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A Study of Constitutive Genetic Differences between Seizure-Prone and Seizure-
Resistant Rat Strains During Embryogenesis.

A Thesis presented to the Faculty of Graduate Studies and Research of Carleton
University in partial fulfillment of the requirements of the degree of Master of
Science in Psychology

Khara M. Sauro
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September, 2003

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**“A Study of Constitutive Genetic Differences between Seizure-Prone and
Seizure-Resistant Rat Strains During Embryogenesis.”**

Submitted by
Khara M. Sauro
In partial fulfillment of the requirements for
the degree of Master of Science

Chair

Thesis Supervisor

Carleton University
September, 2003

Abstract

The immature brain appears to have an increased vulnerability towards seizure activity that is often associated with changes in brain morphology, such as Ammon's Horn Sclerosis. Approximately 65% of adults with temporal lobe epilepsy (TLE) show such sclerosis and other abnormal brain morphologies. However, it has yet to be determined whether these alterations in morphology are a cause or consequence of the seizures. The FAST and SLOW kindling rat strains, developed as animal models favoring or resisting TLE, also exhibit differential brain morphology, with the FAST rats showing a disposition similar to that seen in TLE patients. Given the link between central nervous system (CNS) developmental abnormalities and epilepsy, the primary goal of the current investigation was to compare differences in constitutive gene expression during CNS development in the FAST and SLOW kindling rat strains at several important embryonic time points. In order to assess such differences in gene expression, we employed various molecular techniques, including Differential Display and Superarray analyses. Through the use of these molecular techniques, we found that $\kappa 2$ -macroglobulin ($\kappa 2M$) mRNA was not expressed in the SLOW embryos until embryonic day 13 (E13) while FAST rats demonstrate expression of this gene as early as embryonic day 11 (E11). We also observed an under expression of the voltage-gated sodium channel 22 subunit (Na_v22) and Apolipoprotein E (ApoE) mRNAs in the whole head of FAST rats at embryonic day 21 when compared to SLOW rats sacrificed at the same time point. These results may suggest that the morphological differences observed in the adult brains of FAST and SLOW rats may originate during embryogenesis and that differential expression of a few critical genes during ontogenesis may determine the ultimate seizure susceptibility of the animal.

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Introduction

Epilepsy is a prevalent neurological disorder characterized by recurrent seizures that arise as a result of excessive synchronized neuronal discharge (Kandel, Schwartz, & Jessell, 2000). Although epilepsy affects less than 2% of the population (Freeman, 1995), it is a heterogeneous pathology that is comprised of over 40 clinically defined epilepsies in humans alone (McNamara, 1999). Epilepsy is not selective for race or gender; however, it does appear to be more pronounced in the very young (Freeman, 1995; Glauser, 1995; Holmes & Ben-Ari, 1998). Presently, the underlying etiology for epilepsy is unknown. It has been suggested that deleterious changes in brain morphology (e.g., Ammon's Horn Sclerosis (AHS)) can be seen in up to 65% of Temporal Lobe Epilepsy (TLE) patients (Blumcke *et al.*, 2002). It has yet to be determined whether these alterations in brain morphology are a cause or consequence of the seizures associated with TLE.

Early Central Nervous System (CNS) Development

Development of the CNS begins shortly after fertilization and is initiated by the formation of the neural plate. The neural plate is formed from ectodermal cells that are located in the proneural region and have the potential to evolve into a myriad of neuronal types. The differentiation of ectodermal cells is contingent upon two proteins encoded by both the *notch* and *delta* genes. If *notch* is present in higher quantities than *delta* is, then the cell becomes an immature neuron, whereas if *notch* is present in the same quantity as *delta*, then the cells will become epidermis cells. Thus, once in the neural plate, these cells have the potential to differentiate into either neurons or glia. After the neural plate

has been formed, it begins to exhibit structural changes and produce, via neurulation, a tube-like structure referred to as the neural tube. The neural tube can then be divided into two regions; the caudal region, which is destined to be the spinal cord and the rostral region, and ultimately becomes the brain. It is at this point in development, approximately E13, in which there is a great increase in cell proliferation. (Jessell & Sanes, 2000) Following formation of the neural tube, cells in the rostral region then divide into three vesicles; the forebrain (prosencephalon), the midbrain (mesencephalon) and the hindbrain (rhombencephalon). The position of these cells in the rostral region prior to division appears to be important in determining the cell's ultimate fate. Indeed, different areas of the emerging CNS are exposed to a number of inducing factors, including signaling and adhesion molecules. The positioning of these cells, in combination with the genes that these cells express owing to their developmental lineage, determines the specific fate of that cell. Importantly, the prosencephalon is later divided into the telencephalon, which is the primordial tissue for the cerebral cortex, as well as the amygdala, olfactory bulbs and striatum, and the diencephalons. (Brown *et al.*, 2001)

In the embryonic rodent, cortical development is characterized by growth of the neuroepithelium until approximately E11, which is soon followed by development of the preplate. Following this, emergence of the cortical plate becomes evident at E13 and E14. During this period, neurons that are generated near the ventricles of the developing nervous system migrate to the cortical plate where they form a dense compact cell layer, which splits the preplate into two layers; the marginal zone and subcortical plate. As in the human embryo, migration from the ventricular zone, where these neurons are produced, to their final position in the cortex, is facilitated by radial glia, which express

specific adhesion and signaling molecules such as Neural Cell Adhesion Molecule (NCAM). (Rakic *et al.*, 1974; Edmondson & Hatten, 1987; O'Rourke *et al.*, 1992) As well, a variety of neurotrophic factors, including Nerve Growth Factor (NGF), Brain-derived Nerve Growth Factor (BDNF), Neurotrophin 3 (NT3) and Neurotrophin 4,5 (NT4/5), aid in the development of the CNS by promoting the growth and survival of cells during CNS development and by sustaining neurons that have reached their target, differentiated and matured (Reichardt & Farinas, 1997).

Finally, it should be noted that many regions of the CNS exhibit a distinct developmental profile and develop preferentially at different times in development. For example, in the amygdala, an increase in neurogenesis is noted as early as E13 in the anterior amygdaloid area and as late as E19 in the basolateral nucleus (Bayer, 1980, 1981). Another example of this divergent developmental profile exists in the hippocampus proper, where the Ammon's horn or CA fields exhibit a peak in cell proliferation around E17 until approximately E21 (Bayer, 1980) compared to the granule cells of the Dentate Gyrus, which demonstrate a peak in neurogenesis around the first postnatal week which tapers off by the third postnatal week (Bayer, 1980). Importantly, it is in this period of development that demonstrates an increase in seizure susceptibility (Freeman, 1995; Glauser, 1995; Holmes & Ben-Ari, 1998).

Defects in Neurodevelopment Specific to Epilepsy

In order for proper development of the mature CNS, a number of intricate processes must be precisely orchestrated in the proper sequence. If these processes are disrupted at any time during the development of the embryo, a number of pathologies

may result. For example, many epileptic disorders have been associated with defects in neuronal migration (Palmini *et al.*, 1991). This association has been attributed to morphological abnormalities commonly observed in epileptic patients, including AHS and abnormal granule cell dispersion (GCD), which may arise from a defect in granule cell migration (Houser, 1990; Armstrong, 1993). Interestingly, however, although CNS pathology appears to be widespread among epileptic patients, there does not appear to be a homogeneous pattern of pathology, as is seen in other neurological disorders.

Many childhood epilepsies have been associated with neuromorphological anomalies, which have been linked to various neurodevelopmental defects (Meencke, 1985, 1989; Meencke & Gerhard, 1985; Rosen *et al.*, 1992; Caviness *et al.*, 1995). In fact, it has been proposed that severe neuropathology may predispose an individual to an earlier onset of epilepsy, and as such, all childhood epilepsies may be associated with some neurological morphologic defect (Caviness *et al.*, 1995). In evidence of this, West Syndrome, or infantile spasms, typically affects infants within the first half of their first year of life, and is characterized by a reduction in brain weight; neuronal necrosis; encephalocele (hernia of the brain) and pachygyria (which is hallmarked by abnormally large convolutions of the cerebral cortex) (Meencke, 1985, 1989). Each of these morphological abnormalities has been associated with defects in embryonic development, in particular, cortical development (Meencke, 1985, 1989). Finally, as it has been estimated that abnormalities of neuronal migration are seen in 10-20 percent of epileptic brains (Meencke & Gerhard, 1985), it has been suggested that polymicrogyria, multiple areas of abnormally small brain convolutions, seen in childhood epilepsies, may result

from damage to cortical cells and the radial glia on which they migrate (Rosen *et al.*, 1992).

Reelin: A Control Mechanism in Neurodevelopment

Reelin, an extracellular-matrix protein is also reported to be integral to the proper development of the cerebral cortex (Lambert de Rouvroit & Goffinet, 1998; Rice & Curran, 2001). Reelin is secreted by Cajal-Retzius neurons, the earliest neurons present in the development of the cerebral cortex (Lambert de Rouvroit & Goffinet, 1998; Rice & Curran, 2001). Interestingly, a deficiency of Reelin has been found in Reeler mice, which show pathology similar to that of Ammon's Horn Sclerosis or lissencephaly, and exhibit seizures. Specifically, while Reeler mice appear to develop normally during the initial phases of embryogenesis, at the end of cortical development the Reeler mouse fails to split into the subplate and marginal zone and forms abnormal layering of the cerebral cortex (Lambert de Rouvroit & Goffinet, 1998; Rice & Curran, 2001).

Reelin binds to two receptors, the Very Low Density Lipoprotein Receptor (VLDLR) and the Apolipoprotein Receptor 2 (ApoER2), which are also receptors for apolipoprotein E (ApoE), a protein implicated in the pathology seen in Alzheimer's patients. VLDLR and ApoER2 have also been associated with the activation of Doublecortin (DCX). With respect to epileptic pathology, a defect in DCX, a gene associated with X-linked lissencephaly, has also been implicated in improper cortical development, specifically abnormal cortical migration, and is often seen in persons with intractable seizures (Dobyns *et al.*, 1984; Gleeson *et al.*, 1998; Gleeson *et al.*, 2000; Gleeson & Walsh, 2000). Another gene that has been linked to the development of pathology in 30-40

percent of lissencephalies is LIS-1 (Dobyns *et al.*, 1992). LIS-1 may be involved in intercellular signaling (Walsh, 1995) as well as protein-protein interactions (Neer *et al.*, 1993). Taken together, these findings also advocate the role of genetic factors in the development of epileptic syndromes.

Early Postnatal Life and the High Incidence of Epilepsy

Among many animal species, the early postnatal period marks a critical time in the development of the CNS. Specifically, this period is characterized by a notable increase in synaptogenesis and neurogenesis in a variety of brain regions (Glauser, 1995; Holmes & Ben-Ari, 1998). Although this increase in neuronal proliferation, migration, and differentiation is thought to be integral to the development of the CNS, this period may also present an increased risk for occurrence of epileptic activity (Freeman, 1995; Glauser, 1995; Holmes & Ben-Ari, 1998). For instance, it has been demonstrated that the immature rat brain exhibits an increased vulnerability to convulsions and seizures during both the second and third week of postnatal life (Moshe *et al.*, 1995; Swann *et al.*, 1999). This increase in seizure susceptibility is mimicked in humans as well, where it is estimated that 2-5% of the population experience seizures within the first few years of life (Hauser, 1994). Although the exact mechanism underlying this increase in seizure susceptibility has not been fully elucidated, it has been suggested that such vulnerability may be due, in part, to an imbalance between glutamate excitation and GABA inhibition (Moshe *et al.*, 1995; Flint *et al.*, 1997; Swann *et al.*, 1999). In agreement, it has been observed that immature rat brain exhibits an increase in the expression of NMDA IIA receptors, which result in increased excitatory postsynaptic current duration (Hablitz, 1987; Sutor *et al.*, 1994; Moshe *et al.*, 1995; Flint *et al.*, 1997; Swann *et al.*, 1999).

Paradoxically, this hyperexcitability may actually be required for proper development of the brain. For instance, while synchronized, spontaneous electrical activity during the early postnatal period may create an increased risk for seizure activity in developing brain, it also appears to facilitate the proper development of brain circuitry (Katz & Shatz, 1996; Crair, 1999). Finally, it should be noted that this increase in seizure susceptibility during the early postnatal period may be a result of incomplete synaptic contacts between the cortex and other forebrain structures (Litzinger *et al.*, 1994).

Given the importance of the early postnatal period in the development of the CNS, it may be important to examine the impact of seizures in early life on the brain and its susceptibility to epilepsy in adulthood. In this respect, it appears that a primary risk factor in the development of TLE is the occurrence of a prolonged (i.e., longer than 10 – 15 min.) febrile seizure in childhood (Cendes *et al.*, 1993; French *et al.*, 1993). In a similar fashion, it was shown that febrile seizures in 10 day old rats increased their susceptibility to kainic acid induced seizures later in life in comparison to controls (Baram *et al.*, 2002). It has been proposed that the neuronal damage caused from these prolonged febrile seizures may underlie the increased risk of further seizures (Bruton, 1988; Armstrong, 1993). However, this proposal has not been validated as such neuronal loss has not been observed in rats following prolonged febrile seizures or hypoxia induced seizures (Jensen & Baram, 2000; Baram *et al.*, 2002). Moreover, it must be considered that a large number of children who experience a febrile seizure (~ one in every 20-30 children) never develop TLE.

Seizure-prone (FAST) and Seizure-resistant (SLOW) rat strains

As in many other neuropathologies, genetic factors may impact upon the development of epilepsy. In particular, genetic neonatal epilepsy syndromes, including benign familial neonatal convulsions, are autosomal dominant disorders that begin by the third day of life and often reoccur spontaneously (Berkovic *et al.*, 1987; Malafosse *et al.*, 1994; Marini *et al.*, 2003). Another example is that of childhood absence epilepsy; a disorder thought to be of primarily genetic origin, with a polygenic inheritance pattern (Berkovic *et al.*, 1987; Malafosse *et al.*, 1994). Finally, it has also been reported that febrile seizures carry a strong genetic component (Marini *et al.*, 2003). For example, a recent study has suggested that febrile seizures are inherited in an autosomal dominant fashion with a 75% penetrance (Marini *et al.*, 2003).

In order to further explore those genetic processes that may play a role in the development of epilepsy, an animal model of TLE was created, through the selective breeding of rats for characteristics that are associated with principle features of this disorder, namely susceptibility to seizures. In brief, kindling is the process of progressive epileptogenesis through which an initially subconvulsive electrical stimulation is applied to a given brain region, and with daily repetitions, progressively elicits more provocative seizure behaviour that ultimately culminates in a convulsive seizure (Goddard, McIntyre, & Leech, 1969). Kindling is the most common animal model of human complex partial seizures, especially modeling TLE. Interestingly, it has been suggested that permanent morphological changes occur as a result of kindling (Sutula *et al.*, 1988; McNamara, Bonhaus, Shin, 1993).

The parental populations used to create these two strains were outbred Wistar and Long-Evans Hooded rats (Canadian Breeding Farms, St. Constant, Quebec). As indicated

above, the strains were bred differentially according to their *fast* versus *slow* amygdala kindling rates. Kindling rate was defined as the number of kindling stimulations, applied to a structure like the amygdala, required to generate a stage 5 convulsive seizure (Racine 1972) in each animal. Specifically, animals that were relatively seizure susceptible were bred to create the FAST-kindling strain, while those rats that were seizure resistant were bred to create the SLOW-kindling strain (Racine *et al.*, 1999).

Although the strains were initially bred according to their kindling vulnerability, many co morbid trait differences have subsequently been noted that extend beyond their differential kindling rates, including dissimilar behavioral profiles. For example, the FAST strain fails to habituate over days in an Open Field paradigm and appear to be less fearful in an elevated plus-maze and in a passive avoidance task (Mohapel & McIntyre, 1998). FAST rats are also remarkably impulsive in copulatory behavior with either estrous or non-estrous females, which is not true for SLOW rats (Shin *et al.*, 2002a). It has also been reported that the FAST and SLOW rat strains exhibit differing responses to anxiety/fear, in that they exhibit divergent neurochemical and behavioral responses to a variety of stressors (Anisman *et al.*, 1997). The FAST and SLOW strains also differ in regards to their cognitive functioning. Namely, the FAST rats appear to have greater difficulty learning both the Morris Water Maze and the T-maze, compared to the SLOW rats (McLoed & McIntyre, 1995; Anisman & McIntyre, 2002). Importantly, co morbid conditions, such as those described above, are commonly associated with epilepsy and seizures, and are some of the hallmark symptoms used to diagnose Attention Deficit Hyperactivity Disorder (ADHD) and learning and memory impairments (Hermann *et al.*, 1981).

Considering the hypothesized role that GABA plays in epilepsy, it would seem intuitive that these strains might also exhibit differences in GABAergic mechanisms. Indeed, the FAST and SLOW rat strains do differ in their GABA_A subunit expression and extracellular GABA concentrations post kindling. It has been shown that adult FAST rats over-express the GABA_A subunits genes most commonly seen in embryos (K2, K3, and K5), whereas the adult SLOW rats over-express the subunit (K1) more commonly seen in adulthood (Poulter *et al.*, 1999). Also, the FAST rats exhibit a decrease in extracellular GABA concentrations post kindling in comparison to the SLOW rats (Shin *et al.*, 2002b). Akin to differences seen in human TLE patients, these strains have also been shown to possess significant differences in brain morphology, specifically in the hippocampus and other temporal lobe structures (Gilby *et al.*, 2002; compared to Bertram *et al.*, 1990; Bothwell *et al.*, 2000). These structural differences appear to be consistent with both the learning deficits, and emotional differences observed between the FAST and SLOW rat strains.

Prospective Statement

Given the high incidence of seizures in the immature brain and the hypothesized link between CNS developmental abnormalities and epilepsy, the primary goal of the current investigation was to assess differences in constitutive gene expression during CNS development between the FAST and SLOW kindling rat strains at several important time points. Given that these two strains exhibit differences in seizure susceptibility and differences in brain morphology that are similar to those found in developmentally related epilepsies, it was anticipated that they would demonstrate differences in

genetically controlled developmental mechanisms that may ultimately favor or resist epileptogenesis.

Methods

Subjects

The FAST and SLOW rat strains were established by Racine at McMaster University, Hamilton, Ontario, by breeding Wistar and Long-Evans Hooded rats based on their differential amygdala kindling rates. The animals that required a small number of stimulations to induce a stage five seizure were bred together to produce the FAST rat strain and those that required a large number of stimulations to induce a stage five seizure were bred together to produce the SLOW rat strain. The two strains were maintained at McMaster University until the 11th generation at which time they were moved to Carleton University Neuroscience Institute, Ottawa, Ontario. Since their relocation to Carleton University the differences in seizure susceptibility have been maintained using second cousin mating, so as not to 'fix' the genes in the two strains, which would happen with brother and sister mating.

During the breeding process, the males and females were housed in standard plastic cages with a wire mesh bottom. After mating was complete the animals were separated and housed in standard plastic cages without the mesh bottom. For the duration of the experiment, subjects were maintained on a 12 hour light/dark cycle (light phase: 7A.M.-7P.M.) and received food and water *ad libitum*. All the procedures in the current study were approved by the Carleton University Animal Care Committee and were conducted according to the guidelines established by the Canadian Council on Animal Care.

FAST/SLOW Breeding Procedures

Male and female rats from the FAST and SLOW strains were bred, and the females were observed daily for the release of vaginal plugs, which indicated successful impregnation. The day following evidence of a plug was considered embryonic day 1 (E1) for the developing embryos. At varying time intervals following impregnation (E11, E13, E15, E19 and E21), mothers were sacrificed via standard decapitation procedures and the embryos were extracted for molecular analysis. The selection of these time points was based primarily on the importance of these time points in the development of the CNS as supported by the literature (Bayer, 1980, 1981; Jessell & Sanes, 2000; Brown *et al.*, 2001). Also, these time points span a great deal of embryogenesis allowing us to gain considerable knowledge regarding the development of the CNS during embryogenesis. The whole embryo extracted at E11, 13 and 15 was immediately flash frozen in liquid nitrogen and then stored at -80°C. For embryos extracted at E19 and E21, the entire head was separated from the bodies, flash frozen and then stored at -80°C.

Groups of animals were also sacrificed at various postnatal time points for molecular analysis. Rats were decapitated at Post Natal Day (PND) 6, 12 or 21, their brains extracted, and numerous structures were microdissected, including the amygdala, hippocampus, piriform cortex, prefrontal cortex, parietal cortex, perirhinal cortex, olfactory bulbs and cerebellum. These time points were selected based on the documented importance of the early postnatal life in the development of the Dentate Gyrus, which is linked to TLE (Moshe *et al.*, 1995; Swann *et al.*, 1999), and the reports that the immature brain at two to three weeks postnatally is more susceptible to seizures than in adults (Bertram *et al.*, 1990; Bothwell *et al.*, 2000). The three postnatal data points selected during the latter three week window of time were based on the frequent

use of those three time points in many other experiments on ontogeny, brain development and behavior (e.g., Upender *et al.*, 1997). All tissues were flash frozen in liquid nitrogen and then stored at -80° C.

RNA Extraction

The procedure for RNA extraction was adapted from Sambrook *et al.* (1989). All tissues used for molecular analysis were immersed in 1ml Trizol per 50mg tissue (Gibco BRL) and homogenized. Once completely homogenized, 200µl Chloroform per 1ml Trizol (Fisher Scientific Canada) was added, and incubated at room temperature for 3 min, and then centrifuged at 12000xg for 15min at 4°C. The aqueous phase was removed and placed into a clean tube. Following this, 0.5ml isopropanol, per 1ml Trizol used, was added and the solution was allowed to precipitate at room temperature for 15min, and then centrifuged for 15min at 12000xg at 4°C. The liquid was removed and the RNA pellet was washed using 75% ethanol. After the ethanol wash, the liquid was discarded and the RNA pellet was allowed to dry. Once dry, the RNA pellet was resuspended in 30-50µl DEPC water. The sample was then heated at 65°C for 10min and the concentration of the RNA was measured using the A280 wavelength on a spectrophotometer (BioRad).

Differential Display

The differential display technique is known as a random gene screening tool, in that, the primers used to amplify the complimentary DNA (cDNA), which bind and amplify certain genes expressed in the samples (i.e. FAST and SLOW rats), were

randomly selected from the available 20 primers sets. If all possible combinations of primers were used the entire genome would be represented. The differential display was used in the current study in order to examine a large gene pool in hopes of discovering a differentially expressed gene between the FAST and SLOW rat strains. All time points from which RNA was extracted; E11, E13, E15, E19, E21, PND6 forebrain, PND12 temporal lobe, PND21 amygdala, were examined using differential display. Four samples per group (i.e. E11 FAST) were used to represent that group on the differential display. It should be noted that the Differential Display technique that was used in the current thesis was adapted from Liang & Pardee (1992).

Reverse Transcription

2: g of RNA for each sample was added to 1 μ l (0.5mg/ml) Oligo dT (18mer), and was heat-denatured at 70°C for 3min, and then immediately put back on ice for another 3min. After the mixture was denatured, 2 μ l of 5X first strand cDNA buffer (250mM Tris-HCl, pH8.3, 375mM KCl, 15mM MgCl₂, 50 mM dithiothreitol), 2 μ l 5mM dNTP, and 1 μ l Superscript II RT polymerase (Invitrogen) was added. The mixture was incubated at 42°C for 1hr followed by 5min incubation at 80°C. The, now, single-stranded cDNA was diluted to a final volume of 100 μ l with dH₂O and stored at -20°C.

PCR Amplification

A master mix was created using 3.3: 1 dH₂O, 0.5: 1 10X Klentaq Buffer (ClonTech), 0.05: 1 5mM dNTP, 0.05: 1 K³³PdATP (2000Ci/mmol), 0.25: 1 5' (P) primer (20: M), 0.25: 1 3' (T) primer (20: M), and 0.1: 1 Klentaq polymerase (ClonTech). 4.5: 1 of the master mix was added to 0.5: 1 of each sample of cDNA. The cDNA mixture was put through the following PCR conditions:

94°C for 5min
40°C for 5min
68°C for 5min
94°C for 2min
40°C for 5min
68°C for 5min
repeat steps 4 – 6, one time
94°C for 1min
60°C for 1min
68°C for 2min + 4 sec/cycle
repeat steps 8 – 10, 26 times
68°C for 7min
0°C until ready to use.

Gel Electrophoresis

Once the PCR was completed, 5: 1 loading dye (deionized formamide, 10mM Na₂EDTA; pH 8, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue) was added to each sample. The PCR reaction and loading dye solution was heated for 3min at 95°C and then placed back on ice.

The denatured samples (4: 1) were loaded in a sequencing gel (6% polyacrylamide, 8 M urea gel) in a Genomyx machine under the following conditions, 50°C, 3000V, 100W. The samples were allowed to run in the gel for approximately 4hrs. Once the samples were fractionated in the gel, the gel was dried. The urea was rinsed

from the gel using dH₂O and the gel was transferred to Whatman 3 MM paper and then allowed to dry completely in the Genomyx machine. The dried gel was exposed to Biomax MR film (Kodak) overnight at room temperature.

Superarray

While the differential display is a random gene screening tool, the superarray technique is a more targeted gene screening tool. The superarrays, which contain 112 cDNA spots, were commercially obtained from SuperArray Bioscience Corp. (Frederick, MD, USA). The specific arrays used in the current study were the GEMatrix Q series Human Neuroscience Gene Array, which were chosen, based on the identity of genes that were available on the array. While these arrays, by no means, represent an exhaustive investigation of genes that could be involved in the differences between the FAST and SLOW rats they provide a preliminary look into pathways that might be important.

Prehybridization

Heat-denatured salmon sperm DNA (100°C for 5min) was added to the pre-heated GEMatrix Hybridization Solution (60°C) to a final concentration of 100µg/ml. The array membrane was pre-wetted with 5mls of deionized water in a hybridization tube. The deionized water was then discarded and each array membrane was prehybridized in 3ml of the GEMatrix/prehyb/denatured salmon sperm DNA mixture for 2hrs at 60°C with continuous agitation at 10rpm.

Probe Construction

During the prehybridization period, cDNA probes were constructed. 5: g of RNA (maximum total volume of 10: l) was heat denatured at 85°C for 8min then placed back on ice. 30: l of the Master Mix (5X MMLV buffer, 10mM dNTP (-dCTP), 100: M dCTP,

^{32}P dCTP, RNasin, MMLV) was added to the denatured probe and heated at 42°C for 30min before being placed back on ice. The radioactive cDNA probe was spun in a G-25 spin column at 4°C for 1min at 2800rpm.

Once the cDNA probe was constructed, 1:1 of the probe was combined with 9:1 of DNA loading and fractionated on an acrylamide gel via electrophoresis. This was performed in order to verify equal amounts of total cDNA were present in each probe and that overall incorporation of the radioactive label was approximately equal in each sample.

Hybridization

The entire volume of denatured cDNA probe was added to 0.75ml of the pre-warmed GEAhyb. Once the cDNA probe was added to the GEAhyb it was added to the hybridization tube containing the array membrane. The probe was allowed to hybridize to the array membrane overnight at 60°C with continuous agitation at 5 to 10rpm.

Washing Parameters

The following day the array membranes were washed. The wash solutions were prewarmed to 60°C. Approximately 10mls was used per array membrane. The array membranes were washed twice with Wash Solution 1 (2X SSC, 1% SDS) for 15min per wash, at 60°C with 20 to 30rpm agitation. The array membranes were then washed twice with Wash Solution 2 (0.1X SSC, 0.5% SDS) for 15min per wash, at 60°C with 20 to 30rpm agitation. After the arrays were washed, the membranes were blotted on a piece of filter paper to remove excess Wash Solution. The membranes were then exposed to Kodak BioMax film at -80°C. It should be noted that the superarray protocol used in the present thesis was adapted from Baltathakis *et al.* (2001).

Data Analysis

For both tissue weight and amount of total RNA extracted from the structure, an independent one-way Analysis of Variance (ANOVA) was conducted for each time point examined. In order to normalize the amount of total RNA extracted from the structure, a ratio of total RNA to tissue weight was created. Again, independent one-way ANOVAs were conducted at each time point.

Densitometric analysis was conducted on films obtained from the superarray procedure. Background subtractions were performed to alleviate exposure differences using Quantity One software (BioRad). For the superarrays, the density of a given band was normalized to the housekeeping gene cyclophilin. Each array contained four separate spots for cyclophilin and the average density (OD/mm^2) of these spots was recorded for each array. A ratio of the intensity of signal from a gene of interest to that of the average cyclophilin intensity was calculated in order to determine the relative intensity of expression for that gene.

Results

Tissue Weight

In an examination of the weight of the tissue from embryonic rats (i.e., E11, E13, E15, E19 and E21), independent one-way ANOVA tests at each age ($n = 5$) revealed that the FAST and SLOW rat strains differed from one another only at the earliest embryonic time point examined, E11, $F = 9.503 (1, 9); p < .05$. Specifically, on E11 the FAST rats exhibited lighter *whole body* weights (Mean $\pm$ SEM = $138 \pm 13\text{mg}$) when compared to their SLOW counterparts ($194 \pm 13\text{mg}$). While this is a very crude measure of size, these data may suggest that the SLOW embryos at E11 are further along in development than the FAST embryos.

Insert Figure 1 Here

In order to assess whether differences in tissue weight were also present in a specific brain structures following birth, a separate one-way ANOVA was conducted. Interestingly, as seen in Figure 1, a strain difference was apparent in the amygdala weights, $F = 9.524 (1, 9); p < .05$. In this case, however, the FAST rats exhibited significantly heavier (M $\pm$ SEM = $54 \pm 4.5\text{mg}$) *amygdalae* at PND21 compared to their SLOW counterparts ($34 \pm 4.6\text{mg}$). Again, it should be noted that this is not a particularly accurate measure of amygdala size; however it may imply that the FAST animals have larger amygdala than do the SLOW animals on PND21.

Total RNA

Once the RNA had been extracted from the various tissues, be it whole body or brain structure, the amount of total RNA (μg) in the sample was assessed. It was found, using independent ANOVA tests at each time point, that the two strains had similar amounts of RNA extracted from the given structure, with the exception of the amount of RNA recovered from the E21 head, $F = 79.364 (1, 9); p < .001$. It was observed that the tissue obtained from FAST rats contained significantly lower amounts of RNA ($M \pm \text{SEM} = 602.4 \pm 58.6\mu\text{g}$) than did the tissue extracted from the SLOW rats ($1340.9 \pm 58.6\mu\text{g}$). It should be noted that although the FAST animals had significantly lower amounts of RNA than the SLOW animals, the tissue weight from which the RNA was extracted was not significantly different, $F (1, 9) = 0.11; p > .05$.

Insert Figure 2 Here

The amount of total RNA extracted from each structure was normalized to the gross weight of the structure from which the RNA was extracted. This was accomplished by creating a ratio of amount of total RNA extracted to the weight of the given structure. As mentioned previously, the weight of the E21 head did not differ between the FAST and SLOW rats while the amount of RNA extracted from this tissue was significantly different from each other. Indeed, when normalized, this time point exhibited a significant difference between the FAST and SLOW rat strains, $F (1, 9) = 20.107; p < .01$. The pattern of this difference is similar to that seen within the examination of

amount of total RNA extracted, in that the SLOW rat continued to show a significantly higher ratio ($M \pm SEM = 1.098 \pm 0.123$: g/mg) than the FAST rats (0.518 ± 0.04 : g/mg).

Insert Figure 3 Here

GEArray Q Series Neuroscience Gene Array

While a total of 112 genes were compared between the two strains in the E21 head, only two genes were observed to differ significantly between the FAST and SLOW rats. Importantly, the FAST and SLOW strains did not differ ($F(1, 7) = 0.039$; $p > .05$) in their expression of cyclophilin, a housekeeping gene that is included on the arrays as a way to ensure that an approximately equal number of cells were used in each comparison and that subsequent steps in the procedure were matched. Given that the strains do not differentially express cyclophilin, it is possible to attribute the differences in gene expression, as measured by the arrays, to actual strain differences in mRNA expression. It may be of interest to note that a recent study by Chen *et al.* (2001) has found that cyclophilin is the ultimate control gene as it varies the least between animals.

It should also be noted that the cDNA smear that was created on an acrylamide gel via electrophoresis indicated that all probes were of similar intensities prior to hybridization on the arrays.

Insert Figure 4 Here

Insert Figure 5 Here

With respect to those genes that were found to differ between the strains, it was found that the FAST rats under expressed ($M \pm SEM = 0.386 \pm 0.05OD/mm^2$) the Apolipoprotein E (ApoE) gene, in comparison to the SLOW rats ($0.567 \pm 0.05OD/mm^2$), $F = 6.62 (1, 7); p < .05$.

Insert Figure 6 Here

Similarly, the FAST rats also demonstrated a marked reduction in the expression ($0.377 \pm 0.48OD/mm^2$) of the Sodium Channel, 22 subunit (Na_v22), when compared to the SLOW rats ($2.475 \pm 0.48OD/mm^2$), $F = 9.606 (1, 7); p < .05$.

Insert Figure 7 Here

Differential Display

Of the approximately 750 genes examined as a result of 15 individual differential displays that were performed during embryogenesis as well as approximately 10

differential displays performed on postnatal tissue, only one gene was shown to be differentially expressed between the FAST and SLOW rat strains. The differentially expressed gene fragment was cloned and sequenced (Gilby, personal communication 2003). Through this process, it was revealed that the differentially expressed gene fragment was K2 macroglobulin (K2M); a protease inhibitor. The results from the Differential Display suggest that the K2M is turned on earlier in 100% (4 out of 4) of the FAST rats than in SLOW rats (4 out of 4). While none of the SLOW rats showed K2M at E11, they all expressed it at E13 whereas the FAST rats showed a high level of expression for this fragment by E11. Indeed, K2M continued to be present until E19 in both strains.

Insert Figure 8 Here

Discussion

It has been previously reported that the FAST and SLOW-kindling rats exhibit divergent profiles with respect to seizure susceptibility, neuroendocrine and behavioral response to stressors, spatial learning and the expression of α subunits of the GABA_A receptor (Anisman *et al.*, 1997, 2000; McIntyre *et al.*, 1999; Poulter *et al.*, 1999; Anisman & McIntyre, 2002). In addition, it has also been noted that these two strains demonstrate differences in brain morphology, as realized by reduced hippocampal volume and increased ventricular space in the FAST rats compared to the SLOW rats (Gilby *et al.*, 2002). The latter results are remarkably similar to those seen in human TLE patients, particularly in those patients with hippocampal sclerosis (Bertram *et al.*, 1990; Bothwell *et al.*, 2000). Interestingly, the adult morphological results of the two strains suggest that such difference might arise through ontogenetic development and might predate the appearance of epilepsy *per se*. In other words, the reduced hippocampus and increased ventricle in humans (like the FAST rats) might be genetically determined, and not the result of seizure induced brain damage. Thus in the current investigation, it was of primary interest to assess the early genetic mechanisms that might differentially contribute to the observed differences both in adult brain morphology and behavior in FAST and SLOW rats from a developmental perspective.

Although a number of genes were analyzed during embryogenesis for potential differences in expression between the two strains, only three genes were found to differ significantly between the strains. For example, it was found, via differential display, that the FAST rats expressed the protease inhibitor, K2-macroglobulin (K2M) at an earlier age than did the SLOW rats. Specifically, we found that while the FAST rats expressed K2M

at E11, the SLOW rats did not express it until E13. It should be recalled that K2M is considered a neurite-promoting factor in the CNS (Mori *et al.*, 1990, 1991; Saitoh *et al.*, 1992), and is most highly expressed during early embryonic development and decreases considerably postnatally (Ishiguro *et al.*, 1995). Our findings are consistent with this pattern of development, as the expression of K2M decreased progressively and stopped at the E19 time point in both strains. This gene, which appears to be crucial to neurite growth, has been found to be present in significantly smaller concentrations (Vasil'eva *et al.*, 1989) or not at all in epileptic patients compared to normal individuals (Mora *et al.*, 1977; Moechars *et al.*, 1999). As such, it may be possible that an absence of K2M is partially responsible for, or is at least linked to, the morphological pathology seen in epilepsy patients. This pathology is often seen as reduced dendritic outgrowth in such patients. Interestingly, a similarly reduced dendritic outgrowth was observed by Teskey's group in layer III cortical neurons of FAST versus SLOW rats (personal communication, 2003). This result might suggest that such outgrowth pathology is a result of a genetic predisposition for epilepsy, without the necessity of actually being epileptic *per se*.

While these data suggest that the SLOW rats may be developmentally delayed, other data gathered on these two strains appears to suggest the opposite pattern. For instance, embryonic weights were collected on the strains which showed that SLOW rats are actually heavier than FAST rats at the E11 time point suggesting that the SLOW rats are further developed than the Fast rats by E11. In addition, it had been previously found that the FAST rats were developmentally delayed in their GABA_A subunit expression, relative to the SLOW rats. This was suggested by the observation that FAST rats continued to demonstrate the embryonic GABA_A subunits K2, K3 and K5 postnatally,

rather than the adult phenotype, which emphasizes the K1 subunit. Interestingly, the K1 subunit is actually over expressed in the adult SLOW rats compared to both FAST rats and out bred controls (Poulter *et al.*, 1999).

When RNA was extracted from the rat head of embryos at E21, Superarray analysis revealed that the FAST and SLOW strains differed in their expression of the voltage-gated sodium channel 22 subunit (Na_v 22). Na_v 22 expression, which has previously been shown to alter electrical excitability (Chen *et al.* 2002), was decreased in the FAST strain compared to the SLOW strain. Interestingly, it has been shown that knockout mice which lack Na_v 22, exhibit a similar vulnerability to seizures as the FAST rat strain (McIntyre *et al.*, 1997; Chen *et al.*, 2002). Moreover, the site of action of many antiepileptic drugs (AEDs) is postulated to involve voltage-dependent sodium channels (Ragsdale & Avoli, 1998), which further implies the importance of Na_v22 in the regulation of electrical excitability. As such, it is tempting to speculate that the decrease in Na_v 22 during development may underlie the increased vulnerability of the FAST rats to seizures.

It has been reported that Na_v 22 shows a peak expression 2-3 weeks postnatal in the rat (Ellisman & Levinson, 1982; Wollner *et al.*, 1988; Scheinman *et al.*, 1989). Importantly, perhaps, this period is also the hallmark time for increased synapse formation and myelination, specifically in the granule cells of the dentate gyrus (Bayer, 1980). Thus, in the future, it may be advantageous to assess whether the FAST and SLOW strains differ in their expression of Na_v 22 at that time point too, thus exploring the differences between the two strains in their postnatal development, similar to their prenatal characteristics reported in the present thesis.

We also observed that ApoE, a glycoprotein involved in the transport of cholesterol, differed between the strains at the E21. Indeed, we found that the SLOW rats had a higher expression of ApoE compared to the FAST strain. This finding may have a number of possible consequences when it is considered that ApoE has three major isoforms, ϵ , μ , and η , all of which have differing effects on the CNS. For example, the ApoE η allele has been noted to have deleterious effects on the CNS and is commonly linked to the tangles and plaques seen in Alzheimer's patients. It has also been shown that while the ϵ and μ isoforms promote neurite growth and regeneration, the η isoform has been associated with increased aging process witnessed by decreased cognitive function and memory (Reed *et al.*, 1994; Bondi *et al.*, 1995; Yaffe *et al.*, 1997). Finally, it has been suggested that the presence of the η isoform is commonly expressed in patients with early onset TLE (Breiellmann *et al.*, 2000). Although we are unable to decipher which isoform of the ApoE gene is expressed in our study, it would be consistent with previous findings that the isoform over expressed in the SLOW strain would be either the ϵ or μ isoform, as the FAST strain appears to show characteristics more similar to those reported in the presence of the ApoE η allele. In future experiments, these isoforms will be assessed.

At E21 we also found differences in RNA extracted from the whole head of the embryo. While the tissue weight did not differ between the strains, the amount of RNA we extracted from the FAST embryos was significantly decreased in comparison to that extracted from the SLOW embryos. Indeed, when the amount of total RNA was normalized to the weight of the tissue from which it was extracted this difference remained. While this may have played a role in the over expression of both genes, ApoE

and Na_v22 in the Superarrays, it is unlikely as the cyclophilin expression of the FAST and SLOW rats did not differ significantly. As a possible alternative, it may be that there is a transcriptional silencer acting on the FAST rats at this time point, thus suppressing the production of RNA. An alternate hypothesis, perhaps, may be that by E21 the FAST and SLOW rats are undergoing different phases of development. Preliminary data generated in our laboratory would provide support for this hypothesis. It may be of some interest to note here that this difference in amount of total RNA is not likely to be due to a different number of cells as a result of apoptosis, as there were no differences found between the FAST and SLOW rat strains on a superarray designed with genes related to apoptosis.

The data found in the current experiment suggest that the mechanisms underlying differing susceptibility to seizures in the FAST and SLOW rat strains may be related to mechanisms involved with neuronal migration, differentiation, and proliferation. The genes that were found in this study to be differentially expressed constitutively between the strains during development are intertwined with one another and are involved in the development of the CNS. Indeed, K2M has been found to be identical to the low density lipoprotein receptor-related protein (LRP) (Kristensen *et al.*, 1990; Strickland *et al.*, 1990), which is also a receptor for ApoE (Beisiegel *et al.*, 1989; Kowal *et al.*, 1989; Strickland *et al.* 1990). ApoE also shares two of its receptors, ApoER2 and Very Low Density Lipoprotein Receptor (VLDLR), with the protein Reelin.

Importantly, Reelin is an extracellular matrix protein secreted during early development of the brain by Cajal-Retzius (CR) cells (Lambert de Rouvroit & Goffinet, 1998; Rice & Curran, 2001), which are involved in directing migrating glia and neurons

to their destination (Bar & Goffinet, 1999). The Reelin expressing CR cells are among the first neurons present during CNS development and decrease with maturation (Amaral *et al.*, 1992). Reelin and its receptors are integral to the proper development of the cerebral cortex (Rice & Curran, 2001). Based on research conducted on Reelin and the Reelin deficient mice (Reeler mice) as compared to the FAST and SLOW rat strains, it seems very plausible that the FAST and SLOW rat strains may also show differences in Reelin expression during development. In support of this hypothesis, Reeler mice exhibit altered cortical and hippocampal organization and layer formation (D'Arcangelo *et al.*, 1995; Del Rio *et al.*, 1997) as well as seizures. As indicated below, several of these features are evident in the FAST compared to the SLOW rats. For example, Gilby *et al.* (2002) demonstrated that the FAST strain exhibited a significant decrease in hippocampal volume compared to the SLOW rats. The FAST rats, of course, also are defined by their increased vulnerability to seizures compared to the SLOW rats (McIntyre *et al.*, 1999). While it is unknown whether differences in hippocampal volume arise due to altered organization and layer formation, it has been found that the FAST rats do show altered dendritic branching (Flynn *et al.*, 2002). This pathology is also seen in the Reeler mice, and is similar to many patients with Ammon's Horn Sclerosis (AHS) (Blumcke *et al.*, 2002). Furthermore, it should be noted that humans with a Reelin deficiency show malformations of the cerebellum, hippocampus and brain stem (Hong *et al.*, 2000). Also, it is interesting that knockout mice for the two Reelin receptors, ApoER2 and VLDLR, display defects in fear conditioning (Kim *et al.*, 1993; Chen *et al.*, 1996; Weeber *et al.*, 2002) similar to the FAST rats (Anisman *et al.*, 1997, 2000; Mohapel & McIntyre, 1998). Lastly, these two receptors are also implicated in the activation of DCX, which is similar

to reelin in that it has been shown to be important for proper cerebral cortical development and a lack of DCX is often related to intractable seizures (Dobyns *et al.*, 1984; Gleeson *et al.*, 1998; Gleeson *et al.*, 2000; Gleeson & Walsh, 2000). Interestingly, preliminary immunohistochemistry for DCX in the FAST and SLOW rats suggests that the SLOW rats show more intense staining of DCX during the early postnatal life.

In summary, consistent with previous research on the FAST and SLOW rat strains suggesting different behavioral profiles, seizure susceptibility, and brain morphology, we found several important strain differences with respect to gene expression during development. Through the use of a variety of molecular techniques, we found that K2M was expressed at a later time point in the SLOW rats compared to the FAST rats, which could set in motion a very different cascade of subsequent development in the two strains, leading to their adult phenotypes. We also observed embryonically an under expression of Na_v22 and ApoE in the FAST rats. Accordingly, the data presented in the present thesis are predictive of a seizure profile in the FAST rats that indicates a greater vulnerability to seizure genesis than SLOW rats. Even the morphological data, suggesting epileptic-like pathology in the FAST rats, are consistent with this expectation. While all of these molecular data presented here support our hypothesis that the morphological abnormalities seen as adult FAST rats may be due to differences in developmental processes between the FAST and SLOW rats, it may be valuable in the future to investigate differences in several other genes, including Reelin, which have been more directly related to the abnormal morphology seen in TLE patients.

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Figure 1: Mean weight of the tissue ($\pm$ SE) extracted from FAST and SLOW rats at each of the time points examined. While there is a sample size of five for all time points there is only a sample size of one for the FAST E19 embryos. For E11 through to E15 the tissue weight represents the weight of the entire embryo, whereas for E19 and E21 only the head was weighed. For the postnatal time points, the weight reported was only for the microdissected structure indicated on the graph. An asterisk (*) denotes a significantly ($p < .05$) heavier tissue weight (mg) in the SLOW rats compared to the FAST rats. Two asterisks (**) denotes a significantly heavier tissue weight (mg) in the FAST rats compared to the SLOW rats.

Figure 2: Mean quantity of total RNA (μg) ($\pm$ SE) extracted from FAST and SLOW tissues at the time points indicated ($n=5$). For E11 through to E15 the RNA was extracted from the entire embryo, whereas for E19 and E21 the RNA was extracted from the head only. For the postnatal time points the RNA was extracted for the structure indicated on the graph. An asterisk (*) denotes a significantly ($p<.05$) higher amount of RNA in the SLOW rats compared to the FAST rats.

Figure 3: The amount of RNA extracted from the structures was normalized to the weight of the tissue from which it was extracted. Only a significant difference between the FAST and SLOW rat strains was present in the E21 embryonic head (n=5). An asterisk (*) depicts a significantly higher ($p < .05$) ratio of RNA extracted from that structure in the SLOW rats compared to the FAST rats. It should be noted that the E19 measurement for the FAST rat lacks an error bar due to the fact that there is only a sample size of one for the weight of tissue measurement.

Ratio of RNA Extracted From Tissue to Tissue Weight

Figure 4: Mean density (ODu/mm²) of Cyclophilin mRNA expression ($\pm$ SE), in the head of FAST and SLOW rats at E21 (n=4). There is no significant difference between the FAST and SLOW rats ($p > .05$) indicating that approximately the same number of cells were used in both strains to produce this result.

Cyclophilin Spot on Neuroscience Arrays

Figure 5: Sample Neuroscience SuperArrays probed using RNA from the heads of FAST and SLOW rats at E21. Arrows indicate the location of genes of interest and demonstrate the differential expression of ApoE and Na_v22 as well as the similarity in expression of cyclophilin between the FAST and SLOW strains.

Figure 6: Mean density (ODu/mm²) ($\pm$ SE) of mRNA expression for Apolipoprotein E (ApoE) in the heads of FAST and SLOW rats at E21 (n=4). An asterisk (*) denotes a higher density (therefore expression of ApoE mRNA) ($p < .05$) in SLOW rats in comparison to the FAST rats.

Figure 7: Mean ($\pm$ SE) mRNA expression of the voltage gated sodium channel receptor, 22 subunit (Na_v22), as measured by Density (ODu/mm²), of the FAST and SLOW kindling rats when gene expression in the E21 head was examined (n=4). An asterisk (*) denotes a significantly higher expression ($p < .05$) of Na_v22 in the SLOW rats in comparison to the FAST rats.

Expression of Nav beta2 in the E21 Head

Figure 8: A partial scan of a Differential Display film illustrating the differential expression patterns of K2 macroglobulin (K2M) between the FAST and SLOW rat strains in the E11, E13, and E15 embryos as well as in the E19 and E21 heads (n=4). The arrow points to the band that was ultimately sequenced and identified to be the K2M gene. The lower scan demonstrates a gene fragment that is expressed at the E11 time point in all 4 slow animals which indicates that sample preparation was successful and that the differential expression of K2M is indeed a difference in gene expression and not a failed PCR.