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Differential Seizure Response in Two Models of Cortical Heterotopia

Lisa A. Gabel^{a,b}, Monica Manglani^b, Natalia Ibanez^b, Jessica Roberts^a, Raddy Ramos^c, and Glenn D. Rosen^d

^aDepartment of Psychology, Lafayette College, Easton, PA

^bProgram in Neuroscience, Lafayette College, Easton, PA

^cDepartment of Neuroscience & Histology, New York Col. of Osteopathic Medicine, Old Westbury, NY

^dDepartment of Neurology, Beth Israel Deaconess Medical Center, Boston, MA

Abstract

Malformations of cortical development (MCD) are linked to epilepsy in humans. MCD encompass a broad spectrum of malformations, which occur as the principal pathology or a secondary disruption. Recently, Rosen et al. (2012) reported that BXD29-*Trl4^{lps-2J}/J* mice have subcortical nodular heterotopias with partial agenesis of the corpus callosum (p-ACC). Additionally Ramos and colleagues (2008) demonstrated that C57BL/10J mice exhibit cortical heterotopias with no additional cortical abnormalities. We examined the seizure susceptibility of these mice to determine if the presence (BXD29-*Trl4^{lps-2J}/J*) or absence (C57BL/10J) of p-ACC, in strains with MCD, confers a differential response to chemi-convulsive treatment. Our results indicate that C57BL/10J mice with layer I heterotopia are more susceptible, whereas BXD29-*Trl4^{lps-2J}/J* mice with more severe subcortical nodular heterotopia and p-ACC are more resistant to seizure behavior induced by pentylenetetrazole. These data suggest that p-ACC may confer seizure resistance in models of MCD.

Keywords

FCD; heterotopia; epilepsy; C57BL; BXD29; animal model

1. Introduction

Malformations of cortical development (MCD), including disordered neuronal proliferation, migration and cortical organization, can result in focal or global abnormalities affecting both structure and function. MCD have been linked to both epilepsy and developmental delay in humans. Focal cortical dysplasia (FCD), are the most common group of MCD in patients presenting with intractable epilepsy and epilepsy in children (Blümcke et al. 2009). FCD encompass a broad spectrum of malformations, including cortical dyslamination,

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Address Correspondence To: Lisa A. Gabel, Lafayette College, 303 Oechsle Hall, Easton, PA 18042, Phone: (610) 330-5296, Fax: (610) 330-5349, gabel@lafayette.edu.

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cytoarchitectural lesions and underlying abnormalities of white matter, and can occur as the principal pathology or as a secondary disruption (for review see Blümcke et al. 2011). Understanding the role these malformations play in epileptogenesis is necessary in order to develop new therapies for MCD-related epilepsies. Examination of animal models of the various types of MCD can provide valuable insight into the mechanisms which lead to altered cortical excitability.

Recently FCD have been reported in BXD29- *Trl4^{lps-2J}/J* (Rosen et al., 2012) and a large percentage of C57BL/10J mice (Ramos et al., 2008). The BXD29- *Trl4^{lps-2J}/J* strain exhibit bilateral subcortical nodular heterotopias as well as partial agenesis of the corpus callosum (pACC), whereas the C57BL/10J mice display layer I cortical heterotopia without a callosal defect. Examination of the seizure susceptibility of these two related strains could elucidate whether the presence (BXD29- *Trl4^{lps-2J}/J*) or absence (C57BL/10J) of p-ACC, in strains with MCD, confers a differential response to chemi-convulsive treatment.

The BXD strain family was generated by creating F2 mice from a cross of C57BL/6J and DBA/2J strains, and then subsequent inbreeding to create ~80 different BXD recombinant inbred strains (Pierce et al., 2004; Taylor, 1989). Since their creation, several of these original lines have accumulated new mutations, including the BXD29/TyJ strain. A subset of the BXD29/TyJ strain suffered a mutation in the Toll-like receptor 4 gene (*Tlr4*) rendering them insensitive to inhalation of lipopolysaccharide [lps] (Cook et al. 2006). These mutant mice were renamed BXD29- *Trl4^{lps-2J}/J* mice, and the BXD29/Ty (wildtype) strain was rederived from embryos frozen in 1978. It was recently discovered that the BXD29- *Trl4^{lps-2J}/J* mice exhibit MCD, whereas the wildtype BXD29/Ty strain does not (Rosen et al., 2012). It is important to note that the *Tlr4* mutation was ruled out as the cause of the FCD identified in the BXD29- *Trl4^{lps-2J}/J* strain (Rosen et al., 2012).

Examination of the seizure susceptibility of the parental lines, C57BL/6J and DBA/2J, as well as the majority of the first cohort of BXD strains demonstrated the variable resistance to the chemi-convulsant pentylenetetrazole (PTZ) (Wakana et al. 2000). Wakana and colleagues (2000) confirmed that the C57BL/6J strain is relatively resistant to PTZ treatment, whereas the DBA/2J line is more susceptible to seizure, and the F2 derived recombinant inbred strain, BXD line, display a range of responses to PTZ. More specifically, these data demonstrate that the BXD29/Ty strain exhibit a similar resistance to PTZ induced seizure behavior to the C57BL/6J parental line. In contrast, another BXD recombinant inbred line, BXD9/Ty, and DBA/2J mice were more susceptible to chemi-convulsant induced seizure behavior. However, it is unclear how the BXD29 mutant mice, BXD29- *Trl4^{lps-2J}/J*, would respond to PTZ treatment in light of the presence of bilateral subcortical nodular heterotopia, known to increase seizure susceptibility to chemi-convulsant treatment (Croquelois et al., 2009), but which also have partial callosal agenesis.

The C57BL family is probably the most widely used of all inbred strains, which consists of four major substrains, including C57BL6 and C57BL10. C57BL6 and C57BL10 mice possess a very close genetic relationship; differing at just three loci on chromosome 4 (McClive et al., 1994). Recently Ramos et al. (2008) reported that C57BL6 and C57BL10 mice exhibit layer I neocortical heterotopia, similar to those identified postmortem analysis of patients with developmental dyslexia (Galaburda and Kemper 1979; Galaburda et al., 1985). These malformations have also been identified in several inbred strains of mice which exhibit cognitive impairments (Denenberg et al., 1991; Boehm et al., 1996; Balogh et al., 1998), as well as increased cortical excitability *in vitro* (Gabel and LoTurco, 2001) and increased seizure susceptibility *in vivo* (Gabel and LoTurco, 2002). Based on previous studies, it is likely that C57BL mice with heterotopia will also exhibit an increased

susceptibility to PTZ induced seizure behavior in comparison to C57BL mice without heterotopia; however this hypothesis has yet to be confirmed.

In this study we examined the PTZ induced seizure behavior of BXD29 and C57BL strains to determine if the different neuroanatomical phenotypes influenced the seizure susceptibility of these models of MCD. Based on the higher incidence of heterotopia reported in C57BL10/J mice compared to the C57BL6/J strain (Ramos et al., 2008), we examined endpoint seizure behavior of C57BL/10J mice with and without layer I neocortical heterotopias, but which do not exhibit other cortical defects. Based on previous research examining the seizure susceptibility of mice with similar MCD, we hypothesized that C57BL/10J mice with heterotopia will exhibit seizure behaviors at lower doses of PTZ than mice without heterotopia. However, it was unclear whether the p-ACC would influence seizure susceptibility in the BXD29 mutant mice, BXD29-*Trl4^{ps-2J}/J*, that have large bilateral nodular heterotopias known to increase seizure susceptibility (Croquelois et al., 2009).

2. Results

2.1 Seizure Resistance in BXD29 mice with Heterotopia and Partial Callosal Agenesis

Rosen et al. (2012) recently discovered a 100% penetrant spontaneous mutation that leads to large nodular bilateral subcortical heterotopias with partial callosal agenesis (ACC) in the BXD29-*Trl4^{ps-2J}/J* strain (Figure 1). Based on previous reports that mice with subcortical heterotopias have lower seizure thresholds to chemi-convulsive treatment (Croquelois et al., 2009), we predicted that BXD29-*Trl4^{ps-2J}/J* (mutant) mice with similar MCD would exhibit increased susceptibility to PTZ treatment compared to BXD29/Ty (wildtype) mice. Preliminary analysis revealed no differences between males and females, nor were there any differences between animals acquired from commercial or academic colonies, so the data were pooled across these variables.

Contrary to expectation, mutant mice with bilateral heterotopia and p-ACC exhibited longer latencies to reach stages 2 ($t(21)=-2.53$, $p<0.0125$), 3 ($t(19)=-2.10$, $p<0.0125$), and 4 ($t(19)=-2.47$, $p<0.0125$) compared to wildtype mice (Figure 2A). Similarly, mutant mice required a larger cumulative dose of PTZ to reach stages 3 ($t(19)=-3.23$, $p<0.0125$) and 4 ($t(19)=-2.62$, $p<0.0125$; Figure 2B) compared to wildtype mice. Hence, these results show that mutant mice with bilateral subcortical nodular heterotopias and p-ACC are more resistant to seizure behavior induced by intraperitoneal injection of PTZ in comparison to wildtype mice.

2.2 Increased seizure susceptibility in C57BL/10J mice with heterotopia

Ramos et al (2008) recently reported C57BL/10J mice exhibit layer I heterotopia made up of neurons and glia. Here we examined 40 μ m sections of brains from 24 C57BL/10J mice from frontal to occipital pole in order to quantify heterotopia based on number, location, and size of the malformation. Based on work by Denenberg et al., (1991) layer I heterotopia were characterized as small (containing < 20 cells), moderate (20-50 cells) or large (>50 cells; Figure 3). Sixty-seven percent (16/24) of C57BL/10J mice had heterotopia, a lower incidence than in initial reports (Ramos et al. 2008). A large proportion (7/16, 43.8%) of mice with MCD had two or more heterotopias, which were visible in both hemispheres (Table 1). In one case eleven malformations were identified in a single brain. The majority of mice had malformations located within the somatosensory cortex only (12/16, 75%); the remaining 25% had heterotopia located in both the frontal/motor cortices and the somatosensory cortex. None of the mice examined in this study has heterotopia located in the frontal/motor cortex only. All but one mouse with heterotopia had at least one large malformation (93.8%). These data suggest that C57BL/10J mice have a more severe form of

cortical dysplasia than the other strains with similar spontaneous developing heterotopia (Denenberg et al., 1991; Gabel, 2011; Sherman et al., 1990).

Utilizing similar procedures to those employed with the BXD29 strains, we examined the seizure susceptibility of C57BL/10J mice with neocortical heterotopia. C57BL/10J mice were given an initial bolus of 30mg/kg PTZ (i.p.) followed by 10mg/kg of PTZ every 10 minutes until they experienced a generalized seizure, or a maximum dose of 130mg/kg was reached. C57BL/10J mice received a dose of PTZ that ranged from 30mg/kg -110 mg/kg before a generalized seizure was produced. Heterotopia can only be identified with postmortem histological analysis; therefore the experimenter was blind to which mice had MCD. Of the twenty-four C57BL/10J mice examined, 56% (14/25) experienced a myoclonic jerk (MCJ) before generalized seizure; eight of these mice (8/14) had a heterotopia. Myoclonic jerk behavior occurred at a faster rate ($t(12)=1.80$, $p<0.05$; Figure 4A) and lower cumulative dose ($t(12)=1.80$, $p<0.05$; Figure 4B) in C57BL/10J mice with heterotopia compared to those without MCD. In contrast, generalized seizure behavior was equivalent in both groups based on latency ($t(22) = <1.0$, n.s.; Figure 4C) and cumulative dose of PTZ ($t(22)=<1.0$, n.s.; Figure 4D). Approximately twenty-one percent (5/24) of the C57BL/10J mice died following a generalized seizure, 80% (4/5) of these mice had heterotopia based on postmortem analysis of the brains. These data demonstrate that layer I heterotopia in C57BL/10J mice are associated with increased seizure susceptibility to PTZ treatment.

3. Discussion

MCD has been associated with increased seizure susceptibility in humans and animal models. Previous studies have demonstrated that NZB/BINJ mice with layer I neocortical heterotopia have increased seizure susceptibility *in vivo* (Gabel & LoTurco, 2002). Consistent with previous reports, we find that C57BL/10J mice with identical heterotopia had a lower threshold for myoclonic jerk seizure activity, but not generalized seizure. Additionally, similar malformations have been associated with cognitive impairments (Denenberg et al., 1991; Boehm et al., 1996; Balogh et al., 1998) and auditory processing deficits (Peiffer et al., 2001) in other inbred strains of mice, as well as in individuals with developmental dyslexia (Galaburda and Kemper 1979; Galaburda et al., 1985). Thus, despite the relatively minimal disruption to the cortical architecture (Gabel and LoTurco, 2002; Gabel, 2011), these malformations may account for severe behavioral and sensory impairments in humans.

Of the C57BL/10J mice with heterotopia, all had at least one large malformation located within the somatosensory cortex (15/16, 94%); with slightly less than half (44%, 7/16) having a more than one malformation present in a single brain. Despite the increased incidence of multiple heterotopias present in this inbred line, in comparison to other inbred strains with similar malformations (Denenberg et al., 1991; Boehm et al., 1996; Balogh et al., 1998; Gabel and LoTurco 2002; Gabel 2011), the seizure behavior in response to PTZ treatment was remarkably similar. These data may suggest that the presence of heterotopia may be enough to alter seizure susceptibility in these strains, rather than the severity of dysplasia. However, due to low subject number across conditions, it is still unclear if the severity of the dysplastic tissue and the location of the malformation play a role in seizure susceptibility. Therefore, further examination of the seizure susceptibility in these mice will provide a more in depth analysis concerning the role of size, number and location of malformations on cortical excitability.

Surprisingly, we also demonstrate that BXD29 mutant mice with bilateral subcortical nodular heterotopias have a reduced seizure threshold despite reports of similar

malformations associated with increased seizure susceptibility (Croquelois et al., 2009). Interestingly, the BXD29 mutant mice also have p-ACC which may confer seizure resistance in these animals by reducing the spread of the of the epileptiform activity across hemispheres. For example, the HeCo mouse (Croquelois et al., 2009) has heterotopia similar to BXD29-*Tlr4^{lps-2J}/J* mice, which are in a comparable location, but without reported callosal defects. Unlike the BXD29-*Tlr4^{lps-2J}/J* mice, the HeCo mice exhibit a lower threshold for pilocarpine-induced seizure behavior than control mice. Future research is needed to examine the potential role of the corpus callosum in the spread of seizure activity in this strain.

Previous work has demonstrated that the BXD29/Ty strain exhibit a similar threshold for PTZ-induced seizure behavior to the C57BL/6J parental line (Wakana et al. 2000). In our study, BXD29/Ty mice also exhibit a similar threshold for PTZ induced seizure behavior in comparison to C57BL/10J mice based on the latency and concentration of PTZ needed to generate myoclonic jerk (i.e. stage 2 in the BXD29 study) and generalized seizure (i.e. stage 4) behaviors. However, the PTZ threshold for BXD29 mutant mice, BXD29-*Tlr4^{lps-2J}/J*, with bilateral nodular heterotopias and p-ACC, and C57BL/10J mice with layer I neocortical heterotopia is vastly different. More specifically C57BL/10J mice with MCD displayed seizure behaviors at cumulative doses of PTZ which never induced a seizure response in BXD29 mutant mice. The question remains whether the agenesis of the corpus callosum reduces spread of the seizure, or if the mutation of the Toll-like receptor 4 (Tlr-4) is responsible for inhibiting seizure behavior. However, recent evidence suggests that the Tlr-4 mutation is not responsible for the MCD in these mice (Rosen et al., 2012) and increased susceptibility to seizure behavior is known to occur in response to PTZ treatment in animal models with similar bilateral nodular heterotopias (Croquelois et al., 2009).

Recent work has demonstrated that a C3H/HeJ *Tlr4^{Lps-d}* mouse, with a single nucleotide change in exon 3 of the Tlr4 gene resulting in an amino acid substitution, confers increased seizure resistance to intrahippocampal application of kainic acid compared to C3H/HeOuJ mice (Maroso et al., 2010). Furthermore, intrahippocampal application of a Tlr4 antagonist, prior to kainic acid administration, reduces the seizure susceptibility of control mice (Maroso et al., 2010). However, the question remains whether the large nucleotide insertion in the 3' end of the Tlr4 gene, which results in insensitive to inhalation of LPS in this mutant, can also explain why an animal model with MCD associated with increased seizure susceptibility is now more seizure resistant.

Currently no direct experiments have been conducted on the role of Tlr4 antagonists on seizure activity in animal models of MCD. Tlr4 is indirectly related to the interleukin-1 β (IL-1 β) system which has been shown to be upregulated in glial cells in response to seizure behavior. Previous work examining the expression levels of IL-1 (Ravizza et al. 2006) and Tlr4 (Zurolo et al., 2011) in balloon cells and cortical tubers, have demonstrated an upregulation in neurons and glia within the malformation of these two types of FCD, but not within the surrounding cortex. Therefore, it is possible that there is a connection between Tlr4 function and seizure susceptibility in these animals. However, the Tlr4 is not responsible for the development of the FCD identified in the BXD29-*Tlr4^{lps-2J}/J* mice, therefore further research is needed to examine the role of partial agenesis of the corpus callosum in seizure susceptibility in animal models of MCD.

4. Experimental Procedures

4.1 Subjects

Seizure susceptibility of the mice was assessed at 9-28 weeks postnatal in twelve BXD29/Ty (9 males and 3 females; 143.5 ± 65.4 days postnatal) and twelve BXD29-*Tlr4^{lps-2J}/J* (9

males and 3 females; 85.9 ± 17.9 days postnatal) that were acquired from either Jackson Laboratories (JAX, Bar Harbor, ME; stock numbers 010981 and 000029, respectively) or from a colony (GDR) whose founders were acquired from JAX. Additionally, twenty-four male C57BL/10J mice obtained from JAX (stock number 000665) were tested at 12-15 weeks postnatal. All mice were group housed with free-access to food and water *ad libitum*. Animals were kept on a 12 hour light-dark cycle, with lights on at 8 am; all testing took place during the light cycle. All procedures were approved by the Institutional Animal Care and Use Committee at Lafayette College.

4.2 Pentylentetrazole (PTZ) Paradigm

As described in Gabel & LoTurco (2002), PTZ (Sigma-Aldrich, St. Louis, MO, U.S.A.) at an initial concentration 30 mg/kg was dissolved in 0.9% sterile saline. A 30 mg/kg bolus of PTZ was injected intraperitoneally followed by 10 mg/kg ip every 10 minutes until a generalized seizure was observed, or a maximum cumulative dose of 130 mg/kg was administered. Subjects did not exhibit either spontaneous seizures or behavioral seizure activity after administration of the initial dose of PTZ.

4.3 Seizure Behavior

In experiments conducted using BXD29-*Tlr4^{lps-2J}*/J (mutant) and BXD29/Ty (wildtype) mice, seizure activity was recorded based on the latency and cumulative dose of PTZ required to reach the following stages (Claycomb et al. 2011): stage 0, no behavioral change; stage 1, hypoactivity and immobility; stage 2, two or more isolated, myoclonic jerks; stage 3, generalized clonic convulsions, with preservation of righting reflex; and stage 4, generalized clonic or tonic-clonic convulsions with loss of righting reflex. Experimenters were blind to strain. Seizure activity in C57BL/10J mice was determined by the latency and cumulative dose of PTZ to first myoclonic jerk and generalized seizure. A generalized seizure was characterized as a wild running episode, major generalized seizure without tonus, and/or tonic-clonic seizure.

4.4 Histological Analysis

All mice were sacrificed by transcardial perfusion with phosphate-buffered saline (PBS) and 4% paraformaldehyde/PBS. C57BL/10J brains were cryoprotected and frozen 40 μ m sections were taken from frontal to occipital pole. All sections from C57BL/10J and every 10th section from BXD29/Ty and BXD29-*Tlr4^{lps-2J}*/J mice were stained with cresyl violet, mounted on glass slides with Permount (Fisher Scientific, Pittsburgh, PA), and examined using a Nikon Eclipse 80i microscope with spot camera. Images were obtained using NIS-Elements 3.0 software.

4.5 Statistical Analysis

All statistical analyses were performed using SPSS Statistics 19 (IBM Corporation, Somers, NY). Data are reported as Means $\pm$ SEM. A Student's t-test was used to compare seizure latency and cumulative dose across seizure stages; a Bonferroni Correction was applied.

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Highlights

- Animal models of MCD provide insight into mechanisms of altered cortical excitability
- We examined seizure susceptibility in two genetically related mouse strains with MCD
- BXD29 mutant mice with subcortical heterotopia and p-ACC are resistant to seizure
- C57BL/10J mice with less severe cortical dysplasia are more susceptible to seizure
- Future studies should examine role of p-ACC in seizure resistance in BXD29 mutant mice

Figure 1. BXD29-*Tlr4*^{ps-2J}/J mutant mice have bilateral heterotopia and partial agenesis of the corpus callosum. Nissl stain of section in the coronal plane with bilateral midline neocortical nodular heterotopias. Scale bar (in μm) = 500 μm .

Figure 2. BXD29- *Tlr4*^{Δps-2J/J} mutant mice display increased seizure resistance to PTZ treatment. **(A)** Latency (seconds) to reach different stages of seizure behavior in control (BXD29/Ty) and mutant (BXD29- *Tlr4*^{Δps-2J/J}) mice. **(B)** Cumulative dose of PTZ (mg/kg body weight) across increasing stages of seizure development. Seizure stages: 0, no behavioral change; 1, hypoactivity and immobility; 2, two or more isolated, myoclonic jerks; 3, generalized clonic convulsions, with preservation of righting reflex; and 4, generalized clonic or tonic-clonic convulsions with loss of righting reflex.

Figure 3.

C57BL/10J mice have layer I heterotopia located in multiple regions of the cortex. Nissl stained sections in one C57BL/10J mouse demonstrating bilateral layer I neocortical heterotopia located at midline (A-A'), frontal/motor (B-B') and somatosensory (C-D') cortices. Arrows point to heterotopia. Scale bar (in μm) = 850; 150.

Figure 4.

C57BL/10J mice with heterotopia have an increased seizure susceptibility to chemi-convulsive treatment. **(A)** Latency (seconds) and **(B)** cumulative dose of PTZ (mg/kg) needed to induce myoclonic jerk behavior in C57BL/10J mice (BL10) compared to C57BL/10J mice with MCD (BL10 MCD). **(C)** Latency (seconds) and **(D)** cumulative dose of PTZ (mg/kg) needed to induce generalized seizure behavior in mice with and without MCD.

MCD in C57BL/10J mice**Table 1**

Number of Ectopia	Location	n
1	SS	9 [‡]
	F/M	0
	Mix	0
>2	SS	3
	F/M	0
	Mix	4 [*]

Note ^{*}The location of the layer I neocortical heterotopia were categorized as somatosensory cortex (SS), frontal/motor cortex (F/M), or mixed (Mix) if identified in both the SS and F/M cortices. The size of the MCD was based on the largest malformation in cases where multiple heterotopia were present.

[‡]Fifteen out of 16 mice with heterotopia had at least one large MCD (i.e. >50 cells); one animal with a single heterotopia located in the somatosensory cortex had a malformation of moderate size (i.e. 20-50 cells).

^{*}One subject had two; two subjects had three and one subject had eleven heterotopias located within the SS and F/M cortices.