**2.1 Experimental Animals**

All experiments were performed using young female (postnatal day 30 (P30) to P38) Sprague Dawley rats (RRID:MGI:5651135) obtained from Charles River Laboratories (Quebec, Canada). Rats arrived at P22 and were housed in groups of 4 in polycarbonate cages. Cages contained wood shavings (paper towel strips?) bedding and items for enrichment (wooden block, two orange balls, nyla bone). Animals were provided with standard rat chow and water *ad libitum* and housed at 21°C and 50% ± 10% humidity on a 12-hour light/12-hour dark cycle that began at 7:30 am. Following arrival, the animals were given at least 6 days to acclimate to these conditions before experiments began. All experimental protocols were approved by the Mount Allison Animal Care Committee (protocol #104140) and in accordance with the Canadian Council on Animal Care guidelines.

**2.2 Restraint Stress**

\*equipment and size\* Rats were placed into one of three groups: control, acute stress, or repeated stress. Animals in the acute stress group underwent a single 30-minute restraint, and animals in the repeated stress group underwent five consecutive days of a 30-minute restraint. Restraint was performed at the same time each morning for the five consecutive days, and was kept as close as possible across animals (between time and time).

-schematic?

2.3 Brain Removal and Slice Preparation

Between time and time, immediately following the single (acute) or fifth day of restraint (repeated) for the stress groups, the animal was anesthetized with 5% gaseous isoflurane in oxygen (Dechra Veterinary Products). To confirm anesthesia, a toe pinch reflex test was confirmed. When no reflex was present, the animal was decapitated using a guillotine (World Precision Instruments), and a carotid blood sample was obtained. The brain was quickly removed and immersed in ice-cold artificial cerebrospinal fluid slicing solution (aCSF) that contained (in mM): 75 sucrose, 25 glucose, 87 NaCl, 25 NaHCO3, 2.5 KCl, 0.5 CaCl2 \* 2 H2O, 7 MgCl2 \* 6 H2O, 1.25 NaH2PO4, saturated with 95% O2 and 5% CO2 for a minimum of 15 minutes prior.

After \*transport to lab\*, the brain was placed on a petri dish and the anterior, posterior, and dorsal (cortex) regions were cut off. The brain was glued (Krazy Glue) to a vibratome plate with the posterior side down. A small piece of agar was glued on the ventral side of the brain to prevent movement during slicing. The vibratome plate was placed such that the brain was submerged in the ice-cold slicing solution and a line with 95% O2 and 5% CO2 was inserted. Coronal slices (250 µm) containing the DMH, identified using a rat atlas (Paxinos & Watson, 2009), were obtained using a vibrating slicer (Lecia) and cut into hemi-sections along the midline. Slices were incubated in 32.5°C artificial cerebrospinal fluid (aCSF) that contained (in mM): 10 glucose, 126 NaCl, 26 NaHCO3, 2.5 KCl, 2.5 CaCl2 \* 2 H2O, 1.5 MgCl2 \* 6 H2O, 1.25 NaH2PO4, saturated with 95% O2 and 5% CO2, for a minimum of 60 minutes, until recording began.

**2.4 Electrophysiology**

Hypothalamic slices were submerged in a recording chamber and superfused with aCSF. The solution, continuously bubbled with 95% O2 and 5% CO2, was delivered at flow rate of 1 mL/min and kept at 32.5°C (equipment). The brain slice was kept still using two ~1cm metal weights placed horizontally on each end and was visualized with an upright microscope (Olympus BX51 WI) and Infinity 2 Camera, live footage of which was projected onto a computer monitor. The DMH was located by using the third ventricle as a landmark.

Whole-cell patch clamp electrophysiological recordings were obtained (Fig. 1) using borosilicate glass microelectrodes with tip resistances ranging from 4.0 to 6.0 MΩ (Model P-1000 pipette puller, Sutter Instruments). Two pipettes were used for each recording, one for stimulating and one for recording. Neurons were stimulated extracellularly, using a microelectrode filled with aCSF, while the recording electrode in a neuron of interest was filled with an internal solution that contained (in mM): 108 KGluconate, 8 KCl, 8 NaGluconate, 10 HEPES, 2 MgCl2, 1 K2ETGA, 4 K2ATP, 0.3 Na3GTP, corrected to pH 7.2 with KOH, and between 285-300 mOsm with sterile water. The recording pipette was connected to a positive pressure line, 1-2ish mLs of positive pressure was utilized before the recording microelectrode went into the bath. When a DMH neuron of interest was located, the recording microelectrode was gently pushed next to the neuron where a dimple was formed in the cell membrane, then the positive pressure was released and the neuron sealed around the electrode. When the seal reached the giga-ohm range, the neuron was held at -70mV in voltage-clamp mode, and a suction technique was used to break into the cell. If the initial access (Ra) to the cell was between 1 and 2.5, the recording proceeded. To measure excitatory currents, picrotoxin (50 µM), a GABAA antagonist, was included in all treatment solutions. Picrotoxin blocked all inhibitory GABA currents, allowing for only excitatory, glutamate currents to be recorded.

A close-up of a grey and pink image

AI-generated content may be incorrect.**Figure 1:** **Recording of live neuron.** Stimulating electrode (light blue) stimulates DMH neurons (gray) in the surrounding tissue to release neurotransmitter, while the recording electrode (dark blue) records from the target DMH neuron (pink), visualized at 40X magnification.

Electrophysiological signals were amplified using the Multiclamp700B amplifier (Molecular Devices) filtered at 1 kHz, digitized at 10 kHz using the Digidata 1550B system (Molecular Devices), and pCLAMP acquisition software (Version #, Molecular Devices).

**2.5 Electrophysiological Measurements**

To determine the effects of acute and repeated dress on glutamate transmission in the DMH, the following parameters were measured: evoked currents, paired-pulse ratio, action potential frequency and amplitude…

**2.5.1 Evoked Currents & Paired Pulse Ratio**

EPSCs were evoked at a frequency of 0.2 Hz, and a paired pulse was applied by stimulating twice, 50 msec apart. To study activity-dependent plasticity in the DMH, high-frequency stimulation (HFS) was applied at 100 Hz for 4 seconds, repeated twice, 20 seconds apart (Crosby et al., 2011). The paired-pulse ratio (PPR), calculated as the amplitude of the second peak divided by the first peak.

**2.5.2 Action Potentials**

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Statistical and Data Analysis

-protocols: HFS

-action potentials (?)

-evoked currents (?)