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Prenatal Protein Malnutrition Decreases *KCNJ3* and 2DG Activity in Rat Prefrontal Cortex

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Abstract

Prenatal protein malnutrition (PPM) in rats causes enduring changes in brain and behavior including increased cognitive rigidity and decreased inhibitory control. A preliminary gene microarray screen of PPM rat prefrontal cortex (PFC) identified alterations in *KCNJ3* (*GIRK1*/*Kir3.1*), a gene important for regulating neuronal excitability. Follow-up with polymerase chain reaction and Western blot showed decreased *KCNJ3* expression in PFC, but not hippocampus or brainstem. To verify localization of the effect to the PFC, baseline regional brain activity was assessed with ¹⁴C-2-deoxyglucose. Results showed decreased activation in PFC but not hippocampus. Together these findings point to the unique vulnerability of the PFC to the nutritional insult during early brain development, with enduring effects in adulthood on *KCNJ3* expression and baseline metabolic activity.

Keywords

Prenatal Protein Malnutrition; Prefrontal Cortex; *KCNJ3*; *GIRK1*; Brain Activity; 2-deoxyglucose

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There is strong evidence that exposure to perinatal malnutrition in humans is linked to cognitive impairment and to behavioral and neuropsychiatric disorders later in life, including an increased prevalence of attention deficits, antisocial personality disorders, and schizophrenia (Susser et al., 1996; St Clair et al., 2005; Galler et al., 2012; Galler et al., 2013; Susser and St Clair, 2013). Studies in a rat model of prenatal protein malnutrition (PPM) have shown behavioral, anatomical, morphological and neurophysiological deficits in the adult rat, even after postnatal nutritional rehabilitation (Tonkiss and Galler, 1990; Galler et al., 1996; Morgane et al., 2003). In the hippocampus, PPM leads to neurophysiological and neuroanatomical changes that increase neuronal inhibition (Chang et al., 2003; Lister et al., 2011). In the prefrontal cortex (PFC), PPM leads to alterations in neurotransmitter release (Mokler et al., 2007) and in immediate early gene activation in response to stress (Rosene et al., 2004). Behaviorally, PPM rats have decreased inhibitory control and are more cognitively rigid, two behavioral domains that critically depend on PFC circuitry (McGaughy et al., 2014).

While these long lasting behavioral and neurological deficits have been well documented, little is known about how molecular mechanisms are affected by PPM. To screen for genes that might be altered by PPM, we used a high-density microchip microarray (Galler et al., unpublished results) to compare gene expression in adult rats that had undergone PPM and were challenged with restraint stress to well-nourished controls. This screen identified numerous genes that were up- or down-regulated in the PFC following PPM. (See Table 1 for the genes with highest change). Of these, based on its robust level of change and potential functional importance for inhibitory processes, the gene *KCNJ3* was selected for follow-up investigation. *KCNJ3* is an inwardly-rectifying potassium channel, subfamily J, member 3, also known as GIRK1 and Kir3.1. It belongs to a G protein-gated inwardly rectifying potassium (GIRK) channel family that plays an important role in controlling neuronal excitability by hyperpolarizing the membrane and generating slow post synaptic inhibitory potentials (Hibino et al., 2010). GIRK channels are formed by differential multimerization among the four subunits expressed in mammals (GIRK1–GIRK4), of which only the first three are commonly expressed in the brain (Lusher and Slesinger, 2010). GIRK *loss-of-function* can lead to excessive neuronal excitation, such as in epilepsy (D'Adamo et al., 2013), whereas *gain-of-function* can substantially reduce neuronal activity, such as when GIRK2 is triplicated in a mouse model of Down syndrome (Best et al., 2007). In humans, alterations in expression levels in *KCNJ3* gene have been linked to schizophrenia (Yamada et al., 2011, 2012), epilepsy (Chioza et al., 2002; Lucarini et al., 2007), developmental delays and language impairment (Newbury et al., 2009), and mental retardation in children (Poot et al., 2010).

This study investigated the effect of PPM on *KCNJ3* using quantitative polymerase reaction to quantify the gene expression change and Western blot analysis to verify effects on protein levels. To localize the effect of prenatal malnutrition on brain function, 2-deoxyglucose (2DG) was used as a measure of resting state neuronal activity.

EXPERIMENTAL PROCEDURES

Subjects and Nutritional Treatment

Subjects were male Long-Evan rats derived from two breedings (designated A and B). Adult male and female rats were obtained from Charles River, Wilmington, MA. Nutritional manipulation followed previously published methods (Tonkiss and Galler, 1990). In brief, and as summarized in Figure 1, five weeks prior to mating, nulliparous females were randomized into two groups and placed on one of two isocaloric diets: an adequate protein diet (25% casein) or a low protein diet (6% casein) supplemented by L-methionine (Teklad Laboratories, Madison, WI). Females were mated with males that had been acclimated to these respective diets for one week prior to mating. Dams were maintained on the respective diets throughout pregnancy. Following parturition, litters from both nutritional groups were culled to eight pups (2 females and 6 males) and fostered within 24 hrs of birth as whole litters to dams that had received the 25% casein diet and had given birth within the same 24 hr period. This effectively institutes nutritional rehabilitation for the pups from mothers on the 6% casein diet. For all experiments we used only one pup from each litter to avoid litter effects. Thus, the “n” in each experiment represents both the number of subjects and litters.

Pups born to mothers on the 6% casein diet and fostered to mothers on 25% casein are designated the 6/25 group, while pups born to mothers on a 25% casein diet are designated as the 25/25 group. At day 21, all rats were weaned and placed on a standard laboratory chow diet (Purina Mills Inc., Richmond, IN; Formula 5001). The animal quarters were maintained at a temperature of 23°C ($\pm$ 2°) and at 35–65% humidity on a reverse 12h day/night (8:00–20:00 dark) cycle with red fluorescent lighting during the dark portion providing dim illumination. All procedures were approved by the University of New England Institutional Animal Care and Use Committee (20101005MOK) in accordance with guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Policies on the Use of Animals and Humans in Neuroscience Research.

Euthanasia and Sample Preparation - Fresh Tissue

For tissue harvest of fresh brain samples, animals were deeply anesthetized with CO₂ followed by decapitation. The brains were rapidly removed from the skull and placed on a chilled glass plate. The olfactory bulbs were removed and the hemispheres separated along the median longitudinal fissure and divided into four pieces each: PFC, hippocampus, brain stem/basal ganglia and remaining cortex. The PFC block was dissected coronally just anterior to the corpus callosum and included anterior cingulate, medial and orbital prefrontal cortex and the frontal pole cortex along with some of the head of the caudate. The hippocampus was dissected by cutting the fornix rostrally at the septum and then “unrolling” and separating it as a single piece from the adjacent corpus callosum (dorsal hippocampus) and entorhinal cortices (ventral hippocampus). The basal ganglia and brainstem were then separated from the remaining cortex as one piece. Dissected pieces were immediately frozen on dry-ice and stored at –80°C until processed.

Quantitative real-time polymerase chain reaction (q-PCR)

To test the effect of nutrition on *KCNJ3* expression, RNA from the PFC of 25/25 and 6/25 rats (left hemisphere) was isolated with the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. On column digestion was performed to remove traces of contaminating genomic DNA. cDNA was generated using Superscript II Reverse Transcriptase Kit (Qiagen, Valencia, CA, USA) and random hexamer primers. For *KCNJ3* q-PCR, cDNA was amplified in a total reaction volume of 20 μ l using a SYBR green master mix with 1 μ l of forward and 1 μ l of reverse primers and 1 μ l of the cDNA sample (dilution 1:10). The following specific primers for *KCNJ3* were used: forward (5'-3'): CGAGACCCTCATGTTTAGCGA; reverse (5'-3'): ATTTGAGCAGCTTGCAGCG. Triplicates of each sample were measured and analyzed on an ABI 7500 sequencer (Applied Biosystems, Foster City, CA, USA) using standard amplification conditions set by the manufacturer. Data from q-PCR was analyzed using the comparative $2^{-C(T)}$ method (Livak and Smitten, 2001). *Hprt* and *18s* were used as normalization controls. The following primers were used: *Hprt*: forward (5'-3'): GTTCTTTGCTGACCTGCTGGA; reverse (5'-3'): TCCCCGTTGACTGATCATT and *18s*: forward (5'-3'): CATGGCCGTTCTTAGTTGGT; reverse (5'-3'): GAACGCCACTTGTCCCTCTA. The mRNA levels of the 6/25 rats were expressed as percentage of the mean value of the 25/25 control group. Q-PCR products were controlled by SYBR-Green-based melting curve analysis followed by gel electrophoresis and Sanger sequencing to verify specificity of amplification.

Quantitative Western Blot

Protein lysates were obtained by tissue homogenization in RIPA buffer of the PFC from the right hemisphere of the same animals used in the q-PCR study. Protein concentration was determined according to manufacturer's instructions using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Equal amounts of protein (100 μ g) were denatured in Laemmli buffer at 100°C for 10 min and separated by electrophoresis on 4–15% gradient gel followed by transfer to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were blocked with 5% blotting-grade blocker (Bio-Rad, Hercules, CA, USA) in Tris buffered saline containing 0.1% Tween-20 (T-S-T) at 4°C overnight and incubated for 1 hour at room temperature with the following antibodies: rabbit anti-*KCNJ3* (1:1000, Novus Biologicals, Littleton, CO, USA Cat# NBP1-19393) and mouse anti- β -tubulin III (1:2500, Covance, Princeton, NJ, USA Cat# MMS-435P) as a loading control. After washing in T-S-T, membranes were incubated for 1 hour with HRP-conjugated secondary antibodies (1:20000 goat anti-rabbit for *KCNJ3* or 1:5000 goat anti-mouse for β -tubulin III). Blots were developed by chemiluminescence using Super Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Imaging and quantification were done by band densitometry using Versa Doc Imaging System in conjunction with the Quantity One software (Bio-Rad). Optical densities for *KCNJ3* proteins were normalized by the optical densities for the loading control β -tubulin III.

Euthanasia and Sample Preparation - Metabolic Mapping with ^{14}C -2-deoxyglucose (2DG)

To evaluate baseline brain activity, littermates of rats included in the molecular experiments (Breeding A) were injected with the metabolic activity marker 2DG (100 μ Ci/Kg i.p.;

specific activity=390mCi/mmol; VWR/GE Healthsource, Radnor, PA). After 45 minutes in their home cage, animals were deeply anesthetized (pentobarbital; 65 mg/kg) and perfused through the heart with 250 ml of fixative (2% paraformaldehyde and 15% sucrose in 0.1M phosphate buffer, pH 7.4) for 5 minutes. The brains were removed, coated with albumin, frozen at -30°C in 2-methylbutane and stored at -80°C . Brains were later cut into 20 μm thick coronal sections and one out of every five sections was mounted on subbed cover slips and rapidly dried. Sections were batch-processed by affixing them to Bristol board, and apposing them to high-resolution X-ray film (Structurix, Agfa, Belgium) with ^{14}C microscopes (Amersham, Piscataway, NJ). Sections were exposed at -80°C for 10 days. Films were then developed, fixed, and digitized as previously described (Rushmore et al., 2006). Uptake of 2DG was assessed densitometrically in the PFC and hippocampus, and values were normalized to white matter (Sharp et al., 1983). The sampling regions were chosen to correspond to the PFC and hippocampal samples used in the molecular studies.

Data Analysis

All comparisons were done using standard parametric statistical methods with the significance set at $p = 0.05$. Subjects used in each experiment were unrelated as one rat per litter was randomly selected for each procedure as described above.

RESULTS

Body weight

The use of this model has consistently shown deficits in body weight at birth in the 6/25 group compared to controls. By weaning the weight deficit has declined but remains significant but, by the time the rats reach adulthood ($>P90$), the 6/25 rats catch up with the 25/25 and their weights are similar. (Galler and Tonkiss, 1998; Fischer et al., 2014). A student's t-test comparing the body weight of all subjects in breeding B ($n=8$ 25/25, $n=8$ 6/25) at the time of tissue harvest shows that there is no difference between the two nutritional groups ($t(14)=0.216$, $p=0.832$). The average weight on PND230 for 6/25 subjects was 536.7 ± 10.11 g (mean $\pm$ SEM) while 25/25 subjects weighed an average of 540.0 ± 11.6 g.

Q-PCR for *KCNJ3* Gene Expression

In a cohort of 8 adult males ($n=5$ 25/25, $n=3$ 6/25; Breeding A), results revealed a significant down-regulation of *KCNJ3* in the 6/25 rats relative to 25/25 controls (two-tailed t-test $t(6)=2.69$, $p=0.03$; Fig. 2A). A second cohort of 16 subjects ($n=8$ 25/25, $n=8$ 6/25; Breeding B) was used to replicate mRNA levels of *KCNJ3*. As observed in Breeding A, *KCNJ3* mRNA in the PFC was significantly down-regulated in the 6/25 rats compared to 25/25, confirming the effect of PPM on this gene (two-tailed t-test $t(13)=2.58$, $p=0.02$; Fig. 2B).

To investigate whether the down-regulation of *KCNJ3* secondary to PPM was generalized in the brain or specific to the PFC, q-PCR was performed in tissue samples of hippocampus and of brain stem/basal ganglia in the same Breeding A subjects as the PFC. As shown in Figure 2C&D, *KCNJ3* mRNA levels were not markedly altered in these regions (two-tailed

t-test $t(6)=1.13$; $p=0.30$ in the hippocampus and $t(6)=0.23$; $p=0.82$ in the brain stem/basal ganglia).

Western Blot for Protein Levels

To determine if the changes in gene expression resulted in altered protein levels of *KCNJ3*, we used the PFC samples (right hemispheres) of the same animals used for q-PCR (Breeding A) for Western blot analysis. As shown in Figure 2E&G, consistent with the q-PCR results, Western blot confirmed that protein levels of *KCNJ3* were significantly decreased in 6/25 rats (two-tailed t-test $t(6)=3.08$; $p=0.02$). This confirmed that *KCNJ3* gene expression leads to similar changes in protein.

Assessment of Metabolic Activity with 2DG

Ten naive adult rats ($n=5$ 25/25, $n=5$ 6/25; Breeding A) were injected with 2DG. Of the 10 subjects, 2 rats in the 25/25 group did not exhibit a radioactive brain signal and were excluded from the analysis, leaving 3 subjects in this group. A two-way ANOVA (Hemisphere [2 levels] X Region [2 levels]) revealed no inter-hemispheric difference ($F(1,24)=7.32e-005$; $p=0.993$), no significant interaction between hemisphere and region ($F(3,24)=0.017$; $p=0.996$), but a significant overall effect of region ($F(3,24)=17.29$; $p<0.0001$). Hence, data were collapsed across hemispheres for each region for a two-way ANOVA (Nutrition [2 levels] X Region [2 levels]). Results showed no overall effect of nutrition ($F(1,12)=1.524$; $p=0.240$), but there was a significant interaction between nutrition and region ($F(1,12)=10.02$; $p=0.008$) as well as an overall effect of region ($F(1,12)=20.47$; $p=0.0007$) and. We then ran post-hoc comparisons to determine which region differed between the two nutrition groups. Results revealed a significant decrease in 2DG uptake in the PFC in the 6/25 group (two-tailed t-test $t(6)=2.687$; $p=0.036$) (Figure 3B&C), but no change in the hippocampus (two-tailed t-test: $t(6)=1.681$; $p=0.143$) (Figure 3F&G). Data graphed are the mean metabolic activity collapsed across hemisphere for each region (Figure 3D&H).

DISCUSSION

Summary of Results

In order to explore the molecular basis of observed alterations in a variety of inhibitory processes affected by PPM, changes in the *KCNJ3* gene were investigated. Quantitative PCR confirmed that this gene was down regulated in the PFC but was unchanged in the hippocampus and brainstem. Western blot showed corresponding decreases in the protein levels of *KCNJ3* only in the PFC. To determine if this affected brain function, metabolic activation patterns were explored using 2DG imaging and showed reduced metabolic activity in the PFC but not in the hippocampus, suggesting that the down regulation of *KCNJ3* in the PFC may be associated with decreases in functional activity. The current study, however, does not show a causal relationship between the observed down regulation of *KCNJ3* and lower metabolic activity in the PFC of PPM rats. Overall, these results provide further evidence that PPM leads to alterations in neuronal wiring that persists into adulthood and may be part of the neural substrates of altered processes in the PFC.

KCNJ3, Behavior and Disease

KCNJ3 regulates membrane excitability and plays a role in synaptic plasticity and behavior (Luscher and Slesinger, 2010). In humans, the gene has been linked to several neurological diseases defined by imbalance in excitatory and inhibitory signalling, including epilepsy, Down syndrome and schizophrenia. The direct impact of *KCNJ3* on behavior has been evaluated by transgenic ablation of specific GIRK subunits in mice (Pravetoni and Wickman, 2008). *KCNJ3* knockout (*GIRK1*^{-/-}) mice showed several behavioral differences from the wild type (wt) mice, including elevated motor activity and delayed habituation in the open field test. Furthermore, *GIRK1*^{-/-} mice showed reduced anxiety-related behavior in the elevated plus maze compared to the wt mice. In rats, *KCNJ3* subunit knockdown (using antisense oligodeoxyribonucleotides injections) produced associative learning impairments (Kourrich et al., 2003). Interestingly, our previous studies using PPM rats, have reported lower anxiety and/or higher impulsivity on the elevated plus maze as compared with well-nourished control rats (Almeida et al., 1996).

It is important to consider that *KCNJ3* subunits alone do not form functional homomeric GIRK channels but need to dimerize with other subunits, usually *KCNJ6* (*GIRK2*), to form functional channels (reviewed in Wickman et al., 2002). Moreover, even when *KCNJ3* is completely ablated, *KCNJ6* can still form homomer GIRK channels (Lujan et al, 2014). Thus, even though PPM rats show some behavioral deficits similar to *KCNJ3* knockout rats, namely lower anxiety and higher impulsivity, PPM rats only present a decrease in *KCNJ3* mRNA and protein. However, we do not exclude the possibility that this reduction is part of a network of alterations produced by PPM and hence other changes might contribute to the behavioral deficits. Future studies are needed to determine how the behavioral phenotype in PPM is specifically related to the down-regulation of *KCNJ3*. Such studies would also need to examine the constellation of genes that showed highest changes in the microarray study such as Snap91, Plag1 and Homer1 (Table 1) which are pertinent to synaptic activity.

Published studies of the effects of prenatal malnutrition have identified other genes of interest. Among them, Reelin is reduced in the brains of adult rats whose mothers had diets deficient in methyl donors during pregnancy (Konycheva et al, 2011). In humans, *IGF2* is hypomethylated in adult offspring of mothers that were pregnant during the Dutch Hunger Winter of 1944–1945 (Heijmans et al, 2008). The exact mechanisms by which malnutrition affects the offspring are still not understood, but there is strong evidence that maternal nutrition can have long-lasting effect on gene expression, phenotype, and neurological diseases via epigenetic changes that alter chromatin structure and gene expression, such as histone modifications and DNA methylation (Kirkbride et al, 2012). DNA methylation, in particular, because of its direct link to metabolism, will be of particular interest for future studies in our PPM model.

Decreased metabolic activity in the PFC

Metabolic labelling with 2DG is a sensitive index of neural activity and has been previously used to show alterations in PFC activity in a rat model of attention deficit hyperactivity disorder (ADHD; Barbelivien et al., 2001). In our group, we have recently reported that PPM rats display cognitive rigidity in tests of attentional set shifting and reversal learning.

Same-sex littermates had decreased 2DG uptake in PFC regions critical to these forms of cognitive control (McGaughy et al., 2014). These data support the hypothesis that PPM may selectively diminish activity in the PFC, which then leads to impairments in executive function. The neuroanatomical basis of this hypofunction in the PFC is unclear. It is generally accepted that the 2DG signal predominantly reflects synaptic activity at the axonal terminals of neuronal pathways (Kadekaro et al., 1985). As a result, the decrease in activity in PPM rats could be due to a widespread alteration in PFC connectivity and function, or it could be a result of changes in specific inputs to or within the PFC. Since 2DG labelling makes no distinction between excitatory or inhibitory synapses, the observed decrease in activity could also reflect a population-specific dysfunction in cellular activation in the PFC, with either excitatory or inhibitory populations being more vulnerable to PPM.

However, in previous studies in this model, we observed alterations in a variety of inhibitory processes due to PPM, leading us to hypothesize that the inhibitory cells are functionally more affected by the malnutrition insult. It remains to be seen whether the down-regulation of *KCNJ3* is linked to the dampening of metabolic activity in the PFC of PPM rats but, if the observed decrease in *KCNJ3* occurred mainly on GABAergic interneurons, it would increase their excitability and hence increase inhibitory tone in the PFC, perhaps leading to net lower baseline activity in PFC. Such a selective effect on inhibitory interneurons and processes is congruent with a number of observations in this model of PPM. For example, electrophysiological studies in the hippocampus showed evidence of enhanced inhibition in granule cells of the dentate gyrus (Bronzino et al.; 1991, 1997). In these studies the authors reported a hyperactivation of GABAergic inhibitory interneurons in the dentate gyrus leading to a greater potentiation of inhibition in PPM rats. Future studies using cFos as a marker for activation and double labelling with cell type specific markers could test this hypothesis.

Decreased frontal lobe activation is also observed in human studies in children and adults with ADHD (Rubia et al., 1999; Cubillo et al., 2010) and in patients with schizophrenia (Mueser and McGurk, 2004). The mechanism of these decreases is not well understood, but they have been posited to have early life onset, possibly during the prenatal period (ADHD: Ottoboni and Ottoboni, 2003; Schizophrenia: Räikkönen, 2012). Regardless of mechanism, these observations suggest a unique vulnerability of the PFC to prenatal insults such as malnutrition.

The questions of when these changes occur and whether there are sex differences are also open. In a developmental study, Fernandez-Alacid et al (2011) reported that GIRK subunits, including *KCNJ3*, are already highly expressed in the rat brain at birth (P0), and progressively increase postnatally, suggesting that the gene dysregulation found here was likely established in the prenatal period and continued into adulthood. In this model of PPM, however, it is not known if the deficits observed here are already present at birth (P0) or if they develop postnatally. Also, while our current study only included male subjects, sex differences in behavior have previously been reported in this model (Almeida et al, 1996; Tonkiss et al, 1998). Thus, investigating these effects in females will be important in future studies.

Conclusions

This investigation reports on the down regulation of the expression of *KCNJ3* mRNA and protein in the PFC of adult rats that were exposed to PPM and nutritionally rehabilitated from birth onwards. In parallel, we also report a decrease in baseline PFC activity in the adult littermates from the *KCNJ3* study, though specific causality remains to be determined. Considering implications of our findings in PPM rats for humans, evidence shows that the offspring from the Dutch Hunger Winter and from the Chinese Famine have higher risk for schizophrenia in adulthood (Susser et al., 1996; St. Clair et al., 2005; Xu et al., 2009). There is also strong evidence linking schizophrenia to downregulation of *KCNJ3* in the PFC (Yamada et al., 2011, 2012) and to reduced activation in the PFC (Mueser and McGurk, 2004). Observations reported here of reductions of both *KCNJ3* levels and 2DG activity in the PFC support the hypothesis that the PFC is particularly vulnerable to this nutritional insult and might be linked to neuropsychiatric diseases observed in humans subjected to malnutrition *in utero*.

Acknowledgements

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Abbreviations

PPM	prenatal protein malnutrition
<i>KCNJ3</i>	inward-rectifying potassium channel, subfamily J, member 3
PFC	prefrontal cortex
2DG	2-deoxyglucose
GIRK	G protein-gated inwardly rectifying potassium channel
6/25	prenatally malnourished
25/25	well-nourished controls

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- Maternal malnutrition can have long-term effects on brain function of offspring
- Prenatal malnutrition reduces *KCNJ3* mRNA and protein expression in the prefrontal cortex of rats
- Prenatal malnutrition decreases metabolic activity in the prefrontal cortex of adult rats
- The prefrontal cortex is particularly vulnerable to the prenatal malnutrition insult

EXPERIMENTAL TIMELINE

Figure 1. Experimental timeline of rat model of prenatal protein malnutrition

This model was designed to assure that the malnutrition insult was restricted to the prenatal period. All pups were cross-fostered to lactating dams given the 25% protein diet prior to mating and throughout pregnancy. Pups born to dams provided the 6% protein diet and fostered to dams given the 25% protein diet were designated “6/25” (prenatally malnourished) rats. And pups born from and fostered to dams given the 25% protein diet were designated “25/25” (well-nourished) controls.

Figure 2. PPM causes down-regulation of *KCNJ3* in the PFC

(A&B): *KCNJ3* is significantly down-regulated in the PFC of 6/25 subjects (white bars) from two breedings as compared to 25/25 subjects (black bars). In contrast *KCNJ3* expression was unchanged in the (C) hippocampus ($p=0.30$) and (D) brain stem/basal ganglia ($p=0.82$) of the same breeding. (E) *KCNJ3* protein levels were significantly lower in 6/25 rat PFC compared to controls, consistent with mRNA levels in the same region. (F) Optical density (OD) for the loading control β -tubulin III was equal in the two nutritional groups. (G) Representative Western blots standardized against Beta-tubulin III as loading control. The first three lanes in the blots are representative of 6/25 animals (M1–M3) and the following five lanes of control animals (C1–C5). Cts and ODs are shown as percentage of 25/25 group. * p values shown by each graph when significant (two-tailed t-test). Data presented as Mean $\pm$ SEM.

Figure 3. PPM decreases 2DG activity in the PFC but not in the hippocampus

PFC (A) and hippocampus (E) were outlined according to Paxinos & Watson's *Atlas of the Rat Brain* (2007). Illustrative examples of 2DG uptake in prefrontal (PFC) area and hippocampus (Hipp) of 25/25 rats (B&F, respectively) and of 6/25 rats (C&G). Bar graphs depict the mean ($\pm$ SEM) 2DG uptake in the PFC (D) and in the hippocampus (H) of 25/25 (n=3, black bars) and 6/25, (n=5, white bars) rats. Each bar represents the regional 2DG uptake normalized to adjacent white matter. *p values shown by graph when significant (two-tailed t-test).

Table 1
mRNA species significantly downregulated (left) or upregulated (right) by behavioral stress in the PFC of rats: influence of PPM

Genes were selected on the significant alteration after stress. Statistically significant changes were assessed by ANOVA analysis and examination of false discovery rate.

Gene	Fold change (down)	Gene	Fold change (up)
<i>KCNJ3</i>	-5.8	<i>Plagl1</i>	1.9
<i>Snap91</i>	-1.7	<i>Mta1</i>	1.5
<i>RTIAw2</i>	-1.7	<i>Homer1</i>	1.4
<i>Rnpt4</i>	-1.5	<i>Par3</i>	1.3
<i>Pap3</i>	-1.5	<i>Rasd1</i>	1.3
<i>Vti</i>	-1.4	<i>Scg2</i>	1.3
<i>Opr1</i>	-1.4	<i>Unc13h3</i>	1.3
<i>Birc4</i>	-1.4		
<i>Ptpze</i>	-1.4		

Results obtained from a high-density microchip array (Genome 230A, Affymetrix, Inc., Santa Clara, CA). Subjects in this study were Sprague Dawley male rats age P350 (n=16, n=8 25/25, n=8 6/25) bred and nourished according to the same PPM model described here (Tonkiss and Galler, 1990). Behavioral challenge was conducted as described in Rosene et al. (2004).