EFFECTS OF SEIZURES AND IMMUNE SYSTEM ACTIVATION ON AUTISM-LIKE BEHAVIORS: AN ANIMAL MODEL

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Abstract

Although autism and epilepsy have a high comorbidity rate, the nature of their relationship is unknown, and there have not been any studies that explicitly test for a causal relationship between the two disorders. There has also been a suggestion of the possible contributing role of immune system dysfunction in autism, and there appears to be a possible interplay between the immune system, epilepsy, and autism as evidenced by increased levels of immune system responses in epilepsy and autism. This study uses an animal model to investigate the effects of seizures and the combination of immune system activation and seizures on autism-like behaviors. Seizures were induced chemically and electrically in the anterior piriform cortex in adult rodents, and pregnant mothers were injected with lipopolysaccharide in order to activate the immune system in their offspring. Behavioral tests for social preference and exploration frequency were used to observe the effects of seizures and the combined effect of seizures and immune system activation. This study provides preliminary support for the interaction of immune system activation and seizures in the etiology of some of the behaviors in autism, specifically exploratory behavior. The combination of these two factors affected exploration frequency more than seizures alone. The effects of seizures and immune system activation on social preference were difficult to assess since the mode of seizure induction proved to have the strongest effect on social preference, indicating that different types of seizures occurred depending on whether the anterior piriform cortex was stimulated chemically or electrically.
1. Introduction

Autism is a neurodevelopmental disorder that is identified by the presentation of qualitative impairments in language, communication, and social skills, and stereotypic behaviors before the age of three (DSM-IV, 1994). Epilepsy is defined as two or more unprovoked bouts of abnormal neuronal discharge of unidentifiable cause (Steffenburg et al., 2003). Recent estimates place autism’s prevalence at 1 in 150 (Charman, 2002; Fombonne, 2005), while epilepsy is found in 2 to 3 % of the general population (Tuchman and Rapin, 2002).

Autism and epilepsy are comorbid, although estimates of the presence of epilepsy in autism vary widely, ranging between 14% to 42% (Danielsson et al., 2005; Giovanardi Rossi et al., 2000; Kagan-Kushnir and Roberts, 2005; Kawasaki et al., 1997; Tuchman et al., 1991). Studies that have broken this prevalence down by age indicate that there are lower percentages of epilepsy in children with autism than in autistic adolescents and adults (Danielsson et al., 2005; Tuchman et al., 1991; Tuchman and Rapin, 1997). Research on epilepsy in autism has also found that there are two peaks of epilepsy onset, with one in early childhood, and one in adolescence (Kawasaki et al., 1997). Research investigating types of seizures seen in autism reveals that all types of seizures have been observed and that no one seizure type or pattern is most commonly found (Danielsson et al., 2005; Giovanardi Rossi et al., 2000; Kanner, 2000; Mouridsen et al., 2000; Steffenburg et al., 2003; Tuchman et al., 1991; Tuchman and Rapin, 2002).

There are several theories that attempt to explain the high comorbidity rate between these two disorders (Deonna and Roulet, 2006). As of yet, it is unknown whether the two disorders have the same origin, which could be due to genetics, disease, or a common underlying pathology; whether epilepsy causes autism or some of the behaviors seen in autism, either
through altering normal brain development or causing cognitive dysfunctions; or if having autism can put a child at a risk for developing epilepsy (Deonna and Roulet, 2006; Nelson, 1991). Recent research has highlighted the contributing role of genetics in the vulnerability to autism (Folstein and Rosen-Sheidley, 2001; Korvatska et al., 2002; Muhle et al., 2004). However, even though this research points to the interplay of multiple genes in the genesis of autism, the influence of environmental factors cannot be ruled out (Charman, 2002; Szatmari, 2003).

To date, there have not been any studies that explicitly test for a causal relationship between epilepsy and autism. Thus, those who do not believe that there is a causal relationship base their conclusion on an “absence of evidence” rather than on evidence that disproves causation (Deonna and Roulet, 2006; Kanner, 2000). The lack of information speaks to the need for an experimental study that directly tests whether or not epilepsy causes autism.

The immune system has also been implicated in autism etiology. While many studies have disproved the relationship between the measles, mumps, and rubella (MMR) vaccine and autism (Charman, 2002; Steffenburg et al., 2003), there has been a suggestion of the possible contributing role of immune system dysfunction in autism (Burger and Warren, 1998; Folstein and Rosen-Sheidley, 2001; Jyonouchi et al., 2001; Korvatska et al., 2002; Nelson, 1991). Furthermore, there appears to be a possible interplay between the immune system, epilepsy, and autism. A study by Connolly et al. (2006) found that children with autism and epilepsy had elevated levels of autoantibodies to several different antigens. Additionally, other studies have shown that some cytokine levels increase after seizures (Allan and Rothwell, 2001; Jyonouchi et al., 2001), and that children with epilepsy are at higher risk for abnormal autoantibody levels (Connolly et al., 2006). However, even if the immune system does play a role in the pathology
of autism, it alone cannot cause autism (Jyonouchi et al., 2001). The presence of increased levels of autoantibodies in autism could be a marker of the disorder instead of evidence of a causal role of the immune system (Connolly et al., 2006), but there is not enough research on the topic to definitively identify the relationship between the two.

The ambiguity surrounding autism shows that there is a great need for more research investigating the role of all possible etiologic factors, including epilepsy and the immune system. This study uses an animal model to see if seizures alone or the combination of seizures and immune system activation result in the exhibition of certain autism-like behaviors. Rodent models of autism have been used in other studies (Schneider and Przewlocki, 2005; Tsujino et al., 2007, Walker et al., 2007), but none have tested the behavioral effects of seizures. The area tempestas (in the anterior piriform cortex) was stimulated either electrically or chemically in order to induce seizures. This region was selected because of its sensitivity to bicuculline, a chemoconvulsant, and its propensity to produce robust generalized motor seizures after stimulation (Fornai et al., 2005). As a second part of the present study, the effects of seizures as a second insult after immune system activation in utero due to maternal infection were investigated to see if the combination of these two environmental factors resulted in greater autism-like behaviors than in normal controls, subjects that only experienced seizures, or subjects that only experienced immune system activation. Lipopolysaccharide (LPS) was used in this study because of its role in innate immunity. Since LPS produces proinflammatory and counter-regulatory cytokines which can cross the blood-brain barrier (Jyonouchi et al., 2001), rats born to mothers that were injected with LPS while they were pregnant are considered to have experienced immune system activation.
Four experiments comprise this study. The first two experiments induced seizures chemically by injecting bicuculline into the anterior piriform cortex of adult rats (Bicuculline Single Insult). The third experiment also chemically induced seizures in adult rats, but included some subjects that had experienced immune system activation in utero due to maternal infection (Bicuculline Double Insult). The fourth experiment electrically induced seizures in the anterior piriform cortex in rats that had experienced immune system activation in utero (Electrical Double Insult). In the analyses, all of the subjects that were injected with bicuculline were combined into groups based on whether or not they were born to a mother that had been injected with LPS while pregnant. These two bicuculline groups were then compared with the electrically stimulated double insult group, a control group of rats that had experienced immune system activation in utero, and a sham surgery control group.

2. Materials and Methods

All experimental protocols were in compliance with AALAC standards and approved by the Georgetown University Animal Care and Use Committee.

2.1. Experiment One

2.1.1. Subjects

Eight adult male Sprague-Dawley rats (Harlan Laboratories, IN, USA) were used in this experiment. The rats were housed two to three in cage and were kept in a temperature and humidity controlled colony under standard light conditions (12 hr light/12 hr dark with light on between 6 a.m. and 6 p.m.). Food and water were available ad libitum.
2.1.2. Surgery

All surgeries were performed with a Kopf stereotaxic instrument (David Kopf, Tujunga, CA, USA, Model 900). Each rat was anesthetized with equithesin (1.1 mL/300 g, i.p.), and three reflex tests were performed to verify that the subject was deeply anesthetized. The subject was positioned in the Kopf instrument with the incisor bar 5.0mm above the interaural line, and a midline sagittal incision was made in the scalp. A guide cannula was placed in the area tempestas (ant. 4.0mm, lat. 3.5mm, ventr. 6.5mm) (Pellegrino et al., 1973). Stainless steel jeweler’s screws were attached to the skull, and denture adhesive was used to create a cap that used the screws to anchor the cannula. Stitches were placed in the skin around the cap to aid recovery, and the subjects were placed on a surgical heating pad until they woke up and regained movement.

2.1.3. Seizure Induction

Seizures were induced with the chemoconvulsant bicuculline (0.5 mg/mL). Bicuculline was administered to freely moving animals via a 28 gauge internal cannula that extended 1 – 2 mm below the guide cannula. The cannula was connected via polyethylene tubing to a Hamilton syringe driven by a Sage infusion pump that administered bicuculline at a rate of 22.5 ng/min. This method of infusion is very close to that used by Fornai et al. (2005). Bicuculline was infused until seizure activity occurred, and infusion was stopped immediately after seizure activity was observed. If there was no seizure activity after 20 minutes (450 ng), bicuculline infusion was stopped. This dosage was selected in order to accommodate possible variation between subjects in susceptibility to bicuculline. After each infusion, the internal cannula was left in the guide cannula for one minute before being removed. Each subject was watched for an
hour after infusion to ensure that all seizure activity had ceased. In one instance, 2 cc of diazepam were administered to stop seizure activity after it had continued for 97 minutes.

Seizure activity was rated on a scale of 0 to 5 according to the scale used in Fornai et al. (2005). On this scale, a 0 signified no seizure activity, 0.5 signified jaw clonus, 1 signified myoclonic jerks of one forelimb, 2 signified myoclonic jerks of the head with bilateral forelimb clonus, 3 signified bilateral forelimb clonus lasting longer than 15 seconds, 4 signified bilateral forelimb clonus with rearing, and a 5 signified bilateral forelimb clonus with rearing leading to falling. If a subject exhibited seizure activity rated a 3 or above after an infusion of bicuculline, the subject was considered to have had a seizure, since the ratings between 3 and 5 signify generalized seizures (Engel et al., 1978; McNamara 1986; as cited in Gale).

The number of days of bicuculline infusion varied per subject, ranging between twelve and eighteen days within a thirty day span. One subject’s needle stuck in the cannula on the first day of infusion, resulting in the need to undergo a second surgery to replace the cannula. Thus, this subject was only infused for twelve days. The other subjects varied between sixteen and eighteen days of infusion, differing because of date of surgery. Three of the rats died during the course of the experiment, but subject death never occurred on the same day as a bicuculline infusion. The experiment ended with a sample size of five. Four of the subjects had at least one seizure that was ranked at a 3 or above.

2.1.4. Behavioral Tests

2.1.4.1. Test of Social Preference

The social preference of the subjects was tested by placing them in an apparatus that contained three linearly joined 20.5cm x 10.5cm x 12.2cm boxes (Habitest, Coulbourn
Instruments). The middle box was the starting, or initial chamber [I]. On one side of [I] was the social chamber [S] that contained a novel stimulus rat, and on the other side of [I] was the home chamber [H] that contained bedding from the rat’s home cage. Two plastic partitions with six small holes (.25cm radius) and one large hole (1.25cm radius) in the center minimized direct contact between the subject and the novel rat in [S] and the bedding in [H]. The partitions were placed about a quarter of the way into each chamber so that the subjects could partially enter each chamber.

At the beginning of the test, the subject was placed in [I] and doors between [I] and [H] and [S] were lifted so that the subject could move freely among the three chambers. The subjects remained in the social apparatus for fifteen minutes (900 seconds), and the number and length of visits to both [S] and [H] were tracked by photo beams at the entrance to both the [S] and [H] chambers. This information was recorded and analyzed by a computer (GraphicState, Coulbourn Instruments). In between subjects, the social apparatus was thoroughly cleaned with 70% ethanol and allowed to dry before the next test was conducted.

The first test of social preference was administered the day after infusions stopped, and the subjects underwent a second test for social preference four weeks after the last date of infusion.

2.1.4.2. Test of Exploration Frequency

Exploration behavior was tested by placing the subjects in a 37cm x 25cm x 15cm apparatus with 16 holes (1 cm in diameter) in the floor in a 4 x 4 array. Each hole was filled with beta-cob bedding. Two rings of photocells around the perimeter of the chamber recorded the subjects’ locomotion along the plane as well as the number of nose entries into the holes on
the floor of the plane during a ten minute observation period. The data was collected and analyzed by computer (TruScan, Coulbourn Instruments). In between subjects, the exploration apparatus was thoroughly cleaned with 70% ethanol and allowed to dry before the next test was conducted.

The first test of social preference was administered the day after infusions stopped, and the subjects underwent a second test for social preference four weeks after the last date of infusion.

2.2. Experiment Two

2.2.1. Subjects

Five adult male Sprague-Dawley rats (Harlan Laboratories, IN, USA) were used in this experiment. The rats were housed in conditions identical to those in Experiment One.

2.2.2. Surgery

Experiment Two followed the same surgical procedure as Experiment One.

2.2.3. Seizure Induction

As in Experiment One, seizures were induced with the chemoconvulsant bicuculline (0.5 mg/mL). The bicuculline was administered to freely moving animals via the same method used in Experiment One. Bicuculline was infused for ten days over a fourteen day period. Smaller doses of bicuculline were used in Experiment Two since the large doses in Experiment One did not increase the subject’s susceptibility to seizure induction, and there was a concern that the volume of liquid that accompanied the high doses was damaging to the brain (Fornai, 2005).
Thus, smaller doses of bicuculline were used in Experiment Two. All subjects were infused with 60 ng of bicuculline on the first day, which corresponds to the dosage used in Fornai et al. (2005). Thereafter, the subjects stayed at 60 ng until three days of infusion without seizures passed; then the dosage was increased to 75 ng. Once two days of infusion at 75 ng passed without inducing any seizures, the dosage was increased to 90 ng. This continued for nine days, and on the tenth day (the last day of infusion), all subjects received 120 ng. The bicuculline dosage was gradually increased because its effectiveness in inducing seizures seemed to decline after subsequent infusions at the same dosage. After each infusion, the internal cannula was left in the guide cannula for one minute before being removed. Each subject was watched for an hour after infusion to ensure that all seizure activity had ceased.

The same seizure rating scale from Experiment One was used in Experiment Two. One rat died after surgery to replace a cannula that ripped out during the experimental period, resulting in an ending sample size of four. All four subjects had at least one seizure that was ranked a 3 or above.

2.2.4. Behavioral Tests

2.2.4.1. Test of Social Preference

The subjects were submitted to the same social preference test that was used in Experiment One. The first test of social preference was administered the day after bicuculline infusions ended, the second was two weeks after the last date of infusion, and the third and final social test was administered six weeks after the last date of infusion.

2.2.4.2. Test of Exploration Frequency
The subjects were submitted to the same exploration frequency test that was used in Experiment One. The first test of exploration frequency was administered the day after bicuculline infusions ended, the second was two weeks after the last date of infusion, and the third and final exploration test was administered six weeks after the last date of infusion.

2.3. Experiment Three

2.3.1. Subjects

Six adult Sprague-Dawley rats that were born in the lab from two adult female Sprague-Dawley rats (Harlan Laboratories, IN, USA) were used in this experiment. Two adult females and two adult males were born to a mother that had received intraperitoneal injections of LPS (.5mg LPS/kg; Sigma, St. Louis, MO) diluted in phosphate-buffered saline (PBS; 1mg LPS/1mL PBS) while pregnant. Two adult females were born to a mother that had only been injected with saline while pregnant, making these subjects comparable to the subjects in Experiments One and Two. The rats were housed in conditions identical to those in Experiment One.

2.3.2. Surgery

Experiment Three followed the same surgical procedure as Experiment One. One of the male subjects died following surgery, leaving a sample size of five for the experiment.

2.3.3. Seizure Induction

As in Experiment One, seizures were induced with the chemoconvulsant bicuculline (0.5 mg/mL). The bicuculline was administered to freely moving animals via the same method used in Experiment One. Bicuculline was infused for ten days over a thirty-six day span. One LPS
female was only infused for nine days because of a blocked cannula, and one saline female was
only infused for eight days because the cannula ripped out after eight days of infusion. Subjects
were infused with 60 ng of bicuculline on the first day and with 120 ng on all subsequent days of
infusion. The procedure was changed from Experiment Two because the graduated changes in
nanograms of bicuculline did not affect seizure activity, so it was decided to give one low dose
on the first day of infusion and then increase the dosage for the remainder of the experiment.

One saline subject and one LPS subject had at least one seizure that was rated a 3 or
above; the other three subjects did not.

2.3.4. Behavioral Tests

2.3.4.1. Test of Social Preference

The subjects were submitted to the same social preference test that was used in
Experiment One. The first test of social preference was administered 12 to 15 days after the last
date of infusion. The second test of social preference was administered seven weeks after the
last date of infusion.

2.3.4.2. Test of Exploration Frequency

The subjects were submitted to the same exploration frequency test that was used in
Experiment One. The first test of exploration frequency was administered twelve or thirteen
days after the last date of infusion and the second test was administered seven weeks after the
last date of infusion.
2.4. Experiment Four

2.4.1. Subjects

Five male and three female adult Sprague-Dawley rats were used in this experiment. They were born in the lab from two adult female Sprague-Dawley rats (Harlan Laboratories, IN, USA) that had received intraperitoneal injections of LPS (.5mg LPS/kg; Sigma, St. Louis, MO) diluted in phosphate-buffered saline (PBS; 1mg LPS/1mL PBS) while pregnant. The rats were housed in conditions identical to those in Experiment One.

2.4.2. Surgery

All surgeries were performed in a Kopf stereotaxic instrument (David Kopf, Tujunga, CA, USA, Model 900). Each rat was anesthetized with equithesin (1.1 mL/300 g, i.p.), and three reflex tests were performed to verify that the subject was deeply anesthetized. The subject was positioned in the Kopf instrument with the incisor bar 3.3mm below the interaural line, and a midline sagittal incision was made in the scalp. A platinum electrode was placed in the anterior piriform cortex (ant. 1.8mm, lat. 3.0mm, ventr. 8.0mm) (Paxinos and Watson, 1996). These coordinates followed those used by Ebert and Loscher (1995), who found these coordinates to be a location where electrodes stimulated the area tempestas. Stainless steel jeweler's screws were attached to the skull, and denture adhesive was used to create a cap that used the screws to anchor the electrode. Stitches were placed in the skin around the cap to aid recovery, and the subjects were placed on a surgical heating pad until they woke up and regained movement. Two of the males still responded to the reflex tests after the appropriate dose of equithesin. Even though surgery was not performed, one of the subjects died. The other male was tested.
behaviorally and included with the control LPS group for analysis. Two of the females died after surgery, leaving one female and three males in this experiment.

2.4.3. Seizure Induction

Seizures were induced by electrical stimulation in the anterior piriform cortex. The induction method was based on the methods of Ebert and Loscher (1995). While moving freely, the subjects were stimulated with biphasic 1 ms repeated pulses, 50 Hz, 500 μA peak-to-peak until seizure activity that rated a 3 or above was observed. The same rating scale used in Experiment One was used in this experiment. Stimulation stopped once seizure activity occurred that was ranked a 3 or above. If no seizure activity was present, the stimulation continued for five minutes. At the end of the five minutes stimulation stopped, and was followed by a two minute observational period. This cycle of stimulation and observation was repeated three times, and if no seizure activity was observed, the stimulation was increased by 100 μA for the next five minute stimulation period. This 100 μA increase was continued after each five minute stimulation that did not elicit seizure activity until the subject received stimulation at 800 μA. At the end of the 800 μA cycle, stimulation ceased. Each subject was watched for half an hour after stimulation to ensure that all seizure activity had ceased.

Subjects were electrically stimulated on two consecutive days. One male was only stimulated on one day because the electrode ripped out after stimulation, and this subject did not exhibit any seizure activity. The other three subjects had at least one seizure that was ranked a 3 or above.
2.4.4. Behavioral Tests

2.4.4.1. Test of Social Preference

The subjects were submitted to the same exploration frequency test that was used in Experiment One. The first test of exploration frequency was administered on the last day of electrical stimulation, the second test was administered three weeks after the last date of stimulation, the third test six weeks after the last date of stimulation, and the fourth and final test was administered ten weeks after the last date of stimulation.

2.4.4.2. Test of Exploration Frequency

The subjects were submitted to the same exploration frequency test that was used in Experiment One. The first test of exploration frequency was administered on the last day of electrical stimulation or the day immediately after, the second test was administered three weeks after the last date of stimulation, the third test six weeks after the last date of stimulation, and the fourth and final test was administered ten weeks after the last date of stimulation.

2.5. Controls

Control data for adult subjects that were not born to mothers injected with LPS while pregnant and not stimulated chemically or electrically, but that underwent a sham surgery, were taken from archived lab data. The social preference and exploration frequencies of each experimental group were compared to these controls. Social preference data from the lab was also used to create a LPS control group for analysis, which consisted of rats that were born to a mother injected with LPS while pregnant that never underwent surgery and were never electrically or chemically stimulated. While this data was collected from rats before adulthood,
the mean social index of the LPS control group did not differ significantly from the mean baseline social index of the experimental double insult group, which was taken after surgeries and before stimulation ($t(19) = .035$, n.s.). However, the exploration frequencies between the LPS control group differed significantly from a baseline exploration test of the experimental double insult group ($t(19) = 2.018$, $p < .01$), so the exploration frequencies of the experimental groups were only compared to the control group and not to a LPS control group. The baseline exploration test of the experimental double insult group was not significantly different than the control group ($t(11) = -.111$, n.s.).

2.6. Data Analysis

The social preference of each subject was determined by compiling a “social index.” The social index was determined by dividing the difference between time spent (in seconds) in the [S] and [H] chambers by the total time of the experiment, 900 seconds (formula = $([S] – [H])/900$). The Social Index indicates what percentage more time was spent in [S] than in [H]. Time spent in [I] was not included in the calculation since it was the chamber the subject was placed in at the beginning of the experiment, and the chamber that had to be entered to travel between [S] and [H]. Thus, only time spent in [S] and [H] were evaluated since the subject had to choose to specifically enter both the [S] and [H] chambers. A logarithmic transformation was applied to the data since the social indexes were not normally distributed.

Exploration frequency was defined as the number of nose pokes per minute of ambulation.

Mean values were found for each subject for social index and exploration frequency across all behavioral tests after seizure induction. The mean social index and mean exploration
frequency represent each subject’s social and exploration behavior after exposure to electrical or chemical stimulation.

All of the subjects that were injected with bicuculline were grouped together for analysis. Subjects were divided into separate groups based on whether or not they were born to a mother that had been injected with LPS while pregnant. In the analysis, the animals injected with bicuculline that were born to a mother injected with LPS are referred to as “Bicuculline Double Insult” and animals that were injected with bicuculline that were not born to a mother injected with LPS are referred to as “Bicuculline Single Insult.” The subjects that were born to mothers that had been injected with LPS that were electrically stimulated are referred to as “Electrical Double Insult.” The mean social index (SI) and exploration frequency (EF) of these three groups were compared to each other as well as to the mean SI and EF of the controls, and the SI was compared to the mean SI of the LPS controls. Although some studies have found a gender difference in rates of epilepsy in autism (Danielsson et al., 2005; Tuchman et al., 1991), unpublished research from this lab has shown that there is no gender difference in the animal behaviors of social preference and exploration frequency. Thus, males and females were not evaluated separately in this study.

The aim of the study was to electrically and chemically stimulate subjects in the hopes of inducing seizures, but since there is no uniform number or pattern of seizures in autistic patients, this study was based on the subjects’ exposure to stimulation, not on the number of seizures that occurred. Thus, analyses were only performed on the social indexes and the exploration frequencies of the subjects, and there were no analyses of the number of seizures that were induced. Furthermore, there is evidence that subclinical epileptiform activity occurs in a significant portion (although not the majority) of the autistic community, and the effects of this
epileptiform activity is unknown (Kagan-Kushnir and Roberts, 2005; Tuchman and Rapin, 1997; Tuchman and Rapin, 2002). For these reasons, even subjects that did not exhibit any observable seizure activity have been included in the results.

An analysis of variance (ANOVA) and post-hoc t-tests for paired data were used to compare the data.

3. Results

An ANOVA of the social indexes of the Bicuculline Single Insult, Bicuculline Double Insult, Electrical Double Insult, LPS, and Control groups showed a significant main effect for experimental group (F(4,43) = 7.753, p = .000) (Figure 1). Post-hoc t-tests showed that the control group’s SI was significantly higher than both of the bicuculline groups (t(13) = -3.333, p = .005 when compared to the Bicuculline Double Insult Group; t(19) = -4.533, p = .000 when compared to the Bicuculline Single Insult Group) (Table 1). Additional t-tests were performed that investigated whether the lower social indexes of the experimental groups were due to a decrease in the amount of time spent in [S] or an increase in the amount of time spent in [H]. These tests showed that the difference between the Bicuculline Double Insult Group and the controls was significant due to a decrease in the time spent in the social chamber and a marginally significant trend for an increase in time spent in the home chamber (t(13) = -1.370, p < .1 for home time; t(13) = 2.156, p = .05 for social time). The difference between the Bicuculline Single Insult Group and the controls was due to both decreases in the time spent in [S] and increases in the time spent in [H] (t(19) = -2.696, p <.05 for time spent in the home chamber; t(19) = 4.241, p = .000 for time spent in the social chamber). The means of the
Electrical Double Insult Group and the Control Group were almost identical ($t(13) = -0.016$, n.s.) (Figure 2).

The LPS group’s SI was only significantly higher than that of the Bicuculline Single Insult Group ($t(26) = -3.293$, $p < .005$), and this was due to a decrease in the amount of time spent in [S] by the Bicuculline Single Insult subjects ($t(25) = 1.101$, n.s. for home time; $t(25) = -4.427$, $p = .000$). However, there was a marginally significant trend for the LPS group’s SI to be higher than that of the Bicuculline Double Insult Group ($t(20) = -1.426$, $p < .10$) and lower than the control group’s SI ($t(25) = -1.585$, $p < .10$). There was no significant difference between the LPS group and the Electrical Double Insult Group ($t(20) = 1.262$, n.s.) (Table 2).

The Bicuculline Double Insult Group’s SI was significantly lower than the SI of the Electrical Double Insult Group ($t(8) = 5.969$, $p = .000$) (Figure 3). This was due to a statistically significant decrease in the time spent in [S] and a marginally significant trend for an increase in time spent in [H] ($t(8) = 1.560$, $p < .10$ for home time; $t(8) = -4.449$, $p = .002$ for social time). The Bicuculline Single Insult Group’s SI was also significantly lower than the Electrical Double Insult Group ($t(14) = 5.381$, $p = .000$), which resulted from both a decrease in the time spent in [S] and an increase in the time spent in [H] ($t(14) = 1.865$, $p < .05$ for home time; $t(14) = -6.525$, $p = .000$ for social time). The Bicuculline Single Insult SI was significantly lower than the Bicuculline Double Insult SI ($t(14) = -1.1783$, $p < .05$) (Figure 4). However, a further investigation of the difference only revealed marginally significant trends for a decrease in time spent in [S] and an increase in time spent in [H] ($t(14) = -1.211$, $p < .10$ for home time; $t(14) = 1.520$, $p < .10$ for social time).

An ANOVA of the exploration frequencies of the Bicuculline Single Insult, Bicuculline Double Insult, Electrical Double Insult, LPS, and Control groups also showed a significant main
effect for experimental group (F(4,40) = 9.238, p = .000) (Figure 5). The Control group had a significantly higher exploration frequency when compared to both of the double insult groups (t(11) = 2.284, p <.05 when compared to the Bicuculline Double Insult Group; t(11) = 2.333, p <.05 when compared to the Electrical Double Insult Group). However, the Control group’s EF was only marginally significantly higher than the Bicuculline Single Insult Group (t(17) = 1.455, p <.10), and the Control group’s EF was lower than the LPS control group (t(22) = -2.480, p <.05) (Table 1). There was no statistically significant difference between the Bicuculline and Electrical Double Insult Groups (t(8) = .235, n.s.), nor was there a statistically significant difference between the Bicuculline Single and Double Insult Groups (t(14) = 1.208, n.s.). However, the Electrical Double Insult Group’s EF was marginally significantly lower than the Bicuculline Single Insult Group (t(14) = 1.351, p <.10).

4. Discussion

Under the hypothesis of a causal relationship between epilepsy and autism, the subjects that were chemically and electrically stimulated should have lower social indexes than controls. This was true for subjects that were infused with bicuculline, but was not true for subjects that were electrically stimulated. Since the work of Ebert and Loscher (1995) used an electrode for seizure induction in the area tempestas, an area that is known for its high susceptibility to chemically induced seizures (Fornai et al., 2005), this study presumed that the electrically and chemically stimulated seizures were comparable and that their results could be combined for analysis. However, the statistically significant difference between the social indexes of the chemically and electrically induced groups revealed that the two methods of induction did not result in comparable behaviors, and the results were analyzed separately. The inability to
combine the data is one of the reasons that the sample sizes for the double insult groups are so small. However, the difference in behaviors after chemically and electrically induced seizures is interesting because it shows that these two methods of seizure induction do not result in the same type or pattern of seizure even though the same area of the brain is stimulated. The work of Ebert et al. (2000) and Ebert and Loscher (1995) showed that the piriform cortex can be both electrically and chemically stimulated to induce generalized motor seizures. Although stimulation in the piriform cortex results in generalized motor seizures regardless of the location of stimulation, Ebert et al. (2000) found that different anatomical connections were associated with bicuculline injections in different regions of the piriform cortex. Since electrical stimulation activates all regions of the piriform cortex (Loscher et al., 1995, as cited in Ebert et al., 2000), it is possible that electrically stimulated seizures activate all the anatomical connections associated with the different regions of the piriform cortex, resulting in qualitative differences between electrically induced seizures and bicuculline-induced seizures that are restrained to the anterior piriform cortex.

The difference in the two types of seizure induction can also be seen when the social index of the LPS control group is considered. Immune system activation did not appear to affect social preference, since the LPS control group only differed significantly from the Bicuculline Single Insult group. However, there was a marginally significant trend for the LPS control group’s SI to be lower than that of controls. In contrast with these findings, the Bicuculline Double Insult group’s SI was significantly lower than that of controls, while there was no difference between the Electrical Double Insult Group and the Control Group. At first glance, these results seem to indicate that bicuculline induced seizures made a marginally significant decrease in sociability after immune system activation significantly lower than controls, while
electrically induced seizures reversed this trend. However, this finding is not conclusive since the Bicuculline Single Insult Group also had a significantly lower social index than the Control Group. The fact that both bicuculline groups had a lower social index than controls, regardless of whether they were born to a mother that had been injected with LPS while pregnant, while the electrically stimulated group was comparable with controls, could indicate that the difference in social behavior has more to do with the effects of bicuculline than the effects of seizures and increased immune activity.

While the finding that the social indexes of both bicuculline groups were lower than controls regardless of immune system activation does provide support for the differing effects of electrically and chemically induced seizures, the data can also be looked at from an alternative perspective. Both of the double insult groups had significantly higher social indexes than the Bicuculline Single Insult group, possibly indicating that immune system activation buffered some of the effects of seizures on social preference. However, it is difficult to determine if this is a viable proposal for the changes in behavior seen in this study without an Electrical Single Insult group to compare these results to. A replication of this study with an Electrical Single Insult group would help identify whether immune system activation changes the effects of seizures on social preference, since its effects in the current study are difficult to evaluate independent of the agent of seizure induction.

While the different modes of seizure induction appear to have affected the subjects’ social index in different ways, the two methods had the same effect on exploration frequency. There was no difference in exploration frequency between the two double insult groups, and both groups had significantly lower exploration frequencies than the controls, signifying that the observed decrease in exploration frequency was caused by the epileptic activity, not by the agent
of induction. The combination of seizures and immune system activation decreased exploration frequency more than seizures alone, as the differences between the controls and both double insult groups were significant, while the control group’s EF was only marginally significantly higher than the Bicuculline Single Insult Group. The finding that the double insult groups were significantly lower than controls while the single insult group was not indicates that it was the combination of environmental factors that resulted in decreased exploration. This is a particularly interesting observation since the LPS control group had a higher exploration frequency than controls when measured before adulthood, although this increase in exploration frequency disappeared by adulthood. This suggests that immune system activation increases exploration during development but leaves the subject vulnerable to a greater decrease in exploration if the subject is exposed to another environmental insult. However, the combined effect of immune system activation and seizures on autism-like behavior is not completely corroborated, since there was no significant difference between the two bicuculline groups, although there was a trend for the Electrical Double Insult group’s EF to be lower than the Bicuculline Single Insult Group. Further research is needed to clarify the effects of the combination of environmental factors on exploration frequency.

One of the limitations of this study is that adult rats were tested instead of developing rats because the cannulas and electrodes needed to be placed in a specific location in the brain. In humans, seizures would have to take place in the first few years of life in order to be causative, since autism is diagnosed by the age of 3 (DSM-IV), so future studies investigating a causal relationship between epilepsy and autism could create a more accurate model by inducing seizures in developing rats. The results of this study are important because they show the effect of seizures and immune system activation on two autism-like behaviors, but the full effect of
these environmental factors will not be known until they are studied at the proper developmental stage. Immature brains are more susceptible to seizures but are not as vulnerable to damage as adult brains are (Holmes, 2004), so it cannot be assumed that seizures will affect these behaviors in the same way in developing animals as they do in adult animals. Future research would also benefit from testing additional autism-like behaviors, such as stereotypic behaviors.

Another limitation of this study is that each experiment had a small sample size and followed a different methodology. The variation in the number of days of chemical and electrical stimulation and number of seizures that each subject experienced does not hurt the integrity of this study since seizures in humans are not uniform, but a repetition of this study with larger sample sizes and a standard methodology would confirm these results and strengthen the findings presented here.

Even if these results were to be duplicated, further research is still needed before a causal relationship between epilepsy and autism can be proven. A study that investigates the behavioral effects of seizures induced via a variety of methods would shed more light on whether the results seen here are a result of the seizure or the agent used to induce seizures.

Despite these limitations, this study did find significant behavioral changes in both social preference and exploration frequency, providing preliminary support for the interplay between epilepsy and immune system activation in the etiology of autism, and raises interesting questions about the types of seizures that result from different methods of induction.
Figure 1. Mean Social Index of all experimental groups. An ANOVA found a significant main effect for experimental group (F(4,43) = 7.753, p = .000).
Figure 2. Mean Social Index of the Electrical Double Insult Group and the Control Group ($t(13) = -.016, \text{n.s.}$).
Table 1. Mean Social Indexes and Nose Pokes/Minute of Ambulation compared via a Student $t$-test with the mean of the control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Social Index</th>
<th>Nose Poke/Minute of Ambulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicuculline Single Insult</td>
<td>.008*** (.028)</td>
<td>2.932 (.327)</td>
</tr>
<tr>
<td>Bicuculline Double Insult</td>
<td>.065** (.014)</td>
<td>2.305* (.249)</td>
</tr>
<tr>
<td>Electrical Double Insult</td>
<td>.169 (.010)</td>
<td>2.210* (.314)</td>
</tr>
<tr>
<td>LPS</td>
<td>.120 (.020)</td>
<td>6.942* (.790)</td>
</tr>
</tbody>
</table>

Note: Numbers in parentheses indicate the standard error of the mean (S.E.M.).
* indicates $p < 0.05$ vs. control group, ** indicates $p < 0.01$, and *** indicates $p < 0.001$
Table 2. Mean Social Indexes compared via a Student $t$-test with the mean of the LPS group.

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Note: Numbers in parentheses indicate the standard error of the mean (S.E.M.). * indicates $p \leq 0.05$ vs. LPS group, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$
Figure 3. Mean Social Index of the Electrical Double Insult Group and the Bicuculline Double Insult Group ($t(8) = 5.969$, $p = .000$).
Figure 4. Mean Social Index of the Bicuculline Double Insult Group and the Bicuculline Single Insult Group ($t(14) = -0.1783, p < .05$).
Figure 5. Mean Exploration Frequency of all experimental groups. An ANOVA found a significant main effect for experimental group (F(4,40) = 9.238, p = .000).
References


