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Neurotensin and dynorphin Bi-Directionally modulate CeA inhibition of oval BNST neurons in male mice

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HIGHLIGHTS

- Endogenous NT and Dyn bi-directionally modulate CeA to ovBNST inhibitory transmission through NTR1 and KOR, respectively.
- NT and Dyn are co-localized, co-released, and co-modulate transmission at inhibitory synapses in the ovBNST.
- NTR1 and NTR2 activation have opposing modulatory effects at ovBNST inhibitory synapses.
- First demonstration of a neuromodulatory role of NTR2 at GABA synapses.
- Study highlights importance of neuropeptidergic co-modulation of synaptic transmission.

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ABSTRACT

Neuropeptides are often co-expressed in neurons, and may therefore be working together to coordinate proper neural circuit function. However, neurophysiological effects of neuropeptides are commonly studied individually possibly underestimating their modulatory roles. Here, we triggered the release of endogenous neuropeptides in brain slices from male mice to better understand their modulation of central amygdala (CeA) inhibitory inputs onto oval (ov) BNST neurons. We found that locally-released neurotensin (NT) and dynorphin (Dyn) antagonistically regulated CeA inhibitory inputs onto ovBNST neurons. NT and Dyn respectively increased and decreased CeA-toovBNST inhibitory inputs through NT receptor 1 (NTR1) and kappa opioid receptor (KOR). Additionally, NT and Dyn mRNAs were highly co-localized in ovBNST neurons suggesting that they may be released from the same cells. Together, we showed that NT and Dyn are key modulators of CeA inputs to ovBNST, paving the way to determine whether different conditions or states can alter the neuropeptidergic regulation of this particular brain circuit.

1. Introduction

Neuropeptides are frequently co-expressed in individual neurons within the nervous system; their cooperative role may enable flexible neuromodulation and proper function of neural circuits (Griebel and Holsboer, 2012; Kormos and Gaszner, 2013; Valentino and Aston-Jones, 2010). However, neuropeptides are often studied individually, neglecting the functional outcomes resulting from their coordinated actions (Ptak et al., 2009; Sun et al., 2003). In brain slices prepared from male rats, postsynaptic activation of oval bed nucleus of the stria terminalis (ovBNST) neurons triggers the release of various neuropeptides, such as neurotensin (NT), corticotrophin releasing factor (CRF) and dynorphin (Dyn) which in turn robustly modulate excitatory and inhibitory synaptic transmission onto the same neurons (Normandeau et al., 2018). Notably, endogenously-released NT produces an increase in inhibitory transmission in the rat ovBNST, that is further enhanced by chronic stress (Normandeau et al., 2018). What remains unknown is whether this coordinated modulation of synaptic transmission is circuit-specific.

Although still under debate, the ovBNST may be devoted to energy homeostasis, promoting foraging behaviours and food intake (Jennings et al., 2013; Li and Kirouac, 2008; Moga et al., 1995). Located in the

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dorsolateral region of the BNST, the ovBNST is robustly and reciprocally connected with the central nucleus of the amygdala (CeA), and since these bi-directional connections are exclusively GABAergic, the ovBNST and the CeA most likely inhibit each other (Petrovich and Swanson, 1997). This connection may be integral in the balance between aversive or appetitive behaviours (Davis et al., 2010; Dong et al., 2001; Jennings et al., 2013). Whether neuropeptides are important modulators of this particular brain circuit is largely undetermined. Nonetheless, it is known that the opiate dynorphin (Dyn) released from GABA neurons specifically decreases CeA-to-dorsal BNST inhibitory inputs in male mice (Li et al., 2012). Whether other neuropeptides in the ovBNST regulate CeA inhibition is currently unknown.

Using brain slice electrophysiology in male mice, we triggered endogenous release of neuropeptides and discovered that release of NT and Dyn had opposing effects on CeA-to-ovBNST synaptic inhibition through neurotensin receptors 1 (NTR1) and kappa opioid receptors (KOR), respectively. NT-mediated enhancement of inhibitory transmission in the ovBNST overshadowed the effect of Dyn, paving the way to determine whether changes in condition/state may affect neuropeptidergic regulation of synaptic transmission in this particular neural circuit.

2. Materials and methods

2.1. Mice

46 mice (> 8 weeks old) were group housed on a reverse 12-h light/dark cycle (lights OFF at 8:00 A.M) with *ad libitum* access to chow and water. C57BL/6J adult male mice (n = 29) used for electrophysiology were obtained from Charles River Laboratories (St-Constant, QC, Canada) and V*gat-Cre* adult male mice (n = 17) used for electrophysiology and *in situ* hybridization were bred in house in the McElligott lab (University of North Carolina, Chapel Hill, NC, USA). V*gat-Cre* mice were obtained from Jackson Labs (originally generated by Bradford Lowell, Harvard University), and the virus lot #43140 was generated at UNC Chapel Hill. All experiments were conducted in accordance to the guidelines from the Canadian Council on Animal Care in Science, approved by Queen's University, and were in accordance with the National Institutes of Health guidelines for animal research with the approval of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

2.2. Stereotaxic injections

Mice were deeply anesthetized with 3% isoflurane (vol/vol) in oxygen, placed into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA) and maintained at 1.5–2.5% isoflurane during surgery. A hole was drilled in the skull using CeA coordinates ML: ± 2,95, AP: −1.15, DV: −4.75 from Bregma. Microinjections of 300 nL of virus (AAV5-EF1a-DIO-ChR2-mCherry or AAV5-EF1a-DIO-ChR2-eYFP) were made bilaterally using a 1 µl Neuros Hamilton syringe (Hamilton, Reno, NV, USA) at a rate of 100 nl/minute. After infusion, the needle was left in place for at least an additional 5 min to allow complete diffusion of the virus before being slowly withdrawn. After surgery, all mice were returned to group housing, and recovered for at least 6 weeks prior to the start of experiments.

2.3. Slices preparation and electrophysiology

Mice were anesthetized with isoflurane (5% at 5 L/minute) and their brain removed into ice-cold artificial cerebral spinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 6 CaCl2, 1.2 NaH2PO4, 25 NaHCO3, and 12.5 D-glucose equilibrated with 95% O2/5% CO2. Brains were cut in 2 °C aCSF into coronal slices (300 µm) with a vibrating blade microtome (VT-1000; Leica Canada, Concord, ON, Canada). The ovBNST slice of interest for this study corresponded to −0.26 mm from Bregma. Slices were made of CeA to verify viral injection cites in all *vGAT-cre* animals. Slices were incubated at 34 °C for 60 min and transferred to a chamber perfused (2–3 mls/minute) with aCSF at 34 °C. Remaining slices were kept in aCSF at room temperature until further use. Whole-cell voltage-clamp recordings were made using glass microelectrodes (3–5 MΩ) filled with (in mM): 70 K + -gluconate, 80 KCl, 1 EGTA, 5 HEPES, 2 MgATP, 0.3 GTP, and 1 P-creatine. Electrical stimuli (10–100 µA, 0.1 ms duration) or optical stimuli (490 nm LED intensity 2–100%, 0.1 ms duration) were applied at 0.1 Hz. Inhibitory postsynaptic currents (IPSCs) were evoked by local fiber stimulation with tungsten bipolar electrodes or by a 490 nm LED via the objective while neurons were voltage-clamped at −70 mV. $GABA_A$-IPSCs were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3-dione (DNQX, 50 µM). To induce local endogenous neuropeptide release, postsynaptic neurons were repetitively depolarized in voltage clamp from −70 to 0 mV (100 ms) at a frequency of 2 Hz for 5 min (Normandeau et al., 2018). We quantified peak amplitude and defined 3 possible outcomes to postsynaptic depolarization or drug bath application: 1-long-term potentiation (LTP^{GABA}; > 20% increase from baseline after 20 min), 2-long-term depression (LTD^{GABA}; < 20% decrease from baseline after 20 min) or 3-no change (NC, within 20% deviation from baseline after 20 min) (Normandeau et al., 2018). Recordings were made using a Multiclamp 700B amplifier and a Digidata 1440A (Molecular Devices LLC, San Jose, CA, USA). Data were acquired and analyzed with Axograph X running on Apple computers and Clampex on Windows computers.

2.4. Drugs

Stock solutions of SR142948 (10 mM), Norbinaltorphimine (Nor-BNI, 100 mM), NT (1 mM) and JMV431 (1 mM) were prepred in distilled water. Stock solutions of DNQX (100 mM), and NTRC844 (1 mM) were prepared in DMSO (100%). All drugs were further dissolved in the physiological solutions at the desired concentrations (DNQX 50 µM, SR142948 10 µM, Nor-BNI, 100 nM, JMV431 100 nM, NTRC844 100 nM, SR48692 1 µM, NT 1 µM) and the final DMSO concentration never exceeded 0.1%.

2.5. Fluorescence In situ hybridization (FISH)

Immediately after removal, brains were placed on a square of aluminum foil on dry ice to freeze for 5 min before wrapping to prevent tissue damage. Brains were then placed in a −80 °C freezer for no more than 1 week before slicing. In all, 12-µm slices containing the CeA and ovBNST were obtained on a Leica CM3050S cryostat (Leica Biosystems, Wetzlar, Germany) and placed directly on coverslips. FISH was performed using the Affymetrix ViewRNA 2-Plex Tissue Assay Kit with custom probes for *Pdyn*, *Nts*, *Ntsr1*, and *Ntsr2* designed by Affymetrix (Santa Clara, CA, USA). FISH was also done using the Advanced Cell Diagnostics (ACD) HybEZ(TM) II Hybridization System with custom probes for *Nts* and *Pdyn* designed by ACD (Newark, CA, USA). Slides were coverslipped with SouthernBiotech DAPI Fluoromount-G. (Birmingham, AL, USA). z-Stack (3 × 5 tiled; 8 optical sections comprising 10.57 µm in total) were obtained on a Zeiss 800 confocal microscope. All images were preprocessed with stitching and maximum intensity projection. Quantification of probe colocalization was hand counted using the cell counter plugin in FIJI (ImageJ, NIH, Bethesda, MD, USA). For all studies, cells were classified into three groups: probe 1+, probe 2 + or probe 1 + and 2+. Co-localization ratio was calculated by (probe 1 + and probe 2+/probe 1 + or probe 2+)*100 (McCullough et al., 2018).

2.6. Statistical analyses

Changes in IPSCs peak amplitude were measured from baseline and are shown as percentage change from baseline: