2009; 1-31

# Molecular Differences in the Fischer 344 Rat, A New Animal Model of Autism

Brandyn A. Castro[123] and Dr. Michael Jakowec[23]*

[1]McNair Scholars Program
[2]Department of Neurology, University of Southern California, Los Angeles, California
[3]USC Keck School of Medicine

## Table of Contents

2009; 1-31

# Molecular Differences in the Fischer 344 Rat, A New Animal Model of Autism

Brandyn A. Castro[123] and Dr. Michael Jakowec[23]*

[1]McNair Scholars Program
[2]Department of Neurology, University of Southern California, Los Angeles, California
[3]USC Keck School of Medicine

**Abstract**

The purpose of this study is to examine the neurological differences in protein levels and concentrations in Autism. The following are two aspects that will be introduced to the field: (1) the utilization of the Fischer 344 rat as an animal model of Autism and (2) the evaluation of the protein tyrosine hyroxylase (TH). TH is the enzyme that catalyzes the conformation of L-tyrosine into L-DOPA, the precursor to dopamine. Dopamine is a vital neurotransmitter for normal function within the brain and serves as a precursor for norenephrine (noradrenaline) and epinephrine (adrenaline). Dopamine is produced in the substantia nigra which sends projections to the striatum. Immunocytochemistry (ICC) was performed at the level of the midstriatum, prefrontal cortex, and caudal end of the temporal lobe. It was determined that there are lower levels of TH in the striatal area of Fischer 344 as compared to Sprague Dawley controls. This shows that there are lower levels of dopamine in the striatum in the Fischer 344 which correspond with the dysfunction in the dopaminergic system seen in Autism. No difference was seen in the TH levels of the prefrontal cortex and amygdala. Ongoing studies are further analyzing the Fischer 344 rat as an animal model of Autism. Western blot analysis will be performed with the hope of validating these present findings. ICC and western blots will be assess dopamine transporter to hopefully confirm that the TH levels do in fact correlate with dopamine levels. The glutamatergic system will also be examined in the Fischer 344 because dysfunction in this pathway has been seen in Autism. In conclusion, the Fischer 344 strain may be a useful animal model for identifying neurobiological pathways involved in Autism-related phenotypes.

## INTRODUCTION

Autism is a condition that was first described over 50 years ago. Later in 1998, Rapin et al. defined Autism as a developmental disorder that can be characterized by behavioral symptoms across three general areas: social reciprocity, communication, and restricted and repetitive interests and

behaviors (DSM IV, 1994). Some common symptoms of Autism described in the DSM IV include:

- inability to relate to children or adults
- poor speech or lack of speech
- hyperactivity or passiveness
- inappropriate laughter or crying
- lack of eye contact
- oversensitivity or undersensitivity to touch
- difficulty dealing with changes in routine

The extensive amount of symptoms that classify this disorder is what has made it into Autism Spectrum Disorder. It is an extremely heterogeneous disorder that contains differences in symptoms, onset, and severity. No two cases are exactly the same. Some patients exhibit all of these characteristics while others only show a few. In addition, some express symptoms early on while others do not get diagnosed until adulthood. Because of the variety of symptoms included in the Autism spectrum, multiple etiologies are thought to play a factor. Genetic susceptibility is known to play a role as well as interactions between genetic and environmental conditions. The complexity of the Autism spectrum makes it incredibly difficult to study what is actually causing such abnormal behavior. An additional feature of the disorder is that men are 4.3 times more likely to be affected than women.

For the purpose of this study, we will assume that Autism has a solely genetic basis. Even with this knowledge, there is still very little known about the disorder. Prior studies have been done to

determine what symptoms are typical in Autism and what criterion is necessary to diagnose someone with it. There has been a tremendous increase in the ability to diagnose children with the disorder. According to the U.S. Department of Education, Office of Special Education Programs (OSEP), the number of children diagnosed with Autism has increased from 15,580 children in 1992 to 258,305 children in 2007. This could also be due to a broader range of traits that characterize Autism Spectrum Disorders. The Autism Society of America reports 1 in every 150 children is diagnosed with autism in the United States and 1.5 million children are presently diagnosed as autistic. The annual cost of Autism in the United States is estimated at $90 billion dollars per year. This is a huge fiscal impact on society.

Because so little is known about the disorder it is increasingly more important to conduct research to find out what is happening to cause these behavioral abnormalities in Autistic individuals. The struggle that scientists are faced with is the limited number of ways to study the disorder. MRI scans can be performed in autistic individuals to assess size differences in different areas of the brain. The only mechanistic studies that can be conducted in humans are done in the postmortem brain. This gives us little insight into the development of the disorder and why there are such wide arrays of characteristics.

Animal models provide an outlet to study a disorder like Autism further. They offer a way to discover the mechanism behind a condition and then use that knowledge to create treatments and therapies to reverse that mechanism. There are a three main ways to get an animal model for a specific disease or disorder:

1. Scientists can genetically engineer the animal to express the genetic composition typical of that

disease. This can be seen in the triple knock-out mouse used to study Alzheimer's Disease.

2. Chemicals can be injected that target certain aspects of the body in the animal to reproduce the symptoms of the disease. This can be seen in the Parkinson's Disease model where the neurotoxin MPTP is injected into the animal.
3. The animal can occur spontaneously in nature. This can be seen in the Fischer 344 animal model of Autism used in this study.

The Fischer 344 rat exhibits behavioral traits that can be correlated with characteristics seen in Autism. These include lack of interaction with other individuals, lessened maternal instincts, and high levels of anxiety. For these reasons, the Fischer 344 rat will be introduced as a new animal model for Autism. Differences in the levels and concentrations of proteins will be examined in various aspects of the brain including: prefrontal cortex, striatum, amygdala, substantia nigra, and cerebellum. Studies will be conducted using immunocytochemistry and western blots.

Little, if anything, is known about the molecules and mechanism involved in the pathogenesis of the Autistic disorder. Aside from studies done on the behavioral characteristics of the disorder, the only clue as to the neurological implications of Autism arise from autopsies. These have led to the beliefs that there is dysfunction in the dopaminergic and glutamatergic systems (Salgado-Pineda et al, 2005). The dopaminergic system is comprised of three main pathways Figure 1. The first is the nigrastriatal pathway which is comprised of projections from the substantia nigra to the striatum. Complications of

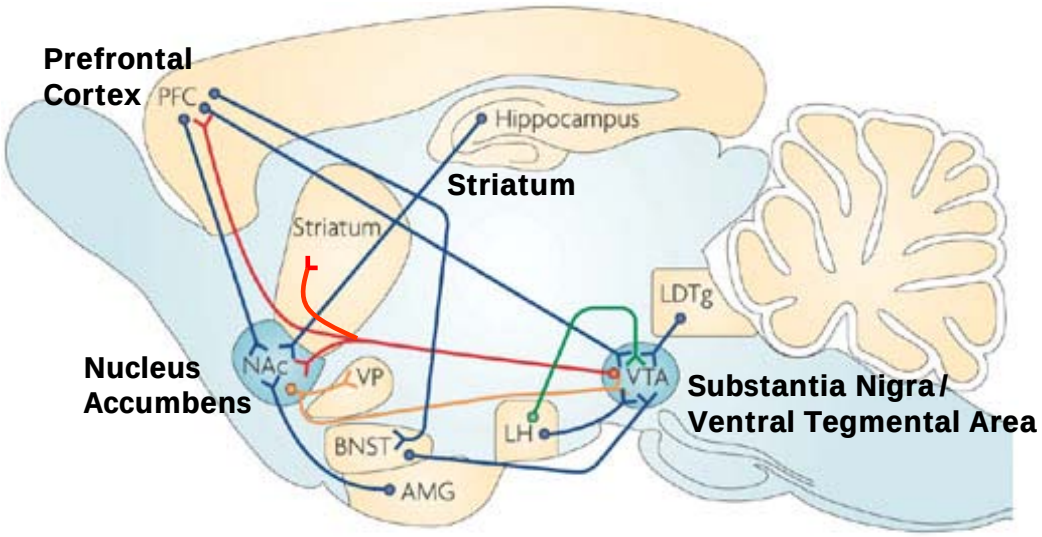


Figure 1. The red pathway denotes the dopaminergic system. The blue pathway denotes the glutamatergic system. The green pathway denotes the GABAergic system.

this pathway could be associated with Parkinson's Disease. The mesolimbic pathway sends projections from the ventral tegmental area to the amygdala. Lastly, projections that stem from the ventral tegmental area and lead to the prefrontal cortex make up the mesocortical pathway. Damage to this pathway could lead to Schizophrenic behavior.

The first protein that will be examined is tyrosine hydroxylase (TH). TH is an enzyme that catalyzes the conformation of L-tyrosine into L-DOPA, which is the precursor for dopamine (DA). Analysis of this protein could give us insight into the mechanism for the dopaminergic dysfunction. It will be looked at in the striatum which is one of the main regions where DA is produced.

## LITERATURE REVIEW

Autism is a complex disorder rooted with a genetic basis. The etiology of the condition is still unknown but a variety of studies have shown neuroanatomical, morphological, and neurochemical differences in Autism that warrant further investigation. Studies have shown that specific areas of the

brain are larger and smaller in size in Autistic subjects. These size alterations accompany specific behavioral symtoms of Autism. Additional studies have shown dysfunction in the dopaminergic and glutamateric systems in Autism. Current animal models try to replicate these findings in order to create another platform with which to study the unknown disorder. The following gives a brief overview for these previous findings which provide the basis for this study.

**Cerebellar Differences in Autism**

Multiple studies have shown that there are differences in the cerebellum between normal individuals and Autistic patients. Autopsies demonstrate that there is a 35%-50% reduction in number of Purkinje cells in autism cerebellum as compared to normal cerebellum (Ritvo et al, 1986, Williams et al, 1980, and Kemper et al, 1998). Along with this, MRI scans have shown that the size of the cerebellum in Autism is smaller than that of a regular individual (Hashimoto et al, 1995 and Courchesne, 1997).

A study done in the laboratory of Purcell et al in 2001 examined these differences in the cerebellum further. They examined postmortem cerebellum samples from 10 autistic individuals and 10 normal or control individuals. By performing RT-PCR, they found higher levels of mRNA from a variety of glutamate related genes which confirm the dysfunction of the glutamatergic system in autism. He found significantly higher levels of mRNA levels from excitatory amino acid transporter 2 (EAAT 2) and the AMPA-type glutamate receptors 1, 2, and 3. In addition, they conducted microarray analysis to look at differences in proteins in the postmordem cerebellum as well. They found higher levels of excitatory amino acid transporter 1 and 2, AMPA-type glutamate receptor 1, NMDA receptor 1, and glutamate receptor interacting protein (GRIP). Using autoradiography they found lower levels

of AMPA-type glutamate receptors in the cerebellum (granule and molecular cell layers) but no significant difference in these levels in the prefrontal cortex or caudate-putamen. There were no significant differences in NMDA is either region of the cerebellum, prefrontal cortex, or caudate-putamen. Pseudocolor images (PI) showed significantly decreased AMPA binding in the cerebellum of a representative autism brain section as compared to a normal individual. No significant differences in PI were seen with NMDA binding.

**Prefrontal Cortex Differences in Autism**

Carper et al conducted studies in 2002 and 2005 examining the frontal lobe in Autistic postmortem brains. Previous studies had shown abnormalities in the size of this region and they examined it further. They conducted MRI scans of Autistic children 2.2-5 years of age and compared the sizes of their orbitofrontal cortex (OFC), medical frontal cortex (MFC), dorsolateral prefrontal cortex (DFC), and precentral gyrus (PCG). The graph shows the deviation from the norm that autistic brains exhibited in this study. Statistical differences were shown with the increase in size of the MFC and DFC. This leads to the assumption that there might be molecular differences in the prefrontal cortex.

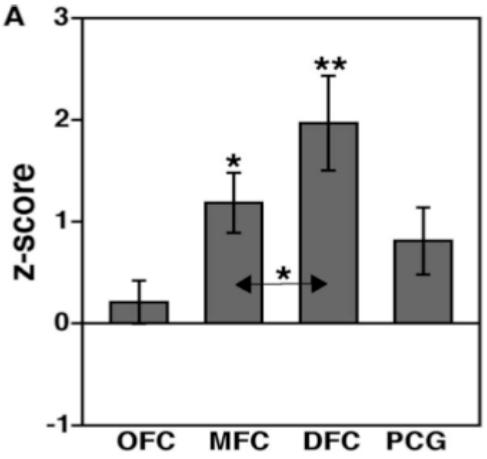


Figure 2 (Carper et al, 2005)

**Basal Ganglia Differences in Autism**

Sears et al conducted a comprehensive study of the basal ganglia in Autism in 1999. They

compared 35 relatively high-functioning persons with autism with 36 healthy controls all ranging from 12 to 29 years of age. A 20-minute high resolution MRI was done on each subject and the size of their caudate, putamen, and globus pallidus was compared. Results show a significant increase in the size of the caudate in the autistic subjects with a p-value of .01. A behavioral analysis was then performed to correlate the differences found with the caudate with specific clinical symptoms. They found that the

**Relationship of Caudate Volume to Items from the ADI Ritualistic-Repetitive Behavior Domain**

| ADI Item (Ever present) | Spearman r | p |
|---|---|---|
| Difficulties with minor changes in routine | -.48 | .003 |
| Compulsions/rituals | -.52 | .001 |
| Complex mannerisms | .49 | .002 |
| Circumscribed interests | -.10 | n.s. |
| Unusual preoccupations | -.02 | n.s. |
| Repetitive use of objects | .30 | n.s. |
| Unusual sensory interests | -.02 | n.s. |
| Abnormal idiosyncratic negative response | .16 | n.s. |
| Unusual attachments | -.20 | n.s. |
| Resistance to change in the environment | -.06 | n.s. |
| Hand and finger mannerisms | -.08 | n.s. |
| Verbal rituals | -.03 | n.s. |

Table 1 (Sears et al, 1999)

autistic subjects showed difficulties with minor changes in routine (p-value = .003), compulsions/rituals (p-value = .001), and complex mannerisms (p-value = .002).

**Amygdala Differences in Autism**

The amygdala has been shown to play an important role in mediating social perception and regulating emotion. For this reason, the amygdala has been a target of interest for studies in Autism. Howard et al conducted a thorough study examining the behavioral and neuroanatomical evidence the

amygdala may have on Autism. They compared 10 males with high-functioning autism (HFA) age 16-40 with 10 healthy normal controls of similar sex and age. They defined HFA as occurring in people with impairments of social interactions, communication and behavioral flexibility, but whose language and intellectual functions are currently normal (Howard et al, 2000). Neuropsychological testing showed that HFA subjects showed impairment in recognition of facial expressions of fear, perception of eye-gaze direction, and recognition memory for faces. They then performed structural MR volumetric techniques to compare the size of the hippocampus, parahippocampal gyri, and amygdala. In the HFA subjects, the amygdala volume was increased bilaterally compared to control subjects. No significant difference was seen in the volumes of the hippocampus or parahippocampal gyri.

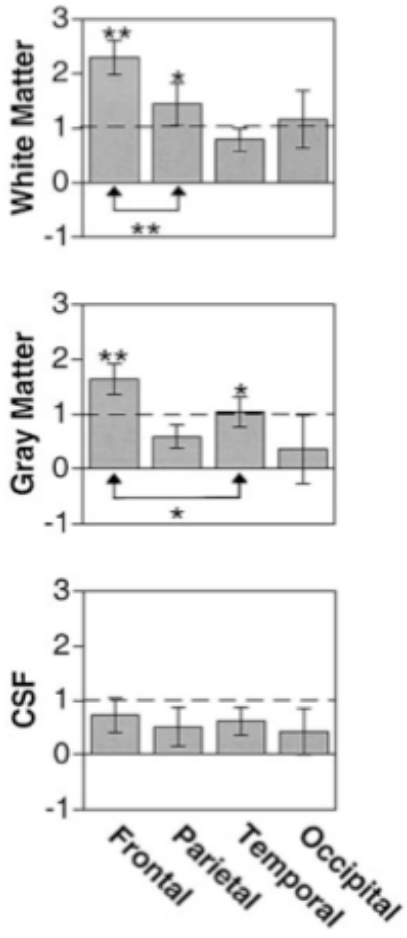


Figure 3 (Carper et al, 2002)

**Differences in Composition of Frontal, Parietal, and Temporal Lobes**

The brain is composed of white matter, gray matter, cerebrospinal fluid, and neuroglia. White matter contains myelin which creates faster neuronal signaling while gray matter does not have myelin. In 2002, Carper et al looked at differences in the composition of the frontal, parietal, temporal, and occipital lobes by comparing Autistic individuals and normal controls (Figure 4). His subjects were under 4 years of age. He quantified the amount of white matter, gray matter, and cerebrospinal fluid and found significantly higher levels of white matter in the frontal and parietal lobes in Autism. In addition, he found that the frontal and temporal

lobes of the Autistic subjects had higher levels of gray matter in the frontal and temporal lobes. The excess amount of white and gray matter in the frontal lobe could explain why the prefrontal cortex is found to be larger in Autism. No significant differences were found in cerebrospinal fluid.

**Hyperactivation of the Dopaminergic System in Autism**

Hyperactivation of the dopaminergic system is seen in Autism. This was shown in the clinical setting when Risperidone, a dopamine antagonist, lessened the severity of many common Autistic symptoms (McCracken et al., 2002; McDougle et al., 2005). Table 2 outlines the symptoms alleviated by risperidone from 101 autistic children age 5 to 17. The table compares those treated with placebo to those treated with Risperidone. Studies done by Nakasato et al in 2007 explored this concept further in the valproic acid (VPA) animal model of Autism. Exposure to drugs such as VPA contributes to the development of autism and prenatally exposing rats to VPA creates a rodent model of Autism (Arndt et al, 2005; Williams et al, 2001). Basal dopamine levels in the frontal cortex were determined in control (n=6) and VPA-exposed (n=5) rats. The VPA-exposed rats showed a significantly (P<.05) higher basal dopamine level than control rats. This abnormality may be associated with depressive and withdrawal behavioral symptoms seen in Autism.

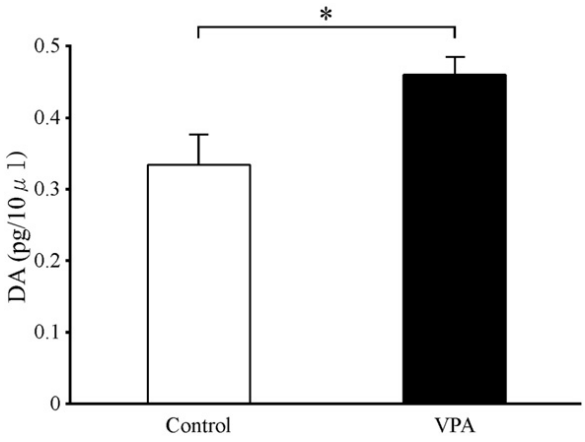


Figure 4 (Nakasato et al, 2007)

TABLE 1. Scores on the Ritvo-Freeman Real Life Rating Scale of Children and Adolescents With Autism in a Placebo-Controlled Risperidone Trial and Open-Label Continuation Study

| Measure From Ritvo-Freeman Real Life Rating Scale | Score in Placebo-Controlled Trial (N=101) | | | | | | Results of Placebo-Controlled Trial | | |
|---|---|---|---|---|---|---|---|---|---|
| | Baseline | | Week 4 | | Week 8 | | End-of-Study Effect Size (Cohen's d) | Interaction of Treatment With Time | |
| | Mean | SD | Mean | SD | Mean | SD | | F (df=1, 87) | p |
| Subscale I: sensory motor behaviors | | | | | | | 0.45 | 10.8 | 0.002 |
| Risperidone | 1.00 | 0.52 | 0.65 | 0.43 | 0.59 | 0.42 | | | |
| Placebo | 0.93 | 0.58 | 0.83 | 0.47 | 0.91 | 0.60 | | | |
| Subscale II: social relationship to people | | | | | | | 0.68 | — | n.s. |
| Risperidone | 0.60 | 0.43 | 0.20 | 0.43 | 0.15 | 0.42 | | | |
| Placebo | 0.72 | 0.43 | 0.47 | 0.51 | 0.46 | 0.52 | | | |
| Subscale III: affectual reactions | | | | | | | 1.10 | 15.4 | <0.001 |
| Risperidone | 1.68 | 0.64 | 1.00 | 0.67 | 0.88 | 0.56 | | | |
| Placebo | 1.84 | 0.64 | 1.64 | 0.64 | 1.60 | 0.71 | | | |
| Subscale IV: sensory responses | | | | | | | 0.77 | 8.5 | 0.004 |
| Risperidone | 1.13 | 0.53 | 0.70 | 0.44 | 0.60 | 0.38 | | | |
| Placebo | 1.21 | 0.53 | 0.98 | 0.54 | 1.07 | 0.54 | | | |
| Subscale V: language | | | | | | | 0.81 | — | n.s. |
| Risperidone | 0.28 | 0.38 | 0.15 | 0.31 | 0.03 | 0.29 | | | |
| Placebo | 0.46 | 0.42 | 0.30 | 0.39 | 0.34 | 0.41 | | | |
| Overall | | | | | | | 1.08 | 15.3 | <0.001 |
| Risperidone | 0.94 | 0.36 | 0.54 | 0.36 | 0.45 | 0.31 | | | |
| Placebo | 1.03 | 0.37 | 0.84 | 0.39 | 0.88 | 0.40 | | | |

Table 2 (McDougle et al, 2005) The Ritvo-Freeman Real Life Rating Scale was used to evaluate the benefits seen in Autistic children treated with Risperidone as compared with placebo treatment. Subscale I: sensory motor behaviors include hand flapping, rocking and pacing. Subscale II: social relationship to people includes appropriate responses to interaction attempts and initiation of appropriate physical interactions. Subscale III: affectual reactions include abrupt changes in affect, crying, and temper outbursts. Subscale IV: sensory responses include being agitated by noises, rubbing surfaces, and sniffing self or object. Subscale V: language includes communicative use of language and initiation of appropriate verbal communication. Significant ($P<.01$) benefit from Risperidone treatment was seen in sensory motor behaviors, affectual reactions, and sensory responses. This thus proves an overall positive effect to the drug for Autism.

**Hypoglutamatergic System Seen in Autism**

Certain brain regions have been shown to be implicated in autism on the basis of neuropathological and/or brain imaging studies. These include areas such as medial temporal structures like the amygdala and the hippocampus (Howard et al, 2000; Raymond et al, 1996) and cortical areas such as the frontal, prefrontal, and parietal cortex (Carper et al, 2002; Carper et al, 2005). The regions explained here area also structures involved in the glutamatergic system causing scientists to come to the conclusion that there is dysfunction in the glutamatergic system in Autism. In exploring

this topic further, they have found that glutamate antagonists result in similar features that are seen in Autism (Carlsson et al, 1998). Comparable symptoms include heightened/distorted visual, auditory, olfactory, and tactile perception, defective proprioception, difficulties estimating time, concrete thinking, defective habituation, depersonalization, rapid fluctuations of mood, anxiety, social withdrawal, hyperactivity, and repetitive movements. This leads to the conclusion that deficient glutamateric transmission is involved in Autism. The study also examined the similarity in effects that were seen in patients treated with serotonin(5-HT)2A receptor agonists. Patients treated with 5-HT2A agonists exhibited most of the symptoms just described. This led to a mechanistic study determining that GABA interneurons in the piriform cortex excite 5-HT2A receptors while inhibiting glutamatergic pyramidal cells. Through this system, 5HT2A stimulation indirectly inhibits the glutamatergic system. This has opened new doors as far as Autism treatment is concerned. A typical counter to the hypoglutamateric system is glutamatergic agonists, however these often result in neurotoxicity. Glutamatergic systems can indirectly be stimulated through 5-HT2A blockers which have less side effects.

**Current Animal Models of Autism**

Animal models relay a high level of specificity because no animal is exactly like a human or entire disease. Animal models can be developed to target one behavioral trait encompassed within the disorder of Autism. There are a few animal models of Autism currently being used. Each expresses specific symptoms of Autism so they can be used to study those particular behavioral traits. The following are mouse animal models of Autism and the symptoms that they exhibit (Halladay et al, 2009):

1. A/J (A): low levels of social behavior in reciprocal social interaction and sociability assays;

deficits in social learning of food preference assay; low activity; high anxiety-like behavior

2. BALBcBy/J (CBy): low levels of social behavior in reciprocal social interaction and sociability assays; high anxiety-like behavior
3. BTBR: low levels of social behavior in reciprocal social interaction and sociability assays; social learning of food preference assay

**The BALB/cJ Animal Model of Autism**

Brodkin began looking at the BALB/cJ mouse as an animal model of Autism due to social behavior abnormalities. In 2007, he conducted behavioral test to compare how BALB/cJ and C57BL/6J control mice interacted with a "stimulus" mouse. First the stimulus mouse was place in a Plexiglas cylinder and then the Plexiglas was removed to observe free interaction. Measures of sociability included the time that the test mouse spent near vs. far from the stimulus mouse that was confined to a Plexiglas cylinder (time spent in social approach), the time spent directly sniffing the stimulus mouse contained in the cylinder (time spent in social sniffing), and the time spent in direct contact between test and stimulus mouse during free interaction (time spent in direct social interaction) (Table 3). Results showed that BALB/cJ showed significantly lower levels of sociability than C57BL/6J controls in all measures. Baseline locomotor activity was equivalent in both strains showing evidence that the differences in sociability were due to behavioral differences caused by the situational apparatus.

| Behavior | Strain comparison |
|---|---|
| Time spent in social approach | C57BL/6J > BALB/cJ |
| Time spent in social sniffing | C57BL/6J > BALB/cJ |
| Time spent in direct social contact | C57BL/6J > BALB/cJ |
| Baseline locomotor activity | C57BL/6J = BALB/cJ |

Table 3 (Brodkin, 2007)

In addition, Brodkin found that the BALB/cJ show less maternal behaviors when caring for their young as compared with C57BL/6J controls. Reduced maternal behaviors of BALB/cJ mothers are part of a more general pattern of low social interaction in BALB/cJ mice causing it to further resemble an Autism model. Autism Spectrum disorders have shown a relatively high prevalence of aggressiveness and self-injurious behaviors (McCracken et al, 2002). This aggressiveness was shown in the BALB/cJ male mice when places in a cage together. These mice tended to fight frequently, sometimes resulting in serious injury.

The BALB/cJ mice have a relatively high brain weight and high brain to body weight ratio in contrast with C57BL/6J controls (Roderick et al, 1973). This corresponds with Autistic subjects, whose brains are typically 10% larger than normal by 2-3 years of age (Hazlett et al, 2005). There was also significant increase in the volume of the forebrain, neocortex, and hippocampus in the BALB/cJ strain. The increase in the forebrain could be due to higher levels of gray and white matter found in the frontal cortex in Autism and thus, the Autism animal model.

**Behavioral Differences in Fischer 344 Rats**

Studies have been conducted in a number of laboratories to examine behavioral differences between Fischer 344 rats and normal rats. There is a trend in this data that lead to the link between Fischer 344 behavior and Autism. Autistic individuals show a lack of interaction with other children or adults. Similarly, young Fischer 344 rats show a decreased rate of play with other rats of their same age as compared to controls. This was seen in a study done by Siviy et al in 2003. They placed two of the same strain of rat in a cage together after being separated for 24 hours. They recorded the amount of play by observing the number of nape contacts and pins. A nape contact was identified when one

partner brought its snout within 1 cm of the other's nape. A pin was characterized if one rat was on its back with at least three paws in the air. Data shows that the Fischer 344 rats show a significantly lower interaction in regards to both nape contacts and pins as compared to Lewis controls. The graph is shown below.

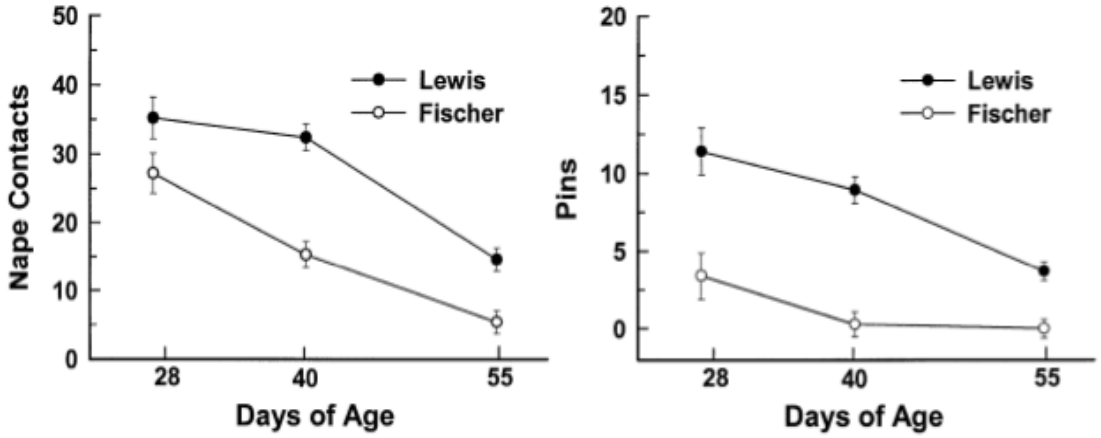


Fig. 1. Mean ( ± S.E.M.) number of nape contacts (left) and pins (right) in Lewis and Fischer rats when tested at 28, 40, and 55 days of age.

Figure 5 (Siviy et al, 2003)

Fischer 344 rats also exhibit less maternal behavior than a normal rat (Siviy et al, 2003). Fischer dams spend significantly less time engaging in typical behavior such as crouching, licking, nursing, and pup retrieval. The data for crouching, or time when dam spend crouching over pups in a nursing-like posture, shows a lower number of occurrences in Fischer 344. In addition, the Fischer dams spend significantly more time out of the nest than did the Lewis rat controls. This again can be related to the tendency for Autistic individuals to isolate themselves from other people.

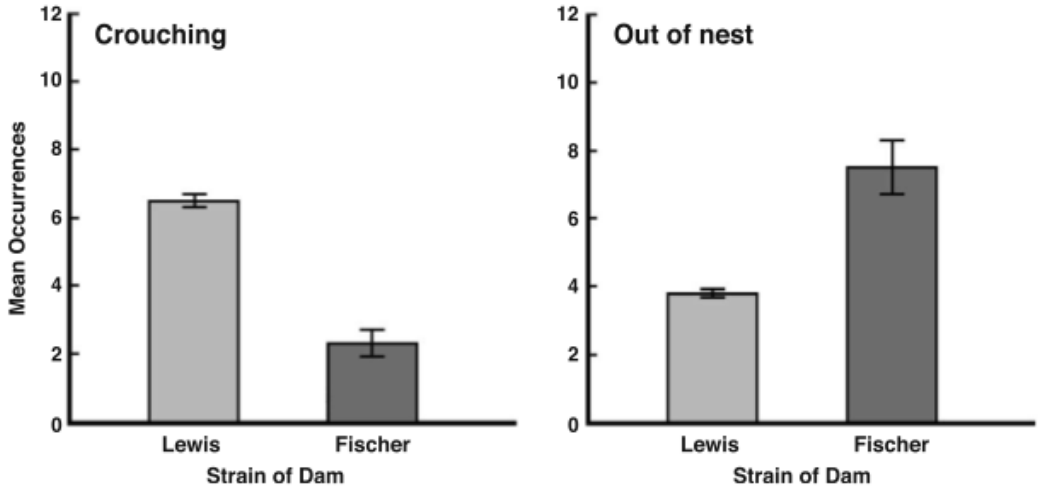


Fig. 4. Mean ( ± S.E.M.) occurrences of two maternal behaviors exhibited by Lewis and Fischer dams toward their respective litters during a 2-h observation period.

Figure 6 (Siviy et al, 2003)

Rex et al conducted further behavioral analysis of the Fischer 344 rats in 1999. He conducted a variety of behavioral tests and compared the Fischer 344 to Harlan-Wistar controls. In the modified open field test, he deprived the rats of food for 20 hours and then placed them in a large white open field containing food in the middle. Results showed that eight of the ten Harlan-Wistar rats ate food under these conditions but none of the Fischer rats began feeding. In addition the Harlan-Wister rats spent more time in the inner area of the open field and had a higher rate of locomotor activity. This shows that Fischer rats have a higher level of anxiety than the controls because time spent in the periphery of the open field has been correlated with higher anxiety.

In the same laboratory, they tested social interaction by placing two rats of the same species in a cage together and recorded the interaction between the two. Fischer 344 rats typically initiated contact with the unfamiliar partner later, showed a lower frequency of contact, and spent less time in contact as

compared to the Harlan-Wistar rats. In addition, the X-Maze test that Fischer 344 rats had a lower exploratory level than Harlan-Wistar controls. Fischer 344 rats also spent more time in the immobilization state (lack of movement) while on the maze.

## HYPOTHESIS

Fischer 344 rats have been shown to exhibit a number of abnormal characteristics such as lack of interaction, reduced level of care for offspring, and high levels of anxiety as compared with normal control rats. These traits are typically seen in Autism. To accompany these alterations in behavior, it is thought that there will also be morphological differences on the molecular level as well.

## METHODS

The animals in this study were Sprague Dawley and Fischer 344 rats. The animals were group-housed in a temperature controlled room under a 12 hour light and 12 hour dark cycle with free access to water and standard rodent chow. The rats were raised to eight to ten weeks of age to a weight of about 150g. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Southern California. A total of 6 rats were used in this study (Sprague Dawley n=3 and Fischer 344 n=3). This assured a large enough n to detect changes within each experimental design.

### Collection of Brain Tissue

When the rats reached the appropriate age, they were fixed by transcardial perfusion. Six of the twelve animals (Sprague Dawley n=3 and Fischer 344 n=3) received an intraperitoneal injections of 500 uL of nebutol to anesthetize the animal. Once the animal had diminished reflexes, the animal was

prepared for perfusion. The right atrium and left ventricle (near the apex) was cut and the animal was perfused with 200 ml of cold-saline followed by 500 ml of 4% and paraformaldehyde/phosphate-buffered saline (PFA/PBS) pH 7.2. Brain tissue was harvested and placed in 4% PFA/PBS solution for 24 hours. The brains were then placed in 20% sucrose solution until they sank to the bottom (48 hours). Tissue was frozen using methyl butane on dry ice and placed in the -80*C freezer for 18 hours.

**Immunocytochemistry**

Fixed tissue from three rats from each group was cut to 25 micron thickness using a cryostat. Slices were placed (free-floating) in a solution of phosphate buffer solution plus thimerisol, an anti-fungal agent, in six well dishes. One slice from each well was placed on subbed slides and nissl stained for correct orientation (i.e. which part of the brain was in each well).

Commercially available antibodies included rabbit polyclonal anti-TH (Chemicon, Temecula, CA). Tissue slices from the mid-striatum, prefrontal cortex, and caudal end of the temporal lobe were selected and washed with Tris-buffered saline (TBS; 50mM Tris pH 7.4 and 0.9% NaCl) and blocked. Slices were then exposed to primary antibody at a concentration of 1:1000 for 18 hours at 4°C. Sections were washed in TBS and exposed to biotinylated goat anti-rabbit IgG secondary antibody (using the ABC Elite kit) at a concentration of 1:500. Antibody staining was visualized by development in DAB/H2O2 to desired darkness. Multiple sections of each animal were stained to ensure that differences in staining intensity were due to differences in antigen expression.

**Image Analysis**

Images from the striatum, prefrontal cortex, and amygdala were taken under a microscope at

low, mid, and high magnifications for visualization. Additional images were taken and digitized. The Image J computer program was used to determine relative optic densities (OD) (expressed as arbitrary units of within the linear range of detection). OD units ranges from the 0-255 (darkest level = 0; lightest level = 255). In the mid-striatal sections, measurements were taken from the cortex, dorsal lateral striatum, dorsal medial striatum, nucleus accumbens, and whole striatum and the relative optic density was determined by subtracting the relative optical density of the corpus callosum as background. The prefrontal cortex was measured from the slices and the relative optical density from around the tissue was subtracted as background. In the slices from the caudal end of the temporal lobe, the relative optical densities of the cortex and amygdala were measured and the relative optical density of the corpus callosum was subtracted as background. The various areas of each section were analyzed and statistical differences, or lacks there of, between the two strains of animals were recorded.

## RESULTS

The pattern of expression of TH protein in the striatum was determined using immunocytochemical staining. This was done in order to examine the anatomical distribution of TH as well as to quantitatively analyze the TH protein levels at different regions of the brain in the Fischer 344 Autism animal model compared with the Sprague Dawley control rat. Immunocytochemical staining of coronal sections at the level of the midstriatum showed intense TH staining within the striatum as well as intense fibrous TH immunoreactivity within the striatum (Figure 7). ICC staining of the prefrontal cortex showed neuronal staining using TH (Figure 9). Staining of the caudal end of the temporal lobe showed TH stained fibers in the area of the amygdala (Figure 11).

### Analysis of TH Expression in Striatum

Analysis of the levels of TH protein in the striatum was done using Image J software to measure

relative optic densities. The cortex, dorsolateral striatum, dorsomedial striatum, nucleus accumbens, and whole striatum were compared between the Sprague Dawley and Fischer 344 rats (Figure 8). No significant difference was seen in the TH protein levels in the cortex. Fischer 344 showed significantly ($P<.05$) less TH in the dorsolateral striatum and dorsomedial striatum. The nucleus accumbens and whole striatum showed even more differentiation ($P<.01$) between the Fischer 344 and Sprague Dawley rats with lower levels of TH being expressed in the Fischer 344.

**A.**

**B.**

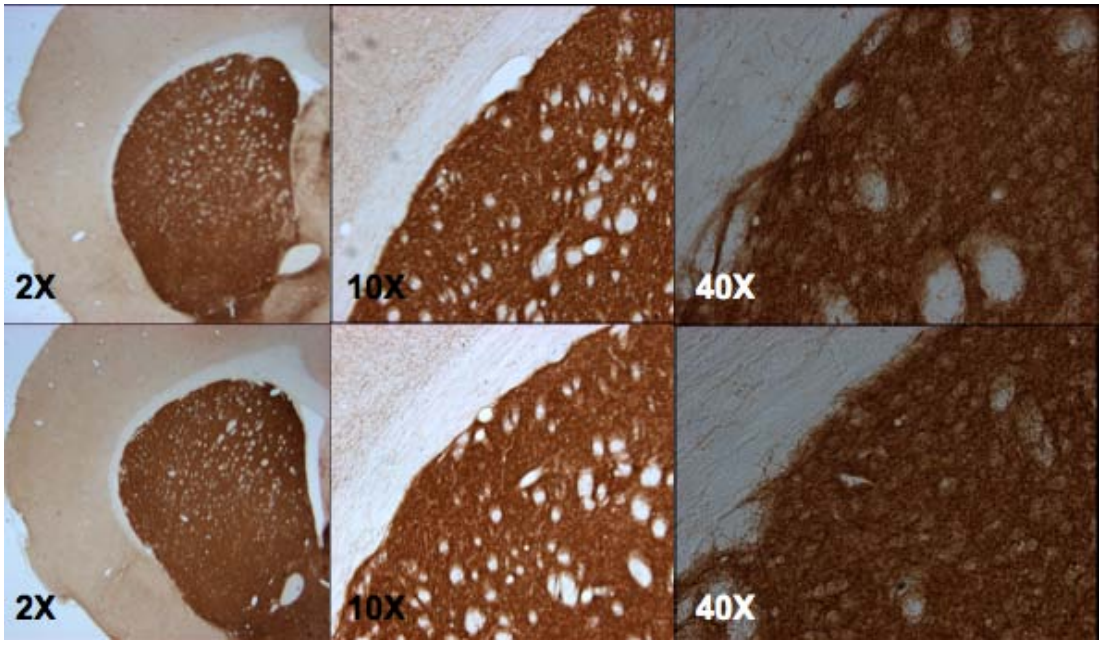


Figure 7. TH protein expression in the striatum of **(A)** Sprague Dawley and **(B)** Fischer 344. Immunocytochemical analysis using an antibody against TH protein demonstrated decreased expression of TH in Fischer 344. Coronal sections at the level of the midstriatum were stained for TH protein and images made at low magnification (2x; left panels), mid magnification (10x; middle panels), and high magnification (40x; right panels). In both the Sprague Dawley and Fischer 344 animals displayed intense TH in the striatal area, as seen by dark staining. Thick networks of TH-positive fibers were seen in both the Sprague Dawley and Fischer 344 animals in the right panels.

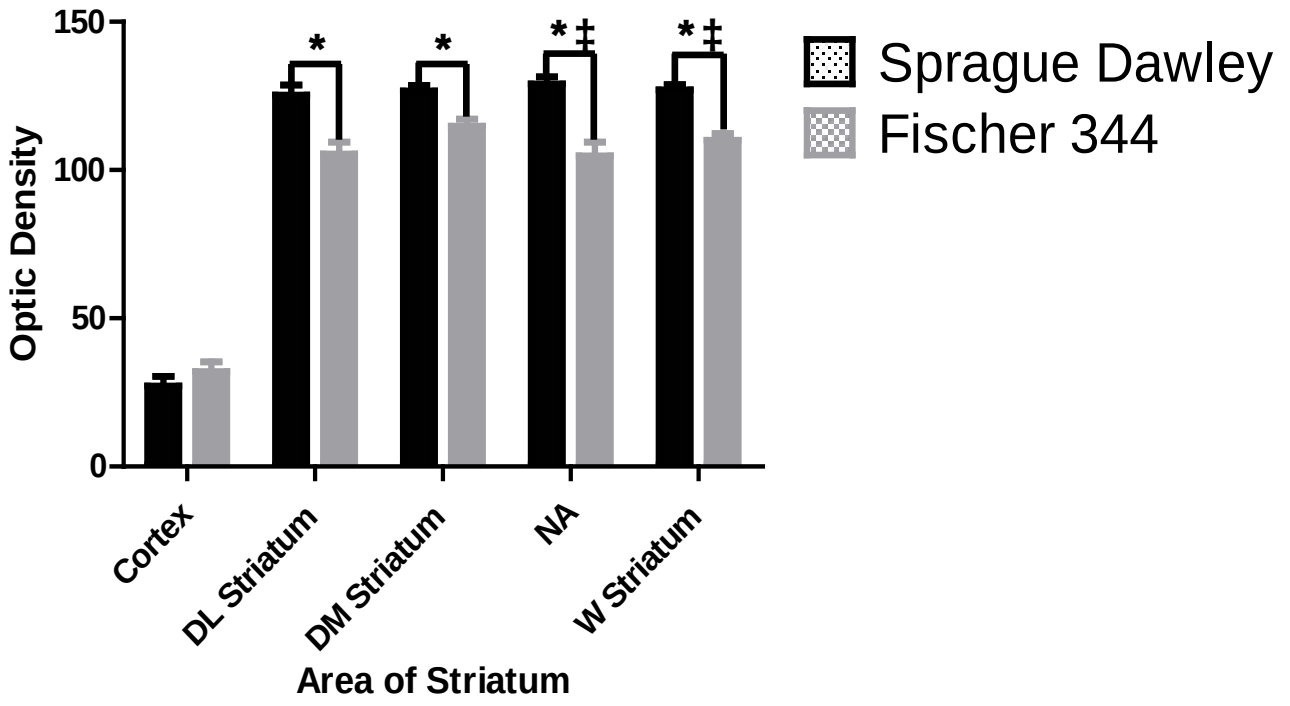


Figure 8. Immunocytochemical analysis of striatal TH protein. Analysis was carried out on coronal sections at the level of the midstriatum of Sprague Dawley (n=3) and Fischer 344 (n=3) rats. No difference in the relative amount of TH protein was seen in the cortex. A significant decrease of striatal TH protein was seen in the Fischer 344 in the DL CPu, CM CPu, NA, and W CPu. Ctx - cortex, DL CPu – dorsolateral striatum, CM CPu – dorsomedial striatum, NA – nucleus accumbems, and W CPu – whole striatum. The * represents statistical significance compared with the Sprague Dawley group ($P<.05$). The ‡ denotes statistical significance with $P<.01$. Error bars are standard error of the mean.

**Analysis of TH Expression in Prefrontal Cortex**

The relative optic densities were measured in the prefrontal cortex and the levels of immunocytochemical TH staining were compared between the Fischer 344 and Sprague Dawley rats. No significant difference of TH expression was seen in the prefrontal cortex of these rats (Figure 10).

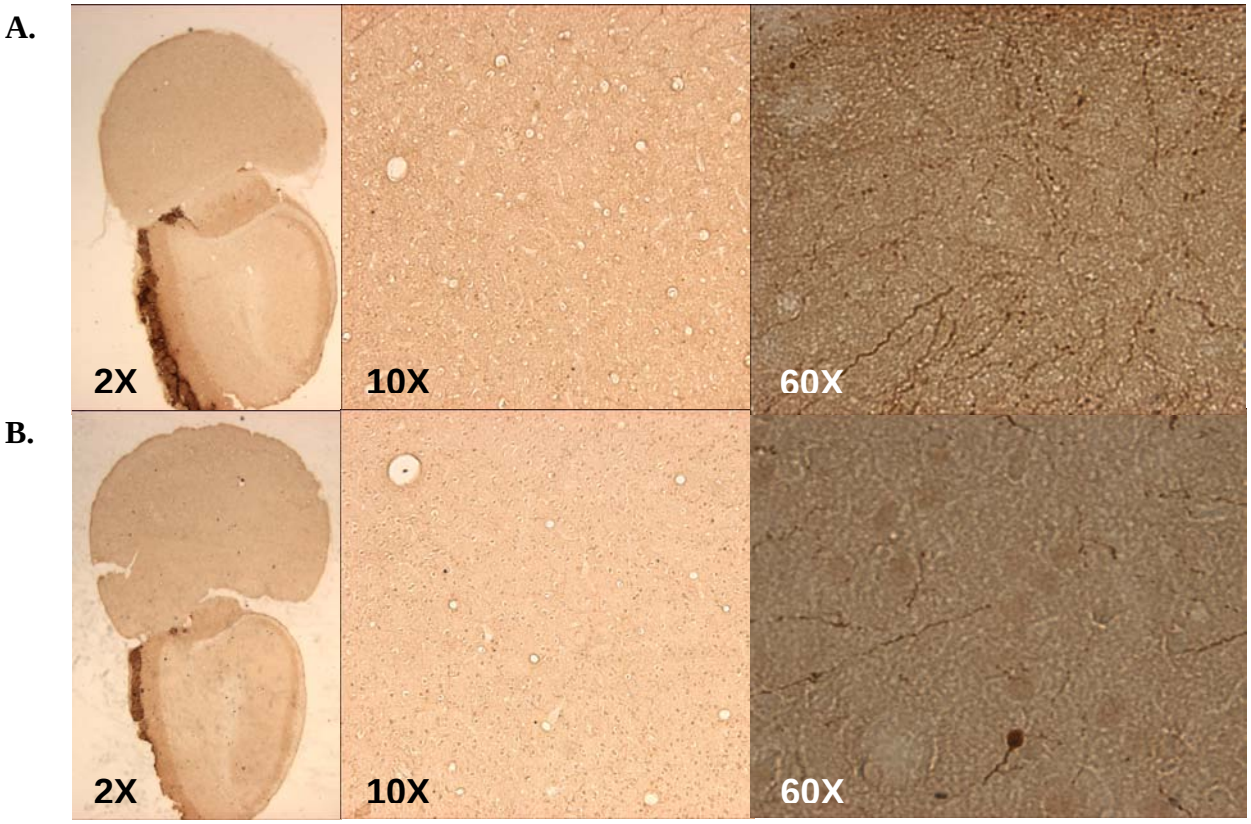


Figure 9. TH protein expression in the striatum of **(A)** Sprague Dawley and **(B)** Fischer 344. Immunocytochemical analysis using an antibody against TH protein demonstrated no difference in expression of TH in Fischer 344. Coronal sections at the level of the prefrontal cortex were stained for TH protein and images made at low magnification (2x; left panels), mid magnification (10x; middle panels), and high magnification (60x; right panels).

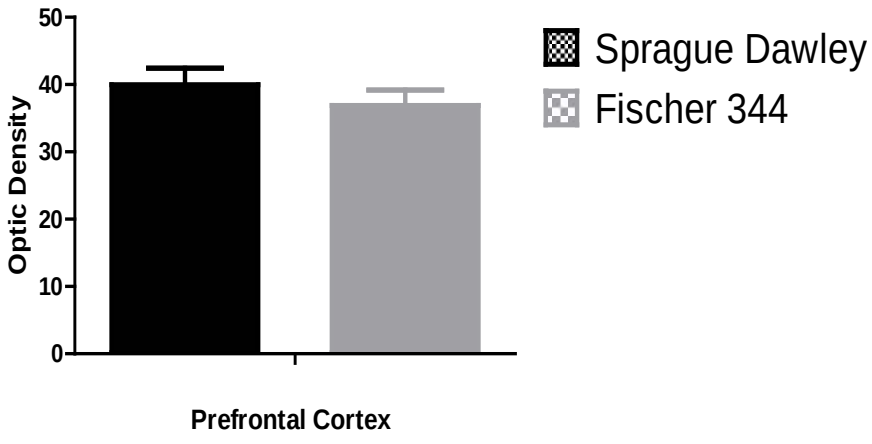


Figure 10. Immunocytochemical analysis of striatal TH protein. Analysis was carried out on coronal sections at the level of the prefrontal cortex of Sprague Dawley (n=3) and Fischer 344 (n=3) rats. No difference in the relative amount of TH protein was seen in the prefrontal cortex. Error bars are standard error of the mean.

**Analysis of TH Expression in Amygdala**

Sections from the caudal end of the temporal lobe were analyzed after being stained with TH using the technique of immunocytochemistry. The TH levels of the cortex and amygdala were compared between the Fischer 344 and Sprague Dawley. No significant differences were found in the cortex or amygdala (Figure 12).

**A.**

**B.**

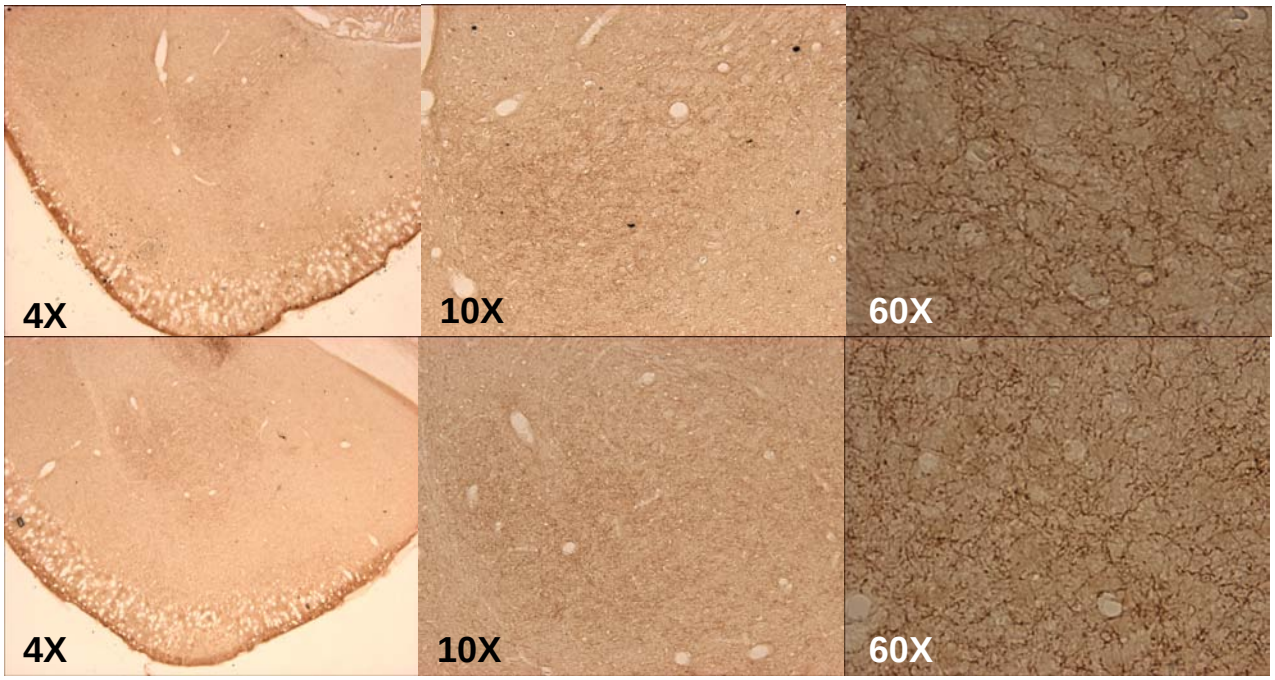


Figure 11. TH protein expression in the striatum of **(A)** Sprague Dawley and **(B)** Fischer 344. Immunocytochemical analysis using an antibody against TH protein demonstrated no difference in expression of TH in Fischer 344. Coronal sections at the level of the caudal end of the temporal lobe were stained for TH protein and images made at low magnification (4x; left panels), mid magnification (10x; middle panels), and high magnification (60x; right panels). Both the Sprague Dawley and Fischer 344 animals displayed darker TH in the amygdala area, as seen by darker staining. Thick networks of TH-positive fibers were seen in both the Sprague Dawley and Fischer 344 animals in the right panels.

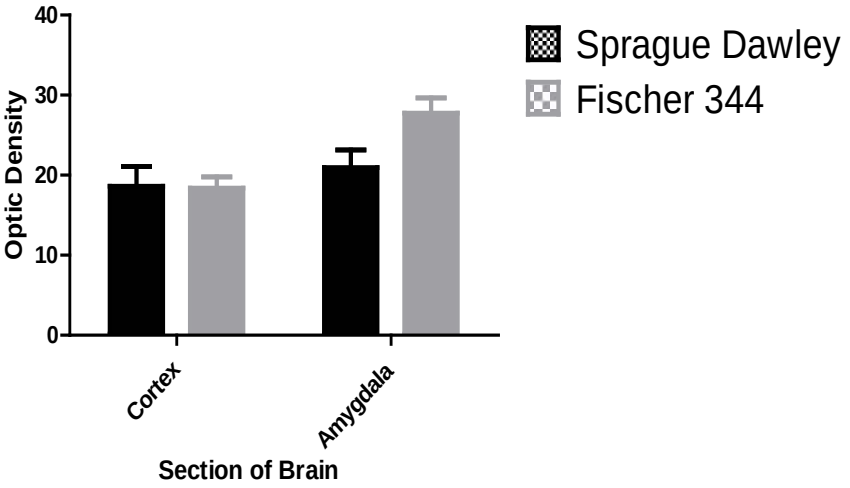


Figure 12. Immunocytochemical analysis of striatal TH protein. Analysis was carried out on coronal sections at the level of the caudal temporal lobe of Sprague Dawley (n=3) and Fischer 344 (n=3) rats. No difference in the relative amount of TH protein was seen in the cortex or amygdala. Error bars are standard error of the mean.

**DISCUSSION**

The Fischer 344 rat has consistently shown behavioral abnormalities similar to Autistic individuals. In this study, the Fischer 344 strain of rat was introduced as an animal model of the Autism disorder. It was anticipated that morphological differences would accompany these aberrant behaviors. This was proven through immunocytochemical staining of TH protein in the various areas of the brain that have shown to be altered in studies of postmortem brains and MRI scans from Autistic subjects. The striatum, prefrontal cortex, and amygdala were analyzed here.

Analysis of this data shows a significant decrease in the levels of TH in Fischer 344 in the dorsal lateral striatum, dorsal medial striatum, nucleus accumbens, and the whole striatum as compared with Sprague Dawley controls. Because TH is an enzyme that catalyzes the reaction to form dopamine, lower levels of TH can correspond with lower levels of dopamine. This shows a relationship with the findings that there is dopaminergic dysfunction in Autism and thus shows more evidence towards the idea that the Fischer 344 rat is an animal that expresses morphological characteristics of Autism in

addition to behavioral characteristics.

The dorsal lateral striatum is responsible for motor behavior. The decrease in the level of TH in this region of the Fischer 344 could correlate with some of the typical hyperactive behavior associated with Autism. This could include the stereotypical repetitive movements or ticks. Non-motor behavior, which is primarily what Autism is characterized by, is seen in the dorsal medial region and nucleus accumbens. The nucleus accumbens is also responsible for reward behavior. Lower levels of TH in these areas in the Fischer 344 rat could represent the delay in developmental characteristics seen in Autism such as communication or interpersonal skills. The abnormal social behavior seen in the Fischer 344 rat and in Autism is further supported by the lower levels of TH in these specific areas of the dopaminergic system/striatum.

Analysis of the levels of TH in the prefrontal cortex using immunocytochemistry shows no difference between the Fischer 344 and Sprague Dawley rats. This means that there is no difference in the dopamine levels in the prefrontal cortex. The prefrontal cortex is involved in the mesocortical pathway of the dopaminergic system. Dysfunction in this pathway is associated with schizophrenia and schizophrenic symptoms are commonly associated with Autism Spectrum Disorders. Because no difference was found in the dopamine levels here, the Fischer 344 rat may not be a good animal to portray Autism with associated schizophrenic behaviors. Because there are projections to the prefrontal cortex from the glutamatergic system, it would be hypothesized that some of the abnormalities seen in the prefrontal cortices of Autism patients may be due to abnormalities in glutamate (Figure 1). If the Fischer 344 is an accurate model of the dysfunction of the glutamatergic system seen in Autism, differentiation between glutamate levels in the Fischer 344 strain would be expected. Ongoing studies

will analyze whether this is the case.

TH levels in the amygdala were analyzed to show no difference between the Fischer 344 and Sprague Dawley rats. The levels of dopamine in the amygdala would therefore also express no difference between the two animal strains. Because there are no dopaminergic projections that extend to the amygdala, no difference in the level of dopamine would be expected in this area. The results validate this theory. Similar to the prefrontal cortex, the amygdala is involved in the glutamatergic system (Figure 1). Ongoing studies will determine whether dysfunction of this system and the enlargement to the amygdala is due to differences in glutamate levels in the amygdala.

**ONGOING STUDIES**

Studies are being conducted in order to further analyze the ability for the Fischer 344 to accurately represent certain behavioral and morphological characteristics of Autism. Additional methods that will be used to hopefully validate the current findings include western blot analysis to compare TH levels in the various areas of the brain. The cerebellum also warrants observation due to the differences seen in Autism subjects when studying the postmortem brain. The substantia nigra will also be looked at since it is also involved in the nigrastriatal dopaminergic system. The substantia nigra produces dopamine and sends projections to the striatum so similarities between the striatum and substantia nigra are hypothesized.

Other proteins are being looked at to compare the morphology of Fischer 344 as compared with Sprague Dawley. Such proteins that merit further study include: dopamine transporter (DAT), glutamate receptor-1 (GluR1), glutamate receptor-2 (GluR2), metabotropic glutamate receptor-1

(mGluR1), and metabotropic glutamate receptor-5 (mGluR5). DAT is a protein spanning the membrane that binds dopamine and transports it from the intersynaptic space into the post-synaptic neuron. It is hypothesized that differing levels of DAT will correlate with the TH levels to show the differentiation in dopamine in the Fischer 344. This will correspond with the dysfunction in the dopaminergic system seen in Autism. The glutamatergic system is necessary for normal brain function and has been compromised in Autism as well as other neurological disorders such as Parkinson's Disease. The glutamate receptors will be analyzed to discover if the Fischer 344 does in fact express glutamatergic dysfunction as well.

## SIGNIFICANCE

### Significance of a New Animal Model

Having an additional animal model for Autism could provide much insight into the neurological mechanism of Autism. The progression of the disorder can be looked at over time. If the morphology and mechanism of Autism is determined, then treatments can be created to reverse the abnormal neuronal interactions. These findings could then be used on animals such as monkeys which are closer relatives to humans. If the treatments succeed in normalizing the developmental disabilities seen in Autism from these animals, they can then be used on humans as a possible treatment or cure. In addition, the new animal model could be used to test drugs and currently available treatments. The success of the drugs could be measured and any side effects could be observed before administration to humans. Succeeding in this aspect could help in treating Autism and will hopefully lead to a cure.

**WORKS CITED**

Arndt TI, Stodgell GJ, and Rodier PM. The teratology of autism. International Journal of Developmental Neuroscience 2005;23:189-199.

Brodkin ES. BALB/c mice: low sociability and other phenotypes that may be relevant to autism. Behavioural Brain Research 2007;176:53-65.

Carlsson ML. Hypothesis: is infantile autism a hypoglutamatergic disorder? Relevance of glutamate – serotonin interactions for pharmacotherapy. Journal of Neural Transmission. 1998;105:525-535.

Carper RA and Courchesne E. Localized enlargement of the frontal cortex in early autism. Biological Psychiatry 2005;57:126-133.

Carper RA, Moses P, Tigue Z, et al. Cerebral lobes in autism: early hyperplasia and abnormal age effects. NeuroImage 2002;16:1038-1051.

Halladay AK, Amaral D, Aschner M, et al. Animal models of autism spectrum disorders: information for neurotoxicologists. Neurotoxicology 2009;1030:1-11.

Hazlett HC, Poe MD, Gerig G, et al. Magnetic resonance imaging and head circumference study of brain size in autism: birth through age 2 years. Arch of General Psychiatry 2005;62:1366-1376.

Howard MA, Cowell PE, Boucher J, et al. Convergent neuroanatomical and behavioural evidence of an amygdale hypothesis of autism. Neurology Report. 2000;13:2931-2935.

Kemper TL, Bauman M. Neuropathology of infantile autism. Journal of Neuropathological Experiments in Neurology 1998;57:645-652.

Kleinhans NM, Johnson LC, Richards T, et al. Reduced neural habituation in the amygdale and social impairments in autism spectrum disorders. American Journal of Psychiatry 2009;166:467-475.

McCracken JT, McGough J, Shah B, et al. Risperidone in children with autism and serious behavioral problems. English Neurology Journal of Medicine 2002;347:314-321.

McDougle CJ, Scahill L, Aman MG, et al. Risperidone for the core symptom domains of autism: results from the study by the autism network of the research units on pediatric psychopharmacology. American Journal of Psychiatry 2005;162:1142-1148.

Nakasato A, Nakatani Y, Seki Y, et al. Swim stress exaggerates the hyeractive mesocortical dopamine system in a rodent model of autism. Brain Research 2008;1193:128-135.

Purcell AE, Jeon OH, Zimmerman W, et al. Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. American Academy of Neurology 2001;57:1618-1628.

Rapin I, Katzman R. Neurobiology of autism. Annals of Neurology 1998;43:7-14.

Rex A, Voigt JP, and Fink H. Behavioral and neurochemical differences between fischer 344 and Harlan-wistar rats raised identically. Behavior Genetics 1999;29:187-192.

Ritvo ER, Freeman BG, Scheibel AB, et al. Lower purkinje cell count in the cerebella of four autistic subjects: initial findings of the UCLA-NDAC autopsy research report. American Journal of Psychiatry 1986;143:862-866.

Roderick TH, Wimer RE, Wimer CC, et al. Genetic and phenotypic variation in weight of brain and spinal cord between inbred strains of mice. Brain Research 1973;64:345-353.

Salgado-Pineda P, Delaveau P, Blin O., et al. Dopaminergic contribution to the regulation of emotional perception. Clinical Neuropharmacology 2005;28:228-237.

Williams G, Kind J, Cunningham M, et al. Fetal valproate syndrome and autism: additional evidence of an association. Developmental Medical Child Neurology 2001;43:202-206.

Williams RS, Houser SL, Purpura DP, et al. Autism and mental retardation: neuropathologic studies performed in four retarded persons with autistic behavior. Arch Neurology 1980;37:749-753.