Animal Care and Cortical Sheet Preparation

This study was approved by the University of Toronto Animal Care Committee and conforms to the care and handling of animals as outlined in the Canadian Council on Animal Care’s Guide to the Care and Use of Experimental Animals, Vol. 2. Animal use protocol number (20012745). Informed Consent Statement: Not applicable.Adult turtles (*Chrysemys picta bellii*) were obtained from Niles Biological and housed in a large aquarium equipped with a freshwater recirculating filter system set at 20 °C, a non-aquatic basking platform, and a heat lamp. Turtles were maintained on a photoperiod of 12 hours of light:12 hours of dark and given access to food three times a week. Turtles were given an intramuscular injection of Alfaxalone 10 mg/mL stock in hydroxypropyl-beta-cyclodextrin [HP-β-CD] and dissolved in a multi-dose preservative solution (Alfaxan®) (Jurox Pty Ltd; Rutherford, Australia) at 20 mg/kg. Once deep anesthesia was achieved, the legs were flaccid, and the eyes were unresponsive to gentle touch, the animals were then decapitated with a guillotine, and the whole brain was excised from the cranium in about 1 minute. The entire dorsal cortex was dissected free and bathed in 3–5°C artificial turtle cerebrospinal fluid (aCSF) composed of (in mmol L –1): 97 NaCl, 2.6 KCl, 1.2 CaCl2, 1.0 MgCl2, 2.0 NaH2PO4, 26.5 NaHCO3, 20.0 glucose, 5.0 imidazole (adjusted to pH 7.4, and osmolarity 290–300 mOsM). Two cortical sheets were cut medially from the visual cortex of each cerebral hemisphere and subdivided into a total of six cortical sheets. Sheets were then lifted out of the chamber and stored in vials of aCSF for no longer than 48 hrs.

Whole-Cell Electrophysiology Techniques

Turtle cortical sheets were placed on a cover slip that forms the bottom of an RC-26 open bath perfusion chamber system, with a P1 platform (Harvard Apparatus, Saint-Laurent, QC, Canada). The chamber was gravity-perfused by a 1-L glass bottle that contained aCSF gassed with 95% O2/5% CO2 to achieve oxidative conditions. Experiments were conducted at room temperature (20 - 22 °C). Whole-cell recordings of neurons from the dorsal cortex and dorsal medial cortex were performed using fire-polished 5-8 MΩ micropipettes produced from borosilicate glass capillary tubes using a P-97 micropipette puller model (Sutter Instruments, Novato, CA, USA). The pipette solution contained the following (in mmol L-1): 8 NaCl, 0.0001 CaCl2, 10 Na-HEPES, 110 Kgluconate, 1 MgCl2, 0.3 NaGTP, and 2 NaATP (adjusted to pH 7.4 and osmolarity 290-300 mOsM). The electrode was filled and inserted into a 1-HL-U electrode holder attached to a CV-4 headstage (gain: 1/100 U, Axon Instruments, Sunnyvale, CA, USA)

Cell-attached 1-20 GΩ seals were obtained using the blind-patch technique. To achieve a GΩ seal, the recording electrode was advanced towards the cell using a PCS-6000 motorized manipulator (Burleigh, Newton, NJ, USA) until the square-wave pulse abruptly decreased, at which point a slight negative pressure was applied to form a seal. To break into the cell, a soft pulse of negative pressure was applied to break through the cell membrane, while the holding potential was voltage-clamped to -70 mV. Once the whole-cell configuration was established, cells were given at least 2 minutes to acclimate to experimental conditions before access resistance was measured, which normally ranged from 20-30 MΩ. Patches were discarded if access resistance varied by >25% over the course of an experiment. Data was collected at 5-10 kHz using a MultiClamp 700B digital amplifier, a CV-7B head stage, and a Digidata 1550B interface (Molecular Devices, Sunnyvale, CA, USA), and stored on a computer using Clampex 10 software (Molecular Devices, Sunnyvale, CA, USA). A liquid junction potential (LJP) was accounted for, and experimentally measured between the aCSF and the pipette solution, supported by LJP calculations using a generalized version of the Henderson equation (Clampex junction potential calculator; Molecular Devices, Sunnyvale, CA, USA).

Electrophysiological Identification and Measurement of Action Potential Parameters

Pyramidal neurons were studied and characterized based on electrophysiological properties. In current clamp mode, when current was injected, pyramidal neurons exhibited spike frequency adaptation in response to a sustained current, which was not seen in stellate neurons.

Action potential threshold (APth) was determined by current-clamping cells and injecting current in 10 pA increments in a stepwise manner from sub-threshold for 500 ms until a spike was elicited. The threshold was recorded at the point at which a sharp elevation in voltage was observed. The full spike amplitude was measured from the point of the APth to the spike tip, while the half-amplitude spike width was measured as the time elapsed between the two-points of the half-amplitude on the spike. The rise time was calculated as the time elapsed between 10% of the full spike amplitude and 90% of the full spike amplitude, while the decay time was calculated as the time elapsed between 10% of the full spike amplitude and 90% of the full spike amplitude. Whole-cell conductance (Gw) was measured as the slope of a voltage-ramp from -120 to -60 mV for 150 ms. Data measurements for all parameters were made with Clampfit software (Molecular Devices, Sunnyvale, CA, USA).

*Impact of acute Alfaxalone application on evoked naïve tissue GABAA Receptor current*

To initiate a GABAA receptor current neurons were voltage-clamped at a holding potential of -100 mV, and 2 mM of GABA were applied for 1-2 seconds. These changes resulted in large outward GABAA- receptor currents that were easily detected and differentiated from other currents. From the GABAA receptor currents produced, we measured the peak amplitude, baseline holding current, decay time as the 90%-to-10% decay time, and the area under the curve as the integrated area between the measured current and the baseline using Clampfit software (Molecular Devices, Sunnyvale, CA, USA). Sheets were then perfused with oxygenated aCSF and 1 µM Alfaxalone for 15 min, and the GABAA receptor current decay time, integrated area under the curve, peak amplitude, and baseline holding current were measured again. This procedure was repeated utilizing gabazine (25 µM), a GABAA receptor antagonist, rather than Alfaxalone, to see if the currents could be blocked to confirm we were recording GABAA receptor currents [26]. Additionally, Alfaxalone-free stock Alfaxalone solution (vehicle, a generous gift from Jurox; Rutherford, NSW, Australia) had no significant impact when applied alone (n = 4).

To construct a dose-response curve, GABAA receptor current measurements following acute Alfaxalone treatment were normalized to pre-treatment values to determine the relative change in decay time, area under the curve, and peak amplitude. The same process to determine relative changes in decay time, area under the curve, and peak amplitude was repeated for tissue sheets perfused with oxygenated aCSF and 0.1 µM, 0.5 µM, 1 µM or 1.5 µM Alfaxalone for 15 min.

Impact of whole-animal Alfaxalone exposure on evoked GABAA Receptor Whole Cell current

GABAA receptor currents were measured as above; however, the recording pipette [Cl-] was increased to 110 mM [Cl-] by equimolar substitution of KCl for Kgluconate, the neurons were voltage-clamped at a holding potential of -100 mV and 2 mM GABA was applied for 15 seconds [26]. This change still resulted in large outward GABAA currents that were detected and differentiated from other currents. The GABAA receptor current decay time, integrated area under the curve, and peak amplitude were determined as described above. The decay time, integrated area under the curve, peak amplitude, and baseline holding current were then normalized to the whole-cell capacitance. The protocol was repeated with cerebral cortex sheets obtained from Alfaxalone-sedated painted turtles at 1 hour, 2 hours, 4 hours, and 6 hours following Alfaxalone administration. The timepoints were binned so that any measurement performed between 1 and 2 hours was considered 1 hour following whole-animal Alfaxalone exposure, any measurement performed between 2 and 3 hours following whole-animal Alfaxalone exposure was considered 2 hours following whole-animal Alfaxalone exposure, any measurement performed between 3 and 4 hours was considered 3 hours following whole-animal Alfaxalone exposure, and any measurement performed between 5 and 6 hours was considered 5 hours following whole-animal Alfaxalone exposure. Measurements made on the 30-minute mark were placed in the latter time group. The aCSF solution bathing the Alfaxalone-treated sheets were replaced every 30 minutes following the completion of the dissection with the first washout occurring 0.5 hours after Alfaxalone application.