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Iron Deficiency Affects Seizure Susceptibility in a Time- and Sex-Specific Manner

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Abstract

Iron deficiency (ID) affects more than three billion people worldwide making it the most common micronutrient deficiency. ID is most prevalent during gestation and early life, which is of particular concern since its impact on the developing central nervous system is associated with an increased risk of a wide range of different psychiatric disorders later in life. The cause for this association is not known, but many of these same disorders are also associated with an imbalance between excitation and inhibition (E/I) within the brain. Based on this shared impairment, we asked whether ID could contribute to such an imbalance. Disruptions in the E/I balance can be uncovered by the brain's response to seizure inducing insults. We therefore tested the seizure threshold under different nutritional models of ID. We found that mice which were postnatally exposed to ID (and were acutely ID) had a decreased seizure threshold and increased susceptibility to certain seizure types. In contrast, mice that were exposed to ID only during gestation had an increased seizure threshold and low seizure incidence. We suggest that exposure to ID during gestation might alter the cellular components that contribute to the establishment of a proper E/I balance later in life. In addition, our data highlight the importance of considering the window of vulnerability since gestational ID and postnatal ID have significantly different consequences on seizure probability.

Keywords

CNS biochemistry, excitatory, inhibitory balance, iron deficiency, neurodevelopmental disorders, neuropathology, neurotransmission, seizure

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Introduction

Despite a concentrated effort by the World Health Organization (2014) to increase awareness and promote iron supplementation, iron deficiency (ID) remains the most prevalent micronutrient deficiency, with the most severe form, ID anemia, affecting over 20% of pregnant women and 23% of children under the age of five. Its effect on developing children is particularly devastating since iron supplementation later in life cannot remedy the learning difficulties, behavioral problems, and psychiatric disorders that are associated with early life ID (Lozoff et al., 2000; Chen et al., 2013; Lozoff et al., 2013; Radlowski and Johnson, 2013). The memory and learning disabilities, which are thought to mainly involve hippocampal function, have been studied extensively and revealed long-term alterations in the expression of parvalbumin and regional monoamines (Callahan et al., 2013), alterations in dopamine synthesis, and deficits in sensorimotor gating determined by prepulse inhibition of the acoustic startle reflex (Unger et al., 2012; Pisansky et al., 2013). In contrast, the association of early-life ID with psychiatric disorders that include a wide range of manifestations such as autism (Schmidt et al., 2014), attention deficit/hyperactivity disorder (Doom et al., 2015), bipolar/unipolar disorder, and anxiety disorder (Chen et al., 2013) is poorly understood and cannot solely be explained by hippocampal impairments.

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A proposed shared feature of the diverse psychiatric disorders associated with early life ID is a suspected imbalance between excitatory and inhibitory synaptic activity (referred to as E/I balance) (LeBlanc and Fagiolini, 2011; Luscher et al., 2011; Bollmann et al., 2015; Rivero et al., 2015; Lener et al., 2017). One of the physiological manifestations of such an imbalance is the susceptibility of the brain to seizurogenic insults (During and Spencer, 1993; Bradford, 1995; Olsen and Avoli, 1997; Alexander et al., 2016).

Analyzing the response to two different seizure models (injection of the seizurogenic drug pentylenetetrazole [PTZ] or exposure to postnatal hypoxia), we found that the impact of ID on seizure response is dependent on time of ID onset (i.e., during gestation or postnatally). In particular, the developmental timing of ID was critical in determining outcomes, as gestational ID (gID) and postnatal ID (pnID) unexpectedly had opposite effects on seizure susceptibility. Our finding that ID can either increase or decrease the seizure threshold, depending on developmental timing, may help to explain the seemingly conflicting results in the literature in human studies. Many such studies suggest that ID increases the risk of febrile seizures (Daoud et al., 2002; Naveed ur and Billoo, 2005; Hartfield et al., 2009; Idro et al., 2010; Sherjil et al., 2010; Zareifar et al., 2012; Fallah et al., 2013; Fallah et al., 2014; Ghasemi et al., 2014; Sharif et al., 2015; Koksal et al., 2016); in contrast, others found that ID with (Bidabadi and Mashouf, 2009; Derakhshanfar et al., 2012) or without (Kobrinsky et al., 1995) anemia protects from seizures; still other investigators found no association between ID and seizure risk (Heydarian and Vatankhah, 2012; Waheed and Butt, 2012; Amouian et al., 2013). On the basis of our carefully controlled animal studies, we suggest that the seemingly contradicting reports in the literature reflect the lack of knowledge about early life iron status in human populations. By separating ID into two groups (a gestationally iron-deficient but postnatally iron-replete group and a gestationally iron-normal but postnatally iron-deficient group), we are able to demonstrate that ID can have opposite effects on the seizure threshold, which is dependent on time of ID exposure, seizure type, and sex.

Materials and Methods

Animal Care

All protocols were approved by the University Committee on Animal Resources at the University of Rochester. Male and female Swiss Webster mice (aged 8 weeks), strain code 024, were purchased from Charles River Laboratories. Animal diets were purchased from Envigo (formerly Harlan Teklad) custom research diets. Iron-normal diet contained 240 μg Fe/g of food (TD.05656). Iron-deficient (ID) diet contained 2–6 μg Fe/g of food (TD.80396). Diets were identical except for iron content. Animals had free access to food and distilled water. All animals were kept on Bed-o′Cob ¼′ corn cob bedding (mice housed on paper bedding will not become iron deficient since paper contains enough iron to supplement the animals). Dams were housed alone 1 week prior to giving birth and offspring housed by sex in groups of 2 to 4 after weaning at P21. Animals were housed at 23°C, 43% relative humidity, and kept on a 12- to 12-hr light-dark cycle.

Gestationally ID mice appear to develop normally and do not show obvious behavioral differences or changes in basal activity levels. However, gID animals remain significantly smaller than age-matched controls (even after complete iron repletion) reaching a maximum size which is approximately 80% to 90% the total size of controls. pnID (which persists through seizure testing) also results in a significant decrease in animal size, but we did not see any obvious change in animal behavior.

Diet Groups

There were three diet groups: a control group, a pnID group, and a gID group (although there were differences in supplementation age between seizure models in the gID group). Animals in all the groups were placed on diet 2 weeks prior to mating. The control group was always fed an iron-normal diet. The pnID group received an iron-normal diet up until the day of birth and was then switched to iron-deficient diet for the remainder of the experiment. These pnID animals were iron deficient to the point of anemia at the time of seizure testing. The gID group received iron-deficient diet throughout embryonic development. The gID group used for the PTZ experiments was switched to an iron-normal diet on P7, whereas the gID group used in the hypoxia experiments was switched to the iron-normal diet on P0. This early supplementation was necessary since the hypoxia experiment can only be performed in young animals (Rakhade et al., 2012; Sun et al., 2016). Animal numbers for PTZ seizure experiments were as follows: control group = 9 males from 5 litters and 8 females from 5 litters; gID group = 7 males from 5 litters and 7 females from 6 litters; pnID group = 11 males from 4 litters and 9 females from 4 litters. Animal number for hypoxia seizure experiments were as follows: control group = 8 animals from 2 litters; gID group = 8 animals from 2 litters.

We originally planned to include a fourth group with animals that received the iron-deficient diet during gestation and remained on the ID diet throughout weaning and postnatal development. However, this regiment resulted in the development of severe iron deficiency anemia (IDA) in the pups and was associated with severely stunted growth and eventual death. We observed

peak death rates around P14 with a nearly 100% mortality rate by P21. It was thus not possible to include this group in PTZ seizure testing at P42. Notably, iron supplementation at P7 (via dam) completely rescued animal mortality, with nearly 100% survival rates in supplemented offspring.

PTZ Seizure Testing

PTZ is thought to induce seizures by increasing the duration of the closed state of the $GABA_A$ Cl^- channel, thereby reducing the effectiveness of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; Huang et al., 2001). On the first day of PTZ dosing, animals were approximately aged P44 (P43.9 gID-males; P43.9 gID-females; P43.6 control-males; P43.3 control-females; P45.1 pnID-males; P45.3 pnID-females). Each mouse was intraperitoneally injected with PTZ at 40 mg/kg body weight, and all seizure types were recorded for the following 20-min observation period. Animals were dosed once per day, between 9 and 11 p.m., for five consecutive days followed by a 2-day rest period. This dosing paradigm was continued until animals had been tested a total of 15 times. Animals that showed three or more Class V seizures during a single 20-min observation were considered fully kindled and were removed from the experiment. Seizures were binned using a modified Racine scale, with the Class I to Class VI subtypes described as follows:

Class I: Myoclonic jerk—muscles of the body tense in unison, no loss of postural control, lasting no more than 1 s. Head typically ducks, eyes widen, and tail lifts/straightens (Straub tail) immediately prior to the onset of muscle contraction.

Class II: Sustained or consecutive myoclonic jerks—similar to "Class I," but mouse remains tense for 1 to 3 s, or it has two consecutive "Class I" seizures separated by less than 1 s. It may also involve flexion of toes and pushing nose/teeth into the floor of the enclosure (though this often indicates an incipient Class IV or V seizure—not counted as Class II seizure if a Class IV or V seizure immediately follows).

Class III: Rearing with forelimb clonus—in seated position, head twists to one side, often exhibit unproductive side-to-side cleaning motion, may partially lose postural control but it is quickly regained, and may include vocalizations. They may also be precipitated by a small/sudden jump into the air. Class III seizures last between 3 and 10 s. The side-to-side cleaning motions are not counted as Class III seizures if they occur as part of a Class IV or V seizure.

Class IV: Tonic-clonic seizure lasting 30–60 s—Stereotypic behaviors described in "Class V" section.

Class V: Tonic–clonic seizure lasting more than 1 min—Often precipitated by vocalization, Straub tail, ears pinned back, flexion of toes, and pushing nose/teeth into the floor of the enclosure. Within a few seconds, there may be another, longer, vocalization as the animal begins the clonic phase of the seizure. This phase typically lasts for 15 to 45 s. It begins as back hunches and animal rolls onto its side or back (loss of postural control). Clonic phase is often characterized by (a) muscles rapidly tensing and relaxing, animal rolls and bounces while the righting reflex attempts to bring animal back onto its feet, this behavior typically lasts for 3 to 10 s; (b) hyperextension of neck (animal stares blankly toward ceiling), extension at shoulder and pushing motion with front feet, and tail held straight and off the ground; (c) tail flagged straight upward, biting or side-to-side motion with face pushed against floor of enclosure, front feet making running/digging motion with little forward movement; and (d) unproductive side-to-side cleaning motion. The clonic phase is then followed by an immobile and unresponsive phase. This phase typically lasts for 15 to 90 s and is often characterized by (a) muscles which are tense and unmoving, (b) a tail which is flexed and held aloft, (c) flexion of toes, (d) rigid posture, (e) no movement or extremely slow movement, and (f) a lack of response to external stimuli. This phase often ends with a jerking movement similar to a Class I seizure.

Class VI: Seizure resulting in death—this seizure type is always precipitated by a characteristic stretching motion in which the front and hind legs stretch in the rostral direction (extension at shoulder joint) and within a few seconds is followed by complete relaxation and death.

No spontaneous seizures were observed in any animal regardless of degree of ID or stage of kindling. Seizures were only observed immediately following PTZ injection or hypoxia.

Calculation of "Seizure Response" and A-E Values for PTZ-Dosed Animals

We focused on seizure Types I and V because these categories collectively make up more than 96% of seizure incidents (exact ratios are Class I, 88.2%; Class II, 0.7%; Class III, 1.7%; Class IV, 0.5%; Class V, 8.4%; and Class VI, 0.4%), though it is important to note that inclusion or exclusion of Classes II, III, IV, and VI does not change our results. Since the pnID group was found to have differing susceptibilities to Class I and Class V seizures, both seizure types were analyzed separately. On each day, the total number of Class I or Class V seizures were tallied, and these values plotted through the experiment (shown by diamonds in Supplemental Figure S1). These daily values were used to calculate a trend line (bound at 0,0) which represents the average seizure kindling rate for each animal. The slope of the trend line represents each animal's overall seizure susceptibility rating

(SSR) for the respective seizure type (Figure 2). Measurement of kindling rate using a trend line, rather than a tally of total seizure number, was necessary because many animals (20 out of 51) either died of a Class VI seizure during the observation period or were removed from the experiment following three or more consecutive Class V seizures. Most of the animals which were lost during seizure testing were in the control group, so these animals could not be ignored without biasing the results. The trend-line method allowed us to estimate each animals kindling rate and assign an overall SSR, regardless of whether the animal died or was removed. In addition, we confirmed the validity of the trend-line method by comparing these results to those obtained using "last observation carried forward" (LOCF) to fill in missing data. The LOCF method represents a conservative estimate of an animals kindling rate since it does not account for increases in seizure responses through time (kindling) when filling in missing data. The comparisons which reached significance (at $p < .05$) using the trend-line method were also significantly different when using LOCF. This supports the validity of the trend-line method. The trend-line method can also be used to estimate an animal's expected daily seizure score and, thus, can be used to calculate an animal's daily deviation from an expected value (A-E value). For example, gID female #6 (shown in Supplemental Figure S1) has a slope of 2.075 for the Class I seizure kindling rate. Her expected seizure value on Day 12 is therefore $2.075 \times 12 = 24.9$ (the trend line value on Day 12). This animal actually had a total of 45 Class I seizures on Day 12, which means her actual seizure score is 20.1 counts above the expected value for Day 12 (A-E value is positive 20.1). A negative actual minus expected value (A-E value) indicates a seizure score below the expected value, and positive A-E value indicates a score above. These A-E values are used for calculations in Figure 3.

Hypoxia Seizure Testing

Dams were placed on diet 2 weeks prior to mating and remained on diet until pups were born. On the day of birth, all groups were given iron-normal diet. At postnatal Day 9, offspring were seizure tested using a hypoxia chamber (Coy lab products, AC100 CO2 controller, Model 2000 O2 controller) heated to 34°C–37°C. Each animal was given a unique tail marking and placed on a heating pad inside of an open-top containment pen. Oxygen was held at 21% while animals were placed into pens and video hardware set up. Animals were observed for 30 min under room air, then nitrogen was pumped into chamber, displacing oxygen, until O_2 levels reached 9% (transition took approximately 10 min). Oxygen was then held at 9% for 5 min, dropped to 6% for 5 min, dropped to 5.5% for 5 min, and finally dropped to 5% for 10 min (transitions took approximately 2 min). Seizures were scored using a modified Morrison scale (Morrison et al., 1996; note that this scale uses a different classification of seizure types and represents distinct seizure types from those used in the PTZ model). Class III seizure behaviors include head shivering, sustained wagging motion of tail, scratching behavior, and running motions while laying on side. Class IV seizure behaviors include muscle spasms of torso and limbs in which at least two feet leave the ground, and there is a partial or complete loss of postural control and are often followed by Class III behavior.

Hematocrit Measurements

Hematocrit, hemoglobin, and red cell volume were measured using the HESKA—HemaTrue hematology analyzer and verified using spin hematocrit. All blood samples were collected after CO_2 euthanasia and decapitation.

Western Blot Analysis

Brain tissue was dissected from E10, E12, P0, and P42 mice. Whole brains were used for the analysis of embryonic tissues and half brains cut mid-sagittally were used for postnatal tissue analysis. Protein quantification was performed using Bio-Rad DC protein assay kit and Perkin Elmer Wallac 1420 multilabel counter. Primary antibody for Ferritin was anti-Ferritin Heavy Chain (FTH1) polyclonal rabbit antibody #3998 from Cell Signaling and was used at 1:1000 for 4 days in a cold room. Secondary antibody was goat anti-rabbit IgG conjugated to HRP (Invitrogen G21234). Ferritin protein has a molecular weight of 21 kDa and beta-actin is 42 kDa. Blots were cut at 35 kDa. Beta-actin antibody was mouse monoclonal IgG1 conjugated to HRP (Santa Cruz Biotechnology sc-47778 HRP).

Statistical Analysis

JMP®, Version 12.2.0. SAS Institute Inc., Cary, NC, 1989–2007, was used for all statistical calculations.

Specific analyses related to Figure 1. Student's *t* test was used to compare brain tissue ferritin at each age.

Specific analyses related to Figure 2. Analysis of variance was conducted on the influence of diet and sex on seizure susceptibility. Some of the data had to be transformed in order to satisfy normal distribution and homoscedasticity; however, these transformations did not change which comparisons were significant with the exception of Class V seizure comparison between gID and controls; untransformed data did not reach significance ($p = .0549$), but transformed data did reach significance ($p = .0435$). For Figure 2(e) and (f), parametric survival model used Weibull distribution.

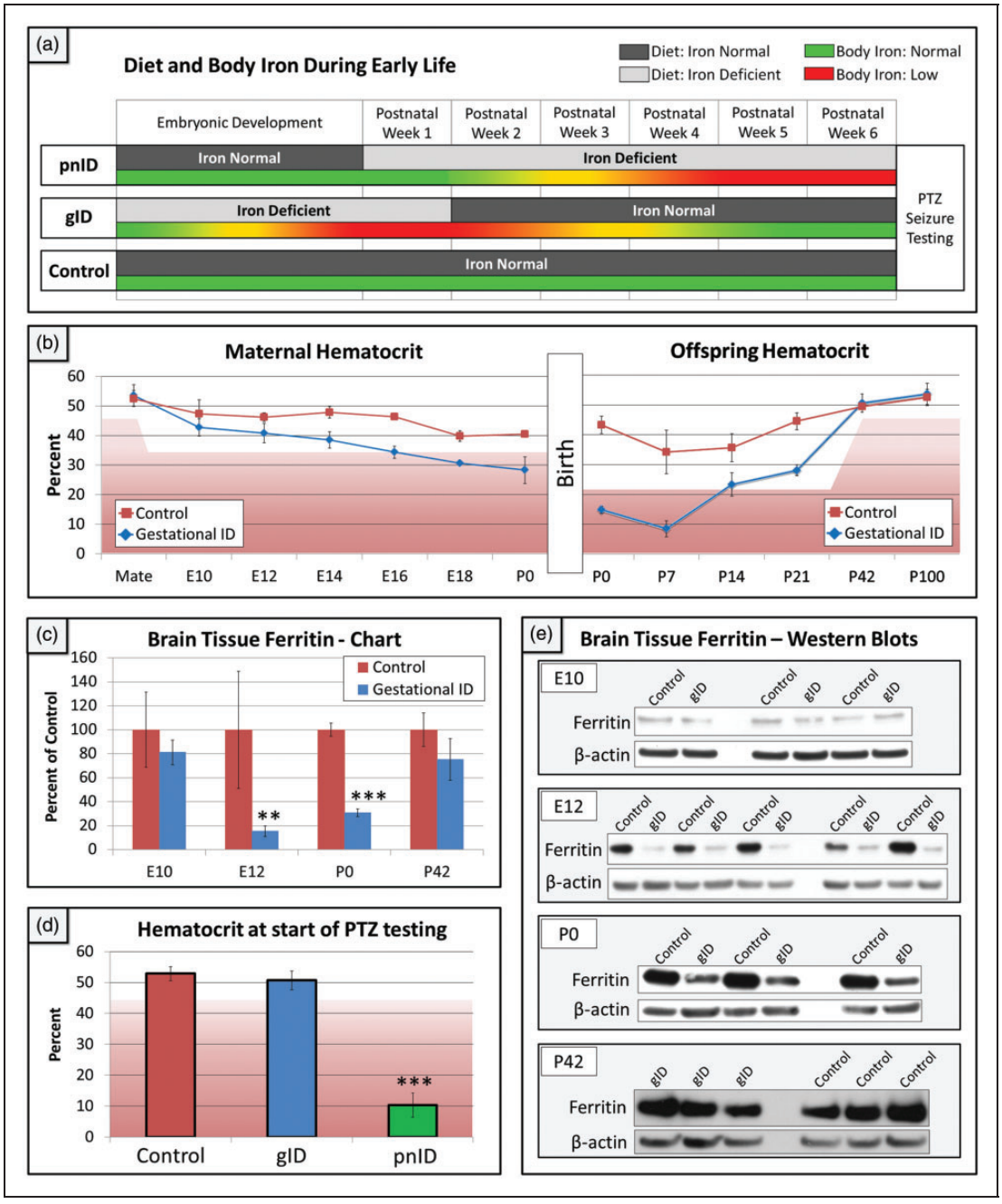

Figure 1. Early life iron status prior to PTZ induced seizure testing. (a) Outline of diet and concurrent iron status under the different iron models: Under pnID (postnatal and ongoing iron deficiency), offspring were iron normal during gestation but were exposed to an iron-deficient diet during postnatal development which continued until the time of testing. Under gID (gestational iron deficiency), embryos were exposed to the ID during gestation but were switched to an iron-normal diet after birth (P7) and remained on iron-normal diet through the time of testing. Control animals received an iron-normal diet only. (b) Hematocrit analysis for dams during pregnancy (E10–E18 representing days of gestation) and offspring after birth from postnatal Day 0 (P0) to P100. Red shading represents anemic hematocrit range during adulthood, pregnancy, and early life (2 SD below control mean), ranging from mild (light red) to severe (dark red). (c) Quantification of brain tissue ferritin in gID and control animals from gestational Day 10 (E10) to postnatal Day 42 (P42) ($n = 3$/age-group). Statistics: Student's t test $^{**}p < .005$; $^{***}p < .0001$. (d) Quantification of hematocrits for each nutritional model at the start of seizure testing (P42) Statistics: one-way analysis of variance, $^{***}p < .0001$. (e) Western blot data corresponding with panel (c). Animal numbers: 3 animals per group per measurement ($n = 86$ for Figure (b), $n = 28$ for Figure (c); $n = 9$ for Figure (d)). gID = gestational iron deficiency; pnID = postnatal iron deficiency; PTZ = pentylenetetrazole.

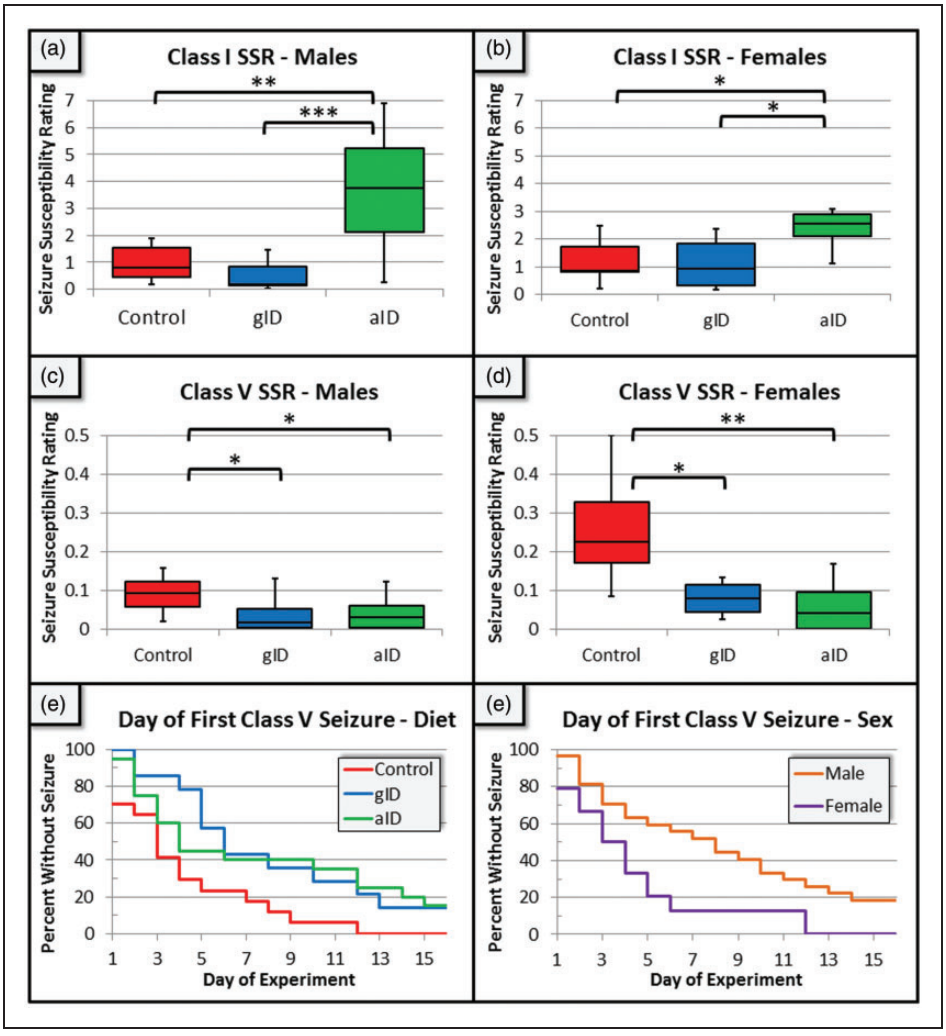

Figure 2. Seizure response to PTZ. Panels (a) and (b) show quantification of Class I seizure susceptibility ratings for (a) pnID, gID, and control males and (b) for pnID, gID, and control females. Panels (c) and (d) show quantification of Class V seizure susceptibility ratings for (c) pnID, gID, and control males and (d) for pnID, gID, and control females. Statistics: Tukey HSD post hoc comparison $^{*}p < .05$; $^{**}p < .005$; $^{***}p < .001$. (e) Analysis of the time until first Class V seizure in pnID and gID groups compared to controls. Statistics: Cox proportional hazards model: pnID = 0.35; gID = 0.32, $p = .0057$. (f) Analysis of the time until the first Class V seizure in males compared to females. Statistics: Effect likelihood ratio, $p = .0012$. Animal numbers: control group = 9 males and 8 females; gID group = 7 males and 7 females; pnID group = 11 males and 9 females. SSR = seizure susceptibility rating; gID = gestational iron deficiency; pnID = post natal iron deficiency; PTZ = pentylenetetrazole; aID = acute iron deficiency.

Specific analyses related to Figure 3. We used a Pearson product–moment correlation followed by pairwise comparisons to assess the relationship between variables.

Specific analyses related to Figure 4. Class III seizure data had to be transformed in order to be normally distributed. Student's t test on untransformed versus transformed data did not affect which comparisons were significant. For Class IV hypoxia seizure data, the presence of multiple zero values in gID group required that we model the data using binomial distribution and link logit. Chi-square goodness of fit statistic ($\chi^2 = 2.19$, DF = 9, $p = .9$; AICc = 9.55) indicates that the model is a good fit. This was followed with a whole model F test.

Results

Establishment of ID Throughout Gestational and Postnatal Life Using a Nutritional Model

We measured seizure threshold using the seizurogenic drug PTZ on two dietary models of ID. The first model was a gestational model of ID (gID) where body iron was deficient during gestation but normal after birth, and the second was an acute model of iron deficiency (pnID)

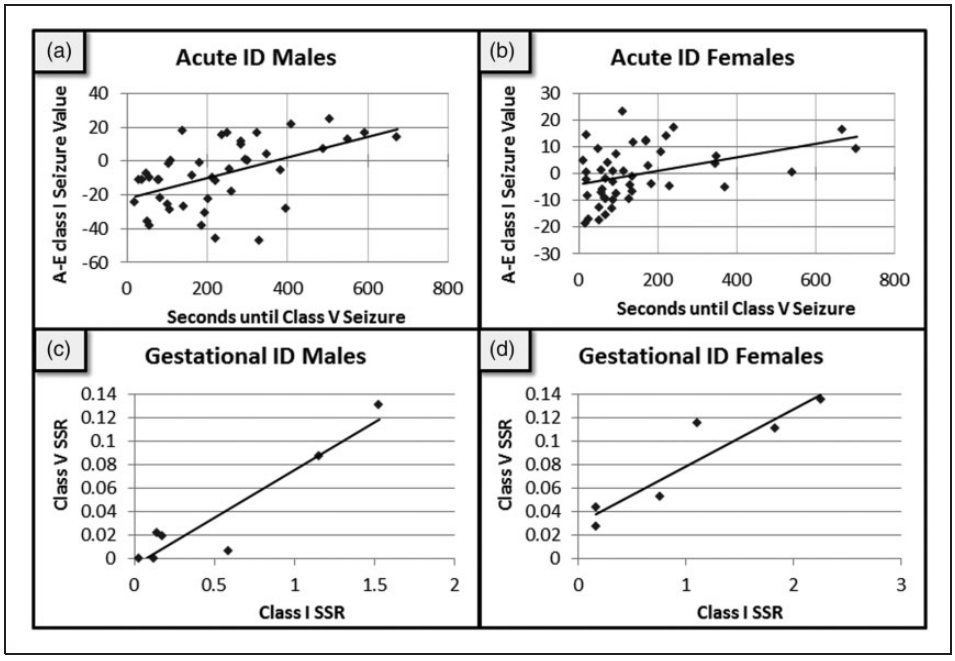

Figure 3. Correlation between seizure types. Pearson correlation between "deviation from expected Class I seizure score (A-E)" and "seconds until Class V seizure" in (a) pnID males ($r = .51$, $n = 43$, $p = .0005$) and (b) pnID females ($r = .39$, $n = 45$, $p = .0074$). Positive Y values represent greater than expected Class I seizure score; negative Y values represent less than expected Class I seizure score. Points represent pool of days on which pnID animals had exactly one Class V seizure. Correlation analysis between the Class V SSR and the Class I seizure susceptibility rating of individual animals in (c) gID males ($r = .94$, $n = 7$, $p = .0016$) and (d) gID females ($r = .93$, $n = 6$, $p = .008$). SSR = seizure susceptibility rating.

where iron was normal during gestation but became progressively more deficient during postnatal life (Figure 1(a)). In the gID model, embryos showed significantly decreased brain tissue ferritin (the iron storage protein) by embryonic Day 12 (E12) but did not show a significant difference 2 days earlier at E10 (Figure 1(c) and (e)). Maternal hematocrit was lower in the gID group but did not reach borderline anemia until gestational Day 16 (E16) and remained in the upper anemic range until birth. Anemic hematocrit range is shown by red shading in Figure 1(b) and (d). Anemia was defined as a hematocrit more than two standard deviations below the control population mean (Raabe et al., 2011) during adulthood (older than P35), pregnancy, or early life (P0–P21). Since fetal brain tissue ferritin was significantly low at least 4 days before maternal hematocrit indicated mild anemia, it suggests that the fetus can be ID before maternal hematocrit indicates a problem. Furthermore, in the ID group, maternal hematocrit at birth was $29 \pm 5\%$ whereas fetal hematocrit at birth was $15 \pm 1\%$; a comparison of this with the control group, where maternal hematocrit was $41 \pm 1\%$ and fetal hematocrit at birth was $43 \pm 3\%$, would suggest that when iron is limited during pregnancy, the fetus can be more iron deficient than the dam. After birth, the gID group was switched to an iron-normal diet resulting in normalized hematocrit levels by P42 (Figure 1(b) and (d)), which was the age of the initiation of PTZ seizure testing. Consistent with the hematocrit data, brain tissue ferritin was no longer significantly reduced by P42 (Figure 1(c) and (e) and Supplemental Figure S3). Therefore, the window of ID in the gID model began around E12 and ended prior to the start of PTZ seizure testing.

In the second dietary model, the pnID model, embryos were iron normal throughout gestation but became progressively more iron deficient after birth, reaching a level of severe IDA ($10 \pm 4\%$ hematocrit) at the start of PTZ seizure testing (Figure 1(d) and Supplemental Figure S3). Taken together, our pnID model is a model of postnatal ID which is ongoing, whereas our gID model is a model of early life ID which has resolved.

gID and pnID Have Different Effects on PTZ-Induced Seizures

Kindling response to the seizurogenic drug PTZ was measured across a total of 15 days, and a seizure susceptibility rating (SSR) was calculated for each mouse (as described in Supplemental Figure S1). We found that seizure response depends on the diet model (pnID vs. gID) as well as sex and seizure type. The pnID group had significantly more Class I seizures than either gID (post hoc Tukey HSD, $p = .0001$) or controls (post hoc Tukey HSD, $p = .0013$; Figure 2(a) and (b)). We also found

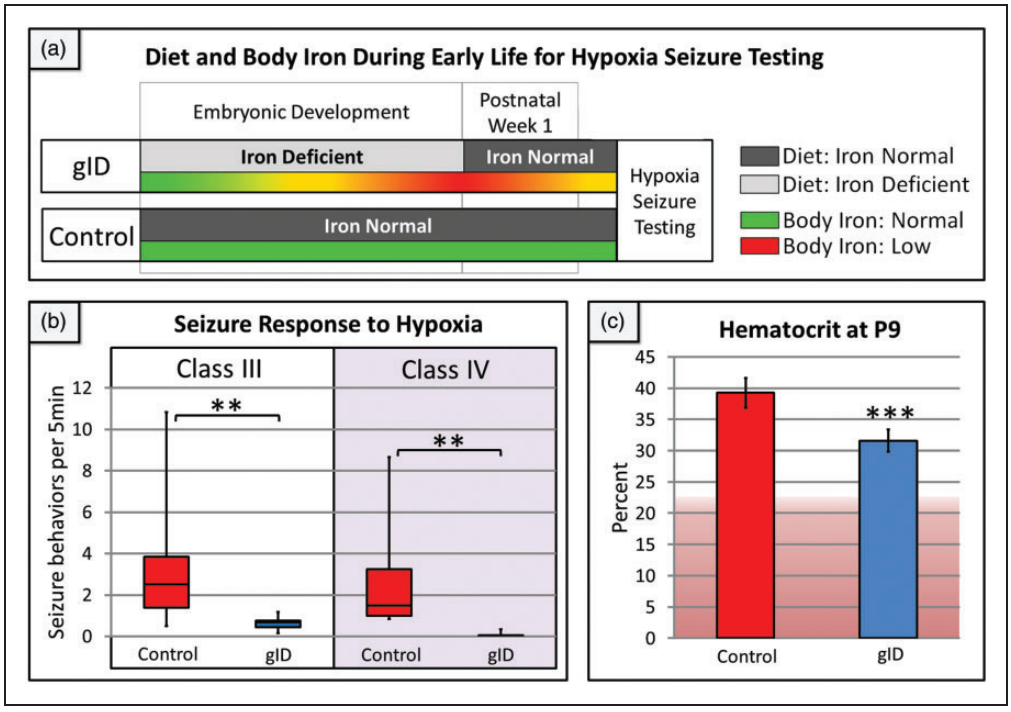

Figure 4. Course of iron deficiency and hypoxia induced seizures. (a) Outline of diet and concurrent iron status for hypoxia experiments: for the gID cohort, animals were exposed to ID during gestation, and dams were switched to an iron-normal diet on the day of birth to allow maximum time for iron replenishment in the suckling pups. The control group always received an iron-normal diet. (b) Quantification of seizure behavior during hypoxia using a modified Morrison scale, where values represent the average seizure count per 5-min interval from 6%, 5.5%, and 5% oxygen, for Class III or Class IV behaviors. Statistics: Class III (Student's *t* test, $^{**}p < .005$) and Class IV (Whole model *F* test, $^{**}p < .005$). (c) Hematocrit for gID and control groups at the time of hypoxia testing. Red shading represents anemic hematocrit range during early life. Statistics: Student's *t* test $p < .001$. Animal numbers: control group = 8 animals; gID group = 8 animals. gID = gestational iron deficiency.

that males within the pnID group were more sensitive to Class I seizures than any other group, including pnID females (post hoc Student's *t* test, $p = .0193$; Supplemental Figure S2A). In contrast, both pnID (post hoc Tukey HSD, $p = .0351$) and gID (post hoc Tukey HSD, $p = .0435$) groups had significantly fewer Class V seizures than the control group (Figure 2(c) and (d)), and males were significantly less susceptible to Class V seizures than females (one-way analysis of variance, $F(1,1) = 14.51$, $p = .0004$; Supplemental Figure S2C). In agreement with the Class V SSRs, using an effect likelihood ratio test, we found that males remained seizure-free for significantly more days of PTZ injection than females (Figure 2(f); DF = 1, $\chi^2 = 19.36$, $p = .0001$), and that gID and pnID groups remained seizure-free (Class V type) for significantly more days of PTZ administration than controls (Figure 2(e); DF = 2, $\chi^2 = 13.46$, $p = .0012$). In addition, a Cox proportional hazards model indicated that both pnID (risk ratio = .35, $p = .0057$) and gID (risk ratio = .32, $p = .0042$) groups had a reduced risk of seizure onset each day when compared to controls. In summary, gID animals tended to have the fewest of any seizure type (Supplemental Figure S2A and S2B); pnID animals had many Class I seizures but few Class V seizures; males were less sensitive to Class V seizures than females; and in the pnID group specifically, males were more sensitive to Class I seizures than females.

The Relationship Between Seizure Types Is Cohort Specific

During PTZ seizure testing, we noted that there appeared to be an inverse relationship between Class I and Class V seizure types in the pnID group, such that many Class I seizures seemed to delay or completely hinder development of the Class V type. To examine this relationship, we pooled all of the days on which a pnID animal had only one Class V seizure and determined whether there was a correlation between the time (in seconds) until onset of that Class V seizure and the amount of deviation between the actual and expected (A-E) Class I seizure count (A-E score is a measure of the degree of positive or negative deviation from the expected daily value). As shown in Figure 3(a) and (b), a Pearson product–moment correlation found a significant positive correlation between these two variables in pnID males ($r = .51$, $n = 43$, $p = .0005$) and in pnID females ($r = .39$,

$n = 45$, $p = .0074$). We saw a weaker correlation in control males ($r = .30$, $p = .0258$), and no correlation whatsoever in control females ($r = .04$, $p = .7983$), gID females ($r = .03$, $p = .8483$), and gID males ($r = .05$, $p = .8339$), suggesting that the inverse relationship between the two seizure types was most pronounced in the pnID dietary model.

We next examined whether specific animals were more or less resistant in general to seizures, by analyzing the correlation between the Class I and Class V SSRs of each individual animal. A pairwise comparison (using Pearson product–moment correlation) showed a very strong positive correlation between Class I and Class V SSRs in both gID males ($r = .94$, $n = 7$, $p = .0016$) and gID females ($r = .93$, $n = 6$, $p = .008$; Figure 3(c) and (d)), but no significant correlation in control males ($r = .59$, $n = 9$, $p = .0944$), control females ($r = .31$, $n = 8$, $p = .4547$), pnID males ($r = .24$, $n = 11$, $p = .4785$), or pnID females ($r = .28$, $n = 9$, $p = .4725$). These data suggest that there are varying levels of overall seizure resistance in individuals from the gID group, but that in the pnID and control groups, an individual who was resistant to one seizure type was not necessarily resistant to the other seizure type.

gID Renders the Offspring Less Sensitive to Hypoxia-Induced Seizures

To examine whether the increased seizure threshold seen in gID animals was specific to seizures induced by PTZ, we used a hypoxia model to test seizure threshold (Figure 4(a)). This model has been shown to elicit seizures in P9 mouse pups in a non-drug-induced manner (Rakhade et al., 2012; Sun et al., 2016), and seizure behaviors that are rated using a modified Morrison scale have been validated using electrophysiological readouts (Rodriguez-Alvarez et al., 2015). Moreover, a hypoxia model is an acute measure of seizure threshold rather than a measure of kindling response, in contrast with the PTZ model. It is also important to note that hypoxia will only induce seizures in young animals, which limits the time of postnatal iron supplementation. As shown in Figure 4(c), gID pups that were iron-supplemented on the day of birth had suboptimal, though not anemic, hematocrit levels by P9 (anemia was defined as hematocrit more than 2 *SD* below the mean of control population during early postnatal growth—P0 to P21). We found, consistent with the PTZ data, that gID animals had a significantly increased seizure threshold for both Class III (Student's t test $t(14) = -3.42$, $p = .0041$) and Class IV (modeled using binomial distribution and link logit: whole model F test $\chi^2 = 8.47$; $DF = 1$; $p = .0036$) seizure types, despite the mild and ongoing ID. Scores from each animal represent the average response per 5-min interval from 6%, 5.5%, and 5% oxygen conditions. We did not see a significant difference between seizure sensitivity at 6%, 5.5%, or 5% oxygen in either control or ID group; and under atmospheric oxygen, neither diet group had a seizure score which was significantly different from zero, for either seizure class. Unfortunately, due to the limitations of a nutritional model, we were unable to induce pnID within 9 days (pnID group) for hypoxia testing, and we were also unable to test gID with ongoing ID in older animals, due to a 100% mortality rate prior to weaning. Despite these limitations, our data would suggest that gID increases the seizure threshold even in the presence of mild ongoing ID and that this change in seizure threshold is not specific to the seizurogenic drug PTZ.

Discussion

We found that the effect of ID on seizure susceptibility depends on whether animals were postnatally iron deficient (pnID) or gestationally iron deficient (gID), and that this response was influenced by sex and seizure type. In the drug-induced model of seizure kindling, we used PTZ (a seizurogenic drug that is thought to decrease the effectiveness of the inhibitory neurotransmitter GABA; Huang et al., 2001) to measure seizure threshold.

We found that pnID increased sensitivity to Class I seizures but paradoxically also decreased sensitivity to Class V seizures. This decreased sensitivity, however, may not be directly caused by ID as we noticed that on days when an animal experienced more than the expected number of Class I seizures, it took longer to have the Class V seizure; conversely, animals experienced fewer Class I seizures on days when the Class V seizure happened quickly (Figure 3(a) and (b)). The significant correlation between the ''time that elapsed between drug injection and the start of Class V seizure'' and the ''deviation from expected value of Class I''(A-E value) supports the hypothesis that Class I seizures prevent the onset of Class V type seizures. Therefore, we suggest that the increased sensitivity to Class I seizures is a response to pnID, while the resistance to Class V seizures may be a result of the increased incidence of the Class I type.

In contrast to pnID animals, gestationally ID (gID) animals did not show an increased sensitivity to either Class I or Class V seizures in response to PTZ. In fact, gID animals had significantly lower Class V SSR, took significantly more time to have the first Class V seizure, and tended to have fewer seizures of any type. Moreover, in gID animals, there was no correlation between ''time to onset of Class V'' and ''deviation from expected Class I'' which suggests that the relative resistance to development of Class V seizures was not due to interference from other seizure types. We found, however, a very strong correlation between Class I and Class V SSRs in individual gID animals, such that an animal which was resistant

to Class I seizures tended also to be resistant to Class V, and likewise an animal which was more sensitive to Class I seizures tended also to be more sensitive to Class V (Figure 3(c) and (d)). This suggests that whatever is increasing, the seizure threshold is affecting all seizure types in the same way.

We also tested the seizure threshold of gID mice at an earlier (P9) time point using hypoxia, a seizure model which is thought to affect glutamate rather than GABA, and does not involve drug metabolism. We found that, once again, gID decreased overall seizure susceptibility compared to control. It is noteworthy that the decreased seizure susceptibility in the gID hypoxia group occurred despite the fact that animals were not yet fully iron replete at the time of testing. This suggests that exposure to ID during pregnancy was the defining factor responsible for the increased seizure threshold, and that it occurred irrespective of whether the acute iron status was normal or significantly low.

Given the disparate response to seizurogenic insults of the pnID and gID animals, we suggest that the disagreement in the literature surrounding whether ID increases, decreases, or is not associated with febrile seizure risk in humans, may be due to a similar effect of gID and pnID in humans. We found that ID can be associated with either increased or decreased seizure risk depending on the time of insult and seizure type, and we found that sex plays a modulatory role. Our data suggest that current iron status is not necessarily a reliable predictor of how seizure threshold will be affected. Gestationally ID animals showed an inhibited response to PTZ and hypoxia and an increased seizure threshold even in the presence of ongoing ID. Conversely, pnID animals that were anemic at the time of testing exhibited a decreased seizure threshold and increased seizure activity. It is therefore reasonable to postulate that, with respect to human studies, individuals who experienced ID during gestation might show increased seizure thresholds and therefore a decrease in seizure incidences; in contrast, individuals who were iron normal during pregnancy and became acutely anemic after birth might present with a decreased seizure threshold and thus an increase in the probability of developing seizures. In humans, it is mostly unknown whether the fetus was exposed to ID and at what stage, particularly in the case of mild anemia (as is seen in our animal model). The lack of such information makes it thus nearly impossible to predict seizure susceptibility in human populations with an unknown history of ID. In addition, the blunted response to PTZ we saw in gID animals persisted long after iron levels had returned to normal, meaning that if acute iron status is the only information available for epidemiological studies, gID and control groups would not be separable, and seizure threshold would be artificially increased in the control group. The lack of knowledge about gestational iron status has been acknowledged as a shortcoming of many human studies; however, recently it has been proposed that maternal ID may serve a protective role in seizure development in offspring (based on data from areas with high or low endemic ID) (Papageorgiou et al., 2015; Koksal et al., 2016). Our results seem to support this hypothesis, although we strongly caution against viewing gID as a preventative measure against seizures or epilepsy since gestational and early life ID, with or without iron supplementation, are known to be associated with an increased risk of behavioral problems (Lozoff et al., 2000; East et al., 2017), learning difficulties (Hurtado et al., 1999), and psychiatric disorders (Chen et al., 2013).

Seizure susceptibility has been reported to vary by sex and seizure type (Thomas, 1990; Hosseini et al., 2013; Dai et al., 2014; Scharfman and MacLusky, 2014), a finding we also confirmed in our work. We found that females were significantly more susceptible to Class V seizures than males irrespective of pnID or gID, having both decreased time to first seizure and more overall seizures when kindled. In contrast, and consistent with increased risk of febrile seizures in ID male children (Sadeghzadeh et al., 2012), we saw in our experiments that pnID male mice were significantly more susceptible to Class I seizures ($p = .0193$ post hoc Student's t test) than pnID females. Moreover, analysis of studies published between 2009 and 2016 on ID and febrile seizure risk revealed that males make up 58% of cases, whereas females only represent 42% of seizure cases (1142 males to 822 females) (Bidabadi and Mashouf, 2009; Hartfield et al., 2009; Idro et al., 2010; Ozaydin et al., 2012; Sadeghzadeh et al., 2012; Zareifar et al., 2012; Amouian et al., 2013; Fallah et al., 2013; Fallah et al., 2014; Ghasemi et al., 2014; Papageorgiou et al., 2015; Sharif et al., 2015; Koksal et al., 2016), suggesting that even studies which did not find a significant sex difference, with regard to febrile seizure risk, still had an overrepresentation of males in the cases.

Our studies on seizure susceptibility in the context of the different ID models also provide a new insight into the possible underlying cellular mechanisms of pnID versus gID. ID anemia is known to affect neurotransmitter synthesis (Coe et al., 2009) and increased seizure susceptibility could reflect these changes (Pfeiffer and Unyi, 1973). In contrast, gID affects the embryonic brain at a time when neuronal progenitors are being specified, undergoing maturation, and migrating to their final positions. Recent evidence suggests that changes in cell composition, integration, and firing pattern within specific neural circuits can have a large effect on E/I balance and thus seizure susceptibility (Coulter et al., 2011). The increased seizure threshold we observed in gID animals, which persists even after postnatal iron-supplementation, suggests that early life ID disrupts the E/I balance

of the brain toward a state of increased inhibitory or decreased excitatory synaptic tone. Incidentally, in humans, a number of the psychiatric disorders that have been associated with early life ID are also thought to affect the E/I balance of the brain. Thus, our animal model demonstrates that there may be a mechanistic link between early life ID and a disruption in the E/I balance which contributes to an increased risk for the development of certain psychiatric disorders in humans. It will be important to characterize the changes within the glutamatergic neurons or GABAergic interneurons which are born during the window of ID and ultimately contribute to the establishment of an appropriate E/I balance in the adult brain.

There are many advantages to using a nutritional model to induce ID. Most importantly, the majority of human ID cases are caused by a nutritional deficiency, making this the most relevant model to humans. In addition, a nutritional model creates a deficiency in iron alone, compared to repeated blood draws or the use of certain iron chelators, which may lead to a deficiency of multiple blood constituents or divalent metals. Moreover, a nutritional model does not require the injection of a chelator which may have adverse effects on the fetal brain which are independent of ID. Despite its strengths, a nutritional model is not well suited to defining which developmental window(s) are specifically disrupted by early life ID because of the relatively large amount of time required to change an animal's iron status. During pregnancy, in our model, it took at least 12 days to see the first signs of ID in embryos, even if the dam is fed ID diet for months in advance (using a diet which is the most severely iron restricted diet currently available, containing 2–6 ppm iron). Resolution of ID is equally gradual, in our case, taking approximately 4 weeks for postnatal pups to once again become iron sufficient. Thus, ID cannot be turned on and off at will in a nutritional model, but rather develops slowly and persistently throughout the course of pregnancy or growth and is reversed in an equally gradual manner. The relatively short gestation time for a mouse (approximately 19 days) and the comparatively long time it takes to induce and then ameliorate ID in mice (approximately 6 weeks) limited this analysis to only three groups: control, gID, and pnID (one group which remains iron normal, one which is ID during development but does not remain ID, and one which gradually becomes ID in the weeks leading up to seizure testing). It should also be noted that a group which is ID through both gestation and postnatal life could not be tested, since the mortality rate for non-iron-supplemented offspring reaches 100% prior to weaning. Thus, though a nutritional model is not useful for further refining the precise developmental windows where ID is affecting neurodevelopment, it is very useful for establishing that gID has a markedly different effect on seizure threshold than pnID, and this is precisely what we found.

Conclusion

Our work shows that pnID and gID have distinct and, in certain seizure types, opposite effects on seizure threshold. For this reason, iron status during early life must be known to accurately estimate seizure risk. Moreover, gID is known to be associated with complex psychiatric disorders that are characterized by a lasting impact on the excitatory and inhibitory balance of the brain. As the response to seizurogenic insults is also modulated by E/I balance and is disrupted in gID animals despite normal iron levels at the time of testing, it is likely that gID affects cellular components early in development that contribute to the establishment of the E/I balance later in life.

Summary

Gestational ID is known to be associated with complex psychiatric disorders that are characterized by a lasting impact on the excitatory and inhibitory balance of the brain. As the response to seizurogenic insults is also modulated by E/I balance, we tested the seizure threshold of ID animals and found that the time of insult (gestational or postnatal) defines seizure threshold.

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Supplementary Material

Supplementary material is available for this article online.

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