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Disruption of the blood–brain barrier exacerbates spreading depression in the locust CNS

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ABSTRACT

In response to cellular stress in the nervous system of the locust (*Locusta migratoria*) neural function is interrupted in association with ionic disturbances propagating throughout nervous tissue (Spreading depression; SD). The insect blood–brain barrier (BBB) plays a critical role in the regulation of ion levels within the CNS. We investigated how a disruption in barrier function by transient exposure to 3M urea affects locusts' vulnerability to disturbances in ion levels. Repetitive SD was induced by bath application of ouabain and the extracellular potassium concentration ($[K^+]_o$) within the metathoracic ganglion (MTG) was monitored. Urea treatment increased the susceptibility to ouabain and caused a progressive impairment in the ability to maintain baseline $[K^+]_o$ levels during episodes of repetitive SD. Additionally, using a within animal protocol we demonstrate that waves of SD, induced by high K^+, propagate throughout the MTG faster following disruption of the BBB. Lastly, we show that targeting the BBB of intact animals reduces their ability to sustain neural function during anoxic conditions. Our findings indicate that locust's ability to withstand stress is diminished following a reduction in barrier function likely due to an impairment of the ability of neural tissue to maintain ionic gradients.

1. Introduction

The extracellular environment of the insect CNS is largely regulated by the blood–brain barrier (BBB) which is composed of overlapping perineurial cells. These specialized glial cells are connected by both septate and tight junctions forming a functional diffusion barrier; the perineurium (Schofield and Treherne, 1984; Treherne and Schofield, 1981). Having a well developed diffusion barrier is essential for insects as the content of their hemolymph is often quite different from the fluid surrounding axons and is subject to large fluctuations in ionic composition (Hoyle, 1954; Pichon, 1970). In addition to serving as a passive diffusion barrier, the perineurium also actively contributes to the maintenance of extracellular ionic gradients through the use of ion pumps such as the Na^+/K^+-ATPase (Kocmarek and O'Donnell, 2011; Treherne and Schofield, 1981). Furthermore, the perineurial cells are coupled by gap junctions to the underlying glial cells surrounding axons, building an architecture well-suited to aid in the regulation of ion levels. The restricted nature of the extracellular compartment in the insect CNS makes them vulnerable to alterations in ion concentrations (Treherne and Schofield, 1981) and thus active maintenance of ionic gradients is critical to ensure optimal neural performance.

A loss in ionic homeostasis is often associated with diminished neural function (Money et al., 2009; Rodgers et al., 2007; Rounds, 1967; Wu and Fisher, 2000). For instance, in response to severe metabolic stress such as anoxia, hyperthermia or hypothermia, locusts experience a silencing of both neural and muscular systems which is paired with a significant increase in extracellular potassium concentration ($[K^+]_o$) within the metathoracic ganglion (MTG) (Rodgers et al., 2007, 2010). In most cases, once the stress is removed $[K^+]_o$ is observed to return to normal levels and this coincides with recovery from the coma-like state (Rodgers et al., 2007). Directly disrupting ionic homeostasis within the locust MTG, using high K^+ saline injections or by inhibition of the Na^+/K^+-ATPase, has similar consequences. In semi-intact locust preparations, continuous bath application of ouabain (Na^+/K^+-ATPase inhibitor) induces repetitive surges in $[K^+]_o$ where the rise and fall of $[K^+]_o$ occur simultaneously with the arrest and recovery of electrical activity (Rodgers et al., 2007, 2009). This ionic disturbance and associated depression in neural activity propagate

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; $[K^+]_o$, extracellular potassium concentration; MTG, metathoracic ganglion; $[Na^+]_o$, extracellular sodium concentration; OUA, ouabain; SD, spreading depression.

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throughout the locust MTG, and thus represent a form of spreading depression (SD) (Rodgers et al., 2007).

SD occurs in both vertebrate and invertebrate nervous systems and it is characterized by a massive redistribution of ions, notably a drastic increase in $[K^+]_o$ and decrease in $[Na^+]_o$ accompanied by an arrest in electrical activity (Leão, 1944; Rodgers et al., 2007, 2010; Rounds, 1967; Somjen, 2001). Although the cellular mechanisms underlying SD are still incompletely understood, it is hypothesized that SD events in the locust are triggered by positive feedback cycles initiated when processes of $[K^+]_o$ accumulation overwhelm the ability to clear $[K^+]_o$ from a restricted extracellular compartment (Armstrong et al., 2009; Rodgers et al., 2009). Recently, we have shown that SD events in the locust can be modulated pharmacologically by targeting glial mechanisms of K^+ homeostasis. For instance, disrupting glial spatial buffering, a mechanism known to help maintain $[K^+]_o$ levels, increases the vulnerability to SD induced by ouabain (Spong and Robertson, 2013). Given the passive and active role of the perineurium in the maintenance of extracellular ion homeostasis and its connectivity with underlying glial cells, we hypothesize that the insect BBB plays an important role during SD in the locust CNS.

The purpose of the present paper is to gain a better understanding of how the insect BBB contributes to ionic homeostasis during stress-induced disturbances in ion levels and to extend our knowledge of the cellular mechanisms underlying SD in the locust. Short exposure to a high concentration of urea reduces the efficacy of the insect BBB and allows increased access of water-soluble cations to neuronal surfaces (Treherne et al., 1973). This disruption in the barrier system is not caused by cellular damage but is thought to result from a change in permeability in either the perineurial cell membranes or tight junctions (Treherne et al., 1973). This is supported by the observation that following urea treatment extracellular tracers are still incapable of penetrating the perineurium and additionally the membrane potentials of perineurial cells and the resting and action potentials of the underlying axons are unaffected by urea exposure (Treherne et al., 1973). Using the technique described above we investigated the contribution of the perineurium to the regulation of ion homeostasis during stress-induced increases in $[K^+]_o$. To examine how a reduction in BBB function affects susceptibility to SD we measured $[K^+]_o$ within the MTG and subjected locusts to a brief exposure to urea prior to inducing repetitive SD with ouabain. Additionally, we investigated the effects of urea treatment on SD propagation by recording DC potential shifts (abrupt negative shifts indicate SD occurrence) at two different locations within the MTG. In these experiments, we induced single SD events by pressure injection of high K^+ saline, before and after treatment with urea. Lastly, we tested the effects of BBB disruption on the intact animal where locusts were subjected to an anoxic treatment following urea injections into the hemolymph. When locusts are submerged under water they enter a reversible coma where the arrest in CNS function coincides with a loss of K^+ homeostasis (Armstrong et al., 2009). We examined how urea affects the time it takes animals to succumb (enter coma) to anoxic treatments, which provided direct information on the ability to maintain ionic gradients during stress.

2. Materials and methods

2.1. Animals

Adult male locusts (*Locusta migratoria*) aged 3–5 weeks past imaginal ecdysis were used for experimentation. Animals were housed in crowded cages under a 12 h:12 h light:dark cycle within a colony located in the Animal Care Facility of the Biosciences Complex at Queen's University. Room temperature was maintained at 25 ± 1 °C. Locusts were fed once daily and raised on a diet consisting of wheat grass and a mixture of bran, skim milk powder, and torula yeast. Animals were randomly obtained from the colony prior to 11:00am each day and held in a ventilated container prior to experimentation.

2.2. Locust dissection

A semi-intact preparation was used in all experiments measuring $[K^+]_o$ and SD propagation. Locusts were pinned dorsal side up on a corkboard after a dorsal midline incision was made and the legs, wings and pronotum were removed. The metathoracic ganglion (MTG) and ventral nerve cord were exposed by clearing the gut, air sacs and fat bodies. The thoracic and abdominal cavities were continuously bathed with standard locust saline which contained (in mM) 147 NaCl, 10 KCl, 4 CaCl, 3 NaOH, and 10 HEPES buffer (pH 7.2; all chemicals were obtained from Sigma–Aldrich). Using a Peri-Star peristaltic pump (World Precision Instruments Inc.) the flow of saline was directed into the anterior region of the thorax and exited through an incision made in the posterior abdomen wall. To gain access to the MTG the second spina situated between the connectives and the tissue covering the nerve cord were removed. A metal plate was placed beneath the MTG to stabilize it and a silver wire was inserted into the anterior region of the thorax to ground the preparation.

2.3. Preparation of potassium-sensitive microelectrodes

K^+ – sensitive microelectrodes were made using 1 mm diameter unfilamented glass capillary tubes (World Precision Instruments Inc.). The capillary tubes were cleaned by washing them with 99.9% methanol and then dried on a hotplate. A programmable glass puller (Sutter Instruments Co.) was used to form microelectrodes with a low resistance tip (5–7 MΩ). The microelectrodes were then placed on a hotplate (100 °C) and exposed to dichlorodimethylsilane (99%) (Sigma–Aldrich) vapor for one hour to make the inner and outer glass surfaces hydrophobic. The microelectrodes were allowed to cool to room temperature and then filled at the tips with Potassium Ionophore-I-Cocktail B (5% valinomycin; obtained from Sigma–Aldrich) and backfilled with 500 mM KCl using plastic syringes. K^+ – sensitive microelectrodes were stored in a glass beaker with their tips suspended in distilled water until needed for experimentation.

2.4. Measuring extracellular potassium concentration

K^+ voltage within the MTG was continuously monitored using a K^+-sensitive microelectrode and a reference microelectrode and was converted to $[K^+]_o$ (mM) using the Nernst equation (Rodgers et al., 2007). Reference microelectrodes were made prior to each experiment from 1 mm diameter filamented glass capillary tubes pulled to form a low resistance tip (5–7 MΩ) and filled with 3M KCl. The K^+-sensitive microelectrode and reference microelectrode were connected to a DUO773 two-channel intracellular/extracellular amplifier (World Precision Instruments) and inserted through the sheath of the MTG adjacent to one another. Electrode pairs were calibrated preceding each experiment using 15 mM KCl + 135 NaCl and 150 mM KCl solutions to obtain the voltage change or "slope". A 10-fold change in $[K^+]$ concentration should produce a voltage change of 58 mV and therefore electrode pairs were discarded if the slope obtained did not fall in the range between 54 and 62 mV.

2.5. Measuring direct current (DC) potential

In experiments investigating SD propagation DC potential was continuously monitored using microelectrodes with low resistance

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