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Gabaergic and Glutamatergic Connections in the Social Neural Network

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A conserved network of brain regions is responsible for mediating social behavior; however, the chemical nature (GABAergic or glutamatergic) of the connectivity in this network is unknown. One node in this network, the medial amygdala (MA), is known to process social stimuli and facilitates appropriate social responses. We hypothesized that a GABAergic population in the posterior MA is responsible for processing both male and female stimuli. In contrast, we hypothesized that both excitatory and inhibitory neurons in the anterior MeA would discriminate between different stimulus types. We estimated the percentage of GABAergic and glutamatergic MA neurons activated by either salient (female) or less-salient (male) social cues in male Syrian hamsters. A significantly greater percentage of GABAergic neurons responded to
all stimulus types in both the anterior MA and the posterodorsal MA, but not in the
posteroventral MA, where equivalent percentages of GABAergic and glutamatergic neurons
responded to all stimuli.

INDEX WORDS: Medial amygdala, Bed nucleus of the stria terminalis, Social behavior, GABA,
Glutamate, Hamster
GABAERGIC AND GLUTAMATERGIC CONNECTIONS IN THE SOCIAL NEURAL NETWORK

by

ASHLEIGH BURNS

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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2014
GABAERGIC AND GLUTAMATERGIC CONNECTIONS IN THE SOCIAL NEURAL NETWORK

by

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2014
DEDICATION

For Zachary.
ACKNOWLEDGEMENTS

I would like to thank the many people who provided guidance and encouragement and made this process more rewarding than I could have ever imagined.

First I would like to thank Aras Petrulis for the invaluable opportunity of working in his lab. I would also like to thank the other members of my thesis committee, Bradley Cooke, Bill Walthall and Tim Bartness for their advice and encouragement.

Special thanks to the Petrulis lab members: Luis Martinez, Marisa Levy and the ever-expanding list of undergraduates. I am truly grateful for your friendship. In particular, thanks Omar Hamki and Megan Moore, you both went above and beyond and I will be forever grateful.

Thank you to Mary Karom and Johnny Garretson, without whom I might have struggled with the in situ hybridization indefinitely. Mary, I’m sorry about that centrifuge.

Finally, I would like to my family and Zachary.
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1 INTRODUCTION

1.1 Overview

Vertebrates possess a highly conserved network of brain regions for making social decisions, which is critical for reproduction (O’Connell & Hofmann, 2012). In many rodents, including Syrian hamsters, generation of appropriate social responses depends on processing of social odor cues in the environment (Baum & Kelliher, 2009; Petrulis, 2013). There are many nodes in the Social Behavior Network, however the medial amygdala (MA) and its major projection target, the bed nucleus of the stria terminalis (BNST), are strongly implicated in initial recognition and orientation towards social odors (Ruth I. Wood, 1997). Considerable functional data demonstrates that both the MA and the BNST are critically involved in processing social stimuli required for appropriate social behavior (Petrulis, 2013). Exposure to social odors increases expression of immediate early genes, such as c-fos, within the MA and BNST (delBarco-Trillo, Gulewicz, & Johnston, 2009; Meredith & Westberry, 2004; C L Samuelsen & Meredith, 2009) that is consistent with single-unit recordings in the posterior MA resulting from social stimuli induced neural activity (Bergan, Ben-Shaul, & Dulac, 2014). Additionally, lesions of the MA (Maras & Petrulis, 2006) and BNST (Been & Petrulis, 2010) eliminate preference for investigating opposite-sex odors. Furthermore, the interaction between these two regions is required for processing volatile sexual odors as functionally disconnecting the MA and BNST, but not MeA, eliminates preference for volatile female odors in male hamsters, without altering copulatory behavior (Been & Petrulis, 2012). This data suggests that the MA and BNST are a critical piece of the brain circuit mediating odor-driven approach and investigation of conspecifics.
The MA is a heterogeneous structure with the anterior part (MeA) receiving primarily chemosensory information, whereas the posterior dorsal section (MePD) is predominately sensitive to hormonal cues; this division is also preserved in the BNST and other structures (Newman, 1999). For example, site-specific implantation of testosterone into either the MePD or BNST restores reproductive behavior in castrated male hamsters (R I Wood & Newman, 1995) while removal of the ipsilateral olfactory bulb counteracts this restoration, suggesting these regions are sites of chemosensory and hormonal cue integration responsible for appropriate reproductive behavior (R I Wood & Coolen, 1997; R I Wood & Newman, 1995).

Despite the importance of the MA-BNST circuit in processing social stimuli, very little is known about the chemical nature of the connections between the different nodes of this part of the Social Behavior Network. The neurotransmitter phenotype of the connections from the different subnuclei of the MA to the BNST has yet to be characterized in detail and it is unclear if one type of MA or BNST neuron (GABAergic or glutamatergic) is more involved in processing information regarding male or female social cues. Consequently, illuminating the excitatory and inhibitory character of these connections is critical to understanding how these nodes interact. The MA and BNST both contain populations of GABAergic (Sun & Cassell, 1993) and glutamatergic (Bian, Yanagawa, Chen, & Luo, 2008) neurons with a larger population of GABAergic neurons found in the MePD compared with the MeA (Simmons & Yahr, 2003). Neurons in these regions with different chemical phenotypes appear to respond to different stimuli and may control distinct behaviors. The posterior MA includes two groups of projection neurons that converge in the ventromedial hypothalamus and respond to reproductive and defensive olfactory stimuli, and are inhibitory and excitatory in nature respectively (Choi et al., 2005). Furthermore, within the MePD, a GABAergic subpopulation controls both aggressive and
copulatory behaviors in males while a glutamatergic subpopulation suppresses on-going social behaviors (Hong, Kim, & Anderson, 2014).

### 1.2 Expected Results

Based on this limited data we hypothesize that a primarily GABAergic population in the posterior MA is responsible for processing both male and female stimuli. Presently, there is insufficient data available to generate a single compelling hypothesis regarding the anterior MA. For example, MeA lesions consistently reduce the number of posterior MA neurons activated by social odors from either sex (Maras & Petrulis, 2010), which suggests that the MeA provides excitatory input to the posterior MA neurons increasing their response to any social stimuli. If this is the case, we would expect to see primarily glutamatergic neurons activated by both male and female stimuli. Alternatively, data in male rats indicates that same-sex odors may activate a greater percentage of GABAergic neurons in the MeA compared to opposite-sex odors (Donato et al., 2010). Therefore, it is also possible that processing social information in the MeA involves both excitatory and inhibitory sub-populations that distinguish between different stimulus types (male vs. female). To test these hypotheses we identified cells (using c-fos as a marker of neuronal activation) in the different sub-regions of the MA that were activated when male Syrian hamsters were exposed to male, female or no stimuli. These active neurons were then identified as either glutamatergic or GABAergic using in situ hybridization for GAD65 or vGlut2 as markers of GABAergic and glutamatergic cells respectively. Finally, to determine the phenotype of neurons in the different sub-regions of the MA that project specifically to the BNST we injected a retrograde tracer into the BNST and colocalized labeled cell bodies with markers for GABAergic and glutamatergic neurons.
1.3 Figures

Figure 2.2.1.1 Projections from the MA to the BSNT. Currently the phenotype of projection neurons between the different sub-regions of the medial amygdala to the BNST is unknown. One of the goals of this research is to determine the character (GABAergic or glutamatergic) of the projection neurons in the MA.
2 EXPERIMENT

2.1 Animals

Experimental subjects were 23 adult (3 to 6 month old) male Syrian hamsters (*Mesocricetus auratus*) that were obtained from Harlan Laboratories (Haslett, MI, USA). A separate group of 4 male and 4 female Syrian hamsters were used as stimulus animals for the purposes of c-fos induction. Experimental and stimulus animals were singly housed in standard rat ACS cages in a climate-controlled facility. All animals were maintained on a reversed 14 hours light/10 hour dark photo period, and food and water were available *ad libitum*. All animal procedures were carried out in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and were approved by the Georgia State University Institutional Animal Care and Use Committee.

2.2 Surgical Procedures

All surgeries were performed under 2% isoflurane gas anesthesia vaporized in an 80:20% oxygen/nitrous oxide mixture. Ketoprofen (5mg/kg subcutaneously delivered, Henry Schein, Melville, NY, USA) was administered intra-operatively to minimize post-operative pain.

2.2.1 Gonadectomy

A minimum of two weeks before use for other procedures, subject and stimuli males’ testes were removed bilaterally via midline abdominal incision and cauterization of the ductus deferens and associated blood vessels. Vicryl sutures and wound clips were used to close surgical incisions. Immediately following gonadectomy, a Silastic capsule containing 20mm length of crystalline testosterone was implanted subcutaneously between the scapulae.
Stimulus females were ovariectomized at least two weeks prior to use for c-fos induction. Ovaries were removed via bilateral flank incisions and the cauterization of the uterine horn and blood vessels. Immediately following ovariectomy, Silastic capsules packed with 5mm length of crystalline estradiol were implanted between the scapulae. Approximately 4 hours prior to testing, females were injected with .1ml of progesterone dissolved in sesame oil (2.5mg/ml Sigma, St. Louis, MO, USA) to induce behavioral receptivity.

2.2.2 Retrograde Tracer Injections

One week prior to c-fos induction, subject animals were injected unilaterally into the BNST with the retrograde tracer Cholera Toxin Beta (CTB) conjugated to Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA) in order to visualize the MeA cell bodies that project to the BNST. CTB was prepared as a 0.5% solution in 0.1 M phosphate-buffered saline (PBS pH 7.4). Subjects were anesthetized and secured in a stereotaxic apparatus such that the skull was level in both the anterior-posterior (A-P) and medial-lateral (M-L) planes. A midline scalp incision was made and the skin and temporal muscles retracted to expose the skull. A-P and M-L measurements were taken in mm relative to bregma and dorsal-ventral (D-V) measurements were taken in mm relative to dura, which was exposed using a hand-operated drill.

90nl of CTB was deposited at rate of 20nl/minute by lowering a 26 gauge microinjection syringe (701N 10µl syringe, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into the BNST (A-P: -1.4mm, M-L: ±0.5mm, D-V: -6.9mm). The syringe was left in place for an additional 15 minutes following the injection to minimize the flow of CTB up the needle tract upon removal. Injections were alternated between the left and right hemispheres between subjects. Following
the removal of the syringe, skull holes were sealed using bone wax and wound clips were used to close incisions.

### 2.3 c-fos Induction

For seven days following injection of CTB, subject males were handled extensively and habituated to both the testing room and testing apparatus (see below.) On the day of c-fos induction subject and stimulus animals were brought to the testing room approximately 1 hour into the dark period and allowed to habituate to the environment for an additional hour. Subjects were then placed into a clean shoebox cage (43cm x 22cm) divided into two equal halves by a perforated acrylic divider and allowed to acclimate for 5 minutes. Following the acclimation period, either a stimulus male, hormonally primed stimulus female, or no animal was added on the other side of the perforated acrylic divider. The subject male was then allowed to investigate (but not contact) the stimulus undisturbed over a period of 70 minutes in order to induce the expression of c-fos. This length of time has been used previously (Been and Petrulis, 2011; Been and Petrulis, 2012) to successfully to generate stimulus-induced expression of c-fos. Immediately following the 70-minute stimulus period, subject males were injected with Beuthansia (.2ml of 390mg/ml pentobarbital sodium, 50mg/ml phenytoin sodium) and deeply anesthetized prior to perfusion.

Subject males were transcardially perfused with 200ml of 0.1M PBS (pH 7.4) followed by 200ml of 4% paraformaldehyde in phosphate buffer. Following removal, brains were stored in 4% paraformaldehyde over night (4°C) before being cryoprotected in 30% sucrose in PBS. Each brain was sectioned coronally at 30µm on a cryostat (-20°C) and collected in a 1:4 series directly on to slides before being stored at -80°C. All post-perfusion procedures were done using RNase free material.
2.4 In Situ Probe Generation

2.4.1 GAD65

In order to identify the chemical phenotype of MeA projection neurons or MeA neurons that responded to different social stimuli, one series of tissue for each animal was processed for *in situ* hybridization for vGlut2 and another for GAD65. Two GAD65 probes corresponding to different regions of the rat GAD65 gene were used concurrently to maximize detection of the mRNA. Plasmids containing these probes were kindly provided by Dr. Shane T. Hentges at Colorado State University.

These plasmids were amplified using XL1-Blue E.coli competent cells (Agilent Technologies, Santa Clara, CA, USA) and DNA was extracted and purified using a Qiagen Plasmid Midi Kit (Qiagen, Limburg, Netherlands). Plasmids were then linearized using the restriction enzyme XbaI to generate an anti-sense strand and HindIII to generate the control sense strand. Linearized DNA was then purified and precipitated. Transcription and labeling of the riboprobe with digoxigenin (DIG) (Roche Applied Sciences, Penzberg, Germany) was achieved using a MAXIscript T7 and MAXIscript T3 kits (Life Technologies, Carlsbad, CA, USA) for sense and anti-sense probes respectively. Resulting RNA was purified using a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) and quantified on a NanoVue prior to being stored in RNase Free tubes at -80°C.

2.4.2 vGlut2

To our knowledge there is not an available vGlut2 probe with sufficient sequence homology to be used successfully in Syrian hamster tissue, therefore we generated a Syrian hamster vGlut2 probe.
Total RNA was extracted from the brain of one adult female Syrian hamster using a TRIzol Plus RNA Purification kit (Life Technologies, Carlsbad, CA, USA). RNA was then converted to cDNA using a Clone AMV First-Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). To amplify the region of interest, primers were designed by entering the hamster sequence for vGlut2 (NCBI Reference Sequence: XM_005083219.1) into the PrimerQuest tool (Integrated DNA Technologies (IDT), Coralville, IA, USA). These primers were used to generate a 496 base pair amplicon that was then inserted into the pCR™4-TOPO® TA, using a TOPO TA Cloning kit (Life Technologies, Carlsbad, CA, USA) before being amplified and linearized using the restriction enzyme NotI to generate an anti-sense strand and PmeI to generate the control sense strand. A DIG labeled vGlut2 riboprobe was generated using methods described above.

2.5 In Situ Hybridization

Five male subjects, injected with CTB, were processed for vGlut2 and GAD65 in order to determine the phenotype of projection neurons from the MeA to the BNST. To determine the phenotype of neurons responding to different stimulus conditions (male, female or clean), an additional 18 animals (n=6 for each condition) were processed for both vGlut2 and GAD65.

Tissue mounted on slides was washed in 2x Saline-Sodium Citrate buffer (SSC) for 10 minutes followed by a 10-minute acetylation wash in an acetic anhydride/triethanolamine solution (1.5% triethanolamine, .5% acetic anhydride in RNase free H₂O). Tissue was then post-fixed in acetone/methanol solution (1:1) for 5 minutes at 4°C followed by a 5-minute 2x SSC wash. All washes were performed at room temperature unless otherwise noted. Tissue was pre-hybridized for 30 minutes at room temperature in hybridization buffer (50% deionized formamide, 1% yeast tRNA, 10% dextran sulphate, 1x Denhardtts solution, 5% 20x SSC). Probes
were added to hybridization buffer at a concentration of 100ng/100µl and denatured at 90°C for 5 minutes. Tissue was hybridized at 58°C for 14-16 hours in a humid chamber for both vGlut2 and GAD65 probes.

The tissue was then subjected to two 10-minute stringency washes in 2x SSC at room temperature followed by a 15-minute digestion with RNase A (10µg/ml in 2x SSC) at 37°C. This was followed by a 30-minute 2x SSC wash at 56°C and two 10-minute 2x SSC washes at room temperature. The tissue was then quenched in 1% H2O2 in 1x SSC for 15 minutes, rinsed twice in 1x SSC with .1% Tween followed by one 5 minute TBS (20mM Tris, 150mM NaCl, pH 7.6) wash. Blocking solution (5% Casein, 5% Normal Sheep Serum in TBS) was applied and tissue was incubated for 30 minutes followed by 2 hour, room temperature incubation with anti-DIG-HRP (1:200, Roche Applied Sciences, Penzberg, Germany). Unbound antibody was washed away with three 10-minutes washes in TBS-T (0.05% Tween in TBS). DIG labeled probes were visualized using a TSA Plus Fluorescein kit (Perkin Elmer). Sections were incubated for 12 minutes in a 1:50 dilution of the Fluorescein working solution followed by three 10-minute washes in TBS.

Tissue used to determine the colocalization of CTB labeled neurons with vGlut2 or GAD65 mRNA was immediately cover-slipped using Prolong Gold (Life Technologies, Carlsbad, CA, USA).
Table 2.4.2.1: Probe Information

<table>
<thead>
<tr>
<th>Probe</th>
<th>Concentration</th>
<th>Hybridization Temperature</th>
<th>Accession ID number</th>
<th>Probe position (bp)</th>
</tr>
</thead>
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<tr>
<td>GAD65 (628bp)</td>
<td>1ng/µl</td>
<td>58°C</td>
<td>NM_008078.2</td>
<td>537-1,207</td>
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<tr>
<td>GAD65 (824bp)</td>
<td>1ng/µl</td>
<td>58°C</td>
<td>NM_008078.2</td>
<td>1,201–2,032</td>
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<tr>
<td>vGlut2 (496bp)</td>
<td>1ng/µl</td>
<td>58°C</td>
<td>XM_005083219.1</td>
<td>726-1,221</td>
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</table>

2.6 Immunohistochemistry

To determine the phenotype of stimulus-responsive neurons, a subset of tissue was double labeled for c-fos immunoreactivity. Immediately following TBS washes at the end of the *in situ* hybridization protocol, sections were incubated overnight with a rabbit polyclonal anti-fos antibody (1:150 in TBS-T, sc-7202 Santa Cruz Biotechnology, Dallas, TX, USA) targeting to the c-terminus of human c-fos. After incubation in primary antibody, the tissue was rinsed in PBS (ten 5-minute washes) and then incubated for 3 hours with anti-rabbit secondary antibody conjugated to AlexaFluor 594 (1:100, Life Technologies) in PBS-T (.4% Triton X in PBS). Sections were then rinsed 10 times in PBS before being cover-slipped with Prolong Gold.

Table 2.4.2.1: Antibody Information

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Manufacturer</th>
<th>Catalogue no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep Anti-DIG-HRP</td>
<td>1:200</td>
<td>Roche Applied Sciences</td>
<td>A21207</td>
</tr>
<tr>
<td>Rabbit Anti-cfos</td>
<td>1:150</td>
<td>Santa Cruz Biotechnology</td>
<td>11207733910</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>1:100</td>
<td>Life Technologies</td>
<td>Sc-7202</td>
</tr>
<tr>
<td>Goat Anti-Rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.7 Data Analysis

2.7.1 CTB + FISH Colocalization

One researcher who was blind to the hypotheses of the experiment conducted all analysis. Five subjects with CTB deposits confined to the BNSTpm and BNSTpi were examined for degree of CTB colocalization with vGlut2 and GAD65. Given the generally unilateral projections between the MeA and BNST (Coolen and Wood, 1998; Wood and Swann, 2005), only MeA sections ipsilateral to the injection sites were counted. One tissue section representing each atlas plate of the MeA (plates 24-29) in the Syrian Hamster atlas (Morin and Wood) was chosen from each animal. Sections were examined on a Zeiss Axioimager M2 epifluorescence microscope and Stereo Investigator software (MBF Bioscience, Williton, VT, USA) was used to generate unbiased estimates of the density of colocalized cell bodies. Tracing of the regions of interest was done at 5x magnification using local landmarks (e.g. optic tract) and a 200\(\mu\)m\(^2\) grid was used to determine the location of each image. Imaging was done at 40x and image stacks with a 2 \(\mu\)m interval between slices were collected through the depth of the tissue section (30\(\mu\)m). All CTB+ neurons that fell in the bounds of the 288\(\mu\)m x 219\(\mu\)m x 30\(\mu\)m volume (0.002mm\(^3\)) that was imaged, were counted and classified as GAD65+, vGlut2+ or neither.

CTB labeled cells were identified as having red fluorescent staining outlining the majority of the cell body while GAD65 and vGlut2 mRNA labeling resulted in punctate staining through the cell body. The average number of CTB+, CTB+/GAD65+, and CTB+/vGlut2+ cells was calculated per sample volume to generate a density for each cell type. The density of double-labeled cells (CTB+/GAD65+ or CTB+/vGlut2+) divided by the density of CTB labeled cells generated the percentage of projection neurons that were either vGlut2+ or GAD65+. 
2.7.2  \textit{c-fos + FISH Colocalization}

One researcher, blind to both the stimulus conditions and the probe type (GAD65 or vGlut2), conducted all the analysis. Images were acquired in same manner as described above and the degree of c-fos colocalization with vGlut2 and GAD65 mRNA was analyzed through the anterior-posterior extent of the medial amygdala. Fos+ cells were identified as having red fluorescent nucleus and GAD65/vGlut2 positive cells as having fluorescent green punctate staining throughout the cell body. The average number of Fos+, Fos+/GAD65+, and Fos+/vGlut2+ cells was calculated per sample volume. The density of double-labeled cells (Fos+/GAD65+ or Fos+/vGlut2+) divided by the density of Fos positive cells generated the percentage of stimulus-activated neurons that were either vGlut2+ or GAD65+ for each animal.
3 RESULTS

3.1 CTB+FISH

Males were included in the BNST injection group (n=5) only if clearly visible fluorescent staining was observed around the deposition site. Deposition site must consist of at least two atlas plates (Morin & Wood, 2011) and extend into both the BNSTpm and BNSTpi. Any animals in which the deposit labeling extended beyond the anterior-posterior limits of the BNST were excluded. The extent of CTB deposits in the BNST for all five animals is shown in Figure 3.1.3.1.

Student t-tests revealed no significant differences between the difference sub-regions of the MA (MeA, MePD, MePV) for either CTB+/GAD65 colocalization or CTB+/vGlut2 colocalization.

3.1.1 Anterior Medial Amygdala

Over all we saw relatively low levels of CTB co-localization with markers for Glutamatergic cells and GABAergic cells in the anterior medial amygdala (25% and 35% respectively). A two-tailed t-test showed that there was a significant difference between the percent colocalization of CTB+ cells with GAD65 compared to the percent colocalization of CTB+ cells with vGlut2 (t(8)=3.02, p=0.017). Representative examples of CTB+/vGlut2+ and CTB+/GAD65+ co-localization are shown in Figure 3.1.3.2 while counting domains for each atlas plate are shown in Figure 3.1.3.3. The average density of CTB+ cells as well as the average density of CTB+/GAD65+ and CTB+/vGlut2+ double-labeled cells are shown in Table 3.1.4.1.

3.1.2 Posterior Medial Amygdala

The posterior medial amygdala was divided into two sub-regions: The MePD and MePV. A two-tailed t-test showed that there was no significant difference between the percent
colocalization of CTB+ cells with GAD65 compared to the percent colocalization of CTB+ cells with vGlut2 in the MePD \( (t(6)=1.77, p=0.13) \) or the MePV \( (t(5)=-0.31, p=0.77) \).
3.1.3 Figures

Figure 3.1.3.1 CTB Injection Sites. Placement and spread of CTB deposition in BNST injected males. Reconstruction of coronal sections of the entire extents of CTB deposition within the
BNSTpm and BNSTpi for all five animals used. Numbers represent the distance posterior to bregma in millimeters.

Figure 3.1.3.2 Representative CTB+ Images.
Fluorescent images of MA sections containing A) CTB retrogradely labeled cells (from animal 2516, MePD, stained for GAD65) B) double-labeled CTB+/GAD65+ cells (from animal 2516, MePD) and C) double-labeled CTB+/vGlut2+ cells (from animal 2516, MePV). White arrows indicate double-labeled cells in each image while grey arrows indicate single-labeled CTB cells. Scale bar = 25µm.
Counts were taken from sections representing six atlas plates of the MA that included three of the MeA, three of the MePD and two of the MePV. Each region was traced by hand using Stereo Investigator software (MBF Bioscience, Williton, VT, USA).

**Figure 3.1.3.3 Counting Domains.**
Figure 3.1.3.4 Percent CTB+ cells that colocalize with GAD65 or vGlut2
A higher percentage of CTB+ cells were double labeled with markers for GABAergic neurons than glutamatergic neurons in the MeA. *p=0.017, two-tailed t-test.
A predominantly GABAergic population of neurons projects from the MeA to the BNST. Approximately equal proportions of GABAergic and glutamatergic neurons project from the MePV to the BNST. An intermediate number of GABAergic neurons project from the MePD to the BNST with a smaller population of glutamatergic neurons projecting from the MePD to the BNST.

3.1.4 Tables

Table 3.1.4.1 Average density of CTB+ and double-labeled cells in the MA.
The mean (± SEM) number of CTB+ cells and CTB+/GAD65 and CTB+/vGlut2+ double-labeled cells within each region.

<table>
<thead>
<tr>
<th>Total/.002mm³</th>
<th>MeA</th>
<th>MePD</th>
<th>MePV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Density</td>
<td>Standard Deviation</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------</td>
<td>--------------------</td>
<td></td>
</tr>
<tr>
<td>CTB+</td>
<td>16.99±1.01</td>
<td>15.92±1.46</td>
<td></td>
</tr>
<tr>
<td>CTB+/GAD65+</td>
<td>6.81±0.74</td>
<td>7.07±1.46</td>
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<tr>
<td>CTB+/vGlut2+</td>
<td>3.45±0.4</td>
<td>3.05±0.72</td>
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</tr>
</tbody>
</table>

### 3.2 c-fos + FISH

#### 3.2.1 Anterior Medial Amygdala

The impact of exposure to social odors on increased c-fos expression in the MA is well documented (delBarco-Trillo et al., 2009; Meredith & Westberry, 2004; C L Samuelsen & Meredith, 2009). The average density of c-fos+ cells in the MeA did not differ between female and male exposed subjects, or baseline (e.g levels found when exposed to a clean cage) (Two-tailed t-test; female vs. clean: t(6) =0.69 ,p = 0.52 male vs. clean: t(7)=1.58, p=0.16, male vs. female: t(4)=1.35, p=0.24). Two-tailed t-test determined that the percent of c-fos+/GAD65+ (Figure 3.2.3.2) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli. Similarly the percent of c-fos+/vGlut2+ (Figure 3.2.3.3) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli.

A significantly greater percentage of c-fos+ neurons in the MeA colocalized with GAD65 with exposure to a male or clean stimulus but not to female stimulus (male: t(5)=3.69, p=0.016, female: t(7)=1.19, p=0.27, clean: t(8)=2.81, p=0.023). The average density of c-fos+ cells and co-localized cells are shown in Table 3.1.4.1.

#### 3.2.2 Posterodorsal Medial Amygdala
The average density of c-fos+ cells in the MePD did not differ between female and male exposed subjects, or baseline (e.g. levels found when exposed to a clean cage). The percentage of c-fos+/GAD65+ (Figure 3.2.3.2) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli. The percent of c-fos+/vGlut2+ (Figure 3.2.3.3) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli. A significantly greater percentage of c-fos+ neurons in the MePD colocalized with GAD65 with female and clean stimulus conditions but not male (male: t(6)=1.69, p=0.144, female: t(8)=2.35, p=0.047, clean: t(7)=3.56, p=0.008).

3.2.3 Posteroventral Medial Amygdala

The percent of c-fos+/GAD65+ (Figure 3.2.3.2) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli. The density of c-fos+/vGlut2+ (Figure 3.2.3.3) double-labeled cells did not differ significantly between subjects exposed to male, female or clean stimuli. There was no significant difference between the percentage of c-fos+/GAD65+ and c-fos+/vGlut2+ cells under any stimulus condition (male: t(4)=0.31, p=0.77, female: t(6)=0.19, p=0.86, male: t(6)=0.59, p=0.57). The average density of c-fos+ cells and co-localized cells are shown in Table 3.2.4.1.
3.2.4 Figures

Figure 3.2.4.1 Counting Domains
Counts were taken from sections representing six atlas plates of the MA that included three of the MeA, three of the MePD and two of the MePV. Each region was traced by hand using Stereo Investigator software (MBF Bioscience, Williton, VT, USA).

Figure 3.2.4.2 Representative c-fos images
Fluorescent images of MA sections containing A) double-labeled c-fos+/GAD65+ cells (MeA of male exposed animal and B) double-labeled c-fos+/vGlut2+ cells (MePV of male exposed animal). White arrows indicate double-labeled cells in each image while grey arrows indicate single-labeled c-fos cells. Scale bar = 25µm.
Figure 3.2.4.3 Percent c-fos+ cells that are GAD65+

The percent of c-fos+ cells that were colocalized with a marker of GABAergic neurons was equivalent for all stimulus conditions and regions. (male: n=4, female: n=5, clean: n=6)
Figure 3.2.4.4 Percent c-fos+ cells that are vGlut2+
The percent of c-fos+ cells that were colocalized with a marker of glutamatergic neurons was equivalent for all stimulus conditions and regions. (male: n=4, female: n=5, clean: n=6)
Figure 3.2.4.5 Colocalization of c-fos in the MeA
A significantly greater percentage of c-fos+ cells were GABAergic in nature than glutamatergic in the MeA. This was true for male and clean conditions. *Male condition: p=0.016, Female condition: p=0.27, Clean condition: p=0.023 (male: n=4, female: n=5, clean: n=6)
Figure 3.2.4.6 Colocalization of c-fos in the MePD
A significantly greater percentage of c-fos+ cells were GABAergic in nature than glutamatergic in the MePD for all female and clean conditions. * Female condition: p=0.048, Clean condition: p=0.008. (male: n=4, female: n=5, clean: n=6)
Figure 3.2.4.7 Colocalization of c-fos in the MePV
The percent of c-fos+ cells that were colocalized with a marker of GABAergic and glutamatergic neurons was equivalent for all stimulus conditions in the MePV. (male: n=4, female: n=5, clean: n=6)
Figure 3.2.4.8 Average density of c-fos+ cells
The average density of c-fos+ neurons in response to a social stimulus did not differ from baseline in any region. (male: n=4, female: n=5, clean: n=6)
### 3.2.5 Tables

Table 3.2.5.1 Average density of c-fos+ and double-labeled cells in the MA.
The mean (± SEM) number of c-fos+ cells, c-fos+/GAD65 and c-fos+/vGlut2+ double-labeled cells in each region for each stimulus group.

<table>
<thead>
<tr>
<th>Total/.002mm³</th>
<th>MeA</th>
<th>MePD</th>
<th>MePV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>c-fos+</strong></td>
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<tr>
<td>Male</td>
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<td>4.76±0.47</td>
<td>6.34±0.87</td>
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<td>Female</td>
<td>6.49±0.5</td>
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<td>8±1.1</td>
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<tr>
<td>Clean</td>
<td>5.03±0.35</td>
<td>3.2±0.32</td>
<td>5.3±0.52</td>
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<tr>
<td><strong>c-fos+/GAD65+</strong></td>
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<td></td>
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<tr>
<td>Male</td>
<td>4.67±0.52</td>
<td>3.26±0.51</td>
<td>3.24±0.65</td>
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<td>Female</td>
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<td>5.23±0.8</td>
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<tr>
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<td><strong>c-fos+/vGlut2+</strong></td>
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<tr>
<td>Male</td>
<td>2.87±0.41</td>
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<tr>
<td>Clean</td>
<td>1.19±0.16</td>
<td>1.06±0.25</td>
<td>2.39±0.56</td>
</tr>
</tbody>
</table>
4 DISCUSSION

4.1 CTB+FISH

CTB has been used previously to retrogradely label MA afferents (Coolen & Wood, 1998; Maras & Petrulis, 2010). In this study we have combined CTB labeling with in situ hybridization for markers of glutamatergic and GABAergic neurons. This method allows us to expand previous findings to include the inhibitory or excitatory nature of these projection neurons.

Sub-regions of the MA are known to have different projection targets, for example, the MeA projects predominantly to the BNSTpi while the MePD projects to the BNSTpm (Gomez & Newman, 1992). Our injections encompassed portions of both the BNSTpm and BNSTpi and so we are unable to differentiate between the specific BNST targets of the retrogradely labeled MA neurons. These data show a predominantly GABAergic phenotype in the MeA and an equal proportion of GABAergic and glutamatergic projection neurons in the MePD and MePV. Subjects where the BNSTpm only or BNSTpi only were targeted with injections of CTB are being analyzed to more accurately determine the destination of these projection neurons. It is possible that coding for different kinds of social stimuli are depending on the target of the projection neurons in the MA.

4.2 c-fos + FISH

The IEG c-fos has been widely used as an indirect marker of neuronal activation, particularly in response to social odor stimuli (Been & Petrulis, 2011; delBarco-Trillo et al., 2009; Meredith & Westberry, 2004; C L Samuelsen & Meredith, 2009). Using a live-animal stimulus model and in situ hybridization for markers of glutamatergic and GABAergic neurons,
we have identified a primarily GABAergic neural response to social stimuli in the MeA and an equal response of GABAergic and glutamatergic neurons in the MePD and MePV.

4.2.1 Methodological considerations

Immunohistochemistry for c-fos provides a useful measure of neuronal activation in response to social stimuli; however, this technique has limitations as a marker of neuronal activation. For example, c-fos does not provide information regarding the temporal dynamics of neuronal response, nor the inhibitory or excitatory nature of the synaptic transmission. Furthermore, measurements of baseline levels of c-fos expression vary according experimental design. In our set up, baseline, (“clean” exposed) subjects are placed into an empty cage, free of odors. Removal from the home cage may result in increased neural activation in the MA, which may reduce the significance of increased c-fos levels to social stimuli. Furthermore, a relatively large range in baseline levels of c-fos expression is possible between animals, so will our small number of subjects, it is possible that the variation between subjects is suppressing differences in c-fos expression to social stimuli.

In situ hybridization is a useful tool for determining the chemical phenotype of these neurons activated by social stimuli. The labeling and identifying of mRNA transcripts allows for colocalization with the nuclear protein c-fos, with the transcripts of proteins that are not localized to the cell body. There are limitations to this technique that must be considered when comparing the degree of labeling between two different target mRNA transcripts. In situ hybridization requires large amounts of transcript for adequate labeling, and depending on the efficiency with which a probe binds a specific transcript, it is possible to underestimate the relative abundance of cells expressing these transcripts. Furthermore, it is important to consider the chosen target transcript. For example, there are two isoforms of glutamic acid decarboxylase (GAD) that are
commonly used as markers for GABAergic neurons, GAD65 and GAD67. In rats, optimum in situ staining for GAD65 was achieved only with increased time in the color reaction (compared to staining for GAD67) indicating possible lower levels of GAD65 mRNA than GAD67 mRNA (Esclapez, Tillakaratne, Kaufman, Tobin, & Houser, 1994). Overall lower levels of GAD65 mRNA may explain some differences in labeling seen in this study, which used GAD65.

4.2.2 MeA

The anterior portion of the MA receives considerable chemosensory input from the accessory olfactory system. Studies using c-fos have show that this region is activated by both conspecific and heterospecific social odors (Meredith & Westberry, 2004). Additionally, the MeA responds equally to same-sex and opposite-sex social odors (Chad L. Samuelsen & Meredith, 2009). Lesions of the MeA significantly decrease levels of c-fos expression in brain regions to which the MeA projects (Maras & Petrulis, 2010). This demonstrates that the MeA plays an important role in conveying information about many types of social odors to other nodes of the social behavior network.

Our data show that in the MeA, greater numbers of GABAergic neurons are responding to male and clean stimuli. However, the percentage of activated neurons that are GABAergic or glutamatergic do not differ between stimulus types. The finding that exposure to male and female stimuli results in equivalent levels of GABAergic responses in the MeA is consistent with previous findings (Donato et al., 2010). However, this method of analysis fails to take into account the targets of these projection neurons and further studies are needed to determine whether the activated GABAergic and glutamatergic populations have different projection targets for specific types of social stimuli.
4.2.3 MePD

The response of the MePD to social stimuli is generally more limited in comparison to the responses seen in the MeA. Increased activity, as measured through c-fos expression, is seen only with exposure to conspecifics and to a greater degree when exposed to opposite-sex versus same-sex odors (delBarco-Trillo et al., 2009; Meredith & Westberry, 2004; Chad L. Samuelsen & Meredith, 2009). Our data shows no difference in the percent of activated GABAergic or glutamatergic neurons in response to females than to males. This contrasts with previous reports of greater colocalization of GABAergic markers with c-fos+ cells upon male stimulus (Donato et al., 2010). The MePD is composed of a heterogeneous neuronal population, with differences in the rostro-caudal axis. Differences in the rostral-caudal distribution of GABAergic and glutamatergic neurons might account for differences seen between this study and previous work.

The percentage of GABAergic neurons activated by female and clean stimulus was significantly greater than the percentage of glutamatergic neurons activated by the same stimuli. Given the large population of GABAergic neurons found in the MePD compared to other sub-regions of the MA (Simmons & Yahr, 2003) this may be an artifact of the predominant phenotype within the region and not a means of coding for specific social stimulus.

Another consideration is that the MePD is composed of populations of hormonally sensitive cells, Appropriate levels of gonadal hormones are required for a male hamster’s attraction to female odors (Been & Petrulis, 2011; delBarco-Trillo et al., 2009; Meredith & Westberry, 2004; C L Samuelsen & Meredith, 2009). This suggests the
coding scheme for processing social information is dependent on hormonally sensitive neurons and the chemical phenotypes of these hormonally responsive cells are the code for different social stimuli.

4.2.4 **MePV**

It has been reported that c-fos expression does not reliably increase in the MePV with social stimulus that includes physical contact (mating or agonistic) (Kollack-Walker & Newman, 1995). However, in the case where males are exposed strictly to social odors, the MePV does show significantly more activation to opposite-sex odors (Maras & Petrulis, 2010). Our exposure apparatus allows for visual, auditory and olfactory stimulus with limited physical contact (through a perforated acrylic sheet). Indeed, our results do not show a significant neuronal activation with exposure to male or female stimuli, above the baseline levels.

The MePV is traditionally studied in the context of defensive behaviors, and neurons in the MePV respond significantly to predator odor (Dielenberg, Hunt, & McGregor, 2001). Additionally, the MePV has predominantly glutamatergic projections to the ventromedial hypothalamus (Bian et al., 2008), a region that is important for both reproductive and defensive behavior (Canteras, Simerly, & Swanson, 1992). We show equivalent levels of glutamatergic and GABAergic neural activation in the MePD, regardless of stimulus type. Future studies are needed to determine the nature of GABAergic and glutamatergic populations of neurons responding to social stimuli have the same target regions when exposed to different social stimuli.
REFERENCES


