Evaluating the role of stress and pain in murine affiliation using a novel behavioral assay

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February, 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

Modeling social interaction is complex, influenced by a number of genetic, psychological, and social factors. While a robust autism literature continues to report deficits in multiple correlates of social behavior among human clinical populations, attempts to evaluate sociability among preclinical models using current behavioral assays have failed to produce new treatments for social deficit disorders. Here I present a novel behavioral assay, the Tube Co-Occupancy Test (TCOT) as a unique way to evaluate rodent affiliative behavior.

Specifically, the TCOT is a way to measure "propinquity," or the tendency for rodents to co-occupy a tube over an extended period of time. Unlike other measures of rodent social behavior, animal tube co-occupation more closely mirrors human social preference (with a demonstrable bias to interact and be closer to familiars than strangers), especially in a stressful environment. Propinquity also serves as a robust and reliable measure of rodent affiliation, a necessary prerequisite for subsequent tests for empathy-like behaviors in laboratory animals. The first aim of this work is to demonstrate that tube co-occupation correlates robustly with familiarity status in outbred mice, before describing an automation method that produces a high through-put measure of rodent familiarity. The second objective of this thesis is to show that our measure of propinquity is deficient in the majority of "autism-like" preclinical models, including relevant inbred strains as well as several mutant knockout models. Perhaps the biggest discovery, however, is that inbred mouse strains, especially C57BL/6 mice that serve as the genetic background for most autism-like rodent models, fail to display a familiar versus stranger propinquity preference, suggesting a potential confound of wildtype genotypes across the entirety of social neuroscience. The third aim of this dissertation is to demonstrate that degree of propinquity among animals correlates with stress and pain status. Specifically, strangers with low tube co-occupation behavior show elevated serum levels of the stress hormone corticosterone and corticotrophin-releasing hormone in the hypothalamus and bed nucleus of the stria terminalis. Likewise, pharmacological interventions that stimulate or suppress corticosterone predictably and reliably modulate concomitant levels of tube co-occupation behavior. Using acute and chronic pain stimuli, I show that pain status impacts propinquity among outbred animals, indicating that pain plays a role in dictating early social interactions in preclinical models.

In summary, this work highlights the need for new ways to model the interplay between stress, pain, and complex social factors. Here we present a novel behavioral assay that can be used to better understand how social factors interact in freely moving animals over an extended period of time. We believe the TCOT can serve as a means to better characterize and measure indices of social behavior in autism-like preclinical models. It is our hope that models like this will add to our collective understanding of autism spectrum disorders (ASD), as well as providing new ways to screen potential therapeutic interventions in an effort to treat these disorders.

Resumé

Modéliser l'interaction sociale est complexe et dépend d'une quantité de facteurs génétiques, psychologiques et sociaux. Bien qu'une solide littérature sur l'autisme continue de montrer des lacunes dans plusieurs corrélats de comportement social au sein de populations humaines, les tentatives d'évaluer la sociabilité des modèles précliniques avec les tests comportementaux actuels n'ont pas réussi à produire de nouveaux traitements pour les troubles du spectre de l'autisme social. Je présente ici un test comportemental inédit, le « Tube Co-Occupancy Test (TCOT) », qui constitue une manière unique d'évaluer le comportement d'affiliation chez les rongeurs.

Plus précisément, le test TCOT est un moyen de mesurer la « proximité physique » (en anglais : « propinquity » ou co-occupance), définie comme étant la tendance de rongeurs à co-occuper un tube sur une longue période de temps. Contrairement à d'autres mesures du comportement social des rongeurs, la capacité d'animaux à co-occuper un tube reflète mieux les préférences sociales humaines (avec un biais manifeste d'interaction et de proximité avec des familiers plutôt qu'avec des étrangers), en particulier dans un environnement stressant. La proximité physique est également une mesure robuste et fiable des comportements d'affiliation chez les rongeurs, une condition nécessaire pour tous tests subséquents mesurant chez les animaux de laboratoire les comportements similaires à l'empathie. Le premier objectif de ce travail est de démontrer que la proximité physique d'un tube est étroitement corrélée au statut de familiarité chez les souris de lignée non consanguines (« outbred mice »), puis de décrire une méthode d'automatisation mesurant rapidement la familiarité chez les rongeurs. Le second objectif de cette thèse est de montrer que la majorité des modèles précliniques pour les troubles du spectre de l'autisme sont déficients pour notre mesure de proximité physique, tant chez les souches consanguines (« inbred mice ») que chez plusieurs mutants knock-out. Cependant, la plus grande découverte reste sans doute que les souches de souris consanguines, en particulier la souche C57BL/6 utilisée comme base génétique dans la plupart des modèles d'autisme, ne présentent pas de préférence envers les familiers par rapport aux étrangers dans les tests de proximité physique en tube. Ceci suggère que les génotypes de type sauvage sont un facteur confondant dans tout le champ de la neuroscience sociale. Le troisième objectif de cette thèse est de démontrer que le degré de proximité physique est corrélé avec le stress et l'état de douleur. Plus précisément, les étrangers dont la proximité physique dans un tube est faible présentent des

niveaux sériques élevés de l'hormone du stress corticostérone et de l'hormone de libération de la corticotrophine dans l'hypothalamus et le noyau de la strie terminale. De façon fiable et prévisible, toutes interventions pharmacologiques stimulant ou supprimant la corticostérone modulent les comportements de proximité physique dans un tube. En appliquant des stimuli de douleur aiguë et chronique, je démontre que la douleur influence le comportement de promiscuité chez les animaux non consanguins, indiquant que dans les modèles précliniques la douleur joue un rôle dans l'élaboration d'interactions sociales précoces.

En résumé, ce travail met en lumière la nécessité de développer une nouvelle manière de modéliser les interactions entre stress, douleur et facteurs sociaux complexes. Nous présentons ici une mesure comportementale inédite pouvant être utilisée afin de mieux comprendre comment ces facteurs interagissent sur des animaux se déplaçant librement et au cours d'une période de temps prolongée. Nous croyons que le test TCOT permet de mieux caractériser et mieux mesurer les indices de comportement social dans des modèles précliniques du spectre de l'autisme. Nous espérons que les modèles comme ceux-ci amélioreront notre compréhension collective des troubles du spectre autistique (TSA) en plus de fournir de nouvelles façons d'évaluer les interventions thérapeutiques potentielles dans le but de traiter ces troubles.

Acknowledgements

I would like to start by thanking my supervisor, Dr. Jeffrey Mogil, for his unwavering encouragement, guidance, and support during my graduate training. I could not have asked for a better mentor or a cooler science dude during my tenure at McGill. I am also grateful for my labmates, who challenged me to be a better scientist and a funnier person. In particular, I would like to thank Drs. Loren Martin, Melissa Farmer, Sioui Maldonado-Bouchard, Boram Ham, and Robert Sorge for their input during my time in the Mogil lab, as well as my colleagues, Jeffrey Wieskopf, Sarah Rosen, and Shannon Tansley. Additional thanks to Jean Sebastien Austin, Susana and Sam Sotocinal for helping me implement the perfect experimental designs and keep track of my mice. I am also grateful to Drs. Nahum Sonenberg, Christos Gkogkas, and Arkady Khourtorsky, for additional animal models and collaborative feedback.

I could not have completed this thesis without the support and encouragement of my family: Leslie, Nick, Gaeton, and Mindell, who have supported me whole-heartedly (no matter how weird my experiments became); my wonderful in-laws, Sarah, Andrew, Cindy, and Tom for their support and interest in my work. Most importantly, I am eternally grateful to my wife, Rebecca, for her help in keeping me focused, sane, and fun during this entire process.

Finally, I would like to dedicate this work to my grandfather, Dr. Harry J. Woehr, who showed me that a good education is the keystone to success and happiness in life.

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Contribution of authors

This thesis is manuscript-based and includes portions of the text and figures from one paper under revision at Nature Neuroscience (Chapters 2-4), one published paper (Chapter 5), as well as one paper in preparation for submission (Chapter 5). In some cases I have reformatted or rewritten the accepted manuscript in order to better comply with doctoral thesis requirements and to better fit the style of the thesis as a whole. A complete reference list for all chapters is included at the end of the dissertation.

Chapter 2-4:

Tuttle, A. H., Dossett, K., Tohyama, S., Khoutorsky, A., Maldonado-Bouchard, S., Tansley, S., Stein, L., Gerstein, L., Crawhall-Duk, H., Pearl, R., Sukosd, M., Leger, P., Yachnin, D., Hardt, O., Austin, J.S., Sotocinal, S., Sonenberg, N., Gkogkas, C., and Mogi1, J.S. Social propinquity in rodents as measured by tube co-occupancy. *Nature Neuroscience*, under revision.

Most of the experiments were conducted by A.H. Tuttle with a few exceptions. K. Dossett, S. Tohyama, S. Tansley, L. Stein, L. Gerstein, Crawhill-Duk, H., R. Pearl, M. Sukosd, P. Leger, and D. Yachnin served as coders and assistants who contributed to data and tissue collection for different experiments under direct supervision of AHT. A. Khoutorsky and N. Sonenberg provided mutant mice and genotyping information for autism-like models. C. Gkogkas and O. Hardt replicated outbred mouse data using rats in a separate testing facility, as well as providing feedback concerning experimental design and the use of relevant autism-like models. S. Maldonado-Bouchard confirmed initial social behaviors outside of the tube in the TCOT assay. J.S. Austin provided training and support for mRNA extraction and RT-PCR analyses. S. Sotocinal was instrumental in developing proprietary software and hardware to automate the TCOT assay. J.S. Mogil was instrumental to the project, serving as graduate supervisor and mentor to A.H. Tuttle for the duration of the project.

Chapter 5:

Martin, L.J., Tuttle, A.H., and Mogil, J.S. The interaction between pain and social behavior in humans and rodents. Current Topics in Behavioral Neuroscience, 20:233-250, 2014

L.J. Martin and A.H. Tuttle were asked to co-write a chapter summarizing the state of preclinical pain research with an emphasis on social behavior. The general background for this chapter is a modified version of this review article. J.S. Mogil served in a supervisory role and provided additional editorial support before submission.

Additional, unpublished data included in this chapter are based primarily on experiments conducted by A.H. Tuttle. Additional support was provided by S. Tansley, J.S. Austin, and J.S. Mogil. Specifically, J.S. Austin provided SNI- injured animals for relevant pain experiments. S. Tansley carried out inflammatory pain testing under direct supervision of A.H. Tuttle. J.S. Mogil served in a supervisory role and provided feedback about experimental design.

Original contributions to knowledge

1. The development of a fully automatable novel behavioral assay, the tube co-occupancy test (TCOT), capable of measuring voluntary proximity behaviors, or "propinquity," in freely-moving rodents over an extended testing session.
2. Validation that the TCOT can detect social behavioral deficits in multiple autism-like mouse models
3. Evidence that inbred mice demonstrate abnormal social behaviors in our test, indicating that current preclinical autism tests may be using poor proxies for "normal" murine social behavior
4. Additional evidence that stress, pain, and animal age significantly modulate social behavior

Chapter 1

General introduction

A recent report by the CDC indicates that rates of autism-spectrum disorders (ASD) are at an all-time high (CDC, 2014). While there are differences in symptom type and severity in ASD, there is a shared pattern of social communication and social interaction deficits that serve as diagnostic criteria (American Psychiatric Association, 2013). Studies to date have failed to identify conserved mechanisms of ASD (Gerlai and Gerlai, 2003; Happe et al., 2006), necessitating new ways to study these related social developmental disorders.

Although autism is a uniquely human developmental disorder, rodents also lead rich social lives and demonstrate complex social behaviors when living with one another. In the course of modeling social behavior, preclinical studies have identified numerous autism-like models. However, finding relevant drugs that mediate social deficits in these models has proven difficult. The first objective of this dissertation is to describe a new way to measure affiliation among mice of various genetic backgrounds, including previously validated autism-like models. In addition to detailing an easily implementable and scalable automated approach to measuring a unique social interaction, this work provides important contrasts to previously published sociability tests. Our results using the tube co-occupancy test (TCOT) suggests that inbred mice, or rodent strains that are used in virtually all neuroscience research, may display a marked inability to differentiate between familiar and unfamiliar partners over a prolonged interaction period. If these results are generalizable then they may help to explain why promising preclinical data to date have failed to work in autistic populations.

1.1 The causes of autism spectrum disorders are poorly understood

Autistic spectrum disorders (ASD) consist of severe impairments in emotional and gestural behaviors, social interaction, and cognitive functioning (Berk, 2003). Currently,

clinicians depend primarily on behavioral observation and standardized interviewing in order to diagnose ASD (Newschaffer et al., 2007). The DSM-V lists ASD as a collective set of neurodevelopmental conditions consisting of impaired functioning in at least two of three relevant behavioral domains: social interaction, communication, and narrowed interests or (repetitive) behaviors (American Psychiatric Association, 2013).

A recent report by the CDC indicates that rates of autism-spectrum disorders (ASD) are at an all-time high, identifying 1 in 68 children as having some form of the developmental disorder (CDC, 2014). The report notes that ASD is reported across racial, ethnic, and socioeconomic groups, and is 5 times more likely to occur in boys (1 in 42) than girls (1 in 189) (CDC, 2014). It is generally believed that prevalence rates of autism account for 1% of the global population, although recent studies using direct screening techniques reveal that small populations may experience higher prevalence rates (Kim et al., 2011). Studies constrained to U.S. pediatric populations estimate that ASD costs between $11.5b- $60.9b per year, reflecting a variety of both direct and indirect costs that include medical care, special education, and lost parental productivity (Cimera and Cowan, 2009; Croen et al., 2006; Ganz, 2007) . An individual with ASD is estimated to need $1.4m during his or her lifespan, with a third of the population requiring up to an estimated $2.4m investment in care with concomitant intellectual disability (Buescher et al., 2014). Increasing prevalence rates, high associated costs, and phenotypic heterogeneity associated with ASD confound efforts to develop diagnostic tools and effective therapies (Lord and McGee, 2001), leading to a bottle-neck in patient care (Jones, 2015).

For many of the intervening years since autism's discovery by Leo Kanner in the 1940's (Kanner, 1943), the disorder remained in relatively obscurity. Since the 1980s, however, the explosion in diagnoses of autism and associated neurodevelopmental disorders has garnered a

high amount of media and public attention. Both governmental and private funding agencies have responded in kind by substantially investing in ASD research over the past two decades, with NIH funding levels increasing five-fold from the mid-1990s through the mid-2000s (Singh et al., 2009) to reach $334 m in public funding with an additional $74m in private funding in 2010 (Dawson, 2013). Analyses of funding priorities reveal that the majority of funded projects focus on "basic science" topics that include neural and cognitive systems, genetics, and other environmental risk factors (Dawson, 2013; Pellicano et al., 2014; Singh et al., 2009). Yet in spite of these unprecedented steps, at this time there is not yet a clear picture of the fundamental molecular pathways involved with ASD that are necessary to develop more effective diagnostic criteria and treatments.

Due to the heterogeneity associated with ASD, it is difficult to isolate specific contributing factors involved. Current diagnostic criteria favor a unified autism spectrum model in part because clinicians have failed to agree on a single way to diagnose ASD. For example, researchers find that different clinicians diagnosed the same children with different disorders, in spite of nearly identical criteria (Lord et al., 2012). In addition, researchers have not found a single dominant neuropathology or treatment that helps only a subset of the larger clinical population, indicating that previously accepted categories of autism (i.e., Asperger's syndrome) are unnecessary (Wing, 2000). In an effort to establish a unifying cause for ASD, researchers have renewed focus on environmental and genetic factors involved with these disorders (Happe et al., 2006). While various studies have identified a handful of viable environmental contributions to ASD (Christensen et al., 2013; Newschaffer et al., 2007), the potential exposure to any of these identified compounds fails to explain the explosion in autism-related diagnoses

over the past thirty-five years. It is far more likely that environmental risk factors interact with genetic factors, leading to the presentation of ASD.

1.2 Genetic risk factors in ASD

The bulk of autism research funding is allocated to further our understanding of the basic biological mechanisms involved with ASD. To date, researchers have made a great deal of progress identifying both inherited and *de novo* variations of genetic risk; additionally, powerful new screening tools and larger autism networks continue to identify contributing sources of variability in the presentation of these disorders (Chen et al., 2015). The rest of this section will provide a brief summary of the evidence showing that there is a strong genetic component of ASD.

Autism is often considered the most heritable of all known neurodevelopmental disorders, with results from twin studies indicating higher concordance rates of ASD diagnoses in monozygotic twins (MZ; 0.36-0.95) compared to dizygotic twins (DZ; 0-0.31), (Hallmayer et al., 2011; Ronald and Hoekstra, 2011). Reported dizygotic twin concordance rates are similar to those reported in familial proband studies, whereby 14.0%-18.7% of siblings born to a family that have an affected older sibling also develop the disorder (Ozonoff et al., 2011).

Although heritability estimates denoting a strong genetic susceptibility exist, finding significant genetic variants contributing to ASD is difficult. On the molecular level, between 200 and 1000 genes are implicated in various disorders on the autism spectrum (Berg and Geschwind, 2012), with multiple modes of inheritance. For example, numerous associated genetic diseases caused by single genetic mutations with high penetrance appear to collectively account for 10-20% of all ASD cases, and include mutations in *FMR1* (fragile X syndrome),

TSC1/TSC2 (tuberous sclerosis complex), *MECP2* (Rett syndrome), *CACNA1C* (Timothy Syndrome) and *UBE3A* (Angelman syndrome) (Berg and Geschwind, 2012; Cook et al., 1997). Scientists have modeled many of these ASD-relevant genetic variants in rodents in order to better understand the underlying pathophysiology of ASD. These models will be discussed further in **Chapter 3**. However, the majority of clinical autism cases are not explained by single gene mutations. It is far more likely that specific constellations of common variants may explain a number of "idiopathic" ASD cases. Although these common variants appear to separately confer small amount of individual risk (<1% of variance), together they can lead to an autism phenotype (Anney et al., 2012).

Recent technological advances using array-based genomics (genome-wide association studies; GWAS) and transcriptomics have made identifying both inherited and *de novo* mutations easier. It is estimated that common inherited variations of single-nucleotide polymorphisms (SNPs) account for 15-40 % of the genetic risk for ASD (Gaugler et al., 2014; Stein et al., 2013). Whereas researchers have identified several genome-wide risk loci (Anney et al., 2012; Wang et al., 2009; Weiss and Arking, 2009), estimated odds ratios (OR) of common variants in ASD (OR <1.2) indicate an overall modest effect of common SNP variants in predicting these disorders (Devlin et al., 2011). Although increasing ASD sample sizes in future studies may yield new (and more reproducible) loci (Chen et al., 2015), failure to replicate common variants across GWAS studies indicates that most cases of ASD are due to polygenic inheritance mediated by a large number of variants (Szatmari et al., 2007).

Likewise, the heterogeneity of ASD makes it difficult to identify rare ASD risk variants. Traditionally, researchers identified inherited risk factors by using linkage disequilibrium analysis to identify common polymorphisms in large families that contain multiple instances of a

disease. However, smaller family sizes (likely due to parents' decisions to stop having children after having one autistic child) and heterogeneity of ASD make it difficult to replicate instances of rare inherited variants (Jones and Szatmari, 1988). Nevertheless, recent studies have used multiplex families (i.e., families that contain more than one ASD member) and consanguineous families with ASD to find evidence of homozygous deletions in particular genes. These efforts identify rare cases of complete gene knockout, compound heterozygous, and X-chromosome mutations and estimate that these mutations account for as much as 5% of ASD cases in males (Lim et al., 2013; Szatmari et al., 2007).

Finally, *de novo* mutations do not factor into heritability estimates of ASD but may still help researchers identify genes implicated in neurodevelopmental disorders. Compared to hereditary variants, *de novo* mutations are rare, affecting DNA in parental germ line cells or somatic cells early on during gestation. Any instances of copy number variants (CNVs) or SNPs observed among ASD offspring and unaffected family members have a high chance of contributing to ASD phenotypes due to their relative rarity (Chen et al., 2015).

A review of studies published using data collected from large patient databases, including the Simons Simplex Collection and the Autism Genetic Resource Exchange notes that frequency of mutations across multiple CNVs is at least 2 times higher in ASD populations than ~1% observed in familial controls (Sanders et al., 2012). Furthermore, both the size and number of genes within CNVs are associated with prevalence rates of ASD, and appear in 5-10% of ASD populations (Pinto et al., 2010; Sanders et al., 2012). Analysis of CNVs in *de novo* cohorts are among the first to show that mutations in synaptic signaling genes *NLGN3, NLGN4*, and *SHANK 3* result in an autism phenotype (Durand et al., 2007; Fischbach and Lord, 2010; Jamain et al., 2003). These genes are particularly relevant to our current work because they affect rodent social

behavior. Unlike CNVs, mutations within a single coding region of an exon (SNPs) provide clear evidence for a gene's involvement in ASD. SNPs are even rarer than CNVs, allowing scientists to systematically characterize these mutations among simplex and multiplex families in an unbiased search for ASD risk genes. Similar to CNVs, SNPs appear to account for 5-10% of ASD phenotypes (Pinto et al., 2014). Recent efforts to identify patterns in *de novo* mutations in ASD populations find almost a dozen recurrently disrupted genes (Michaelson et al., 2012; Neale et al., 2012; O'Roak et al., 2012); preclinical labs are now attempting to model these genetic mutations in new animal models (Zylka, unpublished data).

In spite of recent advances in the sequencing of the human exome, we are only now beginning to understand how inherited and *de novo* mutations are implicated in ASD. To date, many of the exome sequencing studies have produced non-overlapping genetic hits, implicating a multitude of biological pathways in the development of ASD. Based on these results, some researchers believe that there are as many as 1,000 loci underlying these disorders (Buxbaum et al., 2012). As a result, evaluating the relative contribution of highly penetrant mutations, common variants with low penetrance, *de novo* variants, and inherited variants is a difficult task, necessitating ever-larger samples in autism population studies (Chen et al., 2015). Even with sufficient power, association studies are not able to identify other causes of autism, including environmental risk factors, genetic and environmental interactions, and gender differences (Martin-Ruiz et al., 2004). In order to simplify interactions among factors and identify causal mechanisms, animal models of neurodevelopmental disorders are needed.

1.3 Modeling autism-like behaviors in the mouse

Whereas advances in genetic screening techniques have identified a number of genetic risk factors associated with ASD, the mechanisms underlying the manifestation and progression of these disorders are still poorly understood. Ethical considerations, genotypic and phenotypic heterogeneity, and the lack of clear biomarkers all limit the usefulness of human participants in laboratory studies (Hendren, 2014). As a result, basic researchers use animal models to avoid inherent limitations present in clinical research.

Although non-human animals are markedly different than humans in their social behaviors, many believe that the genetics and neurochemistry underlying human social behaviors are conserved across mammalian species, including rodents. If one believes in a phylogenetic continuity of social behavior, it follows that a change in genetics that yields an observable deficit in social interaction among mouse models would likely affect humans in similar ways, with qualitatively similar neurophysiological determinants. Using modern genetic techniques, scientists create animal models that contain specifically targeted mutations (including genes listed in the previous section) as a means to study the impact of genetic factors on subsequent anatomical structures, physiological functioning, and social behavior at the organismic level (Klauck and Poustka, 2006). Furthermore, producing reliable preclinical autism models is a necessary prerequisite to evaluating potential pharmacological treatments. As such, the development of animal models creates a viable pipeline that allows researchers to evaluate the relative impact of genetic and environmental factors, as well as identify possible pharmacological targets for autism-related deficits.

Historically, social behavioral testing used rats more than any other species due to their varied social repertoire (Gamber, 2014; Kas et al., 2014). For example, early attempts to model rodent social behavior, including the Social Interaction test, used rats to study how anxiety levels

impact social behavior among various outbred strains (File and Pope, 1974; File and Seth, 2003). Additionally, researchers are able to demonstrate evidence for juvenile play behavior (and relative deficits) in rat models (for review (Thor and Holloway, 1985). The inherent drive for play among juvenile rats is strong, creating a novel way to model motivational and rewarding aspects of social behavior; unfortunately, this behavior is not evident in mice (Calcagnetti and Schechter, 1992; McFarlane et al., 2008; Trezza et al., 2009).

While exact statistics concerning the use of rodent models in North America are not collected (U.S.D.A., 2014), a review of UK government statistics reveals that in the past decade, *Mus musculus* has become the most common animal for biomedical research, with 2.8-3.0 million mice (as compared to 0.25-0.26 million rats) used in various experimental protocols (U.K.H.O, 2014). If rats demonstrate more varied and robust social behaviors, why is the majority of preclinical autism research concerned with using mouse models? As in other fields, basic autism research uses mouse models for one major reason: the increased scientific interest in genetic factors of ASD. Until very recently (Engineer et al., 2015; Hamilton et al., 2014), using the rat as model organism precluded the possibility of using advanced genome manipulation techniques, including the use of transgenic "knockout" models (Capecchi, 1989). For years, scientists have taken advantage of the mouse's unique ability to produce viable offspring after its embryonic stem cells are targeted for specific genetic modification. Today, new advances in gene editing tools, including the advent of rapid *de novo* mutagenic techniques (including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats (CRISPR) are narrowing the translational gap among animal models by allowing scientists to edit gene expression in fully-formed embryos (Gamber, 2014). This process is more efficient than older gene-targeting techniques, and has the added

bonus of opening up genetic modification to different animal species. In addition, new targeted mutation methods sidestep confounding issues associated with embryonic stem cell manipulation (i.e., linkage disequilibrium) that occurs when implanted transgenes and background genes come from two different organisms with separate genetic backgrounds (Gerlai, 1996).

Besides being the only organisms capable of supporting genetic manipulation for the majority of the last twenty-five years, mice are also uniquely positioned to serve as ideal research organisms because there are a large number (~450) of inbred strains that have been commercially optimized, maintained, and made readily available to scientific researchers for the past eight decades (Beck et al., 2000). In almost all cases, scientists create a new inbred strain by inducing brother-sister mating for at least 20 generations, resulting in nearly global homozygosity in the offspring's genome (99.9% after the first 20 generations). The resulting mice are genetically identical to one another, but genetically distinct from other strains. Because the resulting animals in a strain are essentially clones of one another, geneticists can sequence the entire mouse genome from one specimen in order to identify strain-specific polymorphisms (Frazer et al., 2007). Even without genetic manipulation, researchers can perform highly controlled experiments and ascribe resulting differences in behavior to genetic differences among strains. For example, autism researchers find autism-like phenotypes among several different inbred mouse strains, including the BTBR T + *tf*/J (Moy et al., 2007; Silverman et al., 2012; Silverman et al., 2010a; Yang et al., 2007b) and BALB/c mouse (Brodkin, 2007). Inbred strains will be discussed more in **Chapter 3**.

In addition to serving as ideal targets for genetic manipulation and inbreeding, mice are well-adapted to fit within the structure of the modern research industry. They are small, reproduce quickly and typically yield a large number of viable offspring with each litter. Mice

are easy to handle and transport. They mature quickly and age within two to three years, allowing scientists to easily study phenomena across the animal's lifespan. For all of these reasons, the mouse remains the dominant model in current social neuroscientific studies (Shultz, 2016).

Whereas mice are unable to self-report, one can reliably and objectively score their response to social stimuli. A good animal model must demonstrate three levels of validity: first, the model must demonstrate good construct validity by being relevant to an underlying cause (or causes) of a disease (for example, by having the same sets of genetic mutations that are found in clinical populations). Second, the model must demonstrate strong face validity by exhibiting obvious signs or symptoms of a disease. Third, the animal model should demonstrate predictive validity by responding to treatments that have previously worked in clinical populations. To date, 676 animal models of autism are archived in the SFARI Gene database (SFARI, 2016). However, it is debatable whether any of these models have demonstrated strong construct, face, and predictive validity. Furthermore, preclinical researchers have not yet consistently demonstrated model deficits across all three central diagnostic criteria of autism: abnormal social interactions, deficits in communication, and high levels of repetitive behaviors (Kas et al., 2014).

Researchers interpret the relative paucity of generalizable autism-like rodent models in several different ways. One possibility is that our current understanding of human developmental disorders is insufficient to create accurate animal models. If this is true, it creates an insurmountable challenge for basic researchers. The second possibility is that observed heterogeneity among mouse models is reflective of the heterogeneity of autism in humans. Furthermore, the heterogeneity of genetic factors that influence impairment in human ASD populations precludes the possibility of finding a single parsimonious model of autism-related

behaviors, necessitating the study of multiple different animal models of ASD. A third possibility is that the current failure to find tractable neurobiological pathways of ASD is not due to deficiencies in animal models, but instead is the result of limitations in our ability to model their behavior. To date, no intervention has been able to reverse any of the core symptoms in ASD (Chadman, 2014), making it difficult to evaluate the translational value and predictive validity of current behavioral tests and animal models. Nevertheless, the persistent failure to find effective pharmaceutical targets in ASD have led researchers in academia and industry to call for additional preclinical behavioral tests using rodent ASD models (Murphy and Spooren, 2012).

Mice are ideal models for preclinical autism research because they are naturally social creatures. Over the past 50 years, behavioral neuroscientists have identified a myriad of murine social behaviors, including competition for nursing, play, social approach and interaction, aggression, allogrooming, huddling, parenting, and social learning (Crawley, 2007; Gheusi et al., 1994; Grant and Mackintosh, 1963; Wills et al., 1983). In order to quantify deficits in the three core domains of autism symptomology, behavioral neuroscientists design assays that measure rodent behavior in one of three discrete categories: sociability, communication, and repetitive behavior.

1.3.1 Social interaction tests

To discover deficits in sociability, researchers use specific tests to characterize and quantify reciprocal social behavior among rodents. Whether placed together in standard “home” cages or in laboratory arenas, a pair of mice (called a “dyad”) will engage in discrete bouts of social interaction that includes nose-to-nose contact, nose-to-anogenital sniffing, following, allogrooming, chasing, attacking, mounting, wrestling, and other forms of bodily contact (Kas et

al., 2014). Typically, "blinded" researchers (i.e., investigators who are naïve to animal social status, genotype, or experimental intervention) quantify a pre-defined set of behavioral analogues from a recorded social interaction among animals. The earliest studies focusing on social interaction coopted anxiety tests to identify and quantify social behaviors. The Social Interaction (SI) test, for example, measures social approach, sniffing, chasing and fighting behavior among rats in variably stressful open-field environments (File and Pope, 1974). Today, these tests are still used by some to quantify sociability among rodents. However, the SI can only be scored manually, and thus requires intensive laboratory resources to yield reliable data (Kas et al., 2014). Additionally, the SI lacks a strict experimental protocol, and the resulting failures to reproduce results both within and between laboratories underline the importance of standardizing behavioral procedures and data collection methods (Grange and Collaboration, 2015; Wahlsten et al., 2003; Würbel, 2002). Today, many researchers have replaced the SI with more standardized and automatable social assays, including the three-chambered test (Crawley, 2004).

The three-chambered test is designed to measure social approach behavior between two mice in a controlled testing environment. In the three-chambered test, a behavioral target mouse is habituated to all three attached chambers for 10 min before being placed in the central chamber. A stimulus mouse is then placed into one of the attached companion chambers, while a non-social novel object is typically added to the empty side. The stimuli mouse is in a wired enclosure, so that it cannot initiate contact with the freely moving test animal. The experimenter then raises the doors between chambers and the test mouse explores the entire box (all three chambers) for 10-20 min while experimenters quantify a number of proximity behaviors (i.e., chamber entries, chamber duration, cup proximity, etc.) that occur between the test mouse and the stimulus animal (**Figure 2.2**).

Other behavioral assays use different designs to essentially measure the same set of social approach behaviors, including the two-chambered "partition test" as well as the Y-maze test (Bowers and Alexander, 1967; Kudriavtseva, 1987). In the partition test, researchers place mice into a standard home cage that is separated by a Plexiglas insert. Similar to the holding cells in the three-chambered test, holes in the insert allow mice to sense one another while preventing direct social interaction. Time spent near the partition serves as the dependent measure. The partition is often removed at the end of the test to allow mice to interact with one another directly while observers document additional social behaviors. In the Y-maze test, subject animals begin in a central chamber and choose between two different social stimuli located in separate alleys that branch off in a "Y" configuration. Social approach is quantified based on the number of alleyway entries the mouse demonstrates during a number of trials.

To measure social novelty and social preference, researchers use two- and three-chambered assays with different testing protocols. Instead of exposing a subject animal to a single novel animal, social novelty tests consist of a choice to approach a familiar or novel conspecific in a serial or forced-choice sequence. The three-chambered test is similar to the habituation-dishabituation procedure, where a test animal is exposed to a novel animal (or its odor) before being simultaneously exposed to both the (now familiar) animal as well as a novel animal (Johnston et al., 1991; Johnston et al., 1993; Sundberg et al., 1982). Animals generally explore unfamiliar animals more frequently and for longer periods of time than a familiar conspecific in these assays; other studies replicate this finding using place preference paradigms and operant conditioning tests (Martin et al., 2014; Panksepp et al., 2007).

Using two- and three-chambered assays, scientists have begun to define the quality and extent of social interaction using inbred animal strains, finding that some demonstrate relatively

low levels of social behavior, including BTBR, C3H/HeJ, AKR/J, A/J, and 129S1/SvImJ (Defensor et al., 2011; Moy et al., 2007). Specifically, these strains fail to demonstrate preference for social novelty, as compared to C57BL/6J, C57L/J, DBA/2J,FVB/NJ, and BALB/cByJ mouse strains (Moy et al., 2007).

In addition to measuring degree of social novelty-seeking behavior, researchers vary inter-exposure time in the two- and three-chambered tests to establish measures of social memory among various animal models (Bielsky and Young, 2004; Moy et al., 2007; Spencer et al., 2008). Other methods of measuring social memory involve studying the transmission of social signals between mice, called observational learning. While it is usually assumed that first-hand experience with a task is required to learn new information, studies of observational learning show that new information can be acquired through social facilitation and imitation (Olsson et al., 2007). Researchers evaluating food preference in animals show that an animal is significantly more likely to try a novel food after being exposed to a familiar conspecific that has eaten the same food prior to social interaction. Furthermore, it appears that mice obtain information about food from a conspecific through olfactory cues shared with a proximal conspecific (Wrenn, 2004; Wrenn et al., 2003).

Experiments exploring the neural circuitry underlying observational learning also use classical fear conditioning protocols that involve an acutely painful stimulus (foot shock) and social interaction with ‘‘experienced’’ demonstrators (Knapska et al., 2006). Observational learning in mice is related to social closeness; researchers demonstrate that freezing responses are higher in observer mice when demonstrator mice are socially related (e.g., siblings, mating partners), and impaired by the genetic deletion of Ca_v 1.2 calcium channels in the mouse anterior cingulate cortex (Jeon et al. 2010). The degree of social transmission of fear appears to be strain-

specific, with C57BL/6 mice acquiring the association much more readily than BALB/c mice (Chen et al., 2009), an inbred strain that also displays low levels of social approach behavior (Brodkin et al. 2004). Based on this evidence, a failure to demonstrate observational learning could relate to an underlying failure to perceive relevant social signals.

Finally, antagonistic interactions can serve as a way to detect deficits in animal social behavior. The resident-intruder model (RIM) is a common way to study aggressive behavior among adult rodent dyads. In the RIM, two animals are introduced to one another in the home cage of one of the animals and allowed to interact for a period of time. During the interaction, rodents display aggressive or submissive behaviors toward one another. Generally, the resident attacks quickly and establishes a dominant position in the two-mouse hierarchy, because it is pre-selected based on a number of characteristics, including genetic background, weight, previous displays of aggressive behavior, etc. that will insure that the intruder loses the fight (Martinez et al., 1998). Recently, social neuroscientists using the RIM to characterize aggressive behavior among autism-like models reported that autism-like mice show significantly less aggressive behavior towards an intruder (Cheh et al., 2006; Jamain et al., 2008) and also engage in significantly less social exploration than a wildtype resident (Shahbazian et al., 2002). Furthermore, researchers show that social exploration increases in rats following injections of 2-methyl-6-(phenylethynyl)pyridine (MPEP), a metabotropic glutamate 5 receptor (mGluR5) antagonist that is known to reverse social deficits in several animal models (Spooren et al., 2000), as well as clinical populations (Scharf et al., 2015).

1.3.2 Social communication tests

In addition to transmitting social information using nonverbal cues, mice also vocalize under certain circumstances. However, scientists have only recently begun to systematically investigate vocalizations in mouse models of ASD. Studying murine vocal behavior in ASD models is etiologically relevant because it mirrors deficits observed in autistic populations. Crying, for example, is a salient cue for appropriate caregiving responses (Johnston and Strada, 1986) and is deficient in both autistic populations and relevant animal models (Zeskind et al., 2011). Using ultrasonic sensors to record the number and acoustic profile of emitted ultrasonic vocalizations (USVs), researchers study how changing social conditions modulate these calls in both pups and adult mice.

Pup vocalizations are particularly relevant to ASD because they manifest soon after birth. Pups normally emit distress calls to summon a caretaker (Harmon et al., 2008; Liu et al., 2013), and regulate calls based on the social environment. Hoffer and colleagues show that the presence of a familiar conspecific can decrease pup USVs (a condition called "contact quieting"), whereas removal of the mother from the pup's environment results in an increase in USVs ("maternal potentiation"). Conversely, the presentation of an unfamiliar male odor results in a prolonged suppression of pup-emitted USVs (Hofer et al., 2001).

Adult mice also vocalize, although to a lesser extent than pups (Hahn et al., 1998). Furthermore, although there is general consensus about the function of pup vocalizations (maternal recall), the reason for adult vocalizations is debated (Fischer and Hammerschmidt, 2011). Perhaps the clearest test to elicit adult USVs is to expose a male to a female conspecific. Males will vocalize following direct physical contact with a female; subsequent temporal and spectral analyses indicate that male mouse vocalizations may contain courtship-specific

components (Holy and Guo, 2005). Female mice appear to be attracted to male USVs (Musolf et al., 2010), and prefer to interact with vocalizing males over mute males (Pomerantz et al., 1983).

Currently, there is a debate over whether male calls are specific to courtship, or instead serve multiple purposes, including contact and territorial calling. For example, in the resident-intruder test a resident that has been socially isolated prior to testing emits significantly more vocalizations in the presence of an intruder than socially-housed residents. Unlike courtship assays, intruder tests reveal that female mice emit USVs following 3-4 days of isolation, a significantly shorter period of time than the 3-4 weeks of isolation required for males (Hammerschmidt et al., 2012b). Based on these findings, adult calls may serve as territorial warnings or as a way to establish dominance with an unknown conspecific; subsequent studies have used USVs as a social interaction test to measure social memory among female mice (Scattoni et al., 2009). Clearly, female USVs in these tests are not sexually motivated, but analysis of female calls in the resident-intruder assay and male courtship calls reveals minimal structural differences, suggesting that courtship calls may not be distinct from other call types (Hammerschmidt et al., 2012a).

In addition to the potential for call specificity, other limitations concerning the study of mouse vocalizations include etiological differences between rodent calls and other social species. Unlike human and bird vocalizations, mouse USVs appear to be innate and manifest before the onset of hearing in pups (Hammerschmidt et al., 2012b). A second limitation of murine vocalization studies is a lack of agreement among assays. The majority of studies to date have merely quantified a number of USVs, without characterizing qualitative differences among social conditions. However, there is little consensus among researchers concerning the best way to qualify aspects of mouse calls, including intervals between calls, succession of call types, and

acoustic composition of calls (Scattoni et al., 2009). More research is needed to identify normal calling behavior among inbred strains before potential deficits in calling can be identified in mouse models of ASD.

1.3.3 Tests for behavioral inflexibility and repetition

Similar to clinical reports of ASD (Boyer and Liénard, 2006; Evans et al., 1997), preclinical researchers have observed repetitive (or "ritualistic") behaviors among autism-like mouse models (Bechard and Lewis, 2012; Silverman et al., 2015; Silverman et al., 2010a). Traditionally, assessment of rodent repetitive behaviors consists of either quantifying disturbed fixed action patterns (i.e., excessive self- or other-grooming) or spontaneous stereotypic behaviors (i.e., circular chasing, hindlimb vertical jumping, and backward somersaulting) (Kas et al., 2014; Langen et al., 2011). Similar to qualitative vocalization analyses, a subset of studies not only report quantification of animal grooming behavior, but also include a qualitative assessment of the elements of the repetitive behavior (Berridge, 1990; Kalueff and Tuohimaa, 2005).

In order to qualify attentional switching, researchers use modified operant conditioning protocols to create attentional set-shifting paradigms that evaluate an animal's capacity for reversal learning. Put simply, studies look at whether a trained mouse can adapt an old habit to fit a new set of rules (Bissonette et al., 2013). Multiple studies also use T-mazes and Morris water mazes to demonstrate reversal learning. In these tests, researchers train mice to follow an orientation marker to find a preferred "goal" area. These clues are later switched and subsequent errors made by animal subjects indicate their level of flexibility to change (de Bruin et al., 1994; Mackintosh, 1965)

Restricted or narrow interests are also evaluated by using assays like the marble burying task (Thomas et al., 2009) and related digging assays that first train mice or rats to identify where an item is buried (usually by varying the cage floor medium), before switching where the reward is located and measuring the amount of time it takes for the animal to learn the new rule set (Birrell and Brown, 2000). Unlike attention-shifting tests, restricted interest tests can be performed in naïve animals. .

In spite of recent advances in characterizing repetitive behavior among animal models, the majority of ASD preclinical studies continue to focus on social interaction and communication deficits. Unlike USVs and social approach behavior, repetitive behaviors are complex, not easily identifiable, and require substantial resources in order to produce reliable data. Taken together, these limitations may explain the relative paucity of data concerning cognitive inflexibility in rodent models of ASD.

1.4 Limitations of current animal behavioral assays

After reviewing the state of current preclinical ASD research, several common limitations pertaining to available behavioral assays begin to emerge. The first limitation pertains to rodent models; in the pursuit of reproducibility, virtually all the research in the field has been conducted on inbred mice, or mutant mice bred onto inbred genetic backgrounds. These mice are assumed to serve as adequate proxies for "normal" murine social behavior, but may differ from outbred or wild mice in their social interactions.

A second common limitation pertains to the physical design of many autism assays; many of the tests looking at social, communication, and repetitive behaviors in mice restrict animals from interacting with social conspecifics in a natural way. Instead, stimulus mice are

usually separated by a wire or Plexiglas barrier from target animals, inhibiting their ability to approach or interact with a conspecific. The ability to directly interact with a conspecific is a critical aspect of social behavior that is not currently tested in many social assays, even though aversion to affective or social touch has been documented in both clinical ASD populations (Cullen-Powell et al., 2005; Grandin and Scariano, 1996) and in the lab (Voos et al., 2012).

A third common limitation common in preclinical autism testing is the length of time that animal behaviors are assessed. For example, almost the entirety of standardized sociability data pertain to social behavior captured during short testing periods (5-20 min) with only a few documented exceptions (Shah et al., 2013; Spencer et al., 2008). Similar timeframes are common in communication studies (1-10 min) and are often assessed during sociability testing in adult mice (Hofer et al., 2001). Despite the advantage of being brief and easily quantifiable, studies using popular behavioral assays quantify a limited set of initial interaction behaviors among animals. For example, many sociability tests measure social novelty, a robust social behavior that extinguishes within the first thirty minutes following the first introduction between two animals (Nadler et al., 2004). Furthermore, it is debatable whether social novelty-seeking is the most relevant sociability deficit in ASD populations (Shah et al., 2013). A recent review of the clinical literature states that only 36-40% of autistic children (diagnosed as “aloof”) display a lack of social interest towards strangers (Shah et al., 2013). By only measuring initial social preference during the first few minutes of interaction, current tests do not capture potential deficits in familiar-directed social behaviors that manifest over time. We believe that studying an animal’s preference for familiarity over an extended testing session is more representative of human social behavior; likewise, a failure to differentiate between familiar and stranger is a more profound and clinically relevant deficit. Studies measuring interpersonal distances between both cis-sex

and trans-sex dyads show that humans prefer to interact socially with familiars and maintain closer distances to friends than strangers (Edwards, 1972; Sinha and Mukherjee, 2008). Furthermore, children with ASD maintain the same degree of interpersonal distance after socially interacting with an experimenter, in contrast to typically developing (TD) children who significantly reduce their distance to an experimenter after a brief social exchange (Gessaroli et al., 2013).

By focusing on social novelty as the sole measure of sociability, current studies are constrained by confounding factors, including overall activity and anxiety levels (Moy et al., 2009; Silverman et al., 2010a; Silverman et al., 2010b; Veenstra-VanderWeele et al., 2012). In fact, scientists initially designed sociability tests to measure anxiety levels in both rats and mice. Specifically, data reported using the social interaction test (File and Hyde, 1978) showed that "active" social behaviors, including sniffing, following, walking over, crawling under, and allogrooming, are prevalent in low anxiety conditions and decrease when aversive stimuli are added.

The main practical reason that the majority of preclinical autism testing relies on a handful of brief standardized measures performed on a limited number of inbred mouse strains is to increase replicability and decrease variability in standard measures. However, in doing so, basic researchers risk decreasing the generalizability of their reported findings. Due to their heterogeneity, ASD appear to result when a confluence of different environmental and genetic factors align, leading to a particular set of social and behavioral deficits. By constraining experimental variables to include genetically identical models or a single (brief) measure of sociability, current experimental protocols patently fail to account for changes in social behavior among genetically diverse samples over time.

Recent advances in the automation of behavioral assays are attempting to address some of these limitations. Often, human observers are responsible for collecting complex behavioral data. As Kas et al. (2014) point out, "Manual scoring is labor-intensive, error-prone, and subject to bias as a consequence of individual interpretation." These biases include variability among observer ratings, attention, and response time (Spruijt and De Heer, 2012). The strengths of automated behavioral systems (e.g., CleverSys, ANY-maze, and Noldus IT) include reducing coder workload and producing more accurate measures than human observers, who may be biased or fatigued during coding sessions. Furthermore, because computers evaluate behaviors among animals identically, they have the potential to produce more reproducible results (Spruijt and De Heer, 2012). Finally, by employing RFID sensors (Weissbrod et al., 2013) or infrared and depth cameras (Hong et al., 2015), recent iterations of observation software is beginning to obviate the need for researchers to transfer animals from their home cage, allowing continuous recording of animals as they interact with others in their "natural" environment while simultaneously reducing related confounds, including exploration activity and experiment-induced stress. Theoretically, these improvements will allow for a greater number of high-through-put methods that will allow for longer and less invasive testing protocols.

The problem with automated systems is that the amount of raw data that they generate can often be overwhelming and complex, moving the pre-existing bottleneck from data collection to data analysis. Unlike humans, machines will report raw data that can often be noisy and hard to interpret, especially in characterizing complex behaviors. When they occur, software or hardware errors can be hard for the average user to detect. Even when functioning normally, home cage scanners are not yet sophisticated enough to reliably differentiate among behaviors

from multiple conspecifics of the same strain (Hong et al., 2015) and rely on end users to identify spatial and temporal patterns of behavior.

1.5 Building a better sociability assay to detect autism-like behaviors

Whereas high variability in experimental design can lead to failures in reproducibility, it is equally faulty to rely on too few standard approaches to characterize social behavior in animals. For my main Ph.D. research project, I was interested in developing: 1) a novel social test optimized to examine mouse social behavior over an extended (3-8 h) period of time. 2) An assay design that would allow for a maximum amount of mobility and free interaction among a pair of rodents, rather than keeping them artificially separated from one another. 3) An experimental protocol optimized to detect differences in social behavior among any combination of mice, including inbred and outbred strains, mixed strains, and mutant knockouts. 4) An automatable experimental design that could rapidly produce and categorize data, minimizing user workload.

In **Chapter 2** I describe how we designed the tube co-occupancy test (TCOT) to address each of these principles; the result of our efforts is the discovery of a new form of extended social interaction among animal dyads that we call "propinquity." Furthermore, I demonstrate our test's proof-of-concept using outbred rodent strains. Finally, I outline an automated protocol for the TCOT that allows high-through-put analysis of social behaviors using different animal models.

In **Chapter 3**, I further characterize propinquity in a variety of inbred mouse lines, as well as several genotypes featuring mutations of autism-relevant genes that others have

previously reported show deficits in several measures of sociability. To attain a general understanding of social preference in the mouse species, I also describe results from cross-fostered adult offspring of wild mice (*M. musculus domesticus)* that were trapped in a semi-rural area of Montreal.

In **Chapter 4** I show that animal stress levels negatively correlate with tube co-occupancy in normal testing conditions, and that the mere proximity of a stranger produces a measurable stress response that is significantly greater than a familiar conspecific. I also share data indicating that anxiolytic and anxiogenic pharmacological compounds are sufficient to reverse stranger and familiar behavioral phenotypes, respectively.

Finally, in **Chapter 5** I investigate how the presence of both tonic inflammatory pain and chronic neuropathic pain can significantly alter propinquity behavior in outbred mice. Furthermore, differences in tube co-occupation rates between injured and intact animals do not appear to be related to differences in locomotion or exploratory behaviors between experimental groups.

Chapter 2

Defining propinquity behavior using the tube co-occupation test

2.1 Rationale

The Mogil laboratory previously observed differences in both pain-directed and social behavior among outbred mice based on degree of familiarity among animals (Langford et al., 2010; Langford et al., 2006; Martin et al., 2015). Moreover, studies have shown that familiar outbred spiny mice (*Acomys cahirinus*) demonstrate greater rates of food sharing and huddling behaviors than strangers (Porter, 1981; Porter et al., 1978). Besides these few examples, however, the literature to date has failed to adequately characterize social behaviors over an extended period of time using outbred mice. My first objective was to develop a viable way to measure changes in social behaviors among outbred mice over a period of several hours, in order to identify how these behaviors change with increasing familiarity.

2.2 Introduction

Current behavioral assays are insufficient to study social behavior across an extended period of time. Specifically, the majority of laboratory sociability tests rely on "active" social behaviors, including sniffing, following, walking over, crawling under, and allogrooming (Bowers and Alexander, 1967; Crawley, 2004; File and Seth, 2003; Kudriavtseva, 1987). It is well-known that while these behaviors are prevalent during the first 30 minutes of social interaction, they steadily attenuate over time, making them a poor set of social behaviors to quantify during extended testing sessions (Shah et al., 2013). Surprisingly, although "passive" social behaviors (including passive contact and social proximity) are often reported in current sociability studies during the 10-30 minute testing period, there are few reported studies that have looked at these behaviors for an extended (> 1 h) period of time (Ricceri et al., 2007).

Of the possible persistent passive social behaviors, “huddling” is an easily quantifiable and well-document choice that consists of two or more mice or rats laying together (often in tight conformation) in order to regulate body heat (often during sleep) (Alberts, 1978; Batchelder et al., 1983; Contreras, 1984; Glaser and Lustick, 1975). While huddling is primarily characterized as a homeostatic behavior that mitigates energy expenditure and regulates body heat in a borrow environment, researchers showed that social factors also mediate huddling. When paired with a familiar littermate weanling and two unfamiliar conspecific cage mates, for example, spiny mice (*Acomys cahirinus*) prefer to huddle with siblings. Furthermore, differences in huddling behavior preference persist even after chronic separation (Porter and Wyrick, 1979; Porter et al., 1978).

Rates of huddling among inbred mice appear to be strain-dependent. Using an etiologically relevant visible burrow system, one group found a significant difference between huddling behaviors in BTBR and C57BL/6J mice over a three-day period, with BTBRs showing significantly reduced huddling (Pobbe et al., 2010), especially during the light (inactive) photoperiod. This finding is interesting because numerous studies have found that BTBR mice are deficient in other tests of sociability (Moy et al., 2004; Yang et al., 2007b). However, it should be noted that shorter behavioral assays have failed to find significant amounts of huddling behavior in any inbred strains. For example, Bolivar and colleagues (2007) quantified numerous social behaviors among 7 inbred strains that were placed a neutral home cage environment over a 20-30 min testing session. When one mouse was placed in the cage 15 min prior to the second mouse, coders did not see any instances of huddling in any strain during the 20 min testing session. When both animals were introduced to the neutral home cage simultaneously, the subsequent 30 min of observation revealed that only one strain (DBA/2J) showed significantly more “positive” social behaviors (including huddling) than other strains, including C57BL/6J,

129S1, and A/J. Furthermore, overall rates of positive social behavior were low, on average accounting for less than 30 s of the total 30 min observation time (Bolivar et al., 2007).

Although huddling in mice has been cited as a major and consistent facet of rodent social behavior (Ricceri et al., 2007), only one study has assessed huddling behavior in adult autism models for an extended period of time. Specifically, Lijam and colleagues found that *Dishevelled* knockout mice (*Dvl1)* showed reduced huddling contact during sleep when compared to normal mice in a home cage environment (Lijam et al., 1997). Similar to the outbred mouse literature, several studies using shorter behavioral assays have failed to find significant amounts of huddling behavior in animal models (Crawley et al., 2007; Moy et al., 2008). However, there is some evidence that juvenile autism-like mice may show reduced huddling rates compared to wildtype controls (Yang et al., 2007a).

The paucity of sociability studies using huddling measures is not surprising. Huddling is often associated with nesting and sleep, requiring researchers to test mice over several days in a low-stress environment to see significant amounts of this behavior. Furthermore, with the exception of the social interaction test, sociability assays prevent mice from freely interacting with one another. We were skeptical of the field's current reliance on short-lived sociability tests, believing instead that longer time-course analyses (> 1 h) of freely interacting mice could reveal differences in huddling-like behaviors as they become familiar with one another, without relying on multi-day analyses of home cage data. Specifically, we designed a test to encourage voluntary social proximity over a period of several hours to compare differences in closeness among unfamiliar and familiar mouse dyads.

To name this behavior, we turned to human social psychology studies that characterized correlates of human friendship. These studies find that propinquity, or sense of "personal

closeness" is an important pre-requisite for the formation of friendship for servicemen (or "best buddies" as described by one author) (Loether, 1960; Zeleny, 1947) as well as marriage partners (Abrams, 1943; Bossard, 1932; Clarke, 1952; Davie and Reeves, 1939). More recent media studies compared electronic versus personal modes of interactivity, finding that increasing amounts of communication via technological channels (i.e., telephone, texting, or email) and reduced measures of physical propinquity correlated with a decrease in interactional qualities (including nonverbal behaviors and sense of social presence) and produced less participant satisfaction (Burgoon et al., 2002; Mehrabian, 1981; Short et al., 1976). Finally, studies measuring interpersonal distances between familiar and unfamiliar dyads show that humans prefer to interact socially with familiar people and maintain closer distances to friends than strangers during communication (Edwards, 1972; Sinha and Mukherjee, 2008).

Based on both the human friendship literature and previous accounts of murine huddling behavior, it seemed logical to design a test that would encourage social proximity among familiar rodent pairs by increasing their incentive to remain in close personal contact with one another. We began by comparing propinquity among outbred animal lines, similar to Porter and colleagues' original experimental protocol (Porter et al., 1978). Towards this end, the rest of the chapter will describe a way to measure propinquity behavior exhibited by outbred rodents: the tube co-occupancy test, or TCOT.

2.3 Methods

2.3.1 Animals

In most experiments, naïve male and female CD-1® (Crl:CD-1(ICR)) outbred mice were bred in-house at our animal facility at McGill University. Additional outbred mice of both sexes, including Swiss Webster (SW) and Institute of Cancer Research (ICR) mice were purchased from Charles River (CR:SW; Bourcherville, QC) or Taconic Biosciences, Inc. (Tac: ICR and Tac:SW; Albany, NY) .

Prior to testing, mice were group-housed (3–5 per standard shoebox cage) with same-sex companions (littermates for mice bred in-house). All mice were given tap water and Harlan Teklad 2020x soy protein-free extruded rodent diet *ad libitum* and maintained at 22 °C on a 12/12-h light/dark cycle (lights on at 07:00 h). All protocols and procedures were approved by the Downtown Animal Care Committee of McGill University according to appropriate national regulations for animal use and care.

Mice were assessed beginning in late adolescence or early adulthood (6–10 weeks of age) except in experiments specifically looking at propinquity behaviors in older mice (12-18 weeks of age). Although mice at 6 weeks have previously been reported to show increased social preference and risky behavior, we did not see significant changes in our dependent measure when comparing animals between 6 and 8 weeks of age. All experiments included approximately equal numbers of male and female mice; mice were only used once. Experiments occurred near mid-photoperiod, commencing no earlier than 09:00 h and no later than 16:00 h.

To validate propinquity behavior in rats, one experiment used 5-7 week old male Sprague Dawley rats purchased from Charles River Ltd., UK. Rats were habituated to the facility for a minimum of 2 weeks prior to testing. Rats were kept on a 12/12-hour light-dark cycle (lights on

at 07:00 h), at 22 °C, with *ad libitum* access to food and group housed in cages of four. All protocols and procedures were approved by the UK Home Office and Edinburgh University regulations for animal use and care.

2.3.2 The tube co-occupancy test (TCOT)

Same-sex mouse dyads (or, as a control, a single mouse) were placed, at the same time, into an arena with opaque Plexiglas walls (39 x 26 x 12 cm high). In order to create a stressful open field environment, the arenas were situated on top of a glass shelf 105 cm above the ground (creating a visual cliff), and were brightly illuminated with a 250 W LED light, producing ~3000 lux. Each open field box contained a single opaque polyvinyl chloride (PVC) cylinder (7.5 x 3 cm diameter; or in one experiment, a larger 10 x 3 cm diameter tube) placed against one long wall (see **Figure 2.1a**). In the "two-tube" variant of the TCOT, two 7.5 x 3 cm cylinders were placed 4 cm apart from one another along the long wall of the arena (see **Figure 2.1b**). Mice were tested for 3 h in same-sex pairs, without prior habituation to the room or the TCOT arena (except in one experiment featuring 30- or 90 min-habituation), in one of the following social conditions: 1) *Siblings* – born of the same parents and raised together in a single home cage from birth until testing; 2) *Cagemates* – born of different parents but living in the same home cage from weaning at P21 until testing; 3) *Separated Siblings* – born of the same parents but living in different home cages from weaning at P21 until testing; and, 4) *Strangers* – born of different parents with no contact prior to testing. For stranger habituation experiments, stranger mice from two different cages were put together as a dyad into a clean cage and co-housed for 1, 4, 7 or 14 days prior to testing.

All animals were age-matched and tested only once in the TCOT, except in repeat exposure experiments, where mice were tested multiple (2–4) times. After placing animals in the TCOT arena, male or female experimenters turned on the automated recording system and/or video cameras and then quickly left the room.

For the rat study, the arena measured 60 x 60 x 60 cm, with floors and walls made of white acrylic. A black plastic pipe measuring 14.5 cm long x 11 cm in diameter was fixed to the floor with Velcro. Two bright lights (3000 lux) were shone down onto the arena. Between experiments the arena floor, walls and the tube itself were cleaned using 70% alcohol. Rats were handled for 20 min per cage for 3 days prior to testing. The duration of TCOT testing was 1 h; the experimenter exited the room promptly after rat placement in the arena. TCOT behavior was digitally videotaped and analyzed manually, with the experimenter blinded to animal genotype and social status. The social conditions were identical to the mouse TCOT, except that no separated sibling group was tested.

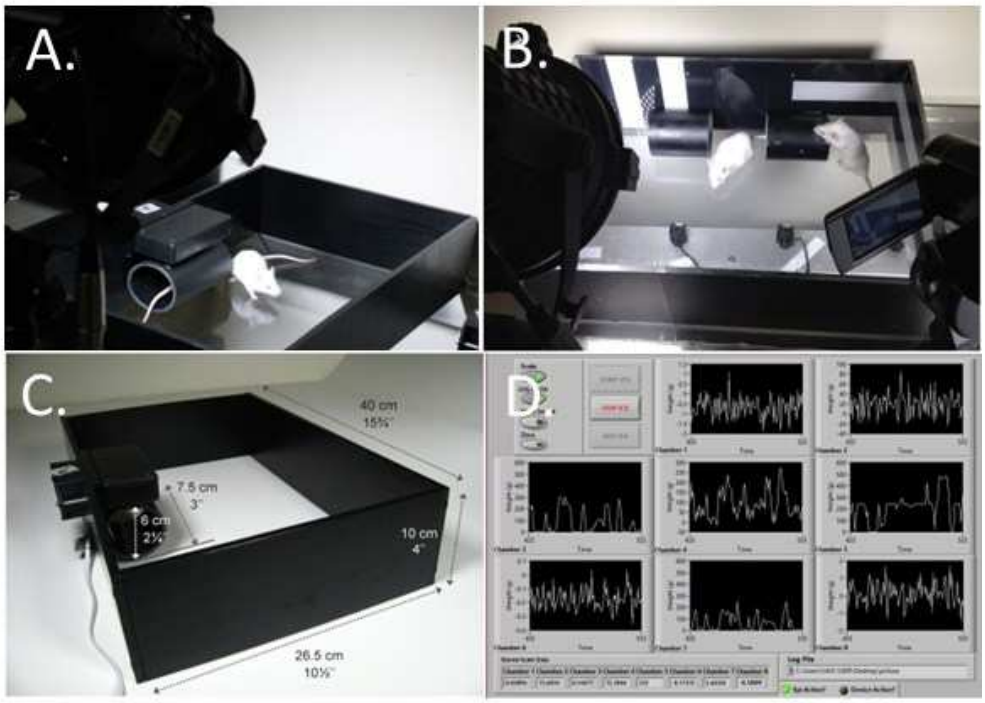

Figure 2.1: A) A snapshot of the manual TCOT setup. Cameras placed above the testing apparatus (not shown) capture mouse behavior during three hours of interaction that occurs in the open field and "safe" tube. Note the placement of bright lights, as well as the presence of a visual cliff underneath the open field. Normally, clear Plexiglas covers are placed over the open field to prevent mice from escaping our testing apparatus (they are removed in this picture). B) The two-tube variant of the TCOT. In this test, experimenters place a second tube along the same wall as the original tube, equidistant from the open field's corners. In this test, mice have a choice to occupy the same tube, or occupy a separate, "safe" place. C) Exact dimensions of the automated TCOT. Note the addition of a pressure sensor above the TCOT tube. In this design, the tube is suspended from the pressure sensor. Any additional weight inside of the tube registers in the automated TCOT software. D) A snapshot of the proprietary software suite associated with the automated system. Our software is currently capable of recording 8 dyads simultaneously in real time, measuring changes in tube occupancy by recording tube weight change (1 measure/s).

2.3.3 TCOT scoring

In the "manual" TCOT test, behavioral scoring was carried out by blinded observer who sampled videos to generate observational bins. Subsequent analysis of these bins produced percentages of tube co-occupancy, single occupancy, or vacancy. Evidence of aggression (chasing, biting, and fighting) were also coded. Sample bins were generated by coding one 10 s sample every other minute. For "manual" scoring, a digital video camera was placed directly over the arena. The resultant video file was also used to score behaviors (e.g., fighting, following, sniffing) occurring outside of the tube.

Data produced by our "automated" TCOT system were based on continuous measures throughout the TCOT run. The automated system contains PVC cylinders magnetically suspended from 780 g-capacity load cells (see **figure 2.1c**). Electrical signals from the load cells were amplified and conditioned for input into a digital processor. The processor output data into a computer programmed to store and present a real time (1/sec) display of the current weight of the cylinder, which was exported to Microsoft Excel® for analysis (see **figure 2.1d**). Tube vacancy, single occupancy and co-occupancy (measured in seconds) were easily inferred from

the weight data. The automated system currently allows for up to 8 simultaneous tests to be conducted simultaneously.

Social interactions outside of the tube were assessed by manually scoring the first 5 min of the TCOT run. A blinded observer then marked the total time an experimental subject spent interacting with a conspecific. Interactions were operationalized as (1) pursuing the naïve mouse and (2) sniffing any part of the naïve mouse's body. A total percent time spent in social exploration was obtained by computing the following: (social exploration behavior/300 s) x 100.

2.3.4 The three-chambered test

To assess whether extended behavioral testing in previously validated sociability tests also show murine familiarity preference over prolonged testing sessions, outbred strangers were tested for 3 hours using the three-chambered system. Specifically, mice were brought from our vivarium to the testing room 10 minutes prior to the start of the experiment in order to acclimate to the environment. The test mouse (purple, **figure 2.2A)** was then placed in central chamber and allowed to explore all three chambers for 10 min. The test mouse was then placed back in the central chamber and doors between chambers were closed. Novel stimulus animal "A" was then placed (in a counterbalanced order) one side of the three-chambered assay before the central chamber doors were raised (red, **figure 2.2B**). The experimenter then started a 10 minute recording session to test for sociability and left the room. At 10 minutes, the experimenter re-entered room and placed the test mouse back in the central chamber (with doors closed). Stimulus animal "B" was then placed in the empty side of the three-chambered assay and doors

were raised (black, **figure 2.2C**). Video recording resumed and proceeded for the subsequent 180 minutes in order to measure potential familiar-directed proximity behaviors.

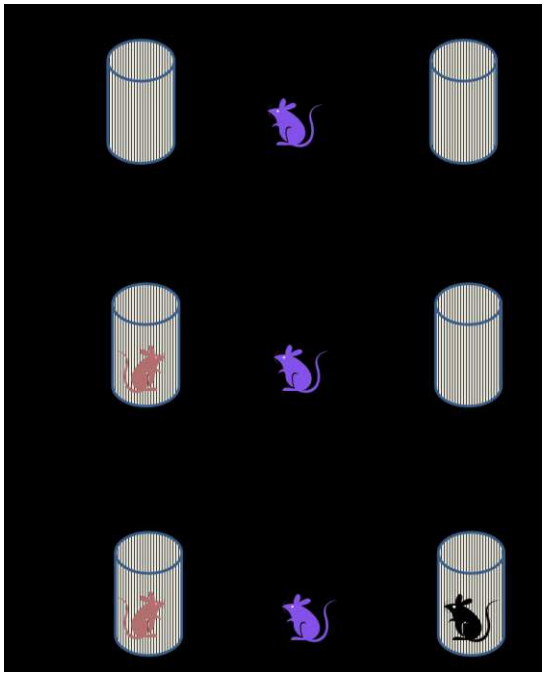

Figure 2.2: A representation of the three-chambered test. In our test an outbred (CD-1®) animal ("test mouse") was pre-habituated to the testing apparatus for 10 min (**A**). A CD-1® stranger was then introduced ("stimulus A"), and the target's subsequent proximity behaviors (entries into stimulus 1's chamber) were recorded and coded off-line (**B**). After 10 minutes, a second CD-1® stranger ("stimulus B") was introduced into the opposite side of the three-chambered apparatus, and target behavior was recorded and coded for 3 hours (**C**).

2.3.5 Three-chambered test scoring

To establish scores for social novelty, videos were manually scored by generating video snapshots (one sample every 60 s) and determining which room the target animal was occupying at the time (familiar room, novel room, or the neutral middle room). Samples were then summed for each 10 min epoch (with a maximum possible score of "10" for each epoch) and compared over the 3 h run (**figure 2.2C**). *N.B.:* We only observed changes in target position during the first hour, after which time targets generally went to sleep in one position in the testing assay. In

addition to quantifying active social exploration among animals in this test during the first hour, we also reported where target mice settled during the second hour of testing.

2.3.6 Statistical Analyses

Coders confirmed that data were normally distributed (Shapiro-Wilk statistic) and featuring homogeneity of variance (Bartlett's test) among groups. Thus, data were analyzed using *t*-test (two-sided), one-way or two-way ANOVA followed by Tukey or Dunnett post-hoc analyses, where appropriate. In repeat exposure experiments, Sidak corrections for multiple comparisons were used as needed. For all analyses, $\alpha=0.05$ was considered significant.

Because of the novelty of the phenomenon, it was not possible to perform power analyses. Sample sizes were determined primarily by breeding success and our experience with other social phenomena in mice.

Behavioral runs were excluded in their entirety if the tube became detached from the load cell or the video camera was unable to record to the end of the behavioral run. In four cases, data were excluded after being identified as statistical outliers (Studentized residuals >2 standard deviations from the group mean).

2.4 Results

2.4.1 Outbred mice will occupy tube alone or with a co-housed partner

When tested alone in the TCOT arena, CD-1® outbred mice showed a strong motivation to occupy the tube, spending the majority of their time inside over the 3 h testing period (**Figure 2.3a**). Same-sex dyadic groups (siblings, non-sibling cagemates, separated siblings [i.e., siblings

housed separately since weaning], and strangers) spent different proportions of their time sharing the tube (co-occupancy), occupying the tube one at a time (single occupancy) or with both mice outside the tube (vacant tube) (**Figure 2.3a**). Stranger dyads displayed significantly less co-occupancy than (identically performing) sibling or cagemate dyads ($F_{3,68}$=5.5, p=0.002). Conversely, strangers and separated siblings spent significantly more time outside of the (vacant) tube compared to sibling or cagemate dyads ($F_{3,65}$=12.0, p<0.001). Given the phenotypic similarities demonstrated by familiar (siblings versus cagemates) and unfamiliar (strangers versus separated siblings) dyads in co-occupation behavior across the 3 h run (**figure 2.3b**) we restricted all subsequent experiments to sibling versus stranger comparisons. This decision resulted in minimum handling during weaning and streamlined subsequent housing protocols: we always weaned siblings to the same cage and tested them with a same-cage partner, whereas we always tested strangers with an inter-cage partner weaned from a different breeding pair.

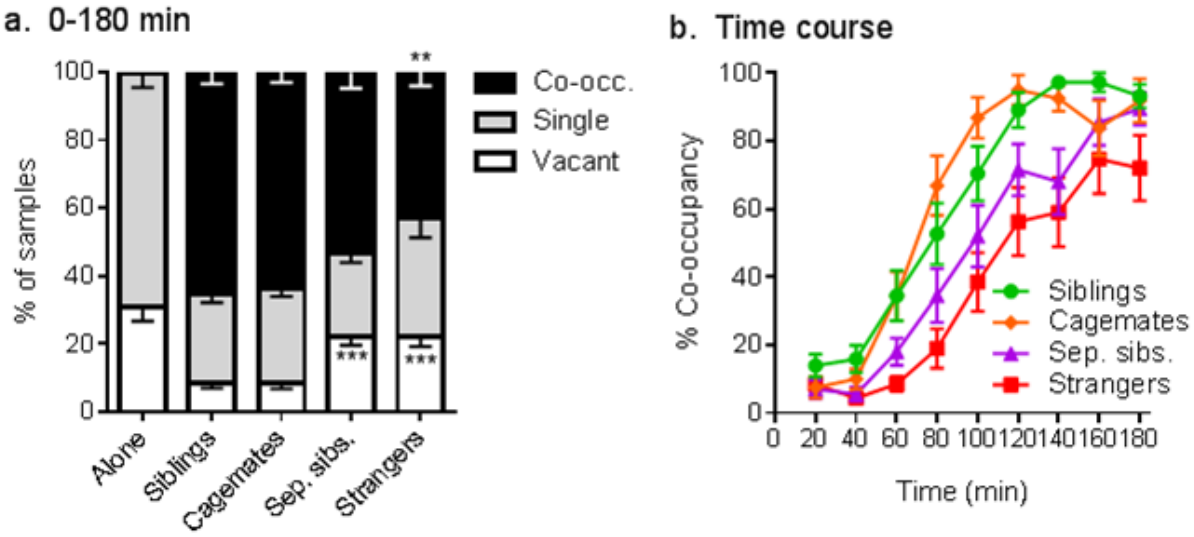

Figure 2.3: Data showing reduced tube co-occupancy in mouse dyads. **a)** Tube occupancy behavior in various social situations (alone, siblings, cagemates, separated siblings, or strangers)

over the full 180 min testing period; n=18 mice or dyads per social condition. Bars represent mean ± SEM percentage of samples featuring tube co occupancy (Co-occ.), single occupancy (Single) and no occupancy (Vacant). **p<0.01, ***p<0.001 compared to sibling groups. **b)** Time course of tube co occupancy behavior in tested animals. Symbols represent mean ± SEM percentage of samples featuring tube co-occupancy (using our bin-sampling method of digital files) per 20 min epoch.

To rule out aggression as a confounding factor for tube co-occupation rates among outbred mice, we also coded for any sign of aggressive behavior during each of the 10 s samples that were manually scored. Aggressive behaviors (bouts of biting or scuffing) in this 3 h assay were extremely rare, with no group at any time point displaying aggressive behavior significantly greater than zero (one-sample t-tests: t_{10} = 1.0–1.5; 0.08<p<0.17) (**Figure 2.4**).

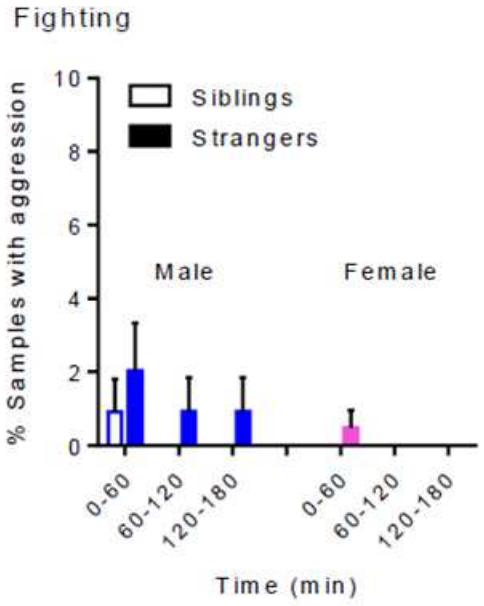

Figure 2.4: Limited fighting behavior in the TCOT. Bars represent mean ± SEM percentage of 10 s/min samples (60 samples per h) featuring aggressive behavior (chasing, biting, and fighting) in a subset of sibling or stranger dyads; n=11 dyads/social condition/sex. No group at any time point displayed aggressive behavior significantly greater than zero (one-sample t-tests: t_{10} = 1.0–1.5; 0.08<p<0.17).

2.4.2 CD-1® mice engage in active exploration during the first hour

A time-course analysis of CD-1® tube occupation reveals that all mice spend > 80% of their time outside of the tube during the first hour, regardless of social status (**figure 2.3b**). There were no differences between sex or familiarity status in single occupancy ratios (a situation we refer to as "tube hogging"); typically, one mouse in the dyad preferred to occupy the tube, spending ≈30% more time alone in the tube compared to the other mouse (**Figure 2.5a**): main effect of sex: $F_{1,44} = 0.4$, p=0.56; main effect of social condition: $F_{1,44} = 0.4$, p=.56; main effect of social condition: $F_{1,44} = 0.0$, p=.98; sex x social condition: $F_{1,44} = 0.0$, p=.84). Social interactions occurring during the first 5 minutes outside the tube did not vary by social status, with all animals engaging in low levels of social investigation regardless of sex or social status (**Figure 2.5b**): No group differences were observed in either anogenital sniffing or pursuit (t_{20}=0.2, p=0.84, t_{20}=1.3, p=0.22, respectively).The high degree of exploration that mice demonstrated in the first hour was not surprising, given the relative novelty of the environment (and social novelty in stranger conditions). As a result, all subsequent analyses skipped the first hour, instead treating it as a prolonged "habituation" period.

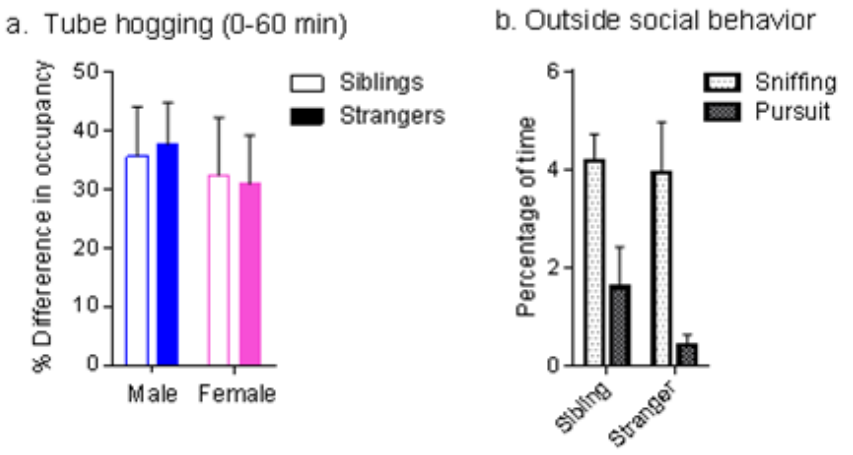

Figure 2.5: a) Differential single occupancy behavior by each member of the dyad (i.e., "hogging" the tube) does not vary by sex or familiarity. Bars represent mean ± SEM percentage difference in single occupancy of one mouse in the dyad compared to the other over the first hour in the TCOT (featuring the highest levels of single occupancy); n=11–13 dyads/social condition/sex. As can be seen, in most cases, one mouse in the dyad dominated the tube (spending ≈30% more time alone in the tube compared to the other mouse), but this did not depend on their sex or familiarity status. **b**) Analysis of early (0–10 min) social interaction behaviors occurring outside of the tube in the TCOT arena. Bars represent mean ± SEM percentage of time engaging in anogenital sniffing and pursuit behaviors of mice in sibling versus stranger dyads; n=10–12 dyads/social condition.

2.4.3 Outbred strangers co-occupy less than familiars in hour 2

Further inspection of the tube co-occupancy time-course data (**Figure 2.3b**) revealed the second hour of testing (60–120 min) shows the largest differences in tube co-occupation behavior among social groups ($F_{3,68}$=6.0, p=0.001) (**Figure 2.6a**), with strangers spending significantly less time in the tube together than siblings or cagemates. We observed sex differences in tube co-occupancy in only one group, with female separated siblings spending significantly more time together than male separated siblings (condition x sex: $F_{3,64}$=5.3, p=0.002) (**Figure 2.6b**). While strangers persisted in demonstrating lower co-occupation rates in the third hour as compared to familiar dyads (**Figure 2.3b**), in the interest of streamlining the TCOT testing protocol we selected the second hour as the "ideal" testing block; we performed all analyses in subsequent experiments on data exclusively collected during this time period.

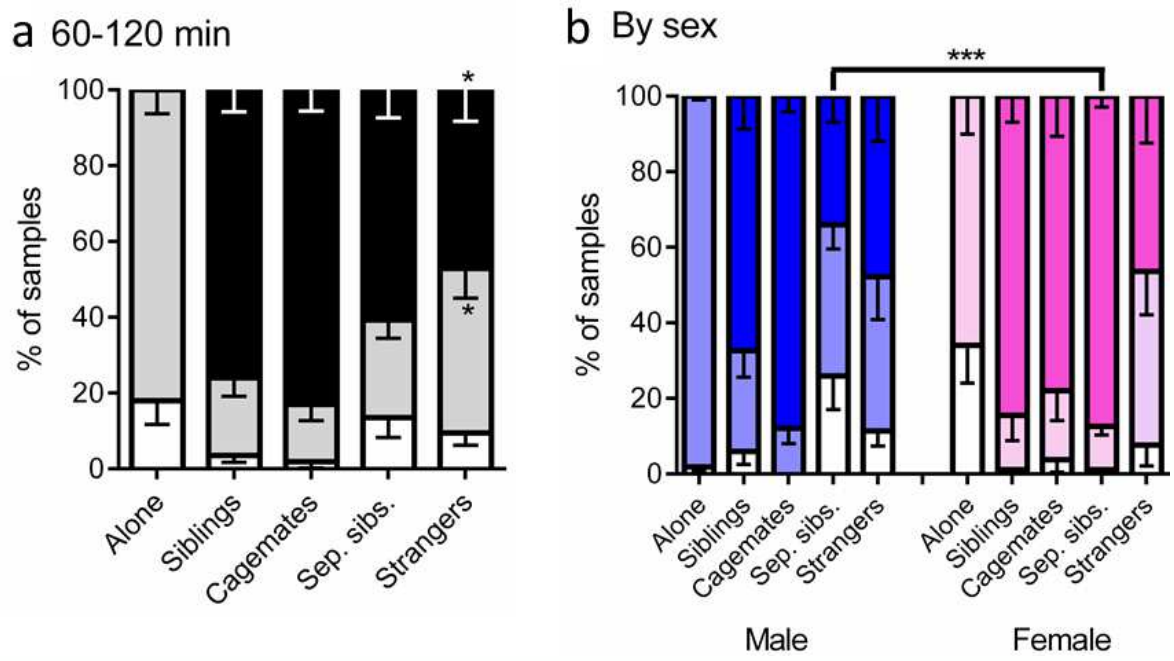

Figure 2.6: a) Tube occupancy in the second hour of testing. Bars as in Figure 2.3 a, but for the 60–120 min period. Bars represent mean ± SEM percentage of samples featuring tube co-occupancy (black), single occupancy (grey) and no occupancy (white). **b**) Tube occupancy by subject sex. Bars are same as in **a**, featuring tube co-occupancy (dark blue/magenta), single occupancy (light blue/pink) and no occupancy (white). n=9 mice or dyads per social condition per sex. *p<0.05, ***p<0.001 compared to sibling groups.

To further investigate the apparent difference in sibling versus stranger preference in outbred CD-1® (Crl:ICR) mice, we tested three additional outbred strain/supplier combinations using the automated TCOT system: ICR mice from Taconic, and Swiss Webster mice from both Charles River and Taconic. Similar to CD-1® animals, the other outbred populations displayed higher levels of co-occupancy behaviors if they were siblings, as compared to strangers (**Figure 2.7a**). ANOVA analysis revealed a significant main effect of social condition ($F_{1,145}$=20.0, p<0.001) but not strain ($F_{1,145}$=1.3, p=0.26), supplier ($F_{1,145}$=0.04, p=0.83) or any interactions (all p's >0.29), confirming that our initial characterization of co-occupancy behavior in CD-1® animals was typical of outbred mice. In order to ensure that the automated TCOT was accurate when compared to our manual sampling method, we compared manual and automated data generated

during the second hour of testing in a subset of animals (**Figure 2.7b**). We found a highly significant correlation between manual and automated TCOT scoring in the second hour, r= 0.96, p<0.001 (**Figure 2.7c**).

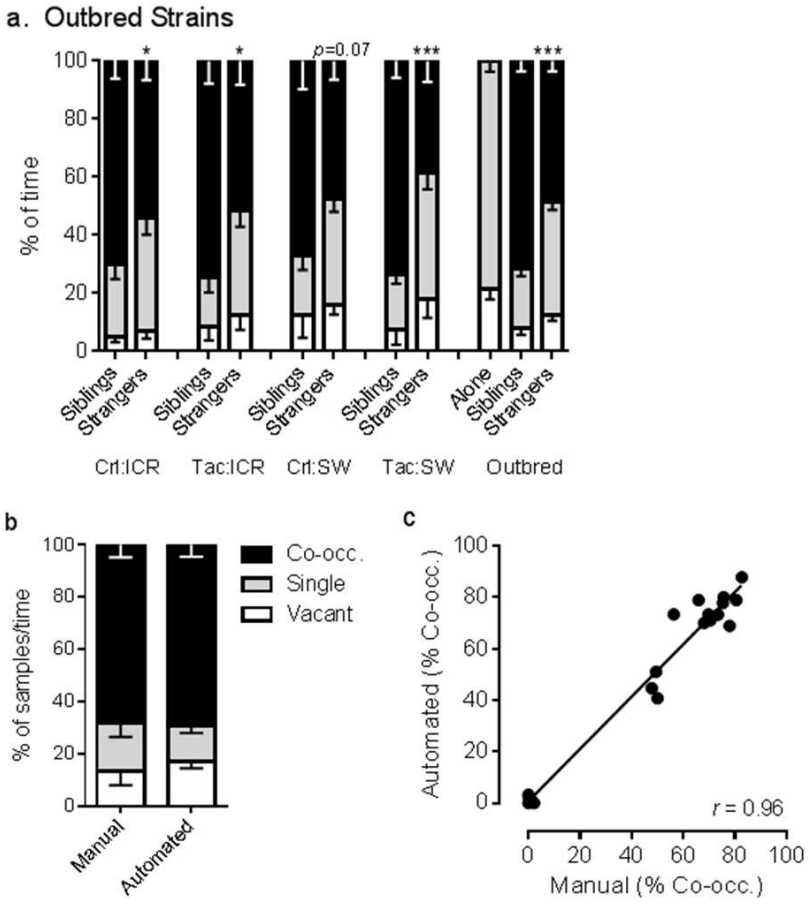

Figure 2.7: Data collected with the automatic TCOT reveal that outbred mice demonstrate familiarity preference in tube co-occupation behavior. **a**) Tube occupancy behavior in six outbred mouse stocks, as well as their cumulative average (Outbred). n=12–26 dyads/social condition/genotype. *p<0.05, **p<0.01, ***p<0.001 compared to sibling groups. **b**) The automated version of the TCOT provides accurate quantification of tube status compared to manual scoring using sampling (repeated measures: $F_{1,18}$ = 1.2, p=0.29). Bars represent mean ± SEM percentage of samples (manual) or percentage of time (automated) featuring co-occupancy (co-occ., black bar), single occupancy (grey bar) and a vacant tube (white bar) from 60-120 min in sibling mice (n=17 dyads/measurement technique). **c**) Correlation between manual and automated TCOT scoring. Symbols (n=19 dyads) represent percentage co-occupancy as in **b**; r= 0.96, p<0.001.

2.4.4 Evaluating environmental and social components of the TCOT

As previously reported in other sociability tests, we found that both environmental and social novelty significantly impacted subsequent tube co-occupation in the TCOT. We began by determining if environmental novelty was necessary for our test by habituating both stranger mice, separately, to the empty arena for 30 or 90 min before dyadic testing. Following habituation, we observed a significant increase in co-occupancy behavior among stranger dyads ($F_{2,45} = 7.6$, p=0.001) (**Figure 2.8a**), but no changes in aggressive behavior (not shown).

We also assessed the importance of social novelty by exposing strangers to one another in repeated daily runs of the TCOT or by co-housing strangers together for a period of time before running them in our test. Repeated measures analysis of stranger dyads run in the TCOT with the same partner for up to 4 days revealed that strangers displayed sibling-like levels of co-occupancy by the third day (repeated measures: $F_{3,63} = 7.5$, p<0.001) (**Figure 2.8b**). Likewise, co-housing strangers for as little as 24 h prior to testing yielded sibling-like levels of co-occupancy behavior in these animals ($F_{5,62} = 14.3$, p<0.001) (**Figure 2.8c**).

Finally, we were curious to see whether modulating aspects of the environment could affect propinquity in mice. Starting first with changes to the social environment, we tested older mice to see if age modulates tube co-occupation. Comparing TCOT data from 12-week and 18-week-old mice to 6-week-old animals showed that only young adult mice demonstrated high levels of propinquity, although older mice still preferred to co-occupy with siblings more than strangers (main effect of age: $F_{(2,65)} = 4.6$, p=0.01; main effect of social condition: $F_{(1,65)} = 7.5$, p=0.008; age x social condition: $F_{(2,65)} = 1.4$, p=0.25) (**Figure 2.8d**). Furthermore, mice did not avoid going into the tube together because the tube was too small: control experiments varying

tube size did not account for age-related decreases in tube co-occupation behavior (data not shown).

In addition to evaluating differences in social stimuli, we assessed whether environmental stressors that we built into the TCOT's design were necessary to drive tube co-occupancy behavior in outbred mice. These stressors included the visual cliff and bright lighting in our testing protocol. Based on our resulting data, it appears that both a visual cliff and bright lights are necessary components for differentiating sibling and stranger behavior in the TCOT (**Figure 2.8e**, **Figure 2.8f**, respectively)

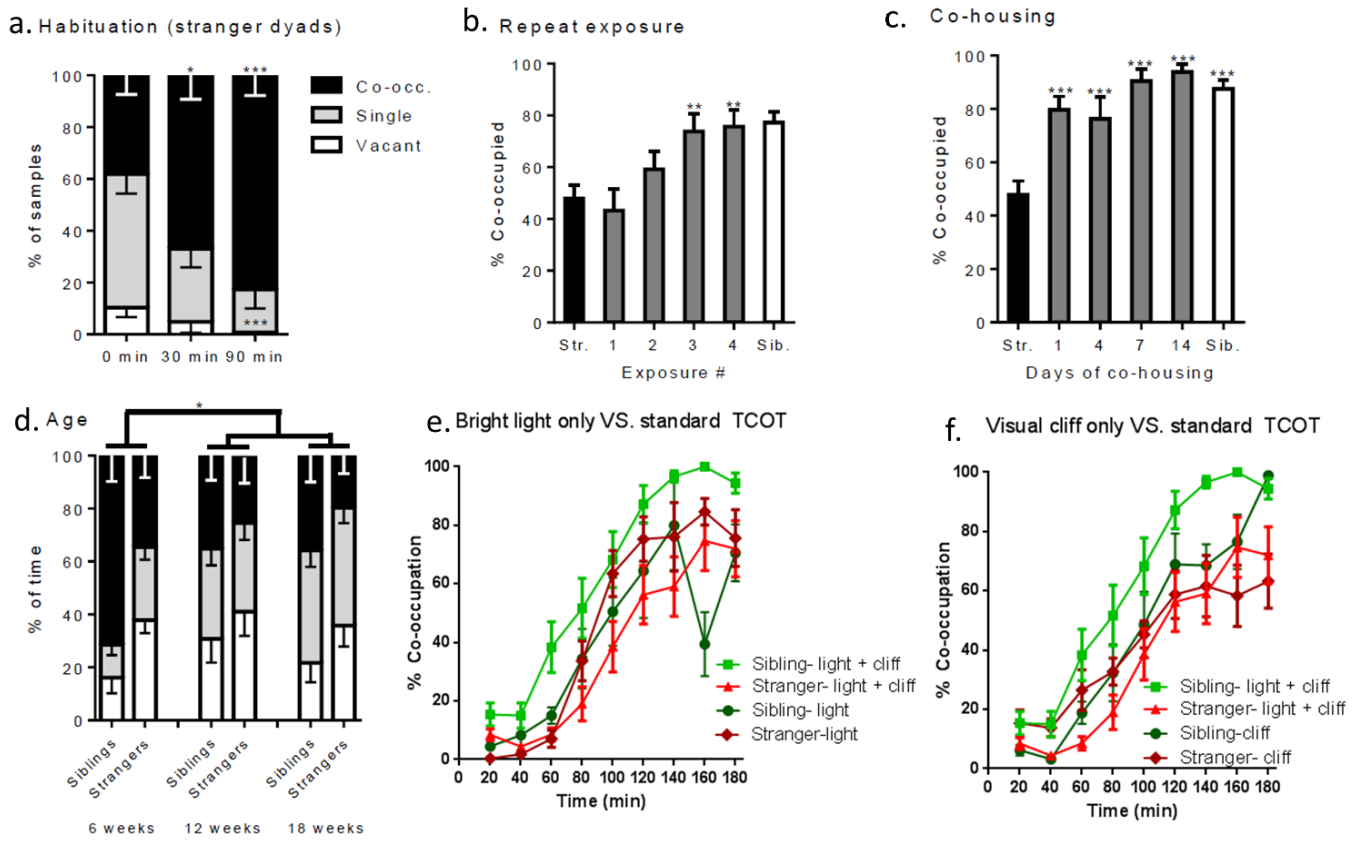

FIGURE 2.8: Parametric considerations affecting co-occupancy in the TCOT. **a**) Habituation of stranger mice to the TCOT before social exposure greatly increases co-occupancy behavior ($F_{2,45}$ = 7.6, p=0.001). Bars represent mean ± SEM percentage of samples featuring co-occupancy (co-occ.), single occupancy and a vacant tube from 60-120 min after social exposure between two stranger mice that were separately habituated to the TCOT for 0 min, 30 min or 90 min (n=16

dyads/habituation time). *p<0.05, ***p<0.001 compared to 0 min group by Dunnett's case-comparison posthoc test. **b**) Repeat exposure of stranger dyads (Str.) to both the TCOT and one another increases co-occupancy behavior (repeated measures: $F_{3,63}$ = 7.5, p<0.001) to the level displayed by sibling dyads (Sib.). Bars represent mean ± SEM percentage of samples featuring co-occupancy of mice exposed to each other 1, 2, 3 or 4 times; n=24 dyads. **p<0.01 compared to first exposure by posthoc testing with Sidak correction for multiple comparisons. **c**) One or more days of co-housing prior to testing yields sibling-like levels of co-occupancy in previously stranger mice ($F_{5,62}$ = 14.3, p<0.001). Bars represent mean ± SEM percentage of samples featuring co-occupancy of stranger dyads (Str.), sibling dyads (Sib.), or stranger dyads having been co-housed for 1, 4, 7 or 14 days before testing; n=6–12 dyads/co-housing time. ***p<0.001 compared to Str. group. **d**) High levels of co-occupancy occur only in young adult mice (main effect of age: $F_{2,65}$ = 4.6, p=0.01; main effect of social condition: $F_{1,65}$ = 7.5, p=0.008; age x social condition: $F_{2,65}$ = 1.4, p=0.25). Bars represent mean ± SEM percentage of samples featuring co-occupancy (co-occ.), single occupancy and a vacant tube from 60-120 min in mice of 6, 12 or 18 weeks of age; n=10–13 dyads/age/social condition. *p<0.05 compared to 12- and 18-week-old mice. Note that although co-occupancy behavior decreased, older mice still preferred to co-occupy with siblings compared to strangers. **e-f**) Time course analyses of tube co-occupation behavior in animals that were tested in a TCOT setup with only bright light (**e**) or a visual cliff (**f**) reveals that both stressors are needed to observe familiarity preference in outbred animals. n= 9-12 dyads/stressor/social status.

2.4.5 Two-tube TCOT control

After optimizing our initial TCOT design, we were uncertain if propinquity, as defined by tube co-occupation in our assay, was an act of volition or merely mutual tolerance expressed by animals in order to escape the aversive open-field environment. To address this potential confound, we tested mice in a two-cylinder version of the test, to determine if mice would remain together in one tube, or separate and occupy different (but equally "safe") spaces (**Figure 2.9**). Although data from the two-tube version of the TCOT show an overall decrease in tube occupation time when compared with the one-tube test (**Figure 2.6a**), the pattern among social dyads in the two-tube test ($F_{3,35}$=6.4, p=0.001) were highly similar to the one-tube version. These results suggest that familiar mice prefer to co-occupy the same tube even if animals have the option to reside separately in the TCOT.

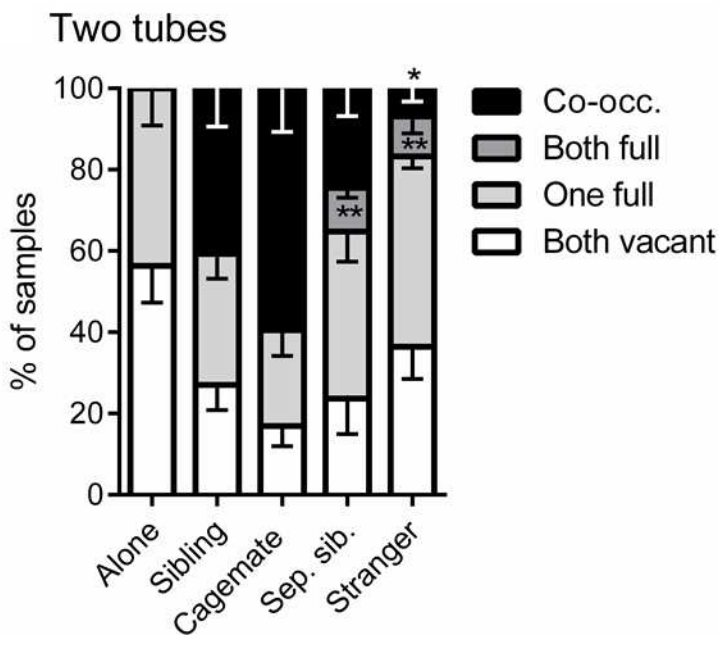

Figure 2.9: Tube occupancy behavior in an arena with two tubes instead of one. Bars represent mean ± SEM percentage of samples featuring co-occupancy of either tube (Co-occ.), simultaneous single occupancy of both tubes (Both full), single occupancy of one tube (One full), or no occupancy (Both vacant); n=8–12 mice or dyads per social condition. *p<0.05, **p<0.01, compared to sibling groups.

2.4.6 Rats show differences in co-occupation behavior in the TCOT

To further test generalizability of the TCOT, we arranged for collaborators (the Gkogkas lab in Edinburgh, Scotland) to run outbred rats in a larger version of the mouse TCOT (see **section 2.3.2** for details). Similar to our observations using outbred mice, our collaborators observed significant differences in tube co-occupation rates among sibling/cagemate dyads versus stranger dyads using outbred Sprague Dawley rats tested for one h in a larger TCOT arena ($F_{2,27}$=60.5, p<0.001) (**Figure 2.10**).

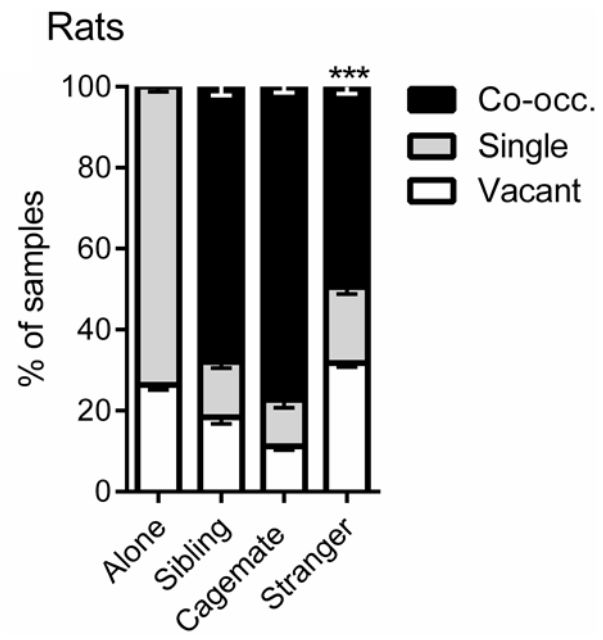

Figure 2.10: Tube occupancy behavior in various social situations in outbred, Sprague Dawley rats in a larger version of the test. Bars as in graph **a**, over a 60-min testing period; *n*=10 rats or dyads per social condition. ***p<0.001 compared to sibling groups.

2.4.7 The three-chambered test does not show propinquity over time

Similar to previously published studies using the three-chambered test to measure inbred stranger novelty-seeking behavior over an extended (> 20 min) period of time, outbred mice in our laboratory fail to show a continued preference for a novel mouse during a 2 h three-chambered test run (**Figure 2.11A-B**). As such, we believe that the three-chambered test (along with other related short-lived tests of rodent sociability) is poorly optimized to study changes in familiarity and associated behaviors, necessitating the additional, complimentary behavioral assays.

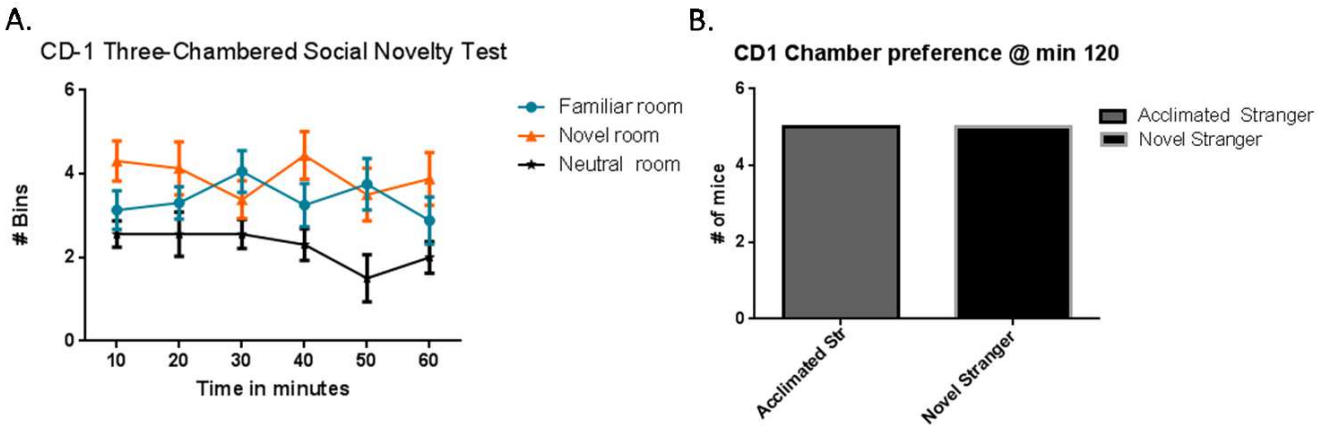

Figure 2.11: Time course of room co-occupancy behavior. Symbols represent mean ± SEM percentage of samples featuring chamber co-residency (using sampling of digital recording) per 10 min period. **A**) While mice appear to demonstrate social novelty behavior in the first 10 min of the test, they spend the remainder of the time frequently switching rooms, without a preference for remaining with a familiar or stranger animal; *n*= 16 triads (test mouse + 2 stimuli mice). **B**) CD1 mice show no preference where they settle down after the first hour of social exploration, neither preferring the acclimated stranger nor the novel stranger animal.

2.5 Discussion

Here we show that outbred mice familiar with one another (siblings or cagemates; **Figure 2.3**), or strangers that are sufficiently exposed to one another prior to testing (**Figure 2.8b/c**) show significantly higher rates of co-occupation behavior in TCOT as compared to unfamiliar animals. Furthermore, we question other experimental design choices that keep animals separated from one another (Bowers and Alexander, 1967; Crawley, 2004; Kudriavtseva, 1987; Yang et al., 2007a). By quantifying multiple behaviors over the 3 h TCOT run, we find that outbred mice initially engage in social novelty-seeking behavior (including following and sniffing; **Figure 2.5b**) and tube swapping (**Figure 2.5a**) before transitioning to co-occupy a "safe" tube after the first hour (**Figure 2.3b**) with virtually no concomitant fighting (**Figure 2.4**). As such, peak differences in propinquity among mice by familiarity status appear to occur during

hour two (**Figure 2.6a**), a significant improvement in testing length when compared with related huddling assays that take between 2-5 days to complete (Pobbe et al., 2010; Porter et al., 1978). Though automated visual tracking systems may offer more standardized, high-through-put assays for huddling and related social behaviors in the future, current home cage observation protocols still require multiple days of testing, have trouble distinguishing social interaction behaviors in tests using multiple subjects, and require complex equipment and a degree of user sophistication in order to analyze and report relevant statistics (Hong et al., 2015; Spruijt and De Heer, 2012). By automating the TCOT, we offer a simplified way to perform high-through-put testing on multiple testing boxes with little need for user analysis. Furthermore, we confirm that continuous (automated) sampling methods are highly correlated and produce almost identical scores to manual sampling methods (**Figure 2.7b/c**).

By allowing researchers to study murine interactions for longer periods of time, the TCOT is uniquely positioned to identify new social behaviors among rodents, including animal propinquity over time. Despite its dominance in the field, the three-chambered test appears to be poorly optimized to show indices of social preference during extended testing sessions (**Figure 2.11A/B**). Additionally, while current sociability tests are helpful in identifying deficits in social novelty behavior, social neuroscience has not yet agreed on a specific set of behaviors that optimally identify deficits in social interaction, allowing for additional assays to identify novel relevant social behaviors (Williams, 2011). Indeed, if the identification of social novelty seeking behaviors is insufficient to adequately describe the complexity and variability of social deficits implicated in autism models then testing with the TCOT may add to the social neuroscientific armamentarium. Furthermore, propinquity behavior in rodents is more consistent with social interaction behaviors in the human literature, which clearly shows that people prefer to interact

socially and maintain closer distances to friends than strangers (Edwards, 1972; Sinha and Mukherjee, 2008). Additionally, children with ASD maintain the same degree of interpersonal distance after socially interacting with an experimenter, in contrast to TD children who significantly reduce their distance to an experimenter after a brief social exchange (Gessaroli et al., 2013).

In addition to adding a new way to screen for autism-like behaviors in mice, our findings also confirm that both outbred mice (**Figure 2.7a)** and rats (**Figure 2.9**) are capable of differentiating between familiar and unfamiliar conspecifics. For the first time, we show that familiar animals prefer to remain in closer proximity to one another than strangers in a stressful environment over a prolonged testing period. While the TCOT does not identify the relevant perceptual and cognitive components necessary for social recognition and motivation among rodents, testing with the two tube variant of the assay suggests that propinquity is a socially mediated act of volition rather than merely a measure of mutual tolerance in an adverse environment (**Figure 2.9**).

Testing reveals that the adverse and novel components of the TCOT testing environment are necessary to show differences between familiar and unfamiliar animals (**Figure 2.8a/e/f**). These observations are consistent with previous social novelty findings. One study, for example, shows that stranger rats demonstrate significantly more social behavior in a 5 min Social Interaction (SI) test if pre-exposed to an open field prior to dyadic testing (Eckman et al., 1969). Conversely, pre-stressing rats for 24 hours prior to the SI, or exposing them to predatory signals during the test leads to a decrease in exhibited social behaviors (File, 1994; Zangrossi Jr and File, 1992). These results help to explain why pre-exposing strangers to our modified open field test leads to an increase in subsequent propinquity behavior during the subsequent run. We propose

that our endeavor to characterize differences in propinquity by social status is more subtle than previous studies that compare social behaviors in the presence of cat urine or highly lit testing conditions (both robust environmental stressors). Nevertheless, the "safe" tube that we include in our test appears to be preferable to the open field because siblings prefer to remain there for the majority of a 3 h test. By reducing the threat of the open field, siblings are less likely to hide, choosing instead to explore the (now familiar) open field. As a result, we observe a convergence between unfamiliar and familiar phenotypes when we remove critical components of the test. It should be noted that previous attempts to study differences in social behaviors by familiarity status have led to conflicting results in the SI test literature (for a review, see (File and Seth, 2003).

One outstanding question regarding propinquity behavior involves the relevant importance of different perceptual modalities to correctly identify social status. As demonstrated in previous social testing, olfactory signatures may play an important role in a rodent's ability to recognize another individual (Sorge et al., 2014; Thor and Holloway, 1982), while visual cues (including facial expressions) (Langford et al., 2010; Langford et al., 2006; Sotocinal et al., 2011) and auditory signals may also contribute to social recognition (Hammerschmidt et al., 2012a; Scattoni et al., 2009). Although we are one of several groups that have demonstrated that social familiarity, or the ability to recognize a familiar conspecific based on prior association (Kareem and Barnard, 1982), is a necessary prerequisite for mice to elicit more complex social behaviors, we do not yet know how mice communicate familiarity status in our test. Our lab's previous efforts suggest that visual communication may be necessary to convey familiarity status. For example, Langford and colleagues were the first to show that mice tested in dyads, and subjected to identical noxious stimuli, displayed increased pain behaviors if the partner

mouse was familiar (Langford et al., 2006). However, this phenomenon was blocked when mice were unable to see one another.

Our results raise another interesting question concerning the general lack of sex differences demonstrated by outbred mice in our test (**Figure 2.6b**), with the exception of the separated sibling group. Our results are not surprising given that inbred mice in the three-chambered test literature do not reliably demonstrate sex differences (An et al., 2011; Sankoorikal et al., 2006). Harder to explain are our observations that males reintroduced after 3-5 weeks of separation act like strangers in the TCOT while similarly-housed females act like siblings. One study measuring rat approach behavior reports that female rats are able to retain social memories for longer intervals than males. Furthermore, castration enhances subsequent social recognition in male animals. However, before applying these findings to our own data, it should be noted that differences in social recognition by sex were measured over 2-3 h, not weeks (Bluthe and Dantzer, 1990). Nevertheless, these data suggest that there are different hormonally-mediated pathways underlying male and female rodent social recognition. The same group has extensively characterized sex differences in neuropeptides associated with social memory formation. For instance, it is known that the social memory of males can be enhanced by vasopressin agonists (Bluthe et al., 1990) and blocked by vasopressin antagonist dPTyr(ME)AVP (Dantzer et al., 1988). The same antagonists have no effect on social recognition in females or castrated males, however, suggesting that male social recognition is dependent on vasopressinergic neurotransmission (Bluthe and Dantzer, 1990).

Researchers have also discovered that oxytocin (OT) is a key component of social recognition in mice. Molecular manipulations using OT null mutant ("knockout") mice reveal similar behavioral patterns. Control conditions reveal that OT knockouts (KO) perform normally

in general learning or memory tasks. Yet these mice do not appear to recognize familiar individuals. Specifically, mice without OT spend significantly more time exploring familiar conspecifics, suggesting that the lack of OT creates a social deficit. Further evidence includes exogenous injections of OT. After receiving exogenous OT in the medial amygdala, for example, OT KO mice show an immediate change in their social responses by recognizing a conspecific and spending significantly less time exploring a familiar conspecific (Ferguson et al., 2000). However, OT appears to be necessary for both male and female social recognition. It is clear that more work is needed in order to understand which neuropeptides, including OT and vasopressin, are responsible for long-term social memory. Studies to date have relied on short-term memory testing to study sex differences in rodents, making subsequent comparisons to our results difficult (Gheusi et al., 1994; Popik and van Ree, 1999).

Based on preliminary results presented in this chapter, one final issue to consider pertains to the marked decrease in propinquity behaviors exhibited by older (12-16 week-old) mice as compared to young adult (6-8 week-old) animals (**Figure 2.8d**). Our findings are particularly surprising given the lack of age-specific differences demonstrated in previous, validated sociability tests that tested 12 week-old inbred mice (Nadler et al., 2004). However, we are aware of at least one study that uses the social interaction test to report a decreasing trend in more complex social behaviors among aging rats as compared to young adults (Spruijt, 1992). Specifically, the author finds that young rats are more influenced by partner behavior; older rats are far less influenced by the behaviors of their social partners. Similar decreases in social interaction behaviors have been reported in older mice (Sankoorikal et al., 2006).

In summary, I propose the TCOT as a novel way to study propinquity, a newly characterized social behavior, in the rodent. The proceeding chapters will describe the characterization of

autism-like models, including both inbred and mutant mice (**Chapter 3**), before describing how stress (**Chapter 4**) and pain (**Chapter 5**) can modulate propinquity behavior.

Chapter 3

Characterizing autism-like mouse models using the TCOT

3.1 Rationale

In **Chapter 2**, we used outbred animals to show that our novel behavioral assay, the (TCOT), was sufficient to measure prolonged voluntary proximity in rodents, a term that we call "propinquity." In order to show that propinquity behavior is not unique to outbred mice, however, a comprehensive characterization of propinquity behavior using inbred mice is needed, similar to previous validation in related sociability tests (Moy et al., 2009; Moy et al., 2008). The discovery of familiarity preference in outbred mice suggests that the TCOT may serve as a new way to measure social behaviors in autism-like models, complementing previously described rodent assays (see **Section 1.3**) To evaluate this hypothesis, we selected several genetic mutant models that have previously demonstrated deficits in social behavior in order to investigate whether these mice also show deficits in propinquity behavior (Silverman et al., 2010b). Finally, we were interested in using the TCOT to determine whether inbred mice are in fact adequate proxies for "normal" murine social behavior by comparing TCOT results among inbred, outbred, and wild mice.

3.2 Introduction: An overview of mouse autism-like models

In the pursuit of identifying specific genetic causes of autism, social neuroscientists use various mouse mutants as a way to both model specific autism-like behaviors as well as elucidate specific genetic pathways implicated in observed social deficits. To date, researchers have developed 676 autism-like models and characterized their behavior using at least one behavioral assay previously discussed in **Section 1.3.1**. These efforts are catalogued in the SFARI database (SFARI, 2016). The reason for the high number of autism-like mouse models stems from the fact

that, until recently, the mouse was the only research organism amenable to targeted genetic manipulation.

3.2.1 Inbred mice as autism-like models

In order to create a genetic knockout (KO) mutant or a genetically hybrid "congenic" animal, researchers relied on inbred mouse strains, or animals that have been bred for at least 20 generations until they are essential clones of one another (Beck et al., 2000). Today, there are 450 inbred mouse lines (Beck et al., 2000; Festing, 1996), with a multitude of inbred mouse strains available for purchase from major suppliers.

Even without genetic modification, researchers can use a combination of social testing and genetic screening techniques to identify differences between behavioral phenotypes and genetic profiles among selected inbred strains. For example, researchers using the three-chambered test reported variation in both sociability and social novelty behaviors among several common inbred strains. In the first of several strain comparison studies, Moy and colleagues found that DBA/2J and C57BL/6J mice demonstrated significantly higher levels of sociability and stranger preference towards a stranger in the three-chambered test than other strains (Moy et al., 2004). In several follow-up papers, the authors observed similar trends for AKR/J, FVB/NJ, and PL/J mice (Moy et al., 2004; Moy et al., 2009). Additional three-chambered testing by the same group and others showed that A/J, BALB/c, BALB/cByJ, BTBR T + tf/J, and 129S1/SvImJ strains demonstrated decreased levels of sociability and stranger novelty (An et al., 2011; Bolivar et al., 2007; Brodkin, 2007; Moy et al., 2007; Nadler et al., 2004; Nadler et al., 2006). Notably, the reliability of the three-chambered test remains a concern; Moy et al., for example, were unable to replicate similar levels of sociability and replicability in several strains across studies, including

DBA/2J. Furthermore, in the most recent strain comparison study, FVB/NJ and SWR/J mice showed signs of aggression while approaching stimulus animals, suggesting that social approach may in some cases be due to territorial or aggressive posturing (Moy et al., 2008).

3.2.2 Mutant autism-like models

While inbred strain surveys have identified potential genetic risk factors for ASD via haplotype mapping, the use of gene editing tools, including older transgenic knockout technologies and newer rapid *de novo* mutagenic techniques now allow researchers to create mouse models of specific genetic mutations found in clinical ASD populations. Although mice are unable to fully emulate the diverse sets of behavioral deficits seen in clinical cases, engineering specific mutations in mouse lines allows researchers to understand how single gene mutations can affect social functioning in a highly-controlled way (Moy et al., 2009).

One of the first Mendelian models used to study autism-like behaviors is the *Fmr1*$^{-/y}$ (fragile X syndrome) mouse. Fragile X syndrome is the most common inherited form of mental impairment and autism, often resulting from an expanded CGG trinucleotide repeat that leads to hypermethylation and subsequent transcriptional silencing of the (human) *FMR1* gene located on the X chromosome. The silencing of (mouse) *Fmr1* prevents the normal production of fragile X mental retardation protein (FMRP), a protein that is normally expressed in the brain and serves as a translational "brake" on pre- and post-synaptic protein synthesis (Gonçalves and Portera-Cailliau, 2013). Mice with the homologous *Fmr1* deletion presented with aberrations in dendritic spine formation (Comery et al., 1997; Irwin et al., 2000) and cognitive impairments similar to those found in clinical populations (D'Hooge et al., 1997; Dobkin et al., 2000; Fisch et al., 1999).

On a synaptic level, *Fmr1* KO animals showed metabotropic glutamate receptor (mGluR5) hyperactivity, resulting in abnormal long-term potentiation (Nosyreva and Huber, 2006) and long-term depression (Huber et al., 2002) in the hippocampus and other brain areas. Pharmacological intervention with 2-methyl-6-phenylethynyl-pyridine (MPEP), a noncompetitive mGluR5 antagonist, normalized phenotypic differences observed in *Fmr1* mutant animals, including aberrant LTP and superfluous dendritic spine formation (Aschrafi et al., 2005; de Vrij et al., 2008; Dölen et al., 2007) Based on research implicating mGluR5 in Fragile X syndrome, FMRP appears to be necessary for maintaining proper excitatory/inhibitory synaptic balance in the brain (Silverman and Crawley, 2014).

Additional alterations of the mGluR5 channel lead to similar disorganization at the synaptic level, producing similar autism phenotypes. One specific set of post-synaptic scaffolding proteins, SHANK family proteins, organize a large protein complex at the postsynaptic density of excitatory glutamatergic synapses. Studies showed that spontaneous deletion of relevant *SHANK* genes (*SHANK1, SHANK2,* and *SHANK3*) are implicated in clinical cases of idiopathic ASD (Berkel et al., 2012; Durand et al., 2007; Sato et al., 2012b), with *SHANK3* mutations being the best characterized mutations in human ASD (Jiang and Ehlers, 2013). To date, five lines of *Shank3* mutant mice have been reported, varying by the number of exons deleted within the *Shank3* gene (Bozdagi et al., 2010; Peça et al., 2011; Schmeisser et al., 2012; Wang et al., 2011; Yang et al., 2012). While a direct comparison of the morphology among these models is difficult due to different experimental protocols among studies, most research shows a general degree of synaptic morphological dysregulation in mouse mutants. Specifically, *Shank3* deletion in mice correlated with a loss of dendritic spines, a reduction in dendritic spine volume, and decreased post-synaptic density thickness. Although a majority of studies described morphological

consistencies, a similar consensus concerning synaptic function does not appear to exist among *Shank3* models (Jiang and Ehlers, 2013).

Closely related to *Fmr1* mutant synaptic dysregulation, an underlying subset of ASD arises from mutations in the mTOR signaling pathway. Normally, the mTOR pathway serves to control cellular growth, with the tuberous sclerosis complexes (TSC1 and TSC2) serving as negative regulators for cell growth. Loss of either (TSC1/2) protein complex leads to tumor formation. Less well-characterized are the mechanisms that underlie the array of neurological phenotypes, including epilepsy, autism, and intellectual impairments that result from TSC-mTOR pathway dysregulation (Bateup et al., 2013). On a synaptic level, dysregulation of the TSC-mTOR (through the mutation of either *Tsc1* or *Tsc2*) changes excitatory synapse structure, function, and plasticity (Auerbach et al., 2011; Bateup et al., 2011; Chévere-Torres et al., 2012).

In an effort to better understand how mTOR pathway dysregulation can lead to neurological phenotypes, researchers began to target specific regulatory components of the mTOR pathway to see how they influence social behavior in animal models. Using a genetic knockin model of one such factor, an mRNA 5' cap binding protein called eukaryotic initiation factor 4E (eiF4E), recent studies reported that knockin mice demonstrate synaptic plasticity deficits (Santini et al., 2013) when compared to wildtype (WT) controls. A collaborator of ours was among the first to identify deficits in social behavior using this model (Gkogkas et al., 2014).

3.2.3 Evaluating social behaviors in mutant autism-like models

A subset of studies evaluated potential deficits in social behaviors using autism-like mouse models. A few studies looking at sociability behavior using *Fmr1* mutant models found reduced social interactions in an open field test (Mineur et al., 2006). Others, however, reported increased

stranger approach behavior in both open field and partition tests (Spencer et al., 2005; Spencer et al., 2008). Using the three-chambered test, McNaughton and colleagues failed to find differences between *Fmr1* knockout (KO) and wildtype (WT) control animals (McNaughton et al., 2008). Background strain, however, may play a role in detecting social behavioral deficits. A follow-up study, for example, reported that *Fmr1* KO mice on an FVB/129 background showed deficits in social preference while *Fmr1* mutants bred on a hybrid background showed comparable levels of social preference (compared to WT controls) in a three-chambered social choice assay (Moy et al., 2009).

A review of the literature reveals a similar lack of agreement concerning *Shank3* knockout animals. Whereas some *Shank3* mutant models show deficits in sociability and stranger novelty scores in the three-chambered test (Peça et al., 2011; Wang et al., 2011), other studies looking at identical or similar isoforms failed to describe sociability deficits (Bozdagi et al., 2010; Yang et al., 2012) (for review, see (Jiang and Ehlers, 2013).

Finally, while some studies showed that *Tsc2* KO animals failed to display social preference to strangers in the three-chambered test (Goorden et al., 2007; Sato et al., 2012a; Tsai et al., 2012), at least one published study found similar levels of reciprocal social interaction between KO and WT animals (Ehninger et al., 2008). Furthermore, an additional study looking at communication differences in *Tsc2* mutant pups reveals that while pup signaling is aberrant, mutant dams show improved maternal behavior and response to pup calls, suggesting that at least some complex social behaviors are improved over WT controls in this mutant model (Young et al., 2010). Taken together, data garnered from mutant models reveal a lack of consensus, creating an opportunity for new standardized social assays to further characterize relevant autism-like behaviors.

Although a complete characterization of mutant autism-like strains is a worthy goal for autism research, a relevant but thus far unanswered question pertains to the generalizability of inbred animals (used to model complex behaviors in virtually all modern behavioral studies). Although no studies have used sociability tests to compare differences in social behavior among inbred mice and outbred controls, a recent high-profile study found that backcrossing a mutant model with wild re-derived controls significantly increased aggressive and active social exploratory behaviors, particularly in female mice. (Chalfin et al., 2014). These results suggest that social behaviors exhibited by inbred animals may not be "normal"; that is, more representative of the species in general. Furthermore, we posit that inbred mice may fail to demonstrate other social behaviors that we find in outbred animals, including a robust pattern of familiarity preference (sibling>stranger). Having completed the initial characterization of propinquity behavior in outbred mice, we were interested in determining whether inbred animals demonstrate the same "familiar versus stranger" preference (**Figure 2.7**). Evaluating the same inbred models that were used in other sociability tests (Moy et al., 2009) allowed us to evaluate whether previous inbred autism models (BTBR, Balb/C) also showed deficits in tube co-occupancy behavior in the TCOT (Yang et al., 2007a; Yang et al., 2007b). In order to determine whether propinquity levels in inbred mice are similar to outbred animals, we compared tube co-occupation behaviors exhibited by inbred mice to both outbred lines as well as cross-fostered adult offspring of wild mice (*M. musculus domesticus*) that were trapped in a semi-rural area of Montreal.

Additionally, we wanted to test whether previously reported autism-like animal models show deficient levels of tube co-occupation behavior in the TCOT. Towards this end, we examined three available genotypes featuring null mutations of autism-relevant genes: *Fmr1*$^{-/y}$ mutant mice

lacking expression of fragile X mental retardation protein, mutant mice with haploinsufficiency of SH3 and multiple ankyrin repeat domains 3 (*Shank3*$^{+/-}$), and *Eif4e*^{Ser209Ala} knockin mice expressing a nonphosphorylatable form of the eukaryotic translation initiation factor 4E (eIF4E).

3.3 Methods

3.3.1 Animals

Male and female inbred mice (129S1/Sv1mJ, AKR/J, BALB/cByJ, BTBR *T*$^{+-}$ *tf*/J, C3H/HeJ, and C57BL/6J) were purchased in equal numbers from The Jackson Laboratory (Bar Harbor, ME). Wild mice were collected using live traps at two different agricultural facilities on McGill's MacDonald campus (45°24'N 73°56'W). Trapped wild mice were bred in quarantine and their offspring were cross-fostered on P2–P3 to a CD-1® dam to minimize exposure to zoonotic agents. Offspring were screened for pathogens at P21 and brought to the laboratory after testing negative for pathogens, remaining in our vivarium until testing between P42-P56. Equal numbers of male and female mutant strains and appropriate control strains were either purchased from The Jackson Laboratory (B6.129-*Shank3*$^{tm2Gfng-/+}$/J, B6.129-*Shank3*$^{tm2Gfng+/+}$/J, C57BL/6J-*Fmr* $^{-/y}$, C57BL/6J-*Fmr1*$^{+/+}$) or supplied by the Sonenberg laboratory (C57BL/6J-*Eif4e*$^{Ser209\rightarrow Ala}$ knockin, *Eif4e*$^{+/+}$) (Furic et al., 2010). Mice procured from other facilities were P21-P28 when they were transferred to the in-house vivarium and tested at P45-P56. Animal care and housing conditions were the same as reported in **Section 2.3.1.**

3.3.2 The TCOT

Testing parameters for all experiments were identical as those described for automated testing in **Section 2.3.2**, with one notable exception during the handling process. Given the

highly active nature of the re-derived wild mice, we found it necessary to dose animals with inhalant isofluorane prior to testing (in order to place them into the testing apparatus). Animals generally recovered within 3-5 minutes after being placed in the TCOT, and were allowed to fully recover (15 minutes) prior to the beginning of testing.

3.3.3 TCOT scoring

Automated scoring methods were identical to those described in **Section 2.3.3.**

3.3.4 The three-chambered test

A subset of animals (AKR/J, C3H/He, CD-1® and BTBR *T*$^{+}$ *tf*/J) were tested in the three-chambered assay in order to determine correlation between sociability and propinquity behaviors. Testing conditions were identical to those described in **Section 2.3.4.**

3.3.5 Three-chambered test scoring

Scoring protocols were identical to those described in **Section 2.3.5.**

3.3.6 Statistical analyses

Since the same tests used to establish outbred propinquity and sociability behaviors were also used to evaluate inbred and mutant models, statistical analyses are identical to those described in **Section 2.3.6.**

3.4 Results

3.4.1. Inbred mice fail to demonstrate familiar preference in the TCOT

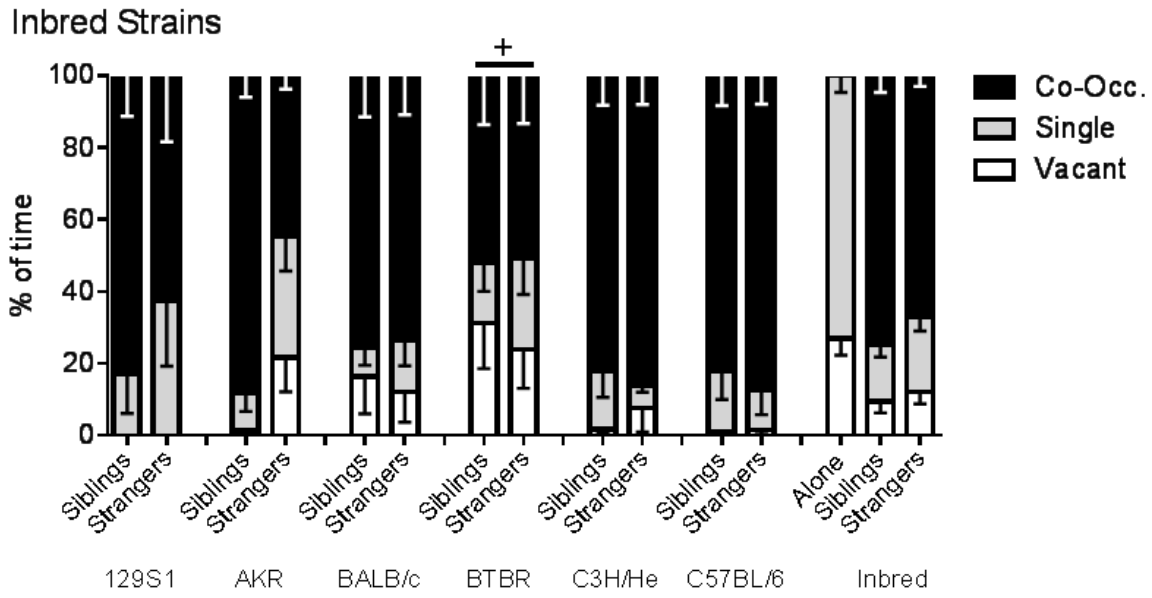

Figure 3.1: Genotype-dependence of tube co-occupancy in sibling versus stranger dyads, and their average (Inbred). Bars represent mean ± SEM percentage of time (60–120 min; using automated measurement described in **Section 2.3.2**) with tube co-occupancy (Co-occ.), single occupancy (Single) and no occupancy (Vacant); n=12 dyads/social condition/genotype except for 129S1 strangers (n=8). + indicates significantly lower co-occupancy rates observed in BTBR mice compared to other strains tested ($F_{5,109}$=2.9, p=0.02).

We designed the first set of experiments to measure propinquity behaviors among a variety of inbred mouse strains (**Figure 3.1**), including three strains that previously demonstrated high levels of sociability on the three-chamber test (AKR/J, C3H/HeJ, and C57BL/6J) and three strains that showed low sociability (129S1/SvImJ, BALB/cJ, and BTBR T^{+} *tf*/J) (McFarlane et al., 2008; Moy et al., 2007; Yang et al., 2007a). Importantly, differences in propinquity behavior did not appear to be due to tube preference: overall inbred, outbred and wild mice displayed equivalent preference for tube occupancy ($F_{2,106}$ = 0.4, p=0.64) when tested alone (data not shown). ANOVA analysis revealed a significant main effect of genotype ($F_{5,109}$=2.9, p=0.02) but

not social condition (sibling versus stranger; $F_{1,109}$=0.5, p=0.47) or their interaction ($F_{5,109}$=0.7, p=0.60). No strain displayed significantly higher co-occupancy levels in sibling versus stranger dyads (0.08<p's<0.94). We found no evidence of sex differences among tested inbred strains in our test.

Notably, BTBR T^{+} *tf*/J siblings displayed the lowest levels of co-occupancy. Although restricted numbers of strains prevented us from demonstrating statistical significance, we found a reasonable correlation between stranger dyad performance during the second hour of the TCOT and previously published sociability scores (time spent with stranger – time spent with empty cage; r=0.61, p=.202) but not social novelty preference (time spent with "new" stranger – time spent with "old" stranger; r=0.05, p=.932) on the three-chamber test (Moy et al., 2007): (**Figure 3.2a-b**). We also observed this pattern after we tested four strains (AKR/J, C3H/He, CD-1® and BTBR T^{+} *tf*/J) with the three-chambered test in our laboratory (TCOT versus sociability: r=0.68, p= .317 ; TCOT versus social novelty: r= -0.82, p=.182) (**Figure 3.2c-d**).

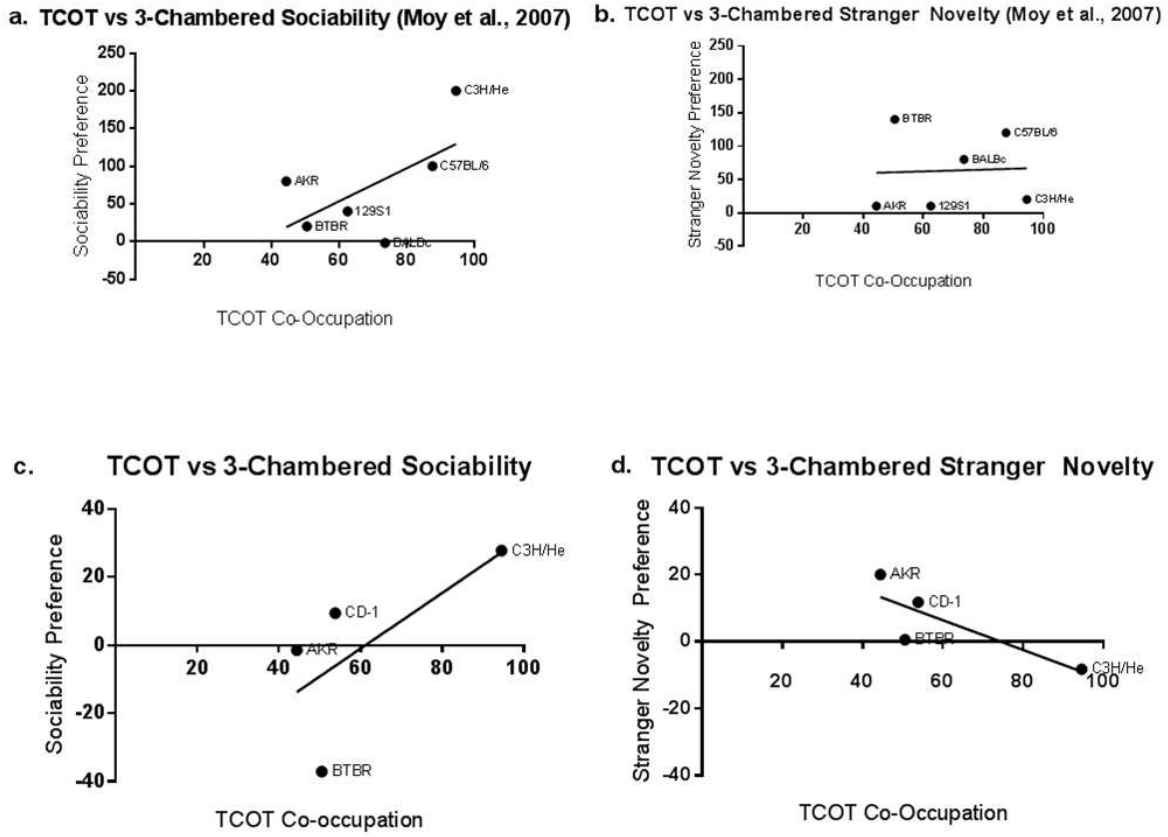

Figure 3.2: Correlation between TCOT co-occupation scores and previously published results (**a-b**) or three-chambered data from our own lab's experiments (**c-d**). Sociability preference = (time spent with stranger – time spent with empty cage) (**a,c**). Stranger novelty preference = (time spent with novel stranger – time spent with "familiarized" stranger), (**b,d**). Note low strain *n* precluded significant statistical values.

3.4.2 Wild mice show familiar animal preference in the TCOT

To evaluate whether inbred or outbred social behavior in the TCOT was more indicative of mice as a species, we also tested cross-fostered adult offspring of wild mice (*M. musculus domesticus*) that were trapped in a semi-rural area of Montreal. Similar to outbred animals, re-derived wild mice also displayed greater co-occupancy in sibling compared to stranger dyads (t_{23}=3.7, p=0.001)

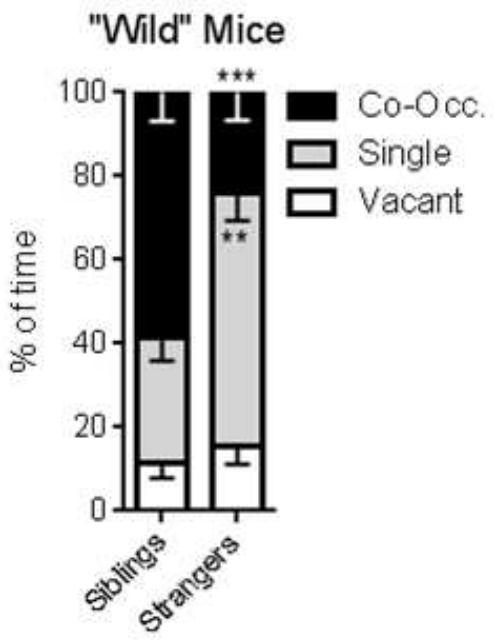

Figure 3.3: Tube occupancy behavior in re-derived wild mice. Bars as in **Figure 3.1**; n=12–13 dyads/social condition. **p<0.01, ***p<0.001 compared to sibling group.

3.4.3. "Autistic" mice show deficits in propinquity and familiar animal preference in the TCOT

In order to evaluate the TCOT as a suitable test for detecting deficits in autism-relevant models, we measured tube co-occupancy behavior in three previously reported autism-like models. *Fmr1*$^{-/y}$ mutant mice and *Eif4e*^{Ser209Ala} knockin mutant mice both demonstrated dramatically reduced tube co-occupancy compared to wildtype controls, regardless of social condition (main effects of genotype: $F_{1,35}$ = 27.5, p<0.001, $F_{1,27}$ = 6.9, p=0.01, respectively, **Figure 3.6a-b**). Testing using mutant mice with a haploinsufficiency of SH3 and multiple ankyrin repeat domains 3 (*Shank3*$^{+/-}$) showed that mutant animals were indistinguishable from WT controls in the TCOT (main effect of genotype: $F_{1,19}$ = 0.2, p=0.68; main effect of social condition: $F_{1,19}$ = 0.1, p=0.76; genotype x social condition interaction: $F_{1,19}$ = 0.2, p=0.66).

(**Figure 3.4 c**). Not surprisingly, the mutant models (all bred onto a C57BL/6 background) failed to demonstrate a familiar versus stranger preference for tube co-occupation in the TCOT, suggesting that both mutant and wildtype controls may not demonstrate robust discrimination between familiar and stranger conspecifics. No evidence of sex differences were apparent in *Fmr1*$^{-/y}$ or *Eif4e*KI mice (data not shown).

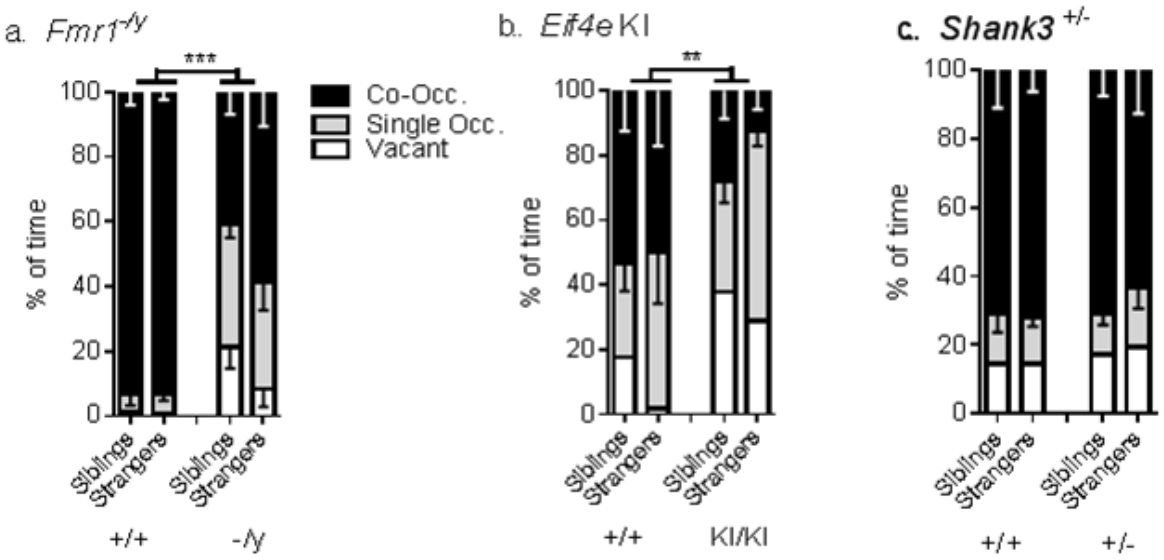

Figure 3.4: Tube occupancy behavior in *Fmr1*$^{-/y}$ mice (-/y) (**a**), *Eif4e* knockin (KI) mice (**b**), and *Shank3*$^{+/-}$ (**c**) compared to controls (+/+). We did not see altered tube occupancy behavior in *Shank3*$^{+/-}$. n=7–12 dyads/social condition/genotype for **a-b**, n=4-7 dyads/social condition/genotype for **c**. *p<0.05, **p<0.01, ***p<0.001 compared to sibling group, or indicated comparison.

3.5 Discussion

Based on our reported findings, the TCOT is a viable complementary tool to current sociability assays; our test is able to identify mouse strains with impaired sociability, including BTBR *T*$^{+}$ *tf*/J inbred mice (**Figure 3.1**), as well as *Fmr1*$^{-/y}$ and *Eif4e* knockin mutants (**Figure 3.4a-b**). We also tested mutant mice with haploinsufficiency of SH3 and multiple ankyrin repeat

domains 3 (*Shank3*$^{+/-}$) and found that these mice are indistinguishable from wildtypes on the TCOT (**Figure 3.4c)**. The lack of propinquity behavior that we observed in the model conforms to several previous findings that showed *Shank3*$^{+/-}$ mutants do not demonstrate deficits in sociability testing (Bozdagi et al., 2010; Yang et al., 2012). Furthermore, a preliminary correlational analysis (**Figure 3.3**) between our own findings using the TCOT and previously-published sociability (but not stranger novelty) preferences suggests that acute social approach and voluntary social proximity behaviors may correlate with one another. However, additional strains are needed in order to verify this claim.

Although our findings suggest that the TCOT may be a useful addition to current rodent sociability screening techniques, mutant model data we present in this chapter may be confounded by the abnormal social behavior of inbred mice. Specifically, inbred mouse strains, especially C57BL/6 mice that serve as the genetic background of most transgenic strains (including all of the transgenic mice included in this analysis), appear to demonstrate unusually high levels of social gregariousness (perhaps as part of their laboratory domestication) such that the familiar versus stranger preference—typical of outbred (**Figure 2.6**) and "wild" mice (**Figure 3.3**)—is absent in them. It is well known that behavioral phenotypes of mutant mice are influenced by the model's background strain. For example, the majority of mutant animals are bred on a 129/sv background and backcrossed to C57BL/6 mice (Skarnes et al., 2011). The lack of social discrimination among inbred lines may partly explain replicability issues using inbred lines and mutant models in other sociability testing (Kas et al., 2014).

Classic laboratory mouse strains, including the background strains used to make mutant models, are the result of controlled breeding over many generations from small founder populations. (Guénet and Bonhomme, 2003). Researchers cite the subsequent lack of genetic

diversity (and the resultant decreased phenotypic variability) a strength in biomedical research, because fewer animals are needed to find statistically significant results (Chia et al., 2005). However, by artificially selecting and genetically fixing inbred strains, scientists reduce the generalizability of mouse models to favor a less robust and more uniform set of behavioral phenotypes, including an attenuation of locomotion, social and aggressive tendencies, and predatory freezing (Blanchard et al., 1998; Guénet and Bonhomme, 2003; Harper, 2008; Price, 1984). In addition to showing a decreased range of behavioral phenotypes, inbred mice also demonstrate genetic defects that are characteristic of their strain. C3H/heJ and FVB/NJ mice, for example, show high rates of blindness (Crawley et al., 1997). Likewise, studies show that BALB/c and C57BL/6 suffer from progressive hearing loss (Erway et al., 1993; Johnson et al., 2000). Inbred mice have shorter lifespans than outbred or wild mice in captivity (Bowman and Falconer, 1960; Phelan, 1992) and display lower fecundity than outbred mice (a phenomenon called "inbreeding depression") (Amos et al., 2001; Bowman and Falconer, 1960; Keller and Waller, 2002). Artificial selection has not only produced dramatic phenotypic differences between wild and inbred lines, but also differences in gene expression when inbred lines are compared with wild specimens (Chalfin et al., 2014).

Due to the fact that inbred animals are essentially clones of one another, an inbred animal's ability to socially differentiate familiar from strangers in the TCOT may be reduced due to less olfactory differentiability among animals (Arakawa et al., 2008). Support for this hypothesis includes a recent study by Pearson and colleagues, who show that C57BL/6J mice fail to demonstrate stranger novelty preference if the stimulus animals are reversed prior to the second phase of the three-chambered trial (Pearson et al., 2010). Furthermore, when the experimenters use CD-1® animals as stimuli (instead of C57BL/6), inbred animals prefer to remain with the

"familiar" CD-1® animals encountered during the first ("sociability") phase of the trial, after these animals are similarly reversed prior to the second trial. These results suggest that inbred animals are unable to reliably differentiate between inbred stimuli mice in the three-chambered test without the aid of additional environmental clues and may prefer to remain with familiar animals when there are sufficient differences between conspecifics.

One additional explanation for the lack of familiarity discrimination among inbred animals pertains to environmental differences between inbred and outbred mice. BTBR pups raised with other inbred peers in an enriched environment do not show subsequent sociability deficits (Yang et al., 2011), indicating that social environment plays a key role in the development of "normal" social behaviors. However, there is still debate on the relative importance of environmental factors in subsequent stranger novelty behavior. A cross-fostering study that placed BTBR pups with C57BL/6J dams fails to show an increase in subsequent sociability behaviors in the three-chambered test when they are tested as adults (Yang et al., 2007b).

Our results suggest that inbred mice are poorly adapted to differentiate between social stimuli during a prolonged social test. A lack of familiarity preference may explain why pharmacological agents that were found to be efficacious in modulating rodent social behavior (e.g., MPEP, fenobam, and STX209, or "arbaclofen") failed to modulate social behaviors in human clinical trials (Berry-Kravis et al., 2009; Berry-Kravis et al., 2012; Henderson et al., 2012). It is possible these drugs are not modulating social behavior, but are instead affecting environmental novelty or stress-related behavior. Alternatively, the failure to translate mGluR antagonist efficacy may be due to current autism-like models, and their reliance on inbred background strains. Fortunately, the emergence of new genetic tools (e.g., CRISPR) will allow scientists to evaluate the relative contribution of genetic background in future sociability

experiments. As neuroscientists begin to transcriptionally silence or overexpress genes of interest in outbred or re-derived animal lines, future experiments will allow us to directly test the contribution of specific genes to autism-like behaviors in more generalizable preclinical models.

It is important to remember that the accurate characterization of an autism model is based on a battery of different behavioral tests that address different aspects of social behavior, repetitive behavior, and murine communication. The TCOT is designed neither as a way to circumvent current experimental methods, nor as a replacement for previously validated tests. Instead, the TCOT should be a complimentary tool used to further understand potential deficits in murine social behavior over an extended testing period. However, we believe that the phenotypic differences displayed by inbred versus outbred/wild strains in the TCOT cannot be ignored. These results demonstrate a set of previously unknown limitations in the use of inbred models in preclinical autism research; by choosing to use outbred mice, future sociability studies may find more consistent and generalizable results.

Chapter 4

Evaluating the role of acute stress in tube co-occupation behavior

4.1 Rationale

It is known that elevated stress levels correlate with lower displays of social behavior in rats (Eckman et al., 1969; File and Hyde, 1978; Haller and Bakos, 2002). Our own lab recently showed that the degree of acute stress is also a critical component of prosocial behavior in both mice and humans (Langford et al., 2011; Martin et al., 2015). Based on these findings, we hypothesize that reduced social interactions between strangers in the TCOT depend on higher overall stress activation, a process that is reduced during interactions between familiar mice. We show here that in addition to decreased rates of tube co-occupancy behavior, outbred strangers also demonstrate increased stress activation when tested in the TCOT. We also show that by modulating acute stress, we can reverse tube co-occupancy behavior in a controlled and predictable fashion. Based on these findings, we believe that the concept of animal "familiarity," measured indirectly by quantifying familiarity-directed behaviors in dyadic interactions, is inversely correlated with activation of the acute stress system. By the same token, aberrant stress signaling may partly explain deficits in social behavior. Indeed, dysfunctional regulation of stress by the hypothalamic-pituitary-adrenal (HPA) axis has been associated with cases of autism in children (Corbett et al., 2006; Jansen et al., 2000; Nir et al., 1995; Richdale and Prior, 1992).

4.2 Introduction

All animals experience stress, a series of reactions to a change in homeostasis (McEwen and Wingfield, 2010). Stressors, or stimuli that activate an organism's biological stress pathways, elicit a number of physiological and behavioral changes. This introduction will first explain how biological systems regulate homeostasis, before discussing how stress can change

an organism's behavioral response. Finally, a concluding discussion will evaluate how social tests currently measure stress in animal models.

4.2.1 An overview of biological stress pathways

The reaction to stressors is called a stress response. The neuroendocrine system evolved to homeostatically regulate an organism's response to stressors and consists of hormone-producing endocrine tissues regulated by the nervous system. A subcomponent of the neuroendocrine system, the hypothalamic-pituitary-adrenal-axis (HPA axis) is composed of a tightly-regulated biological pathway that regulates the release of corticosterone in rodents, or cortisol in humans. (Chrousos, 1998; Kudielka et al., 2007; Tsigos and Chrousos, 2002).

The HPA axis pathway activates when parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) receive input from other areas of the brain. The paraventricular nucleus of the hypothalamus (PVN) releases two core neuropeptides for HPA axis function: corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) (Buwalda et al., 1997). The PVN secretes neuropeptides into the nearby hypophysial portal blood from axon terminals that project from parvocellular cells of the PVN to external zone of the median eminence (Ma et al., 1997)**.** CRH and AVP bind to receptors in the anterior pituitary gland, activating adrenocorticotropic cells that secrete adrenocorticotrophic hormone (ACTH). ACTH diffuses into the circulatory system, where it travels to the adrenal glands. It then binds to melanocortin receptors, stimulating the production and secretion of glucocorticoids (Herman and Cullinan, 1997).

Following activation of the HPA axis, circulating glucocorticoids downregulate the release of additional neuropeptides (Tsigos & Chrousos, 2002). They do so by binding to either

type I mineralocorticoid receptors (MR), or type II glucocorticoid receptors (Nr3c1) in the hippocampus, prefrontal, pituitary, and PVN (Dallman et al., 1994; De Kloet et al., 1998; Kolber et al., 2009; Meaney et al., 1996). MR have a strong affinity for glucocorticoids, while Nr3c1 have a lower affinity. Thus, MR tend to become occupied at lower stress levels, with Nr3c1 remaining unbound until a larger stress response triggers a large release of glucocorticoids. It was shown that MR activation contributes to the initial phase of a stress reaction, while Nr3c1 activation terminates a stress response (De Kloet et al., 1998; Joëls et al., 2008). Nr3c1 inhibits HPA axis activation after binding with glucocorticoids or synthetic antagonists (Bradbury et al., 1991; Lee et al., 2010). Glucocorticoids also mediate glucocorticoid receptor expression levels; increasing levels of glucocorticoids downregulate subsequent GR expression (Hager et al., 2009) In chronic stress-related disorders, GR expression is significantly downregulated in both humans and animals (Palma-Gudiel et al., 2015; Weaver et al., 2004).

Whereas multiple brain areas are responsible for maintaining stress homeostasis, the paraventricular nucleus of the hypothalamus (PVN) functions as the central coordinator in mounting an HPA axis stress response. Glucocorticoid injections into the PVN sufficiently downregulated subsequent CRH mRNA production, as well as decreased ACTH secretions (Whitnall, 1993). Lesions of the PVN markedly reduced secretion levels of stress-induced ACTH and corticosterone (Herman and Cullinan, 1997).

Additional lesion studies revealed that there are other areas that help to propagate an upregulation in the HPA axis. Lesioning the lateral bed nucleus of the stria terminalis (BST), for example, increased expression of CRH mRNA production in the PVN (Herman et al., 1994), whereas stimulating this area increased corticosterone secretion (Dunn, 1987; Kolber et al., 2008). In addition to regulating the HPA axis, the BST provides a link from the central nucleus

of the amygdala (CA) to the hypothalamus (Dong et al., 2001; Van Pett et al., 2000). Together, the BST and CA are referred to as the extended amygdala because they are cytoarchitectonically, neurochemically, and embryologically related (Beckerman et al., 2013; Sun et al., 1991).

Whereas the PVN, BST, and CA are relevant structures in upregulating the HPA axis, it is important to note that they are not the only structures involved in HPA axis regulation. Other brain structures play an inhibitory role in HPA activation, including the hippocampus, lateral septum, and prefrontal cortex (Diorio et al., 1993; Dobrakovová et al., 1982; Herman, 1993). As previously mentioned, GR and MR receptors found within the BST, preoptic area, and hypothalamus also inhibit HPA axis activation when they are bound. Thus, there are multiple, redundant mechanisms in place to ensure that stress levels are homeostatically regulated and that balance is maintained. Modulation of the stress system is adapted to respond to acute stressors; chronic activation of the system is associated with a variety of pathological conditions (Kudielka et al., 2007; Tsigos and Chrousos, 2002), as well as multiple psychiatric disorders (De Kloet et al., 1998; Pariante and Lightman, 2008).

4.2.2 Measuring acute stress in rodent models

The level of circulating corticosterone in an organism is highly variable, influenced by both diurnal factors as well as discrete stressors. Basal HPA axis activity is controlled in part by the suprachiasmatic nucleus (SCN) of the anterior hypothalamus that imposes a circadian rhythmicity (Krieger, 1975). Analyses of circadian cycles revealed that corticosterone concentrations (as well as CRH and ACTH) are at their highest level shortly after an animal wakes, and then the levels decrease during the day until they reach their lowest concentration, or

"circadian trough," during the animal's sleep period (Clow, Thorn, Evans, & Hucklebridge, 2004).

A diversity of neurosensory signals from the limbic, higher cortical, and perceptual systems can also activate the stress system, leading to a multitude of physical and behavioral changes. Researchers believe that these changes are evolutionary adaptive, allowing an individual to quickly meet changing environmental conditions (Cannon, 1939; Chrousos, 1998). To characterize degree of HPA axis activation, scientists directly compared the concentration of relevant hormones (glucocorticoids) and/or neuropeptides (e.g., ACTH, CRH) between animals that were in stressful and nonstressful environments (Benaroya-Milshtein et al., 2004; Roy et al., 2001).

Studies can also measure stress activation by comparing physiological or behavioral differences between animals. The stress literature has identified numerous, test-specific behavioral differences while controlling for stress activation. For example, one of the most common behavioral proxies for stress activation is exploratory activity in a novel, open environment. Rats and mice both naturally avoid open spaces; this avoidance, in turn, correlates with HPA axis activation. Two tests for measuring anxiety, the elevated plus-maze and the open-field test, both measure anxiety levels in rodents by evaluating the amount of time that they spend in a relatively aversive area (Asano, 1986; Hegmann and DeFries, 2014; Lister, 1987; Lister, 1990). In the open field test, the amount of time that a rodent spends in the central area of the testing apparatus is anxiogenic. In the elevated plus maze, animals have the choice between staying in two "closed" (sheltered) arms or exploring two "open" (exposed) arms of an elevated maze. Exploration and time in the open arm are considered to be anxiogenic (Lister, 1987; Pellow et al., 1985). Both tests have excellent sensitivity in detecting changes in an animal's

stress levels, as numerous pharmacological and behavioral studies have shown (Cao and Rodgers, 1997; Crawley, 1985; Dalvi and Rodgers, 2001; Fisher and Hughes, 1996; Homanics et al., 1999). After receiving anxiolytic drugs (benzodiazepines), for example, mice demonstrated increased open field exploration and open arm exploration time in the open field and elevated plus maze, respectively (Cole and Rodgers, 1995; Griebel et al., 2000).

Scientists also utilize relative expression of social behaviors as dependent measures of anxiety. Today, many researchers use the Social Interaction (SI) test as a way to evaluate social deficits in autism-like animal models; however, it was originally designed to study how high-stress open field environments impact rat social approach, sniffing, chasing, and fighting behaviors (File and Pope, 1974). Using the SI, researchers found that rats socially interact more after being pre-exposed to a testing environment. Plasma corticosterone levels were also higher in unfamiliar environmental testing conditions (File, 1980). Follow-up studies using various social and environmental stressors (e.g., brightly lit testing environments) reported that both prolonged administration of anxiolytic drugs (as opposed to acute administration, which yielded drug sedation effects) and acute administration of alcohol resulted in increased levels of social interaction between rat conspecifics (File and Hyde, 1978, 1979; File et al., 1976). Additional findings showed that the mere presence of a familiar rat was anxiolytic in the open field test (Leshem and Sherman, 2006a). Furthermore, repeat exposure to stranger rats yielded increased social proximity behaviors over time and decreased measures of anxiety and fear (Latané, 1969). However, it should be noted that species differences have been reported in the SI test: studies showed that pharmacological compounds proven to be effective anxiolytics in rats do not change mouse behavior in the test (Lister and Hilakivi, 1988; Rodgers et al., 1997).

In spite of the reported species differences, there is sufficient evidence in the literature to suggest that social interactions in rodents are modulated in part by activation of the stress system. Our lab recently showed that the degree of an acute stress response is also a critical component of prosocial behavior in both mice and humans (Langford et al., 2011; Martin et al., 2015). Employing several behavioral assays, we evaluated mice pretreated with metyrapone (MET), a glucocorticoid synthesis inhibitor, and found it elicited empathy-like responses in male strangers. Although we previously showed that male mice do not display increased pain behaviors in the presence of a male stranger (Langford et al., 2006), blocking the acute stress response resulted in increased pain behavior in male strangers (Martin et al., 2015). Furthermore, by simultaneously blocking the glucocorticoid and mineralocorticoid receptor we found similar changes in pain-specific emotional contagion. This is the first evidence that social stress can impact emotional contagion in rodents. Additionally, these findings support our hypothesis that social stress related to degree of animal familiarity can lead to differences in social behavior (Martin et al., 2015).

To further test this hypothesis, we designed a series of experiments designed to characterize the effect of social stress on tube co-occupation behavior in the TCOT. First, we investigated whether lower tube co-occupation behavior in stranger dyads was the result of increased stress activation. We report here differences between familiar and stranger mice in the TCOT, using both measures of systemic plasma corticosterone as well as measures of *Crh* and *Nr3c1* mRNA expression levels in HPA axis-relevant brain regions. Furthermore, by pretreating mice with compounds known to reduce or enhance stress, we show that differential stress activation significantly alters tube co-occupation behavior in both strangers and siblings.

4.3 Methods

4.3.1 Animals

In all experiments, naïve male and female CD-1® (Crl:CD-1(ICR)) outbred mice were bred in-house at our animal facility at McGill University. Housing conditions and testing criteria were identical to those outlined in **Section 2.3.1.**

4.3.2 The TCOT

We performed all stress experiments using the "manual" version of the TCOT. Testing conditions were identical to those in **Section 2.3.2.**

4.3.3 Measuring levels of plasma corticosterone in the TCOT

We tested a separate cohort of CD-1® in the TCOT before euthanizing them, by decapitation under isoflurane/oxygen anesthesia, 30, 60, 120 or 180 min after the start of behavioral testing. Investigators collected trunk blood for corticosterone enzyme immunoassay (EIA). We spun blood samples (15,000 rpm, 4 °C) for 15 min in order to collect supernatant blood plasma. We normalized samples and diluted them 1:800 before running them against a standard curve as part of a validated corticosterone EIA kit (Cayman Chemical).

4.3.4 Evaluating relative expression of *Crh* and *Nr3c1* mRNA

Following TCOT testing, mice were euthanized by cervical dislocation under isoflurane/oxygen anesthesia. Investigators surgically removed the brain from the skull, before blocking off the cerebellum and anterior frontal lobe for further analysis. We placed brain tissue in a 1:10 PBS:water solution, after which time we obtained 300 nm slices of brain tissue with a

vibratome (Leica VT 1000S). Relevant brain sections obtained from each dyadic run were pooled to create a biological sample. We obtained tissue from the paraventricular nucleus of the hypothalamus (PVN), the bed nucleus of the stria terminalis (BST), and the central nucleus of the amygdala (CA) using 21-gauge 1½ needle. Investigators purified RNA solutions with a DNA-free kit (Ambion, USA) and RNeasy Mini kit (QIAGEN, USA). A Bioanalyzer 2100 (Agilent, USA) analyzed the integrity of the total RNA samples. We produced cDNA using TaqMan Reverse Transcription Reagents kit (Applied Biosystems [ABI], USA), carried out on a Peltier Thermal Cycler (PTC-100) (MJ Research, USA).

Relative expression levels of the corticotropin-releasing hormone (*Crh*) and glucocorticoid receptor (*Nr3c1*) genes were measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) using Applied Biosystems TaqMan probes (assay IDs: Mm04206019 m1 and Mm00433832 m1, respectively). Relative expression of *Crh* and *Nr3c1* were compared to expression of control gene, *Gapdh (*cat#4308313) and were made following the ΔΔCt standard curve method.

4.3.5 Stress manipulations

In order to test whether acute stress impacts behavior in the TCOT, we gave a separate cohort of CD-1® one of three compounds: 1) metyrapone (50 mg/kg, *i.p.*), a corticosterone synthesis inhibitor, 2) yohimbine hydrochloride (2.5 mg/kg, *i.p.),* a known anxiogenic compound, or 3) saline (10 ml/kg, *i.p.*) as a control. Investigators injected all drugs while the animal was in the home cage 30 min prior to testing. We randomized mice to one of the three stress conditions by cage prior to testing. Investigators and coders were blinded to drug condition.

4.3.6 Statistical analyses

Description of tube co-occupation analysis is described in **Section 2.3.6.**

We plotted corticosterone data as %B/B0 versus log concentration using a four parameter logistic fit. Sample concentrations were determined using equation obtained from the standard curve plot. We reanalyzed samples with more than 80% or less than 20% of the [corticosterone] described by the range of the standard curve. We performed one-way analysis of variance (ANOVA) on corticosterone results, followed by Tukey post-hoc analyses where appropriate. RT-PCR analysis compared the ratio of the average target gene amount over the average GAPDH amount using multiple t-tests.

For all analyses, we considered α=0.05 to be significant. We excluded statistical outliers (Studentized residuals >2 standard deviations from the group mean); we excluded a total of 3 biological samples from final corticosterone analysis, and 4 biological samples from final RT-PCR analysis.

4.4 Results

4.4.1 Stranger presence leads to higher levels of corticosterone

In order to understand how social stress modulates tube co-occupation behavior in the TCOT, we first showed that the presence of a stranger conspecific produced measureable increases in adrenal stress hormones, confirming our earlier findings (Langford et al., 2011; Martin et al., 2015). Here we show that following social interaction between strangers plasma corticosterone levels are elevated. In contrast, corticosterone levels did not change following interaction between siblings. In the TCOT, plasma corticosterone levels in strangers were significantly higher at 30–60 min after the start of the test than in siblings or mice tested alone,

suggesting that the presence of a stranger induces additional (social) stress above background physical environment (time x condition: $F_{(3,91)} = 6.5$, $p = 0.001$; see **Figure 4.1**). By the start of the third testing hour we failed to distinguish corticosterone levels by social status. We note that converging levels of corticosterone between social groups follows a similar pattern as tube co-occupation behaviors we previously observed (**Figure 2.3a**), despite the fact that strangers do not show the same convergence with siblings in terms of their tube co-occupation behaviors in the third hour (**Figure 2.3b)**.

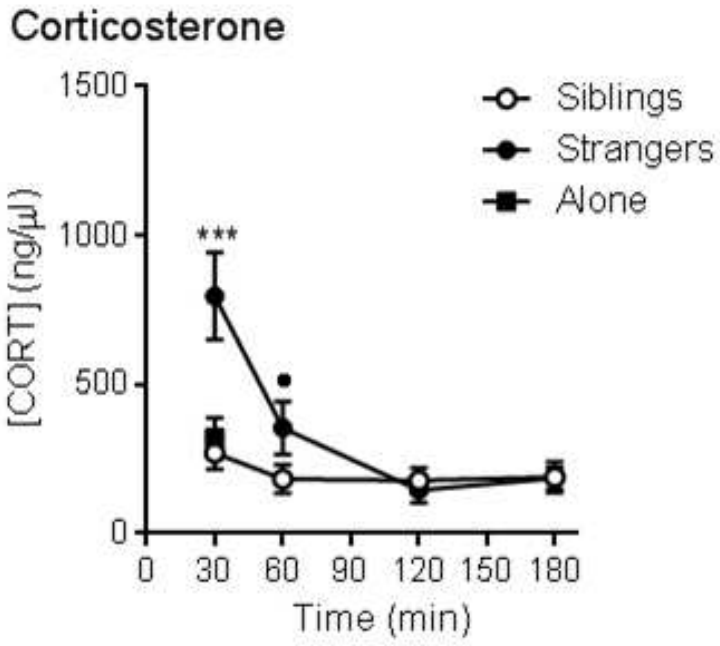

Figure 4.1: Corticosterone expression varies by familiarity status in the TCOT. Compared to mice tested alone, strangers in dyads have elevated corticosterone levels compared to siblings in dyads (time x condition: $F_{(3,91)} = 6.5$, $p = 0.001$). Symbols represent mean ± SEM plasma corticosterone concentration (ng/µl) for mice sacrificed at 30, 60, 120, or 180 min after the start of TCOT testing (n= 11–14 dyads/group/time point). ***p<0.001, ●p<0.1 compared to sibling group at same time point. Note that corticosterone levels are high in all groups, likely due to the stress of the novel environment.

4.4.2 Stranger presence increases *Crh* and decreases *Nr3c1* mRNA expression

To further demonstrate that social stress accounts for decreases in tube co-occupation behavior in the TCOT, we tested whether strangers in the TCOT showed increased mRNA expression of corticotrophin releasing hormone gene (*Crh*), as well as correspondingly lower expression levels of the glucocorticoid receptor gene (*Nr3c1*) in HPA axis-relevant brain regions. After analyzing tissue obtained 3.5- 4 h after the start of the TCOT run, we noticed that strangers displayed significantly higher mRNA expression of the corticotrophin releasing hormone gene (*Crh*) (t_3=13.40, $p < 0.01$) and correspondingly lower expression of the glucocorticoid receptor gene (*Nr3c1*) in the periventricular nucleus (t_5=2.88, $p < 0.05$, see **Figure 4.2a**). We observed similar differences in the BST: strangers showed a strong trend towards higher *Crh* expression (p=.07), as well as significantly lower *Nr3c1* expression in the BST (t_5=3.03, $p < 0.05$, see **Figure 4.2b**). We saw no differences in either *Crh* or *Nr3c1* gene expression in the CA (**Figure 4.2c**).

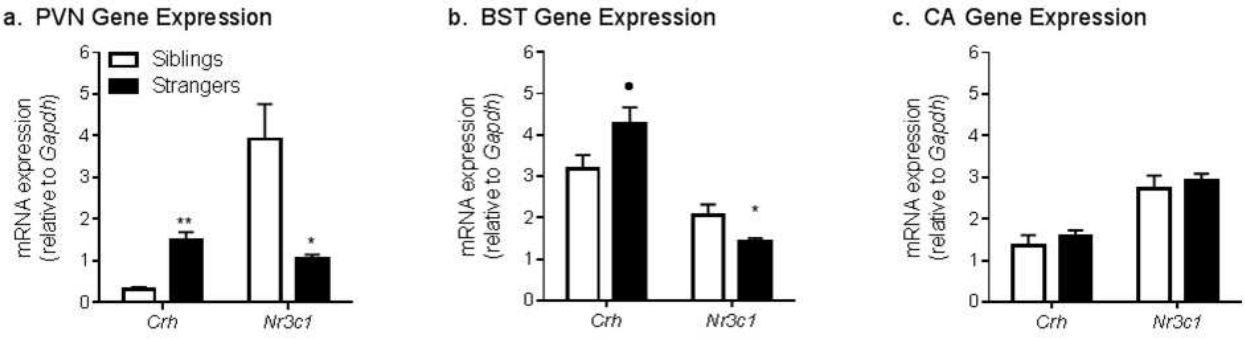

Figure 4.2: Altered expression of stress-relevant genes *Crh* (corticotrophin-releasing hormone) and *Nr3c1*(glucocorticoid receptor) in the periventricular nucleus (PVN) of the hypothalamus (**a**), bed nucleus of the stria terminalis (**b**), and central nucleus of the amygdala (**c**) in stranger dyads compared to sibling dyads. Bars represent mean ± SEM mRNA levels in arbitrary units compared to the housekeeping gene, *Gapdh* (n =3-6 biological replicates/group). ●p<0.1, *p<.05, **p<.01.

4.4.3 Activating/ blocking acute stress response modulates tube co-occupancy

To further define how acute stress activation dictates tube co-occupation behavior, we pretreated mice with compounds known to reduce or enhance stress before we ran them in the TCOT. Whereas the corticosterone synthesis inhibitor metyrapone (50 mg/kg, *i.p.*) showed no effect in siblings, it significantly increased co-occupancy in strangers (main effect of drug: $F_{1,28} = 8.5$, $p=0.007$; main effect of social condition: $F_{1,28} = 0.1$, $p=0.73$; drug x social condition interaction: $F_{1,28} = 3.2$, $p=0.08$; **Figure 4.3a**).

Conversely, the anxiogenic α_2-adrenergic receptor blocker, yohimbine hydrochloride (2.5 mg/kg, *i.p.*), showed no effect in strangers, but significantly decreased co-occupancy behaviors in siblings (drug x social condition interaction: ($F_{(1,31)} = 12.9$, $p=0.001$; **Figure 4.3b**).

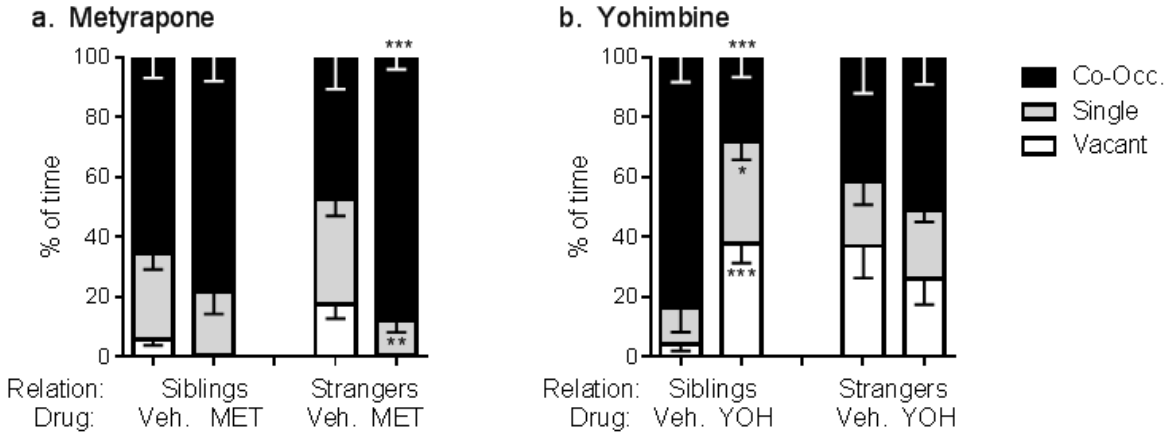

Figure 4.3: Modulation of tube co-occupancy behavior following pharmacological manipulation of the acute stress system. **a**) Stranger dyads show increased co-occupancy behavior after being pretreated with the corticosterone synthesis inhibitor, metyrapone. **b**) Decreased co-occupancy behavior in sibling dyads pretreated with the anxiogenic α2-adrenergic antagonist, yohimbine hydrochloride. **p*<0.05, ***p*<0.01, ****p*<0.001 compared to corresponding vehicle group. Bars represent mean ± SEM percentage co-occupancy in sibling or stranger dyads pretreated (*i.p.*) with vehicle (Veh.) or 50 mg/kg metyrapone (MET) (**a**) or 50 mg/kg yohimbine hydrochloride (YOH) (**b**); *n*=6–10 dyads/social condition/drug.

4.5 Discussion

Using outbred mice in the tube co-occupancy test (TCOT), we present evidence that the presence of a stranger results in higher levels of stress activation when compared to the presence of a social familiar. These data confirm our lab's previous findings using both mice and humans in other social assays (Langford et al., 2011; Martin et al., 2015), and show that social stressors (**Figures 4.1-4.2**) reliably modulate tube co-occupancy behavior. After controlling for environmental novelty, we find that the reticence of mice to co-occupy with strangers appears to be due to stress associated with the stranger itself, over and above the stress associated with the novel open field environment (**Figures 4.1-4.2)**. Furthermore, by pharmacologically controlling for the degree of acute stress activation, we can reliably reverse tube co-occupation behavioral phenotypes in the TCOT (**Figure 4.3**).

Our findings suggest that stress is an integral component of mouse social behavior and can serve as a potential confound in autism behavioral assays. Mice with high anxiety traits, for example, will engage in less exploratory behaviors than low-anxiety models, resulting in a "low" sociability score in the three-chambered test (Silverman et al., 2010b). Discussing their findings, the authors affirm that it is important to evaluate potential explanations for a given social behavior before labeling it a core symptom of autism. Although studies have evaluated anxiety behaviors in inbred autism models using discrete anxiety tests, no studies to date have directly measured physiological correlates of HPA activation (corticosterone, *Crh* expression, etc.) in sociability tests. Furthermore, inbred strains that showed high sociability scores consistently rank as the least anxious in relevant anxiety assays, including the open field test and the elevated plus maze (e.g., (Moy et al., 2008; Moy et al., 2007; Pobbe et al., 2011). Finally, administration of anxiogenic and anxiolytic compounds alters both rat and mouse social behavior. For example,

researchers administering diazepam (a pharmacological anxiolytic compound) or fluoxetine (a selective serotonin re-uptake inhibitor) demonstrated that BTBR mice subsequently showed "normal" levels of sociability behavior in the three-chambered assay (Chadman, 2011; Pobbe et al., 2011). A separate study found that intracerebroventricular injections of Crh directly into the rat brain decreased subsequent social interactions between familiar conspecifics when compared to saline controls (Dunn and File, 1987). In light of these findings, our own characterization of stress activation in the TCOT suggests that environmental and social stressors may play an even greater role in current sociability assays, given their short (~10-20 min) environmental habituation periods and lack of social exposure prior to testing (resulting in potentially higher levels of socially mediated stress response). By allowing animals to interact for 60 min together before we begin recording behavior, we have attempted to minimize stress confounds in the TCOT.

One may be surprised to see that high-stress states fail to produce prolonged social contact between animals in our test, given reported findings in the social buffering literature that show stressed rodents prefer to seek out social proximity. Simply put, social buffering is the amelioration of negative stress-related effects through social support. In the animal literature, both pain and stress behaviors decrease when animals are tested in dyads. For example, one study shows that proximity between animals correlates with subsequent decreases in fear and anxiety behaviors (Kikusui et al., 2006). Social buffering will be discussed more in **Chapter 5**. However, we argue that the TCOT is not directly comparable to social buffering studies for several reasons. First, unlike most of the reported social buffering literature, we vary familiarity status in our test of social proximity behavior. Second, we are looking at prolonged social interactions. Whereas most social buffering studies focus on short interaction periods among

dyads following the presentation of a stressor, the TCOT is designed to look at prolonged social proximity behaviors. Third, we are pharmacologically restricting corticosterone levels to either maintain a very high or very low stress state during our behavioral experiment. In our test, animals do not experience stress reduction and may subsequently approach one another less in a high-stress state. Recent findings in the social buffering literature suggest that an abundance of factors, including the sociability of the species, familiarity of the conspecifics, and type of stress induced during the experiment all significantly predict the effects of social buffering on stress relief (Armario et al.; Hennessy et al., 2009).

In light of our own previous findings, we argue that stress is an inherent component of any social interaction; in a recent study we show that stress plays an important part in dictating social behavior in both people and animals (Martin et al., 2015). Moreover, potentially confounding sources of stress (including environmental exposure, handling, social exposure prior to testing, mouse age, etc.) are of equal, if not even greater concern in other sociability tests. Using rigorous testing methods, we believe we have demonstrated that the TCOT is capable of characterizing potential differences in stress sensitivity in autism-like models without the need to use multiple testing apparatuses. It is important for future studies to determine if autism-like models show abnormal stress responses to social and environmental stressors. Although HPA axis dysregulation is not a ubiquitous clinical feature, several reports have implicated it in ASD cases (Corbett et al., 2006; Jansen et al., 2000; Nir et al., 1995; Richdale and Prior, 1992). In addition, future studies could determine if the administration of typical antidepressants and anxiolytics subsequently alter co-occupation behavior in both gregarious strains as well as autism-like models. Alternatively, blocking GR and MR directly in the hypothalamus or stria terminalis through the use of Mifeprestone (a glucocorticoid antagonist) and RU 26752 (a

mineralocorticoid receptor antagonist) could further elucidate involvement of the HPA axis in social behavior.

One of the primary reasons we designed the TCOT was to measure changes in social status over time. This design feature has allowed us to compare *Crh* and *Nr3c1* mRNA expression over a 4 h testing session. Based on our data, we hypothesize that adult interactions may regulate stress sensitivity over time. Currently, epigenetic regulation of stress is a hot topic in neuroscience; it has been demonstrated, for example, that maternal interaction with pups can significantly influence subsequent stress reactivity in adult animals (Champagne, 2013; Elliott et al., 2010; Murgatroyd et al., 2009; Weaver et al., 2004). Additional work is needed to see if multiple interactions in adulthood produce long-term changes in the expression of stress-specific genes that could in turn predict subsequent levels of propinquity in mice.

Chapter 5

Evaluating the role of pain in tube co-occupation behavior

5.1 Rationale

It is well known that social context can robustly affect pain levels and outcomes in chronic pain patients. One can also demonstrate direct effects of varying social context on laboratory pain sensitivity, although this process proves to be complex. Social contexts and social interactions can also affect pain sensitivity in laboratory animals. The converse is also true: the presence of pain can affect the social interactions of laboratory animals, serving as a useful stimulus in social neuroscience studies. We recently observed that "naïve" mice (not in pain) demonstrated social approach behavior when a conspecific is in pain. Moreover, the degree of proximity significantly correlated with decreased pain behaviors in the affected mouse. That is, the social approach to pain appeared to produce analgesia (Langford et al., 2010). Here we present a series of experiments designed to show how the presence of pain influences social behavior in the TCOT.

5.2 Introduction

As an experience, pain is complex, multidimensional, and contingent on a multitude of different factors. In addition to a physical sensation, pain often precipitates a psychological and behavioral response (Basbaum and Jessell, 2000). Researchers typically rely on the biopsychosocial model (Engel, 1977) to frame the litany of factors that influence an individual's sensitivity and response to pain (Andrasik et al., 2005; Gatchel et al., 2007; Keefe et al., 2002; Turk and Okifuji, 2002). However, the relative contribution of social factors on individual pain sensitivity is still poorly understood. Preclinical researchers have only recently begun to evaluate the role that social context and social interactions play in animal pain sensitivity, for example

(Langford et al., 2006). Conversely, the presence of pain appears to serve as a cue, modulating social interaction among laboratory animals. As such, it can be adapted to serve as a useful stimulus in social neuroscientific studies. In pursuit of developing more etiologically relevant pain models, therefore, new models of animal social interaction are needed (Mogil et al., 2010).

5.2.1 Social effects on pain in rodents

Even the most basic pain-relevant social interaction, simple observation of pain by another, yields a complex pattern of results. The amelioration of aversive stimuli through social interaction (i.e., social buffering) can reduce acute pain ratings. Like humans, rodents also demonstrate social buffering. Rats tested in groups of three demonstrated more approach-withdrawal (Rasmussen, 1939) and less freezing behaviors (Davitz and Mason, 1955) following foot shock compared to rats tested alone. In a more recent study, the presence of a naïve rat significantly reduced freezing behavior in a test rat in response to foot shock, as well as expression of the immediate early gene, *Fos*, in the stress-relevant paraventricular nucleus of the hypothalamus (Kiyokawa et al., 2004).

The mere presence of a social conspecific is sufficient to modulate an animal's response to other (non-painful) environmental stressors. For example, the presence of a familiar cagemate reduced a rat's conditioned avoidance behavior to an anxiolytic stimulus (Baum, 1969; Hall, 1955). Similarly, the presence of a companion lowered a rat's exhibited stress-related behaviors and plasma corticosterone levels in a novel environment (Latané, 1969; Leshem and Sherman, 2006b; Weijers and Weyers, 1998). Scientists also observed social buffering when rats were paired with a physically separated (caged) rat (Latané, 1969) or an anaesthetized animal (Latané

and Glass, 1968), suggesting that physical interaction is not necessarily required for social buffering.

Like environmental stressors (e.g., restraint, forced swimming, foot shock), acute social stressors can also modulate pain behaviors in animals. The classic demonstration of this effect is called "defeat analgesia;" whereby a “losing” intruder that was socially defeated by a dominant animal subsequently displayed opioid-mediated analgesia following a bout of inter-male aggression in the resident-intruder paradigm (Miczek et al., 1982). Subsequent studies demonstrated defeat analgesia in rats, hamsters, and gerbils (Huhman et al., 1991; Raab et al., 1985; Rodgers and Hendrie, 1983). Even without overt aggression, a social situation in which only one male was in pain also produced stress-induced analgesia in the affected mouse, presumably caused by proximity to a potentially threatening (and healthy) conspecific (Langford et al., 2006; Langford et al., 2011) (Fig. 3). This analgesia occurred only when both stranger male mice in the dyad were gonadally intact, or gonadectomized but testosterone-replaced and only when full contact was permitted between the mice. When researchers restricted mice to only limited contact (through vertical metal bars), reducing the threat level substantially, results showed that stress-induced hyperalgesia (Imbe et al., 2006) occurred in same-sex male dyads, but not same-sex female dyads or mixed dyads (Langford et al., 2011). This phenomenon may be related to Rodgers and Hendrie's observation of hyperalgesia in the resident winner of agonistic encounters in the resident-intruder paradigm (Rodgers and Hendrie, 1983).

When rodents are isolated (i.e., devoid of all social contact) their behavior is drastically altered, and pain behavior is no exception. For example, male rats housed in isolation autotomized (i.e., self-mutilated) their denervated limb following dorsal rhizotomy; the presence of a co-housed female rat almost completely prevented autotomy behavior (Berman and Rodin,

1982). A few studies showed that social isolation decreased pain sensitivity and increased analgesic responding by enhancing μ-opioid activity (Becker et al., 2006; Coudereau et al., 1997; Puglisi-Allegra and Oliverio, 1983).

The opioid system is a key analgesic pathway. It has been shown to be altered both by social isolation, as discussed above, and also by social reunion. Following a long period of separation, reunited male mice demonstrated a decrease in pain behaviors; moreover, this analgesia could be reversed with the injection of naloxone, a non-specific opioid receptor antagonist (D'Amato, 1998; D'Amato and Pavone, 1993). The most important stimulus responsible for increased nociceptive threshold among reunited siblings appears to be physical affiliative contact; the authors showed a strong positive correlation between huddling behavior and pain sensitivity (D'Amato and Pavone, 1996). Intriguingly, female siblings separated at weaning did not display any behavioral indices of recognizing their separated siblings, and did not have altered pain thresholds upon reunion. They did, however, recognize unrelated cagemates, and showed opioid-mediated analgesia after being reunited (D'Amato, 1997).

The modulation of rodent behavior by social environment suggests that animals may be able to communicate emotional states to one another, especially those relating to stress. In several studies, rats used ultrasonic vocalizations and chemosignals to communicate stress and were able to recognize and avoid the odor of a stressed rat (Mackay-Sim and Laing, 1981; Valenta and Rigby, 1968). In addition to producing avoidance, the social transfer of stress odors can modulate the pain sensitivity of a non-stressed conspecific. Naïve rats exposed to odors from stressed rats displayed significantly less pain behavior following injections of inflammatory agents; this contagious analgesia was opioid-mediated, being completely reversed by the opioid antagonist naltrexone (Fanselow, 1985). In another experiment, mice that witnessed other mice

being attacked by biting flies displayed both analgesia and self-burying behavior when exposed 24 hours later to flies whose biting mouth parts were removed (Kavaliers et al., 2001). A more recent study published by our lab shows that human chemosignals also appear to produce stress-related analgesia in mice (Sorge et al., 2014).

The most obvious explanation of these findings is that rats and mice recognize the odor of a stressed conspecific and react with stress and subsequent analgesia (Butler and Finn, 2009)). Perhaps most intriguingly, researchers showed that untreated cagemates of an oxytocin (OT)-treated rat displayed reduced pain behaviors during the hot-plate test. Furthermore, this effect was reversed by an OT antagonist (Agren et al., 1997). The contagion mediated by OT is most likely driven by stress (Robinson et al., 2002) and occurs via olfaction (Agren et al., 1997).

In addition to contagious analgesia, our lab has shown that familiarity can modulate pain status among rodents. Specifically, Langford and colleagues found that cagemates, but not strangers, demonstrated contagious pain hypersensitivity; this occurs only if both mice in the dyad are in pain (Langford et al., 2006). Contagious pain hypersensitivity appears to be a different form of contagion that is not mediated by stress because stress levels were actually higher between dyads of strangers, where no hypersensitivity was observed. In fact, mice experiencing inflammatory pain appear to bi-directionally modulate the severity of inflammatory pain behavior in simultaneously tested cagemates; again, no effects are seen among strangers (Langford et al., 2006). Other labs have replicated familiarity-mediated contagious pain hypersensitivity in rodents, using both identical testing protocols (Langford et al., 2011) as well as different pain stimuli (Gonzalez-Liencres et al., 2014).

5.2.2 Pain's effect on pro-social behavior in rodents

Through social communication and emotional contagion, animals may internalize the pain states of those around them. A small but growing consensus suggests that animals will, in some cases, demonstrate "pro-social" behaviors, or actions that (whether deliberately or not) reduce observed suffering (even in unrelated individuals); however it remains unclear whether these actions serve to reduce the suffering of the other, or to reduce an animal's own stress state that accompanies the witnessing of suffering (Rice and Gainer, 1962). For example, after conditioning, rats actively pressed a lever to reduce the suffering of another animal (Church, 1959); a follow-up study replicated this finding using pigeons (Watanabe and Ono, 1986). Furthermore, researchers showed that rats increased allogrooming (pro-social grooming) behaviors towards conspecifics that had recently received an electric shock (Knapska et al., 2010). We recently observed that (unaffected) mice demonstrated increased levels of social approach behavior when a conspecific was in pain (Langford et al., 2010), but only in female mice and only in cagemate dyads. In contrast, male and female strangers appeared indifferent. Furthermore, the degree of proximity significantly correlated with decreased pain behaviors in the affected mouse. That is, the social approach to pain appeared to produce analgesia (Langford et al., 2011). However, the fact that social approach does not imply that the immediate environment surrounding an animal in pain is necessarily preferred; Watanabe also observed that mice approached pain, but later developed a conditioned place aversion to the compartment in which they briefly resided with the afflicted animal (Watanabe, 2012).

Whereas numerous preclinical studies have identified social environmental influences in pain (and related pain behaviors) there is a relative dearth of knowledge as to how social behaviors change in the presence of pain. For example, the studies described above all used short-lasting

pain manipulations. Only one study examined the effect of chronic pain on social interactions in mice. Benbouzid and colleagues showed that a chronic nerve constriction injury produces increased social interaction behavior in C57BL/6J mice (Benbouzid et al., 2008). Similarly, there are few reports of changes in social behavior following neuropathic injury using other animal models. One study showed that rats displayed less dominance behavior and increased submissive behaviors towards a cage intruder following a similar chronic nerve constriction injury, similar to findings reported by Benbouzid and colleagues (Monassi et al., 2003). Conversely, a second study using dominant rabbits injected with formalin (a tissue fixative agent that induces tonic pain after injection) showed that animals displayed increased aggressiveness following injury (Farabollini et al., 1988). The relative paucity of data and conflicting results among animal models suggest that new experiments are needed to understand pain's impact on social behavior. Specifically, we were interested in evaluating whether the presence of different types of pain modulates propinquity behaviors in the tube co-occupancy test (TCOT). Here I present a series of experiments that induces both tonic inflammatory and chronic neuropathic pain in mice to see how their tube co-occupation behaviors subsequently change.

5.3 Methods

5.3.1 Animals

We used naïve male and female CD-1® (Crl:CD-1(ICR)) outbred mice for all of the following pain experiments. We obtained, housed, and tested animals in an identical manner to those described in **Section 2.3.1.**

5.3.2 Pain Testing

To test whether inflammatory pain influences tube co-occupation behavior in the TCOT, we injected a cohort of mice with zymosan A (Sigma, Markham, Canada), a polysaccharide prepared from the wall of *Saccharomyces cerevisiae* (baker's yeast) (Pillemer et al., 1941). Injection of zymosan A ("zymosan") induces maximum inflammation and pain (thermal and mechanical hyperalgesia) within 4 h of injection (Meller and Gebhart, 1997). We delivered zymosan via the plantar surface of the left hind paw (2.5 mg/kg, intraplantar) as described in (Depner et al., 2003). We injected mice 30 minutes prior to the beginning of TCOT testing, in order to achieve sufficient paw allodynia by the second hour of social testing; all dyads used in this experiment consisted of animals in pain. We calculated paw edema by comparing hind paw weight following the TCOT run.

We performed a spare nerve injury (SNI) on a second cohort of mice to characterize social behaviors following a prolonged neuropathic chronic pain state. We compared social behaviors between SNI and naïve animals. The SNI mouse model involves ligation of two of the three branches of the sciatic nerve (the tibial nerve and the common peroneal nerve), while the sural nerve is left intact. The lesion results in marked hypersensitivity in the lateral area of the paw that is innervated by the spared sural nerve (Decosterd and Woolf, 2000). Based on Decosterd and Wolf's characterization and our lab's own observations, marked sensory hypersensitivity of the hind paw ipsilateral to the nerve injury was clearly present 24 h after the surgery and peaked approximately 7 days after the surgery. We selected this particular neuropathic chronic pain assay due to its duration of hyperalgesia and hypersensitivity. Animals were tested 7 days after surgery in order to capture social behavior during peak allodynia; as in the zymosan experiment, both animals in tested dyads were in pain.

5.3.3 The TCOT

We evaluated tube co-occupation behavior in the TCOT using identical methods to those described in **Section 2.3.2.**

5.3.4 TCOT Scoring

We coded tube co-occupancy behaviors using the "manual" coding procedures described in **Section 2.3.3.**

5.3.5 Assessing locomotion in the TCOT

To determine if social co-occupation behaviors in afflicted animals were related to locomotion deficits, we performed a simple line-crossing analysis on videos taken from the first hour of TCOT testing, similar to (van Gaalen and Steckler, 2000). Videos of TCOT runs were arbitrarily separated into a 2 x 1 grid by drawing a line across the center of the open field so that it bisected the tube into two equal parts. Mice were marked as "crossing" the line any time their head passed over the line. Tube occupation did not count as a line cross unless the head was observed emerging out of the opposite end of the tube. We compared male stranger mice that had either received a saline hind paw injection (20 μL, intraplantar), zymosan hind paw injection (2.5 mg/kg, intraplantar*)*, or SNI surgery (7 days prior to testing). We sampled video bins during the first hour of testing in the same manner as previously quantified tube co-occupation behaviors (described in **Section 2.3.3**).

5.3.6 Statistical Analyses

We recorded social behaviors in both paradigms and assessed them offline using a video time-sampling method (10 s/ min) to generate behavioral "bins" as previously described (Langford et al., 2010; Langford et al., 2006). An observer blinded to social and pain status performed video coding. We analyzed data using one-way or two-way ANOVA followed by Tukey post-hoc analyses, where appropriate. For all analyses, we considered α=.05 to be significant.

5.4 Results

5.4.1 Zymosan induces inflammation and increases stranger tube co-occupation

To induce a tonic pain state, we injected a cohort of outbred CD-1® mice with zymosan (2.5 mg/kg, intraplantar) in the left hind paw. The TCOT design prevented the quantification of pain-related behaviors when animals were in the test, due to tube opacity. Instead, we indirectly evaluated pain status in test animals by measuring paw edema following the TCOT run (3.5 h after initial injection of either zymosan or saline) (**Figure 5.1**). As predicted, zymosan injection (but not vehicle) lead to a marked increase in left hind paw weight when compared with the (uninjected) right hind paw, indicating that inflammation (and accompanying hyperalgesia) were present by the end of the TCOT run (t_{142} = -15.48, p < .001).

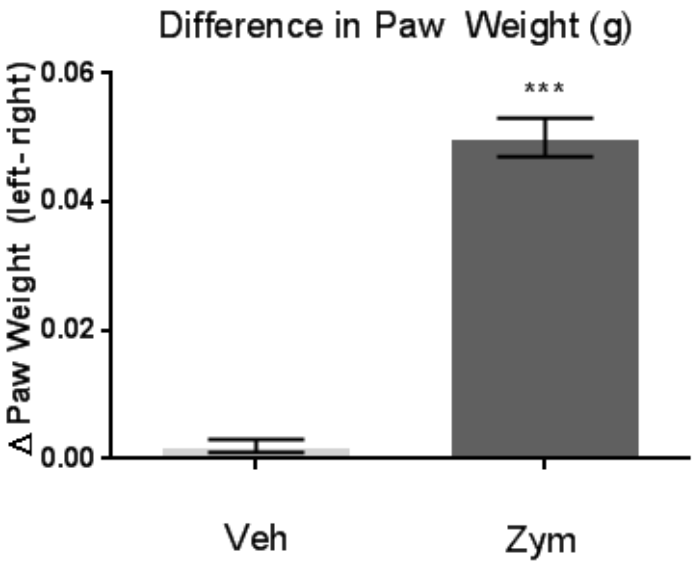

Figure 5.1: Bar graphs depicting differences in paw weight (left paw – right paw) in animals injected with saline or zymosan. Veh= saline-injected animals; Zym = Both animals in pain, (2.5 mg/kg Zymosan A, intraplantar). *n*= 36 dyads/drug. *** $p < .001$ compared to Veh group.

Following zymosan injection, we ran mice in the TCOT in order to determine if the presence of pain modulates tube co-occupation behavior. Here we show that the presence of pain decreases stranger differences in tube co-occupation behavior (pain x relationship interaction: $F_{1,72} = 5.2$ $p < .05$). After receiving an injection of zymosan, strangers significantly increased their tube co-occupation behavior, showing no differences from zymosan-injected siblings. We observed an opposite change in single tube occupation in strangers that received zymosan injection as compared to siblings (pain x relationship interaction, ($F_{1,72} = 6.83$, $p < .05$). To rule out a potential ceiling effect in the sibling testing condition, we looked at tube co-occupation during the first hour. An analysis of data obtained from 30-60 min confirmed that the presence of pain did not significantly modulate sibling tube co-occupancy behavior (data not shown). We found no significant sex differences.

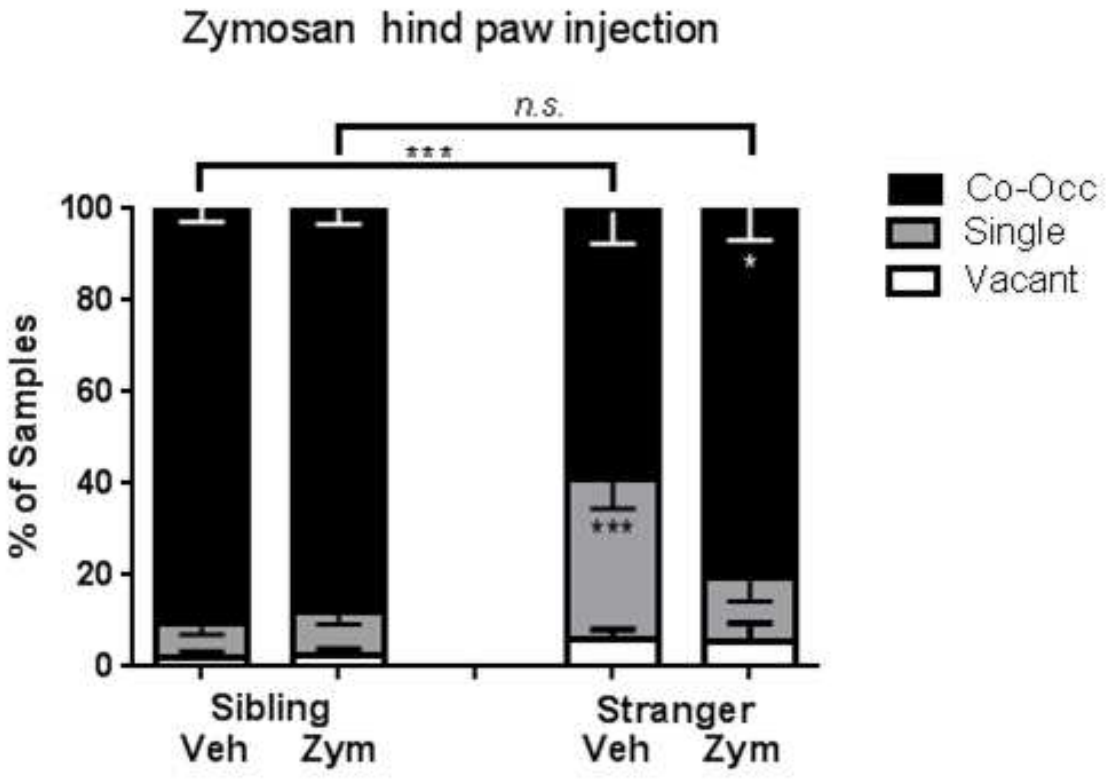

Figure 5.2: Bar graphs depicting co-occupancy behavior between social dyads in the presence or absence of zymosan. n= 18-20 dyads/drug. ***$p < .001$,*$p < .05$ compared to corresponding vehicle group. Bars represent mean ± SEM percentage co-occupancy in sibling or stranger dyads pretreated (intraplantar) with vehicle (Veh.) or 2.5 mg/kg zymosan A (Zym).

5.4.2 SNI increases stranger co-occupation

To induce a chronic neuropathic pain state, we gave a separate cohort of outbred CD-1® mice SNI surgeries 7 days prior to the start of TCOT testing (in order to achieve maximum allodynia). As we observed in zymosan-treated animals (**Figure 5.2**), strangers that received a neuropathic injury significantly increased their tube co-occupation behavior to become phenotypically indistinct from siblings dyads ($F_{1,72}$ =4.2, $p < .05$). These data confirmed our previous findings showing that the presence of inflammatory pain effectively eliminated differences in familiar versus stranger tube co-occupation behavior. Similar to our previous experiments, we did not observe any significant sex differences.

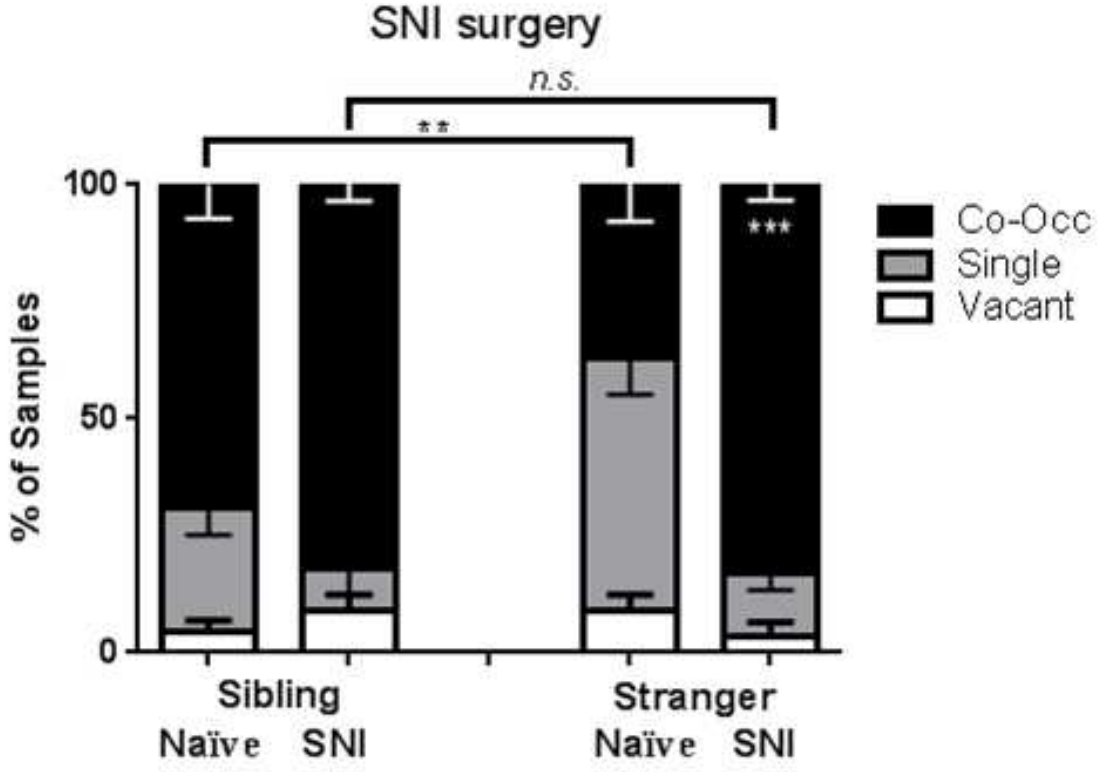

Figure 5.3: Bar graphs depicting co-occupancy behavior between social dyads in the presence or absence of neuropathic pain. n= 14-18 dyads/condition. **$p < .001$ ***$p < .0001$ compared to corresponding vehicle group. Bars represent mean ± SEM percentage co-occupancy in sibling or stranger dyads with no pain (naïve) or 7 d post-SNI surgery (SNI).

5.4.3 Pain does not change exploratory behavior in TCOT

To determine if increased tube co-occupancy behaviors in injured animals were the result of decreased locomotion, we performed a simple line crossing test by analyzing TCOT footage taken during the first hour of testing. Neither zymosan hind paw injections nor SNI surgery resulted in decreased exploratory behavior in the first hour of testing when compared to saline-injected control animals ($F_{2,31}$ =1.91, p =0.17).

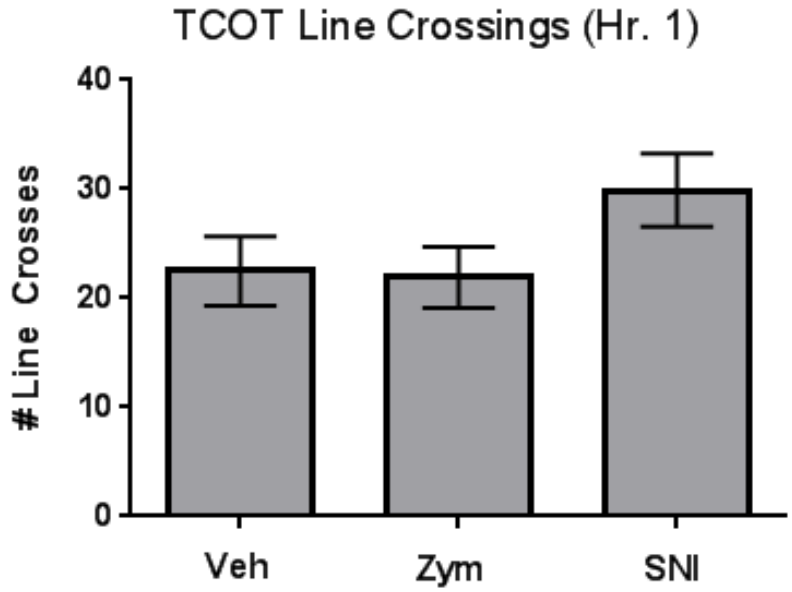

Figure 5.4: Bar graphs depicting total number of line crosses in the TCOT during the first hour of testing. We quantified samples using the same 10 s video bins we previously analyzed for tube co-occupancy behavior. Neither zymosan injection (**Zym**) nor SNI neuropathic pain (**SNI**) significantly altered locomotor activity in animals when compared to saline-injected animals (**Veh**) ($F_{2,31}$ =1.91, p =0.17). n= 10-13 animals/pain test. Bars represent mean ± SEM number of line crosses in the TCOT during the first 60 min.

5.5 Discussion

Here we show that the presence of inflammatory (**Figure 5.2**) or neuropathic (**Figure 5.3**) pain is sufficient to eliminate differences in tube co-occupation behavior between familiar and stranger animals in the TCOT. Specifically, the presence of pain appears to significantly increase stranger tube co-occupation behavior in our test, eliminating previously observed familiarity preference in outbred animals. Additionally, changes in tube co-occupation behavior do not appear to be driven by changes in locomotion, implying that increased tube co-occupation in the TCOT is voluntary. Based on these reported findings, we believe that we provided additional

evidence that pain can serve as a sufficient social cue, modulating subsequent social behaviors in rodents, as we have previously observed (Langford et al., 2010; Langford et al., 2006).

Although our reported findings are encouraging, our understanding of pain signaling in the TCOT has several limitations. First, our data fail to show that nociception is the primary driver of increased tube co-occupancy, rather than concomitant change in stress accompanying pain. Although chronic pain and subsequent changes in stress activation are poorly correlated in rodent neuropathic pain models (Bomholt et al., 2005; Suzuki et al., 2007; Ulrich-Lai et al., 2006), acute pain manipulations can elicit an exaggerated stress response in mice (Benedetti et al., 2012). However, based on our findings of stress's effect on tube co-occupation behavior (**Chapter 4)** it is hard to rectify our current findings (increased stranger tube co-occupation behavior) with a predicted increase in stress response. Nevertheless, we cannot rule out stress as a potential explanatory factor for increased tube co-occupation without further experiments.

Future studies could measure stress in neuropathic and inflammatory models tested in the TCOT. Additionally, we could demonstrate a "rescue" of the familiarity preference phenotype by administering analgesic agents (i.e., Carprofen or morphine) to see if pain is in fact the primary driver of increased stranger tube co-occupation. Finally, in order to determine if tube co-occupation behavior is opioid-dependent, naloxone or naltrexone could be pre-administered to mice to see if an opioid antagonist changes subsequent tube co-occupation behaviors.

Given the physical properties of the TCOT testing assay, we find that quantification of pain-related behavior is impossible once animals are in the opaque testing tubes. As a result, we are unable to determine if tube co-occupation leads to a subsequent reduction in pain behavior. Additional experiments could use video sensors to better capture pain-related behaviors exhibited by animals in the tube. Alternatively, complimentary tests of hyperalgesia and

hypersensitivity could confirm pain status in animals used in TCOT experiments. Finally, our lab has previously reported that the mere presence of a conspecific in pain is enough to elicit increased social approach behavior in intact female siblings (Langford et al., 2010). It would be interesting to determine if tube co-occupation behavior in a "one pain" dyad (one animal receiving a nociceptive stimulus and one receiving a vehicle) shows similar phenotypic patterns.

In spite of these limitations, we believe that the TCOT presents a novel way to assess pain-related changes in social behavior. Perhaps the most outstanding question is whether inbred or mutant autism-like models demonstrate the same behavioral modulation that we observe in outbred animals when both animals are in pain. Although abnormal pain processing has been reported in the clinical ASD literature (Nader et al., 2004; Tordjman et al., 2009), few attempts have been made to characterize pain perception in preclinical autism models. A handful of studies have reported abnormal pain response in one autism relevant model (the rat valproic acid model); however, no similar characterization of pain behaviors has been attempted in mice (Kerr et al., 2013; Schneider and Przewlocki, 2004; Schneider et al., 2008; Schneider et al., 2005).

Similarly, no studies have attempted to characterize social response to pain behaviors using inbred or mutant autism-like models. As the social behaviors described in this dissertation have direct relevance to autism, additional characterization of pain-related social behavior in autism-like models may allow researchers to better understand how individuals with ASD experience pain differently from TD populations.

Chapter 6

General discussion

Collectively, the projects described in this thesis detail a new novel behavioral assay, the TCOT, as a way to measure prolonged periods of voluntary social proximity, or "propinquity," in rodents. Although studies have used social proximity as a proxy for social interaction in open field and home cage environments before (Crawley, 2004; File and Pope, 1974; Kudriavtseva, 1987; Langford et al., 2010; Porter et al., 1978), our study is the first to characterize social proximity between two freely-moving rodents over an extended period of time. In addition to developing a novel behavioral approach, we have created an automatable version of our behavioral test that measures a rodent's continued willingness to co-occupy a "safe" tube in a stressful novel environment. Modulating key factors, including the length of social interaction, familiarity, environmental and social stressors, sex, age, genetic background, and pain status, we also demonstrate that the TCOT can be used to reliably measure social deficits.

Specifically, we show here that our dependent measure, the amount of animal tube co-occupation, changes based on the degree of familiarity between animals, such that animals prefer to spend more time in close proximity to familiar conspecifics than strangers. The TCOT allows us to easily automate tube co-occupation behavior over a prolonged period of social interaction (**Chapter 2**), in a similar fashion to researchers comparing interpersonal distances among human participants (Edwards, 1972; Sinha & Mukherjee, 2008). The following discussion will briefly summarize our major findings before proposing a unifying theory detailing how these factors contribute to tube co-occupation behavior. We conclude this dissertation by discussing the benefits of our new test, as well as implications for the general field of social neuroscience.

6.1 Familiarity significantly alters tube co-occupation in outbred and wild mice

Our lab has previously shown that familiarity is an important determinant of the social modulation of pain. In fact, a standing policy in our laboratory is to co-house animals for at least 21 days prior to testing for socially-modulated changes in pain behavior (Langford et al., 2006; Martin et al., 2015). Like rodent emotional contagion — characterized by hypersensitivity and co-occurrence of pain behaviors among familiar, injured dyads —we show in the TCOT that there is a specific increase in tube co-occupation in familiar, but not stranger, outbred mice (**Chapter 2**). Unlike previous reports, however, the present studies systematically varied the amount of exposure between strangers prior to testing them in the TCOT. Based on our findings, we now know that outbred mice do not require three weeks to become familiar with one another, instead transitioning from "stranger" to "familiar" within 24 hours when sharing the same home cage. Our findings are in line with previous reports showing that animals reduce aggressive behaviors as they become more familiar with one another (Marler, 1976). Similarly, familiar mice are less aggressive towards one another (Parmigiani et al., 1981); indeed, one hour of cage co-occupation is sufficient to show significant reductions in fighting behavior (Parmigiani and Brain, 1983). Furthermore, our comparison of sibling and non-sibling cagemates finds no differences in tube co-occupation behavior, suggesting that affiliation rather than kinship is primarily responsible for rodent propinquity. These observations are in line with our previous assessment of murine social contagion, whereby both cagemates and siblings show emotional contagion of pain when in the presence of a conspecific in pain (Langford et al., 2006).

6.2 Inbred and autism-like models show aberrant behavior in the TCOT

Using a systematic method to compare tube co-occupation rates among familiar and unfamiliar dyads of inbred, outbred, and "autism-like" models, we find that autism-like models show marked deficits in tube co-occupancy behavior in the TCOT when compared to wildtype controls, regardless of familiarity status. To our knowledge, we are the first to compare the effect of social stress on social behavior among stranger and familiar mice over an extended period of time. These models include BTBR T^{+} *tf*/J mice, as well as *Fmr1*$^{-/y}$, *Tsc2*^{tm1Djk}/J and *Eif4e* knockin mutants. Based on our data, we are tempted to conclude that specific "autism" genes alone could explain apparent deficits in tube co-occupation behavior. However, the universally high levels of tube co-occupation behavior we observe among nearly all inbred animals, regardless of social condition, may confound our observations concerning autism-like models. Specifically, inbred mouse strains, especially C57BL/6 mice that serve as the genetic background of most genetic mutant models, appear to have been artificially selected for high social gregariousness. Unlike outbred and re-derived "wild" animals, inbred animals fail to differentiate between familiar and unfamiliar conspecifics in the TCOT (**Chapter 3**). These findings raise doubts concerning inbred animals (and their associated mutant knockout models) as being the best representatives for "normal" rodent social behaviors.

One possible explanation for the distinct lack of familiarity preference among inbred animals is an inability to recognize familiar animals during prolonged social interaction, perhaps due to reduced olfactory differentiability among animals (Arakawa et al., 2008). This finding could not only explain our results, but also those of previous studies showing that the reversal of "familiar" stimuli in the three-chambered test subsequently increased BTBR novelty preference (Pearson et al., 2010). Alternatively, the distinct absence of familiarity discrimination among

inbred animals may be due to the lack of a stranger-mediated stress response over a prolonged testing period, perhaps due to especially high gregariousness demonstrated by the chosen animal strains. Finally, a third potential explanation for deficient familiarity discrimination among inbred lines may be due to differences in the social environment (Yang et al., 2011). That is, inbred mice may experience a relative lack of social novelty in early life, leading to decreased social interest and/or decreased social perception in adulthood. Additional studies are needed to specifically identify why inbred mice fail to show familiarity preference in our test.

6.3 Other factors that modulate tube co-occupation behavior

In addition to familiarity status and genetic background, our findings suggest that at least four other factors appear to influence tube co-occupancy behavior in rodents: length of social interaction, stress, pain status, and age. Together, these factors appear to determine whether or not animals decide to co-occupy a "safe" area in a stressful environment.

6.3.1 Length of social interaction

Although we have outlined a number of methodological differences between the TCOT and other preclinical sociability tests (**Section 1.3.1**), one of the most fundamental is the length of social testing. In the interest of being brief, easily quantifiable, and maximizing the chance for replicability across labs, major sociability tests are designed to measure short bouts of social interaction, and do not characterize social behaviors beyond 20-30 min of testing. As a result, the field has come to quantify a small number of "active" social behaviors, including licking, sniffing, grooming, following, or approaching, to identify autism-like behaviors in animal models.

In order to measure social behaviors over a more extended period of social interaction, we developed the TCOT. The assay's open field design allowed us to compare active social behaviors during the first minutes of testing, although we failed to find significant differences among stranger and familiar dyads. Likewise, tube co-occupation levels did not vary by familiarity status during the first hour of testing. Thus, we have identified that the length of social interaction is a significant component in tube co-occupancy behavior. Only by studying prolonged social interactions were we able to identify a familiarity phenotype in our new test. Indeed, our efforts to measure familiarity preference in the three-chambered test for an extended period of interaction (120 min) failed to show any differences among strangers. However, it should be noted that we did not deviate from social stimuli outlined in the original testing protocols (Crawley, 2004). Perhaps given a choice between a familiar and stranger animal, outbred mice would prefer to remain in close proximity to a familiar animal. As a result, we argue that the TCOT is one of the only social tests that can detect potential deficits in familiar-directed social behavior over an extended (>60 min) period of time. Furthermore, by automating the TCOT we have developed an easy and cost-effective way to measure passive social behaviors in preclinical models

6.3.2 Stress

Designing the TCOT, we purposely chose an aversive environment in order to induce a maximum divergence between familiar and unfamiliar dyads in their levels of tube co-occupation (**Figure 2.8 e/f**). In spite of the high level of environmental stress we show that a reluctance to co-occupy the tube with a stranger appears to be due to stress associated with the stranger itself, over and above the stress associated with the novel environment. After measuring

direct correlates of stress activation (including corticosterone and *Crh* mRNA expression), we find that the mere presence of a stranger conspecific can produce measurable increases in adrenal stress hormones when compared to strangers or mice tested in the same environment. These findings are in line with our lab's previous observations (Langford et al., 2011; Martin et al., 2015). Furthermore, by pharmacologically controlling for the degree of acute stress activation, we show that we can reliably modulate tube co-occupation levels in both strangers and siblings. Specifically, we find that increased stress leads to decreased tube co-occupation behaviors in both siblings and strangers, whereas low stress states lead to increased tube co-occupation in both social groups (**Chapter 4**).

Together, our findings suggest that stress is an important and integral component of tube co-occupation behavior in outbred mice. Furthermore, we posit that a lack of familiarity preference in inbred strains may be explained by a failure to mount a stress response to the presence of a stranger. Follow-up experiments will need to confirm our hypothesis.
Previous reviews have characterized stress as a potential confound in current autism assays (Kas et al., 2014; Silverman et al., 2010b). Although the TCOT is not immune to modulatory effects of stress activation we suggest that stress is not a confound, but instead plays an integral part in social interaction in animals. Even if we concede that stress is a confound in sociability testing, however, we argue that stress most likely plays a greater role in previously validated sociability assays given their relatively short (~10-20 min) habituation period prior to testing; by allowing animals to freely interact for 60 min prior to data collection, we believe the TCOT provides an extended period of habituation than other tests.

6.3.3 Pain

When both animals receive an inflammatory or neuropathic injury, strangers (but not siblings) significantly increase subsequent tube co-occupation behavior. Furthermore, these observed changes are not due to changes in locomotor activity (**Chapter 5**). It is important to note that our initial characterization leaves many questions unanswered. For example, we do not yet know whether the presence of pain in one animal impacts subsequent TCOT interactions, similar to what we observe in murine pain-related social contagion (Langford et al., 2006). We would also like to study how pain changes social behavior among inbred strains and preclinical autism models. Nevertheless, we believe our data demonstrate that the TCOT can be used to study how pain can modulate social behavior.

6.3.4 Age

One final modulator of tube co-occupation behavior is age. Reports using older mice in the three-chambered test are conflicted, with Crawley's group showing that older C57BL6/J mice are no different from younger mice in stranger novelty behavior (Nadler et al., 2004). However, a follow-up study shows that male C57BL6/J mice spend less time interacting with a stranger after they are allowed to freely interact in the three-chambered testing apparatus (the stimulus animal was removed from the cup) (Sankoorikal et al., 2006). Our own data suggest that age leads to a marked decrease in tube co-occupation behavior, with older (12-16 week) mice showing significantly less tube co-occupation than younger (6-8 week) animals. However, older animals still reliably demonstrated familiarity preference.

We do not yet know why older animals displayed decreased social behavior in the TCOT. One potential explanation is a change in older animals' stress activation, so that social and environmental stressors introduced in the TCOT elicit a different response. For example, older

animals may be less intimidated by our testing apparatus over time. One study finds that older (4 month-old) C57BL6/J mice show significantly more exploratory behavior in the open field test than younger (4 week-old) mice (DeFries and Weir, 1964). Alternatively, older animals may simply be less social than younger animals, as shown in similar human studies (for a review, see (Singh and Misra, 2009). In line with this hypothesis, one study used the social interaction test to report a decreasing trend in more complex social behaviors among aging rats as compared to young adults (Spruijt, 1992). Specifically, the author finds that while young rats are more influenced by partner behavior older rats are far less influenced by the behaviors of their social partners. While tube co-occupancy may not be indicative of mature rodent social behaviors, additional studies could further identify mechanisms explaining why older mice are less inclined to stay in close contact with one another over time.

6.4 Factors that do not affect tube co-occupancy behavior

It is surprising that sex differences do not appear to play an important role in the modulation of tube co-occupation behavior in the majority of our experiments (except for our initial characterization of siblings weaned apart and then reintroduced three weeks later in the TCOT), given that our lab's previous findings. Specifically, previous work reveals that female mice demonstrate significantly more social approach behavior to a familiar conspecific in pain (Langford et al., 2010). Furthermore, sex differences in coping mechanisms following acute activation of the stress system are well-characterized in mice (Taylor et al., 2000). Rather than adopting a "fight-or-flight" response originally characterized by Cannon (Cannon, 1939), Taylor and colleagues show that female rodents adopt an alternative "tend-and-befriend" behavioral response to acute stressors (Taylor et al., 2000). The sex-specific stress theory is attributed to

differential drives, whereby females are more likely to group together in response to stress to protect offspring, while males are primed to fight or flee in more antagonistic same-sex interactions. Indeed, several reports show that females find crowded home cage environments to be anxiolytic, in contrast to males (Brown et al., 2003; Brown and Grunberg, 1995). We note, however, that many of the stressors used to characterize sex-specific differences in animal coping mechanisms have relied on environmental stressors, including the presence of a predator or following chronic restraint. We also failed to find any studies that showed sex-specific differences in social behavior following social stress. In contrast, our study uses social stress (the presence of an unfamiliar mouse) to drive observable differences in tube co-occupancy behavior. Although sex differences in other stress-related coping behaviors are well-known, it may be that male and female mice are more alike in propinquity over a prolonged testing period.

Also surprising is the fact that mice do not fight with one another in our test. Our findings contrast with previous reports using other sociability measures (Moy et al., 2008). We believe that the relative novelty and high degree of averseness characterizing our testing environment may contribute to the low levels of observable murine aggression. This was one of the considerations we had in deciding to forego an environmental habituation prior to running mice together in our assay. Indeed, previous reports show that increased environmental novelty leads to diminished levels of aggression (Lister and Hilakivi, 1988; Miczek, 1987). Conversely, familiarity also appears to decrease aggression in mice (Parmigiani and Brain, 1983). In our prolonged testing sessions, the limited levels of aggression diminished further as mice became more familiar with one another.

6.5 A relationship exists among stress, pain, and familiarity-related behavior in outbred mice

Based on our reported results, we believe that familiarity, and the accompanying degree of socially-mediated stress response, dictates tube co-occupation behavior among outbred and re-derived, "wild" mouse lines. Specifically, co-housed animals are more willing to reside in close proximity to one another for extended periods of time in a "safe" tube than strangers, demonstrating a clear preference for familiarity in our assay. By controlling for the degree of stress activation, we find that we are able to selectively modulate subsequent tube co-occupation behavior. Furthermore, the presence of pain increases stranger co-occupation rates, eliminating the otherwise robust familiarity preference that we observe in pain naïve animals. While we do not yet know the mechanism by which pain significantly modulates subsequent social behavior, it is possible that the presence of a pain state affects stress activation. Speculating further, animals may adopt different coping behaviors following injury. For example, the presence of pain may decrease a mouse's willingness to engage in aggressive posturing, leading to lower social stress in stranger dyads.

6.6 The need for new autism-like models

Based on our efforts, we believe that there are several major problems associated with current animal autism assays (**Section 1.3.1**), including short testing sessions, lack of free interaction between animals, and an overreliance on a limited number of social behaviors. Perhaps the most important confounding factor, however involves the sole use of inbred animal models. In an effort to increase experimental reproducibility, virtually all research in modern medical science uses genetically identical inbred mice, or mutant mice based on inbred genetic

backgrounds. Authors assume these mice consistently display “normal” levels of social behavior; as a result these animals are often used as control strains for genetic studies. In our hands, however, inbred animals consistently show high levels of gregariousness, regardless of the social environment. Compared to outbred and wild mice, our results using inbred models suggest that social behavior in these strains is not representative of mice in general. Instead, it appears that inbred animals have been artificially selected for unusual social gregariousness such that the familiar versus stranger preference—typical of outbred and re-derived wild mice—is absent in these strains. As a result, “deficits” observed in autism-like models may in fact be normal levels of social interaction, appearing to be low because control groups demonstrate abnormally high levels of social behavior.

One reason that preclinical autism studies rely on inbred mouse strains is to increase replicability and decrease variability in standard measures (Chia et al., 2005). However, by choosing inbred models, basic researchers decrease the generalizability of their reported findings. As heterogeneous disorders, ASD appear to be the result of a multitude of environmental and genetic factors that impact social interactions over time. By constraining experimental variables to use only genetically identical models or a single (brief) measure of sociability, current research fails to account for changes in social behavior among genetically diverse animals. Fortunately, the emergence of new genetic tools (e.g., CRISPR) allows scientists to evaluate the relative contribution of genetic background in sociability experiments. As neuroscientists begin to transcriptionally silence or up-regulate genes of interest in outbred mice, future experiments will allow us to directly test the contribution of specific genes to autism-like behaviors in more generalizable animal models.

6.7 The TCOT is a new tool for studying social behaviors in animal models

In summary, the TCOT is not designed as a way to circumvent current experimental methods. It is also not a replacement for previously validated tests. Instead, we believe the TCOT is a complementary tool designed to further our ability to characterize potential deficits in murine social behavior over an extended testing period. Our efforts lead us to identify a new measure of social behavior in both mice and rats pertaining to voluntary social proximity, or "propinquity." Although our findings pertaining to the homogeneity of social behavior among inbred mouse lines suggest there is a problem with using inbred models in preclinical autism research, we believe that future studies may find more consistent results by using more generalizable animal models.

Whereas having too many behavioral assays in social neuroscience can make comparisons between labs difficult, we argue that it is equally problematic to rely on a paltry number of standard approaches to characterize social behavior in animals. We believe that the current gold standard for mouse sociability, focused primarily on the exploration of a confined stranger over a brief interaction period, is a poor proxy for social behaviors in ASD populations (Gessaroli et al., 2013; Shah et al., 2013). We designed the TCOT to address several weaknesses we believe are evident in current sociability testing. Our result is a test that allows us to measure a novel social behavior between freely moving animals over an extended period of time. As such, our test shows differences in social behavior among multiple lines of outbred animals, as well as apparent deficits in social interaction among autism-like strains (with the caveat that these "deficits" may in fact be normal for the species, with inbred controls instead showing abnormally high levels of gregariousness). Finally, our automated test design is capable of

creating a high-through-put workflow that allows a user to quickly and reliably measure tube co-occupation over very long testing session among numerous testing rigs.

Although our present data identify a number of novel social phenomena (e.g., preference for separated siblings in females but not males, stress-dependence of social behavior, decreasing tolerance for co-occupancy with age) deserving of further study, we believe we have demonstrated that the TCOT can serve as a means to better characterize and measure indices of social behavior in rodents, including autism relevant preclinical models. Ultimately, methods like ours are needed to characterize new and more representative models of social dysfunction. It is our hope that these models will add to our collective understanding of ASD, as well as providing new ways to screen potential therapeutic interventions in an effort to treat these disorders.

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