Prenatal Maternal Stress from a Natural Disaster and Hippocampal Volumes: Gene-by-Environment Interactions in Young Adolescents

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Abstract

Gene-by-environment interactions influence brain development from conception to adulthood. In particular, prenatal brain development is a window of vulnerability to the interplay between environmental and genetic factors. Rodent and human research demonstrates that prenatal maternal stress (PNMS) alters hippocampal volumes. In Project Ice Storm, it has been demonstrated that increased levels of objective, but not subjective, PNMS are associated with increased right hippocampal volumes in early adolescent females. However, although PNMS affects hippocampal size on average, similar degrees of PNMS lead to different effects in different individuals. This differential susceptibility to the effects of PNMS may be due to genetic variants; however, the role of genes in moderating the effect of PNMS on the hippocampus remains unknown. Therefore, we investigated the role of genetic variants known as single nucleotide polymorphisms (SNPs). SNPs are differences in the DNA sequence that occur frequently across a population and confer phenotypic differences. Here we investigated six SNPs that are candidates to moderate the effects of PNMS on hippocampal volume: 5HT2A (rs6311), COMT (rs4680), CRHR1 (rs110402), GABRA6 (rs3219151), NR3C1 (rs41423247) and BDNF (rs6265). To investigate this, we assessed 53 subjects who were in utero during the January 1998 Quebec ice storm. The mothers had responded to questionnaires about their objective and subjective levels of stress from the ice storm in June 1998. When children were 11½ years old, T1-weighted structural magnetic resonance imaging (MRI) scans were obtained using a 3T scanner and analyzed to determine hippocampal volumes. We also collected and genotyped DNA from the children. Moderation analyses were conducted to determine whether any or all of the six SNPs moderate the effect of PNMS on hippocampal volumes. I

found that objective PNMS was associated with right hippocampal volume and the BDNF and COMT genotypes were associated with left hippocampal volume. In addition, SNPs located on COMT, 5HT2A, CRHR1 and NR3C1 moderated the effect of PNMS on hippocampal volume. Thus, I conclude that an individual's genotype alters their susceptibility to the effects of PNMS.

Resumé

Les interactions gène-environnement influencent le développement du cerveau de la conception à l'âge adulte. Plus particulièrement, la période du développement prénatal du cerveau est une fenêtre de vulnérabilité aux interactions entre les facteurs environnementaux et génétiques. La recherche sur les rats et les humains démontre que le stress maternel prénatal (SMP) altère les volumes hippocampiques. Dans le Projet Verglas, il a été démontré que l'augmentation du niveau de SMP objectif, mais pas subjectif, est associée à une augmentation des volumes de l'hippocampe droit chez les jeunes adolescentes. Cependant, bien que le SMP affecte la taille de l'hippocampe en moyenne, des degrés similaires de SMP engendrent des effets différents chez individus distincts. Cette susceptibilité différentielle aux du SMP pourrait être expliquée par les variantes génétiques; par contre, le rôle des gènes dans la modération des effets du SMP sur l'hippocampe reste inconnu à ce jour. De ce fait, nous avons étudié le rôle des variantes génétiques connues comme les polymorphismes nucléotidiques (SNPs, single-nucleotide polymorphisms). Les SNPs sont des différences dans la séquence d'ADN qui surviennent fréquemment au sein de la population et qui génèrent des différences phénotypiques. Nous avons étudié six SNPs qui sont des candidats pouvant modérer les effets du SMP sur le volume de l'hippocampe : HTR2A (rs6311), COMT (rs4680), CRHR1 (rs110402), GABRA6 (rs3219151), NR3C1 (rs41423247) et BDNF (rs6265). Pour ce faire, nous avons évalué 53 sujets du Projet Verglas dont les mères étaient enceintes pendant la tempête de verglas en Janvier 1998 au Québec. En juin 1998, les mères ont répondu à des questionnaires au sujet de leurs niveaux de stress objectif et subjectif vécus pendant la tempête de verglas. Lorsque les enfants étaient âgés de 11 ans et demi, des scans d'IRM pondérés en T1 ont été obtenus

en utilisant un scanneur 3T, puis analysés afin de déterminer les volumes hippocampiques. Nous avons aussi recueilli and génotypé l'ADN des enfants. Des analyses de modération ont été menées pour déterminer si au moins l'un des six SNPs modérait l'effet du SMP sur les volumes hippocampiques. Nous avons découvert que le SMP objectif était associé au volume de l'hippocampe droit et que les génotypes du facteur neurotrophique issu du cerveau (Brain-Derived Neurotrophic Factor, i.e. BDNF) et de la catéchol-O-méthyltransférase (COMT) étaient associés au volume de l'hippocampe gauche. De plus, les gènes COMT, 5HT2A, CRHR1 et NR3C1 modéraient l'effet du SMP sur le volume hippocampique. Ainsi, nous pouvons conclure que le génotype des individus altère leur sensibilité aux effets du SMP.

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Several other members of the King lab have also played a significant role in this project. In particular, Romane Dufoix analyzed the brain scans that were used for this project and generated the results that inspired the questions addressed below. David Laplante and Lei Cao contributed their expertise and advice and Guillaume Elgbeili kindly assisted with the statistics. In addition, I have greatly enjoyed working alongside the rest of the King lab and others at the Douglas Mental Health Institute: Erin Yong Ping, Sandra Lafortune, Aihua Liu, Teodora Mechetiuc, Alexandra Martinez, Julien Sénécal, Michael Verwey, Angelica Torres-Berrio, Matthew Pokinko and Lauren Reynolds. Finally, my committee members, Dr. Xiangfei Meng and Dr. Jens Pruessner, as well as my mentor, Dr. Lalit Srivastava contributed constructive criticism and valuable suggestions throughout this process.

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have made Montreal home. Lastly, but importantly, I must thank Jesus who I believe has made Science possible and meaningful in creating a coherent and intricate universe.

Preface and Contribution of Authors

Many years of work by many other people went into developing Project Ice Storm and obtaining the genetic and brain imaging data that I have used to write this thesis:

Dr. Suzanne King, my supervisor, developed Project Ice Storm in 1998 and has obtained funding for, and supervised, all of the evaluations of mothers and children since then.
Dr. King has been supported by her research associate, **Dr. David Laplante**, since 2000.
The "Ice Brain Study", which provided the data that were analyzed in this thesis, was funded by a grant to Dr. King from **The March of Dimes Foundation** (#12-FY07-263) entitled *The Effects of Prenatal Maternal Stress on Brain Morphology in 11 Year-Old Children: The Quebec 1998 Ice Storm Project* from 2007-2010, with co-investigators Alain Brunet, Tomas Paus, Jens Pruessner, and Deborah J. Walder.

Dr. Arnaud Charil was the post-doctoral fellow who prepared the protocol for MRI scans at the *Institut de Gériatrie de Montréal*, who supervised all scans, and was responsible for quality control.

Ms. Isabelle Bouchard recruited the subjects into the Ice Brain Study. Students Marjolaine Massé and Maria Papastergiu accompanied families and subjects through the scanning protocol.
Master's student, **Romane Dufoix**, conducted the manual segmentations of the hippocampus for all subjects.

List of Abbreviations

5HT – 5-hydroxytryptamine (serotonin)

5HT2A – 5-hydroxytryptamine (serotonin) receptor 2A

BDNF – brain-derived neurotropic factor

CA – cornu ammonis

COMT – catechol-O-methyltransferase

CRHR1 – corticotropin releasing hormone receptor 1

GABRA6 – gamma-aminobutyric acid A receptor

HPA – hypothalamus-adrenal-cortex

IES-R – impact of events scale-revised

LTP – long-term potentiation

MRI – magnetic resonance imaging

NR3C1 – nuclear receptor subfamily 3 group C member 1

PNMS – prenatal maternal stress

PTSD – post-traumatic stress disorder

SES – socioeconomic status

SNP – single nucleotide polymorphism

TIC – total intracranial

Introduction

The hippocampus is implicated in long-term memory formation, spatial mapping and regulation of anxiety. Given that hippocampal development begins in utero, prenatal events may alter hippocampal development and confer long-term effects on hippocampal structure and function. In particular, the effects of prenatal maternal stress (PNMS) on hippocampal development have been studied by randomly assigning a stressor to pregnant animals and observing the effects on the offspring (Charil et al. 2010). In rodents and primates, high levels of PNMS have been associated with reduced hippocampus volume (Uno et al. 1998; Schmitz et al. 2002; Coe et al. 2003). Animal studies, however, are unable to convey information about the relative roles of the mother's objective stress, subjective stress and cognitive appraisal. In contrast, while human research is able to tease apart the effects of objective, subjective and cognitive stress components, it often lacks random assignment of a stressor. Circumventing many of these methodological problems, natural disasters provide a unique opportunity to study the effects of a randomly assigned stressor on a human population. Accordingly, the laboratory of Suzanne King has studied the effects of the 1998 Quebec Ice Storm on brain development; a longitudinal study known as Project Ice Storm. Here it has been found that objective PNMS, but not subjective PNMS, predicts hippocampal volumes in early adolescent girls (Dufoix, unpublished). These effects were not observed in boys at the same age. Notably, there remains much variance to be explained: children with similar PNMS profiles exhibit different hippocampal volumes, suggesting that individuals are differentially susceptible to the effects of PNMS. Indeed, previous studies have demonstrated that certain genetic traits confer increased vulnerability to the effects of stress (Chen et al. 2015; Qiu et al. 2015). As a result, untested

gene-by-environment interactions may obscure important associations between PNMS and hippocampal volume. My objective is to increase understanding about the role of gene-by-environment interactions in hippocampal development using data from 53 Project Ice Storm children. More specifically, we will determine the extent to which genetic variants moderate the effects of PNMS on hippocampal volume in early adolescence.

Literature Review

1. Hippocampus

1.1 Hippocampal Function

The hippocampus, a brain region located in the medial temporal lobe, is mainly divided into the cornu ammonis (CA), the dentate gyrus and the subiculum. The hippocampus plays a key role in memory formation and learning (O'Keefe and Dostrovsky 1971; Squire 1992). In addition, this region has been implicated in spatial mapping and internalizing behaviours, such as anxiety and depression (O'Keefe and Dostrovsky 1971; McNaughton 1997; McNaughton and Gray 2000). Several neural circuits play important roles in hippocampal function. In particular, the hippocampus receives modulatory input from serotonin, norepinephrine and dopamine neurons, and consists of glutamatergic, GABAergic and cholinergic projections. Cholinergic neurons in the hippocampus are thought to mediate memory, while serotonergic neurons are implicated in mood disorders, anxiety, aggression and impulsivity (Messer 2002; Charil et al. 2010). Interestingly, the relationship between hippocampal function and hippocampal volume is age-dependent (Van Petten 2004). In children, adolescents and young adults, a smaller hippocampus is associated with increased performance in memory tasks; in contrast, some evidence suggests that older adults exhibit a positive correlation between hippocampal volume and memory although the data are difficult to interpret due to high variability between individuals.

1.2. Hippocampal Development

Hippocampal function in adulthood is affected by disturbances during brain development. In rodents and primates the hippocampus develops primarily prenatally, with the peak of neurogenesis in early gestation for primates and late gestation for rodents (Angevine 1965; Seress et al. 2001). During prenatal development, hippocampal neurons originate from the dorsomedial telencephalon, migrate to histologically distinct layers and form functional synapses. Although the hippocampus is largely organized prenatally, neurogenesis in the dentate gyrus, myelination and synaptic pruning continue into adulthood (Benes et al. 2000; Balu and Lucki 2009).

In accordance with this, changes in hippocampal structure and function are observed throughout life (Tottenham 2009; Yang et al. 2012). For example, at postnatal day 18, rodents are able to perform an amygdala-dependent task, to associate a cue with a paired shock, yet they remain unable to perform a hippocampus-dependent task, to associate a context with a paired shock (Rudy and Morledge 1994). Performance in the hippocampus-dependent task increases with age, indicating ongoing development of hippocampal function. Similarly, in children tested periodically between six months and two years of age, performance in a hippocampus-dependent memory task increases over time (Robinson and Pascalis 2004). Development of hippocampal function in early life is paralleled by changes in hippocampal structure. A study that assessed brain development in infants noted a mild increase in hippocampal volume during the first two years of life (Knickmeyer et al. 2008). In addition, a longitudinal study of hippocampal growth from age 4-25 years demonstrated a decrease in anterior hippocampal volume and an increase in posterior hippocampal volume across this period (Gogtay et al. 2006). Taken together,

these results demonstrate that, although hippocampal circuitry is primarily established prenatally, hippocampal structure and function continue to change from birth to adulthood.

During this protracted development period, environmental and genetic factors can influence hippocampal development, thereby inducing long-term effects on brain structure and function. In particular, given the substantial changes in hippocampal organization prenatally, environmental events that occur during fetal brain development have a profound effect on the hippocampus. Prenatal maternal stress (PNMS) is one such event that has been thoroughly researched for its effects on hippocampal development in animals.

2. Prenatal Maternal Stress (PNMS) Affects Hippocampus Development

2.1 Stress

High stress levels during pregnancy have adverse effects on the development of the child in utero (Mulder et al. 2002). Stress occurs when an individual encounters circumstances that require great or impossible adaptation. Potential stressors include divorce, death of a close friend or financial problems. Exposure to a stressor activates the hypothalamus-pituitary-adrenal cortex system (HPA axis) and induces arousal, distress or anxiety. An individual's interpretation of a situation, known as cognitive appraisal, is thought to mediate, or possibly moderate, their physiological response to a stressor (Lazarus 1991). However, despite originating from identical circumstances, the stress response may differ between individuals. The stress response of an individual depends on several factors, including genetics and previous experiences. It is therefore important that stress research measures the objective circumstances that challenge an individual, their

subjective response to stress and their cognitive appraisal of the situation. In PNMS research, many studies have assessed the associations between maternal stress and the offspring's brain development; however, few studies have been able to differentiate the distinct effects of objective stress, subjective stress and cognitive appraisal. This is a limitation of much of the work summarized below, which describes the relationship between PNMS and the hippocampus in animals.

2.2 Animal Research

The effect of PNMS on hippocampal development has often been studied using animal models. In rodents and primates, researchers randomly assign pregnant females to high stress conditions such as repeated restraint or acoustic startle or induce a stress-like response through injections of dexamethasone. The offspring are then assessed to determine the cellular, structural and behavioural effects of PNMS. These studies have provided substantial evidence that PNMS affects hippocampal development (Charil et al. 2010).

Specifically, research in rodents and primates has demonstrated that PNMS is associated with altered hippocampal neuron morphology. Male offspring of stressed mothers exhibit reduced length and complexity of dendrites as well as reduced synaptic density, which suggests that PNMS reduces the number of synaptic sites in the hippocampus (Fujioka et al. 2006; Jia et al. 2009; Martínez-Téllez et al. 2009). Interestingly these effects were not observed in female offspring. In addition, alterations in synaptic sites are combined with impaired hippocampal long term potentiation (LTP), indicating reduced synaptic plasticity as a result of PNMS (Yang et al. 2006; Son et al. 2006). Finally, in both

rodents and primates, PNMS reduces neurogenesis in the dentate gyrus throughout life, thereby leading to a reduced number of hippocampal neurons in adulthood (Lemaire et al. 2000; Coe et al. 2003; Zhu et al. 2004; Van den Hove et al. 2006).

On a macroscopic level, PNMS also influences hippocampal size. MRIs in both rodents and primates demonstrate that PNMS is associated with reduced hippocampal volume (Uno et al. 1998; Schmitz et al. 2002; Coe et al. 2003). Although these studies consistently demonstrate a reduction in hippocampal volume following PNMS, Schmitz et al. 2002 reports a sex-specific effect, with reduced volume only observed in female rats, while other studies find effects in both sexes. Regardless of sex differences, given the effects of PNMS on hippocampal structure, it is expected that PNMS would also affect hippocampal function.

Indeed, many studies have demonstrated that PNMS alters hippocampus-related behaviour in offspring. Rodents that are exposed to PNMS in utero exhibit reduced performance in spatial learning and memory in adulthood, as demonstrated through the Morris Water Maze (Meek et al. 2000; Szuran et al. 2000; Lemaire et al. 2000; Yang et al. 2006). In addition, PNMS is correlated with increased anxiety and depression-like symptoms, which also suggest reduced hippocampal function (Darnaudéry and Maccari 2008). In general, following exposure to PNMS, male offspring are more susceptible to learning deficits than females whereas female offspring are more susceptible to anxiety (Weinstock 2011). These findings from animal studies demonstrate that PNMS is associated with reduced synaptic connections, volume and function of the hippocampus.

2.3 Human Research

Although the effect of PNMS on the hippocampus has been well documented in animals, the evidence remains unclear in humans. Unlike animal studies, human research often lacks a randomly assigned stressor, instead investigating the effect of stressors such as divorce or financial difficulty that may be associated with an individual's traits, such as impulsivity or neuroticism, which may be transmitted to the offspring genetically. In addition, maternal anxiety, the measure often used to assess stress during pregnancy, is a heritable trait. Consequently, while maternal anxiety describes the mother's psychological state, it is not the same construct as PNMS, which is prompted by objective exposure to an independent stressor. In other words, if a pregnant woman experiences stress that she may have induced, in part, by her own temperament (e.g. divorce or job loss), and her child grows up to develop a similarly difficult temperament, it becomes almost impossible to determine the extent to which the association between the stress in pregnancy and the child's difficulties are due to genetic transmission, the intrauterine environment, and the postnatal rearing environment.

The association between maternal anxiety and the offspring's hippocampus has been assessed in humans by Qiu (Qiu et al. 2013). Here, increased maternal anxiety during pregnancy was associated with reduced hippocampal growth in the offspring's first six months of life. Researchers used MRIs at birth and at six months of life to assess hippocampal growth in infants. This study suggests that maternal anxiety during pregnancy predicts differences in hippocampal development in the offspring; however, it

remains unclear whether it is the heritable trait of anxiety or exposure to the stressor that precipitates this effect.

Many studies have also investigated the effect of postnatal stress on hippocampal structure in humans. It has been demonstrated that patients with posttraumatic stress disorder, as a result of adversity in early life or adulthood, exhibit reduced hippocampal volume (Bremner et al. 1995; Gurvits et al. 1996; Bremner et al. 1997; Schuff et al. 1997). However, more recent studies have not replicated these findings (De Bellis et al. 1999; Yamasue et al. 2003; Bonne et al. 2010). A study of Vietnam war veterans with PTSD and their combat-naïve identical twins suggests, in fact, that smaller hippocampal volumes represent a pre-existing risk factor for developing PTSD in the face of trauma (Shin et al. 2006). As a result, the effect of stress on hippocampal volume in humans remains unclear. Taken together, the disparity in findings concerning the effect of stress on hippocampal structure in humans and the different effects of PNMS in males and female animal models suggests that individuals may be differentially susceptible to the effects of PNMS. Differential susceptibility occurs in part due to genetic differences in a population that alter an individual's vulnerability to the effects of life events.

3. Single Nucleotide Polymorphisms (SNPs)

3.1 What are SNPs?

Genetic differences in DNA sequence and gene expression can occur via several mechanisms. Single nucleotide polymorphisms (SNPs) are one source of individual differences in the genome that have been well studied. SNPs are variants of one nucleotide in the DNA sequence that occur frequently across a population and influence gene

expression or function thereby conferring phenotypic differences. SNPs have been correlated with behavioural and physiological differences in humans that, in turn, appear to determine an individual's response to environmental factors and risk of disease throughout life. As a result, SNPs are a strong candidate to confer vulnerability to the effects of PNMS on brain development. In particular, SNPs located on genes that are involved in the stress response or brain development are likely to confer differential susceptibility to the effects of PNMS on hippocampus structure.

3.2 SNPs may Moderate the Effects of PNMS on Hippocampus Volume

Indeed SNPs that affect brain development have been observed to moderate the effect of maternal anxiety during pregnancy on the child's brain structure. Recently, it was shown that a SNP converting a valine to methionine on the brain-derived neurotrophic factor (BDNF) gene, which promotes the growth, maturation and survival of nerve cells, influences the degree to which maternal anxiety induces DNA methylation in the offspring and the relationship between the offspring's methylation and brain volume (Chen et al. 2015). Specifically, this paper found that the met/met genotype in offspring was associated with a greater impact of maternal anxiety on DNA methylation and a greater correlation between DNA methylation and right amygdala volume. Meanwhile the val/val genotype was associated with a greater correlation between DNA methylation and left hippocampal volume. This demonstrates that the offspring's BDNF genotype influences the effect of maternal anxiety on the developing brain.

In addition, Qiu et al. (2015) investigated whether SNPs in the catechol-O-methyltransferase (COMT) gene of offspring could moderate the effects of maternal anxiety

on brain structure, specifically prefrontal and parietal cortical thickness. Indeed they found that among rs737865-val158met-rs165599 haplotypes, the A-val-G haplotype exhibited a positive relationship between maternal anxiety and the offspring's cortical thickness in the right ventrolateral prefrontal cortex and the right superior parietal cortex. Meanwhile, the G-met-A haplotype exhibited a negative relationship between maternal anxiety and the offspring's cortical thickness in the bilateral precentral gyrus and dorsolateral prefrontal cortex. This demonstrates that particular COMT genotypes confer heightened vulnerability of frontal and parietal cortex regions to the effects of maternal anxiety.

While these two studies demonstrate that SNPs moderate the relationship between maternal anxiety and in utero neurodevelopment, further research is required to elucidate this relationship. The research to date has investigated only short-term effects of maternal anxiety on brain structure, demonstrating effects on the children up to six months old. In addition, this research has identified SNPs on the BDNF and COMT genes that play important roles, but many other SNPs that are implicated in stress and brain development may also moderate the effects of stress on brain structure.

3.3 SNPs of Interest

Several SNPs have been linked to an altered stress response and altered hippocampal development. These SNPs are therefore strong candidates to influence the effect of PNMS on hippocampus structure. In particular, rs110402 (CRHR1), rs3219151 (GABRA6) and rs41423247 (NR3C1) are SNPs that are mainly implicated in the stress response. Meanwhile, rs6265 (BDNF), rs4680 (COMT) and rs6311 (5HT2A) are SNPs that are implicated in hippocampal development and circuitry.

Of the SNPs involved in the stress response, rs110402 is located on the corticotropin-releasing hormone receptor 1 (CRHR1) gene, which is a mediator of the endocrine response to stress. This SNP moderates the effects of early life stress and abuse on stress reactivity, neuroticism and major depressive disorder in adolescence and adulthood (Tyrka et al. 2009; DeYoung et al. 2011; Kranzler et al. 2011; Laucht et al. 2013; Sumner et al. 2014). More specifically, the minor homozygote genotype (GG) of rs110402 is associated with increased vulnerability to the effects of early life stress.

Another SNP of interest, rs3219151 is located on the miRNA binding site of the GABRA6 gene, thus affecting expression of this GABA receptor. rs3219151 consists of a A-G substitution, of which the minor homozygote genotype (AA) is associated with reduced stress hormone levels compared to the major homozygote genotype (GG). Specifically minor genotype individuals exhibit lower basal cortisol levels and lower ACTH and cortisol levels following a stressor (Rosmond et al. 2002; Uhart et al. 2004).

Finally, rs41423247 consists of a G-C substitution that alters sensitivity of the glucocorticoid receptor (NR3C1) (Fleury et al. 2003). Glucocorticoid receptors mediate inhibition of the endocrine stress response, a function that is reduced in minor homozygote genotype (CC) individuals (Wüst et al. 2004). In addition glucocorticoid receptors are highly expressed in the hippocampus and the rs41423247 polymorphism has been found to moderate the effect of prenatal solar activity on hippocampal volume in adulthood (Montag 2013).

Other SNPs of interest are thought to alter hippocampal development and circuitry. rs6265, located on the BDNF gene, affects secretion of the BDNF protein that in turn influences memory performance and hippocampal activity and structure (Egan et al. 2003;

Hariri et al. 2003; Karnik et al. 2010). As mentioned above, this SNP moderates the relationship between maternal anxiety, DNA methylation and amygdala and hippocampal volume in newborn offspring (Chen et al. 2015).

A SNP on the COMT gene, rs4680, consists of an G-A substitution that in turn leads to an amino acid valine to methionine substitution. The minor homozygote genotype (AA) for COMT exhibits severely reduced COMT activity, reduced hippocampal activation during a memory task and reduced memory performance (Bilder et al. 2004; Bertolino et al. 2006). In addition, rs4680 is one of the COMT polymorphisms that moderates the effects of maternal anxiety on frontal and parietal cortical thickness (Qiu et al. 2015).

Lastly, rs6311 reduces expression of 5HT2A, a serotonin (5HT) receptor that is widely present in the hippocampus (Burnet et al. 1995). Interestingly, in rats PNMS reduces binding affinity of 5HT2A in the ventral hippocampus (Van den Hove et al. 2006). In addition, the major homozygote genotype (GG) of rs6311 has been identified as a risk factor for PTSD, indicating that this polymorphism affects the vulnerability of an individual to the effects of stress (Mellman et al. 2009; Bar-Shai and Klein 2013).

Overall, these SNPs were chosen because of their association with an individual's stress response and hippocampal development. While certain SNPs are known to modulate the effect of maternal anxiety on brain structure, it remains unknown whether these SNPs interact with objective PNMS, subjective PNMS or cognitive appraisal. To understand the interaction between genes and PNMS, it is necessary to study the effect of an independent stressor, thus eliminating genetic confounds and teasing apart the effects of objective exposure to a stressor and subjective distress following a stressful event. One means by

which this is possible is investigating the effect of natural disasters, an independent, randomly distributed stressor, on a human population.

4. Project Ice Storm

4.1 Project Ice Storm Background

The Quebec ice storm in January 1998 consisted of a series of freezing rain storms in southern Quebec and adjacent provinces and states. As a result of the storm more than three million people in Quebec alone lost power for as long as six weeks, many families were displaced from homes to temporary shelters and 27 people died (King et al. 2012). The storm has been cited as the worst and most costly natural disaster in Canadian history. Following this event, the laboratory of Suzanne King recruited women who were pregnant during the ice storm or became pregnant within three months of the crisis. In June of the same year the participants were sent a questionnaire that included objective and subjective stress questionnaires to measure their level of stress during the ice storm. Since this first questionnaire, parental and child assessments have been collected periodically to assess the effects of PNMS on the offspring's development.

4.2 Advantages of Project Ice Storm to Study PNMS

Project Ice Storm provides a unique opportunity to determine the effects of objective stress, subjective stress and cognitive appraisal of a stressor through a random assignment event. As mentioned earlier, many PNMS studies are unable to differentiate between the objective circumstances that induce stress, the subjective experience of stress by the mother and the mother's cognitive appraisal of the stressor. Animal studies in particular, are highly limited in measuring subjective stress or cognitive appraisal and therefore measure only objective stress exposure. Furthermore, translation of animal research to humans is confounded because rodents are born at a different developmental

stage. While human studies are better able to assess the subjective stress and cognitive appraisal of the mother during pregnancy, they do not easily allow for random assignment and adequate control over type, severity and timing of the stressor. In addition, stressors such as job loss or abuse may affect particular populations more than others and the use of maternal anxiety as an indicator of stress during pregnancy introduces genetic confounds. Overall, these issues limit the ability to infer gene-by-environment interactions from existing PNMS research.

In contrast, natural disasters randomly affect large populations and have a sudden onset that affects pregnant women in a particular stage of their pregnancy. This provides a sample of human subjects that are able to report objective PNMS, subjective PNMS and cognitive appraisal as a result of a single, common stressor. Consequently, we use natural disasters to study the effects of maternal stress on the development of the offspring. This has resulted in several findings concerning both behavioural and structural effects of PNMS on the offspring's brain development.

4.3 Project Ice Storm Findings

In Project Ice Storm, children exposed to high levels of objective PNMS, but not subjective maternal distress, already exhibited differences in behaviour and cognition at a young age. In particular, at 2 years old high objective PNMS was associated with lower Bayley Scale IQ, receptive and productive language skills as well as more stereotypical, rather than functional, play (Laplante et al. 2004; Laplante 2007). Interestingly, subjective PNMS also affected play behaviour but did not affect language. In addition, at 5½ years old, children exposed to high levels of objective PNMS had lower Wechsler IQ scores as well as

lower verbal IQ scores and language abilities compared to children exposed to low or moderate levels of objective PNMS (Laplante et al. 2008). Similar results were found at age 8½ (unpublished data). Taken together these findings demonstrate that objective PNMS results in reduced verbal and nonverbal cognitive function in children.

At age 11½, when the subjects were approaching adolescence or had already attained puberty, the pattern of results changed: for the first time, effects differed by sex, with a significant negative effect of maternal subjective stress, rather than objective stress, on Wechsler IQ scores in boys and no effect of either objective or subjective PNMS in girls (unpublished data). The King lab expected that these behavioural changes would be accompanied by differences in brain structure as a result of exposure to PNMS. Indeed, it has recently been found that at 11½ years old, Project Ice Storm female offspring exhibit a significant, positive correlation between objective stress and right hippocampal volume (Dufoix and King 2015). This effect was not seen in males and subjective PNMS was not associated with changes in hippocampal volume for either sex. It should be noted, however, that in this sample there was no significant associations between hippocampal volumes and either cognitive (IQ, memory) or behavioural (anxiety, depression, aggressiveness) functioning in either boys or girls. As such, for these children on the edge of puberty there are no data to suggest that either larger or smaller hippocampal volumes are associated with better outcomes.

The results collected so far in Project Ice Storm may not reflect the complete story. Behind the null effects of PNMS on hippocampus in boys, and even behind the observed effects in girls, gene-by-environment interactions may increase our understanding of how

environmental and genetic factors may work in combination to explain individual variation in hippocampal volumes.

5. Objectives and Hypotheses

Given that objective PNMS affects hippocampus volume in girls and SNPs are known to moderate the effect of environmental factors on the brain, our goal was to determine the associations among SNPs, PNMS and hippocampal volume. Specifically, we 1) determined the effect of three measures of PNMS (objective PNMS, subjective PNMS and cognitive appraisal) and 6 SNPs on left and right hippocampal volume; and 2) determined the extent to which these 6 SNPs moderate the effect of PNMS on hippocampal volume. In all analyses the sample was be split by sex.

We hypothesized that some of the SNPs will moderate the effects of PNMS on hippocampal volume. Specifically, we hypothesized that the major (GG) genotype of rs4680 (COMT) and the major (GG) genotype of rs6311 (5HT2A) would be associated with increased vulnerability to the effects of PNMS on hippocampal volume; the roles of the remaining SNPs could not be predicted from the existing research, and so their effects remained open questions. The existing literature also did not support directional hypotheses, that is, did not allow us to predict that sensitivity to PNMS would predict an increase or decrease in hippocampal volumes. Since sex is an important determinant for the effects of stress on brain development, we also expected that SNPs would differentially affect males and females.

Methods

1. Subjects

Recruitment: Following the ice storm in January 1998, the laboratory of Suzanne King contacted obstetricians in the Montérégie, a region southeast of Montreal that was highly affected by the crisis. Physicians from four hospitals in the region identified women who met the following criteria: 1) pregnant during or within three months of the ice storm; 2) white French Canadians; 3) 18 years old or older. Those families who gave consent to be followed up have been assessed periodically. The families that responded were significantly better educated and had higher incomes than the regional averages. The protocols were approved by the Research Ethics Board of the Douglas Hospital Research Centre.

Sample: In this study, 53 children who were in utero or conceived within three months of the Ice Storm were assessed to determine the relationship between PNMS, hippocampus volume and genotype. The sample includes 27 boys and 26 girls for whom both brain and genotype data were available. In addition, when the ice storm occurred, the gestational stage at the time of the ice storm (using January 9, 1998 as the ice storm date) was as follows: 14 preconception, 15 first trimester, 14 second trimester and 10 third trimester exposed. We included subjects who were conceived within three months following the ice storm (preconception) because of the long term effects of the ice storm, which continued to affect the mothers after the reference date of January 9, 1998. Finally, the sample includes only right-handed children.

2. Measures

2.1 Prenatal Maternal Stress

Objective PNMS, subjective PNMS and cognitive appraisal measures were collected through maternal questionnaires mailed to the families in June 1998, five months after the beginning of the ice storm.

Objective PNMS: To estimate objective hardship of the mother, the King lab has evaluated four categories of exposure to stress (threat, loss, scope and change) (Appendix A). Questions in each category quantified experiences such as "days endured without electricity" and "displacement from home." Each category has a maximum score of eight points and is summed to create the Storm32 scale. In our sample the Storm32 score ranges from 5-24 and averages 11.55 (SD=4.53).

Subjective PNMS: The subjective distress of mothers was evaluated using the 22-item Impact of Event Scale-Revised (IES-R), which includes questions concerning the severity of posttraumatic stress-like symptoms in three categories (hyperarousal, intrusion and avoidance). The IES-R has good internal consistency (α=0.93) and satisfactory test-retest reliability (r=0.76) (Brunet et al. 2003) and was adapted to relate specifically to the ice storm. In our sample the IES-R score ranges from 0-3.71 and averaged 1.85 (SD=1.09).

Cognitive Appraisal: The mother's cognitive appraisal of the storm was assessed by asking 'Overall, what were the consequences of the ice storm on you and your family?' and providing five response options on a Likert scale ranging from very negative to very positive. In this study, positive and neutral responses have been grouped together and compared to negative responses.

2.2 Hippocampal Volume

MRI Image Acquisition: Magnetic resonance imaging (MRI) was performed at the Unité de Neuroimagerie Fonctionelle (UNF) du Centre de Recherche de l'Institut Universitaire de Gériatrie de Montréal (CRIUGM). 3D, T1-weighted anatomical brain scans were obtained using a 3T Siemens MAGNETOM Trio TIM Syngo (Siemens, Erlangen, Germany), with a 12-channel head coil. Scans were collected from 65 subjects at 11½ years old; however, only 57 of these subjects provided a saliva sample for genotyping. Of these 57 subjects, we have excluded left-handed children, resulting in a total sample of 53 subjects for whom we have both MRI scans and genetic information.

MRI Image Analysis: The raw images have undergone automated correction for intensity, non-uniformity, normalization for signal intensity and automatic registration to a standard, stereotaxic space (MNI) (Talairach and Tournoux 1988). The hippocampus was automatically segmented using the MAGeT pipeline, which includes input from the atlases of Winterburn et al. 2013 (Chakravarty et al. 2012; Pipitone et al. 2014). Finally right and left hippocampus volumes were manually corrected using the Pruessner lab segmentation protocol by Romane Dufoix (Pruessner et al. 2000). To control for changes in total intracranial (TIC) volume that may account for differences in hippocampus volume, we evaluated outcomes of hippocampus/TIC ratio and TIC volume.

2.3 Genotype

When Project Ice Storm children were 8½ years old, saliva samples were collected during the cognitive assessment using Oragene DNA self-collection kit (OG-500) (DNA Genotek) and stored at room temperature until further analysis. DNA extraction was

performed using PrepIT-L2P kit (DNA Genotek) according to the manufacturer's instructions. DNA yield was measured using NanoDrop 8000 Spectrophotometer V2.1 (Thermo Scientific). DNA was stored at -80°C until analysis. rs6265 (BDNF), rs6311 (5HT2A), rs4680 (COMT), rs110402 (CRHR1), rs3219151 (GABARA6) and rs41423247 (NR3C1) were genotyped using Sequenom iPlex Gold Technology (Ehrich et al. 2005) at McGill University and the Génome Québec Innovation Centre.

3. Statistical Analysis

Correlations and ANOVAs were conduced to determine the association between PNMS and SNPs on hippocampus/TIC ratio volume, total intracranial volume (TIC) and uncorrected hippocampal volume. In these analyses the three SNP genotypes were coded to indicate the correlation between the number of major alleles (minor<heterozygote<major; 0, 1, 2) and brain volumes. Moderation and hierarchical regression analyses were conducted to determine whether any of the six SNPs moderate the effect of PNMS on hippocampal volumes. More specifically, analyses tested whether the six SNPs of interest moderate the effect of objective PNMS, subjective PNMS and cognitive appraisal on the ratio of left and right hippocampal volume/TIC volume and TIC volume alone. In these analyses, the SNP genotypes were dichotomized such that genotype groups included either minor merged with heterozygote vs. major or major merged with heterozygote vs. minor. All analyses were conducted by sex: males only and females only.

Results

1. Objective PNMS is Associated with Right Hippocampal Volume in Girls

The King lab has previously found that objective PNMS was correlated with right hippocampal volume in girls, but not in boys (Dufoix, unpublished). Since this paper involves a subset of the sample previously studied (53/68 subjects), we expected similar results here concerning the effect of PNMS on hippocampal volume.

Indeed we found that in boys there was no significant effect of PNMS on hippocampus ratio volume or uncorrected hippocampal volume (Table 1), although there was a nonsignificant trend for greater objective PNMS to predict larger left hippocampus/TIC ratio volume ($r= 0.324$, $p= 0.099$). In addition, there was a significant negative correlation between objective PNMS and TIC ($r= -0.442$, $p= 0.021$), a marginally significant negative correlation between subjective PNMS and TIC ($r=-0.354$, $p= 0.070$) and no effect of cognitive appraisal on TIC, in boys.

In girls, in accordance with our previous findings, objective PNMS was positively correlated with right hippocampus/TIC ratio volume ($r= 0.392$, $p= 0.048$) (Table 1); a weaker, nonsignificant trend was also seen in the left hippocampus/TIC ratio ($r= 0.305$, $p= 0.130$). There was no association between objective PNMS and TIC or uncorrected left and right hippocampal volume alone in girls. In addition, subjective PNMS and cognitive appraisal were not correlated with hippocampus/TIC ratio volume, TIC or uncorrected hippocampal volumes.

2. SNPs of Interest Associated with Hippocampal Volume

2.1 Genotype Frequencies

For five of the six SNPs, all genotypes (minor homozygote, heterozygote and major homozygote) were represented in both girls and boys in our sample (Table 2); however, for the SNP located on the BDNF gene, only the major and heterozygote genotypes were represented in our sample. In addition, we tested each SNP for accordance with the Hardy-Weinberg equilibrium, which indicates whether the genotype frequencies in our sample were representative of the general population. Indeed, the SNPs located on CRHR1, GABRA6, NR3C1, COMT and 5HT2A met the Hardy-Weinberg equilibrium; however, the SNP located on BDNF could not be tested for the Hardy-Weinberg equilibrium because the minor genotype was not represented. Although studying BDNF using our sample was, therefore, limited, we still assessed the effect of having 1 or 2 major BDNF alleles on hippocampal volume because of the importance of this gene in hippocampal development.

2.2 Stress Genes: SNPs on Stress Genes are not Correlated with Hippocampal Volume

Of the SNPs presented in Table 3, three SNPs of interest are located on genes that affect the stress pathway; these genes are CRHR1, GABRA6 and NR3C1. We first assessed the main effect of these SNPs on hippocampal volume. Although having the major genotype was marginally associated with greater right hippocampus/TIC ratio volume in boys ($r = -0.353$, $p=0.071$), there was no statistically significant effect of CRHR1, GABRA6 or NR3C1 on hippocampus/TIC ratio volume in girls or boys. In addition, in boys the major CRHR1 genotype was associated with smaller TIC volume ($r = -0.391$, $p=0.044$); an effect that might

drive observed changes in hippocampus/TIC ratio volume. In other words, having two major CRHR1 alleles may predict larger hippocampus/TIC volume only because it is also associated with smaller TIC volume with no effect on the absolute volume of the hippocampus. Therefore, the SNPs that alter function of the stress pathway do not exhibit a significant main effect on hippocampal volume.

2.3 Neurotransmission and Neurodevelopment Genes: SNPs on COMT and BDNF are Correlated with Hippocampal Volume

The remaining SNPs of interest are located on three genes involved in mediating neurotransmission and neurodevelopment; these genes are COMT, 5HT2A and BDNF. Of these three genes, SNPs located on COMT and BDNF produced significant main effects on hippocampal volume, while the SNP located on 5HT2A was not associated with hippocampus/TIC ratio, TIC, or uncorrected hippocampal volumes (Table 3).

COMT (rs4680): In boys, the COMT major genotype was correlated with increased left hippocampus/TIC ratio volume ($r=0.561$, $p=0.002$) with a similar trend on the right hippocampus/TIC ratio ($r=0.334$, $p=0.089$) (Table 3). There is also a significant positive correlation between the COMT major genotype and the uncorrected left ($r=0.611$, $p=0.001$) and right ($r=0.472$, $p=0.013$) hippocampal volume but there is no correlation between the COMT genotype and TIC ($r=0.284$, $p=0.152$). This pattern of results suggest that there is a real association between the major allele and hippocampal volume that is not due to an effect on TIC. An ANOVA and Tukey post-hoc test demonstrated that in boys the major homozygote genotype is associated with larger left hippocampus/TIC ratio volume compared to the heterozygote, which is in turn larger than that of the minor homozygotes

(p=0.0008, F=5.917, df=2) (Figure 1a) There was, however, no significant difference in right hippocampus/TIC ratio volume between genotypes (p=0.241, F=1.512, df=2).

In girls there is no significant correlation between the COMT genotype and hippocampus/TIC ratio volume or TIC alone; however, there is a significant negative correlation between the major COMT genotype and the uncorrected right hippocampal volume only (r=-0.433, p=0.027) (Table 3), suggesting that, unlike in boys, the major allele is associated with a smaller, not larger, hippocampus. The COMT genotype was not associated with uncorrected left hippocampal volume. There was a nonsignificant trend, however, for the major allele to predict smaller TIC and right hippocampus/TIC volume, which might suggest that the association with the uncorrected right hippocampal volume is part of a larger trend towards smaller brain and hippocampal volumes. Indeed an ANOVA and Tukey post-hoc test demonstrated that there was no significant effect of COMT genotype on left (p=0.871, F=0.139, df=2) or right (p=0.286, F=1.324, df=2) hippocampus/TIC ratio volume in girls (figure1b). Although the ANOVA and Tukey post hoc test revealed a marginally significant effect of COMT genotype on uncorrected right hippocampal volume, in which the number of major alleles was associated with smaller uncorrected right hippocampal volumes (p=0.068, F=3.027, df=2) (data not shown), since this effect in girls did not remain significant when divided by TIC volume, we could not conclude that it was specific to the hippocampus.

BDNF (rs6265): In boys, the BDNF genotype was marginally correlated with left hippocampus/TIC ratio volume (r=-0.369, p=0.058) and left uncorrected hippocampal volume (r=-0.352, p=0.072), but not with right hippocampus/TIC ratio volume, right uncorrected hippocampal volumes or TIC (Table 3). A t-test demonstrated that the major

BDNF genotype is associated with smaller left ($p<0.001$, $t=12.301$, $df=26$) and right ($p<0.001$, $t=12.225$, $df=26$) hippocampus/TIC ratio volume than the heterozygote genotype in boys (figure 2a). Given the lack of association with TIC, this pattern suggests a real effect of BDNF on left hippocampal volume that is not due to generalized effects on total brain volume.

Similarly, in girls the major BDNF genotype is correlated with left hippocampus/TIC ratio volume ($r=0.444$, $p=0.023$) and uncorrected left hippocampal volume ($r=0.437$, $p=0.026$), but not significantly with right hippocampus/TIC ratio, uncorrected right hippocampus or TIC volumes (Table 3). A t-test demonstrated that, in contrast to boys, girls with the major genotype exhibit larger left ($p<0.001$, $t=12.658$, $df=25$) and right ($p<0.001$, $t=12.809$, $df=25$) hippocampus/TIC ratio volume than heterozygotes (figure 2b). As with boys, this effect is stronger with the left than the right hippocampus.

There was no significant association between the 5HT2A genotype and either left or right hippocampal or TIC volumes for either boys or girls. In summary, two SNPs located on genes involved in neurotransmission and neurodevelopment, namely COMT and BDNF, exhibit significant or marginally significant main effects on hippocampal volume in both boys and girls. Remarkably, these genes produce distinct effects in each sex.

3. SNPs Moderate the Effect of PNMS on Hippocampal Volume

3.1 Control Variables

Many factors are known to alter fetal development and thus induce long-term effects on hippocampal volume. To control for this, we tested the effect of some of these factors on hippocampus/TIC ratio, TIC and uncorrected hippocampus volume in boys and

girls (Table 4a, b). We controlled for factors that were correlated with the dependent variable. Accordingly, when testing for effects in both left and right hippocampus/TIC ratio volume in boys, we controlled the number of glasses of alcohol the mother drank per week during her pregnancy. In addition, for left hippocampus/TIC ratio volume in girls we controlled for socioeconomic status (SES). Finally, when testing for effects on TIC in boys, we controlled for birth weight.

3.2 Moderating Effect of Genotypes on Hippocampus/TIC Ratio in Boys (Table 5)

Objective PNMS: For the left hippocampus/TIC ratio in boys, 9.3% of the variance was explained by objective stress levels ($p<0.10$). The addition of the main effects of the genotypes increased variance explained by 10-15%, with significant effects from COMT (minor A merged), 5HT2A (minor A merged), and BDNF (CC vs. CT). There were no significant interactions between objective stress and genotype. The full models, including objective stress, genotype, interactions, and covariates explained 40-42% for the COMT, 5HT2A and BDNF models.

For the right hippocampus/TIC ratio in boys, objective PNMS explained 6.7% of the variance in volume. The addition of any of the genotypes explained very little additional variance (<1%). COMT moderated the effect of objective PNMS on right hippocampus/TIC ratio (R^2-Change =0.122, $p=0.049$), with the full model explaining 37.8% of the variance in hippocampal volume. (Table 5). As shown in Figure 3, for COMT major homozygotes, there was a negative association between objective PNMS and right hippocampus/TIC volume ($p=0.084$) with greater objective stress predicting smaller volumes; however, for minor homozygotes and heterozygotes there was no association between objective PNMS and

right hippocampus/TIC volume. Below objective stress levels of 10.86 there was a significant difference in right hippocampus/TIC volume between genotypes, with major homozygotes having larger volumes than the minor homozygotes.

NR3C1 exhibited a marginally significant moderating effect of objective PNMS on right hippocampus/TIC (R^2-change=0.114, p=0.057), resulting in a total of 37.8% of the variance being explained by the model (Table 5). For minor homozygotes, there was a marginally significant negative correlation between objective PNMS and right hippocampus/TIC (p=0.071) with greater objective stress predicting smaller volumes; however, for major homozygotes and heterozygotes there was no association between objective PNMS and right hippocampus/TIC volume. Below objective stress levels of 13.11 there was a significant difference in right hippocampus/TIC ratio volume between genotypes (minor homozygotes>major homozygotes and heterozygotes).

CRHR1, 5HT2A, GABRA6 and BDNF did not explain any additional variance over and above the variance explained by objective stress, nor did they moderate the effect of objective PNMS on left or right hippocampus/TIC ratio in boys.

Subjective PNMS: In boys, there was no main effect of subjective stress on left hippocampus/TIC. The addition of the COMT genotype increased variance explained by 20% (p=0.015), while the addition of the BDNF main effect increased variance explained by 13.7% (p=0.046). Only CRHR1 significantly moderated the effect of subjective PNMS on left hippocampus/TIC volume, explaining an additional 21.1% of variance (R^2-change=0.221, p=0.007) (Table 5). This effect remained significant following Bonferroni correction for multiple testing (p<0.008) and when controlling for objective PNMS (p=0.020), with the full model explaining 44.5% of the variance in left hippocampus/TIC volume. As shown in

figure 4, the major homozygotes combined with heterozygotes had a significant negative association between subjective PNMS and left hippocampus/TIC volume (p=0.033), while the minor homozygotes had a marginally significant positive association between subjective PNMS and left hippocampus/TIC volume (p=0.057) (Figure 4). When levels of subjective PNMS were below 1.71, there was a significant difference in right hippocampus/TIC volume between genotypes, with the major homozygotes and heterozygotes having larger volumes than minor genotypes. There was a trend for NR3C1 to moderate subjective stress (p=0.099), increasing variance explained by 10%; the total model explained 25.7% of the variance in left hippocampus/TIC volumes.

For right hippocampus/TIC volume in boys, subjective stress explained 9.6% of variance in volumes, and none of the main effects for genotypes improved the model. However, 5HT2A moderated the effect of subjective PNMS on right hippocampus/TIC volume (R^2-change=0.119, p=0.049) (Table 5). For 5HT2A SNP major homozygotes and heterozygotes there was a positive correlation between subjective PNMS and right hippocampus/TIC volume (p=0.015); however, for minor homozygotes there was no association between subjective PNMS and right hippocampus/TIC volume (Figure 5).

Cognitive Appraisal: The main effect of cognitive appraisal explained <1% of variance in right and left hippocampus/TIC volume. CRHR1 (R^2-change=0.097, p=0.086), COMT (R^2-change=0.099, p=0.091) and NR3C1 (R^2-change=0.108, p=0.078) had marginally significant moderating effects of cognitive appraisal on right hippocampus/TIC volume, explaining an additional 10% of variance to each model for total variance explained between 30% and 34%. For CRHR1, while there was a marginally significant interaction effect, there was no significant association between cognitive appraisal and right hippocampus/TIC volume and

no significant difference in right hippocampus/TIC volume between genotypes. For COMT, the major homozygotes exhibited a marginally significant correlation between cognitive appraisal and right hippocampus/TIC volume (p=0.093), with positive cognitive appraisal associated with greater hippocampus/TIC volumes; however, the minor homozygotes and heterozygotes did not exhibit a correlation between cognitive appraisal and right hippocampus/TIC volume (p=0.722). Additionally, there was no significant difference in right hippocampus/TIC volume between genotypes. Finally, for NR3C1, the minor homozygotes had a marginally significant correlation between cognitive appraisal and right hippocampus/TIC volume, with positive cognitive appraisal associated with greater right hippocampus/TIC volumes (p-0.087); however, major homozygotes and heterozygotes did not have a correlation between cognitive appraisal and right hippocampus/TIC volume (p=0.645). There was no significant interaction between genotype and cognitive appraisal for the left hippocampus/TIC ratio in boys. 5HT2A, GABRA6 and BDNF did not moderate the effect of cognitive appraisal on hippocampus/TIC volume in boys.

3.3 Moderating Effect of Genotype on Hippocampus/TIC Ratio in Girls (Table 6)

Objective PNMS: Neither objective stress nor genotype explained significant amounts of variance in left hippocampus/TIC volumes (all R^2<0.10); however, there was a nonsignificant trend for BDNF genotype, which explained 8.4% of the variance in left hippocampus/TIC volume (p=0.119).

For the right hippocampus/TIC volume in girls, objective stress explained 15.4% of the variance, but neither genotype nor gene-by-objective stress interactions increased variance explained significantly.

Subjective PNMS: Subjective stress explained <1% of the variance in left and right hippocampus/TIC volume in girls, and main effects for genotypes did not increase variance explained; the BDNF genotype did increase variance explained by 9% in left volumes, and 11.2% in right volumes, but these increases were not great enough to be considered significant (p=0.112 and p=0.101). COMT moderated the effect of subjective PNMS on left (R^2-change=0.329, p=0.0009) and right (R^2-change=0.211, p=0.023) hippocampus/TIC volume (Table 6). The moderation of subjective PNMS remained significant when controlling for objective PNMS in the left (p=0.001) and right (p=0.007) hippocampus/TIC; however, only the moderation on the left hippocampus/TIC volume remained significant following Bonferroni correction for multiple testing (p<0.008). For COMT major homozygotes, there was a significant negative correlation between subjective PNMS and both left (Figure 6a, p=0.0024) and right (Figure 6b, p=0.040) hippocampus/TIC volume. COMT minor homozygotes and heterozygotes, on the other hand, exhibited no association between subjective PNMS and hippocampus/TIC volume. For the left hippocampus/TIC volume, there was a significant difference in volume between genotypes below subjective PNMS levels of 1.52 (major>minor/heterozygote) and above subjective PNMS levels of 2.77 (minor/heterozygote>major). Meanwhile, for the right hippocampus/TIC ratio, there was a significant difference in volume between genotypes when subjective PNMS levels were above 2.98 (minor/heterozygote>major).

In addition, NR3C1 had a marginally significant moderating effect of subjective PNMS on left hippocampus/TIC volume (R^2-change=0.123, p=0.053), but not right hippocampus/TIC volume. The subjective stress with COMT interaction models explained a total of 53.3% (COMT) and 38.7% (NR3C1) of the variance in left hippocampus/TIC volume.

CRHR1, 5HT2A, GABRA6 and BDNF did not moderate the effect of subjective PNMS on hippocampus/TIC volume in girls.

Cognitive Appraisal: For girls, there was no significant main effect of cognitive appraisal, nor for any genotypes on left or right hippocampus/TIC volumes, although there was a trend towards a main effect of BDNF on right hippocampus/TIC volume (p=0.093). There were no interactions between cognitive appraisal and genotype for left hippocampus/TIC volume. However, COMT moderated the effect of cognitive appraisal on right hippocampus/TIC ratio volume (R^2-change=0.166, p=0.036) in girls. When mothers reported a negative perception of the ice storm in the cognitive appraisal question, minor homozygote females exhibited greater right hippocampus/TIC volume than major homozygotes and heterozygotes (p=0.010) (Figure 7). Meanwhile, offspring of mothers with a positive perception of the ice storm did not exhibit a difference in right hippocampus/TIC volume according to genotype (p=0.894). In addition for minor homozygotes (p=0.134) and for major homozygotes and heterozygotes (p=0.120) there was no significant difference in right hippocampus/TIC volume between maternal negative cognitive appraisal and positive cognitive appraisal groups. Finally, CRHR1, NR3C1, 5HT2A, GABRA6 and BDNF did not moderate the effect of cognitive appraisal on hippocampus/TIC volume.

Discussion

1. Summary

Previous work in our lab has demonstrated that objective maternal stress is positively correlated with the offspring's hippocampal volume at 11½ years old; however this finding contradicts those of animal research, which indicates a negative correlation between PNMS and the offspring's hippocampal volume. Here we wondered whether a child's genotype moderates the effects of PNMS on hippocampal volume, thereby resulting in differential effects of stress on brain structure. While we have observed an effect of PNMS on hippocampal volume, there was no correlation between hippocampal volume and cognitive outcomes such as IQ in the same sample (Dufoix and King 2015). This lack of association between hippocampal volume and cognitive function could be related to the subjects' onset of puberty during which brain scans were collected. Though some evidence suggests that there is a positive correlation between hippocampal volume and memory ability, this relationship remains unclear at the time of early adolescence (Van Petten 2004). As a result, we are unable to conclusively interpret whether a larger or smaller hippocampus results in better or worse outcomes. Rather our interpretation of the results will focus on how genotype can moderate susceptibility to the effects of PNMS.

Our findings support the hypothesis that individuals exhibit differential susceptibility to the effects of PNMS, and that SNPs, genetic variants that alter the function or expression of proteins, underlie these differences. In some cases SNPs, PNMS, and the gene-by-environment interaction combined explained up to half the variance in hippocampal volume. This is significant as it increases our understanding of how genetic

and environmental factors work in combination to affect hippocampal development. While previous research has assessed the interaction between SNPs and maternal anxiety on brain structure, the heritable nature of maternal anxiety limits conclusions from this research concerning the particular environmental and genetic contributions to brain development. Natural disasters provide a unique opportunity to study the effect of an objective, randomly assigned stressor in a human population, thereby overcoming many limitations of previous human gene-by-environment research. Consequently, we have been able to delineate the influences of PNMS and SNPs on hippocampal volume in early adolescent offspring.

Specifically, we found that SNPs located on BDNF and COMT produced main effects on hippocampal volume. Meanwhile, SNPs located on CRHR1, COMT, 5HT2A and NR3C1 exhibited evidence of moderating the effects of objective PNMS, subjective PNMS or cognitive appraisal on hippocampal volume.

This study does have its limitations. Although the results described here further our understanding of the interaction between SNPs and PNMS, given that our sample includes only 53 offspring the power of these findings is limited and further research is required to validate these conclusions. Especially given the importance of the BDNF gene, it is unfortunate that our sample was missing subjects with the minor homozygote genotype for a full investigation of this gene's effects.

2. Genotype moderates the effect of PNMS on hippocampal volume

Overall, four of the six SNPs of interest exhibited significant or marginally significant moderation effects of PNMS on hippocampal volume. These SNPs were located on the genes, COMT, CRHR1, 5HT2A and NR3C1.

In particular, the COMT genotype moderated the effect of objective PNMS in boys and subjective PNMS and cognitive appraisal in girls. In boys, under low objective PNMS conditions the COMT major homozygotes have greater hippocampal volume than heterozygotes and minor homozygotes; however, under high objective PNMS there is little difference between COMT genotypes as the high objective stress is associated with reduced volumes in the major homozygote subjects. Similarly, in girls at low levels of subjective PNMS major homozygotes again exhibit greater left and right hippocampal volumes than heterozygotes and minor homozygotes. As in boys, this difference is reversed or removed in the left and right hippocampus respectively under conditions of higher subjective stress. Thus, high objective and subjective PNMS remove differences in hippocampal volume between genotypes that exist at low PNMS levels. Remarkably, it is the major homozygotes that have a significantly larger hippocampal volume in low objective and low subjective PNMS conditions compared to high PNMS conditions, while heterozygotes and minor homozygotes exhibit little difference between low and high levels of PNMS. The differential effects of PNMS between genotypes indicate that the major homozygotes are more susceptible to the effects of PNMS. Finally, in addition to moderating the effects of objective and subjective PNMS, we found that the COMT genotype can moderate the effects of the pregnant mother's cognitive appraisal of the ice storm on hippocampal volume in girls. For mothers who reported negative cognitive appraisal of the ice storm, minor homozygote

offspring had smaller hippocampal volumes than heterozygotes and major homozygotes; a difference that is removed in offspring of mothers who reported positive cognitive appraisal.

The COMT SNP of interest results in a valine to methionine substitution that in turn reduces COMT enzymatic activity 3-4 fold. Heterozygotes are codominant, which results in an intermediate phenotype. Since the major COMT variant is more active than the minor variant, our findings suggest that greater COMT activity is associated with greater hippocampal volume under low objective and subjective PNMS, and positive cognitive appraisal conditions at least in offspring at the age of 11½.

COMT is an enzyme that metabolizes catecholamines, such as norepinephrine and dopamine. Interestingly, previous findings report that exposure to PNMS affects catecholamine systems: in rats, PNMS has been associated with a reduced concentration of norepinephrine and dopamine metabolites in the cerebral cortex, suggesting reduced turnover of these neurotransmitters (Takahasi et al. 1992). The COMT genotype-specific effects of PNMS observed here may be due to differences in baseline COMT activity between genotypes. Given low levels of baseline COMT activity in minor homozygotes, the enzyme may reach a threshold of minimum activity such that its activity cannot be further reduced. As a result, COMT major homozygotes may be more susceptible to the effects of PNMS.

3. Subjective PNMS affects hippocampal volume when moderated by SNPs

In addition to the SNP on COMT, the SNPs located on both CRHR1 and 5HT2A significantly moderated the effects of PNMS in boys: CRHR1 moderated effects of subjective

PNMS on left hippocampal volumes, while 5HT2A moderated effects of subjective stress on right hippocampal volumes. Overall, all three SNPs that produced significant moderating effects, and four out of six significant results, moderate the effect of subjective PNMS, rather than objective PNMS or cognitive appraisal, on hippocampal volume. In all cases, these effects remained significant when controlling for objective PNMS, demonstrating that they are indeed a function of subjective distress of the mother, independent of her objective circumstances. This is an important conclusion, and could not have been obtained by studying other types of stressors, or maternal anxiety, in which the objective and subjective aspects of the stress cannot be disentangled.

Previously, the King lab found that only subjective PNMS is correlated with the mother's hypothalamic-pituitary-adrenal (HPA) axis activity: higher subjective distress (PTSD-like symptoms) assessed 5 months after the ice storm is associated with lower diurnal cortisol secretion in mothers, as seen in individuals with PTSD. Lower cortisol levels in PTSD may reflect either a pre-existing vulnerability to PTSD, or the result of damage to components of the HPA axis following a surge of stress-induced cortisol. The HPA axis is activated in response to stress, which results in the release of the glucocorticoid, cortisol. Although cortisol is vital for fetal brain development, abnormally high levels of cortisol, induced by increased maternal stress, negatively impacts the fetus (Garbrecht et al. 2006; Seckl and Holmes 2007). Indeed, high levels of cortisol during prenatal development in rats is associated with reduced hippocampal volume at adulthood (Hayashi et al. 1998; Szuran et al. 2000; Schmitz et al. 2002; Coe et al. 2003). Thus, the SNPs of interest may moderate the effects of subjective PNMS in particular because resultant changes in HPA axis activity alter, in turn, hippocampal volume.

Previous gene-environment research in the King lab reported that objective PNMS and cognitive appraisal, but not subjective PNMS, are correlated with the methylation of genes associated with metabolism (Cao-Lei et al. 2014; Cao-Lei et al. 2015). Thus, while SNPs that moderate subjective PNMS in particular may influence hippocampal volume, genetic variants moderating objective PNMS and cognitive appraisal may affect other pathways.

4. Sex Differences in the Main Effect of SNPs on Hippocampal Volume

In addition to the moderating effect of SNPs on PNMS, we identified several main effects of SNPs on hippocampal volume. In particular, SNPs located on COMT and BDNF directly affect hippocampal volume; however, outcomes of these SNPs differed in boys compared to girls. In boys, both the COMT and BDNF major homozygote genotypes, compared to heterozygote or minor homozygote genotypes, were associated with greater left hippocampal volume. Meanwhile, in girls the COMT major homozygote genotype was associated with smaller uncorrected right hippocampal volume, but little change in right hippocampus/TIC ratio volume, and the BDNF major homozygote genotype was associated with smaller left hippocampal volume than the heterozygote genotype. These results suggest that the BDNF and COMT SNPs affect hippocampal development, but that these genes act differently in boys compared to girls.

In contrast to these sex differences in the effect of the COMT and BDNF genotypes on hippocampal volume at early adolescence, it has previously been reported that the minor homozygote BDNF genotype is associated with reduced hippocampal volume in *both* male and female adults (Bueller et al. 2006). Given that we measured hippocampal volume at 11½ years old, rather than adulthood, it is possible that the genotype-dependent sex

differences in male and female hippocampal volume are specific to this age group, which represents the onset of adolescence.

The mechanism underlying the sex-specific effects of COMT and BDNF SNPs on hippocampal volume at early adolescence remains unclear but is likely influenced by sex hormones that are highly expressed in the hippocampus and interact with hippocampal function (Goldsteinn et al. 2001). Sex hormones may influence the role of BDNF and COMT through various mechanisms. For example, the sex hormone estrogen is known to alter levels of BDNF expression, thereby leading to disproportionate regulation of BDNF protein levels in females compared to males (Sohrabji and Lewis 2006).

These same sex hormone differences between adolescent males and females may also explain observed sex differences in the moderation effects. While the COMT genotype moderated the effect of objective PNMS in boys, this SNP moderated the effect of subjective PNMS and cognitive appraisal in girls. Similarly, the SNPs located on CRHR1 and 5HT2A moderated the effect of subjective PNMS in boys, but not in girls. These sex-specific results are in line with our original hypothesis, which postulated that results would differ between males and females, and indicate sex differences in brain development that are particularly prevalent at the onset of puberty.

Conclusion

This work has increased our understanding of gene-by-environment interactions during prenatal brain development. Specifically, we have found that a SNP located on COMT significantly moderates the effects of PNMS on hippocampal volume, resulting in differential susceptibility between COMT genotypes to the effects of PNMS. In addition, the effect of different aspects of PNMS – objective PNMS, subjective PNMS and cognitive appraisal – on hippocampal volume was differentially moderated by the SNPs of interest. When moderated by SNPs located on COMT, 5HT2A and CRHR1, subjective PNMS exhibited greater effects on hippocampal volume than objective PNMS and cognitive appraisal. Overall, in accordance with our hypothesis, these results suggest that a child's genotype can alter their vulnerability to the effects of PNMS; however, these effects are often specific to a particular sex and/or aspect of stress.

Tables and Figures

Table 1. Effect of PNMS on Hippocampal Ratio, TIC and Uncorrected Hippocampal Volume in Boys and Girls

BOYS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
Objective PNMS	r= 0.324^{+} p= 0.099	r= 0.280 p= 0.157	r= -0.442* p= 0.021	r= 0.002 p= 0.993	r= -0.570 p= 0.779
Subjective PNMS	r= -0.140 p= 0.944	r= 0.317 p= 0.108	r= -0.354^{+} p= 0.070	r= -0.203 p= 0.309	r= 0.049 p= 0.808
Cognitive Appraisal	r= -.093 p= .643	r= 0.032 p= 0.873	r= 0.216 p= 0.278	r= 0.067 p= 0.740	r= 0.187 p= 0.351
GIRLS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
Objective PNMS	r= 0.305 p= 0.130	r= 0.392* p= 0.048	r= -0.226 p= 0.267	r= 0.164 p= 0.423	r= 0.267 p= 0.187
Subjective PNMS	r= 0.142 p= 0.488	r= 0.023 p= 0.911	r= -0.100 p= 0.963	r= 0.164 p= 0.424	r= 0.027 p= 0.896
Cognitive Appraisal	r= 0.103 p= 0.618	r= -0.019 p= 0.928	r= 0.048 p= 0.816	r= 0.138 p= 0.500	r= 0.013 p= 0.950

r = Pearson's correlation coefficient; p=significance value ($^{+}$ p<0.10, *p<0.05, **p<0.01).

Table 2. Genotype Frequencies.

SNP	Minor/Major	Minor Homozygote n (%)	Heterozygote n (%)	Major Homozygote n (%)
CRHR1 (rs110402)	G/A	*Boys*: 6 (22.2%) *Girls:* 5 (19.2%)	*Boys:* 12 (44.4%) *Girls*: 12 (46.2%)	*Boys*: 9 (33.3%) *Girls:* 9 (34.6%)
GABRA6 (rs3219151)	A/G	*Boys*: 7 (25.9%) *Girls:* 3 (46.2%)	*Boys*: 15 (55.6%) *Girls*: 12 (38.5%)	*Boys:* 5 (18.5%) *Girls:* 10 (3.8%)
NR3C1 (rs41423247	C/G	*Boys*: 2 (7.4%) *Girls*: 6 (23.1%)	*Boys:* 9 (33.3%) *Girls:* 12 (46.2%)	*Boys:* 16 (59.3%) *Girls*: 8 (30.8%)
COMT (rs4680)	A/G or met/val	*Boys*: 4 (14.8%) *Girls*: 13 (50.0%)	*Boys:* 20 (74.1%) *Girls*: 8 (30.8%)	*Boys*: 3 (11.1%) *Girls:* 5 (19.2%)
5HT2A (rs6311)	A/G	*Boys*: 6 (22.2%) *Girls*: 5 (19.2%)	*Boys:* 12 (44.4%) *Girls*: 14 (53.8%)	*Boys:* 9 (33.3%) *Girls:* 7 (26.9%)
BDNF (rs6265)*	T/C or met/val	*Boys:* 0 (0%) *Girls:* 0 (0%)	*Boys*: 9 (33.3%) *Girls*: 4 (15.4%)	*Boys:* 18 (66.7%) *Girls:* 22 (84.6%)

* rs6265 (BDNF) could not be tested for the Hardy-Weinberg Equilibrium

Table 3. Effect of SNPs on Hippocampal Ratio, TIC or Uncorrected Hippocampal Volume in Boys and Girls

BOYS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
CRHR1 (rs110402)	r= 0.285 p =0.149	r= 0.353^{+} p= 0.071	r= -0.391* p= 0.044	r= 0.018 p= 0.927	r= 0.049 p= 0.810
GABRA6 (rs3219151)	r= 0.064 p= 0.752	r= -0.001 p= 0.994	r= -0.098 p= 0.626	r= -0.033 p= 0.871	r= -0.079 p= 0.694
NR3C1 (rs41423247	r= -0.028 p= 0.892	r= -0.171 p= 0.393	r= 0.280 p= 0.157	r= 0.143 p= 0.476	r= 0.044 p= 0.828
COMT (rs4680)	r= 0.561** p= 0.002	r= 0.334^{+} p= 0.089	r= 0.284 p= 0.152	r= 0.611** p= 0.001	r= 0.472* p= 0.013
5HT2A (rs6311)	r= -0.278 p= 0.161	r= -0.023 p= 0.910	r= -0.016 p= 0.938	r= -0.252 p= 0.205	r= -0.047 p= 0.817
BDNF (rs6265)	r= -0.369^{+} p= 0.058	r= -0.160 p= 0.426	r= -0.062 p= 0.760	r= -0.352^{+} p= 0.072	r= -0.187 p= 0.350
GIRLS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
CRHR1 (rs110402)	r= 0.224 p= 0.272	r= 0.137 p= 0.505	r= 0.039 p= 0.850	r= 0.241 p= 0.236	r= 0.148 p= 0.470
GABRA6 (rs3219151)	r= 0.175 p= 0.402	r= -0.029 p= 0.892	r= -0.202 p= 0.334	r= 0.076 p= 0.719	r= -0.110 p= 0.602
NR3C1 (rs41423247	r= 0.110 p= 0.594	r= 0.057 p= 0.781	r= 0.225 p= 0.270	r= 0.284 p= 0.160	r= 0.222 p= 0.276
COMT (rs4680)	r= -0.099 p= 0.632	r= -0.259 p= 0.201	r= -0.173 p= 0.397	r= -0.239 p= 0.239	r= -0.433* p= 0.027
5HT2A (rs6311)	r= 0.169 p =0.410	r= 0.098 p= 0.634	r= -0.021 p= 0.917	r= 0.187 p= 0.360	r= 0.114 p= 0.580
BDNF (rs6265)	r= 0.444* p= 0.023	r= 0.324 p= 0.106	r= -0.064 p= 0.756	r= 0.437* p= 0.026	r= 0.311 p= 0.122

r = Pearson's correlation coefficient; p=significance value ($^{+}$ p<0.10, *p<0.05, **p<0.01).

Table 4. Effect of Risk Factors on Hippocampal Volume in Boys and Girls

BOYS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
Mother's Cigarettes During Pregnancy	r= -0.179 p= 0.372	r= -0.202 p= 0.312	r= 0.088 p= 0.662	r= -0.099 p= 0.624	r= -0.110 p= 0.584
Mother's Alcohol During Pregnancy	r= -0.389* p =0.045	r= -0.418* p =0.030	r= -0.212 p =0.289	r= -0.423* p =0.028	r= -0.477* p =0.012
Socioeconomic Status	r= -0.333^{+} p= 0.089	r= -0.281 p= 0.155	r= 0.210 p= 0.293	r= -0.151 p= 0.453	r= -0.980 p= 0.627
Birth Weight	r= 0.309 p= 0.117	r= 0.278 p= 0.161	r= 0.485* p= 0.010	r= 0.531** p= 0.004	r= 0.552** p= 0.003
Timing of exposure during pregnancy	r= 0.062 p= 0.758	r= 0.047 p= 0.817	r= -0.114 p= 0.571	r= -0.020 p= 0.920	r= -0.037 p= 0.854
GIRLS	**Left HPC/TIC Ratio**	**Right HPC/TIC Ratio**	**TIC**	**Left HPC**	**Right HPC**
Mother's Cigarettes During Pregnancy	r= -0.151 p= 0.461	r= -0.314 p= 0.118	r= -0.085 p= 0.679	r= -0.216 p= 0.289	r= -0.413* p= 0.036
Mother's Alcohol During Pregnancy	r= 0.155 p= 0.448	r= 0.313 p= 0.120	r= -0.077 p= 0.707	r= 0.120 p= 0.558	r= 0.307 p= 0.128
Socioeconomic Status	r= 0.449* p= 0.022	r= 0.247 p= 0.224	r= -0.213 p= 0.296	r= 0.327 p= 0.103	r= 0.103 p= 0.615
Birth Weight	r= -0.040 p= 0.851	r= -0.014 p= 0.945	r= 0.057 p= 0.778	r= 0.004 p= 0.985	r= 0.031 p= 0.881
Timing of exposure during pregnancy	r= 0.121 p= 0.556	r= 0.106 p= 0.607	r= 0.002 p= 0.993	r= 0.144 p= 0.482	r= 0.135 p= 0.512

r = Pearson's correlation coefficient; p=significance value ($^{+}$ p<0.10, *p<0.05, **p<0.01).

Table 5. Effect of PNMS, Genotype and Gene-by-Environment Interaction on HPC/TIC Volume in Boys, controlling for mother's alcohol consumption during pregnancy with left and right HPC/TIC volume.

Left Hippocampus/TIC Volume

OBJECTIVE PNMS		PRENATAL STRESS			GENOTYPE			INTERACTION			TOTAL
CRHR1		BETA	R2	p	BETA	CH R2	p	BETA	CH R2	p	R2
	MinorG Merged	0,305	0,093	0,099	0,011	0,000	0,956	0,567	0,021	0,435	0,265
	MajorA Merged	0,305	0,093	0,099	0,209	0,041	0,263	-0,792	0,026	0,373	0,310
COMT											
	MinorA Merged	0,305	0,093	0,099	-0,399	0,149	**0,026**	-1,422	0,014	0,473	0,407
	MajorG Merged	0,305	0,093	0,099	0,233	0,047	0,228	0,626	0,002	0,817	0,293
NR3C1											
	MinorC Merged	0,305	0,093	0,099	-0,125	0,012	0,554	-0,928	0,053	0,209	0,308
	MajorG Merged	0,305	0,093	0,099	0,184	0,034	0,312	4,187	0,050	0,216	0,327
5HT2A											
	MinorA Merged	0,305	0,093	0,099	0,414	0,159	**0,021**	0,484	0,016	0,447	0,418
	MajorG Merged	0,305	0,093	0,099	-0,118	0,013	0,526	0,175	0,001	0,855	0,258
GABARA6											
	MinorA Merged	0,305	0,093	0,099	-0,134	0,017	0,480	-0,949	0,038	0,285	0,298
	MajorG Merged	0,305	0,093	0,099	-0,128	0,014	0,521	0,430	0,001	0,844	0,259
BDNF											
	CC vs. CT	0,305	0,093	0,099	0,323	0,103	0,070	-1,045	0,059	0,154	0,405
SUBJECTIVE PNMS											
CRHR1											
	MinorG Merged	-0,021	0,000	0,911	-0,112	0,011	0,595	0,400	0,007	0,661	0,169
	MajorA Merged	-0,021	0,000	0,911	0,271	0,072	0,157	-2,050	0,221	**0,007**	0,445
COMT											
	MinorA Merged	-0,021	0,000	0,911	-0,448	0,197	**0,015**	0,668	0,022	0,394	0,371
	MajorG Merged	-0,021	0,000	0,911	0,267	0,063	0,188	0,495	0,007	0,658	0,221
NR3C1											
	MinorC Merged	-0,021	0,000	0,911	0,088	0,006	0,698	1,017	0,100	0,100	0,257
	MajorG Merged	-0,021	0,000	0,911	0,171	0,029	0,377	-1,235	0,045	0,273	0,225
5HT2A											
	MinorA Merged	-0,021	0,000	0,911	0,365	0,125	0,059	-1,124	0,075	0,125	0,351
	MajorG Merged	-0,021	0,000	0,911	-0,139	0,018	0,481	1,019	0,051	0,243	0,221
GABARA6											
	MinorA Merged	-0,021	0,000	0,911	-0,206	0,039	0,302	0,104	0,001	0,900	0,191
	MajorG Merged	-0,021	0,000	0,911	0,015	0,000	0,942	1,123	0,019	0,480	0,171
BDNF											
	CC vs. CT	-0,021	0,000	0,911	0,380	0,137	**0,046**	-0,497	0,015	0,500	0,303
COGNITIVE APPRAISAL											
CRHR1											
	MinorG Merged	-0,084	0,007	0,657	-0,084	0,007	0,670	-0,241	0,003	0,786	0,168
	MajorA Merged	-0,084	0,007	0,657	0,264	0,069	0,167	0,522	0,015	0,518	0,242
COMT											
	MinorA Merged	-0,084	0,007	0,657	-0,445	0,194	**0,015**	0,882	0,022	0,388	0,374
NR3C1											
	MinorC Merged	-0,084	0,007	0,657	0,052	0,003	0,788	0,429	0,041	0,299	0,202
	MajorG Merged	-0,084	0,007	0,657	0,188	0,034	0,335	-0,883	0,062	0,188	0,255
5HT2A											
	MinorA Merged	-0,084	0,007	0,657	0,360	0,122	0,061	-0,167	0,002	0,825	0,281
	MajorG Merged	-0,084	0,007	0,657	-0,123	0,013	0,549	-0,546	0,015	0,530	0,186
GABARA6											
	MinorA Merged	-0,084	0,007	0,657	-0,191	0,033	0,340	0,750	0,031	0,359	0,223
BDNF											
	CC vs. CT	-0,084	0,007	0,657	0,362	0,131	0,051	0,727	0,041	0,260	0,329

Right Hippocampus/TIC Volume

OBJECTIVE PNMS		PRENATAL STRESS			GENOTYPE			INTERACTION			TOTAL
		BETA	R2	p	BETA	CH R2	p	BETA	CH R2	p	R2
CRHR1											
	MinorG Merged	0,259	0,067	0,158	-0,164	0,023	0,405	-0,441	0,013	0,540	0,278
	MajorA Merged	0,259	0,067	0,158	0,197	0,036	0,293	-0,498	0,010	0,579	0,289
COMT											
	MinorA Merged	0,259	0,067	0,158	-0,121	0,014	0,521	4,154	0,122	**0,049**	0,378
	MajorG Merged	0,259	0,067	0,158	0,124	0,013	0,527	-1,676	0,013	0,545	0,268
NR3C1											
	MinorC Merged	0,259	0,067	0,158	0,121	0,011	0,567	0,307	0,006	0,683	0,259
	MajorG Merged	0,259	0,067	0,158	0,151	0,022	0,411	6,332	0,114	0,057	0,378
5HT2A											
	MinorA Merged	0,259	0,067	0,158	0,054	0,003	0,776	-0,230	0,004	0,749	0,248
	MajorG Merged	0,259	0,179	0,158	-0,039	0,001	0,834	0,276	0,003	0,776	0,246
GABARA6											
	MinorA Merged	0,259	0,067	0,158	0,139	0,018	0,464	0,471	0,009	0,599	0,269
	MajorG Merged	0,259	0,067	0,158	0,122	0,013	0,539	-0,422	0,001	0,847	0,256
BDNF											
	CC vs. CT	0,259	0,067	0,158	0,115	0,013	0,533	0,092	0,000	0,909	0,255
SUBJECTIVE PNMS											
CRHR1											
	MinorG Merged	0,309	0,096	0,089	-0,140	0,017	0,471	0,632	0,019	0,452	0,306
	MajorA Merged	0,309	0,096	0,089	0,222	0,048	0,214	-0,859	0,039	0,261	0,358
COMT											
	MinorA Merged	0,309	0,096	0,089	-0,186	0,034	0,300	-0,566	0,016	0,485	0,320
	MajorG Merged	0,309	0,096	0,089	0,154	0,021	0,418	0,590	0,010	0,578	0,302
NR3C1											
	MinorC Merged	0,309	0,096	0,089	0,081	0,005	0,703	0,472	0,021	0,421	0,297
	MajorG Merged	0,309	0,096	0,089	0,130	0,017	0,471	-1,556	0,071	0,134	0,358
5HT2A											
	MinorA Merged	0,309	0,096	0,089	0,041	0,002	0,824	-1,051	0,066	0,154	0,338
	MajorG Merged	0,309	0,096	0,089	-0,046	0,002	0,804	1,560	0,119	**0,049**	0,392
GABARA6											
	MinorA Merged	0,309	0,096	0,089	-0,004	0,000	0,982	-0,061	0,000	0,938	0,271
	MajorG Merged	0,309	0,096	0,089	0,125	0,014	0,507	1,576	0,038	0,276	0,323
BDNF											
	CC vs. CT	0,309	0,096	0,089	0,079	0,006	0,668	-0,780	0,037	0,290	0,313
COGNITIVE APPRAISAL											
CRHR1											
	MinorG Merged	0,042	0,002	0,821	-0,238	0,054	0,217	-0,156	0,001	0,855	0,232
	MajorA Merged	0,042	0,002	0,821	0,249	0,061	0,187	1,333	0,097	0,086	0,335
COMT											
	MinorA Merged	0,042	0,002	0,821	-0,177	0,031	0,354	-1,866	0,099	0,091	0,306
NR3C1											
	MinorC Merged	0,042	0,002	0,821	0,220	0,048	0,246	0,312	0,022	0,435	0,246
	MajorG Merged	0,042	0,002	0,821	0,135	0,018	0,484	-1,163	0,108	0,078	0,303
5HT2A											
	MinorA Merged	0,042	0,002	0,821	0,020	0,000	0,919	-0,412	0,010	0,610	0,187
	MajorG Merged	0,042	0,002	0,821	-0,075	0,005	0,711	0,530	0,014	0,540	0,196
GABARA6											
	MinorA Merged	0,042	0,002	0,821	0,062	0,004	0,755	0,301	0,005	0,717	0,185
BDNF	CC vs. CT	0,042	0,002	0,821	0,143	0,020	0,453	-0,119	0,001	0,864	0,198

Table 6. Effect of PNMS, Genotype and Gene-by-Environment Interaction on HPC/TIC Volume in Girls, controlling for socioeconomic status with left HPC/TIC

Left Hippocampus/TIC Volume

OBJECTIVE PNMS		PRENATAL STRESS			GENOTYPE			INTERACTION			TOTAL
CRHR1		BETA	R2	p	BETA	CH R2	p	BETA	CH R2	p	R2
	MinorG Merged	0,106	0,008	0,628	-0,125	0,015	0,514	0,786	0,009	0,617	0,234
	MajorA Merged	0,106	0,008	0,628	0,236	0,056	0,210	-0,924	0,014	0,533	0,279
COMT											
	MinorA Merged	0,106	0,008	0,628	0,002	0,000	0,991	0,653	0,014	0,544	0,224
	MajorG Merged	0,106	0,008	0,628	-0,084	0,007	0,664	-0,527	0,020	0,461	0,237
NR3C1											
	MinorC Merged	0,106	0,008	0,628	-0,139	0,017	0,490	0,305	0,005	0,714	0,232
	MajorG Merged	0,106	0,008	0,628	0,265	0,067	0,167	-1,046	0,063	0,171	0,340
5HT2A											
	MinorA Merged	0,106	0,008	0,628	-0,098	0,009	0,625	-0,329	0,002	0,803	0,221
	MajorG Merged	0,106	0,008	0,628	-0,006	0,000	0,974	-2,059	0,043	0,284	0,253
GABARA6											
	MinorA Merged	0,053	0,002	0,811	-0,111	0,012	0,577	0,622	0,005	0,730	0,215
	MajorG Merged	0,053	0,002	0,811	0,074	0,005	0,712	-0,476	0,009	0,639	0,212
BDNF											
	CC vs. CT	0,106	0,008	0,628	-0,315	0,084	0,119	-0,558	0,010	0,595	0,304
SUBJECTIVE PNMS											
CRHR1											
	MinorG Merged	0,049	0,002	0,799	-0,113	0,013	0,557	1,007	0,021	0,453	0,237
	MajorA Merged	0,049	0,002	0,799	0,241	0,058	0,202	-0,594	0,024	0,413	0,285
COMT											
	MinorA Merged	0,049	0,002	0,799	0,002	0,000	0,994	3,266	0,329	**0,001**	0,533
	MajorG Merged	0,049	0,002	0,799	-0,096	0,009	0,617	-0,620	0,033	0,351	0,245
NR3C1											
	MinorC Merged	0,049	0,002	0,799	-0,117	0,013	0,554	0,032	0,000	0,951	0,217
	MajorG Merged	0,049	0,002	0,799	0,251	0,061	0,191	-0,808	0,123	0,053	0,387
5HT2A											
	MinorA Merged	0,049	0,002	0,799	-0,073	0,005	0,713	-0,064	0,000	0,968	0,209
	MajorG Merged	0,049	0,002	0,799	-0,004	0,000	0,983	0,355	0,005	0,720	0,209
GABARA6											
	MinorA Merged	-0,004	0,000	0,985	-0,116	0,012	0,575	2,758	0,005	0,718	0,213
	MajorG Merged	-0,004	0,000	0,985	0,077	0,006	0,707	-0,932	0,047	0,277	0,248
BDNF											
	CC vs. CT	0,049	0,002	0,799	-0,333	0,088	0,112	0,457	0,018	0,466	0,310
COGNITIVE APPRAISAL											
CRHR1											
	MinorG Merged	0,227	0,048	0,236	-0,065	0,004	0,734	-0,474	0,007	0,665	0,260
	MajorA Merged	0,227	0,048	0,236	0,204	0,040	0,280	0,679	0,029	0,358	0,318
COMT											
	MinorA Merged	0,227	0,048	0,236	0,035	0,001	0,857	-0,334	0,003	0,758	0,254
	MajorG Merged	0,227	0,048	0,236	-0,114	0,013	0,539	0,517	0,022	0,432	0,284
NR3C1											
	MinorC Merged	0,227	0,048	0,236	-0,024	0,000	0,905	0,141	0,003	0,791	0,253
	MajorG Merged	0,227	0,048	0,236	0,212	0,041	0,270	0,056	0,000	0,919	0,291
5HT2A											
	MinorA Merged	0,227	0,048	0,236	-0,023	0,000	0,907	0,607	0,018	0,476	0,268
	MajorG Merged	0,227	0,048	0,236	0,053	0,003	0,782	-0,572	0,010	0,596	0,262
GABARA6											
	MinorA Merged	0,194	0,035	0,330	-0,091	0,008	0,641	-0,043	0,000	0,972	0,239
	MajorG Merged	0,194	0,035	0,330	0,064	0,004	0,743	-0,443	0,012	0,582	0,246
BDNF											
	CC vs. CT	0,227	0,048	0,236	-0,270	0,052	0,214	0,582	0,027	0,367	0,329

Right Hippocampus/TIC Volume

		PRENATAL STRESS			GENOTYPE			INTERACTION			TOTAL
OBJECTIVE PNMS											
CRHR1		**BETA**	**R2**	**p**	**BETA**	**CH R2**	**p**	**BETA**	**CH R2**	**p**	**R2**
	MinorG Merged	0,392	0,154	**0,048**	-0,111	0,012	0,568	1,155	0,021	0,464	0,186
	MajorA Merged	0,392	0,154	**0,048**	0,958	0,018	0,490	-1,604	0,063	0,192	0,234
COMT											
	MinorA Merged	0,392	0,154	**0,048**	0,122	0,015	0,529	0,791	0,021	0,462	0,189
	MajorG Merged	0,392	0,154	**0,048**	0,309	0,066	0,177	-0,816	0,049	0,235	0,269
NR3C1											
	MinorC Merged	0,392	0,154	**0,048**	-0,058	0,003	0,775	0,897	0,044	0,281	0,201
	MajorG Merged	0,392	0,154	**0,048**	0,249	0,060	0,197	-0,416	0,010	0,599	0,224
5HT2A											
	MinorA Merged	0,392	0,154	**0,048**	-0,018	0,000	0,926	-0,441	0,007	0,684	0,161
	MajorG Merged	0,392	0,154	**0,048**	0,631	0,006	0,700	-1,562	0,025	0,421	0,184
GABARA6											
	MinorA Merged	0,344	0,118	0,092	0,103	0,011	0,609	1,605	0,034	0,369	0,163
	MajorG Merged	0,344	0,118	0,092	0,023	0,009	0,634	0,130	0,001	0,898	0,128
BDNF											
	CC vs. CT	0,392	0,154	**0,048**	0,269	0,050	0,241	0,046	0,000	0,965	0,204
SUBJECTIVE PNMS											
CRHR1											
	MinorG Merged	0,023	0,001	0,911	-0,068	0,005	0,746	0,843	0,015	0,567	0,020
	MajorA Merged	0,023	0,001	0,911	0,151	0,023	0,472	-0,616	0,026	0,450	0,049
COMT											
	MinorA Merged	0,023	0,001	0,911	0,116	0,013	0,589	2,580	0,211	**0,023**	0,224
	MajorG Merged	0,023	0,001	0,911	-0,316	0,100	0,124	-0,719	0,045	0,296	0,145
NR3C1											
	MinorC Merged	0,023	0,001	0,911	0,067	0,004	0,754	0,505	0,036	0,376	0,040
	MajorG Merged	0,023	0,001	0,911	0,174	0,030	0,405	-0,516	0,050	0,286	0,081
5HT2A											
	MinorA Merged	0,023	0,001	0,911	-0,001	0,000	0,996	0,642	0,006	0,710	0,007
	MajorG Merged	0,023	0,001	0,911	0,172	0,027	0,435	0,301	0,004	0,778	0,031
GABARA6											
	MinorA Merged	-0,063	0,004	0,764	0,106	0,010	0,638	4,385	0,015	0,579	0,029
	MajorG Merged	-0,063	0,004	0,764	0,139	0,018	0,529	-0,881	0,042	0,343	0,064
BDNF											
	CC vs. CT	0,023	0,001	0,911	-0,355	0,112	0,101	0,396	0,014	0,565	0,126
COGNITIVE APPRAISAL											
CRHR1											
	MinorG Merged	-0,019	0,000	0,928	-0,076	0,005	0,726	-0,769	0,019	0,523	0,024
	MajorA Merged	-0,019	0,000	0,928	0,160	0,025	0,454	1,259	0,102	0,124	0,127
COMT											
	MinorA Merged	-0,019	0,000	0,928	0,108	0,011	0,615	-0,571	0,010	0,635	0,022
	MajorG Merged	-0,019	0,000	0,928	-0,317	0,100	0,123	1,403	0,166	**0,036**	0,267
NR3C1											
	MinorC Merged	-0,019	0,000	0,928	0,074	0,005	0,749	-0,494	0,032	0,400	0,037
	MajorG Merged	-0,019	0,000	0,928	0,194	0,035	0,371	-0,139	0,003	0,793	0,038
5HT2A											
	MinorA Merged	-0,019	0,000	0,928	-0,008	0,000	0,972	0,958	0,048	0,303	0,048
	MajorG Merged	-0,019	0,000	0,928	0,167	0,026	0,437	-1,211	0,046	0,306	0,073
GABARA6											
	MinorA Merged	-0,083	0,007	0,693	0,112	0,012	0,603	-1,226	0,039	0,359	0,059
	MajorG Merged	-0,083	0,007	0,693	0,115	0,013	0,590	-0,878	0,059	0,261	0,079
BDNF											
	CC vs. CT	-0,019	0,000	0,928	-0,358	0,118	0,093	0,558	0,025	0,432	0,143

Figure 1. Effect of rs4680 (COMT) Genotype on Hippocampus/TIC Ratio Volume in Boys (1a) and Girls (1b)

a) Boys: ANOVA and Tukey post hoc test demonstrated that in boys, the COMT major genotype is associated with greater left hippocampus/TIC ratio volume (p=0.0008, F=5.917, df=2), but does not affect right hippocampus/TIC ratio volume (p=0.241, F=1.512, df=2). b) Girls: ANOVA and Tukey post hoc test that in girls, the COMT genotype does not affect left (p=0.871, F=0.139, df=2) or right (p=0.286, F=1.324, df=2) hippocampus/TIC ratio volume.

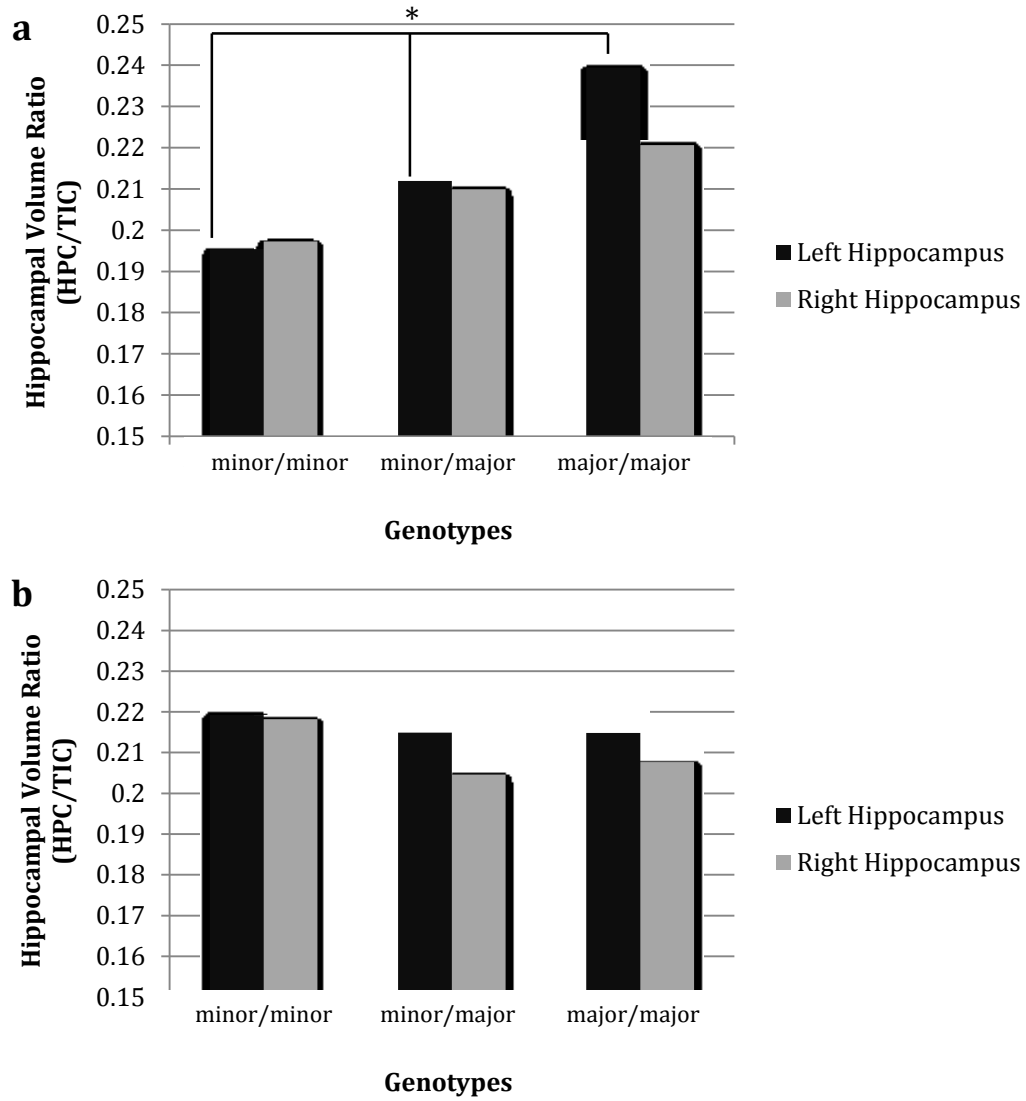


Figure 2. Effect of rs6265 (BDNF) Genotype on Hippocampus/TIC Ratio Volume in Boys (2a) and Girls (2b).

a) Boys: A t-test demonstrated that in boys the major BDNF genotype is associated with smaller left ($p<0.001$, $t=12.301$, $df=26$) and right ($p<0.001$, $t=12.225$, $df=26$) hippocampus/TIC ratio volume than the heterozygote genotype. b) Girls: A t-test demonstrated that in girls, the major genotype is associated with a larger left ($p<0.001$, $t=12.658$, $df=25$) and right ($p<0.001$, $t=12.09$, $df=25$) hippocampus/TIC ratio volume than heterozygotes.

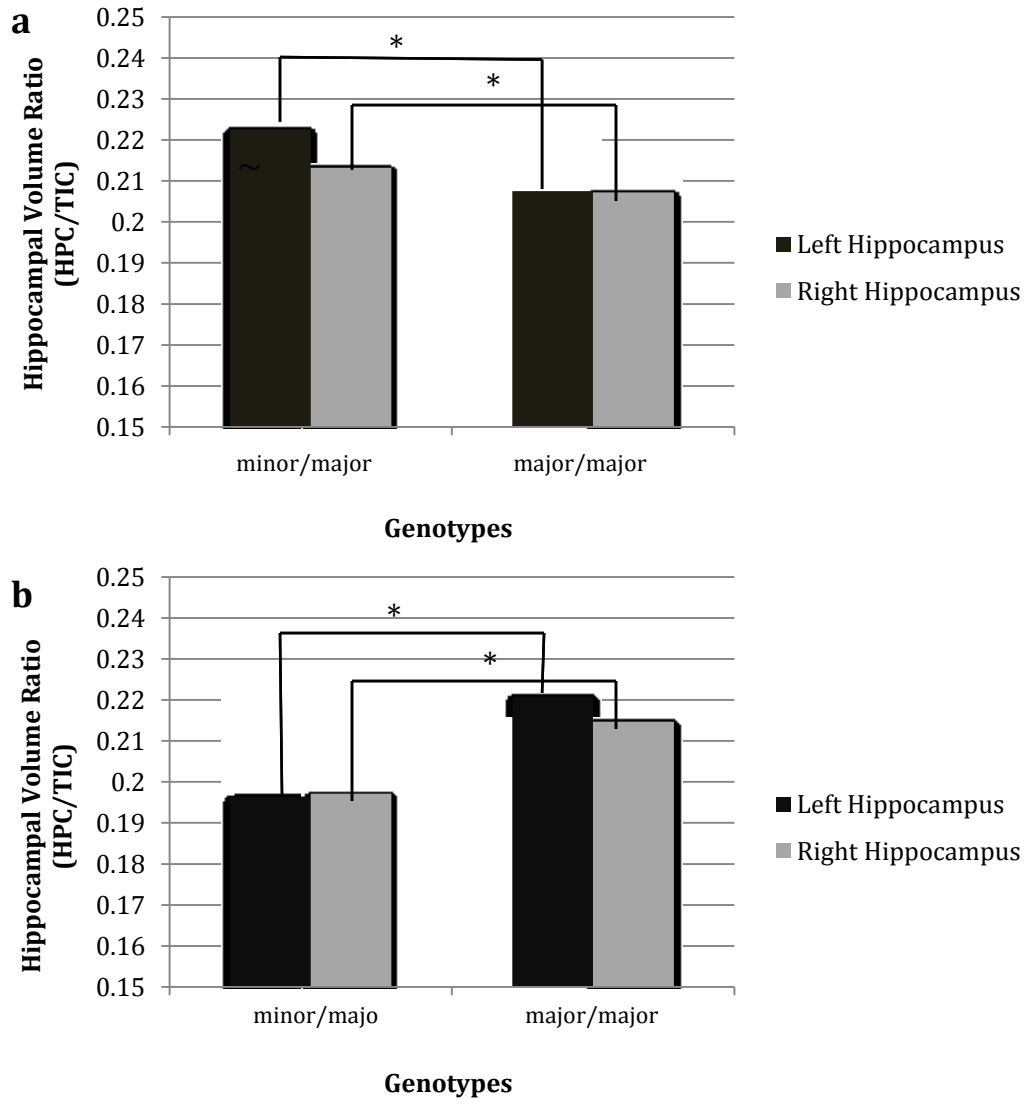


Figure 3. COMT moderates objective PNMS on right hippocampus/TIC ratio volume in boys.

For the major homozygote COMT genotype (black) there is a significant association between objective PNMS and right hippocampus/TIC ratio volume (p=0.0840); however, for the heterozygote and minor homozygote COMT genotypes (gray) there is no association between objective PNMS and right hippocampus/TIC ratio. Moderation analyses demonstrate that there is a significant COMT-by-ObjectivePNMS interaction effect in boys (p=0.0494). There is a region of significance between genotypes at objective PNMS levels below 10.86. The full model explains 37.8% of the variance in right hippocampus/TIC ratio volume.

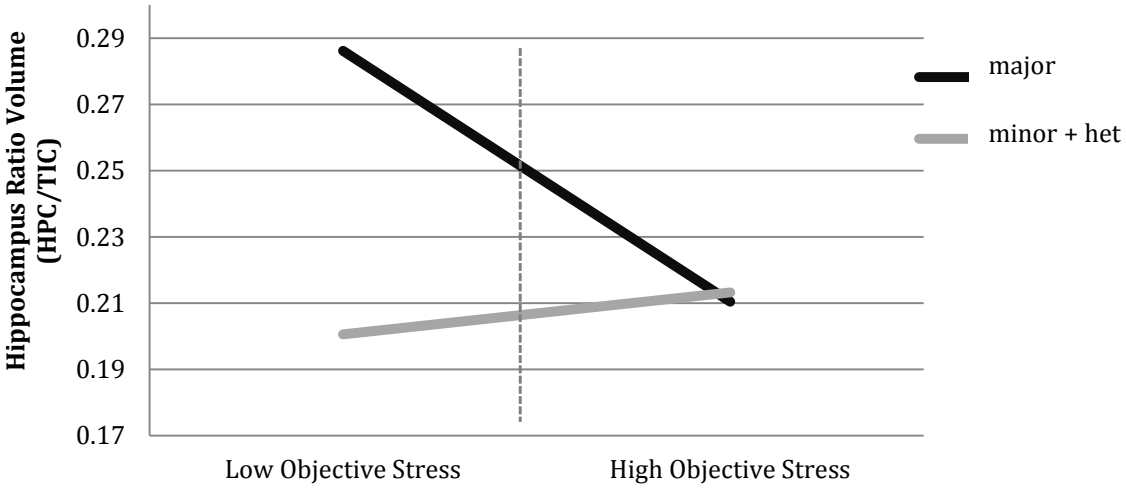


Figure 4. CRHR1 moderates subjective PNMS on left hippocampus/TIC ratio volume in boys.

The major homozygotes combined with heterozygotes (black) had a significant negative association with subjective PNMS (p=0.033), while the minor homozygotes (gray) had a marginally significant negative association with subjective PNMS (p=0.057). Moderation analyses demonstrate that there is a significant CRHR1-by-SubjectivePNMS interaction effect in boys (p=0.0072). There is a region of significance between genotypes below subjective PNMS levels of 1.71.

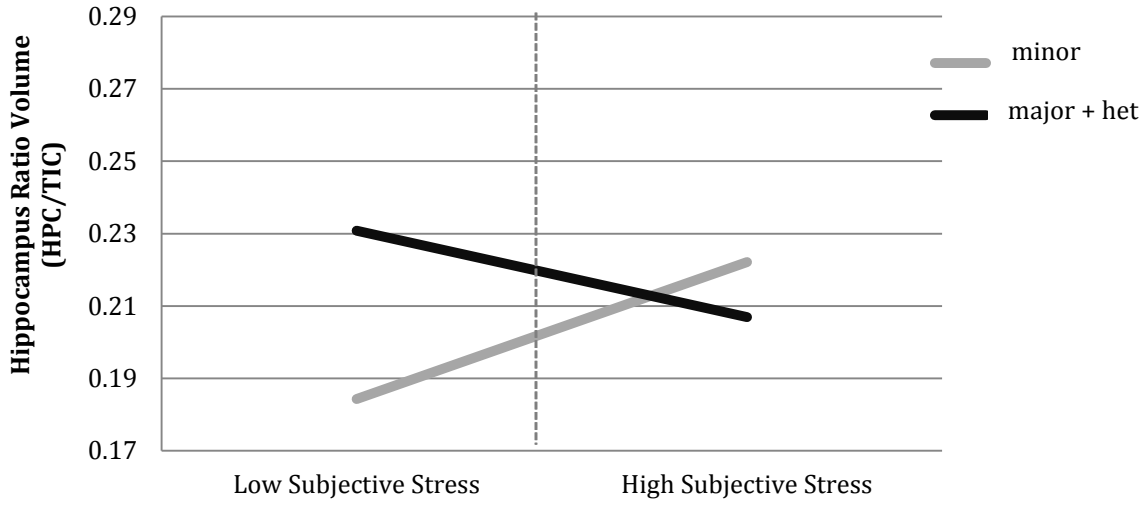


Figure 5. 5HT2A moderates subjective PNMS on right hippocampus/TIC ratio volume in boys.

For major homozygotes and heterozygotes (black) there was a positive association between subjective PNMS and right hippocampus/TIC volume (p=0.015); however, for minor homozygotes (gray) there was no association between subjective PNMS and right hippocampus/TIC ratio volume. Moderation analyses demonstrate that there is a significant 5HT2A-by-subjectivePNMS interaction effect in boys (p=0.049). There are no regions of significance.

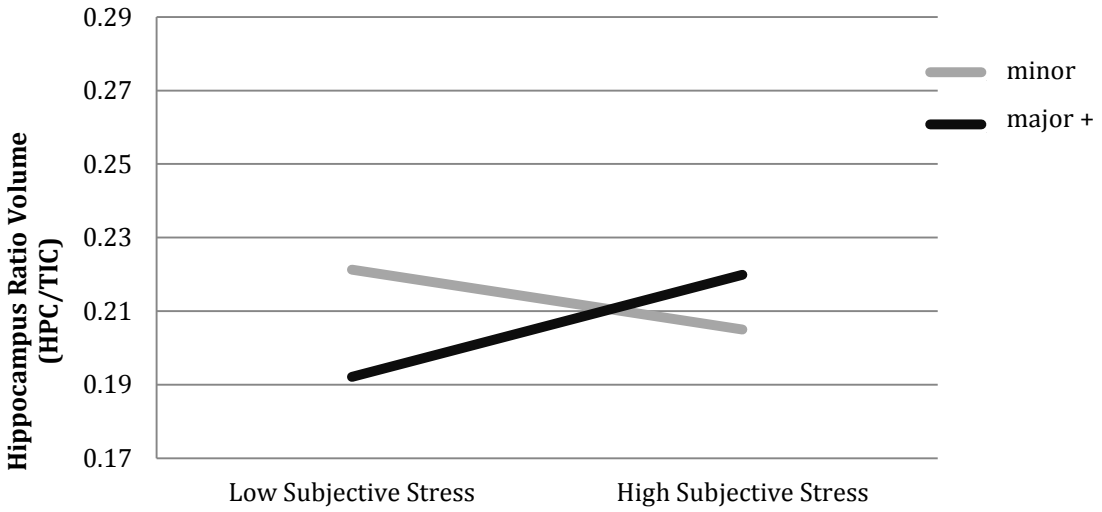


Figure 6. COMT moderates subjective PNMS on left (6a) and right (6b) hippocampus/TIC ratio volume in girls.
a) Left: for major homozygotes (black), there is a significant association between subjective PNMS and left hippocampus/TIC ratio (p=0.0024) but for minor homozygotes and heterozygotes (gray) there is no association between subjective PNMS and left hippocampus/TIC ratio (p=0.129). Moderation analyses demonstrate that there is a significant COMT-by-SubjectivePNMS interaction in girls (p=0.0009). There are regions of significance between genotypes below subjective PNMS levels of 1.52 and above 2.77. b) Right: for major homozygotes (black) there is a significant association between subjective PNMS right hippocampus/TIC ratio volume (p=0.040); however, for minor homozygotes and heterozygotes (gray) there is no significant association between subjective PNMS and hippocampus/TIC ratio volume (p=0.272). Moderation analyses demonstrate that there is a significant COMT-by-SubjectivePNMS interaction effect in girls (p=0.023). There is a region of significance between genotypes above subjective PNMS levels of 2.98.

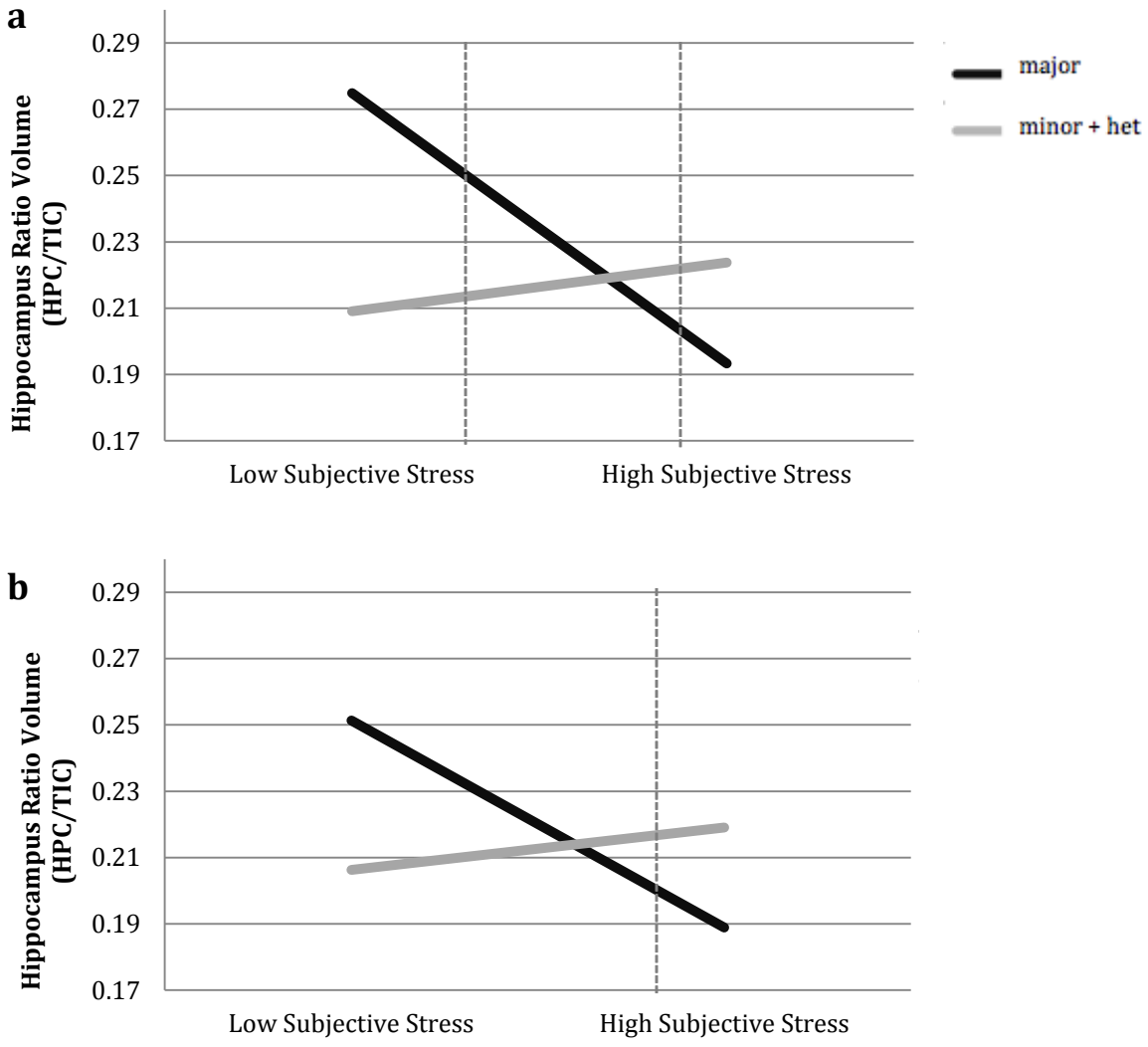
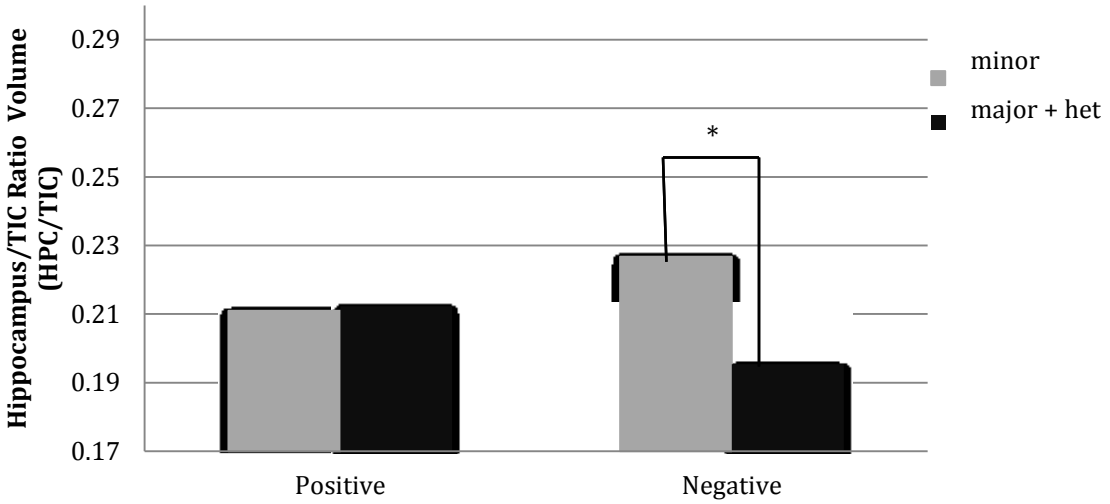


Figure 7. COMT moderates cognitive appraisal on the right hippocampus/TIC ratio volume in girls.

For minor homozygotes (gray) as well as major homozygote and heterozygotes (black) there is no significant difference between offspring of mothers who reported positive cognitive appraisal and negative cognitive appraisal. However when mothers reported negative cognitive appraisal, there is a significant difference between minor homozygotes compared to major homozygotes and heterozygotes (p=0.010). Moderation analyses demonstrate that there is a significant COMT-by-cognitive appraisal interaction effect in girls (p=0.036).



Appendix A

Questions used to assess the four dimensions (Threat, Loss, Scope, and Change) of our Objective Stress Questionnaire that the mothers completed shortly after the ice storm.

	Threat		Loss		Scope		Change
1	Were you injured? No = 0 Yes = 1	1	Did your residence suffer damage as a result of the ice storm? No = 0 Yes = 2	1	How many days were you without electricity? 0 = 0 – 5 days 1 = 6 – 13 days 2 = 14 – 19 days 3 = 20 – 21 days 4 = >22 days	1	Did your family stay together for the duration of the ice storm? Yes = 0 No = 1
2	Was anyone close to you injured? No = 0 Yes = 1	2	Did you experience a loss of personal income? No = 0 Yes = 2	2	How many days were you without the use of your telephone? 0 = 0 days 1 = .01 – 1 day 2 = 2 – 4.5 days 3 = 5 – 7 days 4 = 8+ days	2	Did you spend any time in a temporary shelter? No = 0 Yes = 1
3	Were you ever in danger due to:	3	How much was the total financial loss including income, food, damage to home? 0 = < $100 1 = $100 - $1000 2 = $1000 - $10000 3 = $10000 – 100000 4 = > $100000			3	How often were you required to change residence during the ice storm? 0 = 0 1 = 1 time 2 = 2+ times

3.1 …the cold

No = 0

Yes = 1

3.2 …exposure to downed electrical power lines

No = 0

Yes = 1

3.3 ...exposure to carbon monoxide

No = 0

Yes = 1

3.4 ...lack of potable water

No = 0

Yes = 1

3.5 ...lack of food

No = 0

Yes = 1

3.6 …falling branches and ice

No = 0

Yes = 1

4 Did you take in guests during the ice storm?

No = 0

Yes = 1

5 Did you experience an increase in physical work during the ice storm?

0 = less or same

1 = little or lot more

6 Number of nights away from home:

0 = none

1 = 1 – 7.5 nights

2 = 8+ nights

8 points	8 points	8 points	8 points

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