characterized a nervous system-specific glycoprotein antigen from adult *D. melanogaster* heads corresponding to a β-subunit of a Na^+, K^+-ATPase, and named it Nervana (nerve antigen; Nrv). These authors subsequently described the expression, localization and function of three Nervana isoforms in the nervous system of *D. melanogaster* embryos (Baumann et al., 2010; Sun and Salvaterra, 1995a, 1995b; Sun et al., 1998; Xu et al., 1999). More recently, new functions have been described for these β-subunits related to cell–cell contact (Genova and Fehon, 2003; Kometiani et al., 1998; Paul et al., 2007; Vagin et al., 2006), signal transduction (Kometiani et al., 1998) and epithelial polarity in the embryo (Laprise et al., 2009). Furthermore, roles unrelated to pump function have recently been assigned to *D. melanogaster* Nrv2. Thus, according to a study by Fehon and coworkers (Genova and Fehon, 2003; Oshima and Fehon, 2011), Nrv2 is involved in the formation of septate junctions in embryonic epithelial cells, where both subunits form a core with four proteins, coracle, neurexin, gliotactin and neuroglian, thus forming this paracellular diffusion barrier. However, although Nrv1 and Nrv2 are co-expressed in the septate junctions in a number of *D. melanogaster* tissues, Nrv1 is not required to maintain the paracellular diffusion barrier (Genova and Fehon, 2003; Paul et al., 2007).

The purpose of our work is contributing to understand oogenesis in panoistic ovaries using the cockroach *B. germanica*, a hemimetabolous insect. One characteristic of this cockroach species is that only one batch of basal follicles mature synchronously in each gonadotrophic cycle, which in our laboratory conditions lasts 7 days. At oviposition, eggs are packaged into an egg-case or ootheca that is transported by the female during 18 days, until egg hatching, and during this period the ovarian activity is arrested (Irles et al., 2009). Herein we characterize two isoforms of the β-subunit of Na^+, K^+-ATPase in the cockroach. Our phylogenetic studies suggest that the two isoforms are orthologs of *D. melanogaster* Nrv1 and Nrv2, respectively. We describe the regulatory effects of juvenile hormone (JH) upon them, whereas functional studies using RNAi approaches highlight the key role played by *B. germanica* Nervana 1 in the oogenesis of panoistic ovaries, as well in the resulting embryos that show different defects in their development and never hatch. With the aim to unveil Nervana action on embryo development, wingless and decapentaplegic expression have been measured in embryos from Nervana knockdown females. These two morphogens are important during embryogenesis determining axis development and, in case of dpp. participating in dorsal closure.

2. Materials and methods

2.1. Cockroach colony and tissue sampling

Adult females of *B. germanica* were obtained from a colony fed on Panlab dog chow and water *ad libitum*, and reared in the dark at 29 ± 1 °C and 60–70% r.h (Cruz et al., 2003). Freshly ecdysed adult females were selected and used at appropriate ages. Mated females were used in all experiments; the presence of spermatozoa in the spermatheca was assessed at the end of the experiment, indicating that mating had occurred. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens, under Ringer's saline (1.8 mM $CaCl_2$, 154 mM NaCl, 2.68 mM KCl and 2.38 mM $NaHCO_3$).

2.2. Cloning of B. germanica Nervana cDNA

A 1004 bp fragment of *B. germanica* Nervana 1 (BgNrv1) was isolated from an ovarian cDNA subtractive library previously obtained (Irles et al., 2009). The fragment contains the coding region from the first methionine to poly(A) tail, but lacked the 5′ UTR. To complete it, 5′-rapid amplification of cDNA ends (RACE) was applied to RNA extracted from chorionated ovaries of 7-day-old females, using FirstChoice® RLM-RACE (Ambion, Huntingdon, Cambridgeshire, UK), according to the manufacturer's instructions. The complete ORF of *B. germanica* Nervana 2 (BgNrv2; 975 bp) was obtained from an EST library of *B. germanica* ovary obtained in the laboratory. Both, BgNrv1 and BgNrv2, were amplified, cloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced. Primer sequences used in these experiments are detailed in Table S1.

2.3. Sequence comparisons and phylogenetic analysis

Sequences of Na^+, K^+-ATPase β subunit proteins from arthropods were retrieved from GenBank (Table S2). The search was enlarged by BLAST using the BgNrv and *D. melanogaster* Nervana proteins as queries. As outgroup, we used two Na^+, K^+-ATPase sequences from the nematode *Caenorhabditis elegans*.

Protein sequences were aligned with those obtained in *B. germanica* using the online software MAFFT (http://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2002). The resulting alignment was analysed by the PHYML 3.0 program (Guindon and Gascuel, 2003) based on the maximum-likelihood principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. The data was bootstrapped for 100 replicates using PHYML 3.0 program.

2.4. Juvenile hormone experiments

Two different experimental approaches were used to test the response of BgNrv to juvenile hormone (JH). First, newly emerged adult females were allatectomized (Piulachs et al., 1992), three days later they were topically treated with 10 μg of JH III (Sigma) in 1 μl of acetone, and ovaries were explanted 2–4 h post-treatment. Controls were equivalently treated with acetone. The second approach involved an *in vitro* experiment using UM-BGE-1 cells derived from 4- to 5-day-old embryos of *B. germanica* (Kurtti and Brooks, 1977), which were maintained at 25 °C in Leibovitz-15 medium (Sigma, Madrid, Spain) supplemented as recommended by Munderloh and Kurtti (1989). For JH treatment, 10^6 cells ml^{-1} were seeded into 24-well cell-culture clusters (Costar, Amsterdam, The Netherlands) containing 10^{-6} M of JH III, as described previously (Maestro et al., 2005). Wells of control cells were added with the equivalent volume of acetone (1 μL). They were harvested into buffer lysis 2 and 6 h post-treatment and kept at −80 °C until processing.

2.5. RNA extraction and expression studies

Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma). An amount of 400 ng from each RNA extraction was DNAse treated (Promega, Madison, WI, USA) and reverse