Title: **Activity-dependence of spreading depression in the locust CNS**

Authors: Kristin E. Spong, Tom R. Mazzetti, and R. Meldrum Robertson

Affiliation: Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Corresponding author: Kristin E. Spong

Contact information:

Email: 9ks26@queensu.ca

Phone: (613)-533-6000 ext. 75510 or 77599

Postal address (present address):

Queen's University, Department of Biology

Bioscience complex, Room 3404

Kingston, Ontario, Canada K7L 3N6

List of abbreviations

SD: Spreading depression; CSD: Cortical spreading depression; $[K^+]_o$: Extracellular potassium concentration; $[Na^+]_o$: Extracellular sodium concentration; $[Ca^{2+}]$: Calcium concentration; CNS: Central nervous system; MTG: Metathoracic ganglion; CPG: Central pattern generator; PIDs: Peri-infarct depolarizations.

Abstract

Spreading depression (SD) is associated with large changes in extracellular ion concentrations and can be induced by impairing mechanisms of K^+ ion homeostasis. We tested activity-dependence of SD in the locust model of ouabain-induced SD in the metathoracic ganglion. Wind-activation of thoracic circuitry resulted in small increases of K^+ concentration that took 5-10 s to be cleared from the extracellular space. In the presence of the Na^+/K^+-ATPase inhibitor ouabain, wind stimulation every 30 s halved the latency to the first SD event and increased its duration. Wind stimulation was able to trigger the first event suggesting that local activity could determine the origin of successive SD events. Perfusion with zero-calcium saline blocked neural activity in the ganglion and prevented the occurrence of ouabain-induced SD. We conclude that ouabain-induced SD in the locust CNS is strongly dependent on the existing level of neural activity.

Introduction

Spreading depression (SD) is a neural phenomenon first discovered by Leao (1944) in the cortex of the rabbit but has since been demonstrated to occur in many vertebrate and invertebrate systems. It is characterized by rapid cellular depolarization and an associated arrest in neural activity that slowly propagates throughout neural tissue (Leao 1944; Somjen 2001). The depolarization and disruption in electrical activity is reflective of the massive disturbance in ionic homeostasis that occurs at the onset of SD. Waves of SD are associated with abrupt increases in the extracellular potassium concentration ($[K^+]_o$) and drop in extracellular sodium ($[Na^+]_o$), calcium ($[Ca^{2+}]_o$), and chloride ($[Cl^-]_o$) concentrations (Somjen 2001). Restoration of ionic gradients occurs within minutes following an SD episode and ultimately allows for the recovery of neural activity (Rodgers et al. 2007; Somjen 2001). SD in the mammalian cortex (CSD) is thought to underlie the aura that accompanies migraine and has also been implicated in more severe pathologies such as stroke and traumatic brain injury (Somjen 2001). Invertebrate SD has been best described in the CNS of *Locusta migratoria* and is associated with environmental stress-induced neural shutdown. For instance, locusts enter a reversible coma when exposed to stimuli such as hyperthermia, hypothermia and anoxia during which SD-like events can be monitored within the metathoracic ganglion (MTG) (Rodgers et al. 2007; Rodgers et al. 2010). Interestingly, both the propagation rate (2.4 mm/min) and magnitude of $[K^+]_o$ disturbance (~50 mM) during locust SD (Rodgers et al. 2007) are strikingly similar to that measured during CSD in mammals which is associated with $[K^+]_o$ increases of ~50-60 mM traveling at velocities of 2-5 mm/min (Somjen 2001).

In healthy neural tissue SD can be experimentally induced by disrupting mechanisms of K^+ homeostasis. For example, inhibition of the Na^+/K^+-ATPase with ouabain reliably induces SD in both the vertebrate and invertebrate CNS (Balestrino et al. 1999; Rodgers et al. 2009). In semi-intact locust preparations bath application of ouabain elicits repetitive waves of SD within the MTG characterized by abrupt increases in $[K^+]_o$ where the rise and fall coincide with the arrest and recovery of electrical activity (Rodgers et al. 2009). Due to the robustness and repetitive nature of ouabain-induced SD, it has become a commonly used control procedure for investigations into the cellular

mechanisms underlying SD in the locust CNS. Moreover, ouabain-induced SD in the locust has been proposed as a model for peri- infarct depolarizations (PIDs) which are detrimental depolarizations that spontaneously arise in ischemic brain regions following stroke (Rodgers et al. 2010).

Although the precise mechanisms underlying SD initiation are still not clearly understood, mechanistic models of locust SD suggest that onset occurs once K^+ levels exceed a critical threshold triggering a positive feedback cycle that ultimately leads to the characteristic all-or-none increase in $[K^+]_o$ (Armstrong et al. 2009; Rodgers et al. 2010). It is predicted from these models that increased neural activity would predispose towards the generation of SD, however, direct evidence demonstrating the activity-dependence of SD is lacking. Here we test how increased neural activity, through wind-activation of thoracic circuitry, affects ouabain-induced SD in the locust. Additionally, by manipulating the $[Ca^{2+}]$ in the bathing solution we reduce neural activity and examine how this alters the tissue's response to ouabain. Our results are consistent with the conclusion that ouabain-induced SD in the locust MTG is strongly influenced by existing levels of neural activity.

Materials and methods

Animals

Locusts (*Locusta migratoria*) were housed in a crowded colony located in the Queen's University Animal Facility. The colony was maintained under light and dark cycles of 12 hours at temperatures of approximately 25°C. Animals were fed once daily with wheat grass, and a mixture of milk powder, yeast, and bran. All experiments were performed using adult males aged 2 to 5 weeks past imaginal ecdysis. Locusts were randomly chosen from the colony and held in ventilated plastic containers prior to experimentation. Animals used in the wind stimulation experiments were deprived of food for one day prior to experimentation to increase the probability of reliably activating locomotor circuitry (Davenport and Evans 1984). The thoracic and abdominal cavities of semi-intact preparations (Robertson and Pearson 1982) were continuously bathed in a saline solution to prevent desiccation. Standard locust saline 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, Oakville, ON, Canada). Preparations were grounded by inserting a chlorided silver wire into either the abdomen or the upper thorax. To induce SD, semi-intact preparations were exposed to either 10^{-4} M or 5×10^{-4} M ouabain (Sigma-Aldrich) for 40 min.

Measuring extracellular K^+

The $[K^+]_o$ was continuously monitored within the MTG using K^+-sensitive microelectrodes prepared using 1 mm diameter unfilamented glass capillary tubes. Capillary tubes were rinsed with methanol (99.9%) and dried on a hotplate prior to being pulled. The microelectrodes were then silanized on a hotplate for 1 hour by exposure to dichlorodimethylsilane (99%, Sigma-Aldrich) vapor. The microelectrode tips were filled with Potassium Ionophore I-Cocktail B (5% Valinomycin, Sigma-Aldrich), back-filled with 500 mM KCl and stored in the dark with tips suspended in distilled water until needed for experimentation. Reference microelectrodes were prepared prior to experimentation using 1 mm diameter filamented glass capillary tubes and filled with 500 mM KCl. Tip resistance of both the reference and K^+-sensitive microelectrodes was approximately 5-7 mΩ once filled. Just prior to each experiment the K^+-sensitive and reference microelectrode pair were connected to a DUO773 two-channel intracellular/extracellular amplifier (World Precision Instruments Inc., Sarasota, FL, USA) and calibrated using 15 mM KCl +135 mM NaCl and 150 mM KCl solutions to obtain the voltage difference from a 10 fold-change in K^+ concentration. Following calibration, the microelectrodes were inserted through the sheath of the MTG adjacent to one another and the extracellular K^+ voltage within the neuropil was recorded. Voltage recordings were subsequently converted to $[K^+]_o$ (mM) using the Nernst equation (for details see Rodgers et al., 2007).

Measuring direct current (DC) potential

Abrupt negative deflections in DC field potential are indicative of SD which reflects the massive cellular depolarization that takes place during the events (Somjen 2001). Microelectrodes were prepared using 1 mm diameter filamented glass capillary tubes. Once pulled microelectrodes were filled with 500 mM KCl forming low resistance tips (~5-7 mΩ) and connected to a DUO773 two-channel intracellular/extracellular amplifier (World Precision Instruments Inc.). A single microelectrode was inserted through the sheath of the MTG and DC potential was continuously monitored throughout the experiment.

Wind stimulation protocol

To enhance neural activity wind stimuli were applied to the head of semi-intact preparations. Wind stimulation was applied for a duration of 5 s and was administered through a 5 mm diameter plastic tube situated at a distance of approximately 2 cm from the locust's head. In experiments measuring the extracellular K^+ activity wind stimulation was administered every 30 s until the onset of ouabain-induced SD. In DC potential recordings wind stimulation began following a 5 min baseline period and was administered every 30 s or 1 min for a total of 10 min. The flight central pattern generator (CPG) is one circuit likely activated by wind stimulation (Robertson and Pearson 1982) and thus we monitored flight activity electromyographically by inserting a copper wire, insulated except at the tip, into a dorsal longitudinal wing depressor muscle located in the thorax. To test how wind-activation of thoracic circuitry affects the susceptibility to SD we compared preparations subjected to the wind stimulation protocol to control preparations which received no wind stimulation. In all experiments repetitive SD was induced by bath application of ouabain (5×10^{-4} M) which was administered for 40 min following a 5 min baseline period.

Calcium manipulations

Extracellular Ca^{2+} levels were manipulated by altering the [Ca^{2+}] in the bathing saline solution. Preparations were treated with standard locust saline (as described above), high Ca^{2+} saline or Ca^{2+}- free saline. In the high Ca^{2+} treatment condition the [Ca^{2+}] was increased from 4 mM (standard locust saline) to 8 mM. In the Ca^{2+} free condition zero-Ca^{2+} saline solution was used which also contained the Ca^{2+} chelator, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma-Aldrich) at a concentration of 10^{-3} M. Ouabain (10^{-4} M) was dissolved in standard, high Ca^{2+}, or Ca^{2+}-free saline and administered for 40 min to semi-intact preparations following a 20 min treatment period with the corresponding saline type. Ouabain-induced SD was monitored by measuring the DC potential within the MTG.

Analyses of spreading depression

The latency to onset was measured from the time of ouabain application to the downward inflection point of the first SD event. The duration of the first event was calculated by taking time measurements at half maximum amplitude of the negative shift in DC potential. Total number of events represents the number of SD episodes that occurred within the 40 min treatment period with ouabain.

Statistical analyses

All data were plotted and analysed using SigmaPlot 12.5 (Systat Software Inc., Chicago, IL, USA). Parametric data are displayed using bar charts with columns representing the mean and standard errors of the mean. Non-parametric data are plotted as box plots representing the 25th and

75th percentiles (interquartile range; IQR) with a line indicating medians (Mdn) and whiskers extending to the 10th and 90th percentiles (individual points represent outliers). To determine significant differences between two groups either t-tests or Mann-Whitney Rank sum tests were used for parametric and non-parametric data respectively (P<0.05). A one-way ANOVA was used to determine significant differences between more than two groups and *post hoc* comparisons were performed using Holm-Sidak Multiple comparisons (P<0.05).

Results and discussion

To increase neural activity we applied repetitive wind stimuli to the head of semi-intact locust preparations activating thoracic circuitry including the flight CPG. Flight motor patterns were recorded electromyographically from a dorsal longitudinal wing depressor muscle and the $[K^+]_o$ was continuously monitored from within the MTG (**Fig. 1A**). Wind-activation of thoracic circuitry was associated with 0.2 ± 0.03 mM (n = 10) increases in the $[K^+]_o$ which took 9.8 ± 0.3 s (n = 8) to return to baseline values (**Fig. 1A**) and fit nicely with an exponential $[K^+]_o$ clearance time constant of 3.1 ± 0.4 s (n = 10). Wind alone was able to produce increases in $[K^+]_o$ however the magnitude of increase was greater when flight was induced. The $[K^+]_o$ disturbances reported here are of similar magnitude to the activity-dependent increases in $[K^+]_o$ that occur in the CNS of the cockroach following electrical stimulation to the connectives (Grossman and Gutnick 1981). Such modest increases in $[K^+]_o$ are sufficient to mediate interactions between neurons in close proximity (Yarom and Spira 1982). Furthermore, given the extensive yet restricted volume of the extracellular space, such K^+-mediated interactions are likely to spread, affecting more distant neurons (Spira et al. 1984).

Here we show that wind stimulation could trigger the first ouabain-induced SD event. Repetitive SD was induced within the MTG by bath application of ouabain and monitored by recording the characteristic all-or-none increases in the $[K^+]_o$ (**Fig. 1B**). Upon ouabain wash-in impairment in flight activity and a reduction in emg amplitude was observed. Wind stimulation was applied every 30 s up until the first ouabain-induced surge and in 5/9 preparations the first surge was

clearly triggered by the 5 s wind stimulus (**Fig. 1C**). These results suggest that increases in local activity can determine the origin of successive SD events. This is consistent with recent reports investigating the origins of PIDs in mammalian cortex. Until recently the triggering factors leading to the eruption of PIDs were unknown, however it now has been demonstrated that somato-sensory activation of ischemic cortex reproducibly triggers PIDs and is associated with increased oxygen utilization in the stimulated region (von Bornstadt et al. 2015).

To further investigate the activity-dependence of SD we tested how wind-activation of thoracic circuitry affects susceptibility to SD. Bath application of ouabain induced repetitive SD in 100% of control (**Fig. 2Ai**) and wind-stimulated preparations (**Fig. 2Aii**), however wind-stimulated preparations were associated with shorter latencies to onset compared to control preparations (**Fig. 2Bi**; Wind stimulated: Mdn = 6.8 min, IQR = 5.4, 9.5; Control: Mdn = 15.8 min, IQR = 6.3, 24.1) suggesting that wind stimulation increased the susceptibility to ouabain-induced SD. Additionally, wind stimulation significantly increased the duration of the first SD event from 53.1 ± 8.3 s, without stimulation, to 85.3 ± 10.3 s (**Fig. 2Bii**). A longer duration suggests impairment in the ability to restore ionic gradients. The event duration reported here likely reflects the increased demand on the Na^+/K^+-ATPase in preparations experiencing increased neural activity as a result of wind stimulation.

In addition to investigating how increases in neural activity affect ouabain-induced SD we also tested the effects of reducing neural activity by manipulating the $[Ca^{2+}]$ within the bathing media. We compared ouabain-induced SD under control, Ca^{2+}-free and high Ca^{2+} conditions. Reducing Ca^{2+} levels can be predicted to reduce activity by depressing synaptic transmission. In the current experiments a blockade of neural activity under Ca^{2+}- free conditions was evidenced by a cessation in ventalitory abdomen movements that occurred within 1-5 min following saline application. Bath application of ouabain reliably induced SD in 100% of preparations treated under control and high Ca^{2+} conditions, however, under Ca^{2+}-free conditions ouabain was found to induce SD in only 2/9 (~22%) preparations (**Fig. 2Ci**). The two zero-Ca^{2+} preparations were associated with longer latencies to SD onset (17.61 min, 26.72 min) compared to control (Mdn = 8.9 min, IQR = 3.3, 15.1) and high Ca^{2+} (Mdn = 5.2 min, IQR = 4.4, 11.5) preparations. Additionally, perfusion with zero-Ca^{2+} saline

significantly reduced the number of individual events exhibited within the 40 min treatment period (**Fig. 2Cii**; Zero-Ca^{2+}: 0.9 ± 0.6, Control: 6.7 ± 0.7, High Ca^{2+}: 10.4 ± 1.3). These results demonstrate that a reduction in neural activity suppresses ouabain-induced SD. Reducing neural excitability has previously been shown to protect against stress-induced neural shutdown. For instance, blockade of Na^{+} channels with tetrodotoxin (TTX) delays the onset of hyperthermic-induced neural failure in the locust CNS (Rodgers et al. 2007). In the current experiments preparations exposed to high Ca^{2+} conditions were found to exhibit a significantly greater number of individual SD events compared to controls (**Fig. 2Cii**; data reported above). Interestingly, in the rat brain in vivo increased influx of extracellular Ca^{2+} ions can trigger waves of CSD and facilitate propagation rates suggesting that Ca^{2+} plays an important role in the initiation of CSD (Torrente et al. 2014). Thus the precise role that Ca^{2+} plays in locust SD merits further investigation.

To summarize, we tested the activity-dependence of ouabain-induced SD in the locust CNS. Mechanistic models of locust SD speculate that increased neural activity would predispose towards SD occurrence (Armstrong et al. 2009; Rodgers et al. 2010) however this had seldom been tested. Here we provide direct evidence that increases in neural activity heighten susceptibility to SD while reductions in activity attenuate SD. It is particularly interesting that wind stimulation could trigger the first SD event. Our findings demonstrate that ouabain-induced SD is strongly influenced by existing activity levels and help to substantiate previously proposed models of SD.

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Competing Interests

The authors declare that this research was conducted in the absence of any conflicts of interest, financial or otherwise.

Author contributions

K.E.S., T.R.M. and R.M.R. conception and design of research; K.E.S., T.R.M. and R.M.R. performed experiments and analyzed data; K.E.S., T.R.M. and R.M.R. interpreted results of experiments; K.E.S. and R.M.R. prepared figures; K.E.S. drafted manuscript; K.E.S. and R.M.R. edited and revised manuscript; K.E.S., T.R.M. and R.M.R. approved final version of manuscript.

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Figures

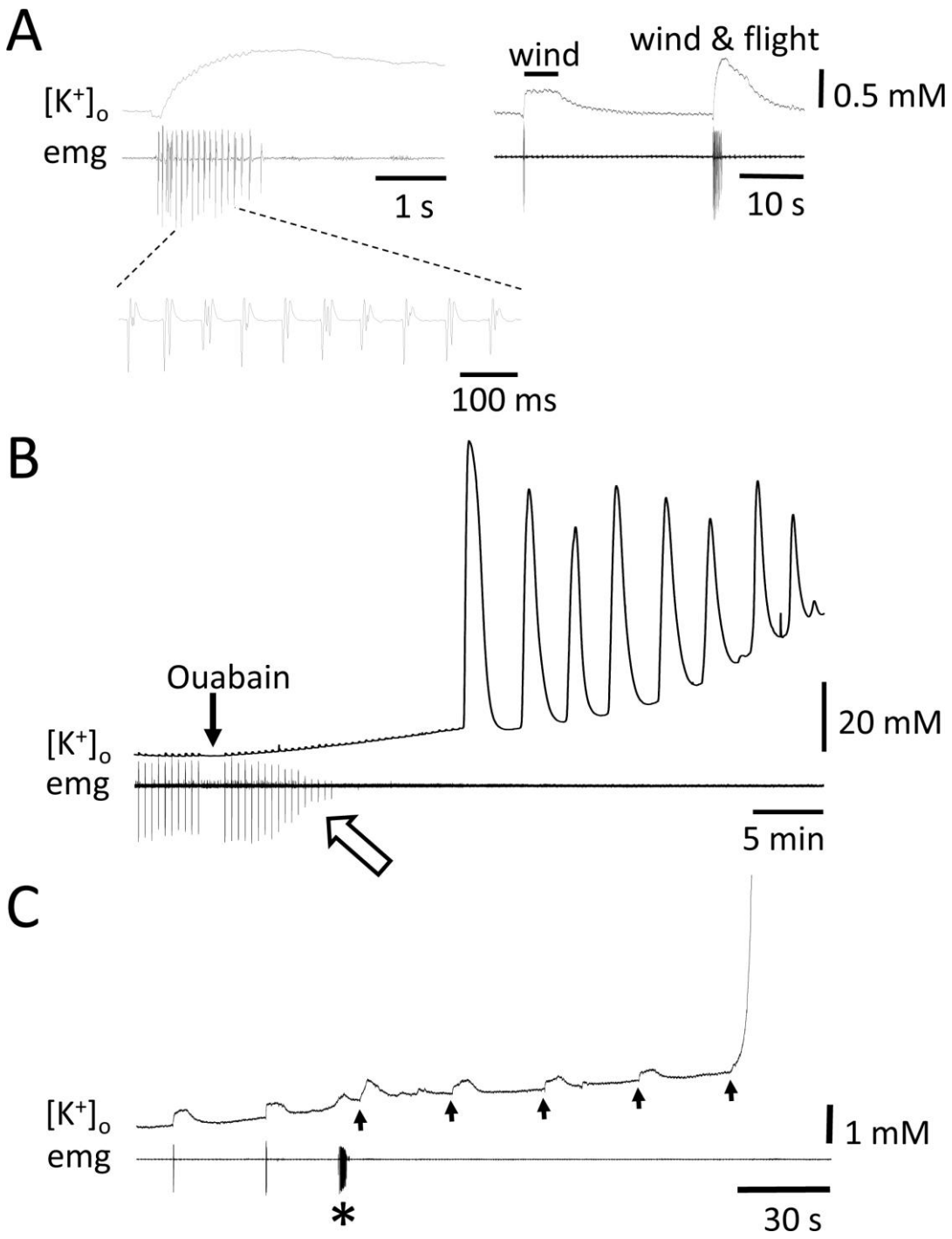

Figure 1. K^+ activity in response to wind stimulation, flight activity and ouabain exposure. A-C. Simultaneous recordings of the $[K^+]_o$ within the MTG and flight activity (emg) induced by wind

stimulation. A. Wind stimulation and flight activity transiently increase $[K^+]_o$. Inset shows an expansion of the flight motor pattern recorded electromyographically from a dorsal longitudinal wing depressor muscle. Note that wind stimulation alone increases $[K^+]_o$ and that such wind-induced $[K^+]_o$ disturbances can be amplified when paired with flight activity. B. Representative recording of ouabain-induced SD in a preparation subjected to wind stimulation (same preparation shown in A). Open arrow indicates impairment of flight and reduction of emg amplitude as a result of ouabain wash-in. C. Representative recording (different preparation from A and B) demonstrating that wind stimulation can trigger the first ouabain-induced event. The asterisk under the emg trace shows a convulsive burst in DL motorneurons as flight CPG fails. The closed arrows indicate times of wind stimulation every 30 seconds after the emg fails. Note that the final wind stimulation triggers the abrupt K^+ surge.

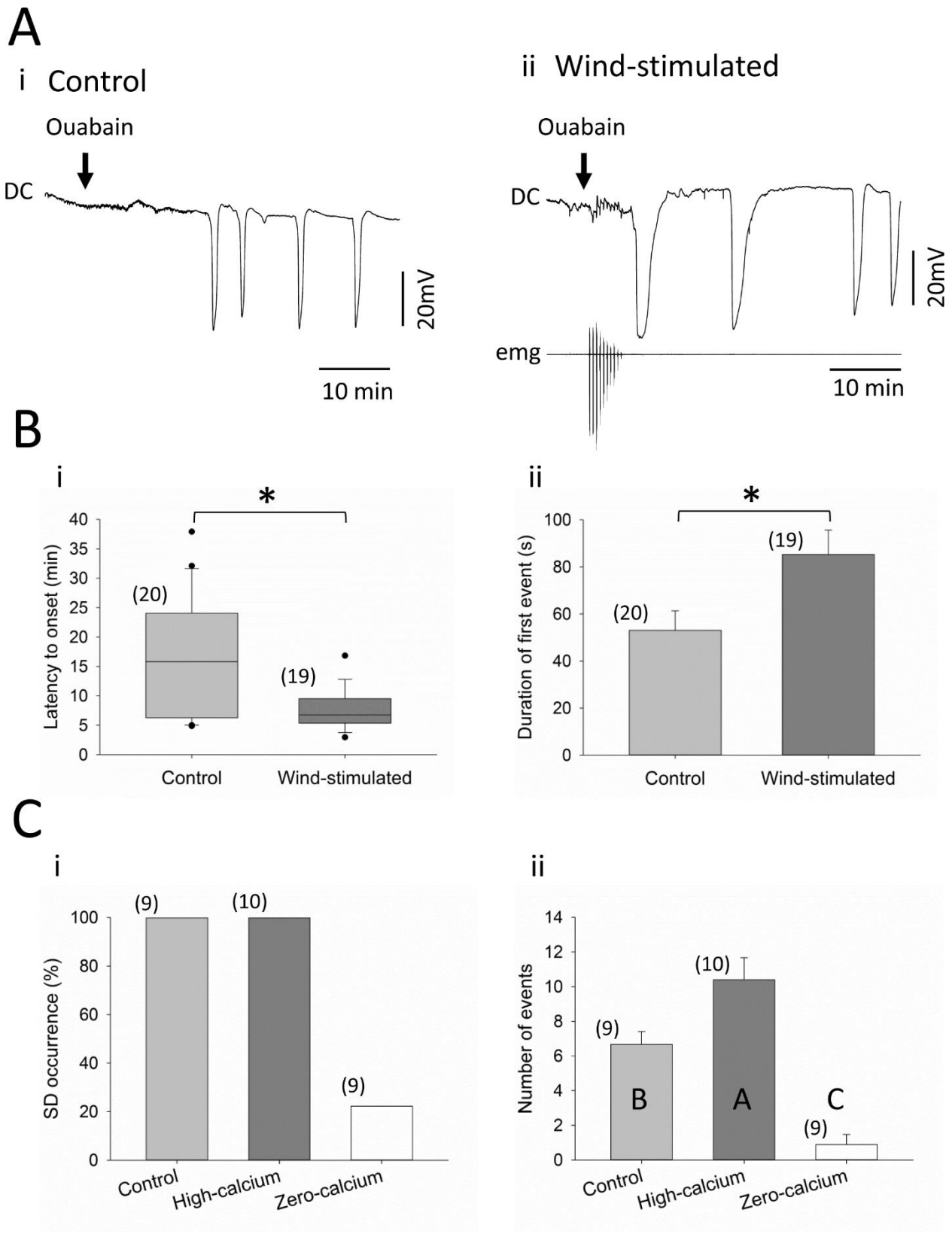

Figure 2. Activity-dependence of ouabain-induced SD. A. Representative traces of the DC potential recorded from within the MTG during bath application of 5×10^{-4}M ouabain under control conditions (no wind stimulation) and experimental conditions (wind stimulation applied) Ai and Aii respectively. Note that following a delay ouabain exposure induces repetitive negative deflections in DC potential indicative of SD. Aii. Simultaneous recordings of the DC potential and flight muscle activity (emg) during wind stimulation. Bi. Wind-stimulated preparations (n= 19) were associated with significantly shorter latencies to SD onset compared to control preparations (n= 20). Data are plotted as the median and upper and lower quartiles. Asterisk indicates a significant difference between conditions (Mann-Whitney U Statistic= 96.000, p= 0.009). Bii. The duration of the first ouabain-induced event was significantly greater, as denoted by the asterisk, in preparations subjected to wind stimulation (n= 19) compared to control preparations (n= 20; Two-tailed *t*-test, t (37) = -2.4, p= 0.019). Data are plotted as means ± s.e.m. Ci. Percent of preparations that exhibited ouabain-induced SD under control (n= 9), high Ca^{2+} (n= 10) and zero-Ca^{2+} (n= 9) conditions. Ca^{2+}-free conditions could prevent SD occurrence. Cii. Number of individual SD events recorded within the 40 min treatment period under control (n= 9), high Ca^{+2} (n= 10) and zero-Ca^{2+} (n= 9) conditions. A one-way ANOVA revealed significant differences between groups (F (2, 25) = 25.8, p< 0.001). Post hoc comparisons using the Holm-Sidak method revealed that there were significantly fewer individual events under zero-Ca^{2+} conditions compared to control and high Ca^{2+} conditions and a significantly greater number of events under high Ca^{2+} conditions compared to control conditions. Data are plotted as means ± s.e.m. Columns assigned different letters are significantly different (Holm-Sidak method, $P<0.05$).