THE INFLUENCE OF INFANT-CAREGIVER EXPERIENCES ON AMYGDALA *Bdnf*, *OXTr*, AND *NPY* EXPRESSION IN DEVELOPING AND ADULT MALE AND FEMALE RATS

by

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ABSTRACT

Previous work with various animal models has demonstrated that alterations in the caregiving environment produces long-term alterations in anxiety-related and social behaviors, as well as amygdala gene expression. We previously introduced a rodent model in which the timing and duration of exposure to maltreatment or nurturing care outside the home cage can be controlled to assess neurobiological outcomes. Here we sought to determine whether our brief experimental conditions produce changes in gene expression within the developing and adult amygdala. Using a candidate gene approach, we examined fold mRNA changes for the *Brain-derived neurotrophic factor* (*Bdnf*), *Oxytocin receptor* (*OXTr*), and *Neuropeptide Y* (*NPY*) genes, which are all highly expressed in the amygdala and play important roles in anxiety-related and social behaviors. In adults, significant group differences were detected for only *Bdnf*, with higher levels of *Bdnf* mRNA for females that had been exposed to maltreatment and males exposed to nurturing care outside the home cage relative to littermate controls. For pups, significant group differences were detected for only *OXTr*, with lower levels of *OXTr* mRNA in females exposed to maltreatment. Finally, for adolescents, maltreated-females showed significant changes in *Bdnf* (decreased), *OXTr* (decreased), and *NPY* (increased) mRNA relative to controls. These data illustrate the ability of brief, but repeated exposure to different caregiving

environments during the first postnatal week to have long-term effects on gene expression within the developing and adult amygdala, especially in females.

Chapter 1

INTRODUCTION

1.1 Early-life Stress and Its Effects on Behavior and the Brain

Rodent models of early-life stress, ranging in depriving offspring of maternal care to exposing them to bouts of unpredictable shock or aberrant caregiving, demonstrate the ability of early-life environmental disturbances to have long-term effects on both social and non-social behaviors and their underlying neurobiology [1-6]. For example, adolescent and adult rats that receive less maternal care or unpredictable shock during infancy express increased anxiety-like behavior and heightened stress responses [7-11]. Alterations in social behavior, increased depressive-like behaviors, and maltreatment of next-generation offspring are also outcomes observed in rodents exposed to changes in the caregiving environment [12-16]. The neural correlates of these behavioral outcomes include structural remodeling, changes in neuropeptide and hormone systems, decreased cell proliferation, and aberrant DNA methylation and gene expression in regions such as the prefrontal cortex, hippocampus and amygdala [3, 5, 17-20].

1.2 Early Maltreatment and Its Effects on DNA Methylation

To better understand the capacity of exposure to maltreatment to alter the developing and adult brain and its relationship to subsequent development of behavior, we previously introduced an experimental model where we expose rat pups to brief but repeated bouts of caregiver maltreatment outside the home cage [13, 21, 22]. Our initial work using this paradigm showed that adults exposed to maltreatment displayed significant alterations in *Brain-derived neurotrophic factor* (*Bdnf*) mRNA and DNA methylation in the prefrontal cortex [13, 21]. We also demonstrated that females with a history of maltreatment displayed significant amounts of aversive behaviors toward their own offspring in the forms of increased stepping on the pups, roughly handling them, and frequently dropping them during transport [13]. More recent work has demonstrated *Bdnf* DNA methylation alterations in additional brain regions, including the hippocampus and amygdala [22].

1.3 Rationale for Current Study

The aim of this study was to further extend our characterization of the developing and adult amygdala by examining gene expresson. Given the role of the amygdala in social and emotional behavior, such data may help to understand mechanisms by which early-experiences contribute to the development of mood- and social-related behaviors later in life. Three genes associated with brain plasticity, stress reactivity, and social behaviors were explored: *Bdnf*, the *Oxytocin receptor* (*OXTr*), and *Neuropeptide Y* (*NPY*). Basal expression (i.e. in animals at baseline

conditions and not in response to any stimulus) of all three genes were assessed at multiple time points (infancy, adolescence, and adulthood) to enable us to compare group differences across development. We also explored whether amygdala gene expression patterns differed between males and females.

Chapter 2

METHODS

2.1 Subjects

Male and female Long-Evans rats were obtained from Harlan and housed in our laboratory's breeding colony. Animals were housed in polypropylene cages with plentiful amounts of wood shavings. Temperature and light were controlled in the colony room (12-hour light/dark cycle with lights on at 6:00 am), and animals were provided *ad libitum* access to food and water throughout the experiments. Prior to beginning experiments, females raised at least one litter so that no first-time mothers were used. Females were bred, and on postnatal day (PN) 1 litters were culled to 5-6 males and 5-6 females. 18 litters were used to generate all PN8, PN30 and PN90 subjects for tissue collection. The sample used here includes subjects that were also used in our recent studies reporting methylation patterns within the prefrontal cortex, amygdala, and hippocampus [21, 22]. The University of Delaware Animal Care and Use Committee approved all procedures.

2.2 Caregiving Manipulations

Behavioral manipulations were always performed during the light cycle using methods as previously reported [13, 21, 22]. Litters were divided in to three equal groups on PN1: maltreatment, cross-foster care and normal maternal care. Beginning on PN1 and continuing through PN7, up to 4 pups (2 males and 2 females) from a litter were exposed daily to a stressed dam outside of the home cage for 30 minutes (maltreatment condition). The maltreatment condition consisted of a lactating female placed in a novel environment with limited nesting material (approximately 100ml of wood shavings scattered on the chamber floor). Additional pups (up to 2 males and 2 females) from the same litter were exposed to a non-stressed dam outside of the home cage (cross-foster condition). The cross-foster caregiver was also a lactating female that was given 1 hour to habituate to the novel chamber and was provided with ample amounts of bedding material (a 2-cm layer across the chamber floor). Remaining pups from the litter (up to 2 males and 2 females) remained with the biological mother in the home cage during manipulations and were deemed the control group. After each 30-minute exposure session, the experimental pups were removed from the exposure chambers and were placed back into the home cage with the biological mother. On PN8 (24 hrs. following the last manipulation), some of the subjects had their brains removed, while others were allowed to reach PN30 (each litter provided one male and one female rat for each experimental condition and age). A separate cohort of rats was

used to generate PN90 animals. At PN21-23, rats were housed in same-sex and same-condition pairs through adolescence and into adulthood.

Caregiving behaviors and pup vocalizations were scored for all 3 exposure conditions by live observations and/or video recordings. As previously reported for this sample [21, 22], pups assigned to our maltreatment condition were subjected to a greater proportion of aversive caregiving behaviors (i.e. being stepped on, dropped, dragged, actively avoided, and roughly handled) than pups assigned to either the cross-foster or normal maternal care conditions. Pups assigned to the maltreatment condition also emitted significantly more audible and ultrasonic vocalizations during exposure sessions.

2.3 Gene Expression Assays

Animals were sacrificed at baseline conditions (i.e. straight from the home cage with minimal stimulation) on PN8, PN30 or PN90. Brains were removed and sliced using a 1-mm brain matrix. They were then flash-frozen on untreated slides with 2-methylbutane and placed in a -80^{o}C freezer until needed for later tissue extraction and processing. The amygdala (basolateral, lateral, and central nuclei were homogenized together to yield sufficient RNA) was dissected on dry ice using stereotaxic coordinates [23] and RNA was extracted using the AllPrep DNA/RNA kit (Qiagen Inc., Valencia, CA) and following the manufacturer's instructions. Quantification and qualification of nucleic acid were determined using

spectrophotometry (NanoDrop 2000). Reverse transcription was performed using a cDNA synthesis kit (Qiagen) and cDNA was then amplified using a real-time PCR system (Bio-Rad CFX96). Taqman probes (Life Technologies) targeted *Bdnf*, *OXTr*, and *NPY* or *tubulin* (for a reference gene) mRNA. Gene assays were always run in triplicates, and product specificity was determined via gel electrophoresis. Comparative C_t measures [24, 25] were used to obtain fold changes for the maltreatment and cross-fostered animals relative to normal care animals.

2.4 Statistical Analysis

One-sample t-tests compared with a hypothetical mean value of 1 were used to analyze fold changes in transcripts in the maltreatment and cross-foster care conditions in comparison to normal care controls. A mean value of 1 would indicate no change in transcript level in comparison to the control group. Additionally, two-way ANOVAs (and t-tests when appropriate) were used to compare fold changes between maltreated- and cross-fostered animals. Differences were considered statistically significant for $p < 0.05$.

Chapter 3

RESULTS

3.1 Gene expression in the Adult Amygdala

Since our prior work with this model revealed long-term changes in *Bdnf* mRNA levels in the prefrontal cortex of adult animals that had been maltreated [13], we first examined *Bdnf* gene expression in the amygdala of our PN90 animals (Figure 1). For *Bdnf*, both the cross-fostered males (t_7=2.47, $p < 0.05$) and maltreated females (t_{13}=2.62, $p < 0.05$) had higher mRNA levels relative to controls. An ANOVA analysis showed no significant main effect of pup condition or sex but a significant sex X pup condition interaction ($F_{1, 39}$=4.76, $p<0.05$).

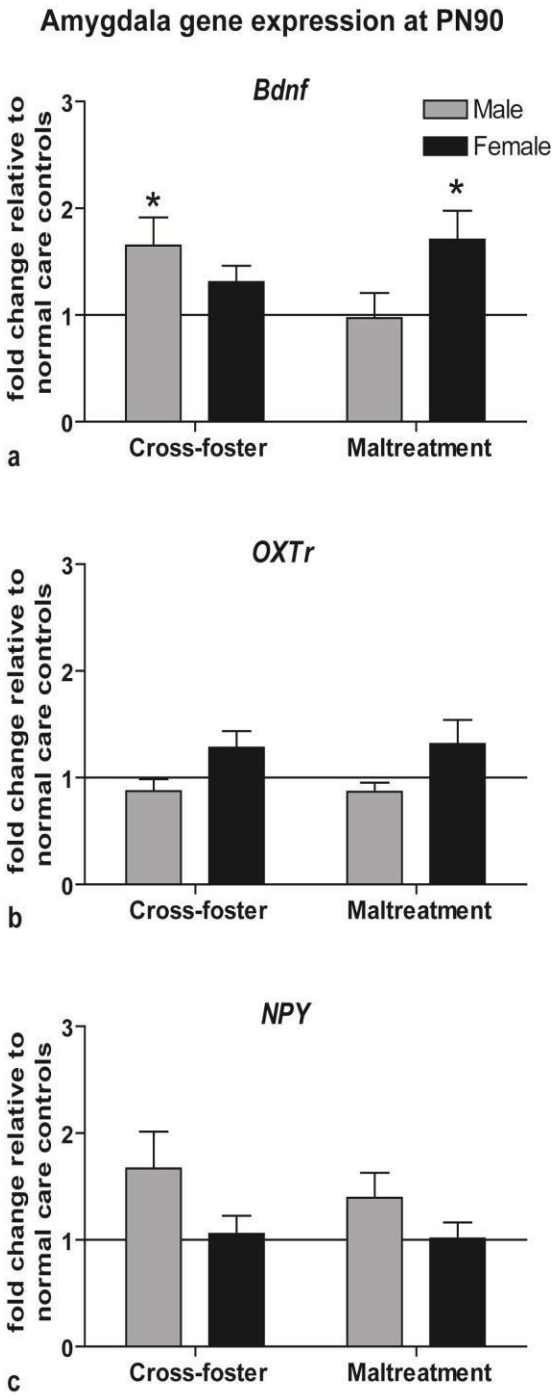

Figure 1 Adult gene expression. Baseline measures of fold changes in (a) Bdnf, (b) OXtr, and (c) NPY mRNA in PN90 amygdala tissue. For all genes, n = 8-14/group; *$p<0.05$ versus normal care controls; error bars represent SEM. The solid line represents a fold change of 1, equal to no difference in expression between experimental and control subjects

We also examined two other candidate gene loci known to be highly expressed in the amygdala and that play roles in social behavior and stress responsivity, *OXTr* and *NPY*. For *OXTr*, maltreated and cross-foster care animals did not statistically differ from controls. An ANOVA showed a significant main effect of sex ($F_{1, 37}$=6.12, $p<0.05$), with females showing higher expression than males, but no significant main effect of pup condition or a significant sex X pup condition interaction. Likewise, one-sample t-tests showed no differences between our experimental and control groups in *NPY* expression. There was again a significant main effect of sex for *NPY* ($F_{1, 34}$=5.24, $p<0.05$) with males showing higher *NPY* expression than females. There was no significant main effect of pup condition or a significant sex X pup condition interaction.

3.2 Gene expression in the Developing Amygdala

To assess the effects of our caregiving environments on pup gene expression patterns, gene assays were performed on tissue collected 24 hrs. after the last exposure session (at PN8; Figure 2). For *Bdnf*, there were no significant group differences across conditions or sex. For *OXTr,* maltreated females (t_7=4.84, $p<0.01$ vs. normal care controls) had significantly less mRNA in comparison to normal care controls. As with *Bdnf*, there were no significant changes for *NPY*. Two-way ANOVAs for each gene locus also did not reveal any significant main effects of pup condition or sex, or any sex X pup condition interactions.

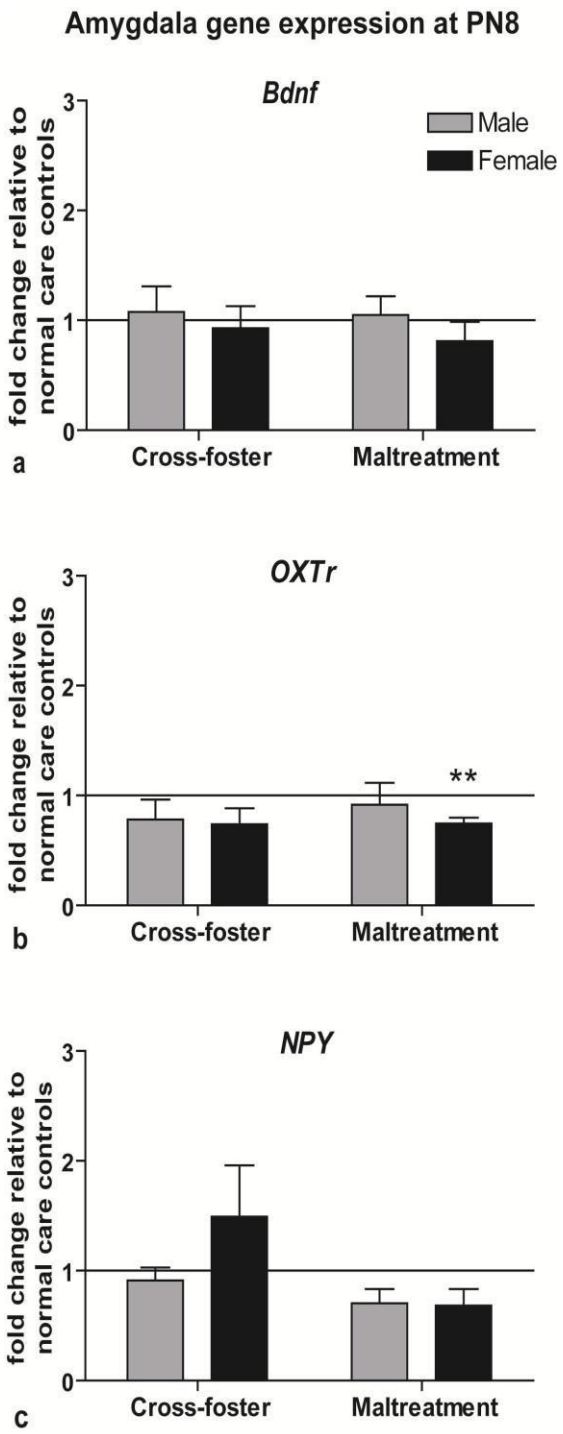

Figure 2 Pup gene expression. Baseline measures of fold changes in (a) Bdnf, (b) OXtr, and (c) NPY mRNA in PN8 amygdala tissue. n = 7-8/group; **p<0.01 versus normal care controls; error bars represent SEM. The solid line represents a fold change of 1, equal to no difference in expression between experimental and control subjects.

Finally, to assess the effects of our caregiving environments on adolescent gene expression patterns, real-time PCR assays were performed on tissue collected at PN30 (Figure 3). Maltreated-females had significantly less *Bdnf* (t_7=3.77, $p<0.01$) and *OXTr* (t_8=2.56, $p<0.05$) mRNA than normal care controls. For *NPY* however, maltreated-females had significantly higher mRNA levels (t_9=2.57, $p<0.05$ vs. normal care controls). Two-way ANOVAs for *Bdnf* and *NPY* did not reveal any significant main effects of pup condition, sex, or sex X pup condition interaction. There was a significant main effect of sex for *OXTr* ($F_{1, 34}$=5.42, $p<0.05$), but no significant main effect of pup condition or an interaction.

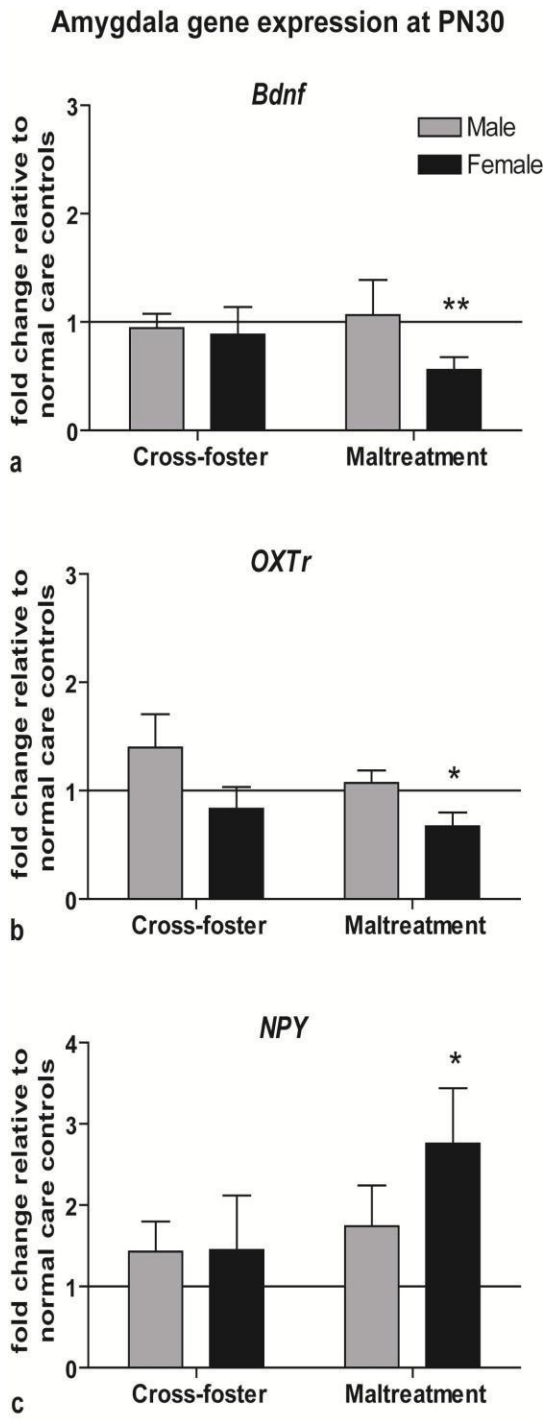

Figure 3 Adolescent gene expression. Baseline measures of fold changes in (a) Bdnf, (b) OXtr, and (c) NPY mRNA in PN30 amygdala tissue. n = 7-10/group; **p<0.01 or *p<0.05 versus normal care controls; error bars represent SEM. The solid line represents a fold change of 1, equal to no difference in expression between experimental and control subjects.

Chapter 4

DISCUSSION

This study was designed to determine whether different caregiving conditions experienced by pups during infancy, particularly maltreatment, produced changes in amygdala gene expression. We examined this in both developing and adult males and females using an experimental design where we distributed pups within the same litter to different and repeated experimental treatments during the first week of life. Our observations of caregiver and pup behavior during the exposure sessions indicate that within the maltreatment conditions pups were subjected to frequent occurrences of aversive caregiving (including frequent stepping on, dragging, and rough handling), and that they differentially responded to this caregiving environment by emitting significant audible and ultrasonic vocalizations [21, 22]. This is in contrast to the cross-foster and normal care conditions, where pups emitted significantly fewer vocalizations and experienced significant levels of nurturing care, including pup licking and grooming and nursing.

The major finding of the present study is that exposure to our maltreatment regimen produced an age-dependent and long-term impact on amygdala gene expression in females. Both PN30 and PN90 maltreated females displayed significant alterations in *Bdnf* mRNA levels (relative to normal care controls). Strikingly, the directional nature of group differences differed in adolescence (decreased) and

adulthood (increased). Isolation from the nest and/or biological mother and variations in the caregiving environment have been shown to produce changes in the levels of *Bdnf* mRNA and protein in the adult prefrontal cortex and hippocampus, with concomitant changes in various realms of behavior [13, 14, 26-30]. Consistent with our age-related observations here, age-dependent effects of maternal deprivation have been previously reported for *Bdnf* in the prefrontal cortex, with stress producing an increase in *Bdnf* levels in PN17 rats while a decrease in levels in PN90 rats [29]. A recent report has shown that periodic bouts of pup separation from the mother produces increased BDNF immunoreactive cells within the adult amygdala [31]. Here we demonstrate divergent developmental effects of maltreatment on *Bdnf* expression within the female amygdala. An increase in *Bdnf* mRNA in adult females may be consistent with observations of dendritic remodeling and amygdala hyperresponsivity that are known to occur with development and stress [32-35].

During both infancy and adolescence, maltreated females also exhibited lower levels of *OXTr* mRNA in comparison to normal care controls. Maternal deprivation and low levels of pup-licking and arched-back nursing have been shown to reduce CNS OXTr levels [36, 37], while high levels of early peer interactions are known to increase OXTr binding in regions of the adult amygdala [30]. OXT is involved in affiliative/social and stress-related behaviors, and a reduction in *OXTr* has been linked to increased anxiety responses, neuroendocrine responses to stress, and decreases in social interactions [38-43]. Our data indicate transient *OXTr* mRNA changes that are present across two life periods when socialization is a major factor driving CNS and

behavioral trajectories. Future work will be necessary to determine whether the observed mRNA reductions have relevance in regard to early affiliative/social behaviors.

Adolescent maltreated females also had greater *NPY* mRNA levels in comparison to normal care controls. Previous work has also shown that early maternal deprivation decreases adult hippocampal NPY levels [44-46] while increasing hypothalamic NPY levels [45]. Furthermore, work has shown that individual susceptibilities to long-lasting neuroendocrine differences are significantly influenced by an interaction between a *NPY* gene variation and an aversive family environment [47]. As alterations in limbic NPY are common observations in models of depression and anxiety [48-50], our data suggest adolescent females could have alterations in stress regulation and stress-related behaviors.

Finally, a manipulation that did not produce any obvious behavior difference in pups (at least in terms of vocalizations), brief and repeated experiences with a nurturing foster dam, also had long-term effects on *Bdnf* gene expression. This effect however was exclusive to adult males. Since we did not see *Bdnf* changes in maltreated-males, this suggests males from the foster-care condition responded to a factor unique to their treatment. This would mostly likely be experiencing nurturing care from multiple dams.

We have previously shown later emerging (by PN30) and age-dependent (decreased at PN30 while increased at PN90) effects of maltreatment on *Bdnf* DNA methylation in the female medial prefrontal cortex [21]. As changes in the caregiving

environment are known to change the developmental trajectory of prefrontal-amygdala connectivity in humans [51], and with the late developing nature of the prefrontal cortex [52] and bidirectional connections between the regions [53], it is possible that our observations of gene alterations in the amygdala are related to those in the prefrontal cortex. A number of other mechanisms may be involved in the developmental changes in gene expressions we observed, including neuroprotection and stress adaption [54-56] and later emerging DNA methylation alterations [21, 22].

Finally, we also note that due to the design of our exposure chambers, we were not able to attribute specific types of caregiving behaviors or vocalizations to individual male or female pups during the exposure sessions. It is possible that caregiving behaviors displayed by dams differed between males and females. It is also known that mRNA and protein levels of epigenetic regulators within the developing amygdala (*Dnmt3a* [57] and MeCP2 [58]) are higher in females than in males. These are both additional factors likely responsible for our different observations in males and females. Future studies will be necessary to determine which specific factors within each condition and mechanisms are responsible for the observed effects.

CONCLUSIONS

In the present study, we have shown that repeated exposure to caregiver maltreatment during the first postnatal week produces long-term and age-dependent alterations in amygdala gene expression. These findings provide further empirical support of epigenetic consequences of exposure to caregiver maltreatment, and the ability of early-life experiences to differentially affect developmental trajectories (of amygdala gene expression) in males vs. females. Extending this work on cellular and behavioral levels will be necessary to ascertain how early-life environmental conditions and social stressors can affect developmental trajectories.

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Appendix A

Permission Letter

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November 14, 2014

This letter is to verify that Kathryn Taylor received all necessary animal training for the experimental work that is the basis of her thesis. Also, I verify that all procedures are consistent with our IACUC protocol #1216.

Sincerely,

Tania L. Roth, Ph.D.
Assistant Professor