THE UNIVERSITY OF TOKYO

# A NEVER-ENDING JOURNEY TO EXPLORE THE GENE AND ENVIRONMENT INTERACTION UPON AUTISM SPECTRUM DISORDERS

by

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I, Emir Turkes, declare that this thesis titled, 'A NEVER-ENDING JOURNEY TO EXPLORE THE GENE AND ENVIRONMENT INTERACTION UPON AUTISM SPECTRUM DISORDERS' and the work presented in it are my own. I confirm that:

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# *Abstract*

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Autism spectrum disorders (ASD) comprise a group of neurodevelopmental disorders that are characterized by impairments in social interaction and language and a restriction of interests with a tendency for unusually repetitive actions. In recent years, the neurohormone oxytocin has been of great interest for researchers, especially as a candidate treatment due to the fact that it can be easily administered in the form of a nasal spray. Abnormalities in the oxytocin system is implicated in ASD and is subject to complex interactions such as polymorphisms and parenting behavior. To examine a potential ASD model, I helped phenotype a mouse knockout model of the oxytocin receptor gene using the Intellicage group housing cage system, which allows for mass data collection while removing error from human interaction.

Looking at the first habituation period as a test of novel environment, we found many statistically significant differences within-group when comparing the first and second day of habituation, specifically when examining corner avoidance, total number of corner visits, tendency for mice to overtake a corner from another, explore all corners, explore all nosepoke areas, and travel diagonally. No statistically significant difference was observed between groups. Lack of significance may be reflection of a common gene-environment paradigm, where genetic risk factors may not present the expected phenotype without an environmental stimulus. As these mice were under no task and raised in low-stress conditions, there is reason to believe that potential ASD phenotypes were mitigated. Further examination of the habituation period is planned to better understand this prospective ASD model.

PyMICE, a new Python library for Intellicage analysis, was used for all results. All data analysis has been hosted on Github for free reuse by others. A Docker container for Intellicage analysis was also made to improve reproducibility of data.

# *Acknowledgements*

I would like to thank Dr. Ohsako for giving me the opportunity to study in Japan as well as Dr. Endo for providing access to and advice for using the Intellicage system. To all the wonderful people I have met here, thank you for the lifetime of memories.

# Contents

# Chapter 1

# Background

## 1.1 Autism Spectrum Disorders

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders that are estimated to have a prevalence of 62/10,000 people throughout the world[1]. The group is categorized by two main symptoms, impairments in social communication and language, and a tendency for repetitive behaviors and a small range of interests, though these two qualities vary wildly in severity [2]. With an unclear etiology and lacking an accepted biomarker, ASDs are currently diagnosed through observation. The disorder is 4.3:1 times more likely to be observed in males rather than females[3] and has been linked to gender dysphoria[4]. Interestingly, gastrointestinal and immune/inflammatory abnormalities have also been observed in patients with ASD[5]. Other commonly comorbid neurological problems are also linked to ASDs, such as epilepsy and mental retardation. As there is yet no cure, and it is ethically debatable whether there even should be[6], a large body of research into treatments for neurological disease have been targeted towards ASD or use ASD models. It is estimated that the cost of lifelong care for an individual with ASD is around $17,000 a year[7], therefore, there is massive incentive not only public health wise but also fiscally, to find ways to improve the independence and quality of life of these individuals.

As it can be reduced into a portable and fast-acting treatment method, the administration of oxytocin (OXT) in nasal spray form holds high promise as a potential avenue of medicine. And as a tool of basic research, OXT along with arginine vasopressin (AVP, together forming the OXT/AVP system) has been shown to be regulated by several important genes implicated in ASD, such as G9a histone methyltransferase[8], and so is of interest to epigenetics researchers as well. Therefore, the opportunity to phenotype a knockout mouse model of the oxytocin receptor (OXTR-KO), which has not been studied as highly, appeared to be a valuable pursuit on multiple fronts.

### 1.1.1 The Transition to a Spectrum

Autism, the foundation upon which its spectrum had grown from, has had awareness for

at least several centuries, though certainly not in the medical light that it is seen today. Neuroscientist Uta Frith has mentioned that autism can be observed throughout history, referenced for example, as the ”Blessed Fools” of Old Russia with peculiarities like insensitively to pain and lack of social awareness[9]. Likewise, seemingly every few months, discussions arise suggesting that so-and-so famous person from an older era was an autist, ranging anywhere from Socrates to Hitler. Interestingly, it can be argued that applying such diagnoses retroactively in this way has led to a distinct ambiguity in the term, which is ironically the very reason medical terminology exists at all! In other words, what is the true intended meaning when one utters the word ”autism”? As some would define it, it may not be a medical disorder in the traditional sense, but rather a part of the broader autism phenotype (BAP)[10].

Leo Kanner is commonly thought to be the ”founder” of autism, which may very well be true, except that as is known, autism was first defined for a symptom that Swiss psychiatrist Eugen Bleuler observed in his schizophrenia patients. That symptom was ”withdrawl from reality”. Not much seems to be known about the term until the work of Dr. Kanner in the 1940s with the seminal publication ”Autistic Disturbances of Affective Contact” (1943)[11], which went on to be one of the most cited in the twentieth century. In this study, Kanner used the term ”infantile autism” to describe eleven children in his care. A key differentiator in his usage was that in his view, autism is a disorder by birth, where the ”withdrawl from reality” prevents relationships from ever forming in the first place whereas schizophrenia is more of a step outside of reality from already existing relationships.

Kanner’s key observations included the children’s extreme tendencies to solitude, speech peculiarities, particularly “echolia”, the repetition of phrases in an inflexible/not-useful manner, and an obsession with sameness. All of these features remain well established trademarks of autism today. Also noteworthy were Kanner’s description of a few subject’s excellent rote memories abilities, having the ability to list objects of the most mundane variety, a trait commonly attributed to savants. Finally, he noted slight impairments in gait and relatively ”larger-than-normal” head sizes, the latter of which is becoming increasingly researched as a screening method for ASD[12]. Kanner’s legacy however, is slightly stained by his suggestion that parental coldness may contribute to

autism, which is a fair theory on its own but nevertheless led to the alarmist and politicized ”refrigerator mother theory”, comparable to modern day commotion over vaccine use.

Vienna pediatrician Hans Asperger had been using the term ”autistic psychopathy” in publications as early as 1944[2]. Ironically, Asperger had not coined Asperger syndrome, which he was immortalized to posthumously. What Dr. Asperger described in his patients was ”a lack of empathy, little ability to form friendships, one-sided conversations, intense absorption in a special interest, and clumsy movements”. Interestingly, some of his patients went on to be quite successful, though Asperger maintained the disorder was more of a detriment than a benefit.

It is likely that Asperger’s syndrome will become a mere footnote in history as time passes on. One of the most talked about aspects of the release of the latest Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) in 2013, was that it grouped together several neuropsychiatric disorders, including autism and Asperger’s syndrome, into one disorder, *autism spectrum disorder*[13]. For completeness, the other disorders are/were pervasive development disorder not otherwise specified (PDD-NOS) and childhood disintegrative disorder. In the previous iteration of the DSM, these disorders shared much in expected presentation and method of diagnoses but remained separate entities. As it seems, the APA has become to feel these differences were insubstantial, though it has been subject to criticism. It appears the new status quo will stick however, especially with studies that start to show biological similarities between the disorders. For instance, Duffy et al. in 2013 used EEG coherence to demonstrate that Asperger’s is more neurophysiologically similar to ASD than neurotypical (NT) populations[14].

For reference, here is the truncated, but word-for-word main diagnostic criteria for ASD in the DSM-5, and the definition for use in the rest of this thesis:

*a:* Persistent deficits in social communication and social interaction across multiple contexts

*b:* Restricted, repetitive patterns of behavior, interests, or activities

### 1.1.2 Diagnosing ASD

As there is currently no verified and accepted biological marker for ASD, behavioral assessments are the only means of diagnosis. As such, there exist a number of checklists

and questionnaires that are used worldwide for the diagnosis of ASD. It is ideal to diagnose ASD as early as possible as early behavioral intervention is associated with more favorable outcomes[15]. However, using current practices has demonstrated that it can be difficult to diagnose a child before the age of 3[16]. Furthermore, it occasionally happens that a neurotypical child is misdiagnosed with ASD, which can have damaging outcomes itself. Part of the problem is a lack of a standardization of methods and many competing standards. And as one review paper suggestions, few tests have been under rigorous testing to ascertain its effectiveness and accuracy. A paper from 2013[16], after reviewing the various methods for diagnosis, mentions the gold standard as being an MDT (multi-disciplinary) assessment followed by clinical judgment using DSM criteria, with the caveat of this being that it is expensive and difficult to coordinate. An alternative, the combination of ADI-R and ADOS, appear to show levels of accuracy comparable to the former and may carry the best cost/benefit ratio of the behavioral assessment. The administration of these sort of tests vary throughout the world. In the US for example, physicians are encouraged to screen for developmental delays at 9, 18, and 24 or 30 months[17].

### 1.1.3 Biological Basis

Findings from molecular, animal, and clinical research on ASD branch out in various ways. Across the different disciplines, approaches to uncover the etiology can be generally divided into something similar to the following fields:

*a:* Genetics *b:* Omics Research *c:* Neuropathology *d:* Neuroimaging *e:* Immune and Inflammation *h:* Hormones *i:* Epigenetics and environment

#### 1.1.3.1 Genetics

Some researchers approach ASD from the mindset of traditional genetics, although most if not all also recognize the influence of environmental factors through epigenetics. Focusing on genetic aspects of ASD is not unwarranted at all, especially if one begins by examining twin studies, which can provide the clearest evidence of heredity in a trait. Several well-known twin studies have approximated ASD's heritability to be around 90% in monozygotic twins[18], though it has been contradicted, for instance in a landmark 192 twin study which estimated a much more modest 38%[19]. Additional support for the genetic basis of ASDs come from separately classified diseases of traditional Mendelian nature that carry strikingly similar phenotypes to ASD. The most famous example, which

is also the most common known form of non-idiological ASD, is Fragile X syndrome (FXS). A sex-linked disorder, FXS is caused by a mutation at the FMR1 gene[20] and has become of the most popular mouse models, especially as a baseline to compare new ones. Other well-known genetic diseases that overlap will ASD include Rett syndrome[21], a mutation of the MECP2 gene, and tuberous sclerosis[22], a mutation in the TSC genes. However, ASD that can be attributed solely to a single gene only account for an estimated 1% of all ASD cases[23], so consensus has shifted to ASD being largely of a polygenic basis. This has promoted sweeping genome-wide association studies (GWAS) in order to uncover as many related genes, polymorphisms, and copy number variations (CNVs) as possible, though it has been believed for years that we are still at the low end of knowledge[24]. To-date, there are 990 genes, 2223 CNV loci, and 1165 animal models that are curated on SFARI, a database for genes implicated in ASD susceptibility.

Twin Studies

Currently, the largest ever autism twin study dates to 2015, which used data from England and Wales to examine an astonishing 258 pairs[10]. As expected, monozygotic twins scored high in heritability estimates, from 56% to 95%. The novel finding of the group however, was their estimation of additive genetic and environmental factors found by analyzing the covariance of ASD diagnostic test scores using a custom bivariate model. Ultimately, it was concluded that both play a role but genetic factors are estimated to have roughly twice the impact of environmental factors, which had a covariance of about 30%. This study made waves to those interested because prior to this, the largest twin study was the California Autism Twin Study (CATS) in 2011[19], by the now famous Hallmayer group, which estimated genetic factors as only contributing about 38% susceptibility while environmental factors accounted for more than half.

Monogenic ASD

It seems ASD is often separated into two factions, in this case, monogenic vs. polygenic ASD which respectively describes syndromatic vs idiomatic autism. There are some interesting patterns found specifically in monogenic ASD, for instance, dysmorphic (body altering) features are more common and the disorders more often have equal male to female ratios (barring sex-linked genes)[25]. Furthermore, single-gene ASDs are often found to be comorbid with ailments like epilepsy and developmental delay. It is also worthy to note that these genes are frequently shown to have variable penetrance. For instance whole-exome sequencing has revealed that around 25% of ASD individuals carry rare genetic mutations associated with syndromatic ASD, however, except in about 1% of cases, they are carried alongside other risk factor genes and may not present the typical

syndromatic phenotype[23]. Despite this, there are notoriously penetrant single-gene varieties of ASD such as neurofibromatosis type 1 (NF1), which was found to contribute to autistic behaviors in half the patients of a 531 patient cohort[26].

Polygenic ASD

The vast majority of implicated ASD genes contribute to a polygenic model of ASD, a situation where specific combinations of genes and mutations are required to bring about the autistic phenotype. Most large genetic screening studies today focus on this area of genetics to tease out common variations that are associated with ASD. In recent years, copy-number variations, the variable repetition of sections of the gnome have also been a popular topic and is linked to ASD as well[27]. The combination of common polymorphisms, copy number variants, and chromosomal aberrations are often described together as genetic risk factors for ASD. In general common variants have a lesser chance of deleterious effects than rare ones, particularly de novo variants due to having never been exposed to evolutionary selection[28].

A key point when discussing polygeneic ASD is that the combination of genes involved typically converge upon an important pathway, typically during early developmental stages. Some common genes in this category include genes that involve synaptic growth like NLGN3, NLGN4X, and SHANK3[28]. Analysis of rare CNVs have also been shown to involve synapse formation and maintenance, causing this pathway to be of leading interest to researchers. In such situations, it is often the case that insufficient pruning can lead to an excess of synapses[29]. Other pathways that are also implicated include those related to neurogenesis, cellular proliferation, ubiquitin signaling, astrocytes, microglia, and inflammation.

#### 1.1.3.2 Omics Research

Omics research includes the fields of proteomics and transcriptomics. Typically, methods like mass spectrometry are used to analyze endogenously produced molecules that may serve as biomarker for ASD[30]. Some groups analyze the blood serum of individuals, for instance, to find raised levels of the proteins apoA1 and apaA4, which are now linked to ASD[31]. This group also found elevations in PONI which is associated with detoxification and the treatment of oxidative stress, providing hints towards that pathway's potential involvement in ASDs. Omics research is often combined with other methods, leading to the term "multi-omics research". An example combining proteomics with traditional

genetics and epigenetics is a protein analysis study done with Fragile X to analyze methylation levels in blood as a potential biomarker[31].

Transcriptomics using methods like quantitative reverse transcription PCR and microarrays have also been valuable tools for measuring gene expression under different time points and conditions. For instance, RNA sequencing was able to demonstrate dysregulated splicing of A2BP1-dependent exons in ASD brains[32]. As A2BP1 is a susceptibility gene for ASD, such studies help identify the mechanism of action of these genes, which in this case is splicing dysregulation. Methods like RNA-seq can also be used to identify differentially expressed genes such as Myelin and Lymphocyte Protein, which has shown altered expression in psychiatric patients[33].

#### 1.1.3.3 Neuropathology

Neuropathology studies typical involve post-mortem analyses of ASD brain tissue. This method has been particularly employed in the study of the cerebellum and its relationship with ASDs. As the cerebellum contains the densest arrangement of neurons and synapses in the brain[22], analyzing its synaptic structure is of high value to a neuropathologist. Abnormalities in cerebellar activity, especially due to the loss of Purkinje cells, is commonly cited to support the excitatory:inhibitory disbalance theory of ASD. As its name suggestions, this theory posits that alterations in glutamate and GABA (released by Purkinje cells) activity can alter calcium signaling in the brain, ultimately altering its function[34]. In a more obvious way, neuropathology studies have also reported larger head size of those with ASD, though debatable[12]. Even more significant are post-motem analyses of gene expression, which has shown for instance, abnormal PTEN and RELN signaling, both hypothesized to overspeed early growth of the brain, an increasingly common proposed etiology for ASD[35].

#### 1.1.3.4 Neuroimaging

Techniques like functional connectivity MRI have revealed associations of oxygenated blood activity (BOLD) to specific regions of the brain such as hypoactivation of the prefrontal cortex[36], an area regarded as playing important roles in executive control. fMRI faces a fair bit of controversy however, as the use of BOLD signal as a proxy for brain activity is by nature indirect and in lieu of a better method. It is also difficult to have patients sit still in the uncomfortable scanner, particularly ASD patients. Nonetheless, fMRI is being frequently researched as a potential method for revealing noninvasive

biomarkers. DTI has revealed some interesting information as well through tract tracing of white matter. This has provided insights such as that higher concentrations of local synapses rather than long range are frequently found in ASD patients[37].

#### 1.1.3.5 Immune and Inflammation

Increased oxidative stress has become of increasing interest to researchers. It is already common knowledge that Reductive Oxidative Species are highly linked to such things as DNA damage but now research is also being conducted that links it to disorders like ASD[38]. Likewise, a large number of genetic risk factors have been traced to immune and inflammation pathways. Interest in this domain have also led to interesting new animal models, such as the Poly I:C model, which mimics viral immune response[39]. Exposure to such chemicals while in the womb or during early development has been linked to ASD-like phenotype. Finally, increases in cytokine activity in the brain has long been held as one of many commonly found traits of ASD[5].

#### 1.1.3.6 Hormones

Some well-known hormones implicated in ASD are oxytocin and vasopressin, which also happen to play a role in the experimental section of this thesis. These hormones are similar enough in form that they sometimes bind each other's receptors[40], hence they are frequently discussed in union. OXT has a reputation for being the sociability hormone and low levels of its expression has been link to abnormal social behavior, aggression, and competitiveness both in humans and rodents[41]. Besides endogenous hormones, exposure to elevated levels of steroidal hormones within the womb, such as progesterone, testosterone, and cortisol have also been linked to ASD [42]. The activity of hormones, whether endogenous or exogenous, can also act upon risk factor genes in addition to typically downstream and secondary messenger effects. An example being RORA, a candidate gene differentially transcriptionally regulated by male and female sex hormones [43].

The Oxytocin Receptor

Gimpl et al. provides the highest cited in-depth review of the oxytocin receptor (OXTR) though it dates to 2001[44]. Classified as a neuropeptide, OXT consists of two amino acid components, a six-amino acid cyclic structure and a three-amino acid tail. AVP differs in only two amino acids, phenylalanine and lysine at positions 3 and 8 in the

amino acid sequence respectively. This relationship is also evolutionarily conserved, with virtually all major vertebrates containing an OT-like and vasopressin-like peptide. One of the many reasons these two neuropeptides are also grouped together functionally is that they are the primary secretions of the posterior pituitary gland (AKA the neurohypophysis) [45]. In the case of OXT, this secretion is implicated in stress, reproductive, maternal, and social bonding, acting primarily upon the hypothalamus and limbic system. OXT also acts locally, in the paraventricular nuclei (PVN) and supraoptic (SON) nuclei within the magnocellular nuclei of the hypothalamus [46]. OXT activity is particularly active in this region during lactation and parturition.

OXTR is considered a type of G-protein-coupled receptor of the rhodopsin-type class I family, while the gene, OTR, is found as a single copy on chromosome 3p25 [47]. Interestingly, while loss of this gene typically results in deleterious effects, ranging from mental retardation to hypotonia, there is a clinical report of two individuals with such a deletion showing only mild clinical effects, a reflection of a greater phenotypic spectrum in the disorder [48]. Rodent research has been instrumental in finding clues to the molecular pathways involved with OXTR, where the receptors have been found in brain regions that are a part of the social behavior neural network (SBNN), the preoptic area (POA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), the periaqueductal gray (PAG), and the bed nucleus of the stria terminalis (BNST) [41]. One theory posits that output of the SBNN, which extends to a variety of social and reproductive linked regions, is regulated by OXT and AVP [41].

In 2016, Heather Caldwell provided an excellent review of the use of knockout mice in exploring OXT and OXTR [49]. She cites several key areas in which KO mice of the oxytocin system have shown phenotypic differences; social recognition memory, maternal behaviors, and aggression. Though less often stressed and cited, there have been reported differences in various nonsocial areas as well, like feeding, anxiety-like behaviors, and nociception. There are several mouse lines from which this data comes from, the first of which were two OXT-KO (Oxt -/-) mice developed from different background strains. The Baylor/Emory line was derived from 129SvEv C57BL/6J[50] mice and the NIMH line from 129X1/SvJ C57BL/6J[51]. Following this, several OXTR-KO (Oxtr -/-) lines were created starting in 2005 with the Nishimori line of 129Sv C57Bl/6J. An NIMH line using this same background strain was developed a few years later[52], along with floxed OXTR lines that use Cre recombinase to primarily target the forebrain (Oxtr FB/FB)[53]. In these lines, Cre recombinase doesn't elevate until

21-28 days after birth, so with the later onset of conditional knockout, the effects of early developmental compensation are somewhat compensated for.

Regarding social memory, both ligand and receptor knockouts show deficits in the form of longer times in the five-trial habituation-dishabituation test[49]. This is a test that measures investigation times when presented with a stimulus mouse, where a familiar mouse should result in a shortened investigation time. These deficits can be seen in both male and female mice, though lower rates of investigation in general in females necessitate different methods be used, like the Bruce Effect [54]. Oxtr FB/FB show a phenotype more similar to wild-type in some respects, such as a decrease in investigation with further habituation. In the shortened and modified two-trial test however, deficits could only be seen in intrastrain social memory, suggesting OXTR's purpose as being more for fine-scale recognition rather than broad [55].

Intermale aggression has been a consistently seen effect in both OXT-KO mice when placed with wild-type mice in recent studies, however early studies reported the opposite[49]. An interaction that resolves this conflict is the finding that OXT-KO mice born to OXT-KO dams display greater aggressive behavior. OXTR-KO mice were also found to not have strain related differences in aggressive behavior, whereas wild-type mice showed greater aggression to different strains [56]. This is supportive of OXT's hypothesized role in humans as being for group bonding and out group competition [57].

#### 1.1.3.7 Epigenetics and the Environment

Epigenetics is the alteration of DNA through means other than mutation, including modifications such as methylation at cytosine sites and acetylation of histone proteins which tightly regulate gene expression[58]. Concerning gene-environment interactions and ASD, it is important to realize that expression of a gene is as important as the gene itself. It is one reason why carrying risk factor genes on their own may not produce the linked phenotype. However, given the right environmental factor, abnormal transcription of certain genes, particularly during sensitive critical periods of early development can lead to permanent abnormalities.

Besides epigenetics, the environment can induce abnormal development through typical toxicological means. For instance, air pollution has been linked to ASD[59]. Finally, the endogenous environment, namely gut microbiota is a currently trending topic in ASD research today. There is increasing evidence that supports the gut-brain axis as having a

powerful influence in brain health. Unusual gut microbiota have been found in ASD patients, [60], but the exact relationship between the two is still not very clear.

Gregory et al. showed in 2009 that OXTR can be silenced by DNA methylation, particularly through hypermethylation of the promotor region which was found in autistic cohorts compared to controls[61]. This finding suggests a very straightforward mechanism to oxytocin deficiency. In 2012, the role of OXTR hypermethylation was further delineated with the finding that it is associated with activity in brain regions believed to be functionally related to Theory of Mind[62]. A very recent study from 2017 found associations with adulthood promotor OXTR methylation and childhood levels of anxiousness [63]. This is a significant finding in epigenetic mechanisms of early life adversity and the sensitivity of critical periods in ASD. Several other studies have also linked specific polymorphisms of the OXTR gene with environment interactions, such as the impact of different cultures[8], and parental stress[64].

# Chapter 2

# Experiment

## 2.1 Objective

This research attempts to reveal phenotypic differences between the oxytocin receptor knockout (OXTR-KO) mouse and wild type mice while maintaining high reproducibility. As a novel method for phenotyping this line, the Intellicage automated home mouse cage was used, as detailed in the Methods section. The scope of analysis was restricted to the mouse's first introduction to Intellicage as a measure of reaction to a novel environment. This time period was chosen for it's lack of studies in comparison to traditional task-related studies in Intellicage. Since few analysis methods exist for this time period, a primary aim was to create reusable pipelines for this study and future studies. These pipelines intend to measure ASD-like phenotypes as well as general baseline activity. It was hypothesized that between group differences, amongst homozygous knockout, heterozygous knockout, and wild-type mice would be either initially, or over the time course of the novel environment situation, significantly different. As this is a situation involving a gene knockout model and an environmental stimulus, it can be considered a study of gene-environment interactions. Within group differences were also of interest as a general measure of the time course to the mouse's introduction to the novel environment.

## 2.2 Materials and Methods

### 2.2.1 Animals and Facilities

All the animal behavioral experiments were conducted at the laboratory of Phenovance Research Technology LLC (Chiba, Japan) and the experimental protocols were approved by the Animal Care and Use Committee in the company. The wild-type, and hetero-, and homozygously oxytocin receptor gene deficient mice with C57BL/6N background were obtained from RIKEN BioResource Center (Strain name: C57BL/6NOxtr<-tm1(KOMP)Vlcg/AC7; *n* = 11 homozygous knockout, *n* = 10 heterozygous knockout, *n* = 11 wild-type). Immediately upon receiving the mice, RFID implantation (Datamars SA) was conducted using 0.2 mL pure isoflurane per mouse as an anesthetic. The RFID chips

provided a unique ID code for each animal. The mice were sorted equally into normal cages and stored in the animal room, housed at 22 ± 1 °C, 50 ± 10% humidity, and 12h LD cycle (lights on 8:00-20:00).

### 2.2.2 Intellicage

Intellicage[65] is a computer controlled automated home mouse cage for data analysis with minimal human intervention. It is basically a large 55 × 37.5 × 20.5 $cm^3$ plastic cage containing four 15 × 15 × 21$cm^3$ corners that function as operant learning chambers. After injection with an RFID tag, a mouse is identified by a unique code and can be tracked throughout the experiment. Upon entry into a corner, a ”visit” is recorded for that mouse, with additional details like the exact time point. Once inside a corner, a mouse then has the option to “nosepoke” into one of two holes, which are also detected. Past each hole is a water bottle equipped with a lick sensor to measure licks. Additional features include the ability to selectivity open and close nosepoke holes, trigger the activation of bright LEDs within the corners, and execute a short, uncomfortable, but harmless puff of air at the mouse within the corners. These features can be combined to simulate traditional behavioral testing methods such fear conditioning.

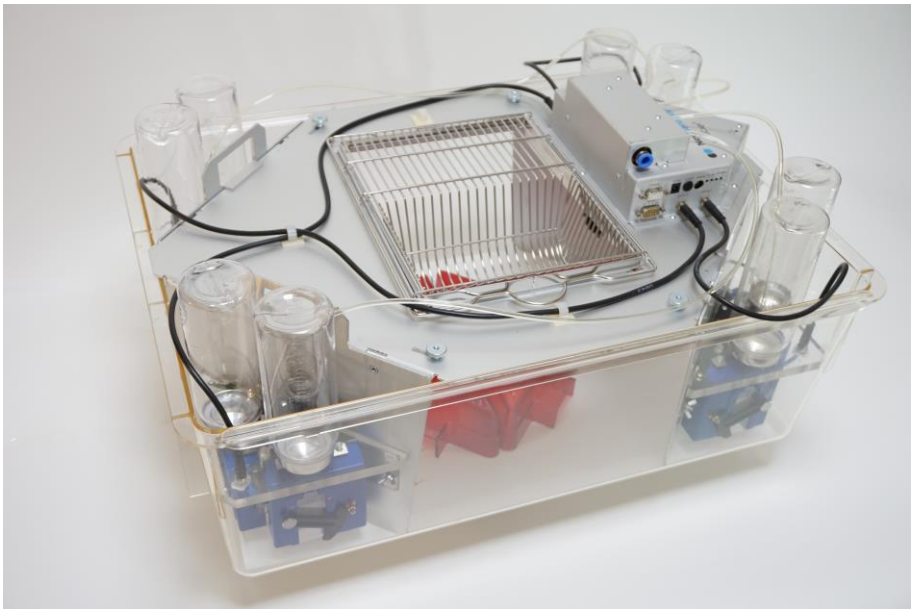

Figure 1. The Intellicage system. Dual water bottles can be seen at each corner and hopper for food is placed in the middle. The red object is enterable by mice and is provided for environmental enrichment.

Figure 2. A mouse entering a nosepoke door within a corner. Beyond the door is a water bottle. LEDs can be seen atop the nosepoke doors and the doors can be closed depending according to experimental protocol.

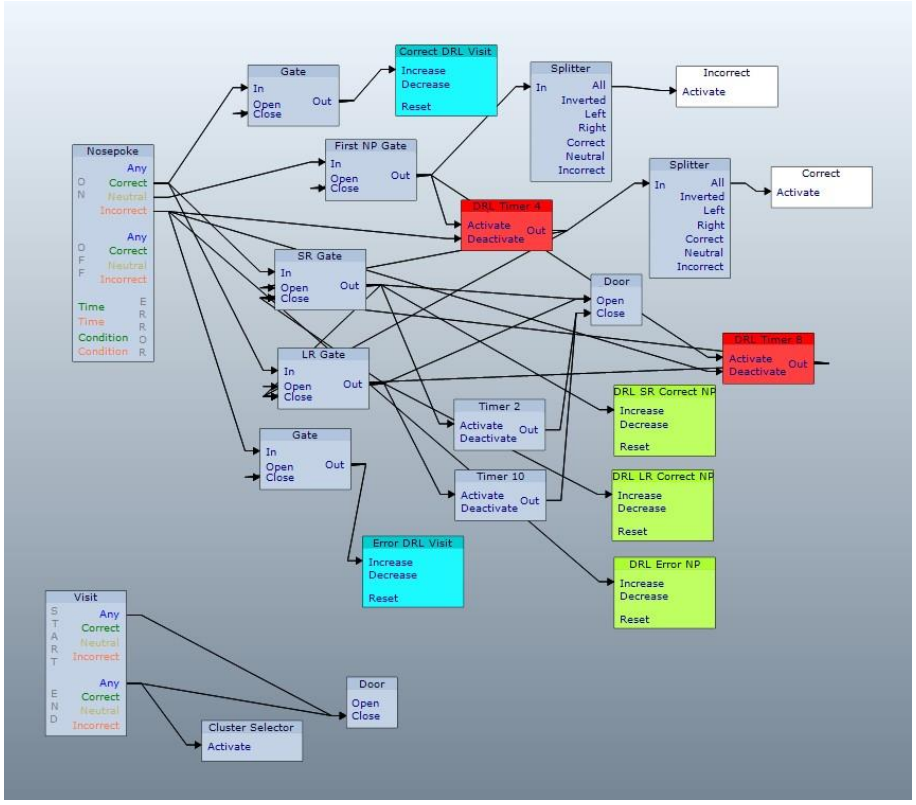


Figure 3. Designer, the program used to setup an Intellicage experiment. In order to illustrate the flexibility of Intellicage, shown here is a complex task.

### 2.2.3 Habituation Protocol

In the Habituation protocol, no distinct task is set for the mice. However, as Habituation 1 represents the animal's first experience within the cage, the protocol effectively represents a novel environment. All standard data collection features are active during this protocol, so corner visits, nosepokes, and licks can all be recorded and analyzed. The exact time period for Habituation 1 was defined as from 2017-03-20 11:02:07 to 2017-03-22 09:02:07. However, in order to obtain a more fine-grained analysis, Habituation 1 was split into two days, essentially: Day 1 = 2017-03-20 11:02:07 to 2017-03-21 09:02:07 and Day 2 = 2017-03-21 11:02:07 to 2017-03-22 09:02:07.

### 2.2.4 PyMICE

Typically, one has the option of analyzing Intellicage CSV files with Intellicage's built in software or using a third-party piece of software called FlowR, which is distributed from the makers of Intellicage. While both are powerful options, they are limited by a lack of user flexibility to customize and edit the workflows provided. There is also no easy way to share the analysis in plain text format, as is common in reproducible science using services such as Github.

This year, a Python programming language library called PyMICE[66] was released under the open-source GPLv3 license. This license forbids the closing of the software into proprietary products, encouraging active involvement from users in its development. Due to the appealing nature of the project and the desire for a 100% reproducible analysis environment, PyMICE was selected to be used for analysis. Only 1 paper had been published using it so far and the authors are the library's developers.

### 2.2.5 Docker Containers for Reproducible Research

A common issue facing data analysis is the "it works for me problem"[67]. Though analyses are frequently released for free access, with good intentions, it is often the case that the analyses cannot be reproduced with ease. In some cases, it may be impossible to operate it at all. In Python development, one solution is to use a tool like virtualenv or conda to set up isolated Python environments. By supplying a list of Python packages needed and versions, one can recreate the original test environment.

For small projects, this may be sufficient, but for larger analyses the cracks begin to show. A common problem is in machine learning environments because here it is necessary to have a properly configured GPU, which can be difficult to do and vary from system to

system. The solution decided on for this thesis' analyses is to do all work within a Docker container. A Docker container is like a virtual machine, or an operating system inside an operation system, but much lighter weight as it shares some low-level system functions. The result is a configurable environment that will run exactly the same on any operating system. To support its use in Intellicage projects, a custom PyMICE Docker container was made and uploaded to DockerHub, free to use for anyone who wants to.

### 2.2.6 Source Code Repository

All source code for this project can be found at the following locations:

https://github.com/eturkes/oxtr-ko_2017.03
https://hub.docker.com/r/eturkes/pymice-notebook/

## 2.3 Results

### 2.3.1 Standard Measurements Using Intellicage

These measurements had already been available for Intellicage through other software packages but were reimplemented here in Python

During Habituation 1, mice are free to roam the cage as they please with no restrictions of water or doors. The first data analysis pipeline created was one to extract the total number of corner visits from each group during Day 1 and then Day 2. SEM was calculated for each mouse individually and hypothesis testing was done at the group level. Each group was found to have a statistically significant drop the next day with the following *p*-values (WT = 0.00044063, HT = 0.00021065, KO = 0.01704563)

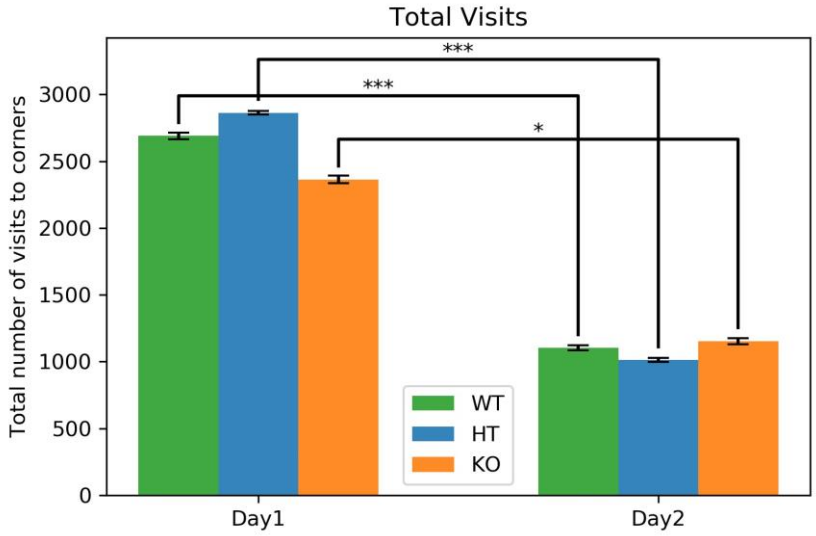


Figure 4. Total visits for all three groups in Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Next, the same analysis was carried out, this time measuring nosepoke events. In this analysis no comparison passed the 0.05 $p$-value threshold.

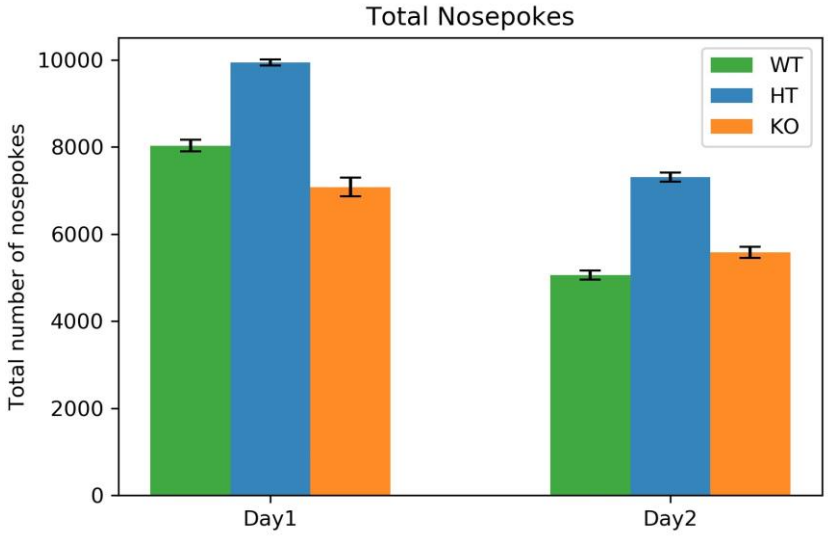


Figure 5. Total nosepokes for all three groups in Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

The next figure shows the total time spent making corner visits, by each group, in order to see if it follows a similar pattern to that of total visits. No comparison passed the 0.05 *p*-value threshold.

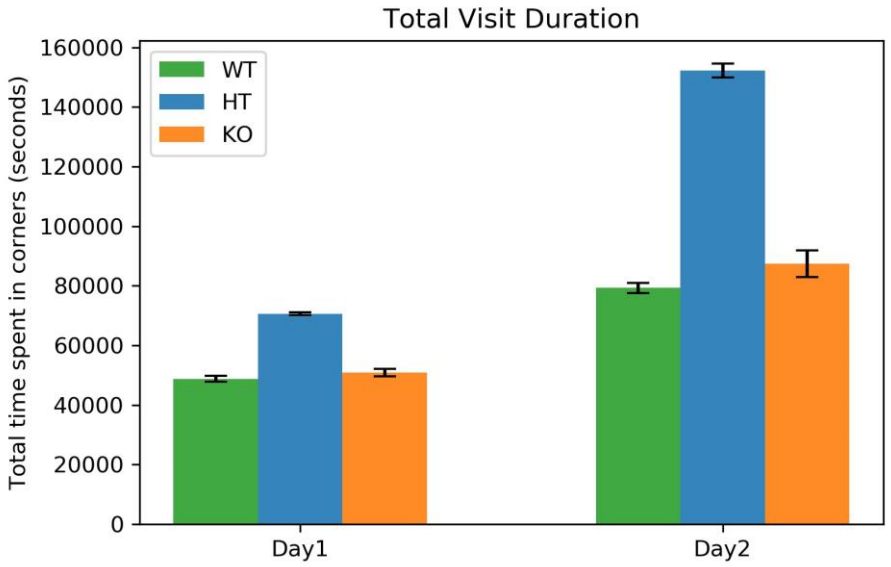


Figure 6. Total time spent visiting corners in Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n$ = 10. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

To see if the total time spent performing nosepokes was significant, we generated a similar figure for that measure. No comparison passed the 0.05 p-value threshold.

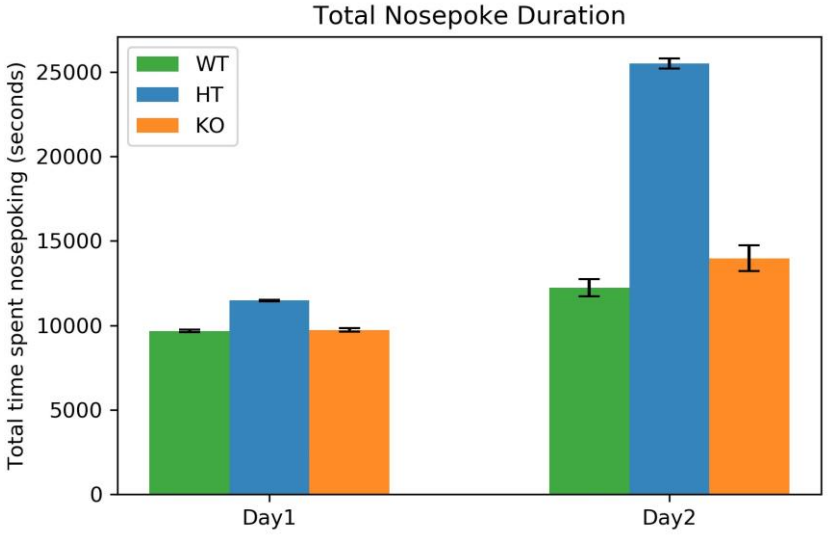


Figure 7. Total time spent nosepoking in Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n$ = 10. Hypothesis test was a Dunn's multiple comparison test, following a

Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

### 2.3.2 Newly Created Measurements for Intellicage

These measurements are new to the Intellicage ecosystem.

As a measure of exploration interest/ability, we measured how long it took for each mouse to visit every possible corner. This measurement showed statistical significance for all within group comparisons (WT = 0.00313921, HT = 0.00017433, KO = 0.00692773)

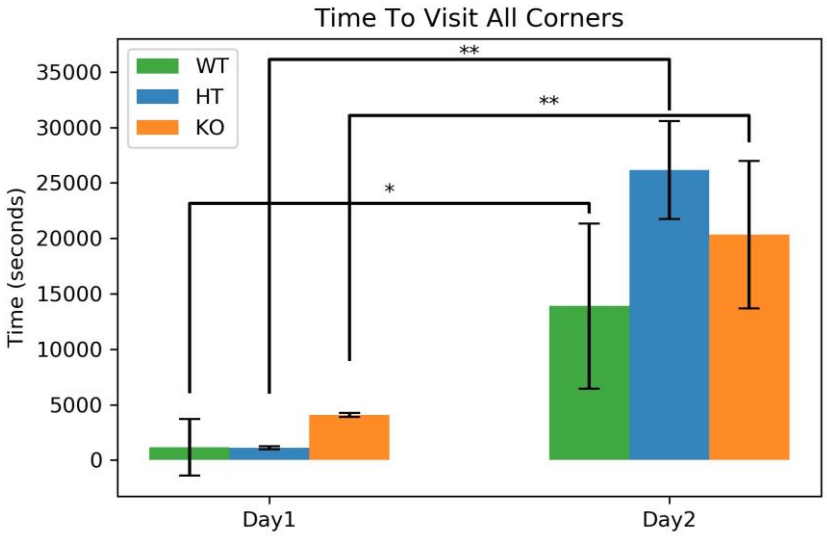


Figure 8. Total time taken until all corners were visited for Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Like previous measures, we extended the last measurement to also look at nosepokes. We found statistical significance for all within group comparisons again (WT = 0.00197925, HT = 0.0028661, KO = 0.04187801).

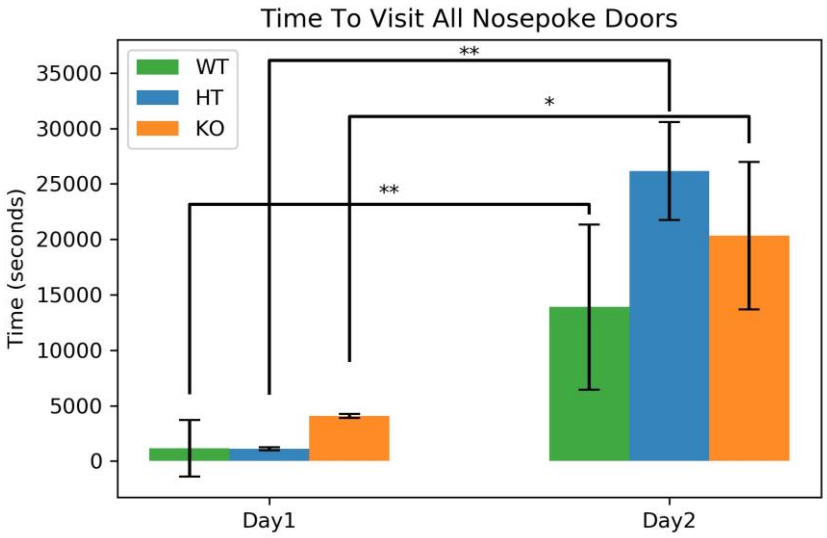


Figure 9. Total time taken until all nosepokes doors were entered for Day 1 and Day 2. Error rate was defined as mean ± S.E.M., $n$ = 10. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Looking at something a bit different, we tried to see if mice held any preferences for particular corners. To measure this, each mouse's most visited corner was found. Then, the ratio of visits to that corner vs. all other corners were calculated. This was presented as the percentage of time spent visiting that corner when making visits. Finally, these percentages were averaged across the group. No comparison passed the 0.05 $p$-value threshold.

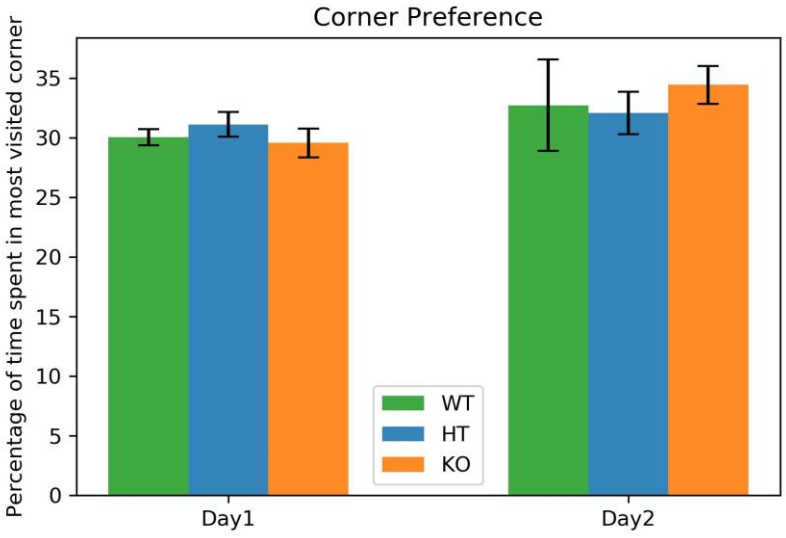


Figure 10. Ratio of visits spent at a mouse's preferred corner, averaged together as a group. Error rate was defined as mean ± S.E.M., $n$ = 10. Hypothesis test was a Dunn's multiple

comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Next, we looked at something very similar, the tendency for a mouse to avoid a corner which it does not prefer. The least visited corner was found and then the ratio of visits spent there vs. total visits were calculated. The percentage was then averaged as a group. There were two significant differences in the WT and HT groups (WT = 0.02412157, HT = 0.04824018)

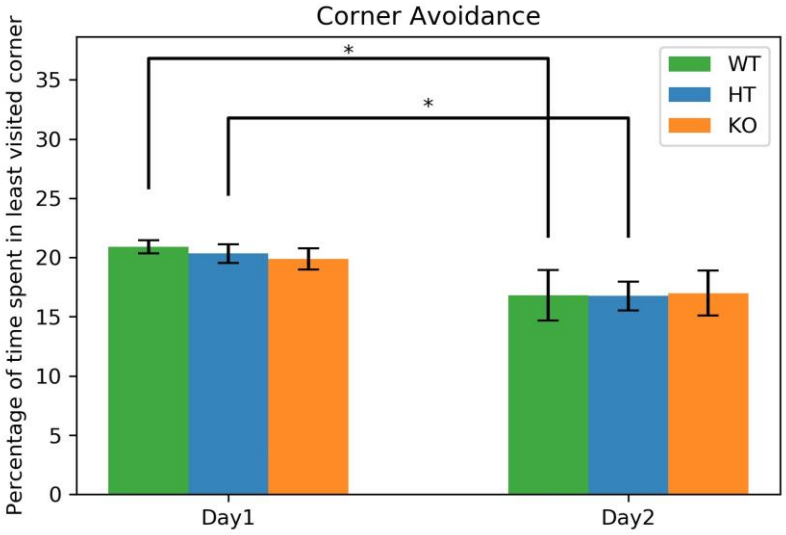


Figure 11. Ratio of visits spent at a mouse's disliked corner, averaged together as a group. Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

We measured diagonal visits as a variation of the open field test. A noted parameter included is that a visit must take place within 60 seconds. This is to prevent the counting of visits where a mouse is not actively trying to travel across the cage (such as eating the food source located in the center). Two significant differences were found (WT = 0.00122881, HT = 0.00855589)

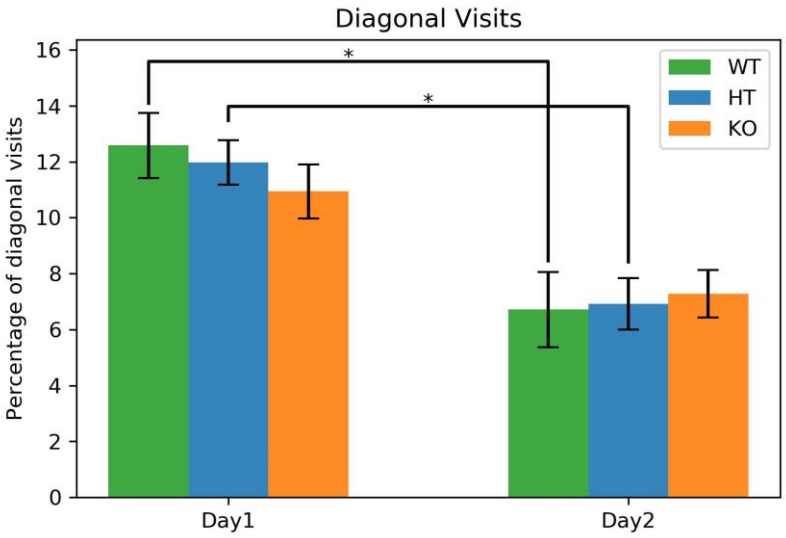


Figure 12. Percentage of total visits where the mice traveled diagonally within 60 seconds. Error rate was defined as mean ± S.E.M., $n$ = 10. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

This measure is effectively the opposite of the last as it looks at visits in a 60 second interval where the mouse either reentered its last corner or visited an adjacent one. The justification is that a more fearful mouse will tend to stay to the outer edges of the cage. No comparison passed the 0.05 $p$-value threshold.

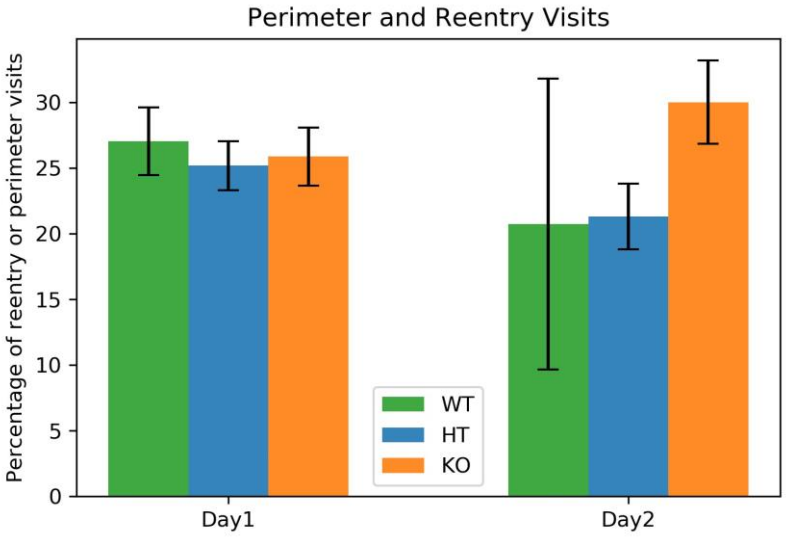


Figure 13. Percentage of total visits where the mice either reentered its last corner or traveled to an adjacent one within 60 seconds. Error rate was defined as mean ± S.E.M., $n$

= 10. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

This measurement attempts to examine aggression in the mice. It shows how many times a mouse entered a leaving mouse's corner within 1 second. All three within group comparisons were statistically significant (WT = 0.00027514, HT = 0.00023451, KO = 0.00449022)

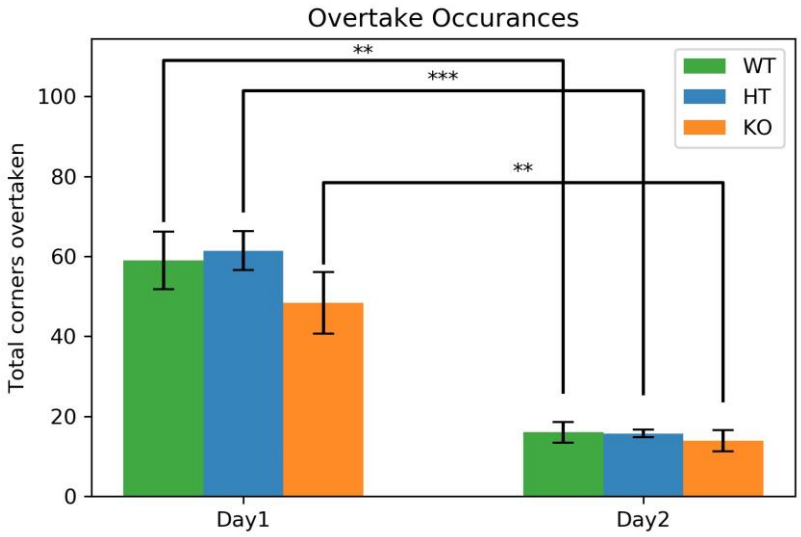


Figure 14. Times where a mouse overtook the corner of a leaving mouse within a 1 second interval. Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

The next measurement shows what percentage of all nosepokes were towards the left door. No comparison passed the 0.05 *p*-value threshold.

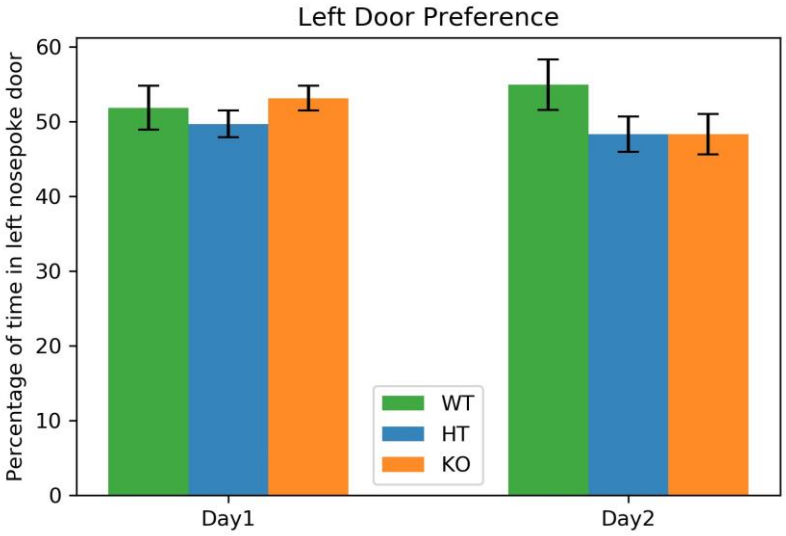


Figure 15. Percentage of nosepokes that were towards the left door Error rate was defined as mean ± S.E.M., $n = 10$. Hypothesis test was a Dunn's multiple comparison test, following a Kruskal-Wallis 1-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

The final measure was much different from the rest, it looks at the first 60 minutes of Habituation 1, cutting the time period into 1 minute bins and then finding the longest duration visit per group. This measurement may have the potential to measure competitiveness in the mice. No hypothesis tests or standard error was calculated for this figure.

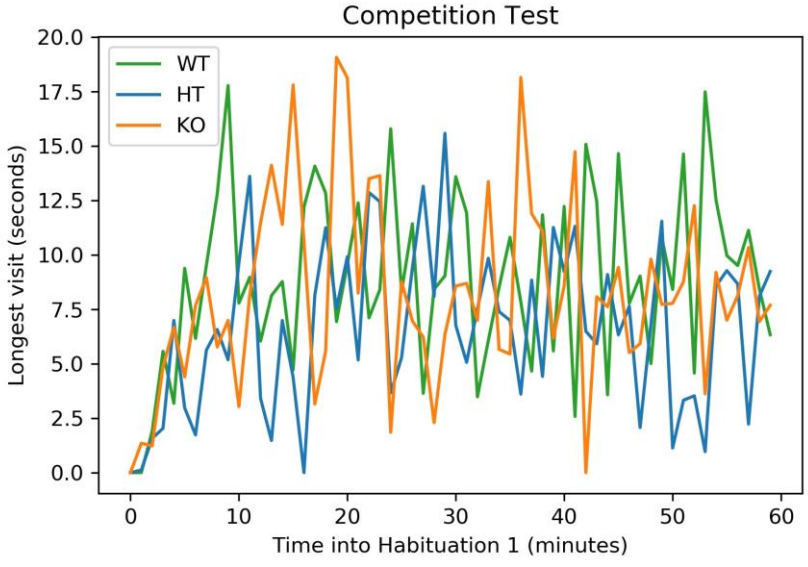


Figure 16. Longest duration visit by group over 1 minute intervals during the first 60 seconds of Habituation 1.

## 2.4 Discussion

The aim of this experiment was to find significant differences between the three groups, in particular to find ASD-like phenotypes. A secondary goal was to utilize the new PyMICE library and implement some common measurements conducted in Intellicage while crafting new ones thanks to the flexibility afforded by using a full programming language.

Regarding goal number 1, at present this study fails due to the fact that no statistical differences were found between the knockout and wild type groups. However, trends in the data could be found, for instance Figure 5 demonstrates a visual difference between the three groups, particularly in Day 1. Due largely to the S.E.M of that figure, there was not enough statistical strength to pass the threshold. An idea might be to look at the data again but using new statistical methods. The current method was believed to be adequate as it is an ad-hoc multiple comparisons test following the Kruskal-Wallis ANOVA, a non-parametric test which does not make assumptions of normality. Some data in this study failed checks for normality however, it is possible that parametric tests which are less reliant on normality, or transformation of the data, may warrant valid use of other methods.

Although it was not intended to be a goal originally, strictly focusing on the acclimation of the mice to Intellicage within the short two-day Habituation 1 period lead to useful results. In 6 of 11 figures where comparisons were made, there were at least one statistically significant result.

Regarding biological explanations of the data, one hypothesis is that there wasn't a suitably stressful environment to bring about a strong ASD-like phenotype in the knockout mice. As these results showed, the KO mice functioned rather similarly to other groups. It is possible however, that the tests performed here are not suitable as an indicator of ASD. Nonetheless, thought was given to try to target ASD-like phenotypes, such as through approximations of the open field test (Figure 12) and competition tests (Figure 14).

Concerning total visits and nosepokes (Figures 4-7), if lameness was a result of the knockout of the oxytocin receptor, then a relatively small amount of nosepokes and visits should be seen, though it could not be detected in our data. This would not be such an unusual situation considering that individuals with ASD are often comorbid with other conditions. Other small details could be subtle signs of ASD symptoms. For example, a mouse with many nosepokes, especially if frequently done to one corner over another could be a sign of repetitive behaviors and a small range of interest. Future examinations

of this data looking into such ideas in a more sophisticated way may reveal these differences.

The ”Time To Visit All” figures (Figure 8 and 9) were specifically created to examine exploration habits. It is imaginable that a healthy, curious mouse would quickly explore all corners. Indeed, the data significantly showed how quickly that task was accomplished in Day 1 compared to Day 2. This may be a robust indicator to demonstrate activity in a novel environment.

Corner preference and avoidance (Figures 10 and 11) also may indicate obsessive tendencies. Though our measurement lacked significance, a more robust way may be to look at preference in both directions within one measurement. For instance, any deviation from a chance visit to a corner (chance being 25% of all visits), can be summed as an absolute value. By effectively combining preference and avoidance, differences between groups should gain statistical power.

Diagonal visits (Figure 12) attempts to be a type of open field test. It is hard to know whether a mouse truly traveled directly across the cage to make the visit, however, by restricting the time period to 60 seconds, direct travels are more likely. In any case, a decrease was seen from Day 1 to 2. This likely represents a relaxation in exploration behaviors as 60 second or less visits become less frequent. The perimeter test (Figure 13) is the opposite form of the diagonal test. It is debatable whether to include reentry visits, but it seems reasonable that a fearful mouse is also likely to retreat into a corner as much as traveling along the sides of the cage.

Overtake occurrences (Figure 14) was measured to look at aggression in the mice. As mentioned, an overtake takes place when a mouse enters a leaving mouse’s corner within 1 second. A mouse that frequently commits overtakes, especially if statistically significant against another group, may likely be due to aggressive behavior, such as fighting over a corner. Such situation seldom happens in Intellicage, but a hypothesis we had was that the KO mice might be particularly aggressive, though it could not be demonstrated in this measurement.

Left door preference (Figure 15) is similar to the corner preference test (Figure 10). It is interesting to see that the WT group visited the left corner slightly higher than 50% of the time compared to all other doors as mice as noted for having leftward preferences. However, hypothesis testing was only done within and between groups so it is not certain that this trend was not due to chance.

The competition test in this study does not appear to reveal anything useful, it may have to be refined to obtain more practical results. In particular no error or hypothesis tests

were used. This plot has actually been generated in the lab before using other means, but it is not part of any standard Intellicage software set.

Conducting this analysis using Python was quite challenging as the PyMICE API requires some abstract thought to use. After a steep learning curve however, the method appears highly flexible. Further analysis of this data set using more sophisticated tests are planned. Another advantage of using Python as an analytical tool is excellent support for machine learning libraries such as Tensorflow. A common competitor, R, is limited in that regard due to not being a complete general purpose programming language and in fact cannot run multicore processes which may become necessary for more computationally difficult tasks.

The use of Docker for this experiment was also a key point. This system can guarantee reproduction of the data and provide quick access to the development environment from any computer. To compliment this technology, other advanced workflow methods were used such as Github which allows fine-grained version control of all work. This means that projects have the potential to become collaborative efforts as users split the work into their own custom branches.

# Chapter 3

# Conclusion

Although this study failed its main intended purpose to show ASD-like phenotypes in the oxytocin receptor knockout group, it was a good opportunity to learn and build good habits as a researcher. One of the key points when it comes to research with Intellicage is reproducibility and reduction of error. A strong effort was made to carry this over to the data analysis side by using scripting tools that can be verified for accuracy. This is true of this thesis as well, which was written in latex, a first for me. Several other technologies were also new, for instance Docker and Github, but I have gained confidence in my ability to employ these effectively in future projects.

This study was limited by several points. First of all, the sample sizes weren't great, with 10 animals per group. Second of all, this analysis only looked at the Habituation 1 period. Although this period has a lot of potential for analyzing baseline activity, especially for a gene-environment interaction models, some kind of stimulus may be necessary to bring about a phenotype that can be measured. In the future, we plan to create more unique tests using PyMICE especially for task-related data.

# Appendix

## PyMICE Dockerfile

```
FROM jupyter/minimal-notebook:2c80cf3537ca

LABEL maintainer="Emir Turkes eturkes@bu.edu"

# Run unprivalaged
# Variable, referring to configured user "jovyan", is derived from base image USER $NB_USER

# Install Anaconda into a new conda environment
# Remove conda-forge for pure upstream Anaconda
RUN conda config --system --remove channels conda-forge \
    && conda create -yq -n pymice Python=3.5 Anaconda

# Install PyMICE into newly created conda environment
# Conda does not support sh, so use bash
RUN /bin/bash -c "source activate pymice \
    && pip install -q --exists-action w PyMICE \
    && source deactivate"

# Configure notebooks to strip output before saving to improve version control
RUN mkdir /home/$NB_USER/.jupyter
COPY jupyter_notebook_config.py /home/$NB_USER/.jupyter/

# Ensure container does not run as root
USER $NB_USER
```

## Code for Figure 1

```
#!/usr/bin/env python3 # -*-
coding: utf-8 -*

import common import statistics import
matplotlib.pyplot as plt import numpy as np

data, start1, end1, start2, end2 = common.load_data(\
    '../data/comp+old-behav-flex/2017-03-20 11.02.07.zip', \ '../data/comp+old-behav-flex/2017-
    03-20 19.58.27.zip', \
    '../data/comp+old-behav-flex/2017-03-21 09.53.27.zip', \ phase1='Day 1', phase2='Day 2')

start = {'start1': start1, 'start2': start2} end = {'end1': end1, 'end2':
end2} dataSets = ['Day 1', 'Day 2']

for i in range(1, 3):
    startStr = 'start' + str(i) endStr = 'end' + str(i)

    visitsByMouse = dict() visitsByGroup = dict()
```

```
        for mouse in sorted(data.getGroup()):
            if mouse != 'Cage9 Pump': mice = [data.getAnimal(m) for m in data.getGroup(mouse).Animals] visits =
                data.getVisits(\ mice=mice, start=start[startStr], end=end[endStr])
                visitorNames = [v.Animal.Name for v in visits]

                if mouse[6:] not in visitsByGroup:
                    visitsByGroup[mouse[6:]] = 0

                for mouse in set(visitorNames): if mouse != '19 WT' and mouse != '13
                    KO':

                        if mouse not in visitsByMouse:
                            visitsByMouse[mouse] = 0

                        visitsByMouse[mouse] = \ visitsByMouse[mouse] + visitorNames.count(mouse)
                        visitsByGroup[mouse[-2:]] = \ visitsByGroup[mouse[-2:]] + visitorNames.count(mouse)

        if i == 1:
            visitsByMouse1 = visitsByMouse visitsByGroup1 = visitsByGroup
            print('%s: %d visits' % ('WT1', visitsByGroup['WT'])) print('%s: %d visits' % ('HT1',
            visitsByGroup['HT'])) print('%s: %d visits' % ('KO1', visitsByGroup['KO']))
        else:
            visitsByMouse2 = visitsByMouse visitsByGroup2 = visitsByGroup
            print('%s: %d visits' % ('WT2', visitsByGroup['WT'])) print('%s: %d visits' % ('HT2',
            visitsByGroup['HT'])) print('%s: %d visits' % ('KO2', visitsByGroup['KO']))

byMouse = [visitsByMouse1, visitsByMouse2]

WT1mice = [0 for x in range(10)]
```

```
HT1mice = [0 for x in range(10)] KO1mice = [0 for x in
range(10)]

WT2mice = [0 for x in range(10)] HT2mice = [0 for x in
range(10)]
KO2mice = [0 for x in range(10)]

w = 0 h = 0 k = 0 for i in range(0,
2):
        for mouse in set (byMouse[i]):
                if mouse[-2:] == 'WT':
                        if i == 0:
                                WT1mice[w] = byMouse[i][mouse]
                                w = w + 1 else:
                                WT2mice[w] = byMouse[i][mouse]
                                w = w + 1 elif mouse[-2:]
                == 'HT':
                        if i == 0:
                                HT1mice[h] = byMouse[i][mouse]
                                h = h + 1 else:
                                HT2mice[h] = byMouse[i][mouse]
                                h = h + 1 elif mouse[-2:]
                == 'KO':
                        if i == 0:
                                KO1mice[k] = byMouse[i][mouse]
                                k = k + 1 else:
                                KO2mice[k] = byMouse[i][mouse]
                                k = k + 1
        w = 0 h
        = 0 k = 0

from scipy import stats

stdevMice = [WT1mice, HT1mice, KO1mice, WT2mice, HT2mice, KO2mice] semMice = [WT1mice,
HT1mice, KO1mice, WT2mice, HT2mice, KO2mice] normality = [WT1mice, HT1mice, KO1mice, WT2mice,
HT2mice, KO2mice]

for i in range(0, len(stdevMice)): stdevMice[i] = statistics.stdev(stdevMice[i])
        semMice[i] = stats.sem(semMice[i]) normality[i] =
        stats.shapiro(normality[i])

variance = stats.levene(WT1mice, HT1mice, KO1mice, WT2mice, HT2mice, KO2mice)

dunns = common.kw_dunn([WT1mice, HT1mice, KO1mice, WT2mice, HT2mice, KO2mice], \
                                        [(0, 3), (1, 4), (2, 5)])

semWT = [semMice[0], semMice[3]] semHT =
[semMice[1], semMice[4]] semKO = [semMice[2],
semMice[5]]

semWT = [semMice[0], semMice[3]] semHT =
[semMice[1], semMice[4]] semKO = [semMice[2],
semMice[5]]
```

```
width = 0.8

WT = [visitsByGroup1['WT'], visitsByGroup2['WT']] HT = [visitsByGroup1['HT'],
visitsByGroup2['HT']]
KO = [visitsByGroup1['KO'], visitsByGroup2['KO']]

indices = np.arange(len(WT))

plt.bar([i - 0.25 * width for i in indices], WT, width = 0.25 * width, \ color = 'tab:green', alpha = 0.9, label = 'WT', yerr =
        semKO, \ capsize = 5)
plt.bar(indices, HT, width = 0.25 * width, \ color = 'tab:blue', alpha = 0.9, label = 'HT', yerr = semWT, \ capsize =
        5)
plt.bar([i + 0.25 * width for i in indices], KO, width = 0.25 * width, \ color = 'tab:orange', alpha = 0.9, label = 'KO', yerr =
        semHT, \ capsize = 5)

plt.xticks(indices,
            ['Day{}'.format(i) for i in range(1, 3)] )

plt.plot([0 - 0.25 * width, 0 - 0.25 * width, 1 - 0.25 * width, 1 - 0.25 * width], \
          [WT[0]+100, WT[0]+300, WT[0]+300, WT[1]+100], lw = 1.5, c = 'k') plt.text(((0 - 0.25 * width) + (1 - 0.25 * width)) *
0.5, WT[0]+275, \
    "***", ha = 'center', va = 'bottom', color = 'k')


plt.plot([0, 0, 1, 1], \
          [HT[0]+100, HT[0]+300, HT[0]+300, HT[1]+100], lw = 1.5, c = 'k') plt.text(0.5, HT[0]+275, \
    "***", ha = 'center', va = 'bottom', color = 'k')


plt.plot([0.25 * width, 0.25 * width, 1 + 0.25 * width, 1 + 0.25 * width], \
          [KO[0]+100, KO[0]+300, KO[0]+300, KO[1]+100], lw = 1.5, c = 'k') plt.text(((0.25 * width) + (1 + 0.25 * width)) * 0.5,
KO[0]+275, \
    "*", ha = 'center', va = 'bottom', color = 'k')

plt.legend()

plt.ylabel('Total number of visits to corners') plt.title('Total Visits')
plt.savefig('number-of-visits.png', dpi = 300) plt.show() plt.clf()

print('normality') print(normality)
print(variance) print('Dunns multiple
comparison test, following a Kruskal-Wallis 1-
way ANOVA') print(dunns)
```

## Code for Figure 13

```
#!/usr/bin/env python3 # -*-
coding: utf-8 -*-

import pymice as pm
```

```
# Files relevent to Habituation 1 (hab1) period.
  dataFiles = ['../data/comp+old-behav-flex/2017-03-20 11.02.07.zip', \ '../data/comp+old-behav-flex/2017-
                                                                                03-20 19.58.27.zip', \
                                    '../data/comp+old-behav-flex/2017-03-21 09.53.27.zip']

# Merge the data. loaders = [pm.Loader(filename) for filename in dataFiles] data =
pm.Merger(*loaders)

print("Done loading data.")

for mouse in sorted(data.getGroup()):
        print(mouse)
print("Ignore Pump group, it is related to another experiment.")

# Read in hab1 period from timeline.ini.
timeline = pm.Timeline('../timeline/hab1.ini') PHASES =
[timeline.sections()[0]] start, end = timeline.getTimeBounds(PHASES)
print("%s:\t%s - %s" % (PHASES, start, end))

# Check for any problems (indicated in the log) during the period of # interest. start, end =
timeline.getTimeBounds(PHASES)

dataValidator = pm.DataValidator(pm.PresenceLogAnalyzer()) validatorReport = dataValidator(data)

noPresenceProblems = pm.FailureInspector('Presence')

if noPresenceProblems(validatorReport, (start, end)): print("Presences OK.")

# Competition test during first 60 minutes of hab1. # Measures corner
occupation among the three groups.

durationPerPhase = [[0 for x in range(61)] for y in range(30)] durationSum = 0

            j    = 0k = 0

for mouse in sorted(data.getGroup()):
        if mouse != 'Cage9 Pump':
                            mice = [data.getAnimal(m) for m in data.getGroup(mouse).Animals]

            visits = data.getVisits(mice=mice, order="Start") visitorNames = [v.Animal.Name for v in
            visits]

            for mouse in set(visitorNames):
                    if mouse != '19 WT' and mouse != '13 KO':
                            durationPerPhase[j][k] = mouse
            k   = k + 1

                            for timePeriod in timeline.sections():
                                    if timePeriod != 'Day 1' and timePeriod != 'Day 2': start, end =
                                            timeline.getTimeBounds(timePeriod) timeVisits = data.getVisits(mice=mouse,
                                            start=start, \
                                                                                    end=end)
```

```
                                    for i in range(0, (len(timeVisits))): adjustTime = timeVisits[i].Duration.total_seconds()
                                        if start > timeVisits[i].Start:
                                            tdelta = start - timeVisits[i].Start adjustTime = \ adjustTime -
                                            tdelta.total_seconds()
                                              if timeVisits[i].End > end:
                                            tdelta = timeVisits[i].End - end adjustTime = \ adjustTime -
                                            tdelta.total_seconds()

                                        durationSum = durationSum + adjustTime

                                    durationPerPhase[j][k] = durationSum durationSum = 0
                                    k = k + 1
                            k = 0 j = j + 1

HTaverage = [0 for x in range(60)]
WTaverage = [0 for x in range(60)]
KOaverage = [0 for x in range(60)]

k = 0 for i in range(0, (len(durationPerPhase))):
        if  durationPerPhase[i][0][-2:]  ==  "KO":  k  =  k  +  1  for  j  in  range(0,
            len(durationPerPhase[i])-1):
                                    HTaverage[j] = HTaverage[j] + durationPerPhase[i][j+1]

for i in range(0, len(HTaverage)):
        HTaverage[i] = HTaverage[i] / k

k = 0 for i in range(0, (len(durationPerPhase))):
        if  durationPerPhase[i][0][-2:]  ==  "HT":  k  =  k  +  1  for  j  in  range(0,
            len(durationPerPhase[i])-1):
                                   WTaverage[j] = WTaverage[j] + durationPerPhase[i][j+1]

for i in range(0, len(HTaverage)):
        WTaverage[i] = WTaverage[i] / k

k = 0 for i in range(0, (len(durationPerPhase))):
        if  durationPerPhase[i][0][-2:]  ==  "WT":  k  =  k  +  1  for  j  in  range(0,
            len(durationPerPhase[i])-1):
                                            KOaverage[j] = KOaverage[j] + durationPerPhase[i][j+1]

for i in range(0, len(HTaverage)):
        KOaverage[i] = KOaverage[i] / k

import matplotlib.pyplot as plt #%matplotlib inline

plt.clf()

plt.rcParams['figure.dpi'] = 150

plt.plot(WTaverage, "tab:green", label = 'WT') plt.plot(HTaverage, "tab:blue", label = 'HT')
plt.plot(KOaverage, "tab:orange", label = 'KO')

plt.xlabel('Time into Habituation 1 (minutes)') plt.ylabel('Longest visit (seconds)')
plt.title('Competition Test')
```

```
plt.legend()

plt.savefig('competition-test.png', dpi = 300)

#xAxis = list(range(11))
#print(xAxis)

#plt.plot((xAxis), (HTaverage))

#plt.plot([1, 2, 3, 4], [1, 4, 9, 16])
```

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