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Review

Coma in response to environmental stress in the locust: A model for cortical spreading depression

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ABSTRACT

Spreading depression (SD) is an interesting and important phenomenon due to its role in mammalian pathologies such as migraine, seizures, and stroke. Until recently investigations of the mechanisms involved in SD have mostly utilized mammalian cortical tissue, however we have discovered that SD-like events occur in the CNS of an invertebrate model, *Locusta migratoria*. Locusts enter comas in response to stress during which neural and muscular systems shut down until the stress is removed, and this is believed to be an adaptive strategy to survive extreme environmental conditions. During stress-induced comas SD-like events occur in the locust metathoracic ganglion (MTG) that closely resemble cortical SD (CSD) in many respects, including mechanism of induction, extracellular potassium ion changes, and propagation in areas equivalent to mammalian grey matter. In this review we describe the generation of comas and the associated SD-like events in the locust, provide a description of the similarities to CSD, and show how they can be manipulated both by stress preconditioning and pharmacologically. We also suggest that locust SD-like events are adaptive by conserving energy and preventing cellular damage, and we provide a model for the mechanism of SD onset and recovery in the locust nervous system.

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1. Introduction

Under conditions of anoxia or high temperature stress many insects enter a reversible coma which is protective. A temporary shutdown of neural and muscular systems can be considered protective by preventing the irreversible consequences of complete energy loss. Until recently the neural underpinnings of coma were little understood. However during the past 6 years we have described the phenomena underlying environmental stress-induced coma in locusts. We found that such coma is associated with dramatic surges of extracellular K^+ concentration ($[K^+]_o$) and

Abbreviations: AD, anoxic depolarization; CNS, central nervous system; CSD, cortical spreading depression; cGMP, cyclic guanosine monophosphate; EMG, electromyographic; E_k, equilibrium potential for potassium; $[K^+]_o$, extracellular potassium concentration; HS, heat shock; HSPs, heat shock proteins; HSP70, heat shock protein with an average molecular weight of 70 kDa; HSR, heat shock response; MTG, metathoracic ganglion; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PIDs, peri-infarct depolarizations; PKG, protein kinase G; V_m, resting membrane potential; NaN_3, sodium azide; SD, spreading depression; TEA, tetraethylammonium chloride; TTX, tetrodotoxin; vCPG, ventilatory central pattern generator.

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temporary electrical silence in the CNS. Remarkably the phenomenon bears all the hallmarks of spreading depression (SD) in vertebrate CNS grey matter. Given the important role of vertebrate SD in a variety of significant neural dysfunctions the ability to address mechanisms in a system that is as eminently accessible and understood as the locust thoracic nervous system is of considerable potential value. The process can be studied in preparations that have intact neuronal architecture allowing the simultaneous investigation of cellular and subcellular neuronal responses with physiological and behavioural responses without anaesthetic.

In this review we describe the generation of comas by environmental stressors in locust and make the case that they are equivalent to the mechanisms of cortical SD (CSD). In addition we show that the onset and severity of locust SD can be modulated both by pre-treatments and pharmacologically, suggesting that CSD may be similarly modifiable. Finally we speculate about the future use of this model and suggest experimental steps to substantiate it.

2. Environmental stress-induced comas in locust

Use of the African migratory locust (*Locusta migratoria*) to investigate stress-induced comas is warranted as locusts are exposed to high ambient temperature as well as flash floods and monsoons in their natural habitat. In response to anoxic conditions locusts experience a silencing of activity in the nervous and muscular systems that we interpret as a stress-induced coma. After return to normoxia, locusts completely recover from up to at least 6 h of anoxia-induced coma (Wu et al., 2002; Armstrong et al., 2009). Given the reversibility of locust comas it is likely that locusts enter this state as a protective strategy to cope with environmental stressors. Ambient temperature is a major stress for insects inhabiting highly variable thermal environments and environmental temperature fluctuation can strongly influence neuronal circuit function and the behaviour of animals. Our locust research has largely focused on the effects of high temperature stress on a locust model of CNS function, the operation of the ventilatory central pattern generator (vCPG).

The vCPG is a reliable neuronal circuit which coordinates a persistent motor pattern that is responsible for delivering and removing oxygen and carbon dioxide via the tracheal system in the locust (Albrecht, 1953). It is an ideal circuit to study adaptations that exist in the nervous system, because any impairment of its function would have severe repercussions for the entire animal. The vCPG is located in the metathoracic ganglion (MTG) and the rhythm is generated within the first three fused abdominal neuromeres (Hustert, 1975; Burrows, 1996; Bustami and Hustert, 2000). We are able to obtain extracellular recordings from both nerves and muscles, as well as intracellular recordings from ventilatory neurons within the ganglion. Semi-intact preparations superfused with saline at room temperature (~20 °C) ventilate at a frequency of around 1 Hz (Newman et al., 2003) and faster and more regular ventilatory rhythms occur during increased activity (e.g. flight), octopamine neuromodulation, and heat stress (Sombati and Hoyle, 1984; Ramirez and Pearson, 1989; Armstrong and Robertson, 2006). As the temperature of the saline is raised the frequency of the motor pattern increases and at 40 °C the frequency is around 3 Hz. At higher temperatures faster ventilatory rates increase heat loss via tracheal evaporation and keep body temperature much below air temperature (Prange, 1990). A continued increase in temperature results in irregularities in vCPG activity and eventually motor pattern activity stops (Armstrong and Robertson, 2006). However if saline temperature is allowed to return to room level, motor pattern activity recovers (Newman et al., 2003; Armstrong and Robertson, 2006). Given the importance of ionic gradients in neuronal signaling it is not surprising that hyperthermia-induced failure of ventilation and the associated neural activity silence is associated with an extracellular K^+ concentration ($[K^+]_o$) disturbance within the MTG.

To measure changes in $[K^+]_o$ associated with vCPG arrest a K^+-sensitive microelectrode can be inserted through the sheath of the MTG into the extracellular space surrounding the vCPG and referenced to an adjacent voltage electrode (Fig. 1A). To monitor vCPG output from a ventilatory muscle an electromyographic (EMG) electrode can be placed on ventilatory muscle 176 in the abdomen of the locust (Fig. 1A and B). To determine the extent of neuronal depolarization associated with vCPG arrest, sharp intracellular microelectrodes are used to record neuronal activity in the MTG concurrently with recordings of the ventilatory motor pattern and $[K^+]_o$ (Fig. 1A and B). Measurements of $[K^+]_o$ made in the MTG revealed a tight correlation between heat-induced arrest of ventilatory motor pattern generation and a surge in $[K^+]_o$ surrounding the vCPG. The rapid rise in $[K^+]_o$ at the moment of heat-induced CNS failure increased from a baseline level of 10 mM to a plateau of around 50 mM (Robertson, 2004b; Rodgers et al., 2007). When the heater was switched off and saline temperature returned to room levels, $[K^+]_o$ returned to baseline levels and vCPG activity resumed.

The abrupt rise in $[K^+]_o$ was not exclusive to hyperthermia-induced vCPG arrest in the locust, but has been found to occur in response to several different cellular stressors (Fig. 1C–E, Fig. 3). Arrest of ventilation in response to O_2 and ATP depletion, Na^+/K^+ ATPase impairment, and neuropile nanoinjections of K^+ was associated with abrupt increases in $[K^+]_o$. Experiments performed in a nitrogen gas-filled chamber (anoxia) or preparations treated with sodium azide (mitochondrial blocker) produced $[K^+]_o$ events in the MTG (Fig. 1C) (Rodgers et al., 2007). The $[K^+]_o$ peaks induced by NaN_3 and anoxia were significantly higher than the peak $[K^+]_o$ induced by hyperthermia (NaN_3 peak: 85 ± 2 mM, Anoxia peak: 79 ± 3 mM, compared to 52 ± 2 mM during hyperthermia). Recovery from the anoxic coma occurred when air was allowed to re-enter the chamber (Fig. 1C) or when sodium azide was flushed out of the preparation. The Na^+/K^+-ATPase plays a major role in maintaining ionic gradients needed for proper neuronal activity. Experiments performed using ouabain (an inhibitor of Na^+/K^+-ATPase activity) demonstrated recurring $[K^+]_o$ events (Fig. 1D) (Rodgers et al., 2007, 2009; Armstrong et al., 2009). Continuous bath application of 10^{-4} M ouabain elicited multiple surges in $[K^+]_o$ where the rise and fall of $[K^+]_o$ was associated with failure and recovery of the ventilatory motor pattern, respectively (Fig. 1D). Finally, another reliable way of inducing coma in the locust is to artificially reduce E_k. This was achieved by delivering nanoinjections of saline containing high KCl content directly into the ventilatory neuropil, which induced an abrupt $[K^+]_o$ surge once a threshold was reached (Rodgers et al., 2007; Armstrong et al., 2009) (Fig. 1E). Raising the $[K^+]_o$ in this manner would decrease E_k and depolarize the V_m. Recovery of neuronal activity following KCl injections might be dependent on clearance of excess K^+ from the extracellular space and restoration of E_k. The stress-induced comas described here and the associated abrupt all-or-none type increases in $[K^+]_o$ in the locust MTG share many of the major characteristics of spreading depression (SD) in mammalian cortical tissue, at least with respect to $[K^+]_o$. The major aim of this review was to describe the occurrence of these events in the locust and their resemblance to SD in mammalian tissue.

3. Locust SD in comparison to cortical SD (CSD)

Inhibition of neuronal activity due to high $[K^+]_o$ in insects has previously been described (Hoyle, 1952), and SD was first proposed

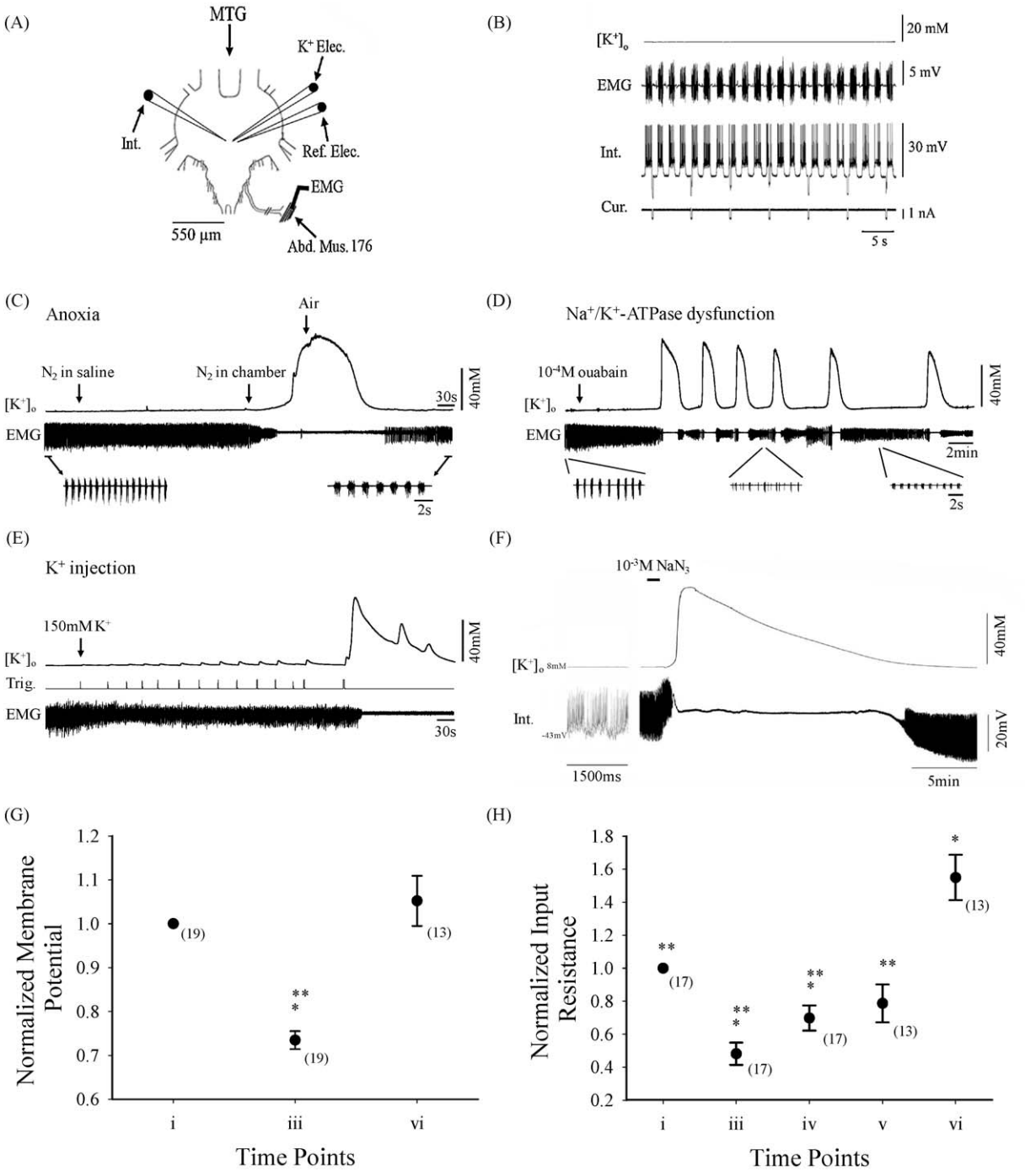

Fig. 1. Key features of spreading depression in the locust MTG. (A) Schematic diagram of the MTG and recording electrodes used to measure extracellular potassium concentration ($[K^+]_o$) and vCPG activity (Int., intracellular electrode; K^+ Elec., potassium ion electrode; Ref. Elec., reference electrode for measuring $[K^+]_o$; Abd. Mus. 176, abdominal muscle 176, one of the ventilatory muscles). (B) Sample recording from a ventilatory interneuron (Int.) and associated motor activity (EMG). Baseline level of $[K^+]_o$ is 8 mM. Lower trace (Cur.) shows −1n *A* hyperpolarizing current pulses (0.2 Hz). (C–E) Simultaneous recordings of the extracellular potassium concentration ($[K^+]_o$) and the ventilatory motor pattern using an electromyographic electrode (EMG). (C) In the locust, we could induce a SD-like surge in $[K^+]_o$ in response to treatments that reduce energy as in cortical SD. In order to deplete oxygen nitrogen gas (N_2) was bubbled into the superfusing saline for 5 min, then blown over the preparation which was in an enclosed chamber until 1 min post-failure. Arrest of the ventilatory motor pattern was reliably associated with an abrupt increase in $[K^+]_o$ surrounding the vCPG. $[K^+]_o$ was restored to normal baseline levels if N_2 gas was cut off and air allowed to enter the chamber, and this was associated with recovery of ventilatory motor patterning. The expansions show the motor pattern trace pre- and post-stress. (D) Abrupt surges in $[K^+]_o$ were reliably triggered by Na^+/K^+-ATPase inhibition using ouabain. Continuous bath application of 10^{-4} M ouabain elicited multiple surges in $[K^+]_o$ where the rise and fall of $[K^+]_o$ was associated with failure and recovery of the ventilatory motor pattern, respectively. On average, time to onset of the first $[K^+]_o$ event was approximately 9.3 min. $[K^+]_o$ increased to 63 ± 5 mM during the initial surge and subsequently returned to a mean baseline level of 10 ± 0.2 mM. (E) SD-like events in the locust could also be induced by artificially increasing the $[K^+]_o$ within the MTG. This figure shows simultaneous recordings of the ventilatory motor pattern (EMG), pressure-injection of a bolus of K^+ within the MTG (Trig.) and the extracellular potassium concentration ($[K^+]_o$). A 35 nl injection of locust saline containing a 15-fold higher [K +] (150 mM compared to 10 mM) was sufficient to bring $[K^+]_o$ to threshold for induction of an abrupt surge. Injection of very small volumes of high K^+ saline triggered a tissue response (indicated by multiple injections), however ignition of the all-or-none $[K^+]_o$ event occurred only when a threshold was reached. (F) To determine the extent of neuronal depolarization during SD-like events, sharp intracellular microelectrodes were used to record neuronal activity in the MTG (Int.) concurrently with recordings of $[K^+]_o$ during bath application of NaN_3. We recorded from neurons that were active in phase with ventilation. The surge in $[K^+]_o$ coincided with membrane depolarization and cessation of neuronal activity. Recovery of vCPG activity occurred when $[K^+]_o$ returned toward normal values and neurons repolarized. (G) There was a statistically significant 30%

to occur in insects over 40 years ago (Rounds, 1967). In mammals, SD has been well documented and studied in the CNS including those of the mouse, rat, rabbit, cat, monkey and human (Leão, 1944; Van Harreveld et al., 1956; Van Harreveld and Khattab, 1967; Aitken et al., 1991; Gorji et al., 2001). When SD occurs in mammalian cortical tissue it is referred to as cortical spreading depression (CSD). The occurrence of SD is not limited to mammals having been observed in the CNS of the frog (*Rana*), turtle (*Pseudemys*), and in the retinas of the chicken (*Gallus*), toad (*Bufo*), and lizard (*Tupinambis*) (Martins-Ferreira and de Castro, 1966; Lauritzen et al., 1988; Streit et al., 1995; Guedes et al., 2005; Hanke and Fernandes de Lima, 2008; for reviews see Somjen, 2001; Smith et al., 2006). SD is initiated following mechanical (contusions), electrical (high frequency stimulation), and chemical insults to the CNS. SD can be evoked by mitochondrial blockers, inhibition of Na^+/K^+-ATPase activity, simulated ischemia, KCl application and hyperthermia (Leão, 1944; Leão and Morrison, 1945; Balestrino et al., 1999; Wu and Fisher, 2000; Müller and Ballanyi, 2003).

SD is believed to be the manifestation of the aura that sometimes accompanies migraine (Hadjikhani et al., 2001), and is also associated with other pathologies including stroke and seizures. Additionally, anoxic depolarization (AD, sometimes called terminal depolarization), which occurs following ischemia and simulated stroke (oxygen/glucose deprivation) is considered to be a related phenomenon that differs in the severity of tissue damage (Obeidat and Andrew, 1998; Joshi and Andrew, 2001; Somjen, 2001). A milder version of AD occurs when brain tissue is briefly exposed to hypoxia (hypoxic SD), and unlike AD, neuronal recovery from hypoxic SD occurs (Müller and Somjen, 2000). SD is thought to be benign when compared to AD which is damaging, largely due to compromised energy production thereby leading to cell swelling and dendritic beading (Obeidat et al., 2000). Recently, SD has been shown to produce local regions of hypoxia as transient increases in the tissue's demand for oxygen outstrip vascular oxygen supply (Takano et al., 2007).

The use of electrophysiological measurements to characterize SD has yielded considerable information about the physiological basis of this phenomenon. The inability to produce action potentials during SD reflects the large redistribution of ionic concentrations inside and outside cells that accompanies these events. Tightly controlled baseline levels of 3.0–3.5 mM extracellular potassium concentration ($[K^+]_o$) rapidly rises to 50.0–60.0 mM $[K^+]_o$ during SD in the rat hippocampus (Vyskočil et al., 1972; Müller and Somjen, 2000). The rise in $[K^+]_o$ is concomitantly associated with a drop of extracellular ion concentrations of sodium (155 mM to around 64 mM), chloride (128 mM to 89 mM) and calcium (1.15 mM to 0.10 mM) (Jiang et al., 1992; Basarasky et al., 1998; Müller and Somjen, 2000). $[K^+]_o$ changes during SD in mammalian cortical tissue are strikingly similar to those in the locust MTG, both in terms of the speed of $[K^+]_o$ increase and the amplitude of $[K^+]_o$ increase. It is likely that the generation of SD-like events in response to these stressors in both mammals and locusts is caused by the inability to provide enough energy required to maintain ionic gradients. Membrane potential, measured from pyramidal neurons in the CA1 sector of the hippocampus, depolarized from baseline membrane potential to zero or close to zero before repolarizing and resuming activity (Snow et al., 1983; Czéh et al., 1993). Occasionally, a brief bust of prodromal spikes preceded the SD wave front (Müller and Somjen, 2000). Prodromal spikes are a characteristic feature during SD-like events and are a product of a short burst of action potentials or intense synaptic noise at the leading edge of SD. Spikes of unpatterned electrical activity frequently occur at the leading edge of SD-like events in the locust. Measurements of membrane potential from cortical interneurons and glia also show a substantial depolarization during SD (Müller and Somjen, 2000). Depolarization of a sizable portion of neurons in the CNS accounts for the strong DC field potential shift used by many researchers to identify SD events. Measurements of input resistance during SD show a substantial collapse in the ability of neurons to regulate ion conductances. Hippocampal pyramidal cells during hypoxic SD have a peak decrease in input resistance between 56.4 and 88.5% from initial levels (see Czéh et al., 1993; Müller and Somjen, 2000). However, decreases in input resistance can be variable, ranging from as little as 11% (Czéh et al., 1993) to as high as 97% (Snow et al., 1983). Glial cells have a more moderate reduction in input resistance as well (Müller and Somjen, 2000). The extent of neuronal depolarization that occurs during SD-like events in the locust has also been investigated using intracellular microelectrodes to monitor activity in rhythmic ventilatory interneurons within the MTG (Fig. 1F). As previously reported a modest depolarization of neuronal V_m (between 11-17 mV) occurs in the MTG at the moment of heat-induced failure (Wu et al., 2001). In the cockroach (*Periplaneta americana*) a similar depolarization occurs during exposure to hypoxia (Le Corronc et al., 1999). During SD-like events evoked by sodium azide, locust ventilatory neurons depolarized modestly by 13 mV coinciding with a surge in $[K^+]_o$ (Fig. 1F and G) (Armstrong et al., 2009). This modest depolarization is in contrast to that of neurons in the mammalian CNS. In the locust, the difference in potential across the neuronal membrane only partially decreased during SD-like events, whereas in mammalian neurons there is a complete depolarization resulting in a membrane potential of 0 mV during SD (Collewijn and Van Harreveld, 1966). The modest depolarizations recorded from locust neurons are similar to those of anoxia-tolerant species of vertebrates (Pamenter and Buck, 2008). It remains to be determined why a limited and not a complete neuronal depolarization of V_m occurred during anoxia and heat stress in this terrestrial insect. There is also a significant decrease in input resistance in neurons during SD-like events induced by sodium azide in the locust (Fig. 1H) (Armstrong et al., 2009). The decrease in input resistance is likely caused by opening of voltage-dependent and other membrane channels during SD-like events.

SD arises in regions of the CNS that contain grey matter; in particular the hippocampus and neocortex are predominantly susceptible to it, whereas the spinal cord is more resistant to it (Czéh and Somjen, 1990). SD was first observed as a propagating wave of depressed neuronal activity over rabbit cortex (Leão, 1944). The depolarization and associated synaptic activity cessation propagates across grey matter at a rate of around 3 mm/min but some studies report speeds as low as 2 mm/min and others as high as 9 mm/min, with propagation stopping at lesions or white matter (Grafstein, 1956; Van Harreveld et al., 1955; Hull and Van Harreveld, 1964; Nicholson et al., 1978). The propagation of AD is infrequently observed as tissue is globally exposed to reduced energy levels. However, during hypoxic SD specific foci of SD initiation have been observed (Aitken et al., 1998). The mechanism of SD wave propagation is poorly understood. The initiation of CSD waves can be prevented using NMDA receptor antagonists and gap junction inhibitors, but once a propagating wave of CSD is evoked it

decrease in membrane potential of MTG neurons during SD-like events evoked by inhibiting mitochondrial function. Neurons depolarized by about 13 mV on average. (H) To monitor changes in input resistance, we delivered hyperpolarizing current pulses through the recording electrode and measured changes in membrane potential. Before the SD-like event, the mean input resistance from neuropil recordings was 9.3 MΩ, which decreased to 4.4 MΩ at the maximal depolarization of each neuron and then recovered to a value of 13.4 MΩ after restoration of the K gradient. (G and H) Values here are shown as normalized changes in membrane potential and input resistance to pre-SD-like event values. (A), (B), (F), (G), and (H) modified from Armstrong et al. (2009) and (C)–(E) modified from Rodgers et al. (2007).

is insensitive to a number of NMDA receptor antagonists and channel inhibitors (Takano et al., 2007). The forward movement of CSD could be driven by K^+ and hypoxia rather than opening of specific channels or activation of transmitter systems. One theory is that the increased $[K^+]_o$ in combination with low energy allows $[K^+]_o$ to diffuse forward along its concentration gradient and propagate across cortex (due to the inability to clear $[K^+]_o$) (Takano et al., 2007). Similar to SD-like events in mammalian cortical tissue, waves of $[K^+]_o$ propagate within the locust nervous system (Fig. 2). In the locust there is a mean KCl-evoked SD-like propagation speed of 2.4 mm/min within the MTG (Rodgers et al., 2007). In mammalian brain tissue grey matter contains neural cell bodies, whereas white matter mostly contains myelinated axon tracts. In the locust, waves of $[K^+]_o$ only propagated locally within the MTG (grey matter) and were unable to propagate through connectives (white matter), an observation that is consistent with SD phenomena occurring in mammals (Van Harreveld et al., 1955) (Fig. 2A and B). Studies of SD in vertebrate cortical tissue have revealed the possible involvement of intercellular gap junctions as a mechanism of propagation (Nedergaard et al., 1995; Largo et al., 1997; Thompson et al., 2006). Glial cells are involved in ion homeostasis, function, and development of the insect brain (Kretzschmar and Pflugfelder, 2002) and thus similar mechanisms of SD propagation may exist in the locust MTG. The mechanism of $[K^+]_o$ propagation and the role of glia during locust SD are currently being investigated.

In cortical tissue compromised by stroke or injury, peri-infarct depolarizations (PIDs) are 'spontaneous' spreading depression waves that propagate through the penumbra region of cortical infarcts into normally healthy tissue thereby increasing the final infarct volume (Fabricius et al., 2005). These repeated spreading depolarizations have been shown to occur during ischemic stroke in the human brain (Fabricius et al., 2005; Dohmen et al., 2008; Dreier et al., 2009). PIDs increase the metabolic requirements for recovery from CSD and lead to an increase in final infarct size that is directly related to the number of PIDs (Dohmen et al., 2008). We suggest that ouabain-induced repetitive or 'spontaneous' SD-like events in the locust MTG (Fig. 1D) represent a good model for investigating PIDs that occur in mammalian cortical tissue. During a continuous bath application of 10^{-4} M ouabain, $[K^+]_o$ returns to baseline and there is recovery of motor pattern generation following each $[K^+]_o$ event. This suggests that 10^{-4} M ouabain does not cause complete inhibition of Na^+/K^+-ATPase and bath application of higher doses demonstrates a concentration-dependence to the effect of ouabain. In comparison to 10^{-4} MM ouabain, 10^{-5} M ouabain had no significant effect, while 10^{-3} M ouabain magnified its effects (Rodgers et al., 2009). 10^{-3} M ouabain shortened the time to onset of the initial $[K^+]_o$ surge, gradually increased baseline $[K^+]_o$ following each $[K^+]_o$ surge, prolonged surge duration, diminished $[K^+]_o$ surge amplitude, and motor pattern recovery was significantly less likely following a surge in preparations treated with 10^{-3} M ouabain compared to 10^{-4} M ouabain (Rodgers et al., 2009). Use of ouabain doses greater than 10^{-4} M (10^{-3}, 10^{-2} M) result in a gradual increase in the baseline level of $[K^+]$ such that a "ceiling" level of $[K^+]_o$ is reached where eventually $[K^+]_o$ does not return to baseline following surges and the motor pattern does not recover. This resembles the effect of repeated PIDs expanding the penumbral region of affected cortical tissue, further contributing to energy compromise and permanent tissue damage.

Interventional strategies such as NMDA receptor antagonists and hypothermia have been shown to decrease the incidence of CSD and PIDs in cortical tissue (Chen et al., 1993). In a study of temperature modulation of cerebral depolarization during focal cerebral ischemia in rats, animals subjected to hypothermia exhibited no infarct volume in comparison to normothermic and hyperthermic animals that had 10.2 ± 12.3% and 36.5 ± 3.4% infarct volumes, respectively (Chen et al., 1993). In locusts, sharp SD-like rises in $[K^+]_o$ occurred in response to hypothermic stress (Fig. 3). In an initial set of experiments the temperature of the superfusing saline was decreased to 5 °C, which induced cessation of motor pattern generation but was not sufficient to induce an abrupt $[K^+]_o$ surge in

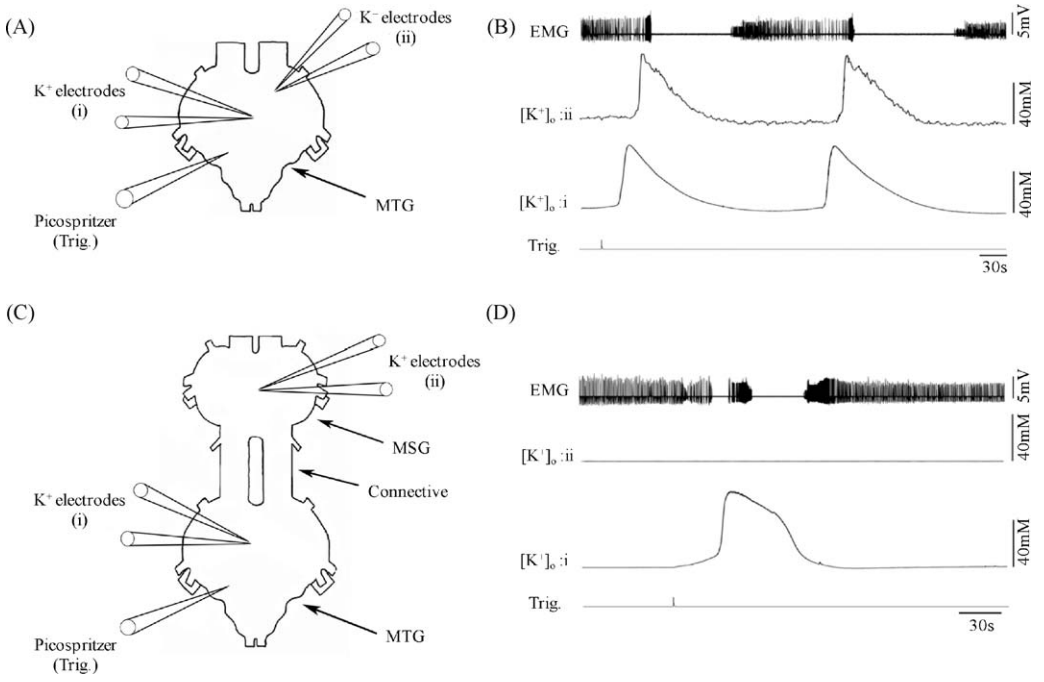

Fig. 2. $[K^+]_o$ waves propagated locally within the locust MTG. (A and B) Abrupt surges in $[K^+]_o$ were generated by injection of high $[K^+]$ saline into the extracellular space and $[K^+]_o$ was measured simultaneously at two different locations in the MTG ~0.4 to 0.6 mm apart. Abrupt surges in $[K^+]_o$ spread locally to other areas of the MTG at a speed of 2.4 mm/min, a similar rate as the depolarizing wave in mammalian brain slices during CSD. (C and D) Abrupt surges in $[K^+]_o$ did not propagate through the connectives to the mesothoracic ganglion and vice versa. (B) modified from Rodgers et al. (2007).

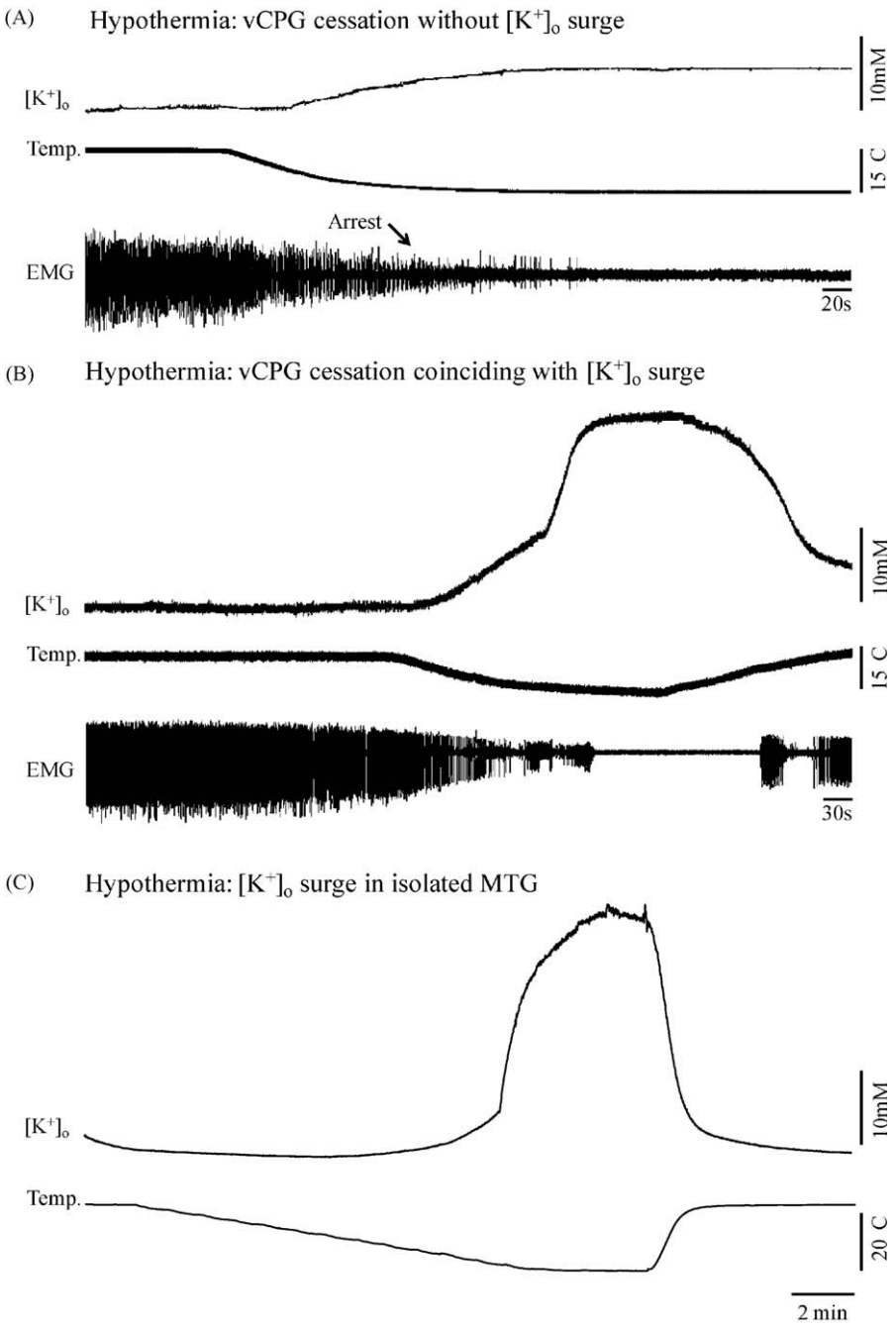

Fig. 3. Cold stress causes vCPG arrest but SD-like events occur only when a critical temperature is reached. (A and B) Simultaneous recordings of the ventilatory motor pattern (EMG), the temperature of the superfusing saline at the MTG (Temp) and the extracellular potassium concentration ($[K^+]_o$). The internal temperature of preparations was decreased to around 5 °C ($N = 12$). (A) A temperature decrease from room temperature (20–22 °C) to 5 °C induced cessation of vCPG operation, but did not induce a SD-like surge in $[K^+]_o$ ($N = 10$). In the experiment shown here $[K^+]_o$ gradually increased from 9.5 mM at room temperature to 15.3 mM at 5 °C, but there was no abrupt SD-like increase in $[K^+]_o$ coinciding with motor pattern silence. (B) In 2 out of 12 preparations, a temperature decrease to 5 °C induced cessation of vCPG operation that coincided with a SD-like surge in $[K^+]_o$ ($N = 2$). In the experiment shown here $[K^+]_o$ increased from 9.2 mM at room temperature to 67 mM at 5 °C. (C) In a separate experiment, the metathoracic ganglion and attached mesothoracic ganglion were dissected out of preparations and pinned onto a peltier plate, which allowed the temperature of the ganglia to be reduced in an incremental manner ($N = 1$). $[K^+]_o$ was measured from the isolated MTG and the temperature was reduced 2.5 °C/min until a $[K^+]_o$ surge occurred, at which point the temperature was held constant. After 3 min, the temperature was increased to room temperature and $[K^+]_o$ recovered.

the majority of cases ($N = 10$) (Fig. 3A). This is an interesting result as it demonstrates that in this case vCPG arrest is independent of the SD-like event and is not the result of the wave in $[K^+]_o$. In contrast to vCPG arrest in response to hyperthermia and the metabolic stressors described above, the inability of the vCPG to operate during hypothermia may be the result of lower O_2 demand rather than a shutdown of neural function. The mean change in $[K^+]_o$ ($\Delta[K^+]_o$) from the beginning of the cold ramp to arrest of motor pattern generation (the point at which continuous bursting could no longer be detected) was 3 mM. This is in comparison to a mean $\Delta[K^+]_o$ of 16 mM from the beginning of a heat ramp to hyperthermia-induced vCPG arrest and a mean $\Delta[K^+]_o$ of 44 mM from the beginning of a heat ramp to the plateau of the $[K^+]_o$ increase. The small increase in $[K^+]_o$ with decreased internal temperature may be due to disregulation of the

Na^+/K^+-ATPase, but not to the point of complete loss of the K^+ gradient. Due to the inability to bring the internal temperature of preparations below 5 °C, a SD-like surge in $[K^+]_o$ was associated with vCPG cessation during hypothermia in only 2 out of 12 preparations (Fig. 3B). In these cooling experiments, the average temperature at vCPG arrest was 9.4 °C and the average temperature at subsequent recovery was 14.7 °C (Fig. 3A and B). In a separate experiment, the locust thoracic ganglia were removed from the preparation and placed on a cooling plate during $[K^+]_o$ measurement within the MTG, enabling a temperature decrease to <5 °C which was sufficient to bring $[K^+]_o$ to threshold for induction of an abrupt surge (Fig. 3C).

4. Locust SD: an adaptive response to conserve energy?

An interesting hypothesis is that SD-like events in the locust can be considered an adaptive strategy to conserve energy and prevent neuronal hyperexcitation (Walter and Nelson, 1975; Rodgers et al., 2007, 2009). Unlike mammalian neurons during CSD, locust ventilatory neurons only partially depolarized during SD-like events (Armstrong et al., 2009) and neuronal operation always recovered following removal of the applied stress, even after ATP depletion using NaN_3 (Rodgers et al., 2007). The frequency and duration of the recovered ventilatory motor pattern became more variable following each $[K^+]_o$ event during ouabain-induced repetitive SD (Rodgers et al., 2007). Although the recovered motor pattern following anoxia was robust, it was not identical to the pre-stress motor pattern, and as in CSD the likelihood and quality of recovery may depend on the severity of the O_2 and energy deprivation, however this has not been investigated in the locust. vCPG operation post-recovery from arrest induced by severe cellular stressors such as anoxia and NaN_3 that result in energy deprivation has not been quantified. CSD in mammals is associated with local tissue hypoxia and neuronal swelling due to energy consumption that is not met with adequate oxygen supply and SD is linked with drops in ATP availability even in the absence of O_2 depletion, resulting in cell death (Thompson et al., 2006; Takano et al., 2007). There is much evidence that vCPG arrest and the abrupt SD-like increases in $[K^+]_o$ in the locust MTG occur before irreversible cellular collapse (Rodgers et al., 2007). A continued increase in temperature beyond hyperthermic vCPG arrest and the associated SD-like event resulted in a second, higher $[K^+]_o$ plateau of 100 mM on average. When the heat stress was removed, $[K^+]_o$ remained elevated and motor pattern generation failed to recover (Rodgers et al., 2007). Interestingly, there was no correlation between ganglionic ATP levels and the occurrence of stress-induced motor pattern arrest and subsequent recovery in the locust (Rodgers et al., 2007). These results suggest that the trigger for SD-like events in the locust is not a fall in ATP (i.e. energetic stress), but the ATP measure by itself does not provide a completely accurate account of cellular metabolic status and changes in AMP, for example, have not been tested. The energy status of a cell can change and trigger signaling pathways without measurable effects on ATP (Lindsley and Rutter, 2004; Hardie et al., 2006). It is tempting to suggest that SD-like events are adaptive; however research on locust SD to date has not addressed this question. Further evidence stems from the fact that SD-like events in the locust are plastic and can be manipulated in many different ways (see below).

5. Modulation of locust SD by pre-treatment

For insects inhabiting hot arid environments temperature variability can be large and at extremes neuronal function can be impaired. Adaptations exist that allow for small extensions in the upper thermal operating range of neuronal function during acute heat stress. A heat shock pre-treatment (HS; 3 h, 45 °C) protects several important neural properties such that the operating range of neural function is extended during exposure to subsequent stress (Robertson, 2004a,b). Extensions in operating range are usually manifested in the hours that follow HS treatment (Whyard et al., 1986) and can last for several weeks (Robertson et al., 1996). Induction of a heat shock response (HSR) is a universal response to elevated temperature which can be characterized by the expression of a suite of heat shock proteins (HSPs) (Parsell and Lindquist, 1993; Kiang and Tsokos, 1998; Feder and Hofmann, 1999; Sharp et al., 1999). The increased presence of HSPs allows cells, tissues, and whole organisms to withstand higher temperatures. The mechanisms by which environmental variables induce adaptive phenotypic changes of crucial neural circuits are also believed to involve induction of neuromodulators such as serotonin and octopamine. In the laboratory the HSR can be induced by briefly exposing animals to heat. Depending upon the species this can usually be observed in as short as 20 min and as long as 3 h. HS protocols for *Drosophila melanogaster* larvae usually consist of 1 h exposure to 36 °C followed by a 1 h recovery at room temperature (25 °C) (Xiao et al., 2007); for *L. migratoria*, 3 h at 45 °C followed by 1 h recovery at room temperature (Whyard et al., 1986); for mouse brainstem slices 20 min at 40 °C followed by a 2 h recovery at 30 °C (Kelty et al., 2002). The protective effects of HS preconditioning has been well established in locust preparations. For example, following HS treatment the operating range of the flight CPG is extended by 6–7 °C (Robertson et al., 1996). Excitatory junction potentials at the hindleg are maintained at temperatures 6.3–8.4 °C higher than in control preparations (Barclay and Robertson, 2000; Klose et al., 2004). Experiments using the descending contralateral movement detector (DCMD) neuron as a model for investigating changes in neuronal properties of the locust following HS have concluded that firing frequency, half-width duration, amplitude and membrane potential are maintained at higher temperatures (Money et al., 2005; Money et al., 2006). Given the essential nature of the vCPG, it is not surprising that this neuronal circuit also displays a robust response to HS treatment. vCPG activity in HS-treated locusts was able to remain in operation 4–9 °C higher than control animals during thermotolerance tests (Armstrong et al., 2006; Dawson-Scully et al., 2007). The length of time taken to recover vCPG activity following cooling was also found to be shorter in HS-treated animals (60 s vs. 180 s) (Dawson-Scully et al., 2007).

The increased ability for the vCPG of heat shocked locusts to function during high temperature stress may reflect an adaptive effect of HS in maintaining ion flow across membranes and ionic gradients necessary for proper functioning. In the locust there is evidence that the HS protective mechanism may induce adaptive modification of ion channel properties. It has been shown that prior stress reduced whole cell K^+ currents from neuronal somata in locust metathoracic neurons (Ramirez et al., 1999). Also, pharmacological block of K^+ channels with TEA mimics the thermoprotective effects of a prior HS on action potentials in locust flight motoneurons (Wu et al., 2001). Downregulation of K^+ currents would be protective by increasing the duration of action potentials and preventing the accumulation of $[K^+]_o$. Given all of this evidence it is reasonable to suspect that HS preconditioning acts by stabilizing K^+ homeostasis.

SD-like events in the locust MTG can be preconditioned by prior HS treatment (Rodgers et al., 2007). HS treatment did not abolish or reduce the degree of SD-like increases in $[K^+]_o$ in the locust but delayed the onset of the heat-induced SD-like event (Rodgers et al., 2007). The delayed onset of the SD-like event allowed for continued vCPG operation at elevated temperature in HS-treated animals. The rate of $[K^+]_o$ clearance following hyperthermic arrest was examined to determine if preconditioning improved $[K^+]_o$ stabilization, and the role of the Na^+/K^+-ATPase in this preconditioning was also tested. HS-treated locusts showed an increase in $[K^+]_o$ clearance rates, which was correlated with a faster vCPG

recovery (Rodgers et al., 2007). There were no changes in steady-state Na^+/K^+-ATPase activity within the MTG, i.e. the ouabain-sensitive fraction of total Na^+/K^+-ATPase activity, following HS pre-treatment. This suggests that the increased rate of $[K^+]_o$ clearance was not linked to changes in total Na^+/K^+-ATPase activity within the MTG, however a protective effect of HS on the Na^+/K^+-ATPase has not been ruled out. Measurements of Na^+/K^+-ATPase activity in the locust MTG did not take into account the possibility of trafficking of pumps in neuronal and glial membranes. The heat shock protein HSP70 has a thermoprotective effect on mammalian muscle sarco/endoplasmic reticulum Ca^{2+}-ATPase (Tupling et al., 2004) and thus HS-induced upregulation of this protein may have a protective effect on Na^+/K^+-ATPase pump activity. HSP70 is synthesized in all cells following ischemia and heat shock, and has been shown to protect the brain against injury produced by ischemia and seizures (Yenari et al., 1998). As a result of ischemia, denatured proteins initiate the process of HSP70 synthesis. Directly outside of the ischemic core, HSP70 promotes the survival of glia and neurons in the area of mild to moderately ischemic tissue where peri-infarct depolarizations occur (Sharp et al., 1999). Thus, HSP70 reduces neuronal damage in the wake of CSD and may have similar protective effects when induced by HS in the locust. Currently the nature of the preconditioning mechanism in the locust is unresolved.

6. Modulation of locust SD pharmacologically

We were able to manipulate SD-like events in the locust using both drugs that act on ion channels and those that target steps along cellular signaling pathways. Tetrodotoxin (TTX), a Na^+ channel blocker, and tetraethylammonium chloride (TEA), a K^+ channel blocker, have been used both in studies of mammalian CSD and locust SD-like events. SD-like events in the locust are delayed by Na^+ channel block using TTX and diminished by K^+ channel block using TEA. These results further demonstrate the similarity between SD-like events that occur in locust neural tissue and mammalian cortical tissue. The effect of activating and inhibiting the NO/cGMP/PKG pathway on the propensity to generate and the severity of SD-like events occurring in the locust CNS has been investigated (Armstrong et al., 2009). Nitric oxide (NO) is a highly diffusible diatomic molecule that acts as a transmitter and modulator of neuronal activity in vertebrates and invertebrates. In the locust nervous system pharmacological manipulation of NO production has been implicated in modulating CPGs (Rast, 2001; Bullerjahn et al., 2006; Newland and Yates, 2007). Nitrergic neuronal modulation of the vCPG has not been reported, although neurons expressing nitric oxide synthase (NOS), the enzyme responsible for producing NO, are present in the MTG near the ventilatory neuropil (Ott and Burrows, 1998; Bullerjahn and Pflüger, 2003). NO stimulates the production of cGMP via augmenting soluble guanylate cyclase (sGC) activity (Elphick et al., 1993). In the locust brain, baseline levels of cGMP are around 200 fM which increase to 800–1600 fM following application of various NO donors to the saline (Elphick et al., 1993). Rises in cGMP concentrations increase the probability of binding to the regulatory domain of protein kinase G (PKG) allowing for increased PKG-dependent phosphorlyation of proteins to occur.

In experiments in which SD-like events were evoked by nanoinjections of KCl it was found that pre-treatment with KT5823, a PKG antagonist, decreased the length of the SD-like event, whereas pre-treatment with 8-Br-cGMP, a PKG agonist, lengthened the duration of the SD-like event (Armstrong et al., 2009). In addition, PKG inhibition using KT5823 shortened the length of time taken for the vCPG to recover, whereas PKG activation resulted in a longer time to recovery of vCPG activity following a KCl-induced SD-like event (Armstrong et al., 2009). These data show that the duration of the SD-like event can be altered by differing levels of PKG activity. Increased PKG activity has been shown to phosphorylate protein phosphatase 2A which then de-phosphorylates specific K^+ channels leading to increased K^+ conductance (Zhou et al., 1996; White et al., 2002). Opposite to increased K^+ conductance, a decrease in K^+ conductance has been argued to reduce the likelihood of initiating heat-induced SD-like events in the locust (Robertson, 2004b). One possibility is that reduced PKG activity reduces the efflux of K^+ to the extracellular space during stress thereby delaying the triggering of the SD-like event by reducing the rate at which $[K^+]_o$ can rise. For this to occur, preconditioning treatments might subsequently attenuate NO production during stress thereby reducing PKG activity in the CNS and reducing K^+ conductance. This implies that lowered NO levels and decreased PKG activity would decrease the severity of SD-like events by reducing their duration. However in mammals NO levels in the affected tissue are increased following CSD (Read et al., 1997). Research from the CNS of rats suggests that increased basal NO levels, produced by endothelial NOS activity, decreases the likelihood of CSD (Petzold et al., 2008). However, when selective neuronal NOS inhibitors are used the occurrence of KCl-induced SD is significantly reduced whereas the duration of SD events are significantly longer (Urenjak and Obrenovitch, 2000). One must be mindful of the complex interaction of vascular supply of oxygen and the propensity to generate SD in mammals. Any role NO might have in regulating SD-like events in the locust would be dependent upon neuronally derived NOS as insects do not possess the same vascular architecture of mammals.

7. A model for SD-like events in the locust MTG

Since the initial intriguing discovery that ouabain induces repetitive SD-like events in the locust, ouabain-induced SD has become a popular control group for many investigations of the mechanisms involved in locust SD. It seems clear that SD-like events in mammals are triggered by positive feedback processes involving an initial ionic disturbance reaching a threshold and caused by neuronal overexcitation or by treatments or conditions that impair ionic homeostasis (Grafstein, 1956; Van Harreveld, 1978; Balestrino et al., 1999; Somjen, 2001; Rodgers et al., 2007). A model describing a possible mechanism for the abrupt increase and decrease in $[K^+]_o$ in response to cellular stressors in the locust MTG has been proposed (Armstrong et al., 2009). The rationale behind this model is that the repetitive nature of $[K^+]_o$ surges induced by ouabain depends on the balance of $[K^+]_o$ accumulation and clearance in the restricted space of the locust MTG (Fig. 4). Extracellular K^+ concentration depends on processes of accumulation (K^+ conductances) and counteracting processes of clearance (transport processes including the Na/K pump and glial buffering/siphoning). At rest these balance to maintain a constant extracellular concentration. As ouabain begins to impair Na^+/K^+-ATPase function neuronal activity outpaces the ability of neurons and glia to sequester accumulating $[K^+]_o$, resulting in increased $[K^+]_o$. This rise in $[K^+]_o$ decreases the potassium equilibrium potential (E_k) and in doing so slowly depolarizes the V_m (V_m is largely dependent upon E_k). During ionic homeostasis E_k is sufficiently large and K^+ driving forces are able to repolarize the neuron. However in situations when E_k is reduced such as during ouabain exposure, K^+ driving forces may be insufficient to restore V_m. Depolarization results in the opening of voltage-gated K^+ and Na^+ channels, further increasing activity and $[K^+]_o$ resulting in further depolarization. This positive feedback cycle could give rise to the explosive rise in $[K^+]_o$ at the leading edge of the surge without invoking special channels. The surge occurs when the build-up in $[K^+]_o$ is already sufficient to shift the equilibrium potential and depolarize membranes (Fig. 4A). Other factors such as HS pre-treatment

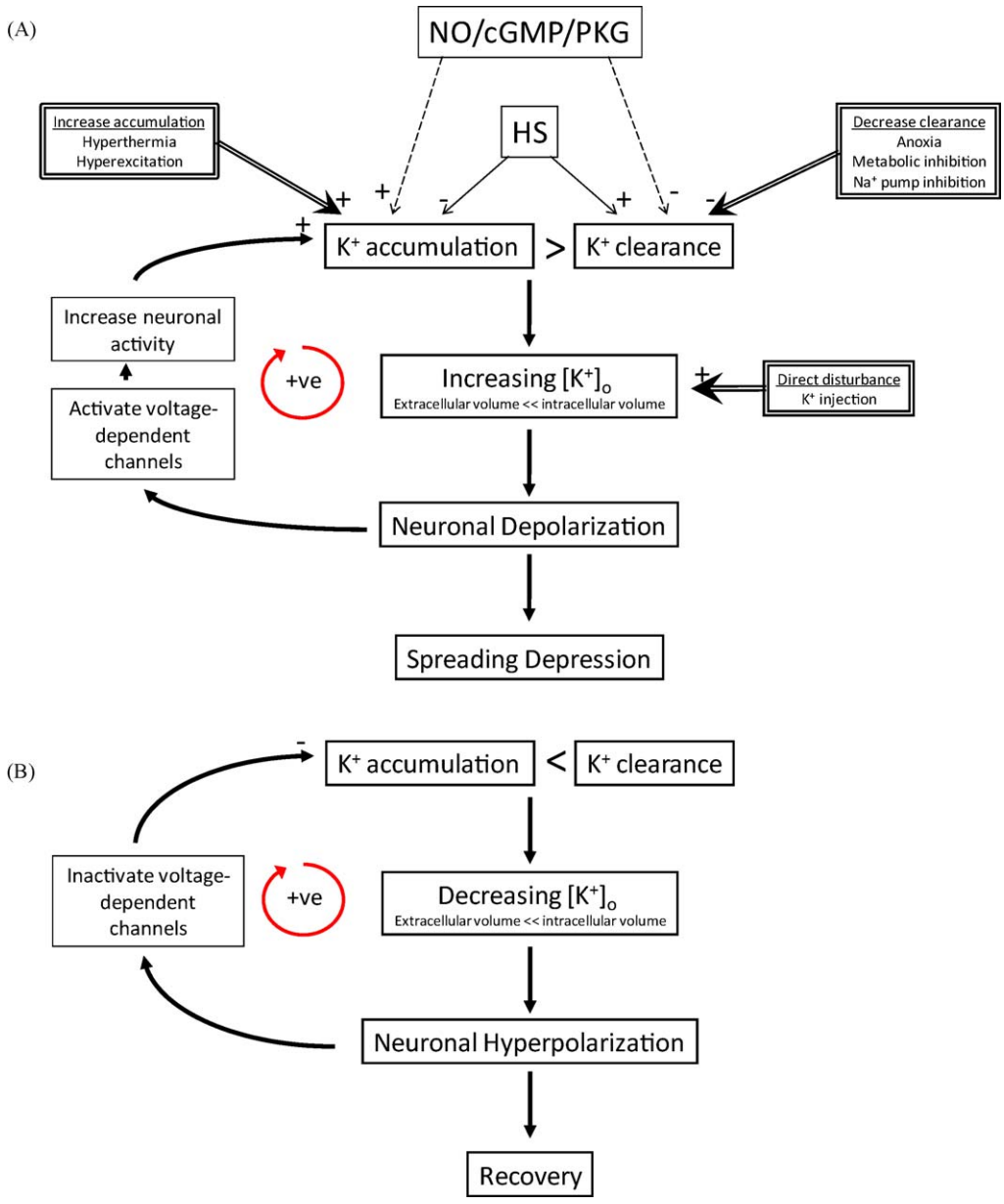

Fig. 4. The repetitive nature of SD-like $[K^+]_o$ surges induced by ouabain depends on the balance of $[K^+]_o$ accumulation and clearance. (A) (Onset) An SD-like increase in $[K^+]_o$ is evoked when extracellular K^+ accumulation exceeds clearance under conditions when $[K^+]_o$ can appreciably affect the K^+ equilibrium potential and cause neuronal depolarization thus entering a positive feedback cycle (+ve). This can be induced by increasing accumulation, decreasing clearance or directly manipulating $[K^+]_o$ (double bordered boxes). HS pre-treatment reduces the tendency to generate SD by decreasing $[K^+]_o$ accumulation (Ramirez et al., 1999) and increasing $[K^+]_o$ clearance (Rodgers et al., 2007). Reduced PKG activity may similarly be protective by preventing $[K^+]_o$ accumulation. (B) Recovery. If cessation of neural activity is sufficient to enable K^+ clearance to predominate then the system enters a positive feedback cycle that restores $[K^+]_o$ and membrane potentials to pre-SD levels. Modified from Armstrong et al. (2009).

and PKG activation or inhibition shift the balance towards $[K^+]_o$ accumulation or $[K^+]_o$ clearance. Recovery from ouabain-induced SD might be afforded through cessation of neuronal activity allowing non-inhibited Na^+/K^+-ATPases to clear excess K^+ from the extracellular space thereby restoring E_k (Fig. 4B).

8. Conclusion: locust SD as a useful model

The discovery that SD-like events occur in the locust MTG was fortuitous as it has allowed an investigation of mechanisms involved in SD-like events while neural circuits are intact and functioning. Given the convenience of the locust preparation and the accessibility of an intact, functioning nervous system, experiments can be conducted with ease and studies on the role of glia, ion channels, and energy-sensing pathways such as the AMP-activated protein kinase (AMPK) pathway in SD-like events are currently underway. It is our hope that our findings will be useful for the treatment of mammalian disorders such as seizures and migraine. At the very least, much knowledge has been gained over the past few years about the mechanisms involved in neural circuit arrest in response to stress. In addition to the many benefits of the locust model for studying SD there are limitations to the extent to which we can draw comparisons to CSD. For example, stroke and ischemia in vertebrate brain tissue are induced via oxygen/glucose deprivation by occluding blood vessels and arteries thereby cutting off blood flow. The locust nervous system lacks vasculature and thus the effects of oxygen and energy stress have a different type of complexity than in mammalian brain tissue. This could be the reason why locust neurons recover from anoxia-induced SD while mammalian neurons do not recover from

anoxic or terminal depolarization. Important questions remain. First, why is it that neurons in the locust CNS only partially depolarize during the SD-like event and does this account for the ability of the locust to survive long periods of anoxic coma? Second, are SD-like events adaptive? Lastly, to what extent are the pathways and mechanisms for heat tolerance in the locust shared by other organisms, and thus the extent to which the findings of this review represent a description of general cellular strategies for neural stress tolerance?

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