# SCHIZOPHRENIA: A CONSEQUENCE OF GENE-ENVIRONMENT INTERACTIONS?

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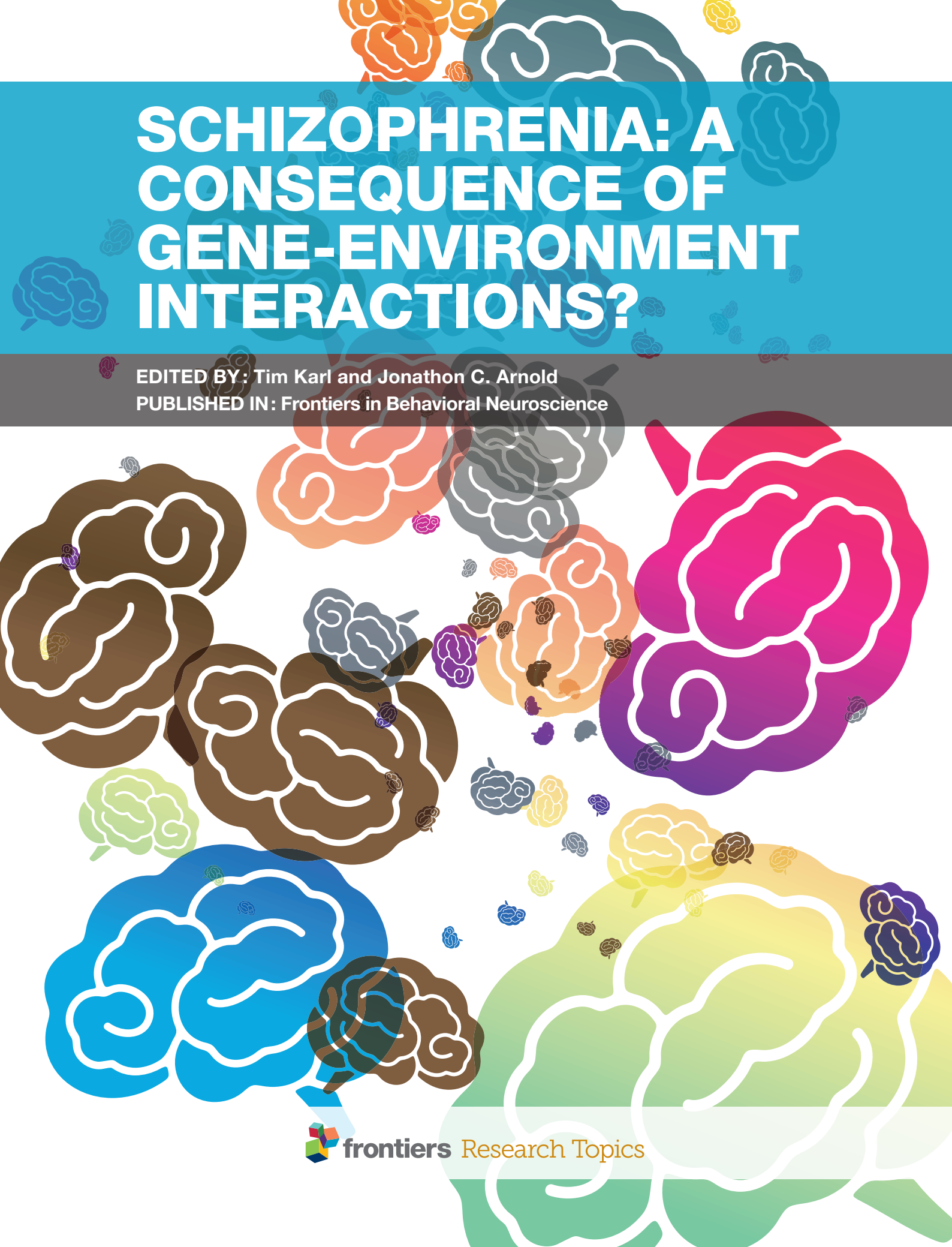

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# SCHIZOPHRENIA: A CONSEQUENCE OF GENE-ENVIRONMENT INTERACTIONS?

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Schizophrenia is a multi-factorial disease characterized by a high heritability and environmental risk factors (e.g. stress and cannabis use). In recent years, an increasing number of researchers worldwide have started investigating the 'two-hit hypothesis' of schizophrenia predicting that genetic and environmental risk factors interactively cause the development of the disorder. This work is starting to produce valuable new animal models and reveal novel insights into the pathophysiology of schizophrenia. Eventually, the research might help advance studies of the molecular pathways involved in this mental disorder and propose more specific molecular medicine. However, the complexity of this multi-factorial line of research has also caused difficulties in data interpretation and comparison.

The intent of our research topic is to cover past and current directions in research that focuses on the understanding and measurement of gene-environment interactions (GxE) in schizophrenia, the neurobiological and behavioural consequences of such interactions as well as the challenges and limitations one encounters when working on complex aetiological systems.

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# Schizophrenia: a consequence of gene-environment interactions?

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**Keywords: Schizophrenia, gene, environment, interaction, GxE, animal model, two-hit hypothesis**

Schizophrenia is a multi-factorial disease characterized by a high heritability and environmental risk factors (e.g., social stress and cannabis use). It is the combined action of multiple genes of small effect size (Owen et al., 2005) and a number of environmental risk factors (McGrath et al., 2004), which causes the development of this mental disorder (Mackay-Sim et al., 2004). This is conceptualized in the "Two-Hit Hypothesis" of schizophrenia, which predicts that genetic and environmental risk factors interactively (GxE interaction) cause the development of the disorder (Bayer et al., 1999; Caspi and Moffitt, 2006). GxE interactions occur when the expression of an individual's genetic predisposition is dependent on the environment they are living in or when environmental influences on a trait differ according to an individual's genome (Tsuang et al., 2004). Human studies have confirmed that nature and nurture are both important in the development of schizophrenia: the concordance rate in monozygotic twins is only around 50% (Tsuang et al., 2001) and genome wide association studies fail to identify major genetic candidates for schizophrenia (Sanders et al., 2008) suggesting an important role of environmental factors in the development of this disorder.

Research into GxE is starting to produce valuable new animal models and has revealed novel insights into the pathophysiology of schizophrenia. This work will help advance our understanding of the molecular pathways involved in this mental disorder and will lead to more specific treatment avenues. Furthermore, understanding GxE interactions will guide the development of new preventative measures (i.e., people genetically predisposed to schizophrenia will be able to minimize exposure to critical environmental risk factors). However, these outcomes are a long way ahead and the complexity of this multi-factorial line of research has also caused difficulties in data interpretation and comparison. Thus, this research topic is intended to cover past and current directions in research dedicated to the understanding of GxE interactions in schizophrenia, the molecular, neurobiological, and behavioral consequences of these interactions, and potential mechanisms involved.

Animal models can incorporate highly standardized genetic and environmental risk factors at critical stages of brain development. These models thereby attempt to mimic the etiology of schizophrenia and help elucidate relevant interactions between GxE and the underlying mechanisms. In this Research Topic, Cash-Padgett and Jaaro-Peled outline how either early immune activation (i.e., PolyI:C treatment) or forms of social stress (i.e., social isolation, chronic social defeat) influence the development of schizophrenia-relevant phenotypes in a number of genetic mouse models for the schizophrenia susceptibility gene *Disrupted in schizophrenia-1* (*DISC1*). Their review concludes that animal models can be instrumental in linking the relevance of GxE interactions to a particular neuropsychiatric disorder. Furthermore, "environmental interaction profiles" can be developed for major risk genes such as *DISC1* (Cash-Padgett and Jaaro-Peled, 2013). Karl as well as Chohan and co-workers focus on the role the schizophrenia susceptibility gene *neuregulin 1* (*NRG1*) might play in the context of GxE interactions. Karl's review overviews a raft of evidence supporting *Nrg1* as a key player in the vulnerability of mice to environmental (risk) factors such as stress, cannabis abuse and housing conditions (Karl, 2013). Following on from recent published work (Chohan et al., 2014a), Chohan et al. describes research in which partial genetic deletion of *Nrg1* modulates the effects of adolescent stress on N-methyl-d-aspartate receptors (NMDAR), with the *Nrg1*-stress interaction tending to decrease NMDAR binding in the medial prefrontal cortex (Chohan et al., 2014b). This is significant as NMDAR is decreased in brains of schizophrenia patients and implicated in the pathophysiology of the disorder. In another research paper, Klug and van den Buuse investigate the effects of young-adult cannabinoid exposure on BDNF deficient mice and find no interaction in this GxE model on learning and memory later in life. Their research highlights that not all GxE models produce interactive effects and that the role of BDNF in conferring vulnerability to the actions of cannabis may be sex-dependent and only evident after pre-exposure to the drug (Klug and van den Buuse, 2013).

Jiang and coworkers provide an excellent review outlining the potential mechanisms behind established GxE interactions using mice with conditional knock down of NMDAR in cortical and hippocampal interneurons during early postnatal development. They suggest that NMDAR hypofunction and social isolation stress interact to disturb parvalbumin-positive (PV) interneurons via oxidative stress mediated by the transcriptional coactivator peroxisome proliferator activated receptor γ coactivator

1α (PGC-1α) (Jiang et al., 2013). Reductions in PV expression have been noted in schizophrenia brain and may help explain asynchrony of neuronal networks (Lewis and Gonzalez-Burgos, 2006). Showing that oxidative stress is responsible for PV interneuron dysfunction resonates with the recent interest in antioxidants such as fish oil as new treatments for mental disorder, particularly prior to disease onset. Extending on the idea of early intervention treatments as the key to treating schizophrenia is the research paper of Van Vugt and colleagues who show that a lack of early maternal care exacerbated schizophrenia-relevant phenotypes in a pharmacological model of the disorder (Van Vugt et al., 2014).

Girardi and coworkers also utilize maternal deprivation to investigate another aspect of the "Two-Hit Hypothesis" of schizophrenia, namely, environment x environment interactions (ExE). They report that maternally deprived rats are vulnerable to the effects of a very mild stress procedure (i.e., saline injection) on corticosterone levels and social behaviors (Girardi et al., 2014). This study is important for studies into GxE interactions, as researchers must be aware that even a relatively minor intervention such as an i.p. injection can modify the stress response in an established environmental model of schizophrenia. Thus, ExE (as well as GxExE) interactions exist and could have a significant impact on established GxE model systems. Within this context, Turner and Burne point out that housing conditions and genetic background both impact on cognitive behaviors of rodents. As cognition is an important domain for the evaluation of face validity of rodent models for schizophrenia, rodent strain and housing conditions must carefully be considered when developing novel model systems for GxE (Turner and Burne, 2013). The last two studies are in line with what is discussed by Burrows and Hannan. Their opinion paper outlines the need for more accurate and sophisticated GxE animal models of schizophrenia and the impact "unexpected" housing conditions can have on genetic mouse models. Environmental factors with clinical relevance should be manipulated to broaden the relevance of preclinical research beyond "standard laboratory housing" and to understand how a decanalized brain produces suboptimal phenotypes' (Burrows and Hannan, 2013).

Godar and Bortolato review evidence suggesting that gene x sex interactions (GxS) are also evident in schizophrenia. They outline that genes encoding enzymes that regulate dopamine levels, such as catechol-O-methyl transferase (COMT) and *monoamine oxidase,* are sexually dimorphic and the impact of sex hormones on their regulation may play a significant role in shaping the course of schizophrenia (Godar and Bortolato, 2014). This is particularly relevant seeing males tend to manifest the disorder earlier than females. Sexual dimorphism also influences GxE as highlighted above in the work of Klug and co-workers (Klug and van den Buuse, 2013) and as noted in various studies reviewed by Karl (2013) in this Research Topic. Importantly, COMT has been shown to be a good candidate for GxE interactions as the gene interacts with adolescent cannabis abuse (Caspi and Moffitt, 2006). Finally, Miller assessed the impact of photic cues on the development of schizophrenia. Her argument for a role for photic cues in the disorder is made more compelling given that single nucleotide polymorphisms have been found in genes relevant to schizophrenia, which are modulated by photoperiod and sunlight intensity (Miller, 2013).

GxE interactions appear to be complex and sensitive to a number of subtle variables, but do exist and justify the need for future research in this area (Van Os et al., 2010). Animal researchers should focus on models with significant relevance to schizophrenia such as cannabis abuse, maternal immunization, or early life stress and consider a number of genetic candidates for GxE interactions. Importantly, some of the articles of this topic suggest that valid GxE animal models will be very sensitive to the laboratory environment, which demands a high level of transparency and standardization of test conditions as well as thorough consideration of housing conditions across research sites. Furthermore, it should be mentioned that there are discussions about the appropriate statistical modeling of GxE interactions, which influence both animal and human research (Zammit et al., 2010).

To conclude, animal models have provided compelling evidence for GxE in schizophrenia based on the greater experimental control possible and the ability to manipulate specific genes and environmental factors. While such studies cannot reproduce the entire complexity of schizophrenia, they do provide simplified multifactorial models of aspects of the condition. These, in turn, allow new molecular and neurobiological insights to be gained and novel drug targets to be discovered.

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# DISC1 mouse models as a tool to decipher gene-environment interactions in psychiatric disorders

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*DISC1* was discovered in a Scottish pedigree in which a chromosomal translocation that breaks this gene segregates with psychiatric disorders, mainly depression and schizophrenia. Linkage and association studies in diverse populations support *DISC1* as a susceptibility gene to a variety of neuropsychiatric disorders. Many *Disc1* mouse models have been generated to study its neuronal functions. These mouse models display variable phenotypes, some of them relevant to schizophrenia, others to depression. The *Disc1* mouse models are popular genetic models for studying gene-environment interactions in schizophrenia. Five different *Disc1* models have been combined with environmental factors. The environmental stressors employed can be classified as either early immune activation or later social paradigms. These studies cover major time points along the neurodevelopmental trajectory: prenatal, early postnatal, adolescence, and adulthood. Various combinations of molecular, anatomical and behavioral methods have been used to assess the outcomes. Additionally, three of the studies sought to rescue the resulting abnormalities. Here we provide background on the environmental paradigms used, summarize the results of these studies combining *Disc1* mouse models with environmental stressors and discuss what we can learn and how to proceed. A major question is how the genetic and environmental factors determine which psychiatric disorder will be clinically manifested. To address this we can take advantage of the many *Disc1* models available and expose them to the same environmental stressor. The complementary experiment would be to expose the same model to different environmental stressors. *DISC1* is an ideal gene for this approach, since in the Scottish pedigree the same chromosomal translocation results in different psychiatric conditions.

**Keywords: DISC1, mouse models, gene-environment, schizophrenia, depression, immune activation, social stress**

## INTRODUCTION

Both schizophrenia and depression have a strong genetic component but are also heavily influenced by the environment. Based on epidemiological studies, environmental schizophrenia risk factors include infections (especially during fetal development), obstetric complications (Mittal et al., 2008), advanced paternal age (Malaspina, 2001) adolescent cannabis use (Henquet et al., 2008), and several forms of stress (reviewed in Brown, 2011). In contrast to the wide variety of factors implicated in schizophrenia, stress in particular stands out as the major environmental risk factor for major depression. Early life stress, involving childhood maltreatment or traumatic events, can be particularly devastating (reviewed in Mann and Currier, 2006). Highly stressful life events, such as death of a family member or divorce are causally associated with onset of episodes of major depression (Kendler et al., 1999).

Here we review a group of studies that utilize mouse models of *DISC1*, a susceptibility gene for psychiatric disorders, to examine the interaction between genetic and environmental risk factors. We start by summarizing human data on the environmental factors most relevant to these studies, infections, and social stress, and their respective experimental paradigms in mice.

## ENVIRONMENT

### ENVIRONMENT: INFECTIONS (REVIEWED IN BROWN, 2011)

Prenatal and early postnatal infections have been implicated in a number of major neurodevelopmental disorders. Direct infection of the fetus can cause serious congenital brain anomalies and mental retardation. Although schizophrenia typically arises in adolescence or young adulthood, it is increasingly regarded as neurodevelopmental in origin (Weinberger, 1996; Cannon et al., 2003; Allardyce et al., 2005; Jaaro-Peled et al., 2009; Shoji et al., 2012). This leads to the hypothesis that subtle perturbations in the developing brain, such as infection of the pregnant mother, may increase the risk of schizophrenia later on in the life of the offspring. Birth cohort studies have established an association between specific prenatal infections and elevated risk for schizophrenia. The largest effects have been attributed to influenza (Brown et al., 2004a), elevated maternal immunoglobulin G (IgG) to the parasite Toxoplasma gondii (Remington, 2006), and periconceptional genital or reproductive infection (Babulas et al., 2006).

A parsimonious explanation of how different viruses/parasites/bacteria increase the risk of the same disorder is suggested by the convergence of their pathogenic mechanism: stimulation of the maternal innate immune response and of

cytokines in particular (Gilmore and Jarskog, 1997). Birth cohort studies support a role for excess maternal cytokines in the development of schizophrenia among offspring (Brown et al., 2004b). As with many proteins originally identified in the immune system, cytokines are also expressed in the nervous system and modulate many aspects of neural development and physiology (Szelenyi, 2001). Transplacental transfer of maternally produced cytokines and production of cytokines at the maternal-fetal interface both lead to an elevation of these molecules in the fetal brain (Meyer et al., 2009). Earlier inflammation is expected to have a more severe effect on brain development due to disruption of fundamental neurodevelopmental processes such as cell proliferation and differentiation, and may predispose the developing nervous system to fail in subsequent cell migration, target selection, and synapse maturation (Figure 6 in Meyer et al., 2007).

Although cytokines are the main suspects in mediating the detrimental effect of the pathogenic interaction between the maternal immune system and fetal brain, other mechanisms do exist. For example, while influenza is not believed to cross the placental barrier, it elicits IgG antibodies which do and subsequently cross-react with fetal brain antigens via molecular mimicry (Wright et al., 1999). Pathogens may also dysregulate brain development and function through a direct interaction between pathogen nucleic acids/proteins and (fetal) brain nucleic acids/proteins or modification of the fetal epigenome (Waterland and Michels, 2007). Some schizophrenia susceptibility genes may affect pathogen virulence, while the pathogens themselves may affect host genes and neural processes (Carter, 2009). For example, DISC1 regulates the microtubule network (Kamiya et al., 2005) that is exploited by viruses for intracellular trafficking.

Although both human and animal studies have mainly focused on maternal/prenatal immune activation, there is also evidence for postnatal infections increasing the risk of schizophrenia. Population-based studies have concluded that childhood viral infections of the central nervous system increase the risk of adult psychotic illness (Dalman et al., 2008; Khandaker et al., 2012).

## ENVIRONMENT: SOCIAL STRESS

Starting as early as prenatal development, environmental stress dysregulates the homeostatic physiological stress response by modulating the hypothalamic-pituitary-adrenal (HPA) axis. This system normally responds to stress by producing glucocorticoids, which can bind receptors throughout the brain and act as transcription factors (Lupien et al., 2009). Neurons in the hypothalamus release corticotrophin releasing hormone (CRH) in response to a stressful environment. CRH triggers secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, in turn leading to production of glucocorticoids (cortisol in humans, corticosterones in mice) by the adrenal gland. Once the stressful situation has passed, negative feedback loops shut down this response and return the HPA axis to a set homeostatic point (O'connor et al., 2000).

A highly stressful social environment can cause maladaptive brain development and function from the prenatal period all the way through old age, and as a result chronic stress is a critical factor in the epidemiology of major mental illness. van Os et al. suggested that "psychotic syndromes can be understood as disorders of adaptation to social context" (Van Os et al., 2010). For example, schizophrenia risk is increased for people raised in an urban environment (Pedersen and Mortensen, 2006). Sub-optimal social conditions have been identified as important mediating factors, in particular low social capital and high social fragmentation (Allardyce et al., 2005; March et al., 2008). Immigrants also suffer from higher risk of psychosis (reviewed in Cantor-Graae and Selten, 2005); two potential explanations of this phenomenon are immigrants' experiences of discrimination and exposure to social defeat, both chronically stressful conditions. Childhood trauma may also increase the risk for psychosis, but more research is needed to establish stronger correlation (Morgan and Fisher, 2007).

The negative effects of chronic environmental stress manifest first in prenatal development, since maternal glucocorticoids circulate freely through the fetal brain. Significantly elevated maternal glucocorticoid levels can result in increased basal HPA axis activity in the offspring up to at least 10 years of age (O'connor et al., 2005). This heightened homeostatic set point results in increased risk for neurological and behavioral disturbances in the offspring (Stott, 1973). Since the human brain undergoes extended postnatal development, the effects of prenatal stress are moderated by the quality of the offspring's environment in early life.

Early life stress, often as a result of childhood maltreatment or traumatic events, decreases mental health in a dose-response fashion (Edwards et al., 2003). It causes dysregulation of the aforementioned stress response systems, leading to increased sensitivity to stress throughout life (reviewed in Heim and Binder, 2012), and has been associated with increased risk for mood and anxiety disorders, posttraumatic stress disorder, unipolar depression, and schizophrenia (Heim et al., 2010). Physiologically, early-life disruption of the HPA axis manifests as an array of abnormalities such as glucocorticoid resistance (Charmandari et al., 2004), increased CRH activity (Cratty et al., 1995), and increased levels of inflammation (McEwen, 1998). Human imaging studies have revealed further effects of early life stress on brain anatomy and function (Papagni et al., 2011). Animal models of early life stress such as variable schedules of maternal separation have provided direct evidence for the causal link between early adverse experience and later brain and behavior abnormalities. Maternal separation has been shown to increase glucocorticoid levels in separated mouse pups and to permanently change glucocorticoid receptor gene expression resulting in excessive glucocorticoid release under stress in adulthood (Anisman et al., 1998).

Detrimental effects on brain and behavior induced by adult chronic stress are reversed after a few weeks of no stress, in contrast to the permanent effects of early life stress (Avital and Richter-Levin, 2005). Exposure to an environmental event during development results in a stable phenotypic alteration in adulthood. This developmental "programming" can alter gene transcription via transcription factors or epigenetic mechanisms (Weaver et al., 2004). In mice, more attentive maternal care of the offspring modulates the selective methylation/demethylation of specific CpG dinucleotides of the glucocorticoid receptor gene (Oberlander et al., 2008). The resulting permanent decrease

in glucocorticoid receptor density, selective to the hippocampus and prefrontal cortex, can increase HPA axis sensitivity to glucocorticoid-mediated negative feedback and lead to a reduction in plasma glucocorticoid levels and stress reactivity throughout the offspring's life (Weaver et al., 2004).

#### STRESS IN ADOLESCENCE (REVIEWED IN ANDERSEN AND TEICHER, 2008; LUPIEN ET AL., 2009)

Both animal and human studies show that adolescence is associated with higher basal and stress-induced activity of the HPA axis compared to both childhood (a stress hypo-responsive period) and adulthood (Vazquez and Akil, 1993; Gunnar et al., 2009). Adolescence is characterized by extensive changes in brain physiology (Andersen, 2003; Jaaro-Peled et al., 2009; Giedd and Rapoport, 2010; O'donnell, 2010), especially among regions involved in social function (Blakemore, 2008). Social stressors during this critical period can therefore potentially have significant long-term consequences on the "social brain" (Van Os et al., 2010). Notably, the brain areas undergoing development and hence most sensitive to stress during adolescence differ between rodents and humans. Rodent hippocampus continues to develop into adulthood, but in humans it is fully developed by 2 years of age (Giedd et al., 1996). While the frontal cortex and amygdala continue to develop in both, the process is more extensive in primates than in other species (Andersen, 2003). Adolescence is the peak age of onset for many psychiatric disorders, including anxiety, mood disorders, eating disorders, psychosis, and substance abuse (Paus et al., 2008).

## GENE × ENVIRONMENT (G × E, REVIEWED IN DUNCAN AND KELLER, 2011)

Gene × environment (G × E) implies synergistic interplay between genes and environment in causing a particular phenotype, where the effect of one is conditional on the other. Genes may modulate sensitivity to the environment, exemplified by a landmark study which demonstrated that a specific serotonin transporter polymorphism moderates sensitivity to life stress contributing to the pathogenesis of depression (Caspi et al., 2003). The environment can impact DNA sequences themselves as well as epigenetic control of risk genes.

Human G × E studies of early life stress and depression have focused mostly on a number of logical candidate genes involved in the serotonin system, neurotrophins, and most of interest to this review, the HPA axis (reviewed in Nugent et al., 2011). Polymorphisms in the glucocorticoid receptor and its regulator FK506 binding protein 5 (FKBP5) as well as in the CRH type I receptor interact with early life stress in predicting depression (Bradley et al., 2008) or in the case of FKBP5, post-traumatic stress disorder (Binder et al., 2008).

First-generation epidemiological G × E studies of psychotic psychiatric disorders have used indirect measures of genetic risk, such as blood relation to a person with schizophrenia, thus representing the complete genetic load including gene × gene interactions. Environmental variables examined in G × E studies include migration, urbanicity, obstetric complications, cannabis use, and stress (Van Os et al., 2008). It is difficult to design human studies that include enough participants to be able to test genetic and environmental risk factors as independent variables alongside any putative gene-environment interaction; any such synergistic interaction might be masked by other genetic and environmental factors. Thus, animal models are utilized in order to rigorously examine the mechanisms and outcomes of G × E interactions.

## ANIMAL MODELS

Epidemiological studies detect correlations which point to potential mechanisms and public health interventions, but causality can only be determined in animal models. These enable the controlled combination of a specific genetic risk factor (on an otherwise uniform genetic background) with a specific environmental risk factor, at a specific developmental time, and detailed observation of the emerging phenotypes over time. Of course, these models have clear limitations of their own; for example, when modeling brain diseases in animals, it is critical to take into account interspecies differences in the timing of brain development. A further complication is that the time course of development differs between different brain regions (cortex, limbic, etc.) and constituents (neurons, glia, blood-brain-barrier, etc.). Most relevant to this review, a large part of gestation in the mouse corresponds to the first half of human pregnancy, and many of the brain maturation events which happen during the second half of human gestation occur postnatally in mice (Clancy et al., 2007).

#### MODELING INFECTIONS IN RODENTS

The effects of specific pathogens have been modeled in animals by prenatal infection with the influenza virus (Shi et al., 2003, 2005) and by adult infection with the Toxoplasma parasite (Vyas et al., 2007). More general effects of immune system activation have been modeled by injecting the bacterial endotoxin lipopolysaccharide (Miyazaki et al., 2004; Nogai et al., 2005) or proinflammatory cytokines (Nawa and Yamada, 2012). A strong innate immune response similar to that caused by viral infection can be induced by injection of a synthetic double-stranded RNA, polyriboinosinic-polyribocytidylic acid (polyI:C) (reviewed in Meyer and Feldon, 2012). Binding of polyI:C to Toll-like receptor 3 stimulates production of many pro-inflammatory cytokines and type I interferons (Okahira et al., 2005; Kumar et al., 2006). Maternal exposure to polyI:C alters cytokine levels in the three compartments of the maternal-fetal interface: placenta, amniotic fluid, and the fetus itself. PolyI:C has been widely used, as it has several advantages over live viruses; it is comparatively safe, and the intensity and timing of the immune response can be tightly controlled (Meyer and Feldon, 2012). However, polyI:C does not induce the full spectrum of immune responses induced by a virus. Prenatal polyI:C has been shown to induce distinct neuropathological and behavioral phenotypes depending on the gestational duration of exposure. For example, Meyer and Feldon hypothesize that the abnormalities induced by immune activation in early/middle pregnancy (embryonic day 9 in mice) are associated mainly with positive symptoms of schizophrenia, whereas the abnormalities caused by immune activation in late pregnancy (embryonic day 17 in mice) are more relevant to negative symptoms of schizophrenia (Bitanihirwe et al., 2010).

## MODELING SOCIAL STRESS IN RODENTS

As described above, social stress is particularly implicated in the pathogenesis of several major mental diseases. Maternal separation can be a devastating stressor, leading to an array of adult social and cognitive dysfunction (Schmidt et al., 2011). Mild chronic stress, entailed by serial exposure to mild but unpredictable stressors, is also a widely used model for depression (Hill et al., 2012). Social stress as applied to rodents can be subdivided to prenatal, early postnatal (until weaning), adolescent or adult.

### *Social isolation*

Agonistic confrontations are not the only mediator of social stress in mammals. Positive social interactions are critical to healthy mental development. In humans, separation and social withdrawal are risk factors for psychiatric disorders (Allardyce et al., 2005). Both maternal separation and long-term social isolation in mice produce a phenotype characterized by cognitive and social-affective dysfunction, such as impaired prepulse inhibition (PPI) and novel object recognition as well as increased anxiety and aggressive behavior (Fone and Porkess, 2008; Niwa et al., 2011).

### *Social defeat*

Social defeat paradigms involve agonistic interaction between two animals. Two mice (usually male) are put into a cage with a removable separator. Upon removal of the separator the two mice fight one another, and a clear "victor" is established in most cases. The defeated animal experiences social stress, and repeated exposures to this stressor have been linked to a robust social-affective dysfunction phenotype similar to depression, characterized by anhedonia, helplessness, hyperactivity, and social-avoidance (Kudryavtseva et al., 1991; Venzala et al., 2012). Exposure to social defeat can be one-time, intermittent, or chronic.

## GENE × ENVIRONMENT × TIMING

The timing of an environmental factor (whether immune activation or stress) is critical and must be taken into account in experimental design and data interpretation. Dynamic, rapid neurodevelopmental processes occur during the pre- and early post-natal periods and to some extent also in adolescence. Each brain region is maximally sensitive to a given environmental factor during critical periods of high plasticity, which differ between species. Analogous to the typical adolescent or adult onset of psychiatric symptoms, behavioral effects of early-life interventions in experimental animals may become evident only in adulthood.

## GENE × ENVIRONMENT STUDIES USING DISC1 MOUSE MODELS

G × E studies in psychiatry are a relatively new field of research, as discovery of risk genes has only started in this millennium and the mouse models based on the genetic findings are not yet fully established. One of the most extensively characterized risk genes for psychiatric disorders is *DISC1*. *DISC1* was discovered in a Scottish pedigree in which a chromosomal translocation that breaks this gene segregates with psychiatric disorders (Millar et al., 2000). Although the acronym *DISC1* means *Disrupted in schizophrenia-1*, members of the pedigree were diagnosed as suffering from a variety of psychiatric disorders: recurrent major depression, schizophrenia, bipolar disorder and adolescent conduct disorder with a combined LOD score of 7.1 (Blackwood et al., 2001). Subsequent association and linkage studies in a variety of populations have confirmed *DISC1* as a susceptibility gene not only to those adult-onset disorders but also to autism (Chubb et al., 2008). DISC1 is a hub protein which regulates many aspects of development and function of the nervous system and as such mutations in it contribute to various brain disorders (Chubb et al., 2008; Brandon and Sawa, 2011; Porteous et al., 2011). The type of DISC1 mutation, mutations in other genes, and/or environmental factors may determine the specific symptoms.

### *DISC1 mouse models*

One of the tools to study the biology of *DISC1* and its relevance to psychiatric disorders is mouse models in which this gene has been manipulated. Numerous *DISC1* mouse models have been generated and characterized for histological, anatomical, neurochemical, and behavioral phenotypes (for review, see Jaaro-Peled, 2009; Brandon and Sawa, 2011; Johnstone et al., 2011). Each model can be considered significantly unique from the others considering the genetic manipulation, the assays used to characterize it, and the particular lab environment; this diversity significantly complicates comparison between models. Theoretically similar assays may yield different results for different *DISC1* models or even for the same model under different lab conditions. Still, each of these genetic models without any environmental intervention show some abnormalities relevant for schizophrenia and/or mood disorders such as: parvalbumin and synaptic deficits, enlarged lateral ventricles, disturbed dopaminergic system, cognitive and emotional behavioral deficits. When looking for a suitable genetic model for G × E studies, it may be useful select G and E conditions which do not *individually* produce impaired phenotypes in the hope of G × E interaction producing a novel, emergent phenotype. There is a substantial pool of *Disc1* mouse models that could be potentially useful for such studies.

So far the *DISC1* G × E studies have been done with either *Disc1* point mutants (Clapcote et al., 2007) or with transgenic mice expressing C'-truncated (as in the original Scottish pedigree) dominant negative (DN) human DISC1 (on top of the endogenous wild-type mouse Disc1) under either the alpha calmodulin kinase II (CaMK) promoter or the prion protein promoter (PrP). The CaMK promoter induces expression of the DN-DISC1 in the pyramidal neurons of the forebrain (Tsien et al., 1996), whereas the PrP promoter induces its expression in most brain regions and cell types (Asante et al., 2002). Presumably due to *DISC1* being one of the most established risk genes for psychiatric disorders, its clear involvement in brain development, and the availability of many mouse models, more G × E studies have been performed using DISC1 mouse models than with any other risk gene. Here we will review five studies, starting with the studies using DN-DISC1 mouse models (**Table 1**) and then moving to the studies using *Disc1* point mutants (**Table 2**).

### *Inducible CaMK-DN-DISC1 × prenatal polyI:C (Abazyan et al., 2010)*

The CaMK-DN-DISC1 genetic model displays brain and behavioral abnormalities including enlarged lateral ventricles, decreased protein levels of endogenous mouse DISC1, decreased

**Table 1 | Summary of G × E in DN-DISC1 mouse models.**

| | Age: | Embryonic | | | Neonatal | | | | Adolescent | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Abazyan et al., 2010 | | | Ibi et al., 2010; Nagai et al., 2011 | | | | Niwa et al., 2013 | | | |
| | **Condition:** | **G** | **E** | **G × E** | **G** | **E** | **G × E** | **G × E drug rescue** | **G** | **E** | **G × E** | **G × E drug rescue** |
| | **Manipulation:** | **DN-DISC1** | **E9 Polyl:C (5 mg/kg i.p.)** | | **DN-DISC1** | **neonatal Polyl:C** | | **Antipsychotics** | **DN-DISC1** | **Social isolation** | | **GR antagonist** |
| Locomotion | OFT | | | | | | | Haloperidol, clozapine | (METH) = | (METH) = | Y | Normalized |
| Cognition and Memory | PPI | = | = | N | = | = | N | | = | = | Y | Normalized |
| | Y maze | = | = | N | = | = | Y | | | | | |
| | NORT | = | = | N | = | = | Y | Clozapine | | | | |
| Depression and Anxiety | FST | = | = | Y | | | | | = | = | Y | Normalized |
| | Sociability | = | = | Y | | | | | | | | |
| | EPM | = | = | Y | | | | | | | | |
| | OFT | = | = | Y | | | | | | | | |
| Anatomy | LV Volume | ↑ | ↑ | Y (smaller increase) | | | | | = | | | |
| | Spine density | = | = | N | | | | | | | | |
| Histology | Nissl stain | | | | | | | | = (FC) | = (FC) | N | |
| | BrdU | | | | = | = | Y (GCL) | | | | | |
| | Parvalbumin | | | | = | = | Y (mPFC) | | | | | |
| Neurochemistry | Cytokine secretion | ↑ IL-1B, IL-5 | ↑ IL-1B, IL-4, IL-5 | Y (IL-2 only) | | | | | | | | |
| | Corticosterone (stress response) | No recovery | No recovery | Y (smaller increase) | | | | | = | = | Y | |
| | Dopaminergic | = | = | = | | | | | = | = | Y (FC, not NAc) | Normalized |

*The down arrow (↓) indicates decrease, the up arrow (↑) indicates increase or augmentation compared to wild-type controls without environmental risk factor; Y indicates a significant G × E interaction (gene-environment interactions exacerbated behavioral deficits unless otherwise specified), N indicates the absence of a significant G × E interaction; G is the gene manipulated condition; E is the environment manipulated condition; G × E is the gene and environment manipulated condition. BrdU, bromodeoxyuridine; EPM, elevated plus maze; FC, frontal cortex; FST, forced swim test; GCL, granule cell layer of the hippocampus; GR, glucocorticoid receptor; IL, interleukin; i.p., intraperitoneal; LV, lateral ventricle; METH, methamphetamine; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; NORT, novel object recognition test; OFT, open field test; PPI, prepulse inhibition.*

**Table 2 | Summary of G × E in point mutant *Disc1* mouse models.**

| | **Age:** | **Embryonic** | | | | | | | **Adult** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Lipina et al., 2013** | | | | | | | **Haque et al., 2012** | | | | | |
| | **Condition:** | **G** | **E** | **G × E** | **G** | **E** | **G × E** | **G × E drug rescue** | **G** | **E** | **G × E** | **G** | **E** | **G × E** |
| | **Manipulation:** | **Q31L** | **E9 Polyl:C (5 mg/kg i.v.)** | | **L100P** | **E9 Polyl:C (2.5 mg/kg i.v.)** | | **Anti-IL-6** | **Q31L** | **Chronic social defeat** | | **L100P** | **Chronic social defeat** | |
| Locomotion | OFT | = | ↑ | N | = | = | N | | = | = | N | = | = | Y |
| Cognition and memory | PPI | ↓ | ↓ | N | = | = | Y | Normalized | = | = | N | ↓ | = | N |
| | LI | = | ↓ | N | ↓ | ↓ | Y | Normalized | ↓ | = | N | ↓ | = | N |
| | NORT | = | = | N | = | = | Y | | | | | | | |
| Depression and anxiety | FST | = | = | N | | | | | = | ↑ | N | = | ↑ | N |
| | SC | | | | | | | | ↓ | ↓ | N | ↓ | ↓ | N |
| | Sociability | | | | = | = | Y | | ↓ | ↓ | N | = | ↓ | N |
| | EPM (% closed arm) | | | | | | | | ↑ | ↑ | N | ↑ | ↑ | Y |
| Neurochemistry | Cytokine secretion | | ↑ IL-6 | N | | ↑ IL-6 | Y | | | | | | | |

*The down arrow (↓) indicates decrease, the up arrow (↑) indicates increase or augmentation compared to wild-type controls without environmental risk factor; Y indicates a significant G × E interaction (gene-environment interactions exacerbated behavioral deficits), N indicates the absence of a significant G × E interaction; G is the gene manipulated condition; E is the environment manipulated condition; G × E is the gene and environment manipulated condition. EPM, elevated plus maze; FST, forced swim test; IL, interleukin; i.v., intravenous; LI, latent inhibition; NORT, novel object recognition test; OFT, open field test; PPI, prepulse inhibition; SC, sucrose consumption.*

levels of cortical dopamine and fewer parvalbumin-positive neurons in the cortex (Pletnikov et al., 2008; Ayhan et al., 2011). Behavioral abnormalities differ between males and females: males show both spontaneous and psychostimulant-induced hyperactivity in the open field as well as altered social interaction, whereas females exhibit impaired spatial memory.

PolyI:C (5 mg/kg) was injected to pregnant dams at embryonic day 9 (E9), modeling the complex scenario of infection affecting the maternal-fetal interface. This timing of polyI:C injection, corresponding to middle/end of first trimester of human pregnancy, has been widely used (Meyer et al., 2006). Abazyan et al. performed intraperitoneal injections—in contrast to the intravenous (i.v.) injections used by previous studies—in order to achieve a lower effective polyI:C dose. Indeed, no effect of the polyI:C alone could be detected in the behavioral tests used although the same i.v. dose has been shown to elicit extensive behavioral deficits (Meyer et al., 2005). DN-DISC1 altered basal levels of interleukin (IL)-1 beta and IL-5 and polyI:C induced secretion of IL-4 and IL-5 in fetal brains 6 h after injection of polyI:C. The other experiments were done on adult males only. The effect of the different manipulations on adult response to acute stress was measured by testing for corticosterone levels at baseline, after a 1 h restraint stress and following 1 h recovery. Although both polyI:C and DN-DISC1 on their own caused a trend of reduced stress-induced corticosterones and no recovery, only the G × E group had significantly lower corticosterones after stress compared to the control group. DN-DISC1 mice have an increased lateral ventricle volume (Pletnikov et al., 2008). Although polyI:C caused increased lateral ventricle volume in wild-type mice, it unexpectedly normalized lateral ventricle volume in the DN-DISC1 mice.

Combination of CaMK-DN-DISC1 × prenatal polyI:C produced new phenotypes not seen with either variable alone: anxiety, as measured by increased peripheral activity in the open field and by open arm time in the elevated plus maze; depression-like behavior, as measured by increased immobility in the forced swim test; and impaired sociability in the three-chamber social test. G × E had no effect on cognitive tests: spontaneous alternation in the Y-maze, object recognition, Morris water maze, PPI.

Finally the authors exploited the Tet-off inducibility of this *CaMK-DN-DISC1* transgene to evaluate the effect of expression timing. In one group, expression of the transgene was induced until weaning (postnatal day 21) and in a second group, expression was induced from weaning onward. The G × E mice behaved normally, implying that continuous expression of the DN transgene is necessary for the G × E effect.

#### *CaMK-DN-DISC1 × neonatal polyI:C (Ibi et al., 2010; Nagai et al., 2011)*

The CaMK-DN-DISC1 mouse model is very similar to the one employed in the prenatal polyI:C study. The difference is that it expresses the transgene in a constitutive manner. The CaMK-DN-DISC1 genetic model on its own has enlarged lateral ventricles, reduced parvalbumin immunoreactivity, dopaminergic abnormalities and relatively mild behavioral deficits (Hikida et al., 2007; Jaaro-Peled et al., 2013). CaMK-DN-DISC1 mice were injected with polyI:C daily from postnatal day 2–6, which corresponds to the second trimester of human brain development (http://translatingtime.net). This paradigm of neonatal polyI:C injection on its own elicits behavioral abnormalities including impairments in PPI, novel object recognition and social behavior in mice tested at 10–12 weeks of age (Ibi et al., 2009). The DISC1 mutant mice (both males and females) were analyzed starting at 8 weeks of age, or young adulthood. In contrast to the previous study (Ibi et al., 2009), polyI:C on its own did not impair performance in the PPI or novel object recognition paradigms, perhaps due to the age difference. Only G × E resulted in significant reduction in parvalbumin-positive interneurons of the medial prefrontal cortex, reduction in spontaneous alternation in the Y-maze as well as in preference for a novel object in the novel object recognition test (Ibi et al., 2010). The other behavioral tests, sensitivity to MK801, fear conditioning and social interaction were not performed on the G or E groups alone, making it difficult to evaluate any potential G × E interaction. In a follow up paper, the same group tested whether antipsychotics could normalize the behavioral deficits in the CaMK-DN-DISC1 × neonatal polyI:C model. The atypical antipsychotic clozapine normalized the above-mentioned preference in the novel object recognition test, while the typical antipsychotic haloperidol did not (Nagai et al., 2011).

#### *PrP-DN-DISC1 × adolescent social isolation (Niwa et al., 2013)*

Niwa et al. (2013) is the first publication to introduce this DISC1 mouse model in which the C'-truncated DN-DISC1 is expressed under the control of the widely expressed prion protein promoter. Mice are typically weaned at 3 weeks of age and then housed 5/cage with same-sex littermates. Pre-weaning isolation is extremely stressful and complicated by the physical survival of the pups still dependent on nursing. PrP-DN-DISC1 mice were subjected to a new, milder social stress paradigm of 3 weeks of isolation from 5–8 weeks of age after which they were tested. This timing corresponds to middle and late adolescence, a sensitive period and may therefore model separation from family or general social isolation in adolescent humans. The combination of the DN-DISC1 gene (G) and mild social isolation (E) led to multiple behavioral, cellular and functional deficits whereas neither the environmental nor genetic factors produced deficits on their own. Both males and females were tested and showed similar phenotypes.

PrP-DN-DISC1 mice with 3-week isolation (G × E) displayed multiple behavioral deficits, including decreased prepulse inhibition, increased immobility in a forced swim test, and an augmented locomotion response to methamphetamine challenge when compared to the control, G, and E groups. These abnormal phenotypes resulted from synergistic gene-environment interaction at the cellular level, rather than any gross anatomical or histological mechanism. Body weight and lateral ventricle volume were similar to controls in the G, E, and G × E groups; Nissl staining and Glial fibrillary acidic protein immunostaining also failed to reveal any significant differences. However, the G × E mice displayed significant dopaminergic alterations at the cellular level. Tyrosine hydroxylase expression and total tissue dopamine levels were significantly decreased compared to control, G, and E groups, as was basal extracellular dopamine in the frontal cortex.

Consistent with their abnormal locomotor behavior, methamphetamine challenge led to significantly higher dopamine release in the nucleus accumbens of the G × E mice compared to the control, G, and E groups.

Notably, treatment with the glucocorticoid receptor antagonist RU38486 normalized all behavioral and dopaminergic cellular abnormalities in the G × E group. The authors therefore hypothesized that an abnormally high stress-induced corticosterone response in the G × E mice might underlie their dopaminergic pathology. Glucocorticoids mediated projection-specific epigenetic changes in the dopaminergic midbrain of the G × E mice. Specifically, mesocortical dopaminergic projection neurons in the ventral tegmental area exhibited robust DNA methylation of the tyrosine hydroxylase gene promoter, while the mesolimbic projection was unaffected. Furthermore, these DNA methylation levels were maintained in the G × E mice until 20 weeks of age after the transient adolescent isolation. RU38486 normalized the increase in DNA methylation in G × E mice.

Only G × E produced neuronal and behavioral abnormalities in the PrP-DN-DISC1 model; neither the DISC1 mutation nor isolation stress produced any observed pathology on their own. As such, the PrP-DN-DISC1 mice are a promising genetic model for exploring synergistic gene-environment interactions underlying the mechanisms of psychiatric pathology.

#### *L100P and Q31L × prenatal polyI:C (Lipina et al., 2013)*

In addition to dominant-negative models, DISC1 point mutants have also been combined with environmental risk factors. These models, identified in a screen of ENU-mutagenized mice, express missense mutations in *Disc1*. The Q31L mutant mice showed depressive-like behavior (deficits in the forced swim test and other measures that were reversed by the antidepressant) whereas L100P mutants exhibited schizophrenia-like behavior (PPI and latent inhibition deficits which were reversed by antipsychotic treatment) (Clapcote et al., 2007). A subsequent study from another group did not reproduce most of these baseline behavioral abnormalities (Shoji et al., 2012) suggesting that the effect of the *Disc1* point mutations is not very robust and may depend on the experimental conditions i.e., subtle environmental factors. The two G × E studies described here used heterozygous *Disc1* point mutants. Heterozygotes exhibit relatively mild phenotypic impairments compared to homozygotes (Clapcote et al., 2007), and as such provide more room for exploring the synergistic interplay between genetic and environmental risk factors. PolyI:C 5 mg/kg i.v. at E9 has been routinely used in environment-only studies and shown to elicit robust phenotypes (Meyer et al., 2005). In this study the authors started with a dose of 5 mg/kg, but pregnant L100P heterozygotes administered polyI:C at 5mg/kg bore no offspring, dead or alive; as a result, they had to decrease the dose to 2.5 mg/kg in order to obtain offspring for behavioral analysis. Litter/offspring size in polyI:C-treated wild type and Q31L mice were unaffected. Thus, they studied Q31L mice with 5 mg/kg polyI:C and L100P mice with 2.5 mg/kg polyI:C. In the assays used, 2.5 mg/kg on its own produced a PPI deficit and 5 mg/kg on its own affected also locomotion in the open field and latent inhibition (LI). Even lower polyI:C dose of 1 mg/kg i.v. may be recommended for G × E studies (Giovanoli et al., 2013).

Since prenatal infection is one of the major epidemiological risk factors for schizophrenia, but not for depression, Lipina et al. (2013) hypothesized that G × E interactions between maternal immune activation produced by prenatal polyI:C would be significantly greater in the L100P mice than the Q31L mice. Heterozygous females were given a single injection of polyI:C on gestational day 9. The Q31L/polyI:C mice exhibited G × E interaction only in risk assessment behavior, meaning that the G × E spent more time scanning the EPM for potential threat than the G or E conditions alone. However, the L100P × polyI:C offspring exhibited a significant exacerbation of schizophrenia-related endophenotypes compared to L100P controls. Deficits in PPI, LI, object recognition, and sociability all demonstrated significant prenatal polyI:C × genotype interactions. Specifically, the L100P × polyI:C group showed a deficit in social motivation but not social recognition—correspondingly, their novel object recognition was intact while spatial object recognition was impaired.

IL-6, a cytokine critical for mediating the effects of prenatal polyI:C, was elevated in the fetal brains of all mice treated with polyI:C; the L100P group, however, exhibited a steeper dose-dependent increase compared to the WT and Q31L groups. It appears that at least PPI and LI behavioral deficits in the L100P mice were causally dependent on prenatal polyI:C, as coadministration of an antibody against IL-6 resulted in normal PPI and LI behavior.

#### *L100P and Q31L × social defeat (Haque et al., 2012)*

Haque et al. also used L100P or Q31L heterozygous mice for low baseline impairments to enable detection of G × E effect. The mice were subjected to chronic social defeat (CSD) as an environmental stressor. CSD on its own has been shown to induce a depressive/anxiety-like phenotype including hypoactivity in the open field, social avoidance, reduced sucrose consumption, and increased immobility time in the forced swim test (Kudryavtseva et al., 1991; Venzala et al., 2012). This makes it difficult to detect G × E effects in these behavioral tests.

CSD resulted in similar depressive phenotypes for all three genetic groups. L100P and Q31L mice did not display significant abnormality in either forced-swim test immobility or sucrose consumption similar to wild-type controls in both the naïve and defeated conditions. Anxiety-related endophenotypes, on the other hand, revealed significant differences between the two mutant lines and controls, as well as between the mutant lines themselves. L100P and Q31L mice spent less time exploring the open arm of the elevated plus maze than did controls. However, Q31L mice did not display any difference in exploration time in response to CSD, whereas CSD significantly decreased exploration time for wild-type and L100P groups. The L100P defeated mice displayed decreased vertical activity in the open field test compared to all G, E, and control groups. A CSD-related decrease in L100P horizontal activity was also observed during the first 5 min of the open field test, but the difference normalized in subsequent time bins. CSD diminished sociability and preference for social novelty in wild-type and L100P mice. Q31L were a social even without CSD, so no further effect could be detected by addition of CSD stress. CSD did not interact with cognitive

deficits in the mutant lines. L100P mice displayed a deficit in PPI, and both L100P and Q31L mice displayed disruptions in LI, but CSD did not exacerbate either endophenotype for any group.

## DISCUSSION

A genetic model with too robust a phenotype may result in a "ceiling effect," whereby the effects of any potential G × E interactions are occluded. It is therefore more practical to use genetic models with relatively weak phenotypes, such as heterozygotes instead of homozygotes (Ibi et al., 2010; Haque et al., 2012; Lipina et al., 2013; Niwa et al., 2013), or to at least focus on specific assays which are not affected by the G condition on its own (Abazyan et al., 2010). The same is true for the choice of the environmental stressor—to detect G × E effects it is useful to choose a regime which is sub-optimal on its own, such as changing the administration route (Abazyan et al., 2010) or reducing the dose (Lipina et al., 2013) of polyI:C, or developing a new, milder form of social stress paradigm (Niwa et al., 2013).

Interestingly, CaMK-DN-DISC1 × *prenatal* polyI:C synergistically induced anxiety and depression-like phenotypes, but cognitive function was not affected (Abazyan et al., 2010). In contrast, CaMK-DN-DISC1 × *neonatal* polyI:C significantly impacted on cognitive tasks (Ibi et al., 2010). Since these two DISC1 models are very similar, such results imply that specific timing of the polyI:C intervention determines which type of behavioral abnormalities emerge. To test this hypothesis, it would be useful to test a single *DISC1* model against several polyI:C injections at different time points, with subsequent behavioral and histological analyses under identical experimental conditions.

Which biological changes underlie the behavioral abnormalities manifested exclusively as a result of G × E? Ibi et al. found decreased parvalbumin immunoreactivity in the prefrontal cortex but not the hippocampus, hinting at a specific fast-spiking interneuron deficiency (Ibi et al., 2010). Abazyan et al. observed decreased spine density in the dentate gyrus. Their most surprising result was that combining polyI:C with DISC1 suppressed the ventricular enlargement phenotype displayed by either the G or E factor alone (Abazyan et al., 2010). Abazyan et al. and Niwa et al. both looked at monoamines and plasma corticosterone response to environmental stress. In the CaMK-DN-DISC1 × neonatal polyI:C model, no changes were detected in dopamine content or turnover in the frontal cortex or hippocampus, while hippocampal serotonin content was increased (Abazyan et al., 2010). Stress-induced corticosterone levels were attenuated in the G × E condition more than in G or E alone. In the PrP-DN-DISC1 × isolation stress model, frontal cortex and caudate-putamen serotonin were not altered while several dopamine-related measures were lower in the frontal cortex but not in the nucleus accumbens (Niwa et al., 2013). These dopaminergic and behavioral phenotypes are at least in part affected by epigenetic silencing of the tyrosine hydroxylase promoter via the elevated glucocorticoids which were detected only in G × E. The isolation stress on its own was mild enough not to affect plasma corticosterones. Abazyan et al. and Lipina et al. detected effect of G × E on cytokines, but each tested for different cytokines (Abazyan et al., 2010; Lipina et al., 2013). Lipina et al. used anti-IL-6 to rescue polyI:C induced PPI and LI abnormalities in L100P mice, confirming the importance of IL-6 as a mediator of the detrimental effects of maternal immune activation.

Can G × E-induced abnormalities be reversed by pharmacological interventions regularly used in psychiatry? Nagai et al. focusing on schizophrenia, tested antipsychotics against the CaMK-DN-DISC1 × neonatal polyI:C model. Increased sensitivity to MK801, an NMDA receptor antagonist, was rescued by both haloperidol and clozapine. Novel object recognition deficit was normalized only by clozapine treatment, but the sociability deficit could not be rescued by either (Nagai et al., 2011). This model may be useful in exploratory screening for new drugs which are especially effective against social deficits. Based on the increased plasma corticosterone levels in the PrP-DN-DISC1 × social stress model, Niwa et al. treated the mice with the glucocorticoid receptor antagonist RU38486 which normalized all behavioral and dopaminergic cellular abnormalities in the G × E group, most likely via normalization of the tyrosine hydroxylase methylation. RU38486 is uniquely beneficial in psychotic depression, major depression with psychotic features (Flores et al., 2006), suggesting that the PrP-DN-DISC1 × social isolation model may be useful in finding better treatments for this disorder. The above drug treatment trials add predictive validity to their respective animal models and suggest that even abnormalities of neurodevelopmental origin may be reversed with appropriate treatment.

As discussed above, timing is critical. Abazyan et al. induced expression of the CaMK-DN-DISC1 transgene either until weaning or starting at weaning. Interestingly, neither group exhibited the depressive-like behavioral deficits detected with constitutive CaMK-DN-DISC1 expression. Elimination of the behavioral phenotypes after turning off expression during the late postnatal period is encouraging; it implies that these phenotypes are reversible despite being neurodevelopmental in origin (Abazyan et al., 2010). Niwa et al. asked whether adolescent stress-induced epigenetic changes in the tyrosine hydroxylase promoter persist into adulthood. They isolated mice from 5 to 8 weeks but then returned them to group housing until 20 weeks of age. The increase in tyrosine hydroxylase promoter methylation was maintained after 12 weeks of group housing supporting the idea that stress in adolescence can impact the brain for life (Niwa et al., 2013). Demonstrating the importance of the timing in which the mice are tested for potential effects of G × E, the detrimental effect of polyI:C on PPI was not detected at the end of adolescence (8 weeks) but only later in adulthood (16 weeks) (Lipina et al., 2013).

Sex differences are an important and under-researched facet of psychiatric disorders. Schizophrenia starts earlier and is generally more severe in men. The risk for depression is doubled in women compared to men. In depression there is human and animal data on differential effect of early life stress on females (Goel and Bale, 2009). In the CaMK-DN-DISC1 × prenatal polyI:C study (Abazyan et al., 2010), only males were tested, although the same group has shown sex-specific phenotypes in the genetic model alone (Ayhan et al., 2011). Ibi et al. tested both sexes together for CaMK-DN-DISC1 × postnatal polyI:C and did not mention anything about potential sex differences (Ibi et al., 2010).

PrP-DN-DISC1 × social isolation was performed on both sexes separately and the results were notably similar for both, suggesting that this paradigm may not be useful to look at sex differences (Niwa et al., 2013). Chronic social defeat is designed to utilize aggressive interactions between males; therefore, only males were tested (Haque et al., 2012).

The recent study combining *Disc1* point mutants with PolyI:C at E9 (Lipina et al., 2013) provides an opportunity to compare the effects of a similar environmental factor on different Disc1 models based on data obtained by the same lab. They found that maternal immune activation interacts with the L100P mutation that causes abnormalities related to schizophrenia while there was no interaction with the Q31L mutation that causes abnormalities related to depression. These results correspond nicely with the epidemiological studies pointing to prenatal infection as a risk factor for schizophrenia but not for depression. The two studies using *Disc1* point mutants also enable us to start to compare the effects of different environmental risk factors on the same model based on data obtained by the same lab. Interestingly, prenatal polyI:C on its own had no effect on sociability, but in combination with L100P it eliminated preference for the stranger (Lipina et al., 2013). On the other hand, CSD reduced sociability both in WT and in L100P mice (Haque et al., 2012). So social impairment can either result from CSD, which is highly relevant for social behavior, or from *Disc1* L100P × prenatal polyI:C interaction—two factors which have no individual effect on sociability.

## FUTURE DIRECTIONS

Exploring a matrix of all risk genes for schizophrenia and their interactions with all environmental risk factors would be exceptionally difficult. Scientists can instead focus on major known susceptibility genes and combine them with environmental factors thought to increase risk, based on epidemiological studies. In order to rigorously dissect the complexity of G × E interactions, both factors must be manipulated independently and the results of G × E must be compared to G and E alone under identical experimental conditions. Ideally, the same genetic model can be exposed to different environmental factors at different times and tested for histological, neurochemical, and behavioral phenotypes without *a priori* assignment of a specific disease model and with equal value assigned to negative results. Such open-ended analysis, although laborious, could suggest how one risk gene can catalyze the emergence of a variety of psychiatric disorders depending on which environmental factor it interacts with (Harvey and Boksa, 2012). DISC1 is an especially attractive candidate for such an approach since it is a risk factor for many different mental disorders. Looking at genetic interactions with an array of environments will still be a simplified model, since additional G × G or E × E interactions probably determine exact outcomes in humans. To further refine our understanding of the *DISC1* × E interaction paradigm, there is a need for greater emphasis on sex differences in mouse models, as well as examination of environmental risk factors such as Toxoplasma gondii exposure, adolescent cannabis abuse and advanced paternal age. In the future, the development of comprehensive "environment interaction profiles" for major risk genes like DISC1 would represent a significant advancement toward the scientific understanding of mental disease pathogenesis.

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# *Neuregulin 1*: a prime candidate for research into gene-environment interactions in schizophrenia? Insights from genetic rodent models

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Schizophrenia is a multi-factorial disease characterized by a high heritability and environmental risk factors. In recent years, an increasing number of researchers worldwide have started investigating the "two-hit hypothesis" of schizophrenia predicting that genetic and environmental risk factors (GxE) interactively cause the development of the disorder. This work is starting to produce valuable new animal models and reveal novel insights into the pathophysiology of schizophrenia. This mini review will focus on recent advancements in the field made by challenging mutant and transgenic rodent models for the schizophrenia candidate gene *neuregulin 1* (*NRG1*) with particular environmental factors. It will outline results obtained from mouse and rat models for various Nrg1 isoforms/isoform types (e.g., transmembrane domain *Nrg1*, Type II *Nrg1*), which have been exposed to different forms of stress (acute *versus* chronic, restraint *versus* social) and housing conditions (standard laboratory *versus* minimally enriched housing). These studies suggest *Nrg1* as a prime candidate for GxE interactions in schizophrenia rodent models and that the use of rodent models will enable a better understanding of GxE interactions and the underlying mechanisms.

**Keywords: schizophrenia, *neuregulin 1*, gene-environment interactions, mouse, rat, stress, enrichment, housing**

## INTRODUCTION

The two-hit hypothesis of schizophrenia states that a combination of genetic and environmental risk factors causes the development of schizophrenia (Bayer et al., 1999; Rapoport et al., 2005; Caspi and Moffitt, 2006). Indeed, twin studies show that nature and nuture are both important in the development of schizophrenia (i.e., concordance rate of monozygotic twins is 50%) (Tsuang et al., 2001) and combined actions of multiple genes of small effect (Owen et al., 2005) and a number of environmental risk factors (McGrath et al., 2004) is likely (Mackay-Sim et al., 2004). Genome wide association studies suggest that it is important to consider the interplay of genes and environment to understand the aetiology of the disorder in more depth (Sanders et al., 2008). In this context, interactions of genetic and environmental risk factors (GxE) occur when an individual's genetic predispositions are expressed dependent on their environment or when environmental influences on a trait differ according to the individual's genome (Tsuang et al., 2004). According to the neurodevelopmental theory of schizophrenia genes and environment together affect brain development negatively during critical periods of neuronal development and thereby induce schizophrenia (Marenco and Weinberger, 2000).

Animal models can incorporate genetic and environmental risk factors at different stages of development, thereby more accurately mimicking the aetiology of schizophrenia, and help elucidate interactions between those factors and underlying mechanism (Burrows et al., 2011). For example, *neuregulin 1* (*NRG1*) is a genetic target for schizophrenia research (Stefansson et al., 2002; Tosato et al., 2005; Munafo et al., 2006) as it influences key neurodevelopmental processes relevant to schizophrenia (e.g., myelination and neuronal migration), and regulates receptors such as N-methyl-D-aspartic acid (NMDA) and γ-aminobutyric acid receptor A ($GABA_A$) (Mei and Xiong, 2008). It has been outlined that there might be genetic subgroups in the population that are more vulnerable to particular environmental risk factors (e.g., cannabis abuse, developmental trauma) (van Os et al., 2010) and *NRG1* might be such a genetic candidate. This review will summarize preclinical data to assess if *Nrg1* might be mediating an increased risk to environmental factors with relevance to schizophrenia (i.e., stress and cannabis) and experimental animal research (i.e., laboratory housing conditions).

## Nrg1 X LABORATORY HOUSING

Environmental enrichment (EE) has a significant impact on animal models of neurodegenerative diseases (van Dellen et al., 2000; Spires and Hannan, 2005). Furthermore, enriched cage structures can modify or even rescue knockout-specific abnormalities of genetic mouse models (Rampon et al., 2000; van Dellen et al., 2000). Thus, the behavioral effects of minimally enriched housing (ME) compared to standard laboratory housing were determined in male transmembrane domain *Nrg1* mutant and wild type-like control mice (Karl et al., 2007). This mutant mouse model

has been shown to have compelling construct, face, and predictive validity for schizophrenia (Stefansson et al., 2002; Walss-Bass et al., 2006; Karl et al., 2007, 2011; van den Buuse et al., 2009; Duffy et al., 2010; Chesworth et al., 2012a). Mice were tested at the age of 3–4 and 4–6 months, as the age of patients has a significant impact on the aetiology of schizophrenia (Thompson et al., 2004). Effects of *Nrg1* mutation on locomotion, exploration and anxiety-like behaviors were age-dependent and interacted with the housing condition males were raised in. *Nrg1* mutants kept in ME developed hyper-exploration in the light-dark test and reduced anxiety-like behavior in the open field test at 3–4 months of age whereas *Nrg1* males kept in standard housing displayed these phenotypes only at the age of 4–6 months. Importantly, well-known explorative and anxiolytic-like properties of cage enrichment (Chapillon et al., 1999; Roy et al., 2001; Benaroya-Milshtein et al., 2004) were more pronounced in *Nrg1* mutant mice than control mice suggesting that mutant transmembrane domain *Nrg1* increased the behavioral sensitivity to ME.

*Nrg1* mutant mice are characterized by hypo-phosphorylation of the NR2B subunit (Bjarnadottir et al., 2007). This is in line with Nrg1's up-regulation of NMDA subunits expression (Ozaki et al., 1997; Stefansson et al., 2004) and the stimulation of Y1472 phosphorylation on the NR2B subunit of NMDA receptors. As NMDA antagonists induce increased locomotor activity (Wong and Van Tol, 2003; Javitt and Coyle, 2004) and as mouse models for NMDA receptors suggest an involvement of the glutamatergic system in rodent hyperactivity (Smith et al., 1998; Dulawa et al., 1999; Mohn et al., 1999; Zhuang et al., 2001), hypo-phosphorylated NR2 subunits may be responsible for the observed hyperactivity. Nonetheless, it should be noted that Hahn and colleagues found that Nrg1 stimulation suppressed NMDA receptor activation in the human prefrontal cortex (Hahn et al., 2006). EE does not impact the behavioral susceptibility to NMDA receptor antagonists, but mRNA expression of specific NMDA receptor subunits was decreased in mice kept in enriched housing (Grilli et al., 2009). This suggests that combined effects of mutant *Nrg1* and ME (i.e., additive GxE) might be responsible for the earlier onset of hyperactivity.

Importantly, hypo-phosphorylation of NR2B subunits in *Nrg1* mutant mice might also support the activation of dopaminergic pathways (Duncan et al., 1999; Kapur and Seeman, 2002) and thereby contribute to their anxiolytic-like and hyper-locomotive phenotype. Indeed, dopamine transporter deficient mice are not only characterized by hyperactivity but also decreased anxiety-like-like responses (Carpenter et al., 2012). In this context, it is important to note that exposure to enriched housing affects the dopaminergic system as enrichment increased the susceptibility of rats to the behavioral and neurochemical effects of amphetamine (Bowling et al., 1993) although another study found reduced dopamine receptor 1 function as a consequence of enriched housing (Del Arco et al., 2007). Further research is needed to pinpoint the mechanisms underlying the differential potency of ME in *Nrg1* mutant and control mice but an involvement of dopaminergic and glutamatergic circuits is likely.

Other genetic mouse models of schizophrenia have been reported to benefit from more complex enriched housing environments (McOmish et al., 2008). Thus, the disease-related phenotype-strengthening effects of ME in *Nrg1* mice are interesting and opposite to reports by others (van Dellen et al., 2000; Olsson and Dahlborn, 2002; Spires and Hannan, 2005). *Nrg1/NRG1* has been described as being critical for how an organism responds and adapts to the environment (Stefansson et al., 2004). Thus, the biological function of *Nrg1* may dictate this disease phenotype-strengthening response to an enriched housing environment, which is different to the effects normally described for EE (i.e., reversing disease phenotypes).

## Nrg1 X STRESS

Stressful life events and changes in HPA axis function are associated with and precipitate the onset of psychiatric disorders (Koenig et al., 2002; Walker et al., 2008). Furthermore, stress plays a role in the development [e.g., behavioral sensitization: (van Os et al., 2010)] and severity of psychotic symptoms (Corcoran et al., 2003) and triggers relapse in schizophrenia patients (Hultman et al., 1997). There appears to be a genetic component to stress vulnerability in schizophrenia: schizophrenia patients are more sensitive to stress (van Winkel et al., 2008), handle negative life events more poorly (Horan et al., 2005), and show impaired cortisol and HPA axis activity in stressful situations (van Venrooij et al., 2010). Importantly, a *NRG1* polymorphism interacts with psychosocial stress thereby affecting reactivity to expressed emotions in schizophrenia patients (Keri et al., 2009) and *NRG1* also interacts with job strain thereby increasing the risk of heart disease (Hintsanen et al., 2007). Furthermore, Nrg1 is expressed in brain regions controlling stress reactivity (Chen, 2007). Thus, a number of research teams have investigated the response of *Nrg1* rodent models to models.

A first study investigated the behavioral and endocrine response of male transmembrane domain *Nrg1* mutant mice to acute restraint stress before and after the onset of the age-dependent hyper-locomotive phenotype (Chesworth et al., 2012b). The suppressive effect of stress on locomotion was evident in all mice regardless of genotype or age. Surprisingly, older *Nrg1* mutants appeared insensitive to anxiety-like-related stress effects in the open field (i.e., center locomotion). All mice displayed robust stress-induced increases in serum corticosterone, although the response was more pronounced in young *Nrg1* mutants compared to WT mice. The study suggested that there is no pronounced effect of mutant transmembrane domain *Nrg1* on the endocrine and behavioral effects of acute restraint stress. Nevertheless, Nrg1 modified corticosterone release in young *Nrg1* mutants and the anxiety-like response of hyper-locomotive older *Nrg1* mice, confirming that the gene plays a role in how an organism responds to environmental manipulations. The phenomenon of a disconnected behavioral and endocrine stress response of older *Nrg1* mice (i.e., no stress-induced anxiety-like response in open field but increased glucocorticoid levels) is consistent with other mouse models (Laarakker et al., 2011; Trainor et al., 2011). Future research should address the impact of chronic stress on *Nrg1* mutant mice and consider additional aspects of HPA functions.

Importantly, recent rat research suggests that *Nrg1* might be involved in stress reactivity downstream from the release of glucocorticoids (Taylor et al., 2011b). More specifically, a rat model for

disrupted Type II *Nrg1* expression was characterized by increased baseline corticosterone levels and improved recovery of corticosterone levels post-acute restraint stress. Importantly, in control rats, Type II *Nrg1* was expressed in the neurocircuitry involved in regulating HPA responses to environmental stimuli. The authors concluded that disruptions to Type II *Nrg1* expression mediated an increased basal HPA axis activity. Elevated levels of glucocorticoid (but not mineralocorticoid) receptors in the hippocampus and pituitary glands of *Nrg1* mutant rats under baseline conditions could then result in a more pronounced negative feedback loop thereby increasing the inhibition of HPA axis activity following acute restraint stress. Interestingly, shifts in the balance of glucocorticoid and mineralocorticoid receptor levels in humans can create a vulnerability to psychiatric disease, especially among genetically predisposed individuals (De Kloet et al., 1998; Zhe et al., 2008). The change in the endocrine stress response of mutant Type II rats was accompanied by altered habituation to an open field environment across test days (Taylor et al., 2011b). Nrg1 is necessary for the establishment of excitatory synapses in GABAergic interneurons and for the development of a balanced excitatory/inhibitory tone in the brain (Ting et al., 2011). As GABAergic mechanisms play a role in controlling HPA axis function (Herman et al., 2004), Nrg1-induced changes to the GABAergic system might present a potential mechanism for the observations in Type II *Nrg1* mutant rats.

In a follow-up study it was found that some of the behavioral and brain characteristics of *Nrg1* hypomorphic rats were highly sex-specific (Taylor et al., 2011a). It should be noted that sex-specificity in rodent models for *Nrg1* is a common phenomenon (O'Tuathaigh et al., 2006; Duffy et al., 2010; Chesworth et al., 2012a) and is in line with gender effects reported for schizophrenia patients (Canuso and Pandina, 2007). Inconsistencies between the stress response of the two investigated *Nrg1* rodent models are most likely due to (1) species differences (Asan et al., 2005), (2) differences in the restraint stress models used (rats were habituated to the general stress procedure whereas mice were naïve), and (3) the particular characteristics of the *Nrg1* mutation [(Harrison and Law, 2006; Mei and Xiong, 2008); for overview on *Nrg1* rodent models see: (Duffy et al., 2008; Karl et al., 2011)]. Adding to the complexity of potential *Nrg1*-stress interactions is a study reporting that Type III *Nrg1* mutant mice display a blunted increase in corticosterone release after mild acute stress (Chen, 2007).

Adolescence is a period of heightened risk to develop schizophrenia (Walker and Bollini, 2002; Costello et al., 2003; Paus et al., 2008) as abnormal adolescent brain development contributes to the aetiology of schizophrenia (Paus et al., 2008; Walker et al., 2008). Furthermore, stress response-relevant neuronal pathways develop during adolescence (Andersen et al., 2000; Spear, 2000; Casey et al., 2008) and HPA axis plasticity appears sensitive to adolescent stress exposure as well (Romeo et al., 2006). Thus, it is important to assess interactions between Nrg1 and stress also during adolescence.

Indeed, Taylor and co-workers investigated the effects of chronic variable stress during adolescence on endocrine and behavioral measures in adult Type II *Nrg1* mutant rats (Taylor et al., 2012). Sex-specific interactions between *Nrg1* genotype and adolescent stress were found. Stress during adolescence reduced baseline corticosterone levels in female control but not mutant rats. Furthermore, stress increased extinction of cued fear conditioning but only in *Nrg1* females. The authors concluded that the findings represent a true *Nrg1* x stress interaction and are consistent with a reduction in sensitivity to environmental stimuli and novelty as described earlier (Taylor et al., 2011a,b). However, *Nrg1* females were the only group susceptible to the effects of adolescent stress on fear extinction. In addition, most earlier findings had been evident in male rats (Taylor et al., 2011b), which failed to be affected by the adolescent stress model chosen.

Social defeat stress models aspect of psychosocial stress in humans, which has been found to interact with a single nucleotide polymorphism of *NRG1* to affect the reactivity of schizophrenia patients to expressed emotion (Keri et al., 2009). Psychosocial stress might also contribute to the development of schizophrenia via sensitization of the pro-inflammatory immune response leading to excessive pro-inflammatory cytokine release. Thus, researchers investigated behavioral and neurophysiological effects of adolescent repeated intermittent social defeat in adult transmembrane domain *Nrg1* mutant males (Desbonnet et al., 2012) and found that *Nrg1* modified the effects of social defeat on several behavioral, immunological and brain measures. For example, psychosocial stress diminished the hyper-locomotive phenotype of *Nrg1* mutant mice without accompanying effects on control littermates. In addition, stress had cognitive-impairing effects in *Nrg1* mice only and decreased sucrose preference (model for anhedonia) in control but not mutant mice. Social defeat also altered the lipopolysaccharide and concanavalin A-stimulated cytokine response of the spleen in a genotype-specific manner (see study for details). In the brain, stress decreased interleukin-beta mRNA levels in the prefrontal cortex of mutant mice only, whereas striatal interleukin-beta was down-regulated in controls and up-regulated in *Nrg1* mice. Finally, hippocampal BDNF mRNA levels were elevated in control mice and reduced in mutant mice whereas tumor necrosis factor-alpha was up-regulated in *Nrg1* mice only. Reduced striatal BDNF levels might have been involved in the disrupting effects of social defeat stress on the spatial memory of *Nrg1* mutant mice (Almli et al., 2000). Importantly, Nrg1 can interact with BDNF in regulating neuronal processes (Mei and Xiong, 2008; Balu and Coyle, 2011), BDNF down-regulation has been reported in schizophrenia (Weickert et al., 2003; Favalli et al., 2012), and BDNF expression changes impact on the sensitivity to social defeat stress (Berton et al., 2006; Krishnan et al., 2007). The authors concluded that the experience of psychosocial stress during adolescence may trigger further pathophysiological features that contribute to the development of schizophrenia in individuals underlying *NRG1* gene abnormalities. The interactive nature of the effects of stress and mutant *Nrg1* resulted in cognitive deficits and an imbalance in BDNF and immunological parameters. On the other side, stress impacted positively on the hyper-locomotive phenotype of *Nrg1* mutant mice, outlining the complexity of GxE interactions in schizophrenia and the need to look at specific disease endophenotypes.

In summary, research teams have started evaluating the role of *Nrg1* in the neuro-endocrine, behavioral, and immunological response of mice to stress. Results so far are inconclusive demanding that future research should focus on schizophrenia-relevant stress models [similar to (Desbonnet et al., 2012)], consider sex and age in experimental designs, and focus on schizophrenia-like behaviors and disease-relevant brain markers.

## Nrg1 X CANNABIS

A review on the role of *Nrg1* in GxE in schizophrenia would be incomplete without mentioning the extensive mouse work on

**Table 1 | Effects of environmental factors on rodent models for the schizophrenia candidate gene *neuregulin 1.***

| | |
|---|---|
| ***Nrg1* × Laboratory housing (i.e., minimal enrichment)** | |
| Transmembrane domain *Nrg1* mutant male mice (Karl et al., 2007) | Minimal enrichment shifted the onset of the hyper-explorative and anxiogenic phenotype of *Nrg1* mice to 3–4 months of age compared to mutant mice kept in standard laboratory housing (onset at 4–6 months of age). |
| ***Nrg1* × Stress** | |
| Acute restraint stress<br>Transmembrane domain *Nrg1* mutant male mice (Chesworth et al., 2012b) | No pronounced effect of *Nrg1* on the endocrine and behavioral effects of acute restraint stress—only subtle, age-dependent modification of stress-induced corticosterone release and anxiety-like behaviors. |
| Acute restraint stress<br>Adult Type II *Nrg1* mutant rats (Taylor et al., 2011a,b) | Altered habituation to an open field environment in *Nrg1* mutant rats.<br>Mutant *Nrg1* resulted in increased baseline corticosterone levels and improved recovery of those levels post stress.<br>Elevated baseline levels of glucocorticoid receptors in hippocampus and pituitary glands. Results are highly sex-specific. |
| Chronic variable stress<br>Adolescent Type II *Nrg1* mutant rats (Taylor et al., 2012) | Female *Nrg1* rats displayed no stress-induced reduction in corticosterone levels and showed increased extinction of cued fear conditioning (no such effects in male *Nrg1* mutants). |
| Social defeat stress<br>Transmembrane domain *Nrg1* mutant mice (Desbonnet et al., 2012) | Stress diminished hyper-locomotion and induced cognitive deficits in *Nrg1* mutant mice without accompanying effects in control mice. *Nrg1* mutant mice were protected against anhedonic properties of social defeat.<br>The effects of stress on the cytokine response of mice were genotype-dependent (for details see study).<br>Stress decreased interleukin-beta mRNA levels in the prefrontal cortex of *Nrg1* mice. Striatal interleukin-beta levels were reduced in control mice and increased in *Nrg1* mice. Hippocampal BDNF mRNA levels were elevated in control mice and reduced in mutant mice whereas tumor necrosis factor-alpha was up-regulated in *Nrg1* mice only. |
| ***Nrg1* × Cannabis reviewed in (Arnold et al., 2012; Karl and Arnold, 2013; Ng et al., 2013)** | |
| Acute treatment with $\Delta^9$-tetrahydrocannabinol (THC)<br>Adult transmembrane domain *Nrg1* mutant mice (Boucher et al., 2007a,b; Long et al., 2010) | *Nrg1* mutants displayed a sex-dependent increased susceptibility to the locomotion-suppressive effects of THC and showed improved prepulse inhibition post THC treatment.<br>THC induced increased neuronal activity in the ventral part of the lateral septum and greater activity in central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus in *Nrg1* mutant mice. |
| Chronic treatment with CP55,940 (CP)<br>Adult transmembrane domain *Nrg1* mutant male mice (Boucher et al., 2011) | *Nrg1* mutants developed a behavioral tolerance to CP-induced hypothermia and hypolocomotion more rapidly, whereas the same mice did not develop a tolerance to CPs anxiogenic effects.<br>Mutant mice showed a selectively increase in CP-induced FosB/ΔFosB expression in the ventral part of lateral septum. |
| Chronic THC treatment<br>Adolescent transmembrane domain *Nrg1* mutant male mice (Long et al., 2013) | THC exacerbated hyperlocomotion 48 h after THC withdrawal. *Nrg1* mutant mice were more resistant to social withdrawal effects of THC.<br>THC promoted genotype-dependent effects on CB1, 5-HT2A and NMDA receptor expression (see study for details). |

*Nrg1* x cannabis interactions. As those studies have been reviewed elsewhere (Arnold et al., 2012; Karl and Arnold, 2013; Ng et al., 2013), this section will only provide a brief summary. It has long been established that cannabis is a component/cumulative cause for the development of schizophrenia (Arseneault et al., 2002, 2004) suggesting interactions with other risk factors (D'Souza et al., 2009). Until recently, Catechol-O-methyltransferase (*COMT*) was the only candidate for a possible interaction between a genetic predisposition for schizophrenia and heavy cannabis abuse [(Caspi et al., 2005; O'Tuathaigh et al., 2010) but see also (Zammit et al., 2011)]. Comprehensive analyses on *Nrg1* x cannabis interactions in transmembrane domain *Nrg1* mutant mice suggest that *Nrg1* increases the susceptibility of an organism to the neuro-behavioral effects of cannabis as well (Boucher et al., 2007a,b, 2011; Long et al., 2010, 2012, 2010). The clinical relevance of this research has recently been highlighted by a genetic study in African Americans, which discovered *NRG1* as a major candidate for the development of cannabis dependence (Han et al., 2012).

## CONCLUSIONS

Recent research utilizing genetic rodent models has revealed an interactive relationship between *Nrg1* and a variety of environmental factors. These interactions appear to be complex and sensitive to a number of subtle variables, but do exist and justify the need for future research in this area (van Os et al., 2010). Researchers should focus on models with significant relevance to schizophrenia including, for example, cannabis abuse (discussed above) and maternal immunization (Ibi et al., 2010; Giovanoli et al., 2013) and consider not only *Nrg1* but also other genetic candidates for GxE interactions. Importantly, the research into *Nrg1*xE outlined above suggests that valid GxE mouse models will be very sensitive to the laboratory environment and other potential test confounders (e.g., age and sex) so that a high level of transparency and standardization of test conditions across research sites will be crucial.

Although the exact nature of *Nrg1*xE and their consequences for schizophrenia have to be evaluated further, an involvement of the GABAergic, glutamatergic and BDNF systems seems likely. Importantly, environmental (risk) factors not always induced adverse (i.e., disease phenotype-strengthening) effects in *Nrg1* mutants, which should be taken into account when looking into GxE interactions [for genotype-specific effects of environmental factors see also (Tucci et al., 2006; Valdar et al., 2006)]. The findings on *Nrg1*xE summarized in **Table 1** are in line with the GxE theory, contribute to the understanding of the pathogenesis of schizophrenia, and might eventually help with possible early intervention programs. Importantly, recent discussions on the appropriate statistical modeling of GxE interactions (van Winkel et al., 2008; Zammit et al., 2010) as well as the limitations of animal model research into schizophrenia (Ayhan et al., 2009) should be considered for future work.

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# Partial genetic deletion of neuregulin 1 and adolescent stress interact to alter NMDA receptor binding in the medial prefrontal cortex

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Schizophrenia is thought to arise due to a complex interaction between genetic and environmental factors during early neurodevelopment. We have recently shown that partial genetic deletion of the schizophrenia susceptibility gene neuregulin 1 (*Nrg1)* and adolescent stress interact to disturb sensorimotor gating, neuroendocrine activity and dendritic morphology in mice. Both stress and *Nrg1* may have converging effects upon N-methyl-D-aspartate receptors (NMDARs) which are implicated in the pathogenesis of schizophrenia, sensorimotor gating and dendritic spine plasticity. Using an identical repeated restraint stress paradigm to our previous study, here we determined NMDAR binding across various brain regions in adolescent *Nrg1* heterozygous (HET) and wild-type (WT) mice using [$^3$H] MK-801 autoradiography. Repeated restraint stress increased NMDAR binding in the ventral part of the lateral septum (LSV) and the dentate gyrus (DG) of the hippocampus irrespective of genotype. Partial genetic deletion of *Nrg1* interacted with adolescent stress to promote an altered pattern of NMDAR binding in the infralimbic (IL) subregion of the medial prefrontal cortex. In the IL, whilst stress tended to increase NMDAR binding in WT mice, it decreased binding in *Nrg1* HET mice. However, in the DG, stress selectively increased the expression of NMDAR binding in *Nrg1* HET mice but not WT mice. These results demonstrate a Nrg1-stress interaction during adolescence on NMDAR binding in the medial prefrontal cortex.

**Keywords: schizophrenia, neuregulin 1, adolescence, stress, NMDA, medial prefrontal cortex, lateral septum, hippocampus**

## INTRODUCTION

Schizophrenia is thought to arise due to a complex interaction between genetic and environmental factors during critical early periods of neurodevelopment that result in disease onset in late adolescence/early adulthood (Weinberger, 1987; Murray et al., 1991; Lewis and Levitt, 2002; Van Winkel et al., 2008; Jaaro-Peled et al., 2009; Van Winkel et al., 2010; Van Os et al., 2010). Ionotropic N-methyl-D-aspartate receptors (NMDARs) mediate activity-dependent plasticity of glutamatergic synapses (Wenthold et al., 2003; Bennett, 2009) and play a key role in normal brain development through regulation of memory, attention and learning processes (Hudspith, 1997; Lieth et al., 2001; Bennett, 2009; Kantrowitz and Javitt, 2010).

Hypofunction of glutamatergic neurotransmission in the form of abnormal functioning of NMDARs in corticolimbic regions of the brain may explain the symptoms of schizophrenia (Carlsson and Carlsson, 1990; Bennett, 2009). For example, the administration of NMDAR antagonists such as phencyclidine (PCP) to humans induces most of the positive and negative symptom, as well as cognitive impairments observed in schizophrenia patients (Javitt, 1987). Similarly, administration of NMDAR antagonists like MK-801 in rodents, particularly during neurodevelopment, promotes lasting schizophrenia-relevant behavioral phenotypes such as locomotor hyperactivity, prepulse inhibition of startle (PPI) deficits, social withdrawal and cognitive dysfunction (Facchinetti et al., 1993; Sircar, 2000; Wang et al., 2001; Harris et al., 2003; Wiley et al., 2003; Andersen and Pouzet, 2004; Stefani and Moghaddam, 2005; Du Bois et al., 2008). Furthermore, post-mortem schizophrenia brain tissue studies have reported an increased binding of the radiolabelled NMDAR ligand MK-801 in the frontal cortex and caudate-putamen (Kornhuber et al., 1989; Newell et al., 2005). Although, reduced NMDAR sub-unit expression has recently been reported in schizophrenia brains which was accompanied by a reduced concentration of NMDA (Errico et al., 2013).

The neurotrophic factor neuregulin 1 (NRG1), is a widely accepted schizophrenia susceptibility gene which plays a significant role in normal brain maturation by influencing neuronal migration, myelination, and synaptic plasticity (Pearce et al., 1987; McDonald and Johnston, 1990; Stefansson et al., 2002; Harrison and Law, 2006; Mei and Xiong, 2008; Barros et al., 2009; Bennett, 2009, 2011). Interestingly, schizophrenia patients

show altered expression of both the ErbB family of receptors for NRG1 and NMDARs (Stefansson et al., 2002; Chong et al., 2008; Alaerts et al., 2009; Hatzimanolis et al., 2013). The shared regulation of neuronal plasticity through the Nrg1-ErbB receptor and NMDARs systems has been demonstrated through an interaction in the post synaptic density (PSD) via the anchoring protein PSD-95 (Garcia et al., 2000; Huang et al., 2000; Bao et al., 2004; Murphy and Bielby-Clarke, 2008). Interestingly partial genetic deletion of *Nrg1* hypophosphorylates NR2B subunits of NMDARs (Bjarnadottir et al., 2007) and promotes subtle changes in NMDAR binding in a number of schizophrenia relevant brain regions in adult rodents (Dean et al., 2008; Long et al., 2013; Newell et al., 2013).

Schizophrenia etiology also consists of an environmental component. Early life stress might be the common denominator linking several environmental risk factors including urbanicity, cannabis use, migration, childhood trauma and obstetric complications (Geddes and Lawrie, 1995; Dalman, 2003; Myin-Germeys et al., 2003; Corfas et al., 2004; Glaser et al., 2006; Henquet et al., 2008; Walker et al., 2008; Van Os et al., 2010). Indeed, adolescence is a period of heightened risk to develop schizophrenia (Walker and Bollini, 2002; Costello et al., 2003; Paus et al., 2008). Increased stress reactivity during adolescence coincides with normal maturation of cognitive abilities, and rapid development of the prefrontal cortex (Leussis et al., 2008; Rahdar and Galvan, 2014) and stabilization of the hippocampus (Leussis et al., 2008). Both the prefrontal cortex and hippocampus are vulnerable to the negative effects of stress (Jinks and McGregor, 1997; Sullivan and Gratton, 1999; Buijs and Van Eden, 2000; McEwen, 2007). Moreover, these regions display schizophrenia brain pathology such as a reduced density of dendritic spines, small protrusions which support excitatory synapses in neuronal circuits (Weinberger and Lipska, 1995; Velakoulis et al., 1999; Eichenbaum, 2000; Glantz and Lewis, 2000; Preston et al., 2005; Von Bohlen Und Halbach et al., 2006; Lawrie et al., 2008; Ebdrup et al., 2010). Stress hormone exposure during adolescence in mice, alters the expression of NMDAR subunits in the prefrontal cortex and hippocampus (Lee et al., 2003; Sterlemann et al., 2010; Buret and Van Den Buuse, 2014), regions that regulate cognitive and sensorimotor gating, and are sensitive to stress-induced loss of dendritic spine density and gray matter losses (Kassem et al., 2013). No prior study has directly examined the effects of adolescent restraint stress on [$^3$H] MK-801 binding in rodents. In adult rats chronic variable stress increased [$^3$H] MK-801 binding in the prefrontal cortex and decreased binding in the hippocampus (Lei and Tejani-Butt, 2010). Given that both neuregulin and stress impact upon NMDARs in their own right, this opens the possibility that neuregulin might confer vulnerability to the effects of stress on NMDAR expression.

*Nrg1* confers vulnerability to the effects of environmental challenges of relevance to schizophrenia. Our laboratory has shown that partial genetic deletion of neuregulin 1 increases sensitivity to the neurobehavioral actions of cannabinoids (Boucher et al., 2007a,b, 2011; Long et al., 2010, 2012, 2013; Spencer et al., 2013) and methamphetamine (Spencer et al., 2012), both of which are drugs of abuse known to activate stress systems in the brain including the HPA axis (Gerra et al., 2003; Huizink et al., 2006; King et al., 2010; Van Leeuwen et al., 2011). We and others have also recently demonstrated that genetic variation in *Nrg1* confers vulnerability to the neurobehavioral effects of stress and modifies neuronal signaling pathways sub serving the stress response. For example, rats heterozygous for type II *Nrg1* display altered expression of glucocorticoid receptors in the pituitary, hippocampus and paraventricular nucleus of the hypothalamus (Taylor et al., 2010). Partial genetic deletion of *Nrg1* conferred vulnerability to the effects of adolescent social defeat stress on spatial working memory function and modulation of inflammatory cytokines in the prefrontal cortex and hippocampus (Desbonnet et al., 2012). We have also recently shown that partial genetic deletion of *Nrg1* altered neurobehavioral responses to repeated adolescent restraint stress (Chohan et al., 2014). Repeated adolescent stress selectively impaired the development of normal sensorimotor gating in *Nrg1* heterozygous (*Nrg1* HET) mice which correlated with a dysregulation in stress-induced corticosterone release. Furthermore, pyramidal neurons in the medial prefrontal cortex of *Nrg1* HET mice exposed to repeated adolescent restraint stress had shorter dendritic lengths and complexity, as well as an increased dendritic spine density. Here we hypothesize that repeated restraint stress, coupled with disrupted Nrg1-ErbB4 signaling during adolescence, might interact to alter NMDAR binding in the mouse brain.

## METHODS

### MICE

Adolescent (PND 35-49) male *Nrg1* HET mice (C57BL/6JArc background strain) and wild-type (WT) littermates were used. The mice were bred in our animal house, sourced from a total of 9 litters and intermixed at weaning on postnatal day (PND) 21. Genotypes were determined after weaning at PND 21 as previously described (Karl et al., 2007). The mice were housed (4–5 animals per homecage) in a room on a 12:12 h light:dark reverse light schedule with food and water available *ad libitum*. Animals had access to environmental enrichment including a cardboard toilet roll, igloo, sunflower seeds, tissue paper and running wheels. Environmentally enriched housing is beneficial when exploring gene and environment interactions (G × E) in mice because it better approximates human cognitive and sensorimotor development than standard housing (Burrows et al., 2011). *Nrg1* HET mice were generated by Prof Richard Harvey (Victor Chang Cardiac Research Institute, Sydney) using a targeting vector in which most of exon 11, which encodes the transmembrane domain, was replaced by a neomycin resistance gene cassette (Stefansson et al., 2002). All research and animal care procedures were approved by the University of Sydney's Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and use of Animals for Scientific Purposes.

### EXPERIMENTAL DESIGN

Male mice were subjected to 30 min/day of restraint stress for 14 days from PND 36 to PND 49 as described in our previous study (Chohan et al., 2014). Restraint stress was chosen as it is a well-characterized physical stressor in rodents that activates the HPA axis and increases anxiety-related behavior

(Eiland and McEwen, 2010; Sutherland et al., 2010; Sutherland and Conti, 2011; Chesworth et al., 2012). Non-stressed animals (WT and *Nrg1* HET) did not receive restraint stress and remained undisturbed in their homecages, similar to prior methods (Eiland and McEwen, 2010; Eiland et al., 2012; Hill et al., 2013; Kwon et al., 2013). Stressed mice were placed in a restraint device (Harvard Apparatus, Holliston, MA, USA), which consisted of a close-ended clear perspex cylinder (9.5 × 2.5 cm). Mice were handled daily for 7 days prior to the commencement of experimentation and randomly allocated to 4 experimental groups: (1) WT-no stress (WT NS, $n = 6$); (2) WT-stress (WT S, $n = 7$); (3) *Nrg1* HET-no stress (*Nrg1* HET NS, $n = 6$), and (4) *Nrg1* HET-stress (*Nrg1* HET S, $n = 5$). Homecage controls and restraint stressed animals were sacrificed by cervical dislocation immediately following their final 30 min restraint stress episode on day 14 (PND 49) and their brains were extracted, snap frozen and stored at −80°C prior to sectioning.

### NMDA RECEPTOR AUTORADIOGRAPHY

NMDAR autoradiography was conducted on brains extracted from the same mice that were used to determine corticosterone levels reported previously by our research group (Chohan et al., 2014). In these mice a differential effect of repeated stress was observed between *Nrg1* HET and WT mice on plasma corticosterone concentrations. The whole brain was coronally sectioned at 20 μm on a cryostat, thaw-mounted onto polysine slides and stored at −80°C until use. Brain regions selected for quantification were identified based on a standard mouse brain atlas (Paxinos, 2004) at bregma levels +1.78 [containing prelimbic (PrL) and infralimbic (IL) cortices); +0.50 (containing anterior cingulate cortex (ACC), rostral caudate-putamen (rCPu), motor cortex (M1-M2), ventrolateral septum (LSV)]; and −1.94 (containing retrosplenial granular cortex (RSG), and subregions of the hippocampus including dentate gyrus (DG), CA1 (cornu ammonis area 1) and CA3 (cornu ammonis area 3) stratum radiatum layers (**Figure 1**). Our prior work showed that Nrg1 hypomorphism alone and in combination with stress affected dendritic morphology in the medial prefrontal cortex and hippocampus (Chohan et al., 2014) and so these regions were consequently analyzed for MK-801 binding in the present study. Furthermore, the medial prefrontal cortex and hippocampus are strongly implicated in the neurobiology of schizophrenia and stress (Michelsen et al., 2007; Radley et al., 2008; Alfarez et al., 2009). The caudal ACC region was examined, as it has been shown previously by our group to be affected by stress (Kassem et al., 2013) and is a point of comparison to another MK-801 binding study performed in *Nrg1* HET mice (Newell et al., 2013). Further, we examined the LSV at it is thought to mediate stress and anxiety-related behavior (Dielenberg et al., 2001; Sheehan et al., 2004) and was shown to be dysregulated in our prior work on Nrg1-cannabinoid interactions (Boucher et al., 2007b, 2011).

The sections were incubated in 30 mM HEPES buffer (pH 7.45) containing 23 nM [$^3$H] MK-801 (specific activity 27.5 Ci/mmol, PerkinElmer, USA), 100 μM glycine, 100 μM L-glutamate and 1 mM EDTA for 2.5 h at room temperature. Non-specific binding was determined by incubating adjacent sections with [$^3$H] MK-801 in the presence of 200 μM (ketamine hydrochloride, National Measurement Institute, Sydney, Australia). Following the incubation, the sections were washed twice for 20 min each at 4°C in 30 mM HEPES containing 1 mM EDTA (pH 7.45) and rapidly dried under a stream of cool air.

### QUANTITATIVE ANALYSIS OF AUTORADIOGRAPHIC IMAGES

Following the binding assays, all sections were placed on Kodak BioMax MR Film along with a [$^3$H] autoradiographic standard (Amersham, UK) for 4 or 6 weeks. Some of the samples from the CA1 region of the hippocampus were re-exposed for 4 weeks due to initial oversaturation of the films, to allow them to fall within the normal pseudolinear response range. Films were developed using Kodak GBX developer/fixer (Sigma-Aldrich, NSW, Australia). Films were scanned using a BioRad GS-800 calibrated densitometer, and quantification of mean density performed in each brain region [average optical density over three adjacent brain sections, for total binding and non-specific binding, using ImageJ (http://rsbweb.nih.gov/ij)]. Using density values for calibrated [$^3$H] autoradiographic standards, radioactive concentrations were derived for all density values using a standard curve, and converted into fmol per mg tissue equivalent (fmol/mg). All regions quantified were analyzed blind to treatment group. Specific *in vitro* binding of [$^3$H] MK-801 was calculated by subtraction of non-specific from total binding values. For each brain region, 6 frozen sections (3 total binding and 3 non-specific binding were selected per animal. Due to sectioning problems some of the sections were torn and unsuitable for processing. Therefore, for some of the brain regions the final value represents an average from five animals only.

### STATISTICAL ANALYSES

Statistical analyses were performed using SPSS (IBM, IL, USA) or Statview (SAS Institute Inc) software. Statistically significant variation in radioligand binding was identified by Two-Way analysis of variance (ANOVA) with genotype or stress as factors. Planned Bonferroni comparisons were conducted to further analyze differences between experimental groups on all measures using the following four specific comparisons (WT-no stress vs. *Nrg1* HET-no stress, WT-stress vs. *Nrg1* HET-stress, WT-no stress vs. WT-stress, and *Nrg1* HET-no stress vs. *Nrg1* HET stress). The results of all analyses were deemed significant at $p < 0.05$.

## RESULTS

In all groups, the highest density of specific binding was distinctly observed in the hippocampus (CA1 & CA3 subregions). Moderately high levels of [$^3$H] MK-801 binding were observed in the PrL, IL and anterior cingulate cortices. The rCPu, RSG, M1-M2, DG and LSV subdivisions displayed moderate-low levels of [$^3$H] MK-801 binding. Two factor ANOVA revealed a significant genotype by stress interaction ($F_{(1, 18)} = 4.53$, $p < 0.05$) in the IL cortex (**Table 1**, **Figure 2A**). A significant effect of stress was found for [$^3$H] MK-801 binding in the LSV ($F_{(1, 19)} = 5.58$, $p < 0.05$) and DG ($F_{(1, 20)} = 15.51$, $p < 0.001$), demonstrating that restraint stress significantly increased NMDAR expression in these regions independent of genotype (**Table 1** and **Figure 2B**). Planned Bonferroni comparisons revealed that stressed *Nrg1* HET

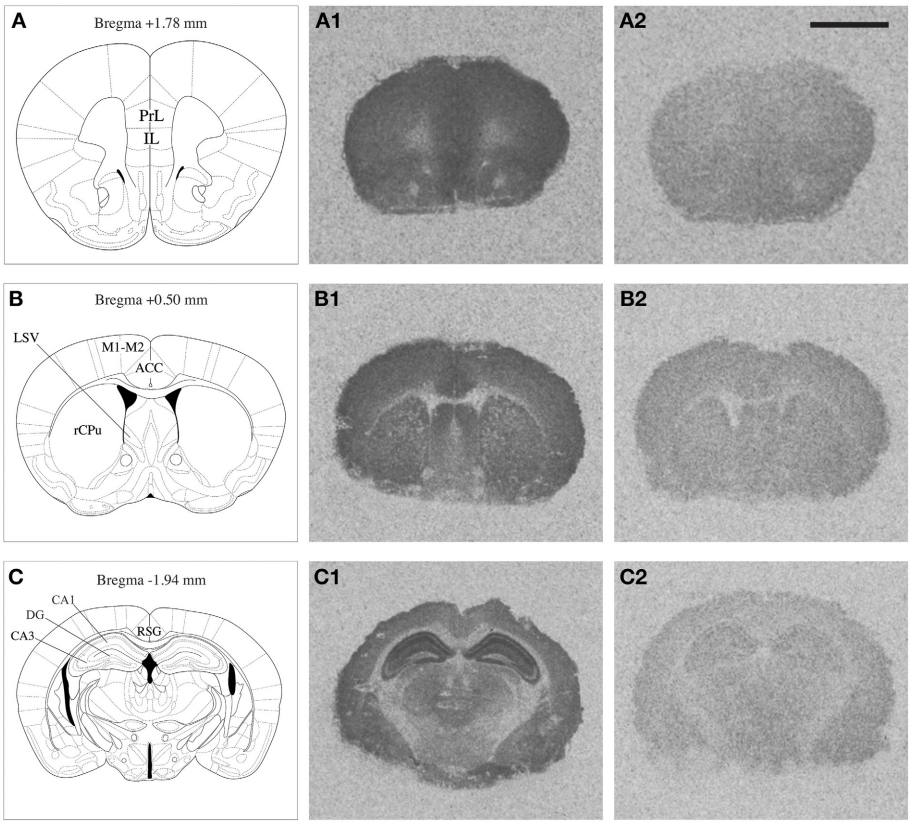


**FIGURE 1 | Mouse brain atlas adapted from Paxinos (2004), indicating the brain regions quantified (A,B,C); PrL: prelimbic cortex, IL: infralimbic cortex, rCPu: rostral caudate putamen, M1-M2: motor cortex, ACC: anterior cingulate cortex, LSV: ventrolateral septum, RSG: retrosplenial granular cortex, DG: dentate gyrus, CA1 and CA3 subregions of the hippocampus.** Representative autoradiograms of coronal brain sections showing total [$^3$H] MK-801 binding ($A_1$,$B_1$,$C_1$) and non-specific [$^3$H] MK-801 binding ($A_2$,$B_2$,$C_2$). Scale bar = 2.5 mm.

mice exhibited greater MK-801 binding in the DG compared with their non-stressed counterparts ($p < 0.01$). There were no main effects of genotype or stress, or genotype by stress interactions for NMDAR binding in any other brain regions examined (**Table 1**). No significant NMDAR binding differences between specific experimental groups in the other brain regions were observed ($p > 0.05$).

## DISCUSSION

Here we show that in adolescence partial genetic deletion of *Nrg1* promoted an idiosyncratic change in medial prefrontal cortex NMDAR binding in response to repeated stress. Repeated stress exposure tended to decrease [$^3$H] MK-801 binding in *Nrg1* HET mice whilst promoting an increase in binding in WT mice in the IL cortex, a subregion of the medial prefrontal cortex. In the DG region of the hippocampus, stress significantly increased NMDAR binding. Interestingly, stressed *Nrg1* HET mice displayed significantly higher NMDAR binding than non-stressed *Nrg1* HET mice, an effect that was absent in WT mice. In addition, we report for the first time that restraint stress increased [$^3$H] MK-801 binding levels in the LSV.

Partial genetic deletion of *Nrg1* failed to significantly alter NMDAR binding in the other brain regions examined (PrL, rCPu, RSG, ACC, motor cortex & CA1/CA3 regions of the hippocampus) when measured in late adolescence (PND 49). Prior studies have shown that adult *Nrg1* HET mice (> PND 60) display unaltered [$^3$H] MK-801 binding in the cortex, caudate putamen, hippocampus and the septum (Dean et al., 2008; Long et al., 2013). Inconsistent with our present findings in late adolescent mice, adult *Nrg1* HET mice exhibited increased NMDAR binding in the ACC and motor cortex compared to WT mice (Newell et al., 2013). The differences observed between the current study and the findings of Newell et al. (2013) may be explained by the different developmental period examined between the studies. As NMDARs undergo significant changes across development (Scheetz and Constantine-Paton, 1994; Cull-Candy et al., 2001; Haberny et al., 2002) and the locomotor hyperactivity phenotype of *Nrg1* HET mice develops over time (Karl et al., 2007), it is possible that the effect of partial genetic deletion of *Nrg1* on NMDAR binding might also follow a developmental trajectory and become significant in adulthood.

Here we show for the first time that repeated restraint stress in adolescence increased NMDAR binding in the LSV. The LSV is responsible for promoting active behavioral responses in stressful situations (De Oca and Fanselow, 2004; Sheehan et al., 2004) and its ablation provoked septal rage and exaggerated defensive

**Table 1 | Specific NMDAR binding densities in repeatedly stressed and non-stressed WT and *Nrg1* HET mice.**

| Brain Regions | WT | | | | *Nrg1* HET | | | | Two-Way ANOVA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No Stress | | Stress | | No Stress | | Stress | | Genotype | | Condition | | G × E | |
| | Mean | s.e.m. | Mean | s.e.m. | Mean | s.e.m. | Mean | s.e.m. | *F-value* | *p-value* | *F-value* | *p-value* | *F-value* | *p-value* |
| PrL | 314.975 | ±26.642 | 342.732 | ± 22.813 | 349.642 | ±13.966 | 308.860 | ±23.018 | $F_{(1,18)} = 0.0004$ | 0.986 | $F_{(1,18)} = 0.090$ | 0.768 | $F_{(1,18)} = 2.493$ | 0.132 |
| IL | 297.697 | ±31.497 | 347.315 | ±23.179 | 348.422 | ±21.457 | 295.171 | ±19.492 | $F_{(1,18)} = 0.001$ | 0.977 | $F_{(1,18)} = 0.006$ | 0.941 | $F_{(1,18)} = 4.527$ | **0.047** |
| rCPu | 160.311 | ±18.842 | 200.519 | ±9.704 | 169.193 | ±11.588 | 160.594 | ±11.154 | $F_{(1,19)} = 1.469$ | 0.240 | $F_{(1,19)} = 1.523$ | 0.232 | $F_{(1,19)} = 3.630$ | 0.072 |
| M1-M2 | 247.933 | ±16.090 | 264.826 | ±9.768 | 236.519 | ±11.668 | 224.474 | ±20.893 | $F_{(1,19)} = 3.223$ | 0.088 | $F_{(1,19)} = 0.028$ | 0.868 | $F_{(1,19)} = 1.007$ | 0.328 |
| ACC | 291.354 | ±26.510 | 324.255 | ±16.283 | 281.255 | ±11.494 | 262.916 | ±14.381 | $F_{(1,20)} = 3.744$ | 0.067 | $F_{(1,20)} = 0.156$ | 0.697 | $F_{(1,20)} = 1.926$ | 0.181 |
| RSG | 171.369 | ±11.506 | 169.808 | ±14.702 | 143.954 | ±9.942 | 184.995 | ±16.380 | $F_{(1,20)} = 0.207$ | 0.654 | $F_{(1,20)} = 2.156$ | 0.158 | $F_{(1,20)} = 2.511$ | 0.129 |
| LSV | 127.992 | ±19.497 | 176.069 | ±14.489 | 128.175 | ±7.725 | 146.736 | ±10.233 | $F_{(1,19)} = 1.068$ | 0.314 | $F_{(1,19)} = 5.584$ | **0.029** | $F_{(1,19)} = 1.095$ | 0.308 |
| CA1 | 610.541 | ±27.062 | 619.457 | ±17.838 | 640.685 | ±23.267 | 678.384 | ±35.213 | $F_{(1,20)} = 3.072$ | 0.095 | $F_{(1,20)} = 0.842$ | 0.367 | $F_{(1,20)} = 0.321$ | 0.577 |
| CA3 | 459.248 | ±42.928 | 458.411 | ±41.408 | 416.045 | ±23.161 | 443.388 | ±32.370 | $F_{(1,19)} = 0.640$ | 0.434 | $F_{(1,19)} = 0.133$ | 0.720 | $F_{(1,19)} = 0.150$ | 0.703 |
| DG | 111.176 | ±10.369 | 137.475 | ±13.169 | 90.114 | ±6.194 | 153.924 | ±13.829 | $F_{(1,20)} = 0.041$ | 0.842 | $F_{(1,20)} = 15.510$ | **0.0008** | $F_{(1,20)} = 2.688$ | 0.117 |

*Data expressed as mean fmol/mg tissue ± s.e.m. for all brain regions examined (n = 5–7/group). Significant values are highlighted in bold. PrL, prelimbic cortex; IL, infralimbic cortex; rCPu, rostral caudate putamen; M1-M2, motor cortex; ACC, anterior cingulate cortex; LSV, ventrolateral septum; RSG, retrosplenial granular cortex; DG, dentate gyrus; CA1 and CA3 subregions of the hippocampus.*




**FIGURE 2 | [$^3$H] MK-801 binding to NMDARs (fmol/mg tissue) in (A) infralimbic cortex (IL), (B) ventrolateral septum (LSV) and (C) dentate gyrus (DG) in repeatedly stressed (S) and non-stressed (NS) wild type-like (WT) and *Nrg1* HET mice; $n = 5$–7/group.** Data are presented as means ± s.e.m. Significant effects are indicated by #$p < 0.05$ and ###$p < 0.001$ (main effects of stress); **$p < 0.01$ [planned Bonferroni comparison, *Nrg1* HET (NS) vs. *Nrg1* HET (S)].

behaviors (Brady and Nauta, 1953). These findings imply that the integrity of the lateral septum is vital for the inhibition of excessive fear and anxiety. New evidence however indicates that the role of the lateral septum in controlling fear and anxiety is more complex than this, as infusion of CRF type 2 receptor agonists or optogenetic transient activation of CRF type 2 receptors in the lateral septum promoted anxiety-related behaviors (Henry et al., 2006; Anthony et al., 2014). Little research has examined the role of NMDARs in the lateral septum in the control of defensive behaviors. NMDAR knockout mice display reduced aggressive behavior and swim-stress induced Fos expression in the lateral septum than WT mice (Duncan et al., 2009).

The ability to directly compare studies examining the effects of stress on NMDARs is limited by factors including the diversity of stress paradigms implemented, differences in post-stress washout periods, and the multitude of methods used to analyse NMDAR expression. Most studies have explored the effect of stress on NMDAR subunit protein & mRNA expression, rather than total NMDAR binding as measured with [$^3$H] MK-801 autoradiography (Sterlemann et al., 2010; Buret and Van Den Buuse, 2014). No prior study has directly examined the effects of adolescent restraint stress on [$^3$H] MK-801 binding in rodents. In adult rats chronic variable stress increased [$^3$H] MK-801 binding in the prefrontal cortex, caudate putamen, nucleus accumbens and basolateral amygdala, while decreasing binding in the hippocampus (Lei and Tejani-Butt, 2010). Here we could only discern measurable effects of stress on the LSV and DG, which might be explained by our use of a relatively mild restraint stress paradigm (30 min per day for 14 days). To resolve the effects of stress on NMDAR binding in other brain regions might require a more intense stress regimen like the classic paradigm of 6 h per day for 21 days that reliably induces retraction of dendrites and loss of gray matter (Radley et al., 2004, 2006, 2008; Magarinos et al., 2011; Kassem et al., 2013). Alternatively, it is possible that earlier application of the stressor (i.e., from PND 28) might have been more effective as recent data suggests that peripubertal stressor exposure (i.e., encompassing the juvenile through to pubertal period, PND 28-42 in rats) is critical to provoking neurobiological changes in stress circuits including increased NMDAR expression (Tzanoulinou et al., 2014).

Using an identical adolescent stress protocol we recently reported that partial genetic deletion of *Nrg1* and repeated stress interacted to offset the normal development of sensorimotor gating and blunted stress-induced corticosterone levels (Chohan et al., 2014). We also provided evidence of abnormal dendritic morphology in the medial prefrontal cortex of *Nrg1* HET mice exposed to stress. Specifically, unlike WT mice whose dendritic morphology was unaffected by stress, repeated stress in *Nrg1* HET mice reduced the length of dendrites and their complexity, and promoted an increase in dendritic spine density in pyramidal neurons of layers II/III of the anterior cingulate and PrL cortices of the medial prefrontal cortex. Given that Nrg1 and stress both influence NMDARs (Garcia et al., 2000; Bjarnadottir et al., 2007; Law et al., 2007; Li et al., 2007; Chong et al., 2008; Bennett, 2009; Cohen et al., 2010; Bennett et al., 2011; Buret and Van Den Buuse, 2014) and that NMDARs regulate the density of dendritic spines (Alvarez et al., 2007; Hayashi-Takagi et al., 2010) we hypothesized that Nrg1 and stress might interact to alter NMDAR binding specifically in the anterior cingulate and PrL cortices.

Therefore, it was surprising to observe in the present study that the Nrg1-stress interaction on NMDAR binding occurred in the IL cortex rather than the PrL cortex. The IL cortex shares reciprocal connections with the PrL cortex (Gabbott et al., 2003, 2005; Jones et al., 2005; Hoover and Vertes, 2007; Gutman et al., 2012) and the IL and PrL regions of the medial prefrontal cortex cooperate to produce an integrated response to stress (McDougall et al., 2004). Therefore, it is possible then that the changes in dendritic morphology in the anterior cingulate and PrL cortices in our previous study (Chohan et al., 2014) may be a cause or consequence of the Nrg1-stress interaction on NMDAR binding in the IL cortex we observed here. Indeed, perturbation of activity in the IL has flow on effects on the PrL cortex, as activation of IL cortex output via optical stimulation in adult rats inhibits PrL pyramidal neurons (Ji and Neugebauer, 2012). Here, there was a tendency toward reduced [$^3$H] MK-801 binding in the medial prefrontal cortex of *Nrg1* HET mice which accords with the general view of NMDAR hypofunction in schizophrenia as well as research showing that NMDAR expression is reduced in the schizophrenia brain (Errico et al., 2013). Although, this contradicts studies that report [$^3$H] increased MK-801 binding in post-mortem schizophrenia brains (Kornhuber et al., 1989; Newell et al., 2005).

Here we report that repeated stress-induced increased NMDAR binding in the DG in *Nrg1* HET mice but not in WT mice, which provides some additional support for *Nrg1* HET mice being more sensitive to the effects of stress on NMDAR binding. However, this must be interpreted cautiously in the absence of an overall interaction between Nrg1 genotype and stress condition. The DG plays an important role in memory and sensorimotor gating function (Reul et al., 2009; Guo et al., 2013), thus the stress induced increase in NMDAR binding specifically in *Nrg1* HET mice observed here may partially explain the spatial memory and PPI deficits observed previously in these mice following adolescent stress (Desbonnet et al., 2012; Chohan et al., 2014). Juvenile stress decreases expression of type III Nrg1 in the hippocampus (Brydges et al., 2014), so it is possible that the effects of stress on an already depleted Nrg1 level in hypomorphic mice is sufficient to then increase [$^3$H] MK-801 binding. Why the DG but not the CA1 or CA3 region is selectively vulnerable to this effect is unclear. It might be partially explained by the DG expressing relatively lower levels of NRG1 than other hippocampal subfields (Law et al., 2004). The mechanisms responsible for the effect of stress on [$^3$H] MK-801 binding in *Nrg1* HET mice will need to be specifically addressed in future research including studies which directly examine the expression, internalization and phosphorylation status of NMDAR, and also whether this effect can be magnified by a more intense stress protocol.

Our findings further reinforce research showing that variation in *Nrg1* confers vulnerability to the effects of stress. Human studies have shown that a *NRG1* polymorphism interacted with psychosocial stress to effect reactivity to expressed emotions in schizophrenia patients (Keri et al., 2009) and that polymorphic variation in *NRG1* interacts with job strain to increase the risk of heart disease (Hintsanen et al., 2007). Reduced type II *Nrg1* expression in rats induced increased baseline corticosterone levels, a disruption in recovery of stress-induced plasma corticosterone concentrations, as well as elevated levels of glucocorticoid receptors in the hippocampus, paraventricular nucleus of the hypothalamus and pituitary gland (Taylor et al., 2010). Further, complex gender specific interactions of type II *Nrg1* genotype and adolescent chronic variable stress were reported on anxiety-related behavior and cued fear conditioning (Taylor et al., 2012). Stress-induced increase in corticosterone was more pronounced in *Nrg1* HET mice than WT mice at the younger (3–4 months) but not the older age group (6–7 months) (Chesworth et al., 2012), highlighting the developmental effect of stress and Nrg1

hypomorphism on the HPA axis. Adolescent social defeat stress has also been shown to selectively impair spatial memory and decrease expression of the inflammatory cytokine interleukin 1β in the prefrontal cortex of *Nrg1* HET mice, but not WT mice (Desbonnet et al., 2012). The latter finding might be related to the present finding of partial genetic deletion of *Nrg1* promoting a unique stress-induced downregulation of NMDAR binding in the prefrontal cortex, as interleukin 1β (IL 1β) has been shown to potentiate NMDA function and reduce the density of synaptic spines (Viviani et al., 2003, 2006). Further, the effects of IL 1β are mediated by interleukin receptor 1 (ILR1) which appear to interact with NR2B subunits of the NMDAR in the postsynaptic density (Gardoni et al., 2011; Viviani et al., 2013).

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# An investigation into "two hit" effects of BDNF deficiency and young-adult cannabinoid receptor stimulation on prepulse inhibition regulation and memory in mice

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Reduced brain-derived neurotrophic factor (BDNF) signaling has been shown in the frontal cortex and hippocampus in schizophrenia. The aim of the present study was to investigate whether a BDNF deficit would modulate effects of chronic cannabis intake, a well-described risk factor for schizophrenia development. BDNF heterozygous mice (HET) and wild-type controls were chronically treated during weeks 6, 7, and 8 of life with the cannabinoid receptor agonist, CP55,940 (CP). After a 2-week delay, there were no CP-induced deficits in any of the groups in short-term spatial memory in a Y-maze task or novel object recognition memory. Baseline prepulse inhibition (PPI) was lower but average startle was increased in BDNF HET compared to wild-type controls. Acute CP administration before the PPI session caused a marked increase in PPI in male HET mice pre-treated with CP but not in any of the other male groups. In females, there were small increases of PPI in all groups upon acute CP administration. Acute CP administration furthermore reduced startle and this effect was greater in HET mice irrespective of chronic CP pre-treatment. Analysis of the levels of [$^3$H]CP55,940 binding by autoradiography revealed a significant increase in the nucleus accumbens of male BDNF HET mice previously treated with CP but not in any of the other groups or in the caudate nucleus. These results show that BDNF deficiency and chronic young-adult cannabinoid receptor stimulation do not interact in this model on learning and memory later in life. In contrast, male "two hit" mice, but not females, were hypersensitive to the effect of acute CP on sensorimotor gating. These effects may be related to a selective increase of [$^3$H]CP55,940 binding in the nucleus accumbens, reflecting up-regulation of CB1 receptor density in this region. These data could be of relevance to our understanding of differential "two hit" neurodevelopmental mechanisms in schizophrenia.

**Keywords: brain-derived neurotrophic factor, prepulse inhibition, schizophrenia, cannabis, memory, mice**

## INTRODUCTION

It is becoming widely accepted that the development of schizophrenia cannot be explained by single gene mutations or environmental factors. Instead, multiple factors, e.g., gene polymorphisms which increase risk, and environmental insults, such as stress or drug abuse, are likely to act synergistically to trigger disease onset, a model commonly referred to as the "two hit" hypothesis of schizophrenia (Bayer et al., 1999; Maynard et al., 2001; McGrath et al., 2003). Several animal model studies are attempting to delineate the molecular mechanisms involved in this synergism, with the ultimate aim to provide new treatment targets, including for early intervention. For example, studies have shown that temporary treatment with low doses of antipsychotics in late adolescence may be effective in preventing the emergence of schizophrenia-like behavioral phenotype in neurodevelopmental animal models of the illness (Piontkewitz et al., 2009). However, the mechanisms involved in such preventative treatments remain unclear.

Brain-derived neurotrophic factor (BDNF) is involved in brain development and neuroplasticity (Monteggia et al., 2004; Lu et al., 2008). Altered BDNF signaling has been implicated in a number of psychiatric illnesses, including schizophrenia and depression (Angelucci et al., 2005; Autry and Monteggia, 2012). For example, post-mortem studies have found significant reductions of BDNF gene expression and protein levels in the brains of people with schizophrenia (Hashimoto et al., 2003; Weickert et al., 2003; Durany and Thome, 2004). Levels of BDNF in the brain are modulated by stress in an age-, sex-, and stress-type-dependent manner (Bath et al., 2013). We have previously used chronic administration of the stress hormone, corticosterone, as a model of late adolescent/early adulthood stress. Corticosterone treatment in BDNF heterozygous (HET) mice caused sex-specific long-term effects in the Y-maze and other effects (Klug et al., 2012).

Cannabis abuse may precipitate psychosis development in vulnerable individuals, much like a "two hit" effect (Van Os et al.,

2002; Henquet et al., 2008; Gururajan et al., 2012). BDNF may be part of the neurochemical mechanism involved in this interaction. Previous studies have shown that cannabis use can alter levels of BDNF in humans as well as in animals (Derkinderen et al., 2003; Jockers-Scherübl et al., 2004; Butovsky et al., 2005; D'souza et al., 2009). For example, injection of $\Delta^9$-tetrahydrocannabinol (THC) has been shown to upregulate BDNF expression in rats and mice (Derkinderen et al., 2003; Butovsky et al., 2005) and BDNF serum levels in healthy humans (D'souza et al., 2009). However, in human studies looking at chronic abuse of cannabis, it is not as clear in which direction cannabis exerts its influence on BDNF. While some have shown that chronic cannabis users have lower basal levels of serum BDNF (D'souza et al., 2009) others could not detect such differences (Jockers-Scherübl et al., 2004; Angelucci et al., 2008). In a study focusing on the val66met BDNF polymorphism, in female psychotic patients, cannabis use was associated with a 7 year-earlier onset of the disease when patients were BDNF-Met carriers but not when they were Val/Val genotypes (Decoster et al., 2011). These results suggest interactions between cannabis use, BDNF and psychotic disorders, even though the mechanism involved remains unclear. We recently investigated maternal separation in rats as a first developmental "hit" and treated the animals with the cannabinoid receptor agonist, CP55,940 (CP) (Klug and Van Den Buuse, 2012). Male "two hit" rats, but not female rats, showed anhedonia-like behavior and increased anxiety, but no deficits in short-term spatial memory (Klug and Van Den Buuse, 2012). Thus, treatment with CP in maternally-separated rats caused markedly different long-term effects than those seen in BDNF HET mice treated with corticosterone. This suggested marked qualitative differences in the two hit effects of either stress (in the form of corticosterone treatment) compared to cannabis abuse (in the form of chronic CP injections). However, species differences could not be ruled out. Therefore, the present study is focused on combining genetically-induced deficiency of BDNF in mice and treatment with the cannabinoid receptor agonist, CP, during adolescence/young adulthood.

We used BDNF heterozygous (HET) mice and treated them with CP from 6 to 9 weeks of age (adolescence/young adulthood) which represents a critical time window for detrimental effects of cannabis abuse (Schneider, 2008). We investigated locomotor activity at the beginning and end of treatment, as well as 2 weeks after treatment had ceased. To investigate whether cognition was affected in the animals, we used the Y-maze and the novel object recognition test. Baseline PPI was compared between the groups and, in addition, in a separate session the mice received an acute injection of CP to see whether differences in reaction to the drug would occur between animals previously treated with the cannabinoid agonist and animals previously treated with vehicle.

## METHODS

### ANIMALS AND PROTOCOLS

Male and female BDNF heterozygous and wild-type control mice were obtained from a breeding colony at the Florey Neuroscience Institutes animal facility and brought over to the Mental Health Research Institute at 4–5 weeks of age, where they were housed in individually-ventilated cages (IVC). All experiments and procedures were approved by the Animal Experimentation Ethics Committee of the Florey Neuroscience Institutes, University of Melbourne, Australia.

The mice received injections with 0.4 mg/kg CP55,940 or vehicle from 6 to 9 weeks of age. CP55,940 ((-)-cis-3-[2-hydroxy-4-(1,1,-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol) was obtained from Tocris Bioscience (Bristol, UK) and first dissolved in 100% ethanol and then diluted in Tween 80 and saline to make a final vehicle solution of 2.5% ethanol, 2.5% Tween 80, and 95% saline. Mice were injected intraperitoneally (i.p.) once a day (at 9:00 am) on weekdays at a dose of 0.4 mg/kg in a volume of 10 ml/kg. The treatment started at 6 weeks of age and continued for 3 weeks until the mice were 9 weeks of age. The mice received no injections on the weekends. Control animals received vehicle solution injections. Thus, there were four male and four female experimental groups: wild-type mice treated with vehicle (WT/veh), wild-type mice treated with CP55,940 (WT/CP), BDNF heterozygous mice treated with vehicle (HET/veh) and BDNF HET mice treated with CP55,940 (HET/CP). Each group consisted of 10–16 animals.

Body weights were obtained at 6 weeks of age (CP treatment start), 9 weeks of age (end of CP treatment) and 12 weeks of age (during behavioral testing). Behavioral testing started at 11 weeks of age and included locomotor activity, Y-maze, novel object recognition, and prepulse inhibition (PPI).

### LOCOMOTOR ACTIVITY

To assess locomotor hyperactivity, mice were placed into individual automated photobeam activity cages (Tru Scan, Coulbourn Instruments, Whitehall, PA, USA; 27 cm l × 27 cm w × 40 cm h). Each cage was fitted out with photobeams which detected an animal's position at any one time during a session. The associated software used this information to calculate the distance moved in centimetres by the animal in 5 min intervals. Mice were placed into the chambers for 60 min to habituate them to the new environment and record baseline activity. Each animal was tested three times for changes in locomotor activity: at the beginning of CP treatment (6 weeks of age), at the end of the treatment (9 weeks of age) and 2 weeks after treatment had ceased (11 weeks of age). During the first two sessions, the mice were placed into the chambers immediately after injection with the daily dose of CP or vehicle and total distance moved was recorded for 12 five min blocks. At 11 weeks of age, locomotor activity was similarly measured but the animals received no injection.

### Y-MAZE

Short-term spatial memory was assessed using the Y-maze (Dellu et al., 1992, 2000), which consisted of three arms (30 cm l × 8 cm w × 16 cm h) with geometric shapes on the wall and a triangular central platform. In the first exposure session, animals were placed facing the wall at the end of one arm (start arm) and allowed to explore the Y-maze for 10 min with one of the two other arms closed-off (novel arm). After a retention period, animals were placed back into the start arm and allowed to explore all three arms of the maze for 5 min. Behavior was recorded on video and analyzed with video tracking software (Ethovision, Noldus, The Netherlands). Behaviors analyzed were time spent in each arm, percentage number of entries to each arm and the overall number of arm entries to measure locomotor

activity. We chose to display percentage number of entries to account for possible differences in locomotor activity. Mice were tested twice in the Y-maze with two different retention periods of 1 or 2 h (Dellu et al., 1992, 2000; Conrad et al., 2003) but because similar results were obtained, only data pertaining to the 2-h interval will be shown here.

### NOVEL OBJECT RECOGNITION

Object recognition was assessed using the novel object recognition task. Two days before testing, animals were habituated to the empty testing apparatus (40 cm l × 30 cm w × 10 cm h) for 10 min each day. On the third day the animals were tested for novel object recognition memory. On the testing day, two identical objects were placed in the right and left corner at the end of the arena. The animals were then placed into the arena facing the opposite wall and were allowed to explore the objects for 10 min (introduction phase). The animals were then removed and returned back to their home cages for 2 h. Following the delay, the animals were placed back into the arena, which now contained one familiar object (from the introduction phase) and one novel object. The animals were allowed to explore the objects for 5 min (recognition phase). Behavior in both phases was recorded on video and analyzed with video tracking software (TopScan, CleverSys Inc., Reston, VA, USA). The time spent sniffing the objects in the introduction phase was analyzed and used as a measure to detect side preference and interaction time. The time spent sniffing the objects in the recognition phase was used as a measure of recognition. Animals that spent less than 10 s exploring both objects were excluded from the analysis. The amount of time spent investigating the novel object was expressed as percentage of total object exploration time.

### PREPULSE INHIBITION OF ACOUSTIC STARTLE

PPI was measured using eight automated startle chambers (SR-LAB, San Diego Instruments, San Diego, CA, USA). Animals were placed individually in a Plexiglas cylinder of 3.8 cm diameter which was secured to a platform with a piezoelectric accelerometer mounted under it to detect whole body startle responses. The cylinders were placed in a ventilated, sound-attenuated and well-lit startle box. Background noise and acoustic pulses were presented through a speaker in the startle box and responses were measured with the SR-Lab software (San Diego instruments) running on a computer in an adjacent room.

A single PPI session included 104 trials and lasted ∼40 min. There was an initial 3 min acclimation period with continuous background white noise only, set at 70 dB, which continued throughout the rest of the entire session. The first and last eight trials were presented at 115 dB. The 88 trials in-between were presented in a pseudo-random order and included sixteen 115 dB startle pulses and 72 prepulse-pulse trials including eight of each prepulse (PP) intensity of 2, 4, 8, 16 dB over baseline and eight "no-stimulus" trials, where no pulse was presented. Each startle pulse was 40 ms in duration, the prepulse was 20 ms and there were either a 30 or 100 ms inter-stimulus interval (ISI) between the prepulse and the pulse. Because the clearest group differences were observed with the 100 ms ISI, only those data will be shown here. Startle amplitude was analyzed as the average of all 115 dB pulse-alone trials. Startle habituation was analyzed by using the pulse-alone data in four blocks of eight trials. PPI was calculated as the percentage difference of the responses to pulse-alone trials minus prepulse trials divided by the response to pulse-alone trials.

The PPI experiment included a saline treatment session and a challenge session with an acute injection of CP (0.4 mg/kg). Saline and CP were administered in a volume of 10 ml/kg, 5 min prior to the PPI session. There were 3 days of drug wash-out in-between the two test sessions. The order of drug administration was randomized to control for the possibility of test habituation.

One week after the last behavioral test, mice were killed by cervical dislocation and the brains stored at −80°C until further use.

### [$^3$H]CP55,940 BINDING

The procedure was essentially as previously described (Chavez et al., 2010) with modifications. Briefly, 20 μm brain sections including the cingulate cortex, caudate nucleus, and nucleus accumbens were cut on a cryostat and thaw-mounted on gelatinized slides. On the day of the experiments, the slides were first washed for 30 min in a 50 mM Tris HCl buffer with 1% BSA (pH 7.4) at room temperature. The sections were then exposed to either the total binding solution, containing [$^3$H]CP55,940 at a final concentration of 2 nM, or the non-specific binding solution containing [$^3$H]CP55,940 and 10 μM of CP55,940 for 2 h at room temperature. The reaction was terminated by three 20 min washes in ice cold buffer. The sections were then air-dried and partially fixed in paraformaldehyde vapor overnight before being placed in Fujifilm BAS 2025 cassettes (Berthold, Australia) and apposed to BAS-TR2025 phosphoimaging plates (Berthold) for 6 days. Autoradiographic images were analyzed using AIS image analysis software (Imaging Research, ON, Canada). Binding densities in the nucleus accumbens and caudate nucleus were quantitated by comparing them to [$^3$H] microscales and expressed as fmol/mg estimated tissue equivalent (ETE) (Pavey et al., 2002; Chavez et al., 2010). It should be noted that this single-point agonist binding method most likely represents high-affinity binding and does not distinguish between possible changes in Kd or Bmax of the receptor.

### DATA ANALYSIS

Data were expressed as the mean ± the standard error of the mean (SEM). Data analysis was conducted with the software program SPSS Statistics GradPack 17.0 (SPSS Inc, Chicago, IL, USA). All data were analyzed using a Three-Way analysis of variance (ANOVA) with repeated measures where appropriate, with factors being sex, first "hit" (BDNF heterozygosity) and second "hit" (CP) as main factors. When interactions were found, separate Two-Way ANOVAs split by one of the independent factors or pairwise comparisons of appropriate group combinations were done to clarify the results (Nieuwenhuis et al., 2011). If $P < 0.05$, differences were considered statistically significant.

## RESULTS

### BODY WEIGHT

At 6 weeks of age (start of drug treatment) female mice weighed significantly less than male mice [main effect of sex: $F_{(1, 98)} = 135.2$; $P < 0.001$] and HET mice had higher bodyweight compared to their wild-type littermates [main effect of genotype: $F_{(1, 98)} = 11.7$; $P = 0.001$]. At 9 weeks of age (end of drug

treatment) statistical analysis revealed a number of main effects [main effect of sex: $F_{(1, 98)} = 290.7$; $P < 0.001$; main effect of genotype: $F_{(1, 98)} = 38.9$; $P < 0.001$ and main effect of CP treatment: $F_{(1, 98)} = 13.2$; $P < 0.001$] and also a significant interaction for sex × genotype [$F_{(1, 98)} = 4.8$; $P = 0.041$]. Two-Way ANOVAs split by sex revealed that male HET mice weighed more compared to male wild-type mice [main effect of genotype: $F_{(1, 49)} = 8.2$; $P = 0.006$] and CP-treated animals had lower bodyweight compared to vehicle-treated mice independent of genotype [main effect of CP treatment: $F_{(1, 49)} = 10.7$; $P = 0.002$]. For female mice there was a significant main effect of genotype with HET mice having higher bodyweights [$F_{(1, 48)} = 36.7$; $P < 0.001$]. At 12 weeks of age (during behavioral testing) the treatment effect of CP had disappeared but a significant genotype effect remained for both males [$F_{(1, 49)} = 11.3$; $P = 0.002$] and females [$F_{(1, 48)} = 42.2$; $P < 0.001$] and with HET mice weighing more than wild-types (**Table 1**).

### LOCOMOTOR ACTIVITY

At the beginning of the CP treatment period at 6 weeks of age, both sexes showed significantly reduced distance moved after the injection compared to vehicle-injected controls [main effect of CP: $F_{(1, 46)} = 109.4$ for male mice and $F_{(1, 43)} = 207.0$ for female mice, both $P < 0.001$]. Additionally, in both sexes a significant interaction for genotype × CP emerged [males: $F_{(1, 46)} = 4.4$; $P = 0.042$; females: $F_{(1, 43)} = 7.8$; $P = 0.008$]. For male mice, pairwise comparisons revealed that HETs were less active compared to wild-type mice when treated with vehicle [$F_{(1, 23)} = 4.8$; $P = 0.040$] but there were no differences after CP treatment (**Figure 1**). For female mice, pairwise comparisons revealed that HETs were more active compared to wild-type controls [$F_{(1, 24)} = 5.4$; $P = 0.029$]. However, when the mice had received CP injection, this phenotype was reversed with HET mice being less active compared to wild-types [$F_{(1, 18)} = 9.5$; $P = 0.007$].

At the end of CP treatment at 9 weeks of age, both male and female mice still showed acute hypo-activity after CP injection compared to saline-injected controls [main effect of CP: $F_{(1, 50)} = 24.5$ and $F_{(1, 48)} = 22.5$ for male and female mice, respectively, both $P < 0.001$] although the extent of the effect appeared smaller than at 6 weeks of age at the start of the CP treatment (**Figure 1**). There were no significant differences between wild-type mice and HET mice (**Figure 1**).

Two weeks after treatment had ceased and animals were tested for baseline locomotor activity without drug injections, male wild-type and HET mice treated previously with CP showed a strong trend for reduced distance traveled [$F_{(1, 50)} = 4.0$; $P = 0.051$]. In contrast, in female mice no main effects or interactions were found (**Figure 1**).

### Y-MAZE

After a 2 h retention period all animals appeared to be able to remember their previous encounter with the Y-maze indicated by a similar preference in the percentage of number of visits to the novel arm [$F_{(2, 82)} = 22.4$; $P < 0.001$, no interactions] and the most time spent in the novel arm [$F_{(2, 82)} = 20.8$; $P < 0.001$, no interactions] (**Figure 2**). The total number of arm entries differed

**Table 1 | Bodyweight (grams) of wild-type (WT) and BDNF heterozygous (HET) mice treated with vehicle (veh) or CP55,940 (CP) at 6 weeks (beginning of drug treatment), 9 weeks (end of drug treatment), and 12 weeks of age (during behavioral testing).**

| | WT/veh | WT/CP | HET/veh | HET/CP |
|---|---|---|---|---|
| **MALE MICE** | | | | |
| Week 6 | 21.4 ± 0.4 | 20.7 ± 0.5 | 21.9 ± 0.3 | 21.9 ± 0.5 |
| Week 9 | 24.3 ± 0.5 | 22.7 ± 0.3 | 25.3 ± 0.4 | 24.1 ± 0.4 |
| Week 12 | 26.4 ± 0.7 | 26.0 ± 0.5 | 28.6 ± 0.5 | 27.7 ± 0.6 |
| **FEMALE MICE** | | | | |
| Week 6 | 16.6 ± 0.5 | 16.8 ± 0.2 | 18.5 ± 0.4 | 17.9 ± 0.6 |
| Week 9 | 18.2 ± 0.4 | 17.9 ± 0.2 | 21.0 ± 0.6 | 19.8 ± 0.4 |
| Week 12 | 19.7 ± 0.5 | 20.0 ± 0.3 | 24.7 ± 1.0 | 23.1 ± 0.6 |

*Data are mean ± standard error of the mean (SEM) of 10–16 animals per group. At all ages, male mice weighed significantly more than female mice and HET mice weighed more than their wild-type littermates. For further details on statistical analysis, see text.*

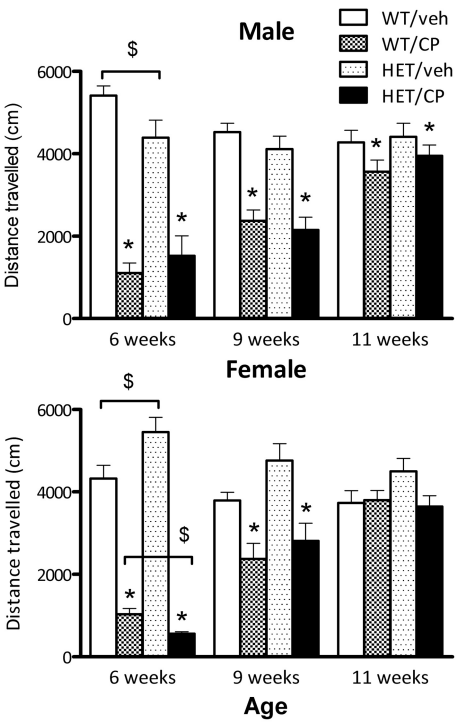


**FIGURE 1 | Total distance traveled (cm) by male and female mice in automated photobeam cages at the start of CP55,940 (CP) treatment at 6 weeks of age, end of CP treatment at 9 weeks of age and 2 weeks after treatment had ceased.** Wild-type and HET mice were either treated with vehicle solution or CP from 6 to 9 weeks of age. CP treatment significantly reduced distance traveled in the first and second session in both sexes and distance traveled was still slightly, but significantly reduced in male mice 2 weeks after treatment had ceased (for statistical analysis see text). Additional effects between groups of animals analyzed with pairwise comparison are indicated in the figure: $^{*}P < 0.05$ for differences between vehicle-treated controls and CP-treated mice based on main effect of CP in ANOVA. $^{\$}P < 0.05$ for difference between genotypes based on pair-wise *post-hoc* ANOVAs.

significantly between genotypes [$F_{(1, 90)} = 4.9$; $P = 0.030$] with HETs having a higher number of arm entries indicating higher general locomotor activity (**Table 2**).

### NOVEL OBJECT RECOGNITION

During the introduction phase there were no differences in the amount of time any of the groups spent investigating the two objects (data not shown). During the recognition phase, analysis of the percentage of time spent with the novel object revealed a main effect of genotype [$F_{(1, 73)} = 8.2$; $P = 0.006$] but no other main effects or interactions. This reflects that HET mice spent less time with the novel object compared to wild-type controls indicating poorer object recognition memory in this genotype. Inspection of the data (**Figure 3**) suggests that this was particularly prominent in male mice but there was no main effect of sex of the animals nor a genotype × sex interaction.

### PREPULSE INHIBITION OF ACOUSTIC STARTLE

Combined analysis of PPI in male and female mice revealed significant main effects of sex of the animals [$F_{(1, 88)} = 5.0$; $P = 0.028$], genotype [$F_{(1, 88)} = 23.8$; $P < 0.001$], acute CP treatment [$F_{(1, 88)} = 22.4$; $P < 0.001$] and an interaction of acute CP × sex × prepulse intensity [$F_{(3, 264)} = 3.8$; $P = 0.010$].

In male mice, ANOVA revealed a main effect of genotype [$F_{(1, 46)} = 7.3$; $P = 0.010$], reflecting that PPI was generally lower in HET compared to wild-type mice (**Figure 4**). Acute CP treatment increased PPI [$F_{(1, 46)} = 14.1$; $P < 0.001$] and this effect was different depending on the prepulse intensity [$F_{(3, 138)} = 4.8$; $P = 0.003$, **Figure 5**] and tended to interact with genotype and young-adult pre-treatment [$F_{(1, 46)} = 3.7$; $P = 0.059$]. Analysis for each prepulse intensity separately revealed that at PP8 and PP16 all groups showed a significant increase in PPI upon acute CP injection [main effects $F_{(1, 46)} = 15.3$ and 28.7, respectively, both $P < 0.001$, no interactions]. In contrast, analysis of PP2 revealed a CP × genotype × pre-treatment interaction [$F_{(1, 46)} = 5.2$; $P = 0.028$] and further analysis showed a marked increase in PPI at the PP2 intensity [$F_{(1, 10)} = 8.1$; $P = 0.017$] in HET mice pre-treated with CP but not in any of the other groups (**Figure 5**). There were no significant effects at PP4.

Inspection of the data (**Figures 4**, **5**) revealed that HET mice pre-treated in young-adulthood with CP had the lowest baseline PPI of all groups which could explain their larger response to subsequent acute CP treatment. However, further analysis in the four groups after acute injection of saline only confirmed the generally lower PPI in HET mice [main effect of genotype: $F_{(1, 46)} = 11.7$; $P = 0.001$] independent of adolescent CP pre-treatment (**Figure 4**). Interestingly, analysis of data obtained after acute CP injection showed no genotype effect, suggesting normalization of PPI in the HET mice by acute CP injection (**Figure 4**).

**Table 2 | Total number of arm entries in the Y-maze after a 2-h retention period.**

| | WT/veh | WT/CP | HET/veh | HET/CP |
|---|---|---|---|---|
| Male mice | 35.5 ± 2.3 | 36.7 ± 4.2 | 35.6 ± 2.3 | 42.3 ± 3.4 |
| Female mice | 39.1 ± 3.3 | 32.9 ± 2.7 | 41.6 ± 3.1 | 40.7 ± 1.5 |

*Data are expressed as mean ± SEM. HET mice had a higher number of arm entries. For statistical analysis, see text.*

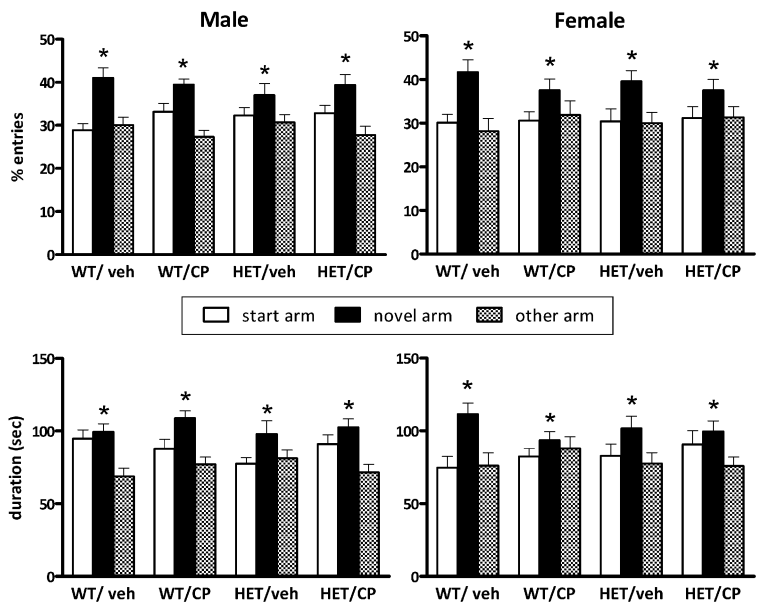


**FIGURE 2 | Y-maze behavior after a 2 h retention period of BDNF heterozygous (HET) and wild-type (WT) mice treated with vehicle (veh) or CP55,940 (CP).** (**Top**) show the percentage number of entries into the start, novel and "other" arm of the Y-maze during the 5 min re-exposure session and the (**Bottom**) show the duration of time spent in each arm. Data are presented for male (**Left**) and female mice (**Right**) and are expressed as mean ± SEM. $^*P < 0.05$ for difference between novel arm, start arm, and "other" arm based on ANOVA. All groups showed significant preference for the novel arm and there were no effects of genotype or CP treatment.

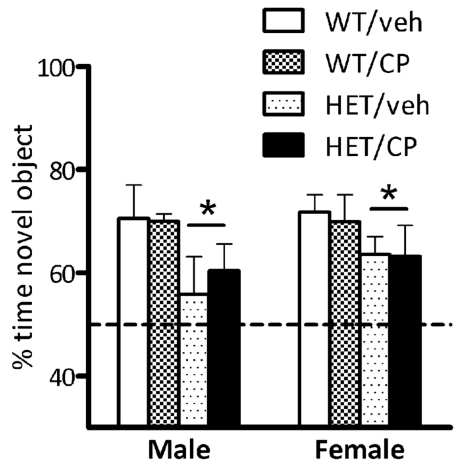


**FIGURE 3 | Percentage of time exploring the novel object in the recognition phase of the novel object recognition test.** Data are for male and female wild-type (WT) and BDNF heterozygous mice (HET) treated with vehicle (veh) or CP55,940 (CP) and are expressed as mean ± SEM. $^*P < 0.05$ for difference between HET mice and wild-type controls based on ANOVA main effect. HET mice spent less time with the novel object compared to wild-type controls but there was no effect of young-adult CP treatment.

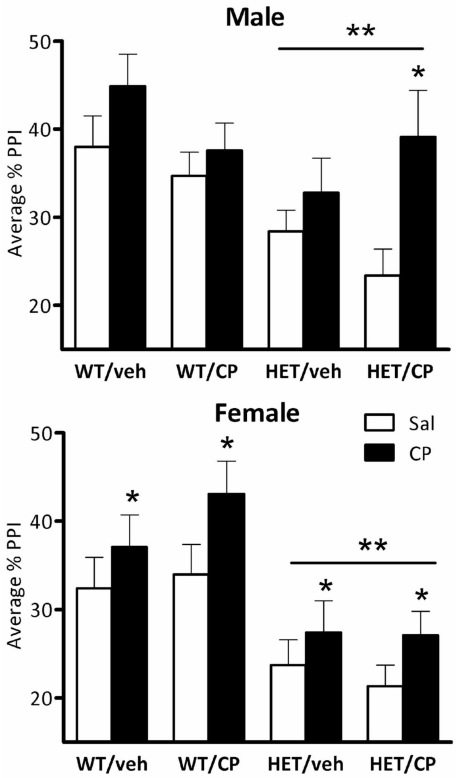


**FIGURE 4 | Average percentage PPI in the four treatment groups: WT/veh, WT/CP, HET/veh, and HET/CP in both sexes.** All groups were administered saline (Sal; open bars) and 0.4 mg/kg CP55,940 (CP; black bars) before the PPI test. HET mice had significantly lower PPI after saline injection compared to wild-type controls. Acute CP treatment increased PPI in male "two hit" mice and in all female groups. $^*P < 0.05$ for difference with acute saline based on genotype × acute treatment interaction and *post-hoc* comparisons (males) or main ANOVA effect (females). $^{**}P < 0.05$ for difference between HET mice and wild-type controls based on ANOVA main effect.

Also in female mice PPI was lower in HET compared to wild-type controls [$F_{(1, 42)} = 18.4$; $P < 0.001$, **Figure 4**]. Acute CP treatment increased PPI [$F_{(1, 42)} = 8.7$; $P = 0.005$, **Figure 4**] and, similar to male mice, this effect was greatest at higher prepulse intensities [CP × prepulse interaction: $F_{(3, 126)} = 11.7$; $P < 0.001$, **Figure 5**]. Further analysis for each prepulse intensity confirmed this observation as a significant main effect of acute CP treatment was found in all groups at PP8 and PP16 [$F_{(1, 42)} = 14.5$ and 20.4, respectively, both $P < 0.001$, no interactions] but not PP4 or, in contrast to male mice, PP2 (**Figure 5**).

### STARTLE RESPONSES

Average baseline startle amplitude was significantly lower in female mice than in male mice [$F_{(1, 87)} = 8.9$; $P = 0.004$] and HET mice had higher startle compared to wild-type mice independent of the sex of the animals [$F_{(1, 97)} = 14.4$; $P < 0.001$; **Figure 6**]. Acute CP injection significantly decreased average startle [$F_{(1, 87)} = 56.7$; $P < 0.001$], an effect which was greater in HET mice [CP × genotype interaction: $F_{(1, 87)} = 6.9$; $P = 0.010$] but was independent of the sex of the animals. However, when analysis was split up by genotype both groups were significantly affected by acute CP injection ([$F_{(1, 50)} = 13.6$; $P = 0.001$ and $F_{(1, 39)} = 41.9$; $P < 0.001$] for wild-type and HET mice, respectively, **Figure 6**). Notably, the acute effect of CP on startle was not affected by chronic young-adult CP treatment in any of the groups (**Figure 6**).

Startle habituation occurred in all groups as indicated by a main effect of block [$F_{(3, 261)} = 104.2$; $P < 0.001$]. Overall, female mice showed less startle habituation than male mice [$F_{(3, 261)} = 4.1$; $P = 0.008$] but there was no difference between the genotypes in terms of startle habituation (**Figure 7**). Acute CP treatment reduced startle habituation in a manner dependent on young-adult CP pre-treatment [$F_{(3, 261)} = 2.9$; $P = 0.037$]. In male mice, there were no effects of acute CP injection on startle habituation (**Figure 7**). In contrast, in female mice, acute CP injection reduced startle habituation [CP × block interaction: $F_{(3, 123)} = 5.8$; $P = 0.001$] and this effect was particularly prominent in animals which had received young-adult chronic pretreatment with CP [$F_{(3, 123)} = 2.9$; $P = 0.039$]. Thus, in female mice previously treated with saline, acute CP had no effect on startle habituation whereas in female mice previously treated with CP in young-adulthood, acute CP reduced startle habituation [$F_{(3, 60)} = 6.4$; $P = 0.001$]. Notably, none of these effects were different between wild-type and HET mice (**Figure 7**).

### [$^3$H]CP55,940 BINDING

Analysis of binding densities in the nucleus accumbens (**Figure 8**) revealed a significant effect of chronic young-adult CP treatment which was dependent on the genotype of the animals [$F_{(1, 40)} = 5.8$; $P = 0.021$] and there was also a sex × genotype interaction [$F_{(1, 40)} = 5.7$; $P = 0.022$]. Further analysis in wild-type mice showed that females had higher CP binding than males [$F_{(1, 22)} =$

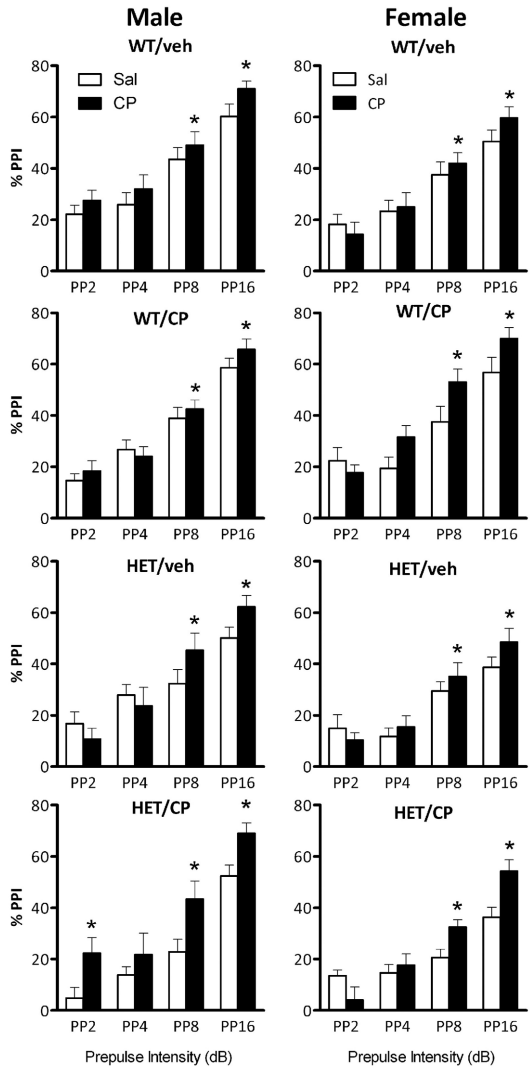


**FIGURE 5 | Percentage prepulse inhibition (PPI) expressed per prepulse intensity in the four treatment groups: WT/veh, WT/CP, HET/veh, and HET/CP in both sexes.** All groups were administered saline (Sal; open bars) or 0.4 mg/kg CP55,940 (CP; black bars) PP2, PP4, PP8, and PP16 indicate prepulse intensities of 2, 4, 8, and 16 dB over the 70 dB background with a 100 ms interstimulus interval. Data are mean ± SEM. $^{*}P < 0.05$ for difference with acute saline treatment based on ANOVA main effect (PP8 and PP16) or CP × genotype × pre-treatment interaction and pair-wise comparison (PP2).

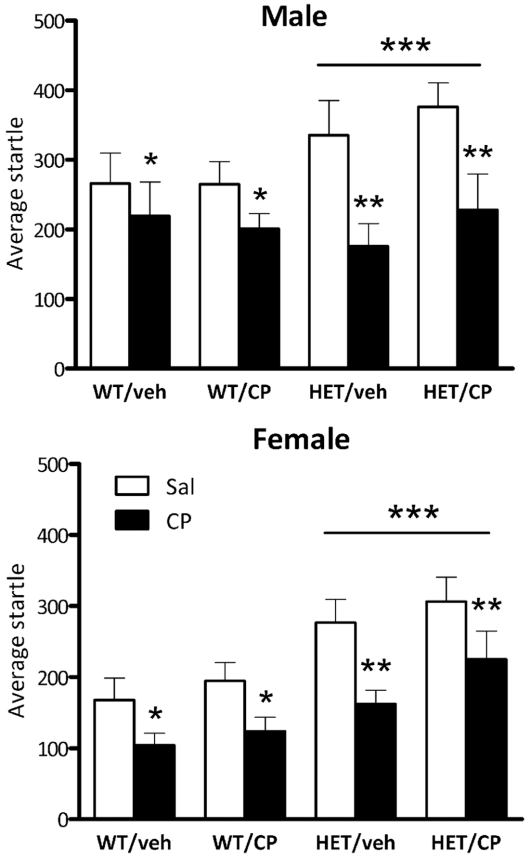


**FIGURE 6 | Average startle amplitude in the four treatment groups: WT/veh, WT/CP, HET/veh, and HET/CP in both sexes.** All groups were administered saline (Sal; open bars) and 0.4 mg/kg CP55,940 (CP; black bars). Data are mean ± SEM. $^{*}P < 0.05$ for difference with saline based on ANOVA main effect. $^{**}P < 0.05$ for greater effect of acute CP injection in HET mice based on ANOVA CP × genotype interaction. $^{***}P < 0.05$ for difference in startle amplitudes between WT and HET mice based on ANOVA main effect.

5.6; $P = 0.027$] irrespective of prior CP exposure (**Figure 8**). In contrast, in HET mice, CP binding was significantly up-regulated [$F_{(1,\ 18)} = 6.5$; $P = 0.020$] and this effect appeared to be greatest in male mice (**Figure 8**) although the sex × CP term did not reach statistical significance. Indeed, analysis of data from male mice showed a significant CP × genotype interaction [$F_{(1,\ 20)} = 4.7$; $P = 0.041$] with no such effect in female mice, suggesting a selective up-regulation of cannabinoid binding density in the nucleus accumbens of male BDNF HET mice, but not wild-type mice (**Figure 8**). There were no sex differences, genotype differences or effects of young-adult CP treatment in the caudate nucleus (**Figure 8**).

## DISCUSSION

The main findings of this study were that chronic young-adult treatment with a cannabinoid receptor agonist did not affect cognition or baseline PPI in adulthood in either wild-type or BDNF HET mice. However, acute injection of CP had differential effects on PPI depending on previous exposure to the drug as well as genotype and sex of the animals. Thus, male BDNF HET mice previously treated with CP showed a large increase in PPI upon acute CP treatment in adulthood whereas the other groups showed smaller or non-significant responses. This effect was paralleled by a significant increase of [$^{3}$H]CP55,940 binding in the nucleus accumbens of male BDNF HET mice previously treated with CP.

In a previous study (Klug et al., 2012), we did not observe the lower baseline PPI seen in BDNF heterozygous mice in the present results. However, in that study, corticosterone was administered

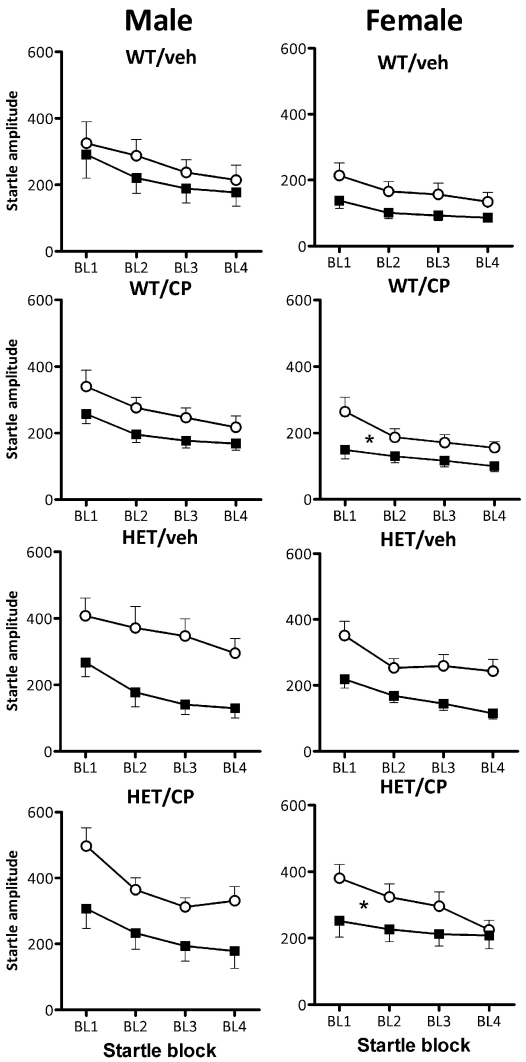


**FIGURE 7 | Startle habituation expressed per startle block in the four treatment groups: WT/veh, WT/CP, HET/veh, and HET/CP in both sexes.** All groups were acutely administered saline (Sal; open circles) and 0.4 mg/kg CP55,940 (CP; black squares). Data are mean ± SEM. $*P < 0.05$ for acute CP injection to reduce startle habituation in female WT and HET mice previously treated with CP in young-adulthood based on ANOVA interaction. Even though acute CP injection reduced startle overall (see **Figure 6**), it had no effect on startle habituation in any of the other groups. For statistical analysis, see text.

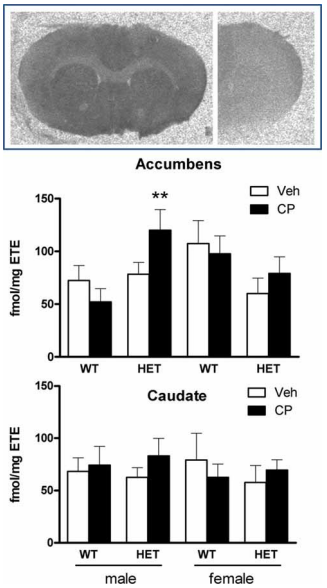


**FIGURE 8 | Top: typical autoradiograms of [$^3$H]CP55,940 binding in the mouse forebrain, showing total binding (left) and non-specific binding (right).** Specific binding densities were expressed as fmol/mg estimated tissue equivalent (ETE) in the nucleus accumbens (**Middle**) and caudate nucleus (**Bottom**) of male and female WT/veh, WT/CP, HET/veh, and HET/CP. Data are mean ± SEM. $**P < 0.05$ for difference with BDNF HET mice which had received chronic vehicle injections.

via the drinking water whereas CP55,940 was chronically injected in the present study. This chronic injection procedure may have induced or unmasked a lasting PPI deficit at baseline in BDNF HET mice. Furthermore, the PPI deficit was accompanied by an increase in startle amplitude which may have confounded the PPI result and was not seen in our previous study in BDNF HET mice (Klug et al., 2012). One explanation could be that BDNF HET mice show increased anxiety which could lead to an increase in startle amplitude. However, only one study found an increase in anxiety-like behavior (Chen et al., 2006) while most other studies did not find an anxiety-like phenotype on BDNF heterozygous mice (Montkowski and Holsboer, 1997; Macqueen et al., 2001; Chourbaji et al., 2004; Ibarguen-Vargas et al., 2009). Another explanation for the enhanced startle amplitude could be the difference in bodyweight between the genotypes. HET mice had significantly higher bodyweights than wild-type mice and this could at least in part account for greater startle amplitudes.

Chronic CP treatment during young adulthood did not lead to a deficit in baseline PPI in adulthood which is similar to what we observed in a recent study in rats (Klug and Van Den Buuse, 2012). Acute injection of CP increased PPI and in male mice this was predominantly in HETs previously treated in young-adulthood with CP. In another study, acute injections of CP was shown to cause a decrease of PPI in wild-type mice but this effect disappeared when the mice were treated chronically (Boucher et al., 2011). It is possible that CP can influence PPI in both directions which would be in accordance with studies in rats where some studies found a decrease in PPI (Martin et al., 2003; Malone and Taylor, 2006) while others reported increased PPI (Stanley-Cary et al., 2002). This may be due to methodological differences between the studies as it has been shown that different solvent agents can influence the behavioral outcome (Stanley-Cary et al., 2002). However, Boucher et al. used a similar method to ours to dissolve their CP and showed decreased PPI in wild-type mice after acute treatment (Boucher et al., 2011). The group of BDNF HET male mice previously exposed to CP which responded with an increase in PPI also had the lowest PPI score of all male groups at baseline. After acute treatment with CP, PPI levels were indistinguishable in this group from the other groups. This seems to be comparable to cannabis self-medication which has been

suggested in people with schizophrenia as a means to alleviate some of their symptoms (Phillips and Johnson, 2001; Schofield et al., 2006). It is possible that CB1 receptor stimulation is effective in reversing disruptions of PPI or other schizophrenia-relevant behaviors, independent of its effect on baseline behaviors. For example, similar to our present results, acute treatment with a CB1 receptor agonist reversed PPI deficits in psychosocially stressed mice (Brzozka et al., 2011) and phencyclidine-treated rats (Spano et al., 2010). On the other hand, in an isolation-rearing stress model, Malone and Taylor (2006) observed a decrease of PPI after acute treatment with THC, which had no effect in normally-housed control (Malone and Taylor, 2006). Future studies could focus on the effects of cannabinoid receptor stimulation in other models of genetic or environmentally-induced PPI disruptions. For example, recently it was shown that BDNF treatment produced long-lasting reversal of PPI deficits in DBA mice (Naumenko et al., 2013) and it would be of interest to study the acute and chronic effects of cannabinoid receptor stimulation in this strain.

Some caution is needed when interpreting the PPI results as acute treatment with CP reduced startle amplitudes which may have confounded PPI. The reduction in startle response is most likely related to adverse effects of the cannabinoid agonist that include hypoactivity and catalepsy (Chaperon and Thiebot, 1999). Other studies have also failed to detect cannabinoid-induced effects in PPI that were not accompanied by impairment in startle reflex (Nagai et al., 2006; Boucher et al., 2011). For example, Martin et al. only found a PPI deficit when rats were treated with the highest dose of CP which also decreased startle amplitudes. When the animals were treated with a lower dose, no effects on startle reactivity were observed but the previously observed PPI deficit had vanished as well, showing that CP treatment does not impair PPI without affecting startle (Martin et al., 2003). This raises the possibility that the effects of CP on PPI are not a real impairment in sensorimotor gating (Swerdlow et al., 2000). Further studies should be considered to see whether a smaller dose of CP would lead to an enhancement in PPI without affecting startle amplitudes. It noteworthy, though that CP only increased PPI in some groups while it reduced startle in all groups, which would suggest that the presently observed selective PPI changes are independent of the effect of CP on startle.

The selective increase in the effect of acute CP in male BDNF HET mice previously treated with the drug, was paralleled by a selective increase in CP binding density in the nucleus accumbens in these mice. The nucleus accumbens plays an important role in PPI regulation (Shilling et al., 2008; Roncada et al., 2009). CB1 receptors are present on fast-spiking interneurons in this nucleus which form inhibitory GABAergic synapses with medium-spiny interneurons and modulate its activity (Winters et al., 2012). These neurons can also modulate behavioral effects of dopaminergic psychotropic drugs (Corbille et al., 2007) and the selective up-regulation of CP binding density in the present study may explain the enhanced effect of acute treatment with the drug on PPI. Similar to the present results, previous studies have found that chronic cannabinoid receptor agonist treatment could influence CB1 receptor levels and function even long after treatment had finished (Zamberletti et al., 2012). However, it is important to note that the single-point agonist binding method used here does not distinguish between possible changes in Kd or Bmax of the receptor. Therefore, future, more elaborate binding studies are needed to extend the present results and ascertain the molecular basis of the changes in [$^3$H]-CP55,940 binding density. These further studies could also include other CB1 compounds as displacing agents.

Another possible mechanism involved in the present results could be the effect of acute and chronic CP treatment on BDNF levels. Previous animal studies have shown that THC increases BDNF gene expression acutely (Derkinderen et al., 2003) and after chronic administration (Butovsky et al., 2005). In human studies, THC increased serum BDNF levels in healthy controls, but not in chronic cannabis users (D'souza et al., 2009). It would be of interest to assess if young-adult CP treatment, as used in the present study, induced long-term reversal of the reduced BDNF levels seen in BDNF HET mice (Hill and Van Den Buuse, 2011). This might also explain the sex differences observed in the present study, as young-adult developmental changes in BDNF levels showed marked male-female differences in normal mice (Hill et al., 2012) and sex-specific alterations in BDNF signaling were found in BDNF HET mice (Hill and Van Den Buuse, 2011).

In addition to its effect on PPI, chronic CP treatment also affected locomotor activity as was expected from previous studies (McGregor et al., 1996; Boucher et al., 2011; Llorente-Berzal et al., 2011). Interestingly, male mice treated with CP during young-adulthood showed reduced locomotor activity even 2 weeks after treatment had ceased. This is similar to what we observed in rats (Klug and Van Den Buuse, 2012) and it seems that male animals are more prone toward the long-term effects that CP can exert on locomotor activity. There were other, more subtle and sex-dependent differences in locomotor activity between the genotypes at 6 and 9 weeks. However, none of these effects were seen in adulthood at 11 weeks of age similar to what has been observed in previous studies (Chourbaji et al., 2004; Saylor and McGinty, 2008; Klug and Van Den Buuse, 2012).

No effects of either genotype, cannabinoid treatment or the combination of the two were observed in the Y-maze tasks. All animals spent more time in the novel arm which is an indicator for intact short-term spatial memory (Dellu et al., 2000). In a previous study, we observed marked deficits in Y-maze performance in male BDNF HET mice treated chronically with corticosterone in young adulthood (Klug et al., 2012). These differential results show that various young-adult "second hits" can have markedly different outcome in adulthood, in this case corticosterone exposure compared to cannabinoid treatment. Novel object recognition was impaired in BDNF HET mice which has been reported before (Seoane et al., 2011). However, similar to Y-maze performance, there was no effect of chronic CP treatment, strengthening the selectivity of its action on PPI. Thus, although previous studies have shown involvement of both the endocannabinoid system and BDNF in memory formation and consolidation (Papaleo et al., 2011; Panlilio et al., 2012; De Bitencourt et al., 2013; Wright et al., 2013), in our protocol including chronic CP treatment followed by a 2-week washout, there were no such effects. Future studies could include the acute effects of cannabinoid receptor stimulation on memory function in BDNF HET mice and controls after chronic pre-treatment with CP, similar to the PPI studies presented here.

In conclusion, this study shows that chronic cannabinoid treatment during young adulthood in mice does not lead to major long-lasting behavioral effects at baseline in adulthood. However, it appears that chronic cannabinoid treatment in male mice with a BDNF deficiency makes the animals more sensitive toward the acute effects of cannabinoid receptor stimulation later on in life. This could have implications for the effects of cannabis abuse in humans in a subset of individuals with low BDNF signaling.

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# Convergence of genetic and environmental factors on parvalbumin-positive interneurons in schizophrenia

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Schizophrenia etiology is thought to involve an interaction between genetic and environmental factors during postnatal brain development. However, there is a fundamental gap in our understanding of the molecular mechanisms by which environmental factors interact with genetic susceptibility to trigger symptom onset and disease progression. In this review, we summarize the most recent findings implicating oxidative stress as one mechanism by which environmental insults, especially early life social stress, impact the development of schizophrenia. Based on a review of the literature and the results of our own animal model, we suggest that environmental stressors such as social isolation render parvalbumin-positive interneurons (PVIs) vulnerable to oxidative stress. We previously reported that social isolation stress exacerbates many of the schizophrenia-like phenotypes seen in a conditional genetic mouse model in which NMDA receptors (NMDARs) are selectively ablated in half of cortical and hippocampal interneurons during early postnatal development (Belforte et al., 2010). We have since revealed that this social isolation-induced effect is caused by impairments in the antioxidant defense capacity in the PVIs in which NMDARs are ablated. We propose that this effect is mediated by the down-regulation of PGC-1$\alpha$, a master regulator of mitochondrial energy metabolism and anti-oxidant defense, following the deletion of NMDARs (Jiang et al., 2013). Other potential molecular mechanisms underlying redox dysfunction upon gene and environmental interaction will be discussed, with a focus on the unique properties of PVIs.

**Keywords: animal model, fast-spiking neurons, GABA neuron, NMDA receptors, oxidative stress, PGC-1$\alpha$, social isolation**

## INTRODUCTION

Schizophrenia is a chronic and complex neuropsychiatric disorder that affects approximately 1% of the population worldwide (Insel, 2010). The characteristic features of schizophrenia have been categorized into three major symptom domains (DSM-IV, American Psychiatric Association, 1994). Positive symptoms are psychotic behaviors not seen in healthy people, such as hallucillations, delusions, disorganized thinking, and movement disorders which, in part, can be relieved through the use of current antipsychotic medications. Negative symptoms are associated with disruption of normal emotions and behaviors, such as motivational impairment (avolition or amotivation), affective dysregulation (depression, mania), and social withdrawal. Cognitive symptoms include poor executive functioning, trouble focusing or paying attention, and problems with working memory. The onset of symptoms usually occurs during late adolescence and early adulthood.

Based on classical family, twin and adoption studies, genetic theories of schizophrenia etiology have been prominent since 1960s (Gottesman and Shields, 1976; Cardno and Gottesman, 2000; Carter et al., 2002; Sullivan et al., 2003; Tienari et al., 2004; Lichtenstein et al., 2009). Early linkage studies produced the first list of gene candidates, and a large body of gene association work identified some of these candidate genes as true susceptibility factors (Owen et al., 2005; Ross et al., 2006; Straub and Weinberger, 2006). Recently, analysis of data generated using genome-wide mass-marker technology (such as genome-wide association studies (GWAS) and next generation sequencing) has raised the possibility that thousands of common polymorphisms (Purcell et al., 2009) and multiple rare copy-number variations (CNVs) in several different genomic areas contribute to schizophrenia risk (Stefansson et al., 2008; Stone et al., 2008; Walsh et al., 2008; Xu et al., 2008; McCarthy et al., 2009; Ingason et al., 2011). Although GWAS from the psychiatric genomics consortium (PGC), a collaboration across more than 300 scientists in 80 institutions and 20 countries, have been carried out to investigate a large number of polymorphisms from thousands of patients (61,220 subjects, Smoller et al., 2013), no single gene or allele has independently been identified as a key risk factor in a large number of people.

One plausible explanation for the weak association of schizophrenia with an individual gene is that genetic risk is critically dependent on environmental context. A considerable body of evidence suggests that an interaction between genetic

vulnerability and environmental factors (GxE) leads to the manifestation of schizophrenia. Even monozygotic twins who share identical genes have a concordance rate of 41–65% (Cardno and Gottesman, 2000). Epidemiologic studies suggest that a diversity of factors, including prenatal infection/immune activation, paternal age, malnutrition, hypoxia-related obstetric complications, and childhood/adolescence social stress and cannabis abuse, are associated with an increased risk for the development of this disorder (see reviews, Barkus and Murray, 2010; van Os et al., 2010; Brown, 2011; Meyer, 2013).

Various theories have been proposed to explain how genes and the environment interact to give rise to complex neuropsychiatric disorders. The "Diathesis-stress" model of schizophrenia, introduced by Rosenthal D. and Bleuler M. in 1963 (Ingram and Luxton, 2005) and recently revisited by Fowles (1992), posits that development of schizophrenia requires both biological vulnerability (diathesis) and stressful life. Weinberger (1987) went on to propose a similar model in the context of brain development and maturation, suggesting that an environmental stressor during early life brain maturation is necessary to trigger the onset of full-blown psychotic behavior. In the framework of the neurodevelopmental theory, a "two-hit" model was proposed and two critical time windows associated with brain early development and maturation during adolescence were identified as sensitive periods for exposure to environmental insults that could account for the emergence of symptoms of schizophrenia (Bayer et al., 1999; Keshavan, 1999).

However, there is a fundamental gap in our understanding of the molecular mechanisms by which environmental factors interact with genetic susceptibility during brain development to trigger psychosis. A large number of neurodevelopmental animal models have been developed to investigate the biological basis for GxE interactions and to evaluate novel pharmacological therapies for their treatment (review, Meyer and Feldon, 2010). For example, inflammatory responses after infection and cytokine-mediated effects on brain development have been studied using polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS) in rodents; protein deprivation and vitamin D deficiency to mimic malnutrition; perinatal/postnatal hypoxia models for obstetric complications; maternal isolation, post-weaning social isolation/chronic restraint stress have been used to study psychosocial stress effects on brain development. Genetic manipulation of schizophrenia susceptibility genes in rodents, such as DISC1, NRG1/ErbB4, and COMT has been largely explored as well.

Interestingly, one of the common findings in both animal models and postmortem tissue from patients with schizophrenia is a reduction of the calcium buffer parvalbumin (PV) mRNA or protein level in cortical fast-spiking (FS) interneurons. PV-positive interneurons (PVIs), which account for ∼40% of the total cortical Gamma-amminobutyric acid (GABA) ergic interneurons in rodents (Rudy et al., 2011) and 25% in primates (Condé et al., 1994) and include basket and chandelier cells, represent a unique class of interneurons. A significant mRNA reduction of PV (Hashimoto et al., 2003, 2008; Mellios et al., 2009; Fung et al., 2010; Volk et al., 2012), GAD67 (Hashimoto et al., 2003; Curley et al., 2011) or GAT1 (Volk et al., 2012) has been found in PVIs in the dorsolateral prefrontal cortex of individuals with schizophrenia. Altered GABA neurotransmission at the synapse between PV-positive chandelier neurons and the pyramidal cell axon initial segment (AIS), such as decreased density of chandelier neuron axon cartridges immunoreactive for GAT-1 (Woo et al., 1998; Pierri et al., 1999), increased $GABA_A$ receptor $\alpha 2$ subunit at AIS of pyramidal neurons (Volk et al., 2002) and increased $GABA_A$ receptor binding (Benes et al., 1992, 1996), was also found in schizophrenic patients. Furthermore, factors required for development of PVI including the transcription factor Lhx6 and brain derived neurotrophic factor (BDNF) and its receptors are reduced in a subset of individuals with schizophrenia (Cobos et al., 2006; Liodis et al., 2007; Zhao et al., 2008, see reviews, Huang et al., 1999; Weickert et al., 2003; Hashimoto et al., 2005; Weickert et al., 2005; Woo and Lu, 2006; Volk et al., 2012).

Dysfunction of PVIs has been indicated in many neurodevelopmental animal models as well. Studies in reverse-translational models using schizophrenia-risk genes, such as ErbB4 (Fisahn et al., 2009; Neddens and Buonanno, 2010), DISC1 (Hikida et al., 2007; Shen et al., 2008; Ayhan et al., 2011), DTNP1 (Ji et al., 2009; Carlson et al., 2011), BDNF (Sakata et al., 2009) and glutamate cysteine ligase modifier (GCLM; Steullet et al., 2010; Cabungcal et al., 2013a), consistently observed a decreased number or impaired function of PVIs in the hippocampus or cortex. Also, intensity of PV immunoreactivity and/or loss of PV-positive cells has been reported in the hippocampus and cortex of mice with developmental inflammation [prenatal poly I:C injection (Meyer et al., 2008; Ducharme et al., 2012; Piontkewitz et al., 2012); neonatal LPS injection (Jenkins et al., 2009)], exposure to hypoxia (Gerstein et al., 2005; Fagel et al., 2009), chronic social isolation stress (Harte et al., 2007; Schiavone et al., 2009, 2012; Filipovic et al., 2013), ventral hippocampal lesions (Tseng et al., 2008; François et al., 2009) or prenatal (E17) methylazoxymethanol acetate (MAM) injections (Lodge et al., 2009; Gastambide et al., 2012). Pharmacological manipulation by NMDA receptor (NMDAR) antagonists, such as repetitive injection of ketamine (Behrens et al., 2007), prenatal exposure to MK-801 (Abekawa et al., 2007) or perinatal phencyclidine (PCP) injection (Wang et al., 2008), all resulted in a decrease in the number of cortical PVIs. These data suggest that PV immunoreactivity and PVI cells themselves are particularly sensitive to neurodevelopmental insults.

PVI cell loss and dysfunction have serious consequences for cortical and hippocampal function. PVIs produce sustained, high-frequency trains of brief action potentials with large and fast after-hyperpolarization and little spike frequency adaptation. They have the lowest input resistance and the fastest membrane time constant among all interneurons, features that ensure fast synaptic responses (Connors and Gutnick, 1990; Markram et al., 2004; Ascoli et al., 2008; Goldberg et al., 2008). FS PVIs are interconnected via chemical and electrical synapses (Galarreta and Hestrin, 1999; Gibson et al., 1999) and have a highly divergent synaptic output to principle neurons (Gulyás et al., 1999). Inhibitory synapses between PVIs synchronize action potential activity within basket cell network, whereas inhibitory synapses between basket cells and principle neurons distribute this synchronized activity to the principle neuron

population. Accordingly, multiple lines of evidence indicate that FS PVIs are essential for the generation of gamma oscillations (Csicsvari et al., 2003; Hájos et al., 2004; Mann et al., 2005; Cardin et al., 2009; Sohal et al., 2009), which provides a temporal structure for information processing and contributes to cognitive functions including attention (Fries et al., 2001, 2008; Siegel et al., 2008), perception (Rodriguez et al., 1999) and working memory (Howard et al., 2003). Cognitive impairments such as deficits in working memory, attention, and executive function are particularly evident in patients with schizophrenia (Elvevåg and Goldberg, 2000), and abnormalities in gamma oscillations may contribute to these deficits. Schizophrenic patients exhibit decreases in the power or synchrony of gamma oscillations during responses to sensory stimulation or cognitive tasks (Gallinat et al., 2004; Spencer et al., 2004; Symond et al., 2005; Wynn et al., 2005; Cho et al., 2006; Ford et al., 2007, 2008; Haenschel et al., 2009; Uhlhaas and Singer, 2010). Thus, abnormalities of FS PVIs may underlie the cognitive disturbances associated with schizophrenia.

Considering the substantial evidence for interneuron dysfunction and NMDAR hypofunction in schizophrenia, we investigated the impact of NMDAR deletion specifically from interneurons using a Cre/*loxP* system in which early postnatal ablation is restricted to 40–50% of the cortical and hippocampal interneurons, with the majority of cre-targeted cells being PV-positive [NMDAR (GluN1) knockout mouse strain (Ppp1r2-cre/fGluN1 KO mice; Belforte et al., 2010)]. In this mouse, NMDARs were functionally eliminated in the early postnatal period (Cre recombination was detectable in the cortex and hippocampus firstly at postnatal day seven and almost completed by postnatal three weeks). Reduced GAD67 and PV protein levels and reduced GABA release were observed from GluN1-depleted interneurons in mutant animals, and mutant mice exhibited cortical disinhibition evidenced by increased firing of cortical excitatory neurons and reduced neuronal synchrony. At the behavioral level, this mutant mouse reproduced positive, negative, cognitive and anxiety-like behavioral phenotypes that resemble the symptoms of human schizophrenia. Most mutant behavioral phenotypes were first observed >12 weeks of age, suggesting a latency period between GluN1 knockout and the emergence of these phenotypes (Belforte et al., 2010; Nakazawa et al., 2012). Interestingly, social isolation initiated during adolescence exacerbated the expression of these phenotypes in the mutant (Jiang et al., 2013).

Importantly, schizophrenia-like pathophysiological and behavioral phenotypes were not observed when genetic GluN1 ablation in the same subpopulations of GABAergic (gamma-aminobutyric acid) neurons occurred after adolescence (Belforte et al., 2010), suggesting that GluN1 deletion is most detrimental during the postnatal maturation of PVIs. Furthermore, a prominent increase of oxidative stress was observed in KO mice, particularly in cortical PVIs, with post-weaning social isolation sharply exacerbating redox dysfunction. Chronic treatment with apocynin (APO), an antioxidant and reactive oxygen species (ROS) scavenger, abolished oxidative stress signs and partially alleviated schizophrenia-like behavioral phenotypes in KO mice (Jiang et al., 2013).

In the context of this new data and the substantial evidence for PVI dysfunction in schizophrenia, we propose that social isolation in development exacerbates schizophrenia-like phenotypes via cortical oxidative stress in PVIs (Jiang et al., 2013). These data are in line with the "diathesis–stress" and neurodevelopmental theories for the etiology of schizophrenia and suggest that oxidative stress is one of central factors linking genetic and environmental risks to GABAergic dysfunction (**Figure 1**). Below, we discuss in detail the evidence for the involvement of oxidative stress in the pathophysiology of schizophrenia, the specific properties of PVIs that render them vulnerable to oxidative stress, and a potential molecular pathways which could account for environmentally-induced oxidative stress in PVIs.

## OXIDATIVE STRESS AS A CONVERGENCE POINT FOR GENETIC AND ENVIRONMENTAL SUSCEPTIBILITY TO SCHIZOPHRENIA

Growing body of evidence suggests that oxidative stress plays a significant role in the pathogenesis of schizophrenia (see reviews, Behrens and Sejnowski, 2009; Do et al., 2009; Yao and Keshavan, 2011). Oxidative stress occurs when ROS or reactive nitrogen species (RNS) are over-produced or antioxidant defense mechanisms fail to counterbalance endogenouse ROS/RNS generated from normal oxidative metabolism or from pro-oxidant environmental exposure. Excessive ROS or RNS can lead to DNA damage and membrane damage due to lipid peroxidation and protein dysfunction. Mammalian antioxidant defense system include ROS detoxifying enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (Gpx), and nonenzymatic antioxidant components such as albumin, uric acid, bilirubin, glutathione (GSH), ascorbic acid (vitamin C) and a-tocopherol (vitamin E). Reduced levels of ROS detoxifying enzymes and antioxidants have been reported in schizophrenia (Suboticanec et al., 1990; Reddy et al., 1991; McCreadie et al., 1995; Mukerjee et al., 1996; Yao et al., 1998, 2000; Do et al., 2000; Ranjekar et al., 2003; Reddy et al., 2003; Yao et al., 2006; Dadheech et al., 2008; Raffa et al., 2009; Gawryluk et al., 2011; Coughlin et al., 2013), in addition to increased levels of lipid peroxides in blood (Zhang et al., 2006; Al-Chalabi et al., 2009; Padurariu et al., 2010), platelets (Dietrich-Muszalska et al., 2005), plasma (Mahadik et al., 1998) and urine (Dietrich-Muszalska and Olas, 2009a). Increased protein modification has been also demonstrated in plasma (Dietrich-Muszalska et al., 2009), platelets (Dietrich-Muszalska and Olas, 2009b) and postmortem brains (Wang et al., 2009).

### GENETIC ORIGIN OF OXIDATIVE STRESS

Interestingly, a number of genes involved in antioxidant defense have been linked to susceptibility for schizophrenia. These include MnSOD (Akyol et al., 2005), glutathione S-transferases (Saadat et al., 2007; Nafissi et al., 2011), and a subunit of the key GSH-synthesizing enzyme GCLM (Tosic et al., 2006; Gysin et al., 2007, 2011). A mitochondrial DNA sequence variation affecting a subunit of NADPH-ubiquinone reductase (complex I), a component of the electron transport chain responsible for generating superoxide, has also been associated with schizophrenic patients and with increased superoxide levels in postmortem brain samples (Marchbanks et al., 2003). Furthermore, abnormal proteins

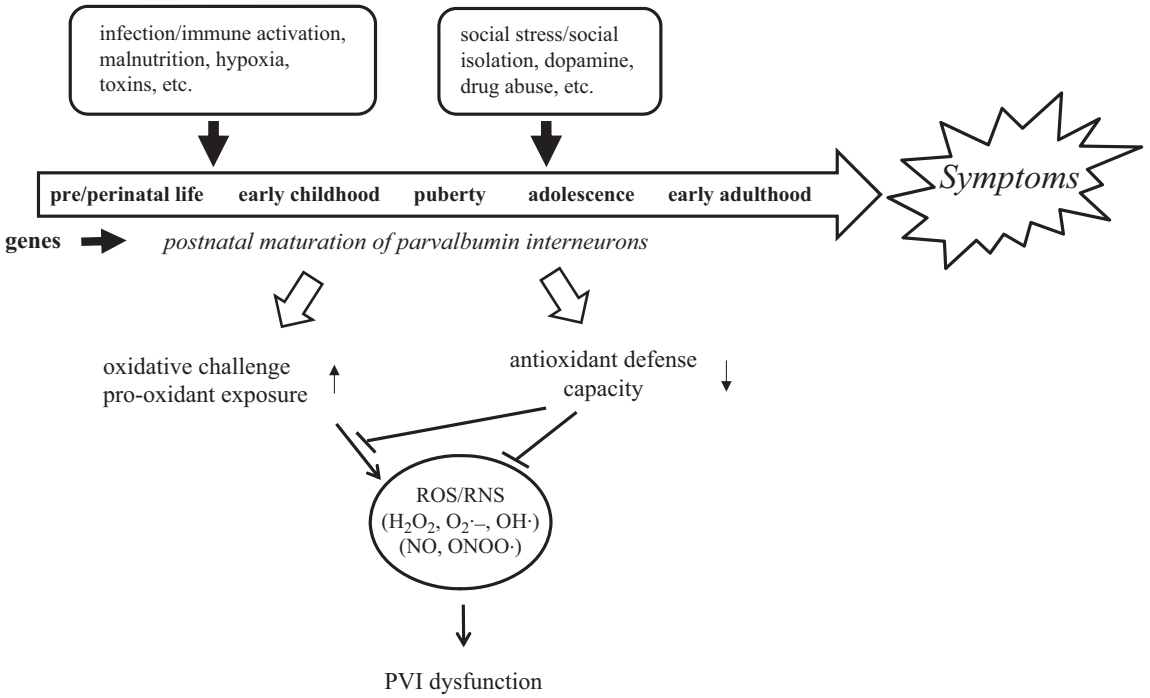


**FIGURE 1 | PVIs are vulnerable to oxidative stress induced by GxE during development.** An interaction between genetic susceptibility and environmental insults disrupts normal brain development and leads to the manifestation of schizophrenia. Unique properties of PVIs make them vulnerable to redox imbalance induced by GxE.

encoded by schizophrenia susceptibility genes (PRODH, DISC1, DAOA, NRG1, G72) cause oxidative stress and/or hypersensitivity to oxidative stress (Goldshmit et al., 2001; Krishnan et al., 2008; Drews et al., 2012) or mitochondrial dysfunction (Park et al., 2010).

### OXIDATIVE STRESS INDUCED BY ENVIRONMENTAL INSULTS

In addition to our own evidence for social isolation-induced oxidative stress, other studies have demonstrated links between environmental stressors and oxidative stress. ROS elevation was found after restraint stress (Madrigal et al., 2001; Chakraborti et al., 2007; Sahin and Gumuslu, 2007), and social isolation rearing in rats induces behavioral changes akin to schizophrenia, mediated by an oxidative stress following NADPH oxidase (Nox-2) elevation in pyramidal neurons (Schiavone et al., 2009, 2012) or associated with decreased superoxide dismutase activity and oxidized/reduced glutathione ratio in the cortico-striatal area (Möller et al., 2011). As in our model, APO treatment alleviated the signs of oxidative stress and behavioral deficits following social isolation (Schiavone et al., 2009, 2012). Furthermore, oxidative stress has been speculated to be an important downstream mechanism of inflammation-mediated immune responses. Maternal immune activation by LPS in rodents triggers oxidative stress (Lanté et al., 2007; Kaneko et al., 2012; Oskvig et al., 2012), and maternal N-acetyl cysteine (NAC, a glutathione precursor) treatment prevents LPS-induced adverse developmental outcomes including elevation of cytokines in maternal and fetal compartments (Xu et al., 2005; Beloosesky et al., 2006), fetal death, preterm labor (Buhimschi et al., 2003), hypomyelination (Paintlia et al., 2004), and impairments in spatial memory and hippocampal long-term potentiation in the offspring (Lanté et al., 2007). Protein malnutrition (Feoli et al., 2006a,b) and hypoxia (Dafre et al., 2003; Sheldon et al., 2008; Niatsetskaya et al., 2012) can also increase oxidative stress.

### GxE-INDUCED OXIDATIVE STRESS

A number of studies have investigated whether prenatal and/or postnatal environmental insults interact with specific genetic factors to cause oxidative stress and increase the risk of long-lasting neurodevelopmental brain dysfunction. Using mice which exhibit a 60–70% reduction in GSH due to ablation of the GCLM gene, Kim Q. Do and her colleagues have shown that impaired GSH synthesis is associated with morphological, neurochemical anomalies and behavioral phenotypes similar to those in schizophrenia patients (Steullet et al., 2010; Kulak et al., 2012). They also found elevated oxidative stress indicated by increased levels of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-OH-dG), a marker of oxidized DNA, in ventral hippocampus and anterior cingulate cortex (Steullet et al., 2010; Cabungcal et al., 2013a). An exogenous dopamine stress induced by a dopamine reuptake inhibitor, GBR-12909, led to exacerbated 8-OH-dG labeling in the anterior cingulate cortex of GCLM KO mice but not wild-type mice. Treatment with NAC fully prevented the increase in 8-OH-dG labeling induced by GBR-12909 in KO mice (Cabungcal et al., 2013a).

In light of our recent study indicating an interaction between NMDAR hypofunction and oxidative stress (Jiang et al., 2013), it is important to note that the relationship of NMDAR hypofunction to oxidative stress was initially observed in the rodent models treated with NMDAR antagonists. Chronic perinatal PCP administration reduced glutathione levels and produced long-term alteration of antioxidant defense in the corticolimbic areas of the rat brain (Radonjic et al., 2010). Similarly, repetitive exposure to ketamine, another NMDAR antagonist, activated the superoxide-producing enzyme Nox-2 in mouse brains (Behrens et al., 2007; Zhang et al., 2008). Elevation of cortical ROS level was also found in our Ppp1r2-cre/fGluN1 KO mice when NMDAR was eliminated in cortical PVIs during early postnatal period. Interestingly, post-weaning social isolation sharply exacerbated ROS level in KO mice. Chronic treatment with APO abolished oxidative stress signs and partially alleviated schizophrenia-like behavioral phenotypes in KO mice. These indicated that oxidative stress does contribute to behavioral impairments of this mutant line and social isolation exacerbated schizophrenia-like phenotypes via cortical oxidative stress (Jiang et al., 2013).

## PV INTERNEURON-SPECIFIC VULNERABILITY TO OXIDATIVE STRESS

It has been observed in several lines of animal studies that PVIs are vulnerable to oxidative stress during development. Behrens et al. (2007, 2008) found that repetitive adult exposure to the NMDAR antagonist ketamine increases the levels of the proinflammatory cytokine interleukin-6 (IL-6) in brain which, through activation of the superoxide-producing enzyme Nox-2, leads to the loss of the GABAergic phenotype of PVIs. Pretreatment of animals with APO, or with the SOD-mimetic C3 reduces superoxide production and prevents the loss of PV immunoreactivity (IR) induced by ketamine. Nox-2 deficiency completely prevented the loss of PVIs in the prelimbic regions (Behrens et al., 2008). The reversible effects in adult exposure (Behrens et al., 2008) were not observed for the animals with perinatal exposure of ketamine, suggesting that perturbation of the excitatory/inhibitory balance during early life produces oxidative stress that has profound effect on PVI maturation. In fact, exposure of wild type mice to ketamine on postnatal day 7, 9, and 11 is sufficient to cause a reduction in PV-IR in adulthood without death of interneurons (Powell et al., 2012), suggesting that blockade of NMDAR signaling is sufficient to alter the expression profile of FS interneurons.

The idea that PVIs are more susceptible to oxidative stress during development is also supported by the evidence that early postnatal PCP injection can selectively reduce PVI numbers (Wang et al., 2008; Nakatani-Pawlak et al., 2009) and cause redox dysregulation (Radonjic et al., 2010) in corticolimbic areas. Cabungcal et al. (2007) showed that a transient brain GSH deficit induced by BSO (Lbuthionine-(S, R)-sulfoximine) during early postnatal period is sufficient to cause cognitive impairment as well as decreased numbers of PVIs in adulthood. Using GCLM KO mice, Cabungcal et al. (2013a) demonstrated that impaired synthesis of glutathione delays maturation of PVIs in the anterior cingulate cortex as indicated by reduced PVI numbers, but not calretinin and calbidin positive interneuron numbers, and a reduction in the density of perineuronal nets (PNNs), specialized extracellular matrix components concentrated around PVIs. These effects of reduced glutathione synthesis were only evident during early development but not in later stages. More interestingly, they found that an additional oxidative challenge induced by the dopamine reuptake inhibitor GBR-12909 in preweaning or pubertal but not in young adult GCLM KO mice reduces the number of PVIs in anterior cingulate cortex. Additionally, PVIs in rats with a neonatal central hippocampal lesion exhibit oxidative stress prior to symptom onset as indicated by increased 8-OH-dG marker intensity in most PVIs in the prefrontal cortex (O'Donnell, 2012). Treatment with the GSH precursor NAC during juvenile and adolescent periods reverses the loss of PVIs and restores deficits in prepulse inhibition (O'Donnell, 2012). Collectively, these data suggest that PVIs are vulnerable to oxidative stress, particularly during their development. In our Ppp1r2-cre/fGluN1 KO mice, we observed the reduction of PV-IR and oxidative stress increase on cortical PVIs, not calretinin or calbidin positive interneurons, following the post-weaning social isolation. Reduction of PV-IR upon social isolation was prevented by chronic treatment of APO, suggesting that reduced PV protein level is associated with elevated oxidative stress in PVIs (Jiang et al., 2013).

### POSTNATAL DEVELOPMENT AND MATURATION OF FAST-SPIKING PVIs

Specific vulnerability of PVIs to developmental stressors that cause oxidative stress could result from their relatively late time course of maturation. The mammalian GABAergic system develops slowly during early postnatal life; in the neonatal brain, GABA is the principal excitatory transmitter that induces depolarization and that are important for the early development of the neural network (Ben-Ari et al., 2004). Conversion of GABAergic transmission from depolarizing to hyperpolarizing during the first postnatal week in rodents depends on the expression of the K+/Cl- cotransporter (KCC2) and the Cl- electrochemical potential (Ben-Ari et al., 2004, 2012). GABA signaling not only regulates interneuron axon branching and synapse formation during the maturation of inhibitory innervations (Chattopadhyaya et al., 2007) but also regulate synapse elimination and axon pruning (Wu et al., 2012).

Studies on multiple mammalian species have shown that cortical FS PVIs develop dramatically during early postnatal life. In rodents, transcriptional and electrophysiological maturation of neocortical FS interneurons from slow (low gamma band frequency) into fast (upper gamma band) signaling devices occurs over the first four weeks (Doischer et al., 2008; Okaty et al., 2009; Goldberg et al., 2011). PVIs start to express parvalbumin mRNA and protein by postnatal first week (de Lecea et al., 1995; Itami et al., 2007). The electrochemical properties of PVIs change around the same developmental age; the K+ channel subunits Kv3.1 and Kv3.2, sodium channels Scn8a and Scn4b, and two-pore K+ leak channels TWIK1 and TASK1 are developmentally regulated and contribute to the establishment of the FS behavior characteristics of PVIs (Du et al., 1996; Rudy and McBain, 2001; Tansey et al., 2002; Grieco and Raman, 2004; Grieco et al., 2005; Levin et al., 2006; Okaty et al., 2009; Goldberg et al., 2011). It was also found that the synaptogenesis

of both electrical and GABAergic connections among FS PVIs and PVI-pyramidal neuron microcircuit in rodent was not established until the second postnatal week (Pangratz-Fuehrer and Hestrin, 2011; Yang et al., 2012), and maturation of perisomatic innervations by PVIs is activity and experience dependent (Chattopadhyaya et al., 2004; Jiao et al., 2006). Mature PVIs are wrapped by PNNs, and removal of PNNs increased the excitability of interneurons (Dityatev et al., 2007). Recently it was revealed that PNNs protected mature PVIs against oxidative stress and excessive ROS challenge during early life disrupted PNNs maturation around PVIs and made them more susceptible to oxidative stress (Cabungcal et al., 2013b).

A critical period for PVI maturation is adolescence. Studies in rodents, primates and human subjects indicate that expression of PV protein or mRNA and the density of PVIs processes increase during the pre-pubertal period, peaking during adolescence (Anderson et al., 1995; Erickson and Lewis, 2002; Fung et al., 2010; Caballero et al., 2013). FS PVIs in PFC undergo dramatic changes in the composition of glutamatergic receptors during adolescence: the NMDA/AMPA ratio is dramatically decreased in adolescence, but returns to juvenile levels in adults (Wang and Gao, 2009) and more FS PVIs express calcium permeable AMPA receptors during adolescence (Wang and Gao, 2010). Dopamine modulation of FS PVIs changes dramatically during adolescence (Tseng and O'Donnell, 2007; O'Donnell, 2010, review). Interestingly, in a human study, Uhlhaas et al. (2009) found that cortical networks undergo a transient destabilization from late adolescence to early adulthood, reflected by significant reductions of phase synchrony and induced gamma-band power.

### THE HIGH ENERGY DEMAND OF PVIs

The majority of PVIs are FS (86%, Pawelzik et al., 2002) and PVIs receive extremely dense excitatory innervations (Gulyás et al., 1999), suggesting that PVIs are often in a depolarized state. As a consequence of intense neuronal firing, the constant requirement for maintenance of sodium and potassium balance puts a high demand on cellular energetics. Consistent with this idea, PVIs have particularly larger numbers of mitochondria and higher cytochrome c content than other neurons (Gulyás et al., 2006). Also, it was recently reported that gamma oscillations are also especially energy demanding and require both high level of complex I and strong functional performance of mitochondria. Kann et al. (2011) found that the amount of oxygen consumption of gamma oscillations reaches that of seizure-like events, and gamma oscillations utilize mitochondrial oxidative capacity near limit. Groups of studies indicated that gamma oscillations are exquisitely sensitive to mitochondrial dysfunction (Fano et al., 2007; Huchzermeyer et al., 2008; Hájos et al., 2009; Pietersen et al., 2009; Whittaker et al., 2011) under conditions with a variety of mitochondrial respiratory chain inhibitors through an effect on FS interneurons. Interestingly, Kann et al. (2011) found that gamma oscillation power, oxygen consumption and complex I subunits are particularly higher in hippocampal subfield CA3. Kim Q. Do and her colleagues observed a selective increase of oxidative stress and a decrease of PV-IR interneurons in ventral CA3/DG in GCLM KO mice with a deficit of antioxidant capacity in the brain (Steullet et al., 2010). This may be related to particularly high energy demanding in these areas.

In line with a high metabolic rate of PVIs, these neurons (and transcription of PV itself) are especially vulnerable to stimuli that compromise mitochondrial function. For example, PVIs in the striatum are sensitive to 6-hydroxydopamine (Salin et al., 2009), quinolic acid (Giampà et al., 2006), rotenone (Lapointe et al., 2004), and methamphetamine (Zhu et al., 2006) and developmental injections of 3-nitropropionic acid (3-NP) cause a long-term loss of PV protein (Gibson and Clowry, 2003). Of particular interest is the vulnerability of cortical, hippocampal, and striatal PVIs to perinatal anoxia/hypoxia (Dell'Anna et al., 1996; Van de Berg et al., 2003; Gerstein et al., 2005; Wang et al., 2011) and global ischemia (Guan et al., 2000; Meade et al., 2000), considering that perinatal complications and/or hypoxia could be significant environmental factors contributing to the development of schizophrenia.

## PGC-1$\alpha$ AS A MOLECULAR TARGET OF PVI-SPECIFIC GxE

Recent evidence suggests that a critical regulator of antioxidant capacity in PVI could be the transcriptional coactivator peroxisome proliferator activated receptor gamma (PPARgamma) coactivator 1$\alpha$ (PGC-1$\alpha$).

### PGC-1$\alpha$ DRIVES DEVELOPMENTAL TRANSCRIPTIONAL PROGRAMS FOR INCREASED ANTIOXIDANT CAPACITY, PARVALBUMIN TRANSCRIPTION, AND MITOCHONDRIAL FUNCTION

PGC-1$\alpha$, initially coined the "master regulator of metabolism," is highly expressed in tissues with high energy demands, such as brown adipose tissue, heart, liver, skeletal muscle and brain (Puigserver et al., 1998). It has been found to serve as a central component of the transcriptional regulatory circuitry that coordinately controls the energy-generating functions of mitochondria (**Figure 2**). It is capable of driving transcriptional control of mitochondrial biogenesis through direct interaction with, and coactivation of, PPARs (Madrazo and Kelly, 2008), estrogen-related receptors (ERRs; Schreiber et al., 2004; Eichner and Giguère, 2011), nuclear respiratory factors (NRF-1/NRF-2; Wu et al., 1999; Scarpulla, 2011) and the transcription factor yin-yang one (YY1; Basu et al., 1997; Seelan and Grossman, 1997; Cunningham et al., 2007; Xi et al., 2007), which are important nuclear transcription factors controlling mitochondrial metabolism (Scarpulla et al., 2012). PGC-1$\alpha$ is also an inducible responder to cellular energetic and metabolic stress, such as cold exposure (Puigserver et al., 1998; Uldry et al., 2006; Fisher et al., 2012), nutrient deprivation (Herzig et al., 2001; Yoon et al., 2001; Handschin et al., 2005; Rhee et al., 2006) and exercise (Baar et al., 2002; Handschin and Spiegelman, 2008) and is dynamically regulated in response to a variety of signaling pathways involved in cellular growth, differentiation and energy metabolism. Additionally, a large amount of evidence suggests that PGC-1$\alpha$ links mitochondrial biogenesis and the response to oxidative stress. PGC-1$\alpha$ has been shown to be a powerful regulator of ROS metabolism (St-Pierre et al., 2006; Cunningham et al., 2007).

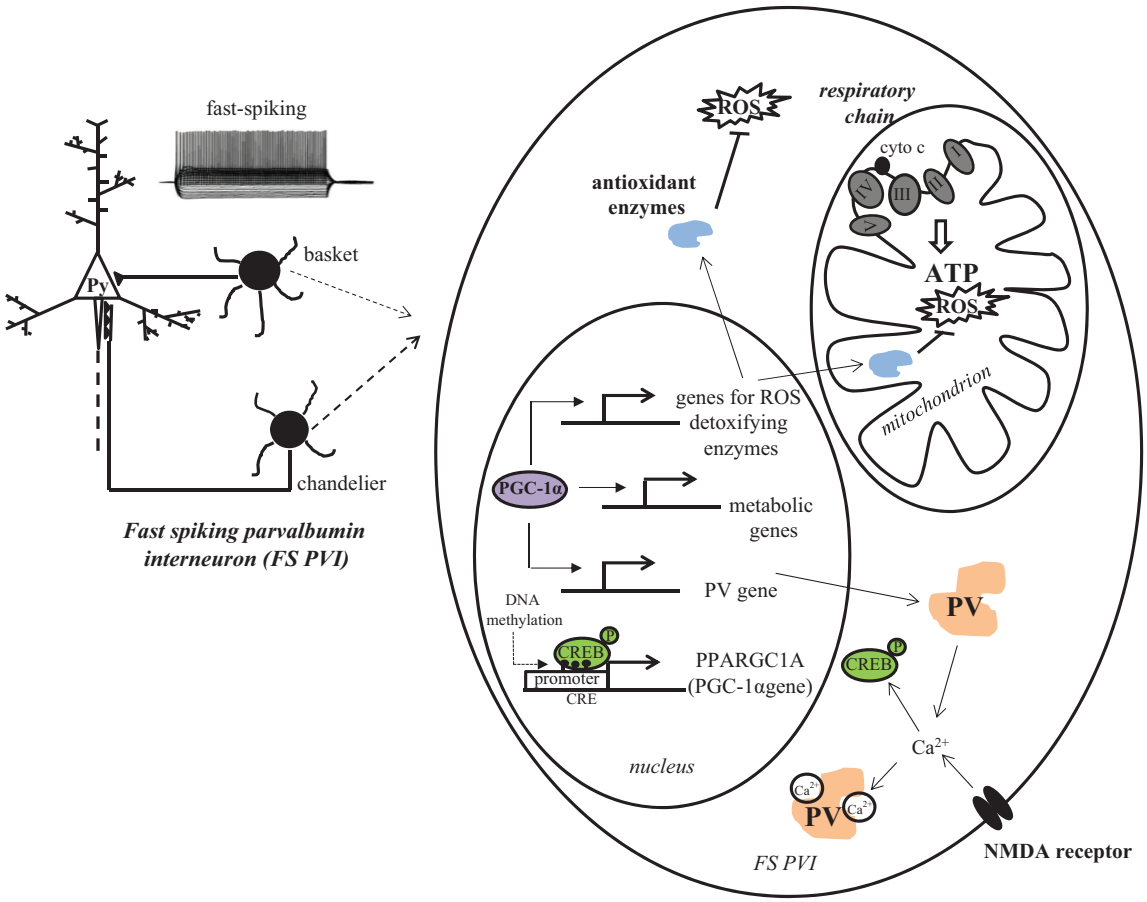


**FIGURE 2 | PGC-1α is a critical contributor to the antioxidant capacity of FS PVIs.** The high energy demand of FS PVIs makes them vulnerable to mitochondrial dysfunction and oxidative stress. PGC-1α, a master regulator of transcriptional programs involved in mitochondrial energy metabolism and antioxidant defense, is highly concentrated in PVIs. In concert with other proteins, PGC-1α increases the transcription of antioxidant enzymes and PV. Transcription of PGC-1α itself is intracellular $Ca^{2+}$ influx-dependent and can be regulated by epigenetic modifications.

In the central nervous system, PGC-1α protein is expressed most highly in GABAergic neurons (Cowell et al., 2007) with a particularly high concentration in cortical PVIs (Jiang et al., 2013). Reductions in PGC-1α mRNA or protein level and its activity have been implicated in several neurodegenerative diseases, such as Huntington (Cui et al., 2006; Weydt et al., 2006), Alzheimer's (Qin et al., 2009), and Parkinson's Disease (Zheng et al., 2010; Shin et al., 2011). A complete absence of PGC-1α in mice causes profound motor impairment and spongioform neurodegeneration in the striatum and cortex (Lin et al., 2004) by six weeks of age that is entirely attributable to a loss of PGC-1α in the central nervous system (Lucas et al., 2012). Of note, motor impairments are still observed in PGC-1α heterozygous mice, albeit to a lesser extent (Lucas et al., 2012); it is possible that the heterozygous mice would provide a better model to investigate the relevance of PGC-1α reductions for understanding its roles in pathophysiology.

The neuroprotective effect of endogenous PGC-1α has been explored in multiple different studies. Mice lacking PGC-1α are more sensitive to neurodegeneration induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and kainic acid, and this degeneration was associated with greater levels of stable markers of oxidative damage, such as nitrotyrosylation of proteins and 8-OH-dG in DNA. Elevation of PGC-1α levels above those of wild-type nerve cells give an increased resistance to death by the oxidative stressors $H_2O_2$ and paraquat (St-Pierre et al., 2006), and overexpression of PGC-1α in culture increases mitochondrial density and ATP levels (Wareski et al., 2009), drives the expression of transcripts involved in antioxidant defense, glucose transport, and mitochondrial fusion and protects cells from hydrogen peroxide-induced cell death and caspase-3 activation (Cowell et al., 2009). In the context of NMDAR activation (Hardingham and Bading, 2010), knock-down of endogenous PGC-1α mediated by siRNA increases extrasynaptic NMDAR(EX) activity and vulnerability to excitotoxic insults in rat cortical neurons, while exogenous expression of PGC-1α cDNA results in a neuroprotective reduction of extrasynaptic currents without affecting synaptic NMDAR activity (Puddifoot et al., 2012).

Interestingly, PGC-1$\alpha$ protein is selectively localized to interneuron nuclei by the second week of postnatal life in rodent brain (Cowell et al., 2007), although its mRNA and protein are observed in neurons throughout the embryonic forebrain. This temporal pattern of expression coincides with the postnatal developmental switch of GABA from an excitatory to inhibitory neurotransmitter and the developmental induction of PV. In fact, mice lacking PGC-1$\alpha$ are deficient in PV-IR, without a loss of other interneuron markers such as GAD67, GAD65, calbindin, calretinin, cholescystokinin and somatostatin (Lucas et al., 2010). Changes in mRNA level of the PVI specific potassium channel Kv3.1 were not observed, either, suggesting that FS interneurons are intact in PGC-1$\alpha$ null mice, just lacking expression of PV protein. Furthermore, overexpression of PGC-1$\alpha$ cDNA in neuroblastoma cells robustly induces transcription of PV promoter, implicating PGC-1$\alpha$ as a critical factor for the developmental induction of PV gene transcription in cortical PVIs. In support of this hypothesis, we found substantial overlap of PV and PGC-1$\alpha$ mRNA in the cortex, with almost all PV mRNA-containing cells being PGC-1$\alpha$ mRNA-positive in S1 cortex and most of the total population of PGC-1$\alpha$ mRNA positive cells being PV mRNA-positive (Jiang et al., 2013). These observations raise the possibility that PGC-1$\alpha$ coordinately regulates programs of gene expression for the developmental induction of PV and an increased antioxidant capacity in PVIs (**Figure 2**).

With this in mind, we investigated whether PGC-1$\alpha$ mRNA or protein is affected by the developmental deletion of NMDARs in our Ppp1r2-cre/fGluN1 KO mice (Jiang et al., 2013). Cortical PGC-1$\alpha$ mRNA and protein levels were down-regulated and post-natal social isolation exacerbated the reduction. In addition to a reduction in PV-IR, mutant mice exhibited a reduction in mRNA levels of several key ROS-detoxifying enzymes including CuZn-SOD, MnSOD, CAT and Gpx, and an impairment in antioxidant capacity and cortical oxidative stress. All these results suggest that PGC-1$\alpha$ plays a critical role in PVI antioxidant capacity that could have relevance for the understanding of schizophrenia patho-physiology. While it is currently not known whether PGC-1$\alpha$ expression is affected in schizophrenia, genetic association studies on chromosome 4p implicated the PGC-1$\alpha$ gene locus in bipolar disorder and schizophrenia (Blackwood et al., 1996; Christoforou et al., 2007, 2011). Integration of genetic data across human and murine studies also suggests PGC-1$\alpha$ as a potential susceptibility gene for anxiety-related disorders (Hettema et al., 2011).

Concerning the potential roles for PGC-1$\alpha$ in the maintenance of mitochondrial function, there is substantial evidence for mitochondrial dysfunction in schizophrenia. Using $^{31}$phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) to measure ATP and phospholipids (Fujimoto et al., 1992; Volz et al., 1997), and positron emission tomography (PET) scans with [$^{18}$F]-fluoro-deoxy-glucose (FDG; Buchsbaum and Hazlett, 1998), altered energy metabolism has been observed in cerebral cortex of schizophrenic patients. A significant decrease in mitochondria number and density in the prefrontal cortex and caudate nucleus of postmortem brains of subjects with schizophrenia was observed compared with control subjects (Uranova et al., 2001). A lower number of mitochondria were found in medication-free patients compared with those taking antipsychotics or control medications, suggesting drug treatment normalizes the number of mitochondria (Inuwa et al., 2005). Combining a parallel transcriptomics, proteomics and metabomics approach, Prabakaran et al. (2004) explored the molecular signatures in brain tissue of schizophrenia. Almost half of the altered proteins identified were associated with mitochondrial function and oxidative stress responses. Critical role of PGC-1$\alpha$ in mitochondrial biogenesis and metabolism highlights it as an interesting candidate for further study in the context of schizophrenia.

## POTENTIAL BIOLOGICAL MECHANISMS UNDERLYING CONVERGENCE OF GxE ON PGC-1$\alpha$

It is notable in our study that no significant change in PGC-1$\alpha$ mRNA or protein level in Ppp1r2-cre/fGluN1 KO mice was observed until the post-adolescent period (eight week old). Post-weaning social isolation exacerbated the reduction of PGC-1$\alpha$ only in KO mice. This implicates that PGC-1$\alpha$, as a master regulator of mitochondria energy metabolism and anti-oxidation, with a selective expression pattern in FS PVI, could be a potential converging factor for GxE. However, the mechanisms by which GxE regulate PGC-1$\alpha$ transcription and trigger oxidative stress on PVIs remains to be clarified.

### *Early postnatal NMDAR hypofunction in cortical PVI*

Notably, PGC-1$\alpha$ transcription and activity itself can be regulated by alterations in intracellular calcium concentrations and the activation of calcium calmodulin-dependent kinase IV (Handschin et al., 2003) that often occur with activation of the NMDAR. In fact, in neurons, transcription of PGC-1$\alpha$ is activity-dependent (Liang and Wong-Riley, 2006; Meng et al., 2007; Yu and Yang, 2010) and increased by NMDAR activation (Lee et al., 2007; Luo et al., 2009; Liang et al., 2010; **Figure 2**). Thus, it is not surprising that early postnatal KO of NMDAR in PVIs is associated with a down-regulation of PGC-1$\alpha$.

### *Epigenetic regulation of PGC-1$\alpha$*

Another mechanism of PGC-1$\alpha$ regulation that could account for environmental and genetic convergence on PVIs may be the modulation of PGC-1$\alpha$ transcription and/or activity by epigenetic mechanisms. Increasing evidence suggests that external environmental factors, such as nutritional, chemical, physical, even psychosocial factors can modify gene expression through epigenetic processes (Jirtle and Skinner, 2007). Epigenetics refers to a set of mitotically heritable and reversible changes in gene expression that occur without a change in the genomic DNA sequence. Epigenetic changes have been associated with a number of paradigms involving social behavior in animal models, such as maternal care (Weaver et al., 2004), early life adversity (Murgatroyd et al., 2009), animal models of posttraumatic stress disorder (PTSD; Chertkow-Deutsher et al., 2010) and chronic social defeat (Tsankova et al., 2006).

Furthermore, epigenetic changes in interneuron-specific transcripts have been documented in schizophrenia; a significant reduction of GABAergic protein (GAD67 and reelin) are accompanied by increased methylation of the GAD67 and Reelin

promoters and increased DNA methyltransferase (DNMT) one in the same interneurons in the cortex of schizophrenic patients (Guidotti et al., 2000; Grayson et al., 2005; Veldic et al., 2005; Huang et al., 2007; Ruzicka et al., 2007). Mill et al. (2008) published the first epigenome-wide study of psychosis using postmortem tissue obtained from frontal cortex of patients with schizophrenia or bipolar disorders. This study enriched the unmethylated fraction of genome DNA and used a CpG island microarray to assay DNA methylation at approximately 12,000 sites across the genome. Their gene-ontology analysis highlights epigenetic disruption to loci involved in mitochondrial function, stress response and brain development.

Recently, another study utilizing genome-wide methylation profiling of blood samples from monozygotic twins discordant for schizophrenia linked aberrant DNA methylation of PGC-1$\alpha$ gene to schizophrenia (Dempster et al., 2011). Indeed, one CRE (CREB binding site) was found in mouse PGC-1$\alpha$ promoter region and it encompassed a CpG site (**Figure 2**). It has been reported that methylation of CpG in CRE blocked pCREB binding (Iguchi-Ariga and Schaffner, 1989; Sunahori et al., 2009). CREB is an important regulator for PGC-1$\alpha$ gene transcription (Herzig et al., 2001; St-Pierre et al., 2006). Barrès et al. (2009) provided evidence that PGC-1$\alpha$ hypermethylation is concomitant with reduced mitochondrial content in type 2 diabetic patients, and links DNMT3B to the acute fatty-acid-induced non-CpG methylation of PGC-1$\alpha$ promoter. Although studies by Dempster et al. (2011) and others assessed PGC-1$\alpha$ methylation status in peripheral samples, and the methylation response in peripheral cells may not accurately reflect methylation status in the brain (Provençal et al., 2012), these data suggest that a differential epigenetic response does occur on PGC-1$\alpha$ in schizophrenic individuals compared with normal subjects. There is also evidence that PGC-1$\alpha$ gene expression and activity are influenced directly by inhibitors of histone deacetylases (HDACs), enzymes that epigenetically modify histones to block transcription. Overexpression of HDAC5 reduces PGC-1$\alpha$ in the heart (Czubryt et al., 2003) by blocking the developmental induction of PGC-1$\alpha$ by myocyte enhancing factor 2 (MEF2), and exposure of neuroblastoma cells to the HDAC inhibitors trichostatin A or valproic acid (a mood stabilizer given as adjunct therapy to schizophrenia patients) increases mRNA level of PGC-1$\alpha$ and its target gene glucose transporter four (Cowell et al., 2009). Hypoxia exposure itself can cause an increase in the expression of the DNA methylating enzymes DNMT1 and DNMT3b mRNA that is sustained in adulthood and accompanied by increased methylation of the SOD2 promoter (Nanduri et al., 2012); taking into account the role of PGC-1$\alpha$ in tissue responses to hypoxia (Arany et al., 2008; Zhu et al., 2010; Pino et al., 2012; Zhao et al., 2012), it is attractive to speculate that environmental stimuli can modulate PGC-1$\alpha$-dependent pathways and PVI antioxidant capacity by epigenetic mechanisms.

Hypothalamic-pituitary-adrenocortical (HPA) hyperactivity by social isolation/stress could involve into PGC-1$\alpha$ regulation through epigenetic mechanisms as well. Activation of the HPA axis represents a common reaction to many environmental insults, including infection, malnutrition, hypoxia, and psychosocial stress (Oitzl et al., 2010). Most of rodent studies showed that chronic early life social isolation is associated with higher basal levels of glucocorticoids and increased HPA response to acute stressors (Weiss et al., 2004; Chida et al., 2005; Perelló et al., 2006; Ruscio et al., 2007; Williams et al., 2009; Weintraub et al., 2010; Toth et al., 2011). Glucocorticoids have been reported to influence DNA methylation in genes containing glucocorticoid response elements (GREs), such as fkbp5 and tyrosine hydroxylase gene (Lee et al., 2011; Niwa et al., 2013). Studies in early postnatal maternal care/separation have linked epigenetic mechanisms with alteration of genes involved in the regulation of the HPA axis. Early life experience influences DNA methylation state of glucocorticoid receptor (GR) gene in human studies (Oberlander et al., 2008; McGowan et al., 2009; Labonte et al., 2012) and animal models (see review by, Weaver, 2009). Epigenetic-mediated changes in GR could contribute to PGC-1$\alpha$ alteration. Further studies need to be done to clarify how GxE influence the transcription of PGC-1$\alpha$ and PV and antioxidant capacity in cortical PVIs.

## SUMMARY

The interaction between genes and environment (GxE) has been postulated to be critical to trigger the onset of schizophrenia. Epidemiological observations and neuropathological studies all support that schizophrenia is a consequence of abnormal brain development. As an essential player in the generation of gamma oscillations, corticolimbic PVIs have a key role in higher cognitive processing. Considering the evidence for PVI dysfunction in patients with schizophrenia and in various neurodevelopmental animal models, the vulnerability of PVIs during early life development and the mechanisms by which these neurons respond to environmental stimuli are particularly relevant for our understanding of schizophrenia pathophysiology. Here, we presented evidence for a convergence of genetic and environmental factors on oxidative stress in PVIs and suggest that PGC-1$\alpha$ is a critical regulator of PV transcription and antioxidant capacity in these neurons. The delayed maturation during postnatal period and high energy demanding of FS PVIs make them susceptible to environmental insults, particularly redox imbalance, during early life experience. Oxidative stress could be one of the important mechanisms integrates GxE on PVIs disturbance. PGC-1$\alpha$, as a master regulator of mitochondrial metabolism, which is highly and selectively expressed in PVIs, could be one of the key factors responsible for the antioxidant capacity of PVIs. Our study using NMDAR hypofunction animal model revealed that redox dysregulation due to a decrease in PGC-1$\alpha$ abundance in cortical parvalbumin interneurons exacerbated schizophrenia-like phenotypes following social isolation stress. This implicates PGC-1$\alpha$ as a potential converging factor for GxE. Further studies on the impact of GxE on PGC-1$\alpha$ transcription and function would not only help identify neuropathophysiological mechanism underlying GxE in schizophrenia, but importantly, aid in the development of targeted interventions for this illness.

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# Maternal care affects the phenotype of a rat model for schizophrenia

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Schizophrenia is a complex mental disorder caused by an interplay between genetic and environmental factors, including early postnatal stressors. To explore this issue, we use two rat lines, apomorphine-susceptible (APO-SUS) rats that display schizophrenia-relevant features and their phenotypic counterpart, apomorphine-unsusceptible (APO-UNSUS) rats. These rat lines differ not only in their gnawing response to apomorphine, but also in their behavioral response to novelty (APO-SUS: high, APO-UNSUS: low). In this study, we examined the effects of early postnatal cross-fostering on maternal care and on the phenotypes of the cross-fostered APO-SUS and APO-UNSUS animals later in life. Cross-fostered APO-UNSUS animals showed decreased body weights as pups and decreased novelty-induced locomotor activity as adults (i.e., more extreme behavior), in accordance with the less appropriate maternal care provided by APO-SUS vs. their own APO-UNSUS mothers (i.e., the APO-SUS mother displayed less non-arched-back nursing and more self-grooming, and was more away from its nest). In contrast, cross-fostered APO-SUS animals showed increased body weights as pups and reduced apomorphine-induced gnawing later in life (i.e., normalization of their extreme behavior), in line with the more appropriate maternal care provided by APO-UNSUS relative to their own APO-SUS mothers (i.e., the APO-UNSUS mother displayed more non-arched-back nursing and similar self-grooming, and was not more away). Furthermore, we found that, in addition to arched-back nursing, non-arched-back nursing was an important feature of maternal care, and that cross-fostering APO-SUS mothers, but not cross-fostering APO-UNSUS mothers, displayed increased apomorphine-induced gnawing. Thus, cross-fostering not only causes early postnatal stress shaping the phenotypes of the cross-fostered animals later in life, but also affects the phenotypes of the cross-fostering mothers.

**Keywords: cross-fostering, early-postnatal stress, schizophrenia, apomorphine-susceptible/apomorphine-unsusceptible rats, maternal care, apomorphine-induced gnawing, novelty-induced locomotor activity, (non-) arched-back nursing**

## INTRODUCTION

Schizophrenia is a severe and complex mental illness with a neurodevelopmental origin. It affects approximately 1% of the general population (Insel, 2010), and is thought to result from an interplay between genetic and environmental factors (Sullivan et al., 2003; Tsuang et al., 2004). Previous studies have suggested that exposure to environmental risk factors during childhood, such as disrupted families and malnutrition, may lead to an increased risk of developing schizophrenia in adulthood (Galletly et al., 2011; Fryers and Brugha, 2013). Gene-environment interactions are difficult to study in humans due to the relatively large genetic variation, the timing of the events (i.e., early-life adversity determines late onset of the disease), the variability in the environment and the ethical issues involved in changing the environment (van Os et al., 2010). Animal models allow the study of such interactions in a well-controlled experimental setting at both the genetic and environmental level (Ayhan et al., 2009; Karl, 2013).

In order to investigate the interplay between genetic factors and postnatal stressors, we have used the apomorphine-susceptible (APO-SUS) rat line and their phenotypic counterpart, the apomorphine-unsusceptible (APO-UNSUS) rat line (Ellenbroek et al., 1995). APO-SUS and APO-UNSUS rats have been bred on the basis of their behavioral response to a subcutaneous (s.c.) injection of the dopamine D1/D2 receptor agonist apomorphine (Ellenbroek and Cools, 2002). With respect to this gnawing response, both APO-SUS and APO-UNSUS rats are extremes within a normal Wistar rat population; compared to Wistar rats, APO-SUS rats show a high behavioral response to apomorphine, whereas APO-UNSUS rats show a

low response to apomorphine (Cools et al., 1990). APO-SUS and APO-UNSUS rats are also extremes in their locomotor response to novelty (Cools et al., 1990). Importantly, APO-SUS animals display various other schizophrenia-relevant features as well (for review: Ellenbroek and Cools, 2002), including an increased behavioral response to amphetamine (van der Elst et al., 2007), disturbed prepulse inhibition (Ellenbroek et al., 1995; Chung et al., 2011), diminished latent inhibition (Ellenbroek et al., 1995) and an increased endocrine, dopamine, and subsequent behavioral response to (environmental) challenges (Cools et al., 1990; Rots et al., 1995; van der Elst et al., 2005).

Maternal care has been shown to change behavioral and endocrine responses to stressors in various animal models (Caldji et al., 1998; Francis et al., 1999; Sequeira-Cordero et al., 2013). Ellenbroek et al. (2000) have previously shown that cross-fostering reduces apomorphine-induced gnawing in APO-SUS, but not in APO-UNSUS rats. However, cross-fostering-induced changes in the maternal care and phenotypes other than the gnawing behavior of these rats have not been investigated. Given the individual-specific behavioral responses to environmental stressors, we hypothesized that exposure of APO-SUS and APO-UNSUS mothers to pups of a different phenotype results in individual differences in maternal care. Here, we performed a detailed cross-fostering study, including a systematic analysis of the maternal care provided by APO-SUS and APO-UNSUS mothers while cross-fostering, and received by the cross-fostered pups. In addition to the effects of cross-fostering on apomorphine-induced gnawing, we also explored the effects of cross-fostering on novelty-induced locomotion. Finally, we studied whether cross-fostering alters the phenotypes of the APO-SUS and APO-UNSUS mothers.

## MATERIALS AND METHODS

### ANIMALS

All experiments were performed in accordance with institutional, national and international laws and guidelines for animal care and welfare and the experiments were approved by the Radboud University Nijmegen Ethical Committee on Animal Experimentation (RU-DEC). Rats were bred in the Central Animal Laboratory of the University of Nijmegen according to methods described by Cools et al. (1990). Briefly, Wistar rats were subjected to a 1.5 mg/kg apomorphine injection (s.c.), after which they were exposed for 45 min. to a box designed to measure stereotyped gnawing (see Cools et al., 1990). This so-called "gnawing box" was slightly modified from the gnawing box described initially by Ljungberg and Ungerstedt (1978) and contains 32 holes surrounded by concentric ridges. Gnawing, on these ridges caused a characteristic vibration that was detected by a microphone, amplified and converted to digital pulses. The nine highest-gnawing animals per sex were selected to obtain the first APO-SUS generation, whereas the nine lowest-gnawing animals per sex were selected to obtain the first APO-UNSUS generation. This selection procedure was repeated for 15 generations, after which the lines were considered stable and no additional apomorphine selection tests had to be performed anymore. In this study, Nijmegen Wistar rats (APO-SUS and APO-UNSUS) of the 33rd generation of the replicate lines were used; in the previously performed cross-fostering experiment (Ellenbroek et al., 2000), the 20th generation of the original lines were used. The average gnawing score of APO-SUS animals is ∼1750 gnaws in the present compared to ∼800 gnaws in the previous study by Ellenbroek et al. (2000). Water and food was available *ad libitum* except during behavioral testing. The animals were housed in Makrolon type III cages (42 × 26.5 × 18 cm) with wood chip bedding located in temperature and humidity controlled rooms with a standard 12 h light/dark cycle (lights on from 07:00 h to 19:00 h).

### BREEDING

One male and one female rat were placed for one night together in a special mating cage containing a wire-mesh floor. The next morning this cage was checked for a copulation plug. When the plug was found, that day was labeled embryonic day (E) 0. The pregnant females were housed in isolation and the cages were cleaned at E15 with great care to avoid stress. From E19 onwards, cages of pregnant rats were checked three times a day (at 08:00, 12:00 and 16:00 h) for the presence of pups. The day mothers gave birth to the first pup of their nest (E22 ± 0.03) was labeled postnatal day (PND)0. Nests which were born after 16:00 h were considered born at 08:00 h on the following day. Cages were again cleaned at PND14 and PND21 by only renewing the dirty bedding outside the nest. Pups were weaned at PND28, after which they were housed with one or two other rats of the same sex and nest. The breeding animals were housed together with one or two other rats of the same rat line and sex.

### CROSS-FOSTERING PROCEDURE

In the un-/cross-fostering experiment, at 16:00 h the dams were removed from their cages for a short period of time (5 min.) and were put back with their own pups (unfostered) or with pups from their phenotypic counterpart (cross-fostered) (Ellenbroek et al., 2000). At the day of weaning (PND28) the pups were weighed. The average nest sizes of APO-SUS and APO-UNSUS animals over the last four generations were checked (APO-SUS: average = 7.5 ± 0.27 pups, $n$ = 113; APO-UNSUS: average = 10.8 ± 0.26 pups, $n$ = 124). Extreme APO-SUS and APO-UNSUS nests (i.e., nests that varied more than two pups from their average nest size) were excluded from the experiment.

### SCORING OF MATERNAL CARE

To enable blind scoring of maternal behavior, the identification labels on the animals' cages were replaced by non-descriptive codes. Maternal care scoring was performed five times a day from PND2 to PND8 during the light phase at 09:00, 12:00 and 16:00 h, and during the dark phase at 06:00 and 20:00 h for a 60-min. period, using previously described procedures (Myers et al., 1989; Champagne et al., 2003). Within an observation session, the ongoing behavior of each litter was observed every 3 min. (20 observations per session). Immediately after each of these observations, the behaviors seen were recorded on a checklist. In this way, each mother-litter combination was observed 700 times from PND2 to PND8 (7 days × five sessions per day × 20

observations per session). Red lights were used during the dark phase to enable behavioral assessment. The following behavioral features were scored (see also: Myers et al., 1989; Champagne et al., 2003): mother away from the nest (i.e., the mother is not in contact with the pups), mother licking the pups (i.e., the mothers display a licking or grooming spell directed at the pups), mother self-grooming (i.e., the mothers display a self-licking or grooming spell), non-arched-back nursing (i.e., the pups have access to the nipples, but the mother did not extend her legs, includes passive nursing and blanket nursing), arched-back nursing (i.e., the mother presents her nipples to the pups by arching over the pups).

### BEHAVIORAL STUDIES

The cross-fostered and unfostered pups (at ∼PND 60) and the cross-fostering and unfostering mothers (at ∼2 weeks after weaning) were subjected to an open field test and the apomorphine susceptibility test. For the open field experiment, rats were placed in the middle of a square black table (160 × 160 cm; 95 cm elevated above the floor) surrounded by a white background illuminated by white light of 80 Lux at the middle of the open field (Verheij et al., 2008). The activity was recorded with a video camera for a period of 30 min. and analyzed with a computerized tracking system, as described by Cools et al. (1990). Two weeks after the open field test, the animals were subjected to the apomorphine susceptibility test, as described by Cools et al. (1990). The animals received an injection of 1.5 mg/kg apomorphine (s.c.) and were placed in a gnawing box (69 × 69 × 25 cm) with a central cubical (25 × 25 × 25 cm) which contained 32 holes (diameter approximately 3 cm), each of which was surrounded by five concentric ridges (Ljungberg and Ungerstedt, 1978). A microphone was placed underneath the central cubicle to allow registration and automatically scoring of the gnawing counts (Cools et al., 1990). Since the gnawing of APO-SUS rats facing one of the corners, instead of the gnawing holes, could not be automatically recorded, we excluded APO-SUS animals that spent more than 12% of the total observation time (45 min.) in the corners (Cools et al., 1990).

### STATISTICS

Maternal care was statistically analyzed using a two-way ANOVA with the factors rat type (APO-SUS and APO-UNSUS) and treatment (cross-fostered and unfostered), followed by *post hoc* Student's *t*-tests where appropriate. The level of significance was set at $p < 0.05$. Given that open field locomotor activity of the pups at adulthood ($t_{(141)} = -6.3$, $p < 0.05$) and weight at PND28 ($t_{(134)} = 2.5$, $p < 0.05$) significantly differed between control males and females, the results regarding these two measures were analyzed per gender (see **Figures 2**, **4** below). Maternal care received by the pups was analyzed in groups containing at least six mothers per group. The groups in which the measurements in the (grown-up) pups were obtained (weight, open field and apomorphine-induced gnawing score) consisted of at least 20 animals and were derived from at least four different nests. Data are expressed as average ± standard error of mean (SEM).

## RESULTS

### APO-SUS- AND APO-UNSUS-SPECIFIC EFFECTS OF CROSS-FOSTERING

APO-SUS- and APO-UNSUS-specific effects of cross-fostering were found for all measures of maternal care (except for licking of the pups). A two-way ANOVA revealed significant rat type × treatment interactions for non-arched-back nursing ($F_{(1,40)} = 36.7$, $p < 0.05$), arched-back nursing ($F_{(1,40)} = 4.89$, $p < 0.05$), the frequency of the mother away from the nest ($F_{(1,40)} = 12.37$, $p < 0.05$) and the self-grooming frequency of the mother ($F_{(1,40)} = 12.94$, $p < 0.05$). In addition, cross-fostering differentially affected the weights of the APO-SUS and APO-UNSUS pups (rat type × treatment interaction: males $F_{(1,162)} = 33.47$, $p < 0.05$, females $F_{(1,171)} = 7.61$, $p < 0.05$). Rat-type-specific effects of the cross-fostering were also found at adulthood (gnawing: $F_{(1,38)} = 5.16$, $p < 0.05$). The results of the *post hoc* analyses are described below.

### MATERNAL CARE RECEIVED BY THE APO-SUS AND APO-UNSUS PUPS

Cross-fostered APO-SUS pups received significantly more non-arched-back nursing ($t_{(22)} = -5.2$, $p < 0.05$, **Figure 1A**), which was accompanied by a similar decrease in arched-back nursing ($t_{(22)} = 4.4$, $p < 0.05$, **Figure 1B**). The other maternal behaviors (mother away: $t_{(22)} = 1.2$, n.s., **Figure 1C**; mother self-grooming: $t_{(22)} = 1.2$, n.s., **Figure 1D**; mother licking the pups: $t_{(22)} = 0.5$, n.s, data not shown) were not different between cross-fostered and unfostered APO-SUS nests. In contrast, cross-fostered APO-UNSUS pups received significantly less non-arched-back nursing ($t_{(18)} = 3.6$, $p < 0.05$, **Figure 1E**), but arched-back nursing was not significantly different ($t_{(18)} = 0.8$, n.s, **Figure 1F**). The APO-SUS mothers fostering APO-UNSUS pups were more frequently away from their nests ($t_{(18)} = -3.9$, $p < 0.05$, **Figure 1G**) and showed more frequently self-grooming ($t_{(18)} = -3.3$, $p < 0.05$, **Figure 1H**). Licking of the pups by the mother was not significantly different between cross-fostered and unfostered APO-UNSUS nests ($t_{(18)} = 0.1$, $p < 0.05$, data not shown).

### WEIGHTS OF APO-SUS AND APO-UNSUS PUPS AT PND28

As previously shown (Degen et al., 2005), unfostered APO-SUS pups were heavier than unfostered APO-UNSUS pups (male: $t_{(67)} = 2.7$, $p < 0.05$, correlation with nest size $p < 0.05$ (Pearson's analysis); female: $t_{(64)} = 6.3$, $p < 0.05$, correlation with nest size $p < 0.05$ (Pearson's analysis). Cross-fostered APO-SUS pups weighed significantly more than unfostered APO-SUS pups at PND28 (male: $t_{(89)} = 4.2$, $p < 0.05$, **Figure 2A**; female: $t_{(87)} = 2.5$, $p < 0.05$, **Figure 2C**), whereas cross-fostering resulted in a significant (male-specific) reduction of body weights in APO-UNSUS pups at PND28 (male: $t_{(73)} = -4.6$, $p < 0.05$, **Figure 2B**; female ($t_{(84)} = -1.5$, n.s., **Figure 2D**).

### BEHAVIOR OF UNFOSTERED/CROSS-FOSTERED APO-SUS AND APO-UNSUS ANIMALS AT ADULTHOOD

Cross-fostered APO-SUS pups showed a decreased apomorphine-induced gnawing score at adult age compared to unfostered APO-SUS animals ($t_{(186)} = -2.0$, $p < 0.05$, **Figure 3A**), whereas cross-fostered APO-UNSUS pups displayed no change in

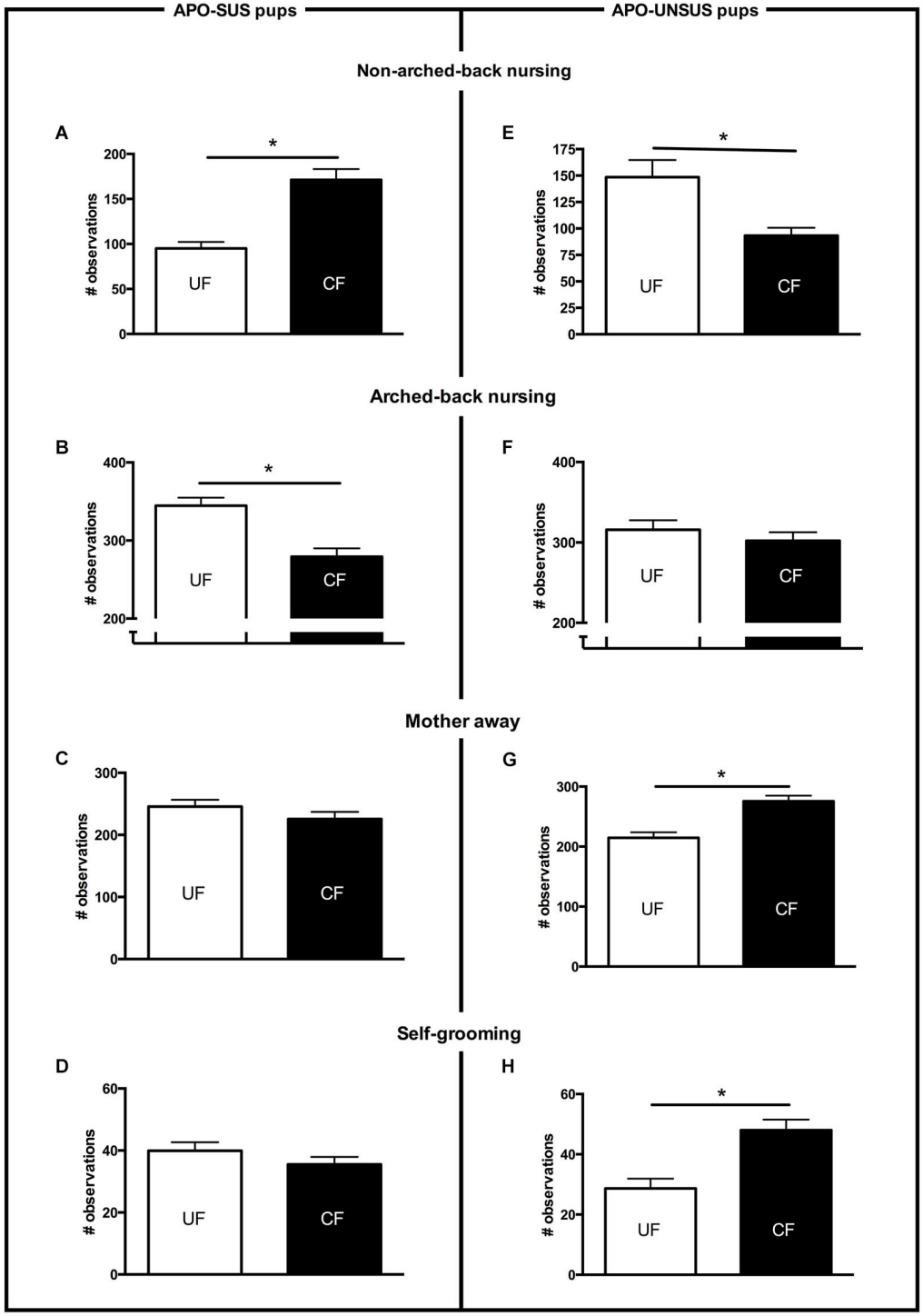


**FIGURE 1 | Maternal care received from PND2 to PND8 by APO-SUS (left panel) and APO-UNSUS pups (right panel) when being unfostered (UF; white bars) or cross-fostered (CF; black bars)**. **(A** and **E)**: non-arched-back nursing; **(B** and **F)**: arched-back nursing; **(C** and **G)**: mother away; **(D** and **H)**: self-grooming. The total number of observations per nest was 700. * $p < 0.05$. Note: To avoid nest disturbance, gender-specific maternal care was not analyzed (see Section Discussion).

apomorphine-induced gnawing at adulthood ($t_{(93)} = -0.83$, n.s., **Figure 3B**). A small, but significant, (male-specific) decrease of novelty-induced locomotor activity was observed in cross-fostered APO-UNSUS pups at adult age (male: $t_{(88)} = -2.1$, $p < 0.05$, **Figure 4B**; female: $t_{(97)} = -1.4$, n.s., **Figure 4D**). In contrast, cross-fostering had no effect on locomotor activity on

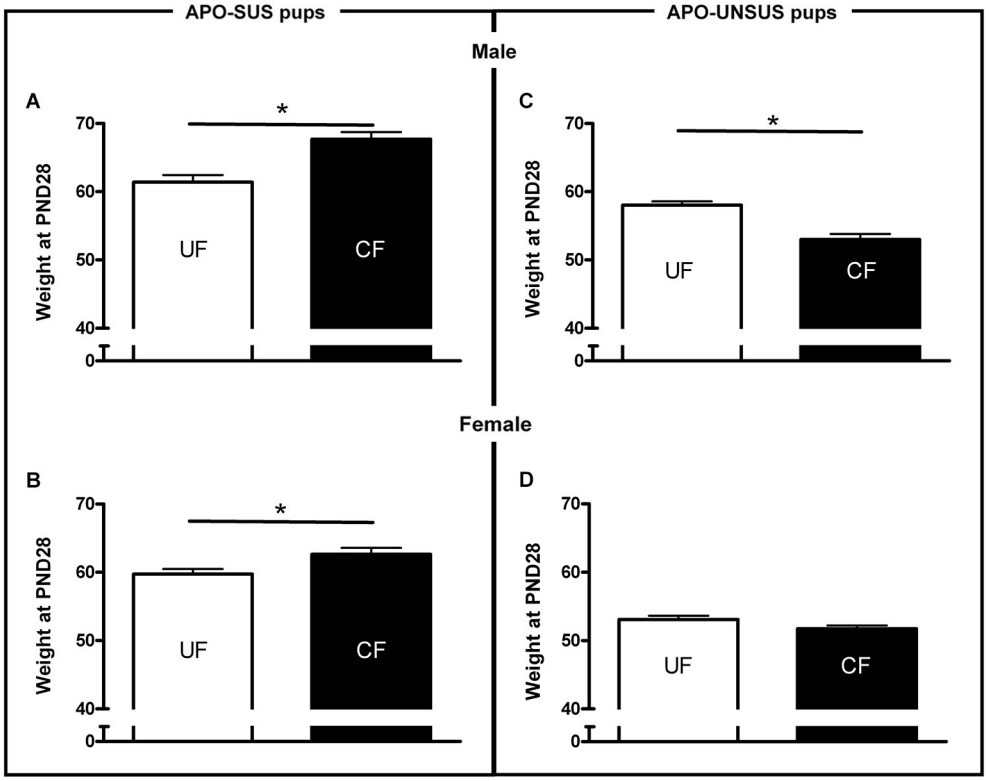


**FIGURE 2 | Weights at PND 28 of male (A) and female (B) APO-SUS, as well as male (C) and female (D) APO-UNSUS pups after being unfostered (UF; white bars) or cross-fostered (CF; black bars).** * $p < 0.05$.

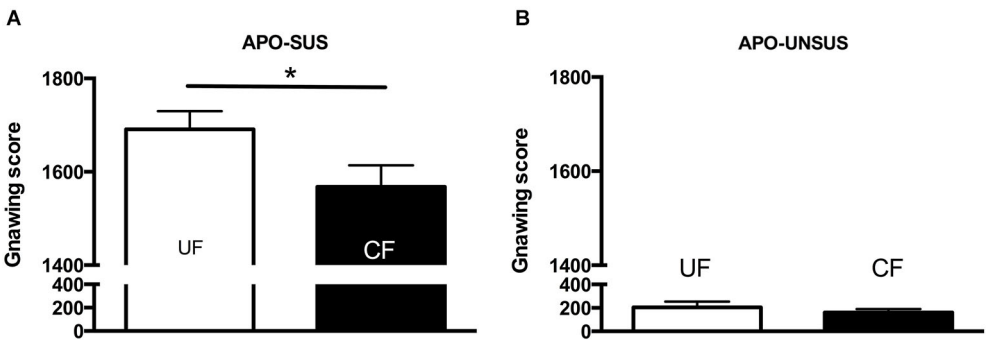


**FIGURE 3 | Apomorphine-induced gnawing of APO-SUS (A) and APO-UNSUS (B) rats after being unfostered (UF; white bars) or cross-fostered (CF; black bars) in their early life.** * $p < 0.05$. Note: No gender differences were observed.

the open field in APO-SUS rats at adulthood (male: $t_{(99)} = -0.17$, n.s., **Figure 4A**; female: $t_{(78)} = -1.1$, n.s., **Figure 4C**).

### BEHAVIOR OF THE APO-SUS AND APO-UNSUS MOTHERS AFTER WEANING

Two weeks after the mothers were separated from their pups, APO-SUS mothers having fostered APO-UNSUS pups showed a significant increase in apomorphine-induced gnawing when compared to APO-SUS mothers that had fostered their own pups ($t_{(21)} = 2.4$, $p < 0.05$, **Figure 5A**), whereas cross-fostering had no effect on the gnawing behavior of the APO-UNSUS mothers ($t_{(17)} = 0.2$, n.s., **Figure 5B**). The novelty-induced locomotor activity on the open field did not significantly differ between mothers having fostered their own pups and mothers having fostered pups of their phenotypic counterpart (APO-SUS mothers: $t_{(16)} = -1.1$, n.s.; APO-UNSUS mothers: $t_{(16)} = -0.4$, n.s., data not shown).

A summary of the results from our cross-fostering experiments with APO-SUS and APO-UNSUS rats is presented in **Table 1**.

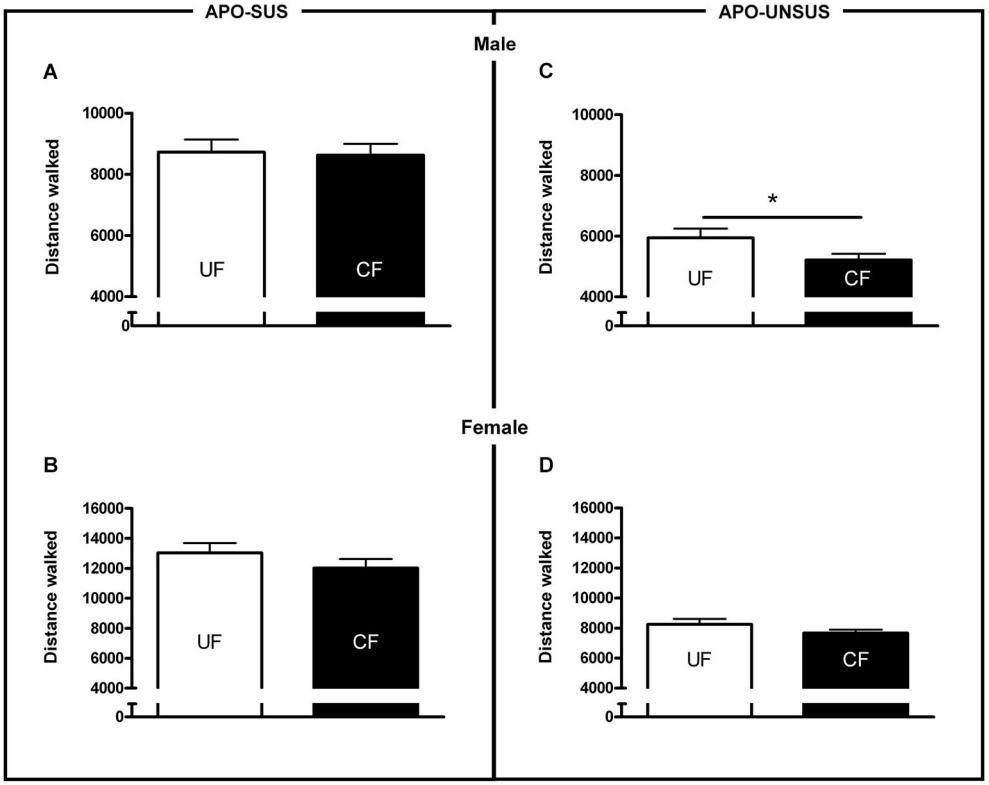


**FIGURE 4 | Novelty-induced locomotion in male APO-SUS (A) and APO-UNSUS (B) pups as well as female APO-SUS (C) and APO-UNSUS (D) pups after being unfostered (UF; white bars) or cross-fostered (CF; black bars).** * $p < 0.05$.

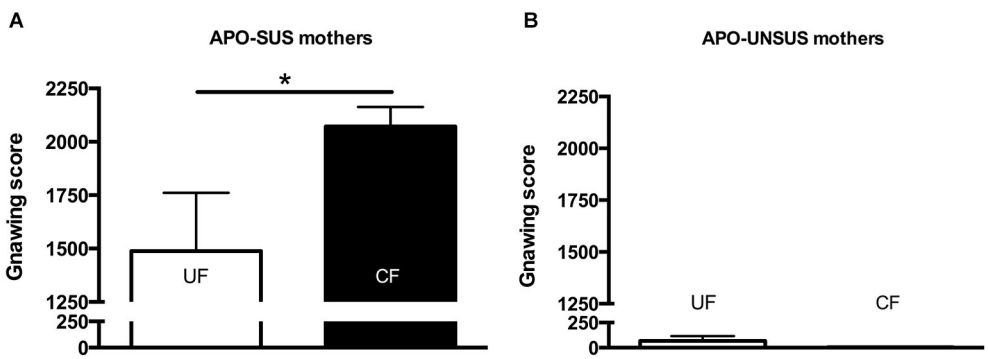


**FIGURE 5 | Apomorphine-induced gnawing of APO-SUS (A) and APO-UNSUS (B) mothers after fostering their own pups (white bars) or fostering pups from their phenotypic counterpart (black bars).** * $p < 0.05$.

## DISCUSSION

In this study, we performed cross-fostering experiments to determine whether a change in the maternal care of pups may alter their later-life phenotypes and what the effects of disrupted mother-pup interactions are on the dams. An important indicator reflecting the quality of maternal care received by a pup constitutes its body weight. The body weight of a pup is known to be heavily determined by the amount of nutrients and body warmth provided by the mother (Stone et al., 1976; Smart et al., 1983). With respect to nutritional supply, it is important to note that maternal care can be provided by arched-back or non-arched-back nursing. Despite the fact that the non-arched-back position is generally considered to be less favorable for feeding than the arched-back position (Lonstein et al., 1998), we found that in cross-fostered APO-SUS pups an increase of non-arched-back nursing, together with a similar decrease of arched-back nursing, was accompanied by increased body weights. Apparently, despite this form of

**Table 1 | Overview of the results from the cross-fostering experiments with APO-SUS and APO-UNSUS rats.**

| | PND2—8 | PND28 | Adulthood (>PND60) | |
|---|---|---|---|---|
| | Maternal care received | Weight | Open field | Gnawing score |
| CF vs. UF APO-SUS rats | Non-arched-back-nursing ↑<br>Arched-back nursing ↓ | ↑ | = | ↓ |
| CF vs. UF APO-UNSUS rats | Non-arched-back nursing ↓<br>Mother away ↑<br>Mother self-grooming ↑ | ↓ (males) | ↓ (males) | = |

*Maternal care was scored from PND2 to PND8. At weaning (PND28), the weights of the cross-fostered (CF) and unfostered (UF) pups were determined. The phenotypes of the grown-up pups were determined after PND60.*

behavioral competition (i.e., non-arched-back nursing replaces arched-back nursing), the APO-SUS pups were still able to suckle the nipples to receive enough milk even when the APO-UNSUS mother was in a less favorable position. In addition, the more pronounced skin-to-skin warmth provided by the higher level of non-arched-back nursing of the APO-UNSUS mothers may also have contributed to the observed increased body weights of the cross-fostered APO-SUS pups. The decrease in non-arched-back nursing received by APO-UNSUS pups when they were fostered by APO-SUS mothers likely results not only in less milk received by the pups but also in less skin-to-skin warmth (Kojima and Alberts, 2011). The decreased non-arched-back nursing received by cross-fostered APO-UNSUS pups was indeed accompanied by a decrease in their body weights. These observations indicate that APO-SUS mothers fostering APO-UNSUS pups provided less maternal care than cross-fostering APO-UNSUS mothers. Cross-fostering APO-SUS mothers showed indeed more self-grooming and more time away from the pups. The lower quality of maternal care provided by cross-fostering APO-SUS vs. APO-UNSUS mothers may be explained by the fact that APO-SUS rats show a stronger and longer endocrine, neurochemical, and subsequently behavioral response to environmental challenges (i.e., in this study exposure to pups of their phenotypic counterpart) than APO-UNSUS rats (Rots et al., 1995).

Ellenbroek et al. (2000) have previously found that cross-fostering reduced apomorphine-induced gnawing in APO-SUS, but not in APO-UNSUS rats. The more pronounced decrease of cross-fostering-induced gnawing observed in the previous study may be due to the fact that here we used an APO-SUS rat line resulting from a different pharmacogenetic selection and bred for 12 more generations than the previous line. Importantly, in the present study we performed additional behavioral studies showing that cross-fostering reduces novelty-induced locomotion in APO-UNSUS but not in APO-SUS rats. The observed decrease in body weight and the more extreme phenotype (i.e., reduced locomotion) in cross-fostered APO-UNSUS rats may be attributed to the less appropriate maternal care of APO-SUS mothers (i.e., less non-arched-back nursing, more observations of the mother being away and more self-grooming). In contrast, the observed increase in body weight and the normalization of the phenotype (i.e., reduced gnawing) in cross-fostered APO-SUS rats may very well be explained by the more appropriate maternal care provided by APO-UNSUS mothers (i.e., more non-arched-back nursing, and no change in the number of observations of the mother being away or self-grooming).

In order to disturb the nests as little as possible, we performed whole-nest observations, which implied that maternal care could not be analyzed per pup. In contrast, in other studies the nests have been disturbed to examine mother-pup interactions per pup—either by labeling the pups individually (Champagne et al., 2003) or by adjusting the gender composition of the litter to obtain either single-sex (male or female) or mixed-sex (half male and half female) litters (Hao et al., 2011). Interestingly, in the latter study mothers were found to spend more time licking male than female pups, presumably explaining that maternal deprivation had opposite effects on neurodevelopment between males and females (Oomen et al., 2009). The sex-specific effects of cross-fostering on pup body weight and adult open field behavior that we observed, suggest that cross-fostering APO-SUS and APO-UNSUS mothers may also have taken care of male and female pups differently.

To our knowledge, the effects of cross-fostering on the mothers themselves have not been described before. In this study, we observed 2 weeks after weaning an increase in apomorphine-induced gnawing in APO-SUS mothers that fostered APO-UNSUS pups, whereas APO-UNSUS mothers were unaffected by cross-fostering. We hypothesize that this long-lasting increase in apomorphine-induced gnawing (reflecting a more extreme phenotype) is due to the exposure to pups of a different phenotype, resulting in a stronger and longer-lasting stress response in APO-SUS than APO-UNSUS mothers (see also Rots et al., 1995). Indeed, chronic stress has been found to increase apomorphine-induced stereotypic behavior (Cabib et al., 1984).

## CONCLUSIONS

Although most mother-pup interaction studies have focused on the role of arched-back nursing (Caldji et al., 2003; Champagne et al., 2003; Daskalakis et al., 2012), we demonstrate that non-arched-back nursing is also important for the proper growth of the pups. In addition, the more stress-sensitive APO-SUS rats (Rots et al., 1995) provide less maternal care when they foster APO-UNSUS pups, resulting in pups with reduced body

weights and more extreme adult phenotypes. The finding that the body weights of APO-SUS pups increased and the adult phenotypes of these animals became less extreme after being fostered by relatively stress-resistant APO-UNSUS mothers opens the intriguing perspective for early-life environmental intervention approaches (e.g., prevent exposure to stressful events) in humans predisposed to schizophrenia. Finally, cross-fostering not only influences the quality of maternal care, but may also have a long-lasting impact on stress-sensitive mothers. This may very well affect the phenotype of the next litter of these mothers. In conclusion, the effect of cross-fostering is not only determined by the background of the pups and their mother, but also by mother-pup interactions.

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# Neonatal stress-induced affective changes in adolescent Wistar rats: early signs of schizophrenia-like behavior

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Psychiatric disorders are multifactorial diseases with etiology that may involve genetic factors, early life environment and stressful life events. The neurodevelopmental hypothesis of schizophrenia is based on a wealth of data on increased vulnerability in individuals exposed to insults during the perinatal period. Maternal deprivation (MD) disinhibits the adrenocortical response to stress in neonatal rats and has been used as an animal model of schizophrenia. To test if long-term affective consequences of early life stress were influenced by maternal presence, we submitted 10-day old rats, either deprived (for 22 h) or not from their dams, to a stress challenge (i.p. saline injection). Corticosterone plasma levels were measured 2 h after the challenge, whereas another subgroup was assessed for behavior in the open field, elevated plus maze (EPM), social investigation and the negative contrast sucrose consumption test in adolescence (postnatal day 45). Maternally deprived rats exhibited increased plasma corticosterone (CORT) levels which were higher in maternally deprived and stress challenged pups. Social investigation was impaired in maternally deprived rats only, while saline injection, independently of MD, was associated with increased anxiety-like behavior in the EPM and an impaired intake decrement in the negative sucrose contrast. In the open field, center exploration was reduced in all maternally-deprived adolescents and in control rats challenged with saline injection. The most striking finding was that exposure to a stressful stimulus *per se*, regardless of MD, was linked to differential emotional consequences. We therefore propose that besides being a well-known and validated model of schizophrenia in adult rats, the MD paradigm could be extended to model early signs of psychiatric dysfunction, and would particularly be a useful tool to detect early signs that resemble schizophrenia.

**Keywords: early life stress, animal model, schizophrenia, corticosterone, anxiety-like behavior, social behavior, anhedonia**

## INTRODUCTION

Mental illnesses are among the most frequent causes of functional debilitation. Schizophrenia is a markedly devastating psychiatric disorder, affecting approximately 1% of the global population (Freedman, 2003). It is challenging to precisely outline the causes of schizophrenia, because a multitude of genetic and environmental factors are thought to interact in the course of the disease, resulting in the onset, maintenance and evolution of schizophrenic symptoms (for a review on the multifactorial aspect of mental disorders, see Akdeniz et al., 2014; Uher, 2014).

One of the most explored aspects on the environment-gene interaction prompting schizophrenic phenotype is the impact of harsh early life conditions, leading to the neurodevelopmental hypothesis of schizophrenia (Fatemi and Folsom, 2009; Piper et al., 2012), which postulates that neurochemical disturbances induced by adverse early life events could permanently affect brain function, producing abnormalities that would ultimately underlie the emergence of the disorder. The early stages of life are characterized by an intense neural development, in which there is a dynamic process of synaptic shaping and pruning, making this period highly vulnerable to damaging disturbances (Martínez-Téllez et al., 2009; Stolp et al., 2012).

The impact of childhood experiences on the development of emotional behavior and their influence on psychopathology has received vigorous scientific support in the past decades (Heim et al., 1997, 2000; Bernet and Stein, 1999; Heim and Nemeroff, 2002; McEwen, 2003). However, studies that aim at correlating early life events to schizophrenia or to other psychopathologies are rather speculative and based on retrospective subjective reports. Manipulations of the early environment for subsequent assessment of emotional state in humans are operationally and ethically unfeasible. Therefore, animal models are imperative to comprehend how early life adverse events affect behavioral and neurobiological aspects that reflect the basic underpinnings of mental disorders such as schizophrenia.

Developmental stress models used to investigate long-lasting consequences on schizophrenia-related emotional and cognitive

behavior range from prenatal maternal immune (Meyer et al., 2006) and psychological challenges (Kinnunen et al., 2003; Lee et al., 2007), to maternal vitamin D deficiency (Kesby et al., 2006), maternal deprivation (MD; Ellenbroek et al., 1998; Garner et al., 2007) and socially unstable conditions (Möller et al., 2011). An acute 24-h MD on post-natal day (PND) 9 exerts profound effects on a myriad of affective and cognitive features in the offspring that have been linked to major processes underlying clinical manifestations of schizophrenia (Ellenbroek et al., 1998; Ellenbroek and Cools, 2002b; Ellenbroek et al., 2005; Takase et al., 2012). The rationale for the putative MD effects is the fact that during the first 2 weeks of life, the infant hypothalamus-pituitary-adrenal (HPA) axis shows a blunted basal activity as well as a marked hyporesponsiveness to external noxious stimuli (Sapolsky and Meaney, 1986; Rosenfeld et al., 1992; Schmidt et al., 2003). This period is named stress hyporesponsive period (SHRP) and core elements of the maternal care were identified as pivotal for SHRP maintenance (Cirulli et al., 1992; Suchecki et al., 1993). Prolonged maternal absence disinhibits basal and reactive adrenocortical activity to mild stressors, producing high circulating plasma level of corticosterone (CORT) during a stage when it is naturally upheld low (Levine et al., 1991; Suchecki et al., 1995; Faturi et al., 2010). Despite the strong body of evidence showing that high circulating levels of CORT can affect brain structures that orchestrate cognitive and emotional behavior (Woolley et al., 1990; Mitra and Sapolsky, 2008), it is not yet completely understood whether CORT action on neural development is the mechanism involved in the long-lasting MD effects that resemble symptoms of schizophrenia.

The DSM-V lists three main clusters of signs that are required for schizophrenia diagnosis, comprising the so-called positive, negative and cognitive symptoms (American Psychiatric Association, A.P.A.D.S.M.T.F., 2013). Although it is puzzling to mimic hallucinatory and delusional behaviors in animal studies, positive symptoms of schizophrenia are still easily modeled in animals because pharmacological validation tools can be used to investigate rodent analogs of psychosis (such as prepulse inhibition (PPI) impairments and hyperlocomotion). Because overactive dopamine is a key-candidate mechanism to underpin positive symptoms (Howes and Kapur, 2009; Lodge and Grace, 2011), animal models that aim to mimic these symptoms evaluate behaviors that are mainly affected by a magnification of the dopaminergic transmission, such as hyperlocomotor activity in novel environments (Powell et al., 2009; Le Pen et al., 2011) and hypersensitivity to dopaminergic drugs (Ellenbroek and Cools, 2002a). Also extensively employed are animal models that explore the negative symptoms, which include affective flattening, anhedonia, avolition and asociality (Ellenbroek and Cools, 2000; Moser, 2014).

By all means, most of the developmental interventions aim at behavioral assessments that have been considered to have specific translational relevance to schizophrenia. Evaluation of a more comprehensive range of emotional behaviors would help clarify whether the abovementioned effects of MD are restricted to schizophrenic-like features that emerge in adulthood or if there is an overall effect on a broader spectrum of emotionality that manifests likewise earlier in life. Furthermore, these aspects are typically tested in early adulthood, when the schizophrenic-like phenotype is assumed to be fully established. However, the initial signs may include poor sociability and anxiety symptoms followed by the onset of typical psychotic outbursts (Agius et al., 2010). Hence, aside from testing typical schizophrenia-like behavior restricted to adulthood, current investigation approaches should expand the assortment of behaviors and anticipate the age of interest, ensuring that early alterations such as augmented anxiety and mild social impairment in adolescence would be included in the scope of developmental animal models of schizophrenia.

One additional step for a full characterization of MD as an unambiguous developmental animal model for schizophrenia would be to match the effects of MD to the effects of different early life stressors. If one could dissociate the effects of maternal care disruption and that of different stressors, this would pave the way to the understanding on how specific the association is between MD and schizophrenia-like phenotype in animal models. Alternatively, if disruption of maternal care prompts the HPA axis to a reactive state, adding an extra stress challenge to the maternally-deprived infant rat, with an external noxious stimulus, would trigger a robust neuroendocrine response, which, in turn, could be responsible for more intense and impacting developmental and behavioral effects.

Taken together, the aforementioned evidence indicates that experimental approaches of concurrent early life events and earlier characterization of behavioral effects are necessary to better illustrate the time course and the full range of symptomatology witnessed in the clinical scenario. Therefore, we employed the MD paradigm and a mild stress challenge or a combination of both to characterize, in adolescence, a wide range of behavioral effects that might unravel early signs of psychiatric dysfunction in this developmental stress animal model.

## MATERIALS AND METHODS

### SUBJECTS AND EXPERIMENTAL DESIGN

Fifteen pairs of Wistar rats were bred, generating a total of 96 male and 24 female Wistar rat pups, of which, only males were used in this study. Male-female pairs were obtained from the Center for the Development of Animal Models for Biology and Medicine (CEDEME), Universidade Federal de Sao Paulo. All procedures were approved by, and conducted in accordance with the Research Ethics Committee of the Universidade Federal de Sao Paulo, approval protocol #0366/12.

Litters were obtained from mating pairs maintained together in Plexiglas cages under controlled temperature (22 ± 2°C) and a light-dark cycle of 12 h, with lights on at 7 a.m. 10 days after the onset of breeding, couples were separated and females were individually housed until the end of experiments. 15 days after the onset of breeding, sawdust and paper towels were provided for nest building. Inspection for births started on day 18 and continued twice daily, at 9:00 a.m. and at 5:00 p.m. The day of birth was considered postnatal day 0 (PND 0) and litters were culled to approximately 6 males and 2 females, whenever possible, on PND 1. When the number of newborns in a litter did not reach the minimal of 6 males or 2 females, additional males or females (when available) were maintained at culling or

surplus pups were adopted from litters born in the same day, in order to preserve the standard number of 8 pups per litter. Litters with less than 8 pups were not used in the experiment ($n$ = 3 litters). Births occurred in a range of 4 days (22–25 days after mating onset). The 12 remaining litters were assigned to Maternal Deprivation (MD = 6 litters) or Control non-deprived (CON = 6 litters) groups. For MD, the dam was removed from the home-cage and housed in a separate room for 24 h, from PND 9 to 10. CON litters were left undisturbed, with their mothers, during this period. Twenty-two hours after the onset of MD, or at the corresponding time for CON litters, each group was subdivided in two: a subgroup was submitted to a stress challenge, consisting of an intraperitoneal injection of 0.3 mL of a 0.9% saline solution (STR) and another subgroup was kept unstressed, with no saline injection (UNS). UNS pups were briefly handled in the same room in which injections occurred. In order to counterbalance birth date among groups, each new delivery was assigned *a priori* to one of the groups, in the following order: CON UNS (3 litters), CON STR (3 litters), MD UNS (3 litters) and MD STR (3 litters). Two hours after the saline injections, 1–2 pups/litter were decapitated and trunk blood was collected for determination of plasma CORT levels. Additional litters were included in this phase in order to increase the number of blood samples for acute blood analysis. The remaining pups returned to their mothers and were left undisturbed until weaning and cages were then cleaned every 4 days, so that half of the bedding material was removed and replaced with fresh one and nesting material was supplemented as necessary. On PND 21 (weaning), male offspring was individually identified and transferred to another animal room, under the same environmental conditions as described above, housed in regular Plexiglas cages in groups of 2–3 siblings per cage. Behavioral testing started on PND 45. One day before the onset of behavioral evaluation, each rat was individually housed with water and food ad libitum.

#### DETERMINATION OF PLASMA CORT LEVELS

Trunk blood was collected in cooled EDTA-containing vials and centrifuged at 2300 rpm, 4°C for 15 min. Plasma was stored at −20°C for determination of CORT levels by radioimmunoassay, using a commercial kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA) with a modification of the original method, using a reduced sample volume of 5 μl (Thrivikraman et al., 1997). The sensitivity of the assay was 3.125 ng/ml and intra and inter-assay variations were, respectively, 7.1% and 10.3%.

## BEHAVIORAL TESTS

#### SUCROSE NEGATIVE CONTRAST TEST (SNCT)

To test the intake response to a sudden decrease in concentration of a palatable sucrose solution, two identical bottles were provided for each single-housed rat, during 3 days. One bottle contained 200 mL of sucrose solution and the other an equal volume of water. On each day, bottle positions were interchanged to avoid an effect of side preference. Each bottle was weighed every 24 h (at 11:30 a.m.) to estimate liquid intake. On testing days 1 and 2, sucrose solution concentration was 15% and on day 3, it was 2.1% (Matthews et al., 1996). The sucrose preference index was calculated as the percentage of sucrose intake in relation to the total intake of liquid, according to the equation: Sucrose preference index = (sucrose intake/(water intake + sucrose intake)) × 100.

#### OPEN FIELD

On the afternoon after the end of the SNCT (from 12:00 p.m.–16:00 p.m.), each rat was individually placed in the center of a circular arena (80 cm diameter) surrounded by 50-cm high walls, and left undisturbed for 10 min. The luminosity during the test was limited to the lights placed on the top of the open field (25 lux). The arena was thoroughly cleaned with 70% ethanol between animal sessions to eliminate olfactory cues. The tests were recorded using a digital video camera and an off-line analysis was performed using the software Ethovision XT (Noldus, The Netherlands). The parameters used were distance and time traveled in each virtually defined compartment (center and periphery) as well as total ambulated distance by each rat. Center was defined as a 52-cm diameter (14 cm from the wall) concentric inner circle.

#### ELEVATED PLUS MAZE (EPM)

In the next morning after open field test (from 9:00 a.m.–12:00 p.m.), rats were tested on the Elevated Plus Maze (EPM). The maze is elevated 60 cm above the floor and consists of four arms: two of them are enclosed by 40 cm high walls, and the other two are open arms (with no enclosing walls). Each arm is 50 cm long and 10 cm wide. Procedures were based on those described elsewhere (Pellow et al., 1985). Briefly, each animal was individually placed in the central segment of the maze facing one of the open arms, interchangeably on consecutive animals. Each test session lasted 5 min. The apparatus was thoroughly cleaned with 70% ethanol between sessions. The tests were carried out in a dim environment (11 lux). We manually scored the number of entries and the time spent in open and closed arms. Data are expressed as percentage of time and percentage of entries in the open arms.

#### SOCIAL INVESTIGATION

In the afternoon of the same day as the EPM test (from 12:00 – 16:00 p.m.), rats were submitted to the social investigation test, which was conducted in the same arena and same environmental conditions as the open field test so that the animals were familiarized to the environment. Inside the arena two identical metal grid cylindrical cages (20 cm in diameter and 25 cm in height) were placed on opposite sides of the arena. One cage was empty and the other contained a naïve rat. Each experimental animal was individually placed in the center of the arena and left undisturbed to explore the environment for 10 min. Tests were recorded using a digital video camera and an off-line analysis was manually performed. Cage exploration was computed when the rat approached and pointed its nose towards one of the cages and as close as possible to the cage (approximately 1 cm or as closer as it was possible to judge from the video image). We quantified the time of exploration of the empty cage and the cage containing the naïve rat.

### STATISTICAL ANALYSIS

Each parameter was analyzed by two-way ANOVA, with Maternal Deprivation (CON vs. MD) and Saline Injection (Unstressed [UNS] vs. Stressed [STR]) as main factors. The SNCT was analyzed by repeated measures ANOVA, in which the within subject factor was Day (day 1 vs. day 2 vs. day 3) and MD and Saline Injection as between factors. The level for statistical difference was set at $p < 0.05$. When a significant main effect or an interaction was detected, the Newman-Keuls *post hoc* test was applied for between-group comparisons.

## RESULTS

### CORT PLASMA LEVELS

There was a significant effect of MD factor ($F_{(1,50)} = 64.767$, $p < 0.001$), and Saline Injection ($F_{(1,50)} = 15.215$, $p < 0.0003$) as well as an interaction between these factors ($F_{(1,50)} = 13.418$, $p = 0.0006$). *Post-hoc* analysis revealed that MD increased plasma CORT levels (CON UNS vs. MD UNS, $p = 0.008$) and this effect was further increased by the saline injection (MD UNS vs. MD STR, $p = 0.0001$) (**Figure 1**).

### SUCROSE NEGATIVE CONTRAST TEST

UNS, but not STR animals, drank less 2.1% than 15% sucrose solution (day 3 < [day 1 = day 2 ($p$'s < 0.005)]); effect of day $F_{(2,52)} = 7.395$, $p = 0.001$). When the low concentration of sucrose was offered, STR rats drank more than UNS rats, regardless of MD (interaction between Day and Saline Injection ($F_{(2,52)} = 7.107$, $p < 0.002$; UNS vs. STR on Day 3, $p < 0.03$) (**Figure 2**).

### OPEN FIELD

Ambulation, measured as total distance traveled in the open field, was not affected neither by MD ($F_{(1,30)} = 0.053$, $p = 0.818$) nor by saline injection $F_{(1,30)} = 2.253$, $p = 0.143$) (**Figure 3A**). Conversely, there was a clear effect of the interaction between

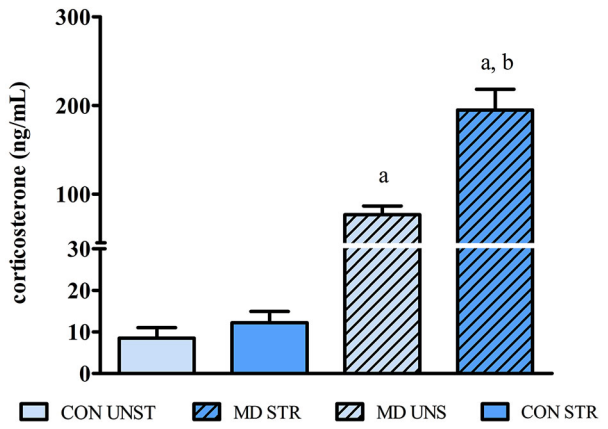


**FIGURE 1 | CORT plasma levels on PND10 (24 h after maternal deprivation onset/2 h after saline injection) expressed as mean ± S.E.M.** CON = group not maternally deprived; MD maternal deprivation on postnatal day 9. UNS = no stress challenge; STR = saline injection stress challenge. a = significantly different from respective CON; b = significantly different from respective UNS ($n$ = 11–16/group).

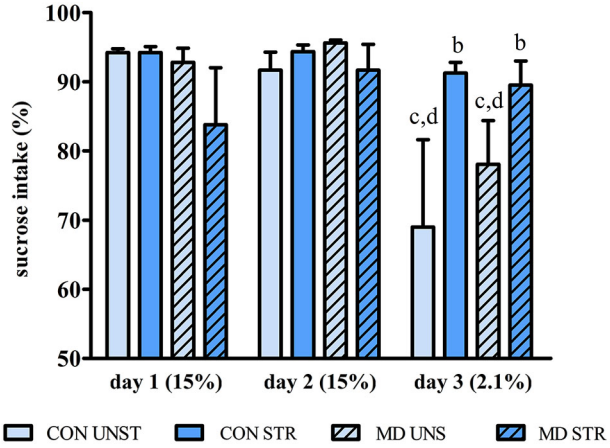


**FIGURE 2 | Mean ± S.E.M. percentage of sucrose intake during the course of 3-day testing relative to total liquid intake in each day**. CON = group not maternally deprived; MD maternal deprivation on postnatal day 9. UNS = no stress challenge; SAL = saline injection stress challenge. b = significantly different from respective UNS; c = significantly different from respective intake on day 1; d = significantly different from respective intake on day 2 ($n$ = 8–10/group).

MD and Saline Injection for both distance traveled and time spent in the center portion of the open field (Distance traveled: $F_{(1,30)} = 5.860$, $p = 0.021$ and time spent in the center $F_{(1,30)} = 5.848$, $p = 0.021$). CON STR and MD UNS groups ambulated significantly less ($p$'s = 0.012) and spent less time (**Figure 3B**) in the center of the open field than CON UNS rats ($p < 0.015$ and $p < 0.009$, respectively).

### ELEVATED PLUS MAZE

Rats that received a saline injection on PND 10 entered less and spent less time in the open arms than their UNS counterparts (main effect of Saline Injection for percentage of entries ($F_{(1,30)} = 5.928$, $p < 0.020$ and for percentage of time in the open arms $F_{(1,30)} = 9.761$, $p < 0.004$, respectively). No significant effect was observed on the percentage of closed arms exploration time or number of closed arm entries, indicating that no effect on locomotor activity on the EPM was observed (**Figure 4**).

### SOCIAL INVESTIGATION

Rats submitted to MD spent less time investigating the rat-containing cage than CON rats (main effect of MD ($F_{(1,30)} = 4.812$, $p < 0.04$)) (**Figure 5A**), whereas no significant difference was found on the exploration of the empty cage (**Figure 5B**). No significant saline injection ($F_{(1,30)} = 1.795$, $p = 0.190$) nor an interaction between these two factors ($F_{(1,30)} = 0.211$, $p = 0.648$) was observed.

## DISCUSSION

The present results confirmed previous findings that 24 h MD disinhibits basal and reactive adrenocortical activity to mild stressors, such as a saline injection (Levine et al., 1991; Suchecki et al., 1995; Faturi et al., 2010). The middle-term effects of MD included reduced ambulation in the center part of the open field

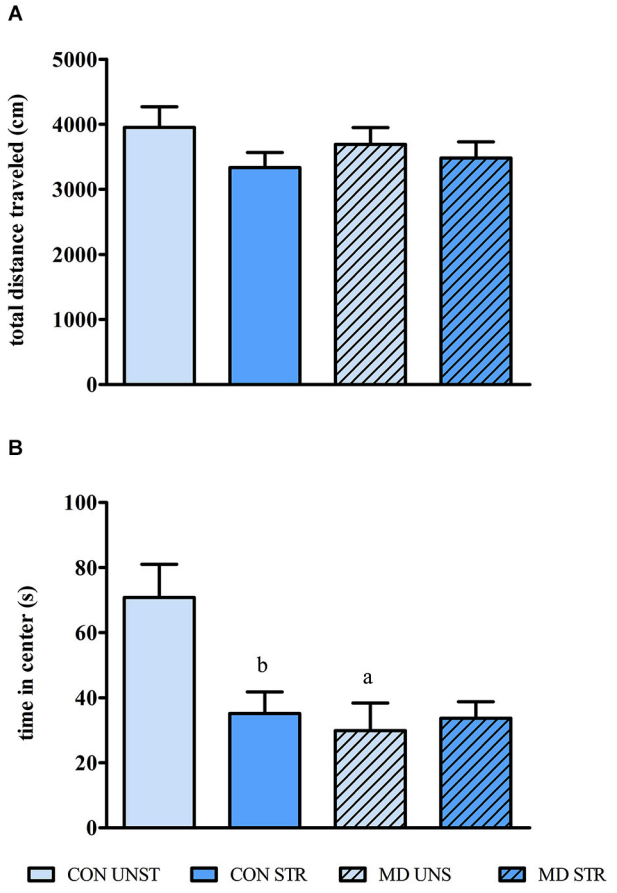


**FIGURE 3 | Mean ± S.E.M. total distance traveled (A) and time spent in the central region of the open field (B)**. CON = group not maternally deprived; MD maternal deprivation on postnatal day 9. UNS = no stress challenge; SAL = saline injection stress challenge. a = significantly different from respective CON; b = significantly different from respective UNS (*n* = 8–10/group).

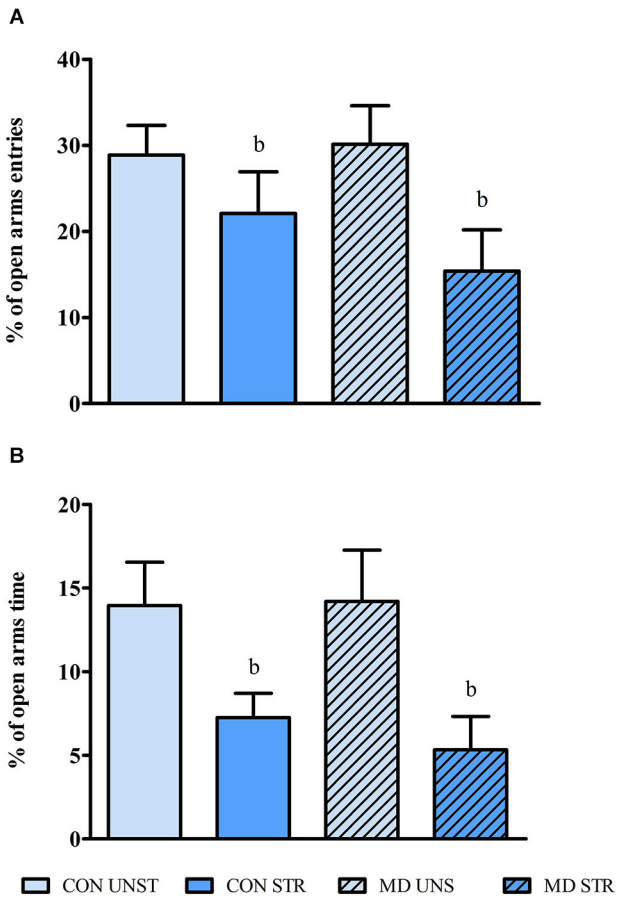


**FIGURE 4 | Mean ± S.E.M. percentage of open arms entries (A) and of open arms time (B) in the EPM test**. CON = group not maternally deprived; MD maternal deprivation on postnatal day 9. UNS = no stress challenge; SAL = saline injection stress challenge. b = significantly different from respective NSAL (*n* = 8–10/group).

and less social interaction than non-deprived rats. On the other hand, the saline injection on PND10 resulted in increased anxiety-like behavior and impairment in the sucrose negative contrast test (SNCT). Interestingly, there was no evidence of interaction between these two early events, inasmuch as MD STR neonates presented a four-fold increase in CORT levels after the saline injection compared to MD UNS rats, but behaviorally these groups exhibited similar alterations.

The open field test is useful to assess the locomotor activity and, in the case of animal models of schizophrenia, motor hyperactivity presents face (Powell et al., 2009) and construct validity (van den Buuse, 2010) to the positive symptoms, reflecting exaggerated release of dopamine in the mesolimbic pathway (Powell et al., 2009; Le Pen et al., 2011). This apparatus is also employed for the assessment of anxiety-like behavior, which is translated by reduced ambulation in the center portion of the arena (Miller et al., 2002), reduced vertical exploration, increased grooming and defecation (Denenberg, 1969; Masur et al., 1980). The results of the open field test indicated that MD was linked to an increased anxiety-like behavior, but surprisingly, a single saline injection in CON 10 day-old pups was also related to a similar behavioral alteration. These results are not in complete consonance with those observed in the EPM, a widely used apparatus to test anxiety-like behavior in rodents. In this test, the fewer the entries and the shorter the time spent in the open arms is interpreted as higher expression of anxiety-like behavior (Pellow et al., 1985). This test shows predictive validity for it is sensitive to anxiolytic drugs that act on $GABA_A$ receptors, such as benzodiazepines, increasing the visits and time spent in open arms (Pellow and File, 1986). We observed that saline injection on PND10, regardless of maternal deprivation-induced sensitized CORT response, led to greater avoidance of the open arms, being thus, considered an anxiogenic stimulus. This outcome suggests that increased anxiety during adolescence is independent of the magnitude of the CORT response to stress in infancy, but that saline injection may activate other systems, as has been shown with restraint stress-induced CRH and AVP mRNA levels in the paraventricular nucleus of the hypothalamus

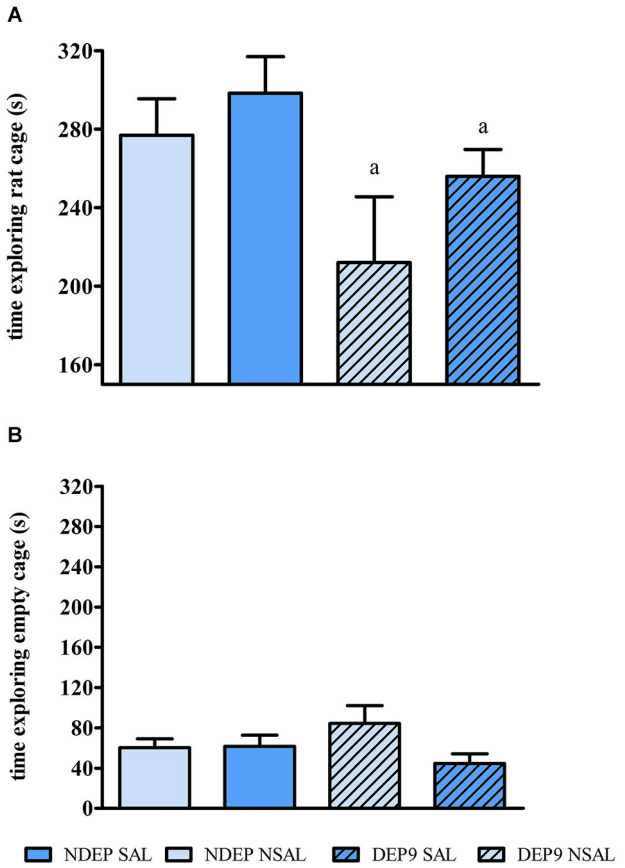


**FIGURE 5 | Mean ± S.E.M. time exploring the rat-containing cage (A) and the empty cage (B) on the social investigation test**. CON = group not maternally deprived; MD maternal deprivation on postnatal day 9. UNS = no stress challenge; SAL = saline injection stress challenge. a = significantly different from respective CON ($n$ = 8–10/group).

(Dent et al., 2000a,b) and of CRH in the septum (Vazquez et al., 2006) during the SHRP. Therefore, even in the absence of a pituitary-adrenal response to stress, the central component of the HPA axis is active during the neonatal period and does not seem to require a previous disinhibition. Regarding the effect of MD on anxiety-like behavior, the present results replicate previous studies, which have shown that adolescent rats submitted to MD at PND9 do not display increased anxiety-like behavior in the EPM (Marco et al., 2013). However, the fact that PND11 maternally-deprived adolescents display greater exploration of the center part of the open field (Suchecki et al., 2000) and a similar exploration of the open arms in the EPM than their non-deprived counterparts (unpublished data), strongly suggests an age-dependent effect of MD.

Suppression of reward seeking by chronic stress is a well-known phenomenon (Anisman and Zacharko, 1982). This characteristic behavioral change has been used as an index of anhedonia (Muscat and Willner, 1992), a feature that reflects the lack of motivation to engage in pleasurable activities, which represents one of the core symptoms of depression (Willner et al., 1992). The negative contrast sucrose test has been used to test motivational behavior and is based on the fact that reductions in expected incentives lead to behavioral adjustments characterized as depression effects (reduced motivation), that reflects anticipatory frustrative emotionality (Amsel, 1958). Therefore, when animals perceive the shift in the salience from a highly (15% sucrose solution) to a less rewarding stimulus (2.1% sucrose solution), there is a reduction in consummatory behavior, which represents a hedonic feature (Verma et al., 2010). It has being shown that adult male and female rats submitted to maternal separation present smaller contrast, i.e., variation of intake from the 15% to 2.1% sucrose solution, than their respective control rats (Matthews et al., 1996). The diminished responsiveness to changing reward salience of STR groups, irrespective of MD, could be interpreted as an analog of the blunted hedonic responsivity seen in human depression (Matthews et al., 1996). However, evaluation of predictive validity is required prior to further speculation. Alternatively, the results may be interpreted as (1) an inability of saline injected adolescents to attribute differential salience values to different concentrations of the sucrose solution, thus higher or lower concentrations would represent the same rewarding value; or (2) these animals attributed higher palatable value to the stimulus, likely reflecting an anxiety trait, in which rats maintain the sucrose intake to alleviate this affective state. This supposition is corroborated by findings that increased intake of sucrose reduces corticotropin releasing factor mRNA in the paraventricular nucleus of the hypothalamus, suggesting that carbohydrates can counterbalance augmented activity of the stress response (Dallman et al., 2003). Reduction of the HPA axis activity could be a mediator of the anxiolytic-like effect of sucrose intake, since rats genetically selected for high levels of anxiety exhibit exacerbated HPA axis stress response (Wigger et al., 2004). Conversely, it has been shown that benzodiazepine anxiolytic drugs induce faster recovery from the suppressed intake after the downward shift in sucrose concentration (Becker, 1986; Flaherty, 1990). However in the present study we did not see the expected downward shift in sucrose intake in STR rats.

Schizophrenia is a psychiatric disorder that emerges in stages, with a typical course of symptoms, beginning with nonspecific clinical features, including depression, anxiety, social isolation and school/occupational failure. The early phase, which manifests in pre-adolescence until young adulthood is also marked by comorbid anxiety disorders, including panic and social anxiety, although the comorbidity has been overlooked for many years (Pallanti et al., 2013). According to the DSM-V, diminished emotional expression, anhedonia, asociality and avolition are prominent negative symptoms of schizophrenia and mild forms of such altered behaviors may manifest early in life (for review, see Azorin et al., 2014; Foussias et al., 2014). These behavioral alterations are relatively feasible to model in animals, particularly social interaction tests that have been widely employed (Moser, 2014). Among the several animal models of schizophrenia-like behavior, based on the neurodevelopmental hypothesis (Fatemi and Folsom, 2009), there seems to be a general consensus of reduced social interaction in adolescent animals, represented by increased latency to begin and shorter time engaging in social contact (Shi et al., 2003), reduced active

interaction (Flagstad et al., 2004) and less time in contact (Blas-Valdivia et al., 2009), very much in consonance with the present results, in which a clear maternal deprivation-induced avoidance of social investigation of a naïve rat was observed. The fact that adult rats maternally deprived on PND9 exhibit a deficit of pre-pulse inhibition (Ellenbroek et al., 1998, 2005) prompted us to test these animals during adolescence, in order to explore if this paradigm could also serve to detect early signs that would predict a posterior emergence of schizophrenic-like traits. The present results pointed out to a broader spectrum of psychiatric dysfunction, ranging from a likely increase in anxiety-like traits, to reduced social interest, suggesting a lack of specificity as a schizophrenia model, while saline injection was linked to anxiety-like alterations, which can be viewed as a non-specific affective effect. On the other hand, it is still conceivable to propose the MD paradigm on PND9 as neurodevelopmental model of schizophrenia. MD was associated with impaired social interest, which is a valid approach for the negative symptoms of schizophrenia in rats (Moser, 2014) and this neurodevelopmental model is widely employed for investigating neurobehavioral alterations that resemble schizophrenic aspects in adult rats.

Taken together, our data support that the early emergence of MD-specific behavioral alterations can be regarded as a compelling tool to investigate brain changes and possible strategies of treatment or prevention in an earlier phase of schizophrenic-like features.

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# Interaction of genotype and environment: effect of strain and housing conditions on cognitive behavior in rodent models of schizophrenia

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Schizophrenia is associated with many genetic and environmental risk factors and there is growing evidence that the interactions between genetic and environmental "hits" are critical for disease onset. Animal models of schizophrenia have traditionally used specific strain and housing conditions to test potential risk factors. As the field moves towards testing gene (G) x environment (E) interactions the impact of these choices should be considered. Given the surge of research focused on cognitive deficits, we have examined studies of cognition in rodents from the perspective of GxE interactions, in which strain or housing manipulations have been varied. Behavior is clearly altered by these factors, yet few animal models of schizophrenia have investigated cognitive deficits using different strain and housing conditions. It is important to recognise the large variation in behavior observed when using different strain and housing combinations because GxE interactions may mask or exacerbate cognitive outcomes. Further consideration will improve our understanding of GxE interactions and the underlying neurobiology of cognitive impairments in neuropsychiatric disorders.

**Keywords: background strain, cognition, enriched environment, schizophrenia, animal model**

## INTRODUCTION

Schizophrenia is a complex group of disorders in which genetic vulnerability may lead to greater sensitivity to adverse environmental conditions (Bayer et al., 1999; van Os et al., 2008, 2010; Tost and Meyer-Lindenberg, 2012). Psychiatric epidemiology has provided clues about biologically plausible combinations of genetic and environmental risk factors for the neuroscience field to examine (Caspi and Moffitt, 2006; Meyer and Feldon, 2010). For example, being raised in an urban environment has repeatedly been linked to an increase in psychotic symptoms, however this risk is amplified in individuals with a genetic predisposition to psychosis (van Os et al., 2004; Krabbendam and van Os, 2005; Spauwen et al., 2006; Weiser et al., 2007). Unravelling the neurobiological changes that lead to vulnerable or resilient phenotypes may provide important information about how gene (G) x environment (E) interactions occur and provide clues for the research community. Rodents have been used to model biologically plausible risk factors and we are beginning to appreciate the complexity of GxE interactions on outcomes relevant to schizophrenia. With the recent focus on measuring cognitive deficits in rodent models (Jentsch, 2003; Kellendonk et al., 2009; Young et al., 2009; Keeler and Robbins, 2011; Bussey et al., 2012) and the known influence of strain and housing conditions on cognitive measures (Chapillon et al., 2002; Harker and Whishaw, 2002; Wolff et al., 2002; Pena et al., 2009; Simpson and Kelly, 2011), it is important to consider whether schizophrenia-related outcomes are dependent on the strain or housing conditions used.

Currently there is a lack of animal models of schizophrenia investigating these GxE interactions on cognitive outcomes. For example, a PubMed search using the terms "strain", "housing", "schizophrenia", "cognition" and "animal model" returned no results; substituting "strain" for "gene", and "housing" for "environment" or "enrichment" only returned seven research articles although none in which housing conditions were compared. Guidelines for cognitive testing in rodents have been established to improve the progression of novel drug treatments, and the use of animal models to examine GxE interactions on established cognitive tests are needed to bridge the translational gap. The next challenge is, therefore, to develop animal models to test the hypothesis that GxE interactions affect cognitive behavior in animal models of schizophrenia. This article focuses on the consequences of strain and housing conditions on cognitive outcomes in rodent models of schizophrenia and how these factors may be useful in modeling GxE interactions.

## MODELING THE COGNITIVE DEFICITS IN SCHIZOPHRENIA

Cognitive deficits are a core symptom group associated with schizophrenia and are the strongest predictor of functional patient outcomes (Green et al., 2000). While cognitive remediation techniques are beneficial, current drug treatments to improve cognitive deficits are largely ineffective and the failure to translate drug findings from animal models to clinical settings has impeded progress (Pratt et al., 2012). To guide future research an initiative of the NIMH was formed, Measurement and Treatment Research

to Improve Cognition in Schizophrenia (MATRICS), to make suggestions for the development of cognitive testing in animal models of schizophrenia (Green et al., 2004; Young et al., 2009). Based on the core cognitive deficits found in patients with schizophrenia seven cognitive domains were identified including working memory and attention/vigilance (Green et al., 2004). From these domains various clinical tests were selected by the follow-up group, Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia (CNTRICS), to be used in validating drug efficacy and to improve consistency between research groups (Carter and Barch, 2007). In order to bridge the translational gap, tests used in animal models have also been considered and selected for future use and development (Gilmour et al., 2012; Lustig et al., 2012). Domains such as verbal learning and memory cannot be translated to rodents, however processes such as attention, memory and executive control can be measured in a number of ways (Powell and Geyer, 2007) The tests selected for rodents that best reflect the cognitive constructs measured in patients include the five choice serial reaction time task (5C-SRTT) (Robbins, 2002) and sustained attention task (dSAT) for measuring attention (Lustig et al., 2012), the attentional set-shifting task (ASST) (Birrell and Brown, 2000) and reversal learning (Izquierdo and Jentsch, 2012) as measures of executive control and the radial arm maze (RAM) and delayed match to position (DMTP) task (Dudchenko, 2004), which provide the best assessment of working memory.

The CNTRICS panel reviewed the use of these tests in both rats and mice, however the selection of species and strain should be determined based on suitability for the experimental manipulation and the cognitive test being implemented (Young et al., 2009). The use of non-human primates may also be warranted where processes need to be defined differently for humans and rodents, such as in tests of working memory (Castner et al., 2004). For example, GxE interactions were examined on cognitive outcomes in an animal model of schizophrenia using catechol-o-methyl transferase (COMT) knockout mice and the 5C-SRTT (Papaleo et al., 2012). At baseline there was no effect of sex or genotype on cognitive performance, however by manipulating the inter-trial interval, measures of impulsivity were found to differ by sex and genotype. After a mild stressor, males had impaired performance in terms of accuracy and impulsivity measures and this was particularly so for males with reduced (+/−) and absent (−/−) COMT. Other measures were found to differ based on sex and genotype only after reducing motivation. This study illustrates that phenotypes based on sex or genotype may not be readily apparent, however, differences were revealed after manipulating environmental conditions. These findings are in agreement with the suggestion that genes alone do not lead to schizophrenia, but they may predispose an individual to greater vulnerability following exposure to certain environmental insults or "hits".

## ENVIRONMENTAL CONDITIONS AND COGNITION

Epidemiological evidence for the role of environmental factors suggests that housing environment may be an important factor for modeling schizophrenia in rodents (Mcdonald and Murray, 2000). Housing conditions can have a significant influence on rodent behavior and have been used to induce stress or anxiety and to alter cognitive development (van Praag et al., 2000; Nithianantharajah and Hannan, 2006; Burrows et al., 2011; Simpson and Kelly, 2011). Environmental enrichment has been incorporated to enhance sensory and motor experience through the inclusion of novel objects, expanded caging and larger social groups (van Praag et al., 2000). Environmental enrichment has been linked to a number of brain-related outcomes, such as increased brain weight, increased branching and synapse formation in the cortex, increased expression of brain-derived neurotrophic factor (BDNF), glial-cell-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) and increased acetylcholine levels (see review by van Praag et al., 2000). Many of these factors are likely to affect cognitive functioning, for example NGF and BDNF are both known to play a role in learning, while acetylcholine levels have been shown to correlate with attentional performance in rodents (St Peters et al., 2011). Housing conditions have been difficult to standardise across research groups, particularly when enrichment is used. Rather than viewing this noise as a nuisance, it could be seen as an opportunity to investigate how environmental conditions interact with proposed risk factors (Toth et al., 2011).

Rodents reared in more stimulating conditions often acquire tasks after fewer trials (Park et al., 1992), have reduced age-related deficits (Soffie et al., 1999; Harati et al., 2011) and recover from injury faster (Hicks et al., 2002; see Pena et al., 2009). This may indicate phenotypes are being rescued in enriched environments or that deficits only develop in a deprived environment. In some cases, such as animal models of depression, standard housing may be largely contributing to the phenotype, possibly by reducing an animal's compensatory ability to deal with additional challenges (Brenes et al., 2009). The brain may require stimulation beyond that provided in standard housing to develop sufficient connectivity and functionality to detect higher order cognitive deficits. Whether enrichment should be considered as a therapeutic intervention or the standard conditions required for developing a "normal" brain continues to be debated (Wurbel, 2001).

## GENETIC BACKGROUND AND COGNITIVE PERFORMANCE

Mutant mouse models have been used to investigate other key candidate genes linked to schizophrenia (Chen et al., 2006). Despite the availability of tasks for cognitive testing in rodents, a recent review by Arguello and Gogos (2010) did not report any mutant mouse models in which the "top 30" genes linked to schizophrenia had been tested on an attentional paradigm. Considering the need to investigate cognitive symptoms in animal models, there is an obvious gap that needs to be addressed. The genetic risk for schizophrenia is likely to be the result of hundreds or even thousands of genes of small effect (Wray and Visscher, 2010). Systematically testing each individual mutation is unlikely to replicate the disorder, nor is this approach feasible. However, specific genetic mutants may be useful for identifying the origin of cognitive endophenotypes of schizophrenia (see review by Kellendonk et al., 2009). While using single gene mutants provides information about a particular gene of interest (Papaleo et al., 2012), the polygenic nature of schizophrenia may be better modeled by comparing different strains.

Strain-dependent changes in behavior have been observed on many cognitive tasks and in response to drugs; but these changes are also dependent on the manipulation applied (Andrews et al., 1995; Schmitt and Hiemke, 1998; Mirza and Bright, 2001; Harker and Whishaw, 2002; Wahlsten et al., 2003; Zamudio et al., 2005; Higgins et al., 2007). For example, a widely used task in animal models of schizophrenia, pre-pulse inhibition (PPI) of the acoustic startle response, is a well validated test of sensorimotor gating but results are known to vary depending on the background strain (Rigdon, 1990; Glowa and Hansen, 1994; Varty and Higgins, 1994; Varty et al., 1999; Swerdlow et al., 2001; van den Buuse, 2003). Given the variability on this pre-attentive task, it is not surprising that strain differences have also been reported using more sophisticated cognitive tasks, such as the 5C-SRTT (Didriksen and Christensen, 1993; Mirza and Bright, 2001; Higgins et al., 2007; Auclair et al., 2009). These studies also demonstrate the variability between studies using the same strain, which may be due to variation in the protocol used or the source of the strain (Andrews, 1996; Karl et al., 2011).

Rat models of schizophrenia have been developed predominantly using two albino strains, however the reasons for these selections are not always obvious. Furthermore, studies of schizophrenia-related manipulations comparing rat strains are lacking. The neonatal ventral hippocampal lesion model was compared in the outbred Sprague-Dawley (SD) and two inbred strains, Lewis and Fischer 344, which differed in stress responsivity (Lipska and Weinberger, 1995). For example, SD and Lewis rats show habituation of the hypothalamic-pituitary-adrenal axis (HPA) response to a repeated restraint stress paradigm, whereas F344 rats do not habituate within or between stress-inducing sessions (Dhabhar et al., 1997). As predicted the hyper-responsive F344 strain showed greater behavioral vulnerability to the neonatal lesion, while the hypo-responsive Lewis rats showed greater resistance when both were compared to the SD strain. Thus, stress responsivity is a critical consideration both for models utilising stressful manipulations and for the interpretation of behavioral results from different strains (Faraday, 2002). Spontaneous and amphetamine-induced hyper locomotion varied across development with strain, indicating genetic predisposition has a critical role in determining the phenotype derived from this neurodevelopmental model, although cognitive outcomes were not assessed in this study (Lipska and Weinberger, 1995).

## GENE X ENVIRONMENT INTERACTIONS AND COGNITIVE ENDOPHENOTYPES

The focus of GxE interaction studies in animal models of schizophrenia has taken advantage of the genetic tools available in mice, comparing mutant and control animals after adverse environmental exposures such as immune activation, stress or drug administration (Kannan et al., 2013). The influence of enriched housing conditions on rodent models of schizophrenia has been addressed by only a few studies (Karl et al., 2007; McOmish et al., 2008; Ishihama et al., 2010). However, the neurological and behavioral effects of environmental enrichment have been assessed in a range of other animal models including Huntington's disease, Alzheimer's disease, Parkinson's disease, Epilepsy and drug addiction (Bezard et al., 2003; see review by Nithianantharajah and Hannan, 2006; Laviola et al., 2008). For example, the influence of environmental enrichment has been shown using the transgenic mouse model of Huntington's disease (van Dellen et al., 2000). This neurodegenerative condition has a genetic cause, yet mice housed in enriched cages show delayed onset and progression of both the motor and cognitive deficits compared to standard housed controls (Hockly et al., 2002; Nithianantharajah and Hannan, 2006; Pang et al., 2006). Using animal models of schizophrenia, it will not only be important to address the detrimental effects of the environment, but also conditions that have a protective influence (Takuma et al., 2011; Pang and Hannan, 2013).

With the aim of developing biologically-relevant animal models of schizophrenia, studies using a GxE approach are rapidly emerging (Millstein et al., 2006; Millstein and Holmes, 2007; Oliver and Davies, 2009; Desbonnet et al., 2012; Hida et al., 2013; Petrovszki et al., 2013). Prenatal stress followed by acute stress during adulthood was used in three rat strains to examine how genetic background interacted with adverse environmental conditions to alter hippocampal gene expression (Neeley et al., 2011b). Five relevant genes (*Nr3c1, Chrna7, Grin2b, Bdnf, Tnfα*) were found to be altered by either strain or stress treatments, however changes were inconsistent across strains indicating a modulatory role of genotype. A second experiment comparing these strains using a stress protocol found that changes in *Bdnf* expression and associated pathways were also strain dependent (Neeley et al., 2011a). These studies demonstrate the importance of strain selection and genetic diversity in understanding GxE interactions.

In another recent study, rats were exposed to two commonly used risk factors, post weaning social isolation and chronic ketamine treatment, and selectively bred based on behavioral deficits relevant to schizophrenia to produce a vulnerable sub-strain (Petrovszki et al., 2013). After 15 generations, four groups were compared on three behavioral tests and the results were accumulated into an overall score. Rats with a standard genetic background raised under standard conditions were used as a control group. The environment-only group consisted of genetically-naïve rats that were then isolated and treated with ketamine. Rats from the selectively bred vulnerable sub-strain that were raised under standard conditions were used as the genetic-only group. And finally rats from the vulnerable sub-strain that also underwent social isolation and chronic ketamine treatment were used to investigate the GxE interaction. The GxE group scored the highest on schizophrenia-relevant deficits and the control group scored the lowest, indicating that both genetic and environmental insults were important. The behavioral tests used assess nociception, sensorimotor gating and recognition memory, which do not address the key cognitive domains identified by CNTRICS and therefore further work would be required to understand the influence of these manipulations on cognitive deficits relevant to schizophrenia. Nevertheless, this study does present a new way of investigating previously tested risk factors.

## FUTURE RECOMMENDATIONS

A recent review of mouse models of GxE interactions relevant to schizophrenia has discussed a comprehensive list of weaknesses to be addressed by future studies (Kannan et al., 2013). The

authors suggested standardising strain and housing conditions to reduce variability between studies. However, genetic and environmental choices clearly alter outcomes relevant to schizophrenia and phenotypes may only be detected under specific strain or housing conditions. Furthermore, the way genetic and environmental conditions interact to protect or exacerbate phenotypes is of key importance in understanding the pathways that lead to schizophrenia.

Investigating genetic changes, such as mutant mouse models, may be easily replicated across laboratories, however environmental manipulations are more difficult to standardise. For example, wild type mice show different behavioral phenotypes when tested under similar conditions but at different laboratories (Crabbe et al., 1999). More recently, heterozygous neuregulin mutant mice showed different behavioral phenotypes when tested in different laboratories, despite being on the same genetic background (Karl et al., 2011). Although these differences may be unavoidable, it is recommended the housing conditions of rodents be clearly stated in research methods. Unfortunately many articles do not list the forms of enrichment used (such as type of bedding, shelters, wood chews and tubes) however these should be indicated even if considered to represent "standard" housing conditions. Recommending a standardised enrichment protocol would reduce variability between experiments, but would also limit the scope of enrichment studies (Wurbel, 2002). Protocol design should take into consideration the species-specific relevance of environmental changes, the timing and duration of exposure, the ethical implications and the reproducibility of the chosen design. Therefore, optimal enrichment conditions should be selected based on experimental aims.

Future studies could take a number of directions, including the use of GxG and ExE studies to identify the influence of genetic and environmental factors; as well as understanding the mechanisms that lead to increased vulnerability (Giovanoli et al., 2013). To more fully assess the effects of GxE interactions on cognitive endophenotypes, the field also needs to improve the range of the behavioral tasks available. The potential therapeutic benefit of improved animal models may be limited by the sensitivity of the behavioral measures employed. Incorporating GxE clues from epidemiology into our animal models, and improving assessment techniques will advance our understanding of schizophrenia.

## CONCLUSION

There is clear evidence to show that genetic and environmental conditions alter cognitive outcomes in rodents. However, the lack of studies comparing cognitive deficits in rodent models of schizophrenia using different strain and housing conditions is surprising. Schizophrenia develops from the complex interaction of GxE and we need to incorporate this complexity into animal models to understand the etiology of schizophrenia. Although it is difficult to recapitulate complex disorders, such as schizophrenia, in a rodent model, the use of endophenotypes in carefully controlled experiments may allow us to better understand some of the mechanisms behind GxE interactions. Current animal models are falling short of replicating the complex suite of risk factors implicated in schizophrenia and using different strains or housing conditions may provide an accessible stepping stone towards understanding altered brain development. Given the infancy of GxE interaction research in animal models of schizophrenia, manipulating these factors in existing and novel animal models will be informative in terms of GxE interactions. GxE interaction models will be particularly informative for understanding the role of vulnerable and resilient phenotypes in determining the influence of secondary "hits" on cognitive outcomes in schizophrenia.

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# Decanalization mediating gene-environment interactions in schizophrenia and other psychiatric disorders with neurodevelopmental etiology

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## OVERVIEW

Schizophrenia provides a striking example of a disorder in which complex genetic and environmental factors combine to produce abnormal brain development and function. In order to fully understand the disorder, and develop more effective and targeted treatments, more accurate and sophisticated animal models are required, which incorporate genetic and environmental variables and their associated gene-environment interactions. We discuss key considerations in modeling gene-environment interactions, with a focus on the recent proposal that schizophrenia involves "decanalization," whereby "experience-expectant" brain development can have its trajectory derailed when particular genotypes (and associated cryptic genetic variants) are exposed to "unexpected" environmental conditions. This has broader implications for the modeling of schizophrenia and other brain disorders involving neurodevelopmental etiology, including autism spectrum disorders (ASDs). We propose that it is insufficient to examine animal models expressing particular genetic variants or mutations only in the single environmental context of "standard" housing conditions. The exploration of disease-associated polymorphisms and mutations under housing conditions in which environmental factors of clinical relevance are systematically manipulated will facilitate the testing of specific hypotheses associated with pathogenic gene-environment interactions and decanalized development.

## INTRODUCTION

Over the past several decades, significant advances in genetics and neuroscience have transformed our understanding of how the brain produces adaptive behavior and the ways in which normal functioning becomes disrupted in neurodevelopmental disorders, such as schizophrenia and ASD. Nevertheless, we have only begun to comprehend how particular combinations of genomes and environmental histories combine to produce a given set of clinical symptoms.

A major challenge for translating these findings to specific, effective treatments has been the heterogeneity that exists both in clinical presentation and in the genetic associations that have been uncovered. Both schizophrenia and ASD exhibit high heritability and significant research efforts have been geared toward uncovering genetic variation in a bid to explain cause. Genome-wide association studies (GWAS) have identified hundreds of common variants associated with complex diseases, however, the overall genetic risk explained by these loci remains modest (Manolio et al., 2009; Eichler et al., 2010). There is evidence for both common genetic variation and rare DNA sequence variants contributing to genetic susceptibility in both disorders (State and Levitt, 2011; Mowry and Gratten, 2013). This contribution of common and rare alleles is thought to be variable among cases, making it difficult to reliably detect an association signal among the heterogeneity and genetic noise. Adding further complexity, the genetic architecture may include epistatic (gene-gene) effects among interacting loci (Phillips, 2008). Even with sophisticated approaches to resolve gene–gene interactions acting within whole genome contexts, we are still faced with the conundrum of "missing heritability" (Manolio et al., 2009; Hannan, 2010; McGrath et al., 2011).

The problem of missing heritability may be at least partly addressed by improving the discovery rate of genetic variants via statistically well-powered cohorts of individuals that are better characterized for disease phenotypes, genetic background, and environmental exposure (Manolio et al., 2009; Eichler et al., 2010). This approach is dependent on the idea that a combination of common variants of small effect, rare variants of large effect and environmental factors will lead to disease (Manolio et al., 2009; Eichler et al., 2010; Gibson, 2011). A focus on genetic risk factors alone has limited heuristic value due to the interdependent interactions between genetic and environmental factors that play key roles in pathogenesis. An alternative view suggests that gene-environment (G × E) interactions can account for a substantial proportion of disease risk (Svrakic et al., 2013). There is strong evidence that G × E interactions are ubiquitous, accounting for the greater part of phenotypic variation seen

**Abbreviations**: ASD, autism spectrum disorder; G × E, gene environment; GWAS, Genome-wide association studies; PLC-β1, phospholipase C-β1

across genotypes (Grishkevich and Yanai, 2013). Research utilizing model organisms has identified that not all genes are equally likely to exhibit G × E interactions; promoter architecture, expression level, regulatory complexity, and essentiality correlate with the differential regulation of a gene by the environment (Grishkevich et al., 2012). In fact, genes that exhibit G × E interactions may confer evolutionary advantage in that they facilitate phenotypic plasticity and provide an organism with the flexibility to adjust its phenotype with respect to the specific environmental conditions experienced (Via et al., 1995; Lande, 2009). This plasticity could represent a substantial advantage in unpredictable environments but could also, when combined with genetic susceptibility, underlie disruptions in normal brain function.

## DECANALIZATION AS A DEVELOPMENTAL MANIFESTATION OF GENE-ENVIRONMENT INTERACTIONS

Humans are far from being at genetic equilibrium, owing to marked changes in population size, admixture, and environment in the past few thousand years. Principle among these environmental changes are dietary shifts, modern hygiene, altered pathogen exposure, urban living, stress, and departure from natural circadian rhythm through artificial lighting. These, and other, environmental perturbations may alter the genetic contributions to phenotype by revealing cryptic genetic variation, especially among individuals with existing genetic vulnerability (Gibson, 2009; McGrath et al., 2011).

The capacity of a population to produce the same phenotype, regardless of variability in environment or genotype has been termed canalization (Waddington, 1942). Canalization is proposed to have arisen from millions of generations of stabilizing genetic selection, thus ensuring that crucial mammalian physiological mechanisms, for example, glucose metabolism, immune function, and cognitive performance remain at an optimum (Gibson, 2009). When an organism moves out of the adaptive niche, the capacity to buffer the developmental trajectory can be compromised. Decanalization, a particular class of gene-environment interaction, has been proposed as a conceptual framework for understanding how complex genetic disorders, including schizophrenia, arise (Gibson, 2009; McGrath et al., 2011). Decanalization allows cryptic genetic variation to be expressed. These mutations or polymorphisms generally don't contribute to the normal range of phenotypes observed in a population, but are thought to have a role in modifying a phenotype that arises after environmental change or the introduction of novel alleles (Gibson and Dworkin, 2004).

Human brain development may be particularly susceptible to decanalization due to rapid evolution of brain structure and function in the relatively recent *Homo sapiens* lineage (McGrath et al., 2011). Both innate genetic information and experience-dependent neural activity play critical roles in brain development. The "experience expectant" brain extracts information from the environment and uses this information to dynamically shape and refine synaptic connections. This has been clearly demonstrated by pioneering experiments showing the fundamental role of sensory input during critical periods of development for appropriate formation of connections in the visual cortex (Hubel and Wiesel, 1970). Indeed, brain development is characterized by a series of sequential gene-environment interactions; critical periods of exposure to the environment are necessary for the appropriate cortical connections to form and genetically influenced traits to be expressed. There is a consensus of evidence to show that environmental stress specifically during these critical periods of brain development in combination with a genetic predisposition increases the vulnerability to developing schizophrenia. Decades of epidemiological studies have highlighted the importance of environmental contributions to the development of schizophrenia. Several risk factors have been identified during pregnancy including season of birth, vitamin D deficiency, urbanicity or population density, and maternal viral infections (Cannon and Clarke, 2005; Brown, 2006; Patterson, 2007; McGrath et al., 2010). In addition, during adolescence, stress and cannabis abuse have been identified to negatively impact on an individual's risk of developing schizophrenia (van Os et al., 2002; Henquet et al., 2008; van Winkel et al., 2008). Animal models utilizing environmental manipulations that parallel the results of epidemiological studies in psychiatry have been critical to advancing our understanding of how the environment can modify brain function and thus the biology of psychiatric conditions. However, it is animal models incorporating gene–environment interactions that more accurately mimic etiologic factors and help to elucidate underlying pathogenic mechanisms (Gray and Hannan, 2007; Burrows et al., 2011; Kannan et al., 2013). Recent work has unmasked new phenotypes in mouse models expressing disease-associated gene mutations exposed to adverse environments, including toxins, maternal infection, stress, and drug exposure (Connor et al., 2012; Desbonnet et al., 2012; Haque et al., 2012; Abazyan et al., 2013; Karl and Arnold, 2013). Exposure to these adverse environments may lead to G × E interactions that divert brain development away from its normally canalized developmental trajectory (McGrath et al., 2011). Conversely, protective environmental factors (such as cognitive stimulation, physical exercise and a healthy diet) can induce neuroprotection and functional compensation (Nithianantharajah and Hannan, 2009). Environmental enrichment has been demonstrated to exert a range of beneficial effects on both wild-type laboratory rodents as well as animal models of brain disorders (Nithianantharajah and Hannan, 2006; Sale et al., 2009). Recent evidence suggests that an enriched environment can ameliorate behavioral traits resembling schizophrenia symptoms in genetic mouse models (McOmish et al., 2007; Harper et al., 2012), although the exact nature of the behavioral changes depends upon the gene mutations and environmental manipulations involved (Karl et al., 2007; Karl, 2013; Turner and Burne, 2013). An alternative interpretation to environmentally driven improvements is that standard housing conditions (which generally provide minimal opportunities for cognitive stimulation and physical exercise) may be actually modeling adverse environment and could interact with genetic factors to produce misleading phenotypes of reduced translational potential. Consistent with this concept, the endophenotypes

modeling schizophrenia in phospholipase C-β1 (PLC-β1) knockout mice (McOmish et al., 2007) were most pronounced under standard housing (i.e., conditions of increased sensorimotor deprivation), consistent with the null mutation leading to decanalized brain maturation within this environmental context. Specific behavioral abnormalities were found to be absent when these PLC-β1 knockout mice were raised under enriched conditions.

This conceptual framework also has implications for the way in which preclinical trials in animal models are designed and implemented. We have recently proposed that environmentally enriched conditions could be used as a "secondary screen" for those preclinical studies initially conducted under standard housing conditions (Burrows et al., 2011).

## NEW DIRECTIONS IN MODELING G × E INTERACTIONS IN NEUROPSYCHIATRIC DISORDERS

An additional implication of this theoretical framework is that brain development may be canalized in appropriately stimulating enriched environments, but may become decanalized under conditions of sensorimotor deprivation or environmental stress. Thus, the analysis of animal models in a single "standard" housing environment has limited heuristic value (Richter et al., 2010). Animal models exploring multiple genetic and environmental forces that modify the development of pathological traits may inform mechanisms mediating decanalization and associated gene-environment interactions. Improving the validity and accuracy of animal models, including G × E interactions of clinical relevance, will increase the capacity for translation and development of new treatments (Burrows and Hannan, 2013).

The potential importance of decanalization as a mechanism mediating G × E interactions in schizophrenia is supported both by epidemiological evidence and animal models (McGrath et al., 2011). However, it would be expected that decanalization also contributes to the etiology of other complex polygenic disorders involving neurodevelopmental abnormalities.

ASDs involve abnormal brain development and contributions from both environmental and genetic factors. Furthermore, there is evidence of overlapping genetic etiologies amongst neurodevelopmental disorders such as schizophrenia and ASD (Cristino et al., 2013; Smoller et al., 2013). It is clear that the majority of ASD cases (with the exception of monogenic disorders such as fragile X syndrome) result from complex and heterogeneous genetic etiologies (Geschwind, 2011). A variety of genetic mutations and polymorphisms have been implicated in the causation of ASD, however, they are insufficient to explain key clinical and epidemiological features. This suggests that early environmental exposures also contribute. Pregnancy complications, perinatal exposure to air pollutants, maternal infection and advanced paternal age among others have all been associated with increased risk of ASD (Garbett et al., 2008; Hultman et al., 2011; Lyall et al., 2012; Roberts et al., 2013). ASD are therefore another group of major neurodevelopmental disorders that could involve decanalization. ASD can be diagnosed within the first few years of postnatal life, therefore environmental exposures *in utero* or in mothers prior to conception could be particularly important in precipitating such decanalized brain development.

Applying the decanalization framework to clinical and preclinical research may help to identify clusters of neuropsychiatric disorders that emerge from shared decanalization events. This approach has been partially utilized in the human genetics field with a recent genome-wide analysis leading to the identification of risk loci with shared effects on five major psychiatric disorders (Smoller et al., 2013). If these psychiatric disorders share early decanalization events then the current approach based on the premise that diagnoses represent distinct diseases will not differentiate the signal from the noise. Many studies currently taking this reductionist view, separating complex genetic factors from environmental exposure may in fact be missing the primary cause of these complex disorders.

A major limitation in applying this framework is the feasibility of incorporating the staggering complexity that underlies psychiatric disorders into research designs. Furthermore, simple models are not likely to be sufficient to unravel this complexity. There has been a realization that disease entities that appear to be a single disorder actually have separate developmental trajectories arising from distinct genetic vectors that influence the epigenetic landscape (McGrath et al., 2011). Recently, the director of the National Institute of Mental Health (NIMH, US), Tom Insel, and colleagues have proposed an alternative approach to address complexity in psychiatric disorders by developing a research classification system to reflect ongoing advances in genetics, neuroscience and cognitive science (Cuthbert and Insel, 2013). The Research Domain Criteria (RDoC) project, aims to support research that moves beyond descriptive syndromes in psychiatry, and toward a nosology informed by disease cause. For example, clinical trials might study all patients in a mood clinic rather than those meeting strict major depressive disorder criteria. There is scope to include gene-environment interactions in future research designs, which may provide further insight into biological mechanisms underlying psychiatric disorders.

## CONCLUSIONS

Understanding how evolved genetic programs and biological systems are dynamically sculpted by G × E interactions is the next frontier in the analysis of complex traits and in understanding the origin of neurodevelopmental disorders such as schizophrenia and ASD. In the past, G × E interactions driving variation in complex traits have been regarded by some as a nuisance, leading to difficulty in replicating results across cohorts and to the rejection of interesting genetic effects that are significant in specific environments but exhibit diminishing significance when averaged across constellations of differing environments. The realization that G × E interactions via decanalization may be integral to the development of major disorders such as schizophrenia will motivate research aimed at elucidating mechanisms as well as identifying and modifying key environmental risk factors. Furthermore, incorporation of G × E interactions into our models of neuropsychiatric disorders will provide us with the powerful tools to understand how the decanalized brain produces suboptimal phenotypes

and to develop more effective therapeutic approaches.

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# Gene-sex interactions in schizophrenia: focus on dopamine neurotransmission

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Schizophrenia is a severe mental disorder, with a highly complex and heterogenous clinical presentation. Our current perspectives posit that the pathogenic mechanisms of this illness lie in complex arrays of gene × environment interactions. Furthermore, several findings indicate that males have a higher susceptibility for schizophrenia, with earlier age of onset and overall poorer clinical prognosis. Based on these premises, several authors have recently begun exploring the possibility that the greater schizophrenia vulnerability in males may reflect specific gene × sex (G×S) interactions. Our knowledge on such G×S interactions in schizophrenia is still rudimentary; nevertheless, the bulk of preclinical evidence suggests that the molecular mechanisms for such interactions are likely contributed by the neurobiological effects of sex steroids on dopamine (DA) neurotransmission. Accordingly, several recent studies suggest a gender-specific association of certain DAergic genes with schizophrenia. These G×S interactions have been particularly documented for catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), the main enzymes catalyzing DA metabolism. In the present review, we will outline the current evidence on the interactions of DA-related genes and sex-related factors, and discuss the potential molecular substrates that may mediate their cooperative actions in schizophrenia pathogenesis.

**Keywords: schizophrenia, dopamine, catecholamine-O-methyltransferase (COMT), monoamine oxidase (MAO), gene-sex interactions, sex hormones**

## INTRODUCTION

Schizophrenia is a chronic and severe neurodevelopmental disorder, characterized by a highly complex and heterogeneous set of perceptual, cognitive and emotional deficits (Breier, 1999; Rowley et al., 2001). According to the current diagnostic criteria, the pathognomonic manifestations in schizophrenia are clustered into three groups of symptoms: (1) *positive symptoms*, which encompass hallucinations and delusions; (2) *negative symptoms*, including flat affect, alogia, anhedonia and social deficits; and (3) *cognitive symptoms*, which reflect impairments of attention, memory, perception and thought. Converging evidence has revealed that the primary deficits in schizophrenia are likely mediated by dopamine (DA), in cooperation with other key neurotransmitters, such as glutamate, γ-aminobutyric acid (GABA) and serotonin. Nevertheless, the quest to understand the pathogenic mechanisms of schizophrenia has not yet led to a conclusive theory, and its pathophysiology remains frustratingly elusive.

A wealth of genetic data has identified a number of vulnerability factors that are not inherently pathological, but predispose an individual to develop schizophrenia in the presence of critical environment determinants. These findings have prompted a shift in the conceptual framework of schizophrenia, and underscored the importance of gene-environment (G×E) interactions in this disease (Van Os and Murray, 2008; Van Os et al., 2008, 2010; Van Os and Rutten, 2009).

Multiple lines of evidence have also highlighted that sex-related factors play a potentially important role in shaping the clinical trajectory of schizophrenia. Indeed, males have a higher risk for schizophrenia than females, with earlier age of onset and greater severity of negative and cognitive symptoms (Markham, 2012). Based on these premises, it is possible to theorize the existence of specific gene × sex (G×S) interactions that may also contribute to schizophrenia pathogenesis.

Numerous preclinical studies support that the DAergic system is one of the key mediators of sex differences in schizophrenia (Bay-Richter et al., 2009; Arime et al., 2012; for a detailed presentation of this issue, see Sanchez et al., 2010); accordingly, genetic investigations point to a clear involvement of the key metabolic enzymes of DA, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), in the underpinnings of G×S interactions in schizophrenia. In the present review, we will discuss how the emerging evidence on the genes encoding these enzymes and their interactions with sex-related factors may provide fundamental clues to unravel the essence of the biological bases of schizophrenia.

## THE ROLE OF DA IN THE PATHOPHYSIOLOGY OF SCHIZOPHRENIA

The role of dopamine in the pathogenesis of schizophrenia was originally postulated following the discovery that $D_2$ dopamine receptor antagonism was a fundamental pharmacological requisite of antipsychotic drugs, and that the therapeutic efficacy of these agents was correlated with their inhibitory potency (Seeman and Lee, 1975; Creese et al., 1976). While several studies support

the concept that stimulation of $D_2$ receptors in subcortical areas (and particularly striatum and nucleus accumbens) results in psychotic manifestations, other lines of evidence strongly suggest that negative and cognitive symptoms (which are generally not affected by $D_2$ receptor antagonists) may be underpinned by the insufficient activation of $D_1$-like receptors in the prefrontal cortex (PFC) (Goldman-Rakic and Selemon, 1997). These findings have led to the view that schizophrenia may be underpinned by mesolimbic hyperactivity and mesocortical hypoactivity (Weinberger, 1987; Davis et al., 1991). Although studies in the last two decades have documented the fundamental roles of other neurotransmitters in schizophrenia, particularly glutamate and GABA (Benes and Berretta, 2001; Tsai and Coyle, 2002), the DAergic hypothesis still affords the best-validated theoretical framework for this disorder. Recent imaging and post-mortem studies have lead to a refinement of this hypothesis, indicating that the dysregulations of DA neurotransmission in cortex and elevations in presynaptic DA content in the striatum may be the main biological signatures of psychotic disorders (Howes and Kapur, 2009; Fusar-Poli et al., 2011; Howes et al., 2011, 2012, 2013; Allen et al., 2012; Egerton et al., 2013; Stokes et al., 2013; Lataster et al., 2014; see Kuepper et al., 2012 and Smieskova et al., 2013 for more thorough reviews on dopaminergic dysfunctions in brain imaging studies in schizophrenia). The increase in presynaptic striatal DA may disrupt informational salience and help contribute to other schizophrenia symptoms (Rosier et al., 2013; Winton-Brown et al., 2014).

The bulk of the evidence suggests that the DAergic deficits in schizophrenia are underpinned by functional, rather than constitutive, abnormalities. Indeed, the majority of studies on post-mortem tissues have failed to identify consistent alterations in the expression of DAergic targets (Harrison, 2000). Accordingly, multiple large-scale genetic analyses have found no robust association for DAergic genes and schizophrenia (Hoogendoorn et al., 2005; Alvarez et al., 2010), and instead point to a predominant involvement of glutamatergic targets (Collier and Li, 2003). In contrast with this evidence, the notion of functional dysregulation of DAergic circuits in schizophrenia is strongly supported by neuroimaging findings, which point to multiple patterns of dysconnectivity between intracortical and subcortical networks (Laruelle, 2003). These dynamic alterations of DAergic neurotransmission are thought to play a key role in the adaptive and neurodevelopmental processes of this system, which are particularly active throughout childhood and adolescence (Teicher et al., 1995; Spear, 2000). These developmental periods may be especially critical for the interactions of DAergic genes with environmental and sex-related vulnerability factors in schizophrenia. In fact, preclinical experiments have shown that sex hormones have a profound influence on the development of the DAergic system throughout early developmental stages (Anderson et al., 2005).

To establish a conceptual framework for the role of DA in schizophrenia, it is necessary to consider that one of the fundamental functions of this system is the extraction of salient information from the environment, through the stimulation of output neurons of cortical and striatal regions integrated within cortico-striato-thalamo-cortical (CSTC) circuits. In particular, the role of mesocorticolimbic DAergic neurons is consistently influenced by the action of glutamatergic and GABAergic cells, which surround and interface with the somata in the ventral tegmental area, as well as the axons and presynaptic boutons in the efferent areas (nucleus accumbens, striatum and PFC). In line with the role of DAergic pathways as neural mediators of informational salience, both the adaptive plasticity and modalities of neurotransmitter release by these neurons are finely regulated by multiple factors; changes in these variables, particularly if occurring during developmental periods, may therefore have long-standing implications on the integrity and coherence of the perceptual process. The modulatory role of DA on processing informational salience is extremely critical during adolescent stages, in which the DAergic system alters cortical innervation and undergoes synaptic maturation and pruning of its glutamatergic and GABAergic connections (Andersen, 2003; O'Donnell, 2010; Burke and Miczek, 2013; Penzes et al., 2013).

The natural corollary of these premises is that the DAergic system may be directly involved in G×S interactions during postnatal development, while prenatal and inborn elements of predisposition may be more directly related to the glutamatergic system (which in turn governs DAergic function through direct and indirect dynamic interactions). This idea is in line with the "multiple hits" hypothesis for the pathogenesis of schizophrenia, which postulates that the disorder may result from the progressive accumulation of deficits from prenatal to juvenile stages, due to different, yet concurring, causes. In the next sections, we will present an overview of the role of sex hormones on schizophrenia, followed by a detailed discussion on the available evidence on the G×S interactions involving DAergic genes.

## THE ROLE OF SEX FACTORS IN SCHIZOPHRENIA

### GENDER DIFFERENCES IN SCHIZOPHRENIA

The existence of gender differences in schizophrenia has been recognized since its first nosographic description by Kraepelin. The best-characterized difference concerns arguably the earlier age of onset in male patients, which typically ranges from 15 to 24 years. In comparison, females exhibit their first overt clinical manifestations between 20 and 29 years, with an average difference of 3–5 years from males (Angermeyer and Kuhn, 1988). It is widely assumed that this divergence in age reflects different developmental trajectories in the DAergic system throughout adolescence across both genders.

A comprehensive and systematic analysis of sex differences in schizophrenia is a complicated undertaking, in view of several methodological issues that may generate spurious results, such as recruitment bias, different metabolic responses to antipsychotic drugs and gender diversity in social adjustment with respect to psychiatric disorders (Markham, 2012). The awareness of these issues, and the numerous discrepancies in the literature have led several authors to cast a skeptical eye on other potential sex differences in schizophrenia, such as prevalence and symptomatic presentation (Häfner, 2002; Jablensky, 2003). Nevertheless, more recent studies, performed with more accurate and tighter controls, have actually found that the gender differences in schizophrenia may encompass several aspects of this disorder, including: (1) a higher risk of schizophrenia in males

(∼40%) (Markham, 2012); (2) poorer premorbid adjustment in males; (3) a greater severity in clinical course in males, characterized by higher frequency and intensity of negative symptoms, as well as more rapid cognitive deterioration and greater predisposition to relapse (Larsen et al., 1996; Markham, 2012). Current studies investigating the role of sex differences in neuropsychiatric disorders have also highlighted the potential impact of stress and hormonal influences on epigenetic phenomena, which may result in enduring behavioral changes across subsequent generations (see Goel and Bale, 2009; McCarthy et al., 2009; Bale, 2011; McCarthy and Nugent, 2013).

### ROLE OF ESTROGENS IN SCHIZOPHRENIA

The prevalent line of interpretation of this sex-related disproportion lies in the neuroprotective role of estrogens in women (Seeman, 1996). Indeed, women display greater severity of their psychotic symptoms in conditions associated with lower concentrations of β-estradiol, the main estrogen hormone, such as fluctuations within the menstrual cycle (Bergemann et al., 2007; Rubin et al., 2010), and menopause (Häfner et al., 1993). Furthermore, plasma β-estradiol levels are reduced in schizophrenia female patients across all phases of the menstrual cycle (Riecher-Rossler et al., 1994), and the age of disease onset in women is inversely related to the age of puberty (Cohen et al., 1999). Accordingly, several clinical trials have shown that additive treatment with estradiol substitutes improves and accelerates the therapeutic response of patients (Kulkarni et al., 1996, 2002; Akhondzadeh et al., 2003; Kulkarni et al., 2008). A number of clinical studies have also shown associations between estrogen receptor polymorphic variants in psychotic-related phenomena (Weickert et al., 2008; Min et al., 2012; Wang et al., 2013). In general, it appears that the sex factors do not induce specific qualitative differences in symptoms, but rather dampen the severity or delay the onset of the same manifestations. The biochemical nature of the neuroprotective effects of estrogens has not been fully qualified yet, but a number of studies point to a direct implication of the DAergic system (Sumner and Fink, 1993; Fink et al., 1998), in addition to glutamate and GABA. In general, the relations between estrogens and DA are supported by a host of clinical and preclinical evidence (for a thorough and detailed presentation of this issue, see Sanchez et al., 2010).

### ROLE OF ANDROGENS IN SCHIZOPHRENIA

The involvement of sex steroids in schizophrenia is not likely limited to estrogens, but may also include androgen hormones. These steroids appear to exert a multifaceted influence on the neurobiological substrates of schizophrenia; in particular, the complexity of this role stems from the fact that testosterone, the main gonadal androgen, is also converted into β-estradiol via aromatization. Men with schizophrenia tend to exhibit lower levels of testosterone, and testosterone levels are inversely correlated with the severity of negative symptoms (Akhondzadeh et al., 2006; Ko et al., 2007). Furthermore, this hormone has been shown to exert therapeutic properties for negative symptoms in schizophrenia (Ko et al., 2008). In contrast, the role of other androgens in schizophrenia is less clear. For example, schizophrenia patients exhibit high levels of the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione (Ritsner and Strous, 2010); in addition, DHEA has been found to attenuate the extrapyramidal symptoms induced by antipsychotic drugs (Ritsner et al., 2010).

In general, it is possible that androgenic metabolites of testosterone may facilitate the development of schizophrenia-related symptoms. The conversion of testosterone and androstenedione into their androgenic metabolites dihydrotestosterone (DHT) and androstanedione, respectively, is mediated by 5α-reductase (Paba et al., 2011). Notably, this process competes with the aromatization of the same substrates to β-estradiol and β-estrone. In males, 5α-reductase activity is enhanced during puberty; thus, it is possible that the increased rate of conversion of testosterone and androstenedione into their 5α-reduced androgenic metabolites (instead of estrogens) may contribute to the greater schizophrenia vulnerability and earlier age of onset in males. Our group has tested this intriguing hypothesis in rodent models of schizophrenia; our results indicate that inhibition of 5α-reductase leads to marked anti-DAergic actions on endophenotypes relevant to schizophrenia, such as sensorimotor gating deficits and stereotyped behavior (Bortolato et al., 2008a; Paba et al., 2011; Devoto et al., 2012; Frau et al., 2013). In addition, we recently found that inhibition of another key androgen-synthetic enzyme, CYP17A1, elicits similar, albeit less potent, anti-DAergic effects in the same schizophrenia-related behavioral paradigms (Frau et al., 2014). Collectively, these findings highlight that, in addition to testosterone, other androgens may have a role in the pathogenesis of schizophrenia-related features, through the mediation of DA neurotransmission (for a more detailed description of this issue and its potential therapeutic implications, see Paba et al., 2011).

## THE ROLE OF DAergic GENES IN G×S INTERACTIONS IN SCHIZOPHRENIA

### COMT

COMT catalyzes the methylation of the 3O group of catecholamines. The methyl group is donated by S-adenosylmethionine (SAM), and DA is directly converted by COMT into metanephrine. Other catechol-containing structures are substrates of COMT, including norepinephrine, epinephrine and the DA precursor l-DOPA.

COMT has a soluble form (S-COMT) and a membrane-bound form (MB-COMT), both of which are encoded by the same gene (Lundstrom et al., 1991), located on chromosome 22q11.2. COMT expression is controlled by two promoters in the third exon of the gene (Salminen et al., 1990; Lundstrom et al., 1991). The P1 promoter regulates the expression of a shorter transcript, which can code for S-COMT only (Tenhunen et al., 1993), whereas the more distally located P2 promoter can encode both transcripts. S-COMT is generally dominant in most tissues, with the only exception being in the human brain, where 70% is MB-COMT, and 30% is S-COMT. In the brain, S-COMT is mostly found in the glia and is not likely to serve a primary function in DA metabolism (Rivett et al., 1983; Naudon et al., 1992); conversely, MB-COMT is abundantly localized in postsynaptic terminals of neurons and in perisynaptic locations (Bertocci et al., 1991; Lundstrom et al., 1991; Schott et al., 2010). This form is likely to play a key role in DA degradation, particularly in regions

with low DA transporter (DAT) expression, such as the PFC, or, alternatively, in conditions of DAT inhibition (Karoum et al., 1994; Sesack et al., 1998; Huotari et al., 1999; Matsumoto et al., 2003).

Notably, the effect of COMT on DA metabolism may be particularly dominant in males; indeed, only COMT knockout males exhibit a significant (3-fold) increase in DA levels in the PFC (Gogos et al., 1998). MB-COMT is generally localized intracellularly, but not in the cell membrane (Ulmanen et al., 1997). This distribution implies that its function in DA metabolism is secondary to DA uptake in the postsynaptic terminal (**Figure 1**), which may be served by either the organic cation transporter 3 (OCT3; SLC22A3) or the plasma membrane monoamine transporter (PMAT; SLC29A4) located in the postsynaptic neuron or glia. PMAT is highly expressed in the forebrain (Engel et al., 2004; Dahlin et al., 2007), including brain regions with sparse DAT expression.

The perisynaptic location of COMT suggests that this enzyme may be important for volume transmission of DA, which plays an important role in the PFC (Paspalas and Goldman-Rakic, 2004). Given the relevance of volume neurotransmission in the PFC for the acquisition of certain informational aspects, such as the perception of salience and the dynamic regulation of signal-to-noise ratio, alterations in COMT activity may result in cognitive changes, particularly with respect to PFC-mediated functions. In addition, COMT may be a crucial element in differentiating the temporal patterns of tonic and phasic DA action (Bilder et al., 2004).

A host of studies have proposed the *COMT* gene as a potential candidate for psychosis and related phenomena (Egan et al., 2001; Williams et al., 2007). In particular, numerous investigations have focused on rs4680, one of the best-characterized single-nucleotide polymorphisms of the *COMT* gene, resulting in the substitution of a valine (*Val*) for a methionine (*Met*) residue at position 108 of S-COMT and 158 of MB-COMT (*Val-Met*) (Lachman et al., 1996; Harris et al., 2005; Wahlstrom et al., 2007). The *Val*-allele confers a higher intrinsic COMT activity than the *Met*-allele (Männistö and Kaakkola, 1999), leading to an overall reduction in DA levels in the PFC. Indeed, COMT serves as the primary enzyme for DA metabolism in this region (Egan et al., 2001; Schott et al., 2006; Tan et al., 2007; Diaz-Asper et al., 2008). Accordingly, individuals harboring the *Val* allele exhibit low DA levels predominantly in the PFC, which may result in a region-specific dysregulation of DA receptors (and particularly $D_1$, the most abundant DA receptor in the cortex). Conversely, striatal DA levels and $D_2$ receptor availability appear to be unaffected by alterations in COMT activity (Yavich et al., 2007; Hirvonen et al., 2010). Moreover, *Val*-allele carriers have been associated with impaired physiological responses across several functional domains, including cognitive flexibility, working memory, attentional control and emotional resilience (Malhotra et al., 2002; Goldberg et al., 2003; Blasi et al., 2005; Smolka et al., 2005).

The role of COMT in schizophrenia has been extensively studied, yet results have unequivocally shown that neither genetic variants nor the catalytic activity of the enzyme have great intrinsic influence on schizophrenia risk (Chen et al., 1996; Daniels et al., 1996; Riley et al., 1996; Wei et al., 1996; Karayiorgou et al., 1998; Wei and Hemmings, 1999; De Chaldee et al., 2001; Semwal et al., 2001; Strous et al., 2006). Nevertheless, multiple lines of evidence indicate that high-activity *COMT* variants is robustly

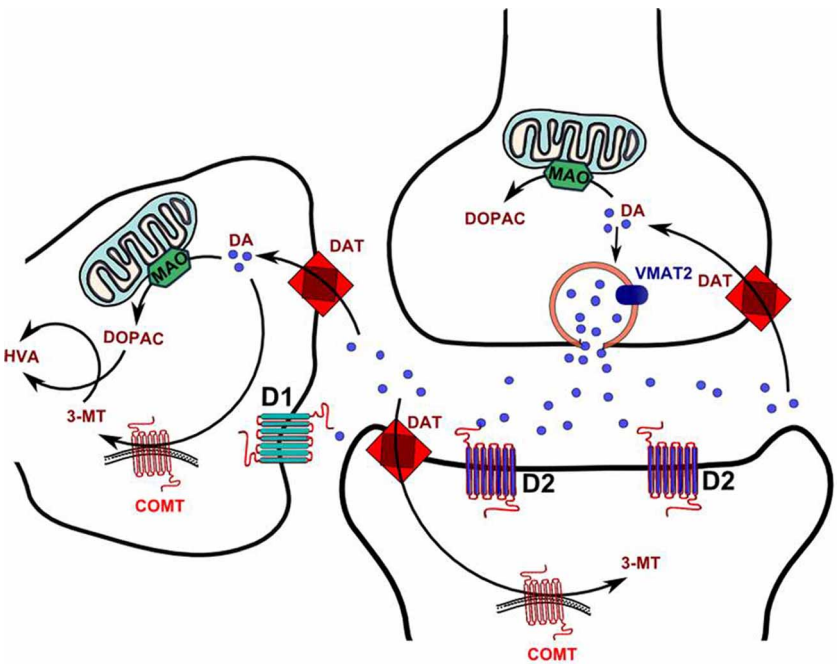


**FIGURE 1 | Schematic diagram of DA synaptic metabolism.** Abbrevations 3-MT, 3-Methoxytyramine; COMT, Catechol-o-methyltransferase; DA, DA; DAT, DA reuptake transporter; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, Homovanillic acid; MAO, Monoamine oxidase.

associated with a greater severity of negative and cognitive symptoms in schizophrenia patients, as well as specific endophenotypic impairments related to functional deficits of the PFC (Egan et al., 2001; Herken and Erdal, 2001; Weinberger et al., 2001; Weinberger, 2002). Specifically, the *Val* allele has been associated with poorer performance in schizophrenia patients across several neuropsychological tests for executive functioning (Goldberg et al., 2003; Nolan et al., 2004; Ohnishi et al., 2006; Diaz-Asper et al., 2008; Opgen-Rhein et al., 2008; Ira et al., 2013), as well as sensorimotor gating deficits in comparison to carriers of the *Met* allele (Quednow et al., 2010). Individuals harboring the *Val* variant also exhibit greater prefrontal noise, corresponding to the electromagnetic activity in the region (Winterer et al., 2006). In contrast, multiple studies have ascertained that the *Met* variant is associated with a slightly lower schizophrenia risk, as well as less severity of attentional, cognitive and information-processing deficits (Egan et al., 2001; Bilder et al., 2002; Bray et al., 2003; Gallinat et al., 2003; Tunbridge et al., 2006; Ehlis et al., 2007; Lu et al., 2007).

Although the aforementioned studies indicate that the *Val* variant confers at best a very modest enhancement of schizophrenia risk, recent investigations suggest that the interaction of this haplotype with other genetic or environmental vulnerability factors may lead to schizophrenia (Schenkel et al., 2005; Stefanis et al., 2007; Collip et al., 2011; Pelayo-Teran et al., 2012). In particular, the interaction of the *Val* variant with cannabis abuse in adolescence has been shown to increase schizophrenia risk (Caspi et al., 2005; Henquet et al., 2006; Estrada et al., 2011), but the neurobiological bases of this interaction remain poorly understood.

While most of the research on *COMT* genotypes and schizophrenia has been focused on the impairments associated with the *Val* variant, emerging lines of evidence have also pointed to the possibility that the *Met*-variant may predispose schizophrenia patients to aggression and violence (Strous et al., 1997; Lachman et al., 1998; Nolan et al., 2000; Liou et al., 2001; Han et al., 2004, 2006; Kim et al., 2008; Tosato et al., 2011; Bhakta et al., 2012; Singh et al., 2012). Interestingly, this predisposition appears to be specific for males, pointing to a potential G×S interaction (Nolan et al., 2000; Soyka, 2011; Singh et al., 2012).

A summary of the main studies that have identified G×S interactions concerning *COMT* polymorphisms is reported in **Table 1**. Although these data should still be regarded as preliminary, several studies suggest that male patients with high-activity COMT may have greater severity of endophenotypes associated with prefrontal deficits in schizophrenia, such as eye movement disturbances (Rybakowski et al., 2002) prefrontal noise (Winterer et al., 2006) and schizotypal traits (Ma et al., 2007). Similarly, Hoenicka et al. (2010) found that the effects of the *Val*$^{158}$*Met* polymorphism on schizophrenia vulnerability are more directly related to male patients, possibly through an epistatic interaction with $D_1$ receptor (Hoenicka et al., 2010) (see below). Other studies indicate that only female carriers of the *Val/Met* alleles exhibit high propensity to engage in risky behaviors (Amstadter et al., 2012) and alterations in emotional processing (Domschke et al., 2012).

COMT activity has been reported to be higher in males than females (Boudikova et al., 1990). This gender difference may reflect the ability of testosterone and DHT to increase COMT expression (Purves-Tyson et al., 2012). Alternatively, estrogens have been found to reduce the transcription and expression of COMT (Männistö et al., 1992; Xie et al., 1999). An additional mechanism that may predict a lower COMT activity in females may be afforded by the function of catecholestrogens. These 2- and 4-hydroxylated metabolites of β-estradiol (Ball and Knuppen, 1980; Zhu and Conney, 1998) compete with DA for COMT-mediated metabolism, and may act as inhibitors of the enzyme at high concentrations. Accordingly, catecholestrogens have been shown to modulate the turnover of catecholamines (Parvizi and Wuttke, 1983). While the specific role of catecholestrogens on G×S interactions of *COMT* in schizophrenia remains to be investigated, the reduction of COMT activity in females may explain the lower susceptibility of this gender for the phenotypic effects of the *Val* variant on PFC function. At the same time, this mechanism could also account for the higher proclivity of female carriers of the *Met* allele to engage in risky behaviors (Amstadter et al., 2012).

A number of preclinical studies have found sex-specific neurochemical and behavioral differences associated with COMT (Gogos et al., 1998). In particular, heterozygous male COMT-deficient mice exhibit impaired object recognition (Babovic et al., 2008). Conversely, COMT overexpression was found to be associated with blunted stress responsiveness, as well as impairments in working memory and attentional set-shifting (Papaleo et al., 2008). These data are in agreement with evidence showing that COMT alterations may negatively impact prefrontal functions in both humans and rodents (Papaleo et al., 2012). In a recent study, Risbrough and colleagues found that male mice carrying the COMT158*Val*-variant exhibit marked reductions in spatial working memory and disruptions in sensorimotor gating; conversely. female mice carrying the COMT158*Met*-variant displayed alterations in fear-related behavioral responses (Risbrough et al., 2014). Collectively, these preclinical findings further support the role of sex-specific influences of COMT genetic variations on prefrontal DAergic systems.

### MAO A AND B

MAO A and B are mitochondrial-bound enzymes (Greenawalt and Schnaitman, 1970) differing by substrate affinity. MAO A has high affinity for serotonin and norepinephrine, while MAO B metabolizes the trace amine phenylethylamine. DA can be degraded by both isoforms; however, the primary enzyme differs across species. In humans and primates, MAO B is the major metabolic enzyme of DA, whereas MAO A serves this role in rodents (Garrick and Murphy, 1980; Cases et al., 1995; Fornai et al., 1999). The metabolism of DA mediated by MAOs occurs, for the most part, in the presynaptic terminal, following reuptake by the DAT. The difference between MAO A and MAO B also concerns their anatomical localization. MAO A is localized in catecholaminergic neurons (and in particular in the locus coeruleus, nucleus accumbens, hypothalamus and mammillary complex), whereas MAO B is found in serotonergic neurons, as well as histaminergic cells and astrocytes (Westlund et al., 1988; Saura et al., 1994; Luque et al., 1995, 1996; Jahng et al., 1997; Bortolato et al., 2008b).

**Table 1 | List of major studies documenting an interaction between COMT polymorphic variants and sex in schizophrenia and related symptoms.**

| Study aim | Polymorphisms (SNP if applicable) | Total subjects | Male: female ratio | Finding | References |
|---|---|---|---|---|---|
| Clinical response of risperidone in Chinese schizophrenic patients | 10 SNPs rs9606186 | 130 | 45:85 patients | Increased efficacy of risperidone efficiency in males | Zhao et al., 2012 |
| Gender effects of COMT polymorphisms on cognitive function in children | Val/Val | 8707 children | Numbers not indicated | Val/Val genotype scored lower on selective attention and executive functioning than Met/Met in males | Barnett et al., 2007 |
| COMT genetic polymorphisms association with Chinese schizophrenic patients | rs740603 and rs4818 | 604 (284 patients and 320 controls) | 203:81 patients 140:180 controls | Significant association with negative symptoms in females | Li et al., 2012 |
| COMT genotypes in schizophrenia risk | rs165774; rs174675; rs4646316; rs4680; rs6267; rs737866; rs740603 | 410 (160 patients and 250 controls) | 138:22 patients 148:102 controls | Significant genotype assocation with schizophrenia in males | Voisey et al., 2012 |
| COMT polymorphisms association with tardive dyskinesia | rs737865; rs6269; rs 4633; rs4818; rs4680; rs165599 | 226 (90 positive for Tardive dyskinesia) | 140:73 patients | Higher association of antipsychotic-induced tardive dyskinesia occurance in males | Zai et al., 2010 |
| Role of D1 dopamine receptor polymorphisms and its interaction with COMT genotype in schizophrenia | Val/Val | 701 (337 patients and 364 controls) | 226:111 patients 171:193 controls | D1 dopamine receptor polymorphisms and COMT Val/Val genotype associated with schizophrenia in males | Hoenicka et al., 2010 |
| Impact of COMT genotype on sensorimotor gating in healthy colunteers | Val/Val and Val/Met | 107 healthy controls | 54:53 controls | Serotonin 2A receptor polymorphisms and males with COMT Val/Val or Val/Met have sensorimotor gating deficits | Quednow et al., 2009 |
| Association between schizotypal traits and COMT in a healthy Chinese population | Val/Met | 465 healthy controls | 231:234 controls | Met alleles showed increased schizotypal personality questionnaire scores in males | Ma et al., 2007 |
| Association of prefrontal electrophysiologic "noise" and COMT genotype in schizophrenia patients | Val/Val | 282 (83 patients; 87 siblings; 112 controls) | 65:18 patients 31:56 siblings 66:46 controls | Val/Val-allele males exhibit higher prefrontal "noise" | Winterer et al., 2006 |
| Role of COMT genotype in schizophrenia on performance on wisconsin card sorting test | Val/Val | 124 patients | 60:64 patients | Male Val/Val alleles displayed best (lowest) scores in Wisconsin card sorting test; Female Val/Val carriers had worst (highest) scores | Rybakowski et al., 2006 |
| COMT genotype on psychosis in Alzheimer's disease | Val/Met | 373 patients | 130:243 patients | Female Val/Met carriers with Alzheimer's disease had a higher risk of psychosis | Sweet et al., 2005 |

*(Continued)*

**Table 1 | Continued**

| Study aim | Polymorphisms (SNP if applicable) | Total subjects | Male: female ratio | Finding | References |
|---|---|---|---|---|---|
| Role of COMT in schizophrenia vulnerability in Arabic population | Val/Val | 332 (255 patients and 77 controls) | 161:94 patients 31:53 controls | Female Va/Val carriers display higher risk for schizophrenia, while male Val/Met carriers have higher risk | Kremer et al., 2003 |
| COMT genotype on eye movement disturbances in schizophrenia patients | Met/Met | 177 (117 patients and 60 controls) | 74:43 patients 29:31 controls | Male schizophrenia patients with Met/Met genotype had lower oculomotor disturbances | Rybakowski et al., 2002 |
| Role of COMT in homicidal behavior in schizophrenia patients | Met/Met | 507: 30 violent patients; 62 nonviolent patients; 415 controls | 28:2 violent patients 30:32 nonviolent patients 159:256 controls | Higher Met/Met male carriers in violent schizophrenic patients | Kotler et al., 1999 |
| Role of COMT in schizophrenia vulnerability in Jewish population | 12 SNPs: Val/Val rs165599 and Val/Val rs165599-rs165688 | 12906 (2188 patients and 10718 controls) | 1383:775 patients 7947:2771 controls | rs165599 Val/Val and rs165599-rs165688 higher in female schizophrenia patients | Shifman et al., 2002 |
| Role of COMT genotype in cognition in children | Haplotype (rs6269; rs4633 and 4s4818) rs2075507 (previously rs2097603); rs6269; rs4818; rs4680; rs165599 | 8173 children | 4211:3962 children | ValB/ValB (lowest COMT activity) haplotype with highest Verbal IQ; Val/Val in rs165599 show lower working memory in males | Barnett et al., 2009 |

Several studies have documented that *MAOA* gene polymorphisms are associated with different psychiatric disturbances (Ozelius et al., 1988; Black et al., 1991; Hotamisligil and Breakefield, 1991; Hinds et al., 1992; Shih and Thompson, 1999). Most of the genetic studies on MAOA have focused on a variable number tandem repeat (VNTR) polymorphism, which is located 1.2-kilobase upstream of the transcription initiation site and has been associated with changes in gene expression (Sabol et al., 1998). Of the six different allelic variants characterized in humans, the most common display 3 repeats (3R) and 4 repeats (4R) (Sabol et al., 1998; Deckert et al., 1999; Jonsson et al., 2000). The 3R variant has been associated with behavioral features linked to low MAO A activity, such as impulsive aggression and antisocial personality (Oreland et al., 2007; Buckholtz and Meyer-Lindenberg, 2008). In contrast, the 4R variant has been associated with higher *MAOA* gene transcription and enzyme activity (Sabol et al., 1998; Denney et al., 1999). Neuroimaging imaging studies have found a link between the VNTR variants of *MAOA* promoter and structural and functional differences in the PFC (Meyer-Lindenberg et al., 2006).

In general, the majority of genetic studies have failed to find a straightforward association between *MAOA* and schizophrenia (Coron et al., 1996; Sasaki et al., 1998; Syagailo et al., 2001; Norton et al., 2002; Iwata et al., 2003; Li and He, 2008; Wei et al., 2011). Nevertheless, other analyses found preliminary results in support of a sex-specific effect of *MAOA* with respect to schizophrenia diagnosis or select symptomatic aspects of the disorder (Jonsson et al., 2003; Qiu et al., 2009; Camarena et al., 2012; Sun et al., 2012b).

A summary of the main findings on potential G×S interactions involving the *MAOA* gene is reported in **Table 2**. Although the evidence on sex-dependent effects of MAOA is still preliminary and inconclusive, it was recently reported that male schizophrenia patients exhibit abnormal patterns of methylation of the *MAOA* promoter, pointing to the possibility that the effect of sex may be directly dependent on epigenetic alterations (Chen et al., 2012). In addition to the evidence on schizophrenia, several findings have documented that genetic variations of *MAOA* may play a central role in neuropsychiatric disorders in a sex-dependent fashion. This concept is best highlighted by the elegant study conducted by Caspi and colleagues, showing that males, but not females, harboring low MAO A activity polymorphic variants and subjected to early childhood maltreatment exhibit a significantly higher vulnerability to develop antisocial and aggressive behaviors in adulthood (Caspi et al., 2002; Foley et al., 2004; Kim-Cohen et al., 2006). Indeed, subsequent studies have found that testosterone levels in the cerebral spinal fluid paralleled aggressive responses in carriers of the low MAO A activity polymorphism (Sjoberg et al., 2008). In contrast, DAergic metabolic levels were inversely associated with testosterone in low

MAO A activity carriers (Sjoberg et al., 2008). Although both low-activity *MAOA* variants and testosterone have been independently shown to affect aggression, it remains unclear how this genotype may predispose individuals to higher androgen synthesis and how these two properties may interact to influence aggression. It is worth noting that males have a markedly higher frequency of low MAO A activity variants than females (Sjoberg et al., 2008).

Although the mechanism is unclear, females harboring the high-activity *MAOA* variant display higher baseline cortisol levels than males with the same polymorphism than females carrying low-activity alleles (Jabbi et al., 2007). Furthermore, both females and males harboring low-activity *MAOA* variants exhibited a sexually dimorphic increase in stress response, which was dependent on *COMT* genotype (Bouma et al., 2012). In particular, male carriers of the *Met/Met COMT* allele displayed a significantly higher cortisol response to stress than both females with the same allele and males with other genotypes. Conversely, females carrying the *Val/Val COMT* allele in combination with low-activity *MAOA* variants showed higher stress responses than their male and female counterparts.

Although no common polymorphisms have been reported in the gene's coding region, *MAOB* allelic variants may possess different enzymatic activities (Balciuniene et al., 2002; Costa-Mallen et al., 2005). Indeed, several groups have reported the association of polymorphic variants of *MAOB* gene with several neuropsychiatric disorders characterized by DAergic dysfunction. In particular, *MAOB* allelic variations have been associated with bipolar disorder (Lin et al., 2000) and higher schizophrenia susceptibility (Hovatta et al., 1999; Gasso et al., 2008; Carrera et al., 2009; Piton et al., 2011); these results, however, have been not been consistently replicated (Coron et al., 1996; Sobell et al., 1997; Matsumoto et al., 2004; Bergen et al., 2009).

A direct implication of *MAOB* in schizophrenia is supported by several studies (Coron et al., 1996; Bergen et al., 2009; Carrera et al., 2009; Piton et al., 2011; Wei et al., 2011; Sun et al., 2012a) and may be reflective of the greater contribution of this enzyme to the metabolism of DA in humans. In particular (see **Table 2**), numerous articles have recently reported that different *MAOB* variants may predispose to schizophrenia in women (Gasso et al., 2008; Wei et al., 2011) or in men (Wei and Hemmings, 1999). In addition, other studies highlighted that *MAOB* variants may moderate several symptomatic aspects of schizophrenia, including flat affect (Camarena et al., 2012) or paranoid manifestations (Sun et al., 2012a). Although little is currently known on the potential interaction of sex hormones with MAO B, females have been reported to display significantly higher MAO B activity in platelets in comparison with males (Snell et al., 2002).

A plethora of studies has shown that sex hormones differentially affect MAO activity and expression in specific brain regions. Androgens increase MAO transcription in the substantia nigra (Ou et al., 2006; Purves-Tyson et al., 2012) and in the striatum (Thiblin et al., 1999). Chronic administration of anabolic androgenic steroids, however, reduces MAO activity in the caudate and amygdala, as well as DA metabolites in the nucleus accumbens shell (Birgner et al., 2008). Similarly, gonadectomy also increases MAO A activity in the PFC (Meyers et al., 2010), suggesting that acute treatment with androgens may enhance MAO activity, while chronic treatment exerts the opposite effect. In contrast to androgens, estrogen administration to neonatal, but not adult males elicits an increase in hypothalamic MAO activities (Vaccari et al., 1981). In females, estradiol reduces MAO A activity in the hypothalamus and amygdala (Luine et al., 1975; Ma et al., 1993; Gundlah et al., 2002).

### OTHER DAergic TARGETS

The current evidence on the implication of the other DAergic targets in G×S interactions is scant and mostly limited to DAergic receptors. Interestingly, several studies have shown that polymorphic variants of the genes encoding $D_1$, $D_2$, and $D_4$ receptors are linked to different responses to antipsychotic medications in a gender-sensitive fashion. For example, variants of the *DRD2* gene, which codes for the $D_2$ receptor, may predispose females to a greater prolactin increase in response to antipsychotics (Mihara et al., 2000, 2001); however, this difference may not be dependent on an actual G×S interaction, but rather on the higher baseline levels of prolactin in females (Yasui-Furukori et al., 2008). Variants of the *DRD1* and *DRD4* genes (coding for $D_1$ and $D_4$ receptors) may also predispose to different responses to antipsychotic treatment (including side effects) (Hwu et al., 1998; Potkin et al., 2003; Hwang et al., 2007; Popp et al., 2009). Only few studies have pointed to a direct role of these genes in specific symptomatic aspects. Different variants of the *DRD2* gene may be associated with higher perseverative responses in female schizophrenia patients (Rybakowski et al., 2005), while VNTR variants of the *DRD4* gene may predict for differences in age of onset in female patients (Goncalves et al., 2012). Notably, the *DRD1* gene has been recently found to establish an epistatic interaction with the *COMT* gene, which predicts schizophrenia risk in males, presumably due to the functional association of $D_1$ receptors and COMT in the PFC (Hoenicka et al., 2010).

Independent investigations have reported that different variants of the *DRD3* gene may be associated with schizophrenia predisposition in males (Asherson et al., 1996; Griffon et al., 1996) and females (Aksenova et al., 2004; Godlewska et al., 2010). Interestingly, preliminary results in our animal models suggest that the behavioral responses elicited by agonists of $D_3$, but not $D_2$ receptors, may be under control of neurosteroids with respect to the regulation of sensorimotor gating (Frau et al., submitted). Future work is warranted to establish the nature of this intriguing neurobiological finding.

## CONCLUDING REMARKS

As mentioned above, the attempts to identify a genetic basis of schizophrenia have revealed a picture of extreme complexity and high heterogeneity of heritable bases. This view has gradually replaced our "genome-centric" perspective with a broader framework, in which genetic vulnerability is a piece of a much greater mosaic, consisting of complex interactions with environmental factors. In this perspective, sex hormones may also play a significant role in shaping the course of schizophrenia and modifying the developmental trajectory of the neurobiological alterations of DA and other neurotransmitter systems underpinning this disorder.

**Table 2 | List of major studies implicating the interaction between MAO polymorphic variants and sex in schizophrenia and related symptoms.**

| Study aim | Polymorphisms (SNP if applicable) | Total subjects | Male: female ratio | Finding | References |
|---|---|---|---|---|---|
| Role of MAO A and B polymorphisms in negative and positive schizophrenia symptoms | MAOA: 3-repeat and 4-repeat uVNTR MAOB: rs1799863 and rs1137070 | 468 (344 patients and 124 controls) | 209:135 patients 60:64 controls | Higher affective flattening in female schizophrenic patients homozygous for MAOA 4-repeat uVNTR and MAOB/rs1799836 (GG) | Camarena et al., 2012 |
| Role of MAO A gene polymorphisms in paranoid schizophrenia in a Chinese population | MAOA 3-repeat and 4-repeat uVNTR and 41 SNPs | 1122 (555 patients and 567 controls) | 284:271 patients 308:259 controls | VNTR 3-repeat-rs6323, VNTR 3-repeat-rs1137070 and VNTR 3-repeat-rs6323-rs1137070 haplotypes associated with paranoid schizophrenia in females | Sun et al., 2012a; Sumner and Fink, 1993 |
| Association of MAO A/B genes and schizophrenia in a Chinese population | MAOA: rs6323 MAOB: rs1799836 | 1073 (537 patients and 536 controls) | 294:243 patients 284:252 controls | MAO A rs6323 and MAO B rs1799836 haplotype associated with schizophrenia in females | Wei et al., 2011 |
| Association between antipsychotic-induced restless legs syndrome and MAO polymorphisms in schizophrenia | MAO A: 3-repeat and 4-repeat uVNTR MAO B: A644G SNP | 190 patients | 106:84 patients | Males patients with MAO A 3-repeat uVNTR and MAO B A644 genotype has higher association with antipsychotic-induced restless leg syndrome | Kang et al., 2010 |
| Association of MAO gene microsatellites with schizophrenia | MAO A: (AC)n repeats MAO B: (TG)n repeats | 89 nuclear families with schizophrenic offspring | Not indicated | Families of male schizophrenia patients had higher frequency of transmitted MAO B $(TG)_{24}$ repeats | Wei and Hammings, 1998 |
| Association of MAO A gene variants and schizophrenia in a Chinese population | MAO A uVNTR 3-repeat and 4 repeat and -941G/T and -1460C/T restriction fragment length polymorphisms | 355 (234 patients and 121 controls) | 156:78 patients 76:45 controls | Haplotype association of schizophrenia with 3-repeat uVNTR and -941T allele in males | Qiu et al., 2009 |
| MAO platelet activity relationship to auditory hallucinations and paranoia in schizophrenics | MAO platelet activity (MAO B) | 237 (101 patients and 136 controls) | 64:37 patients 65:71 controls | Decreased platelet MAO activity associated with paranoid subtype and presence of auditory hallucinations in male schizophrenia patients | Meltzer and Zureick, 1987 |

The findings summarized in this review indicate that, although the role of G×S interactions in schizophrenia is still inconclusive, sex hormones might affect brain substrates through a multilayered set of mechanisms, which appear to have a particular impact on the catabolic apparatus of DA.

The diagnostic definition of schizophrenia (as based on the DSM-IV and DSM-5) is only related to symptomatic descriptors, rather than biomarkers and quantitative endophenotypes. This scenario raises the possibility that this disorder may actually correspond to an array of diverse clinical conditions which share a common "final pathway" accounting for the pathognomonic manifestations of schizophrenia. Accordingly, a greater understanding of the role of DA neurotransmission in schizophrenia may have important repercussions also with respect to a better nosographic classification of this disorder.

The integration of preclinical research with neuroimaging and genetic studies will play a critical role in enabling us to identify central neurobiological networks that underpin gender-specific neurobehavioral endophenotypes of schizophrenia. Additionally, the contribution of these studies and a greater understanding of sex-dependent epigenetic mechanisms of transcriptional regulation will be fundamental to qualify premorbid signs and

symptoms, and chart the developmental trajectory of psychosis in males and females.

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# Evidence for phenotypic plasticity in response to photic cues and the connection with genes of risk in schizophrenia

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Numerous environmental factors have been identified as influential in the development of schizophrenia. Some are byproducts of modern life, yet others were present in our evolutionary past and persist to a lesser degree in the current era. The present study brings together published epidemiological data for schizophrenia and data on variables related to photic input for places of residence across geographical regions, using rainfall as an inverse, proxy measure for light levels. Data were gathered from the literature for two countries, the former Yugoslavia and Ireland, during a time in the early 20th century when mobility was relatively limited. The data for Yugoslavia showed a strong correlation between hospital census rates for schizophrenia (by place of birth) and annual rain ($r = 0.96$, $p = 0.008$). In Ireland, the hospital census rates and first admissions for schizophrenia (by place of permanent residence) showed a trend for correlation with annual rain, reaching significance for 1st admissions when the rainfall data was weighted by the underlying population distribution ($r = 0.71$, $p = 0.047$). In addition, across the years 1921–1945, birth-year variations in a spring quarter season-of-birth effect for schizophrenia in Ireland showed a trend for correlation with January-March rainfall ($r = 0.80$, $p \leq 0.10$). The data are discussed in terms of the effect of photoperiod on the gestation and behavior of offspring in animals, and the premise is put forth that vestigial phenotypic plasticity for such photic cues still exists in humans. Moreover, genetic polymorphisms of risk identified for psychotic disorders include genes modulated by photoperiod and sunlight intensity. Such a relationship between phenotypic plasticity in response to a particular environmental regime and subsequent natural selection for fixed changes in the environmentally responsive genes, has been well studied in animals and should not be discounted when considering human disease.

**Keywords: schizophrenia, pyschoses, epidemiology, photoperiod, natural light, prenatal, melanotropin, vitamin D**

## INTRODUCTION

The epidemiology of psychiatric disease represents an invaluable resource for new insights into gene-environment interactions as a cause of mental illness. That epidemiological variation in incidence must occur across space and time is consistent with known principles for all human disease, be the cause predominantly environmental or genetic. Although no field of endeavor is so fraught with potentially confounding variables, the perceived difficulties in interpretation should not lead to a blanket rejection of such work. As evidence builds for a consistent trend between studies, and as data mounts from research avenues in genetics and pharmacology that support the epidemiologic results, the resulting knowledge can be used to more productively design future research. Such is the case with three epidemiologic outcomes for schizophrenia that are likely related: the effect of latitude on rates of disease in the indigenous population, the effect of immigration from southern to northern latitudes and the late winter-to-spring quarter season of birth effect, a modest but consistent finding that has survived mathematical challenges (Lewis and Griffin, 1981; Dalén, 1990; Pulver et al., 1990; Watson, 1990), and questions as to its relevance in the Southern hemisphere (McGrath and Welham, 1999), where the effect is much less robust.

One key environmental link between the epidemiological studies and related genetic/pharmacologic results is photic input (McGrath et al., 2002), a factor also of relevance to the melanotropin genes shown to be associated with psychotic disorders including schizophrenia (Severinsen et al., 2006; Miller et al., 2009; Demontis et al., 2012) and of relevance to pharmacological results that pertain to the function of those genes (Miller, 2013). If photic input is the key variable, the less pronounced Southern hemisphere results actually bolster the season-of-birth theory because a much lower percent of populated land mass occurs at the higher latitudes in the southern hemisphere than in the Northern. Importantly, Brisbane, the most populous city in the McGrath et al. study of Australia (2002), rests at latitude 27.5°S. Dublin, in contrast, is at latitude 53.4°N. A meta-analysis of published season of birth studies demonstrated that the effect does go up with increasing latitude (Davies et al., 2003). Furthermore, variation in overall schizophrenia incidence would be expected to vary with latitude, and a meta-analysis by McGrath and colleagues (Saha et al., 2006) demonstrated unequivocally that a

gradient exists. This conclusion conflicts with an earlier report sponsored by the World Health Organization (WHO) discounted any correlation with latitude (Jablensky et al., 1992), but their results were somewhat compromised by selective inclusion of one study site in the final report but not another (Chandigarh, an area with a large Sikh population was included, but not Agra, an area with a large Muslim population). Data from their preliminary report (Sartorius et al., 1986) showed a nearly 10-fold difference between the two sites, but the final report (Jablensky et al., 1992) deemed that only suspected methodological differences could explain such geographical variation in incidence on such a small scale despite the fact that differences in racial composition alone could be the basis for those differences. For the study site in Ireland, a country previously reported to have a very high incidence of schizophrenia (Walsh, 1968; Kelleher et al., 1974), the WHO study team selected Dublin as a center for data collection, a region in Ireland not reported to have the high 1st admission rates for schizophrenia more characteristic of the west of Ireland (Kelleher et al., 1974).

When examining the effect of an environmental variable, it is always helpful to first look at the extremes. Nowhere has lack of photic input exerted more effects on humans over time than in the high latitude country of Ireland, where comparatively low dietary vitamin D and the lack of sunlight-induced vitamin D selected for the fairest skin type in the world, as reported in dermatological surveys (Gibson et al., 1997). Yet, although most Swedish residents live at higher latitudes than the Irish, their proportion of skin type 1 and 2 is not as high (Karlsson et al., 2000; Rodvall et al., 2007). Even in the far reaches of populations in the Arctic Circle, the impact of low light on skin type prevalence (Karlsson et al., 2000) was not so extreme, most likely because a diet rich in vitamin D from seafood helped to mitigate the lack of sun. The relationship between a diet rich in fatty fish and serum levels of vitamin D is clear (Burgaz et al., 2007).

McGrath and Welham (1999) have proposed that vitamin D availability may modulate the eventual development of schizophrenia, and Kinney et al. (2009) have extended that theory to propose the risk for schizophrenia around the world is related to levels of vitamin D from fish in their current diet. A complementary hypothesis is that a diet rich in fatty fish actually changed the evolutionary trajectory for some populations. From the time of the Vikings on, the Nordic cultures developed such a robust fishing enterprise that they exported their products to many other European destinations (Sicking and Abreu-Ferreira, 2008). The Irish, in contrast, failed to develop an historically strong sea-faring and fishing industry (Donnchadha et al., 2002), in part because the coastline toward which they were pushed during British occupation (beyond "The Pale"; McManus, 1931) was dangerously rocky and difficult to trawl (Woodham-Smith, 1962). The dire impact of this situation was most apparent during the potato famine, when a marine diet might have saved millions of lives (Donnchadha et al., 2002; Woodham-Smith, 1962). But over the generations, lacking readily available nutrients from the sea meant the resulting deficiency of vitamin D from either diet or the sun selected for an extremely fair skin type, which helped their descendents avoid rickets.

Clearly, the relative lack of sunlight had an evolutionary impact on genes affecting vitamin D generation from sunlight, but what evidence is there that it might also have selected for a change in the prevalence of a disease such as schizophrenia? Might there be vestigial phenotypic plasticity that provides a window into the forces that shaped our evolutionary past? In the animal kingdom, photic cues are crucial to survival, and animals that are adapted to life in regions of low light have evolved to have different light-responsive genotypes than those that evolved near the equator. Yet phenotypic plasticity can also be found, as evidenced by the well-studied effect of photoperiod on gestation, an effect which can not only determine coat color at birth but also neo-natal behavior (Hoffman, 1978; Reppert, 1985; Stetson et al., 1986; Weaver et al., 1987; Lee and Zucker, 1988; Nagy et al., 1993; Bellavía et al., 2006; Butler et al., 2007). Perhaps, then, the season-of-birth effect in schizophrenia could well be evidence of vestigial phenotypic plasticity in response to seasonally varying levels of light.

The approach taken in the present study was to analyze the correlation between rates of schizophrenia and a proxy measure for photic input, rainfall. Two countries were selected for inclusion, one at a more extreme latitude, Ireland, and one at a more moderate latitude, the former Yugoslavia. No attempt was made to compare the two, as a meta-analysis of numerous country-to-country differences has been well performed by others (Saha et al., 2006). Rather, the question being asked was whether small scale geographical differences in prevalence and incidence exist within each country and whether those differences might relate to variations in rainfall. These two countries offered the advantage of weather extremes they encompass, as well as the availability of detailed schizophrenia epidemiology during the early to mid-20th century, at a time when mobility was limited compared to today's world and when the chances were good that someone born in an area would be quite likely to grow up in the same town. The former Yugoslavia represented an opportunity to examine annual rainfall extremes within one country, as its western coastal range home to a region that receives more rainfall than any other in continental Europe, a district in the current Montenegro (Papp and Erzberger, 2007) and the nearby town of Crkvice, Croatia (Krause and Flood, 1997; Marinkovic et al., 2012), while the southern and eastern-most regions of the former country were quite dry. Ireland, on the other hand, offered the unique opportunity to investigate how photic input might relate to the season of birth data reported for birth years with the highest second quarter season-of-birth effect ever documented (O'Hare et al., 1980). As prior research has shown that rainfall 3 months before birth is significantly associated with the risk of becoming schizophrenic (Messias et al., 2001, 2006), this study focused on rainfall during the months encompassing what would have been the third trimester of gestation for births in the second quarter of the year.

## MATERIALS AND METHODS

### THE FORMER YUGOSLAVIA

The hospitalization rates for schizophrenia by place of birth in Yugoslavia were derived from Crocetti et al. (1964). Those authors published a detailed map of the data put together by Kuljzenko (1933). During the course of one year (1931), hospitalization

records had been obtained by Kuljzenko and co-workers for the whole of Yugoslavia and for each patient, the place of birth was noted. The scale of their plotted data was on the order of 100 sq. km. For the purposes of this study, the epidemiological map (Crocetti et al., 1964) was digitized by a draftsman using the program AutoCad (**Figure 1**, top panel) to enable digital overlay of epidemiological and meteorological data. The present study relied completely on the interpretation by Crocetti et al. (1964) of Kuljzenko's publication.

The mean annual precipitation data (equivalent to rainfall) for Yugoslavia was provided by the Yugoslavian Hydrometeorological Institute (**Figure 1**, bottom panel). The data was for a 15 year period (1925–1940) encompassing the year of the hospital census, but not necessarily the year of birth of the patient. However, the decade-to-decade variation in rainfall is quite low (<7%; personal communication from the former Yugoslavian Federal Hydrometeorological Institute), and more importantly, the variation in rainfall totals is unlikely to have changed the pattern of rainfall in any significant way. Data was digitized for the author by the Yugoslavian Hydrometeorological Institute and the mean annual rainfall values were calculated for 0.5 degree cells, constituting roughly 2000 sq. km. The boundaries of each cell were then superimposed on the schizophrenia hospitalization map. For each level of hospitalization rate, the corresponding cells for rainfall were tallied by the author and mean values were calculated. Where the boundary of a mapped schizophrenia hospitalization rate excluded a portion of a rainfall cell, the rainfall data was weighted by the landmass for the proportion of the cell that was included. To exclude the potentially confounding effect of different ethnic populations, regions with non-Slavic ethnic groups that represented from 5 to ≤100% (**Figure 2**) were excluded from the final analysis.

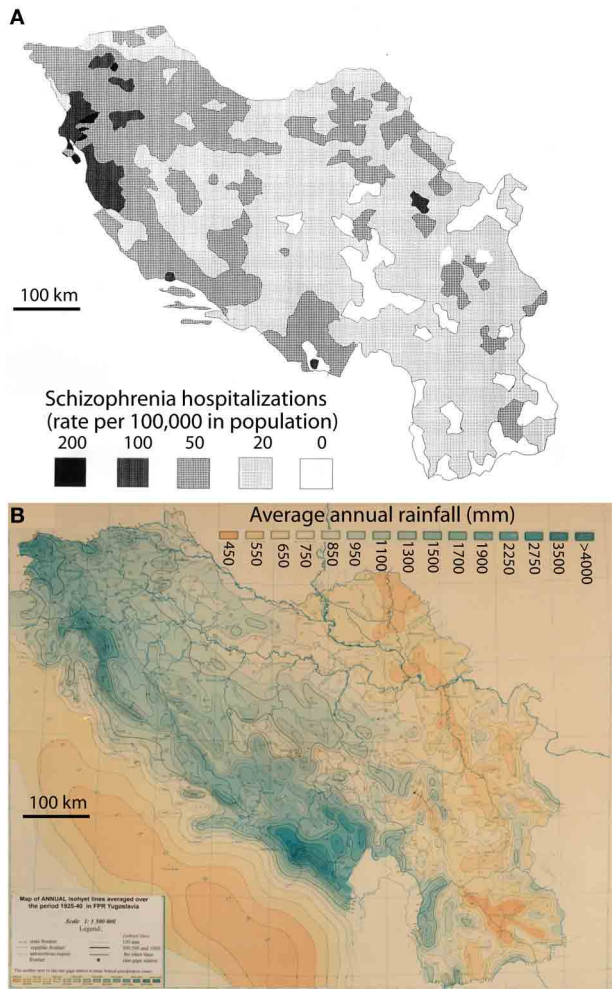


**FIGURE 1 | Rates of schizophrenia hospitalization in the year 1931 (A) vs. the average annual rainfall, 1925–1940 (B) for the former Yugoslavia.** The hospitalization rates are plotted by place of the patient's birth (derived from Crocetti et al., 1964).

## IRELAND

The Irish Health Research Board publishes yearly compilations of mental health data, including first admission rates for schizophrenia by health board catchment area, and for 1991, a country-wide census was compiled for hospitalization at midnight on March 31, 1991. The period 1982–1991 (including the census year) was used by the author to calculate the mean first admission rates for each catchment area. The catchment area sizes were approximately 4600 sq km and up.

Maps of the catchment areas for Ireland were digitized with the program AutoCad, encoded with the hospitalization rates and the 1st admission rates for schizophrenia (**Figure 3**, left panel). The catchment areas were then superimposed on a map of the mean annual rainfall for Ireland (**Figure 3**, right panel). The mean annual rain map was digitized by AutoCad from a map provided by J. J. Logue of the Irish Meteorological Service (Logue, 1984), representing rainfall data collected 1941–1970. Those years most probably included the year of birth of a good portion of the patient population creating the hospitalization and first admission rates used in this study. Since the isohyets of mean rainfall obviously did not match the boundaries of the maps for schizophrenia rates, the area covered by a given isohyet interval was digitally calculated. The percent of each catchment covered by a given isohyet interval was determined, and the mean rainfall for the region identified by summing the contribution of each area for a given isohyet interval, weighted by its percent contribution to the catchment area. The correction for population distribution (1986 census) was carried out by weighting the rainfall data by the population (as a percent of the total population in the particular catchment area) in the towns with 5,000 or more residents and assuming that the remaining land area exhibited a uniform population distribution.

Season of birth data for schizophrenia for Ireland as a whole (not available per catchment area for different years) was obtained from O'Hare et al. (1980) for births in the years 1921–1955. The data was available in 5 year increments: 1921–1925, 1926–1930, etc. January through March (Jan–Mar) rain for those years was available only for the years 1921–1945 (with the exception of the war year 1941), obtained in map form as a percent excess of average, from the annual publication "British

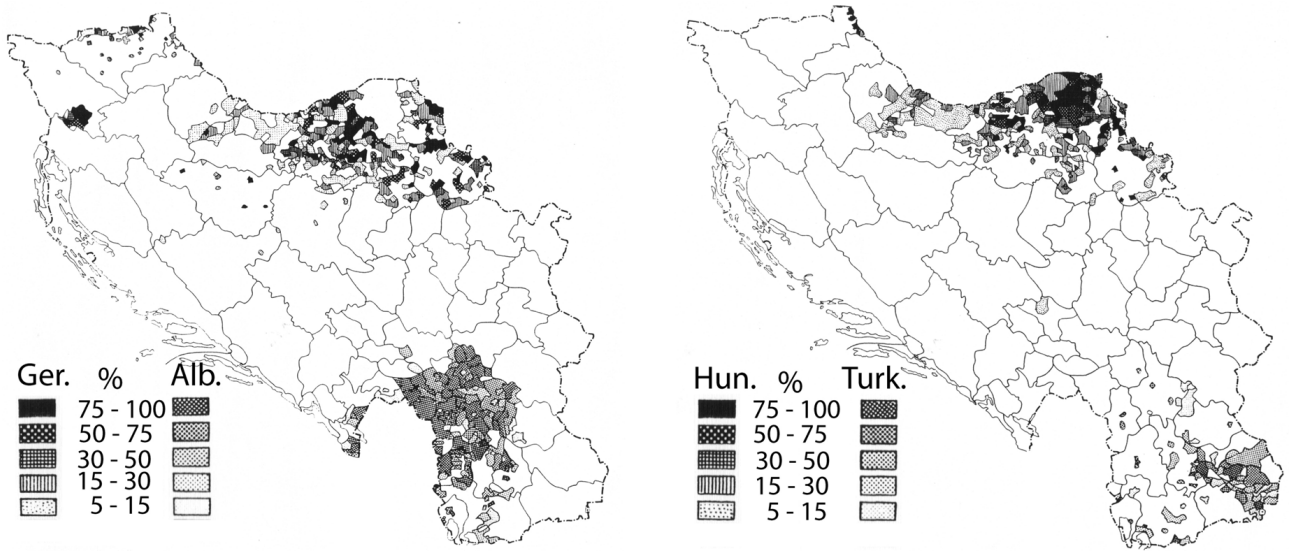


**FIGURE 2 | Maps showing the distribution of non-Slavic ethnic groups within the former Yugoslavia (reprinted from Banac, 1984 with permission from Cornell University Press).** The **left panel** depicts regions where Germans (Ger.) and Albanians (Alb.) constitute from 5 to 100% of the population. The **right panel** depicts regions where Hungarians (Hun.) and Turkish communities (Turk.) constitute 5–100% of the population. In the **left panel**, the regions with significant Germanic communities lie exclusively within the top half of the map, and the regions with significant Albanian communities within the bottom half of the map. In the **right panel**, the regions with significant Hungarian communities lie within the top half of the map and the regions with significant Turkish communities within the bottom half of the map.

Rainfall." The percent of the country covered by a given isohyet interval was calculated, and the correction for population distribution was carried out by weighting the rainfall data by the population (in this case, as a percent of the total population of Ireland) in the towns lying within a given isohyet interval (only for those with 5,000 or more residents) for the appropriate census year, and assuming that the remaining land area exhibited a uniform population distribution. For the birth years 1921–1930, the census data was derived from the 1926 Ireland census; for the birth years 1931–1940, the census data was derived from the 1936 Ireland census; and for the birth years 1942–1945, the census data was derived from the 1946 Ireland census. The relationship between the population-corrected rain data and the 2nd quarter season-of-birth data was then analyzed (**Figure 4**).

### STATISTICS

Where mean values were calculated for rainfall and for rates of schizophrenia, standard deviations are not reported because no group-wise comparisons are made of the means. The program Linear regression was carried out using the program SigmaStat to test for correlation between mean rates of schizophrenia and mean values of rainfall, and the resulting $r$ value with the associated significance level ($p$ value) is reported.

### GENE AND PEPTIDE SYMBOLS

*Alpha-MSH* represents alpha-melanocyte-stimulating hormone.
*MC5R* represents melanocortin receptor-5 (for which alpha-MSH is an agonist).
*MCH* represents melanin-concentrating-hormone.
*MCHR1* represents melanin-concentrating-hormone receptor-1.
*MCHR2* represents melanin-concentrating-hormone receptor-2.

## RESULTS

### THE FORMER YUGOSLAVIA

A 16 year period of mean rainfall values (1925–1940) was considered representative of the pattern of rainfall normally experienced in Yugoslavia during the late 19th and early 20th centuries, according to the Yugoslavian Federal Hydrometerological Institute. A visual comparison of the weather map for the former Yugoslavia (**Figure 1A**) and a map of hospitalization rates for schizophrenia in the year 1930 (**Figure 1B**; after Crocetti et al., 1964 and Kuljzenko, 1933), revealed a striking similarity in the patterns. To quantify this apparent relationship, the mean annual rainfall was calculated for regions experiencing a given rate of hospitalization (**Table 1**). The correlation between the hospitalization rate for schizophrenia by place of birth (Crocetti et al., 1964; after Kuljzenko, 1933) and mean annual rainfall was determined to be $r = 0.96$ ($p = 0.008$).

Crocetti et al. (1964), confirmed the patterns revealed in Kulzjenko's work for Croatia only. However, apart from the methodology of the patient ascertainment, an additional concern in a country as diverse as Yugoslavia is the confounding effect that different ethnic groups would pose for a disease that is thought to be partly genetic in origin. The bulk of the former Yugoslavia was composed primarily of Southern Slavs (Banac, 1984) who were either Christian or Muslim in religion. Small pockets of German, Hungarian, Albanian, and

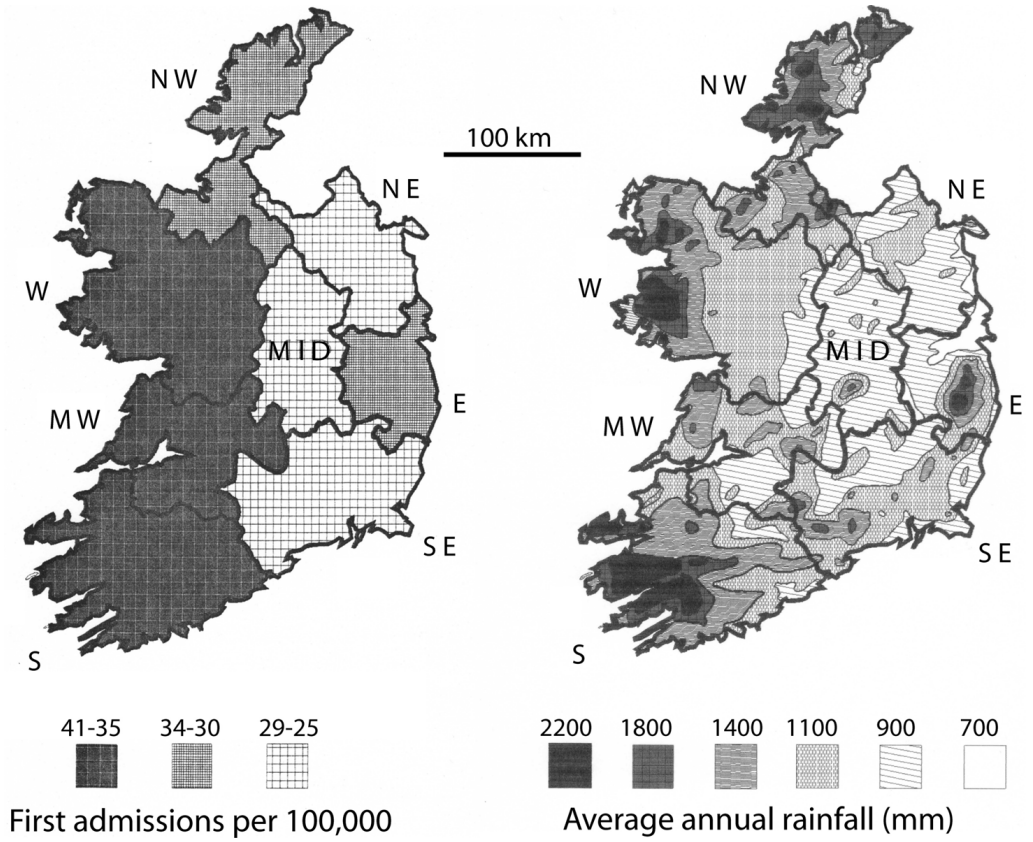


**FIGURE 3 | Rates of schizophrenia 1st admissions (left panel) vs. annual rainfall (right panel) in Ireland.** The 1st admissions digital plot represent means of the yearly values in the outlined health catchment area (S, MW, W, NW, MID, NE, E, and SE), calculated for 1982–1991 from data published by the Health Research Board for Ireland (Activities of Irish Psychiatric Hospitals and Units Dublin, Ireland). The annual rainfall digital plot (derived from the Irish Meteorological Service, Galway and Logue, 1984) represents means of the yearly totals for 1941–1970, which would have included the birth years for many of the 1st admission patients.

Turkish communities populated Yugoslavia in 1921 (**Figure 2**, after Banac, 1984). The most direct way to assess the impact of these ethnic groups was to remove from the analysis those regions in which non-Slavs were a high percentage of the population (**Table 1**, right column). Doing so had no effect on the direction of the correlation or the significance level ($r = 0.97$; $p = 0.008$); thus, ethnic differences do not appear to be responsible for the variation in rates of schizophrenia within Yugoslavia.

## IRELAND

The data for schizophrenia in Ireland (**Table 2**) was derived from yearly publications of the Irish (Health Research Board, 1972–1994), which provide a variety of hospital statistics including first admission rates and comprehensive censuses taken for point hospitalization rates.

The hospital census data presented are for the year 1991. Mean first admission rates were calculated for a 10 year period (1982–1991, inclusive), selected to include a comprehensive census year (1991) and selected as a time of relative stability in terms of the grouping of the hospital reporting system. The exception is 1991, when: (1) in the NE, Cavan and Monaghan began to report as a unit, (2) in the S, a new psychiatric unit was formed in Tralee (joint reporting with the hospital in Killarney) and (3) in the NW, a new psychiatric unit in Letterkenny began reporting with St. Conal's (also in the town of Letterkenny). The latter event may be responsible for the apparent jump in first admission rates for the NW at that time. However, it is also possible that the 1991 jump in NW first admission rates reflects compensation for under-reporting in the previous years, or is due to some other unknown factor.

For the number of patients hospitalized with schizophrenia, the correlation with mean annual rain is $r = 0.52$, $p = 0.19$. For first admissions of schizophrenics, the correlation with mean annual rain is $r = 0.65$, $p = 0.084$. Thus, there is trend toward a correlation between rainfall and 1st admission rates of schizophrenia, but the trend does not reach statistical significance. One difference between Ireland and Yugoslavia was that both the range of rates for schizophrenia and the range of rainfall values were smaller in Ireland,

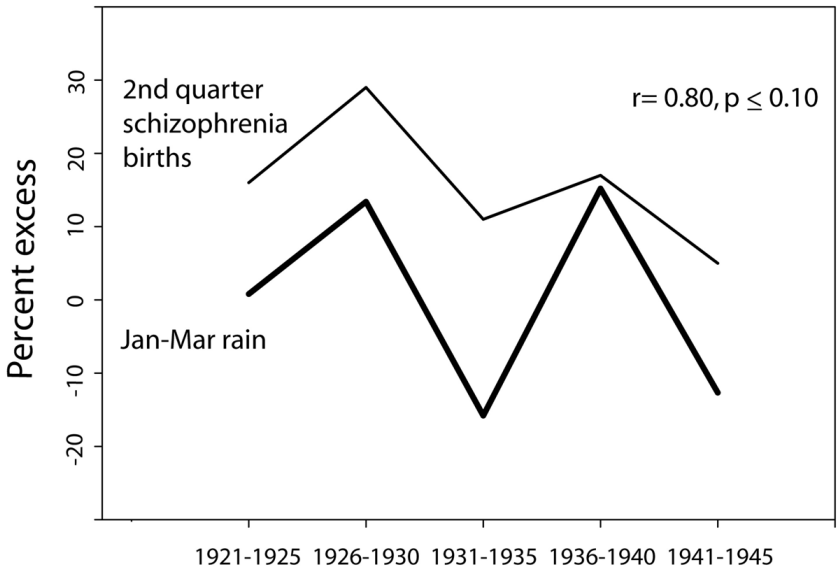


**FIGURE 4 | The relationship between the percent excess (of average for weather stations in Ireland) of Jan–Mar rain and the percent excess of 2nd quarter schizophrenia births (as compared to the year-round quarterly average for 5 year period) for time periods spanning the years 1921–1945 (season-of-birth data derived from O'Hare et al., 1980).** Note that Jan-Mar rain was not available from the series "British Rainfall" for the war year 1941, and thus the data point for the rainfall does not include 1941, though the schizophrenia data does include that year. To the extent that the weather in Ireland typically parallels the British Rainfall data mapped for Wales/West Midlands/Southwest England, the Jan–Mar rain would be expected to have been lower than average in 1941.

**Table 1 | Rates of hospitalization and annual rainfall, Yugoslavia.**

| Hospitalized Schizoprenic Patients (per 100,000)[a] | Mean annual rain (mm)[b] | Mean annual rain (mm) wi diverse ethnic regions removed |
|---|---|---|
| 200 | 1780 | 1780 |
| 100 | 1470 | 1483 |
| 50 | 906 | 917 |
| 20 | 948 | 932 |
| 0 | 764 | 766 |

[a] *Data obtained from Crocetti et al. (1964) for hospitalization rates in 1931.*
[b] *Data provided by the Yugoslavian Hydrometeorological Institute for the years 1925–1940.*

which decreases the power of the analysis. In addition, the scale of the data maps was more detailed for Yugoslavia than for Ireland. Thus, small scale variations in rainfall affecting a non-uniform distribution of population were more likely to confound the Irish data. For example, in County Donegal, the mean rainfall is 1455 mm, but relatively small proportion of the population lives in regions with rainfall values of that magnitude.

Thus, to more accurately represent the amount of rainfall the average person in a catchment area experiences, the rain data was weighted by population distribution (**Table 2**; Materials and Methods). This analysis assumed a similar distribution of population existed during the time period when rainfall might have exerted an effect. The results show that if the rain data is weighted by the exposure of the most populous cities in a catchment area (**Table 2**), the 1st admissions for schizophrenia correlated significantly with mean annual rainfall ($r = 0.71$, $p = 0.047$), and hospitalization rates showed a trend to correlate with mean annual rainfall ($r = 0.65$, $p = 0.082$).

## SEASON OF BIRTH EFFECT IN IRELAND

In 1980, O'Hare et al. published a study tracking the season of birth effect for births over a 35 year period in Ireland (1921–1955), reported for 5-year intervals. When compared to the expected number of births of individuals who would go on to develop schizophrenia, based on the total number of births in each quarter, the spring quarter showed a marked 29% excess of future schizophrenics born in the 5 year period of time 1926–1930. The rainfall data available for that time period also showed some notable trends. To quote from the publication British Rainfall (which covered Ireland during those years), "1928 was the 6th successive year in which the rainfall over the British Isles as a whole was in excess of the average…we have to go back to the 'seventies to find so long a run of wet years.… A run of six consecutive years each with an appreciable excess is, however, unprecedented."

Of the years encompassed by the season of birth study, maps of rain data for Ireland were available from the British Meteorological Service spanning the years 1921–1945, with the exception of 1941. The mapped isohyet lines made possible the easy calculation of the surface area covered by particular rainfall patterns. **Figure 4** illustrates the similarity in patterns between the national Jan–Mar quarterly excess in season of birth for schizophrenia in those years and the national excess or deficit in January to March rain for those years.

**Table 2 | Rates of hospitalization, first admissions and annual rainfall, Ireland.**

| Catchment area | Hospitalized Schizophrenic patients[a] (per 100,000 gen. population) | 1st Admissions for Schizophrenia (per 100,000 gen. population) | Mean annual rain[b] (mm) | Mean annual rain weighted by pop. exposed[c] |
|---|---|---|---|---|
| W | 166.6 | 34.7 | 1274 | 1248 |
| MW | 122.4 | 40.7 | 1134 | 1134 |
| S | 124.4 | 37.5 | 1441 | 1360 |
| NW | 102.0 | 32.7 | 1378 | 1317 |
| SE | 99.0 | 29.4 | 1063 | 1013 |
| MID | 103.4 | 27.8 | 935 | 935 |
| E | 65.9 | 30.5 | 1045 | 933 |
| NE | 84.4 | 24.6 | 986 | 912 |

[a] *Hospital census in 1991 (Health Research Board, Ireland).*

[b] *Mean annual rain in Ireland for the years 1941–1970.*

[c] *Population based on the 1986 census for Ireland.*

When the annual rain data was weighted by the underlying population distribution affected by each particular rain pattern, the degree of correlation with season of birth effect was high ($r = 0.80$) and trended toward significance ($p \leq 0.10$).

The possibility of a correlation was explored between 1st admissions for schizophrenia (**Table 2**) and degree of season-of-birth effect as reported by O'Hare et al. (1980), but no correlation was found ($r = 0.0034$, $p = 0.994$).

## DISCUSSION

The data assembled and analyzed for this study are entirely consistent with a role for photic cues in human development and behavior, in this case behaviors as pronounced as those seen in schizophrenia. The observed patterns could reflect differences in genetic traits of the populations, gene-environment interactions specific for certain genotypes in certain environments, and/or phenotypic plasticity that can occur for all genotypes.

The season-of-birth pattern strongly supports work by Messias et al. (2001, 2006) who demonstrated a remarkably similar finding for schizophrenia season-of-birth in Brazil, where the main variation in seasonal weather is limited to a January through March rainy season. In those studies, a significant association was found between rainfall during a given month and the number of individuals with schizophrenia with birth dates 3 months later. Similarly, McGrath et al. (2002) found a significant association between variations in perinatal sunshine duration and the season of birth effect in schizophrenia. Furthermore, a recent report demonstrates an equivalently strong season-of-birth effect in multiple sclerosis with peaks in April and May and in parallel with schizophrenia (Torrey et al., 1997; Davies et al., 2003), deficits in births of future multiple sclerosis patients during October and November (Dobson et al., 2013), a finding the authors attribute to variations in sunlight during gestation and to the resulting variations in vitamin D availability.

Although the association between schizophrenia and the influence of heavy rainfall on photic input represents a potentially important avenue of research, this study outcome does not preclude the involvement of other environmental variables influenced by rainfall. The critical variable that rainfall represents could also include lower temperature (although rainfall does not always correspond to lower temperature) and infectious disease (spread more easily when people must spend more time indoors). Others have shown (Hare and Moran, 1981; Kinney et al., 1993) that the degree of the season of birth effect for schizophrenia was proportional to the severity of weather near birth, but in that case an association was found with cold temperatures during the last trimester. Similarly, Kendell and Adams found an association with low temperature 6 months prior to birth (1991) and Gupta and Murray (1992) report an association between environmental temperature and the incidence and outcome of schizophrenia. Data presented here for Ireland would argue against infections as underlying the association between rainfall and schizophrenia because the rates for 1st admissions are highest in the rural south and west, where infectious disease spread through crowded indoor quarters was less likely than in the eastern urban areas. Furthermore, an aspect of the season-of-birth effect that has been somewhat overlooked (Torrey et al., 1997) is the consistent deficit in schizophrenic births occurring in the late summer and early fall, particularly evident at higher latitudes (Davies et al., 2003). The excess/deficit finding is more compatible with cyclic, seasonal decreases and increases in light than with spread of any single infectious agent. Despite the fact that peaks in specific infectious diseases certainly do occur for particular months, those peak months are not usually matched by a large deficit in a couple of months at the opposite end of the year.

### THE PROCESSING OF PHOTIC STIMULI IN ANIMALS

The availability of sunlight is undoubtedly one of the most important environmental factors that influence survival. Animal physiology is accordingly geared to respond to changes in both the sunlight intensity and duration, i.e., photoperiod. Photoperiod is defined as the length of time a given species perceives photic stimuli during the day and is obviously specific to the season of the year and to latitude. The response of the pineal to changes in the photoperiod involves regulation of melatonin production, a hormone integrally involved in setting the circadian clock (Bartness and Goldman, 1989). From the survival standpoint, a change in photoperiod is more informative for long term conditions in the postnatal environment than is temperature and for most animals,

the photoperiod determines whether reproduction occurs or not. In species that can reproduce at different times of the year or year round, the effects are more subtle and relate to postnatal hormonal levels, circadian entrainment and somatic measures of development.

Photoperiod is sensed via projections along the retino-hypothalamic pathway to the pineal gland, operating as a step function (Prendergast and Zucker, 2012). Below a given intensity range, there is no response and above that range, the response is constant until the light level drops again. Suppression of melatonin synthesis in the pineal progresses in the early dawn with the first faint signal that the sun will be rising soon. The sensitivity of response is species specific, such that 1 lux is reported to be sufficient to significantly suppress melatonin synthesis in the Syrian hamster (Brainard et al., 1982). But at 119 lux, a level comparable to a clear summer sunrise at northern temperate latitudes (Didrikas and Hansson, 2009), the human pineal will generally have downregulated melatonin production by only 50% (Zeitzer et al., 2000) with full suppression by ∼2500 lux (Coetzee et al., 1989; Arendt, 1998).

The relevant cues provided by photoperiod can be delivered both pre-and postnatally (e.g., voles, Lee and Zucker, 1988; collared lemmings, Nagy et al., 1993; Siberian hamsters, Stetson et al., 1986; Shaw and Goldman, 1995; and Prendergast et al., 1996; sheep, Ebling et al., 1989; and red deer, Adam et al., 1992). Perhaps of greatest importance to the behavior of interest, postnatal dopaminergic tone is influenced by the photoperiod experienced *in utero*. Dopamine controls prolactin levels, the most obvious expression of which is coat thickness and/or color in animals (Hoffman, 1978; Lee and Zucker, 1988). A short photoperiod upregulates hypothalamic dopaminergic activity, inhibiting prolactin release and initiating the development of a winter coat. Postnatal administration of dopaminergic antagonists can block development of the winter coat, whereas dopaminergic agonists promote a winter coat (Badura and Goldman, 1992; Gower et al., 1993). To what extent such striking gene-environment interactions are controlled by epigenetic changes is not known for mammals, though epigenetic modifications in response to photoperiod have been well documented in plants (Kim and Sung, 2010). Many photoperiod effects controlled by the pineal are encoded by the peptide hormone *alpha-MSH* (Kastin et al., 1967a,b), which is upregulated in response to long photoperiods, predominantly expressed in the cells of the intermediate pituitary and centrally, in the arcuate nucleus of the hypothalamus (O'Donohue and Dorsa, 1982; Hadley, 1984; Khachaturian et al., 1985).

The timing of photoperiod effects relevant to behavior could theoretically include events as early as the time of conception. As proposed by Jongbloet (1975) and Pallast et al. (1994), increased light duration during the summer could lead to release of ova that are over-mature and predisposed to defective development. However, the pre-natal critical period for the major photic effects on animal behavior and development probably lies closer to the equivalent of the last trimester in humans (Hoffman, 1978; Reppert, 1985; Stetson et al., 1986; Weaver et al., 1987; Lee and Zucker, 1988; Nagy et al., 1993; Bellavía et al., 2006; Butler et al., 2007). The types of behaviors influenced by photoperiod are species and gender dependent, and include the more bold behaviors observed for female Brazilian guinea pigs born in spring, whereas males do not show such clear differences (Guenther and Trillmich, 2013). Other behavioral effects are induced by a short postnatal photoperiod and include elevated measures of anxiety and depression seen in adult Siberian hamsters, collared lemmings, and nocturnal rodents, as well as reductions in learning and memory capacity seen in male white-footed mice (as reviewed by Walton et al., 2011).

In addition to photoperiod response, there are responses to sunlight intensity, some of which are not mediated by the pineal. In contrast to most other animals, humans have a large cutaneous surface area that is responds to sunlight by proportional (not stepwise) adjustments to sunlight intensity for both vitamin D (Chen et al., 2007) and melanotropin production (Farooqui et al., 1993; Chakraborty et al., 1996; Hiramoto et al., 2003).

Although sunlight-induced vitamin D is not an important source of vitamin D for lower animals, it has been shown that prenatal dietary vitamin D in Sprague-Dawley rats has significant effects on postnatal anxiety and social behaviors (Pan et al., 2013).

Therefore, at issue is which of these photic response processes might relate to observations that rates of schizophrenia vary with latitude, season of birth and rainfall? Photoperiod could certainly underlie a phenotypic response to latitude and season, but unlike processes modulated by sunlight intensity, it is not affected by weather. Rather, entraining the photoperiod is strongly tied to the calendar date and is seemingly independent of year to year fluctuations in precipitation or cloud cover. One of the most informative observational studies in this regard involved the coat color change in the snowshoe hare, in which it was demonstrated that a year with an unusually heavy spring snowfall pattern (and hence, cloud cover) did not change the date at which coat color changed from white to brown, leaving some animals brown against a white background (Mills et al., 2013).

Although weather does not alter photoperiod entrainment by the pineal, there may nevertheless be neurophysiological consequences resulting from weather changing the rate and the degree to which pineal melatonin is suppressed during the daylight hours. Thus, even the snowshoe hare study described above revealed a possible role for sunlight intensity, in that the hares began the development of a brown coat at the correct calendar time in a snowy spring but completed the transition from white to brown at a slower rate than during a less snowy spring (Mills et al., 2013). A pineal-mediated effect exerted by low light intensity occurring during a relatively long photoperiod has also been directly examined in birds, for which Kumar et al. (2007) found a delay in reproduction, explaining why in wild bird populations, heavy rainfall can similarly delay reproduction (reviewed by Small and Moore, 2009). The magnitude of the impact of a heavy rainy season on sunlight intensity has been quantified for the Tibetan plateau, where consistently heavy rain in summer decreases both the daylight duration and the sunrise to sunset light levels equivalent to those seen in spring (Liu et al., 2012). For humans, the effect of rain on photic input is further complicated by the need to be indoors during heavy rain and for much of the time period covered by the present study, the populations in question would have had limited alternatives for indoor lighting. Even with

optimal "daylighting" strategies seen now in modern building design, the maximum indoor sunlight levels achieved midday on a clear Stockholm day in December, for example, barely reaches above 200 lux (De Carli and Valeria De Giuli, 2009), with values less than ∼50 lux for at least some of the working hours after sunrise but before sunset. Cloud cover and rainfall could be expected to further decrease those levels by 70 and 83%, respectively (Luccini et al., 2003), depending on the thickness of cloud cover and the intensity of rain (Calbo et al., 2005).

With respect to the possible role of sunlight intensity in behavioral phenotypes, it has been proposed that cutaneous vitamin D generation may be involved in gestational effects that modulate the eventual development of schizophrenia (McGrath and Welham, 1999). In addition, other hormones of interest are produced in the skin in response to the intensity of the natural spectrum, including the melanotropin *alpha-MSH* (Farooqui et al., 1993; Lin and Fisher, 2007). Any matching CNS elevations of *alpha-MSH* via cues from the pineal would be expected to have important effects on learning and memory (LaHoste et al., 1980; Beckwith et al., 1989; Machado et al., 2010; Shen et al., 2013), the processing of sensory information (Miller et al., 1993) and feeding behaviors (Nahon, 1994, 2006).

## PHENOTYPIC PLASTICITY, GENETIC TRAITS AND GENE-ENVIRONMENT INTERACTIONS

The season-of-birth effect is clearly an example of phenotypic plasticity but it also offers a window into forces that may have selected for genetic change. There are many examples in animal evolutionary history of phenotypic plasticity giving way to related "hardwired" traits (Van Buskirk et al., 1997), a phenomenon that some evolutionary biologists term the "flexible stem hypothesis" (Wund et al., 2008; Tebbich et al., 2010; Muschick et al., 2011). The physiology of light-responsive genes normally seen with seasonal environmental changes in light levels would also be engaged when year round light levels become different, as happens for individuals migrating from southern to northern climates. But over evolutionary time, the more completely adapted physiologies will exhibit permanent genetic traits that have been selected for by the new environment. A potentially relevant example would be the phenotypic plasticity identified in monozygotic twins discordant for bipolar disorder, who carry epigenetic methylation differences in the receptor for the functional antagonist of *alpha-MSH*, the melanotropin receptor known as *GPR24* or *MCHR1* (Dempster et al., 2011). Yet, hardwired differences in that gene were also selected for and have been found to be associated with bipolar disorder across unrelated individuals (Miller et al., 2009).

The need for vitamin D may have played an important role in selecting for polymorphisms in a variety of light-responsive genes, including the melanotropins. Any genetic polymorphism that enhances the ability of vitamin D to be generated from light would have been advantageous in low-light regimes, except when vitamin D was easily obtained from the diet. It is well known that a lack of vitamin D causes rickets, which would have had a negative impact the ability to perform the physical work necessary to survive in historical times, but more importantly, frequently caused fatal outcomes during delivery because of the improper configuration of pelvis in severe rickets (Harrison, 1966; Cruickshank, 1967; Konje and Ladipo, 2000). Genetic polymorphisms that increase risk of fatal outcomes prior to successful reproduction are under intense negative selective pressure, readily apparent within a few generations (reviewed by Miller, 2009). In such a manner, certain polymorphisms in melanotropin genes may have become more prevalent in low-light environments if they positively affected the natural synthesis of vitamin D by reducing the synthesis and sequestration of melanin (Valverde et al., 1995). The evolutionary trade-off in this case would have been an increased prevalence of polymorphisms in melanotropin genes (*MCHR1, MC5R, MCHR2*) of risk for schizophrenia (Severinsen et al., 2006; Miller et al., 2009; Demontis et al., 2012).

Although no genetic associations between vitamin-D receptors or enzymes involved in its formation or degradation have yet been identified for schizophrenia, that outcome does not necessarily mean that vitamin D is without effect in modifying the phenotypic plasticity that is obviously present in the disease. An interaction between vitamin D and the melanotropin system during development has been demonstrated by Eyles et al. (2007) who found that prenatal vitamin D deficiency in rodents leads to elevations in the functional antagonist of *alpha-MSH*, the melanotropic peptide *MCH*. However, McGrath and colleagues have also shown that the relationship between vitamin D in gestation and subsequent schizophrenia may be complex, in that those with low maternal vitamin D are at increased risk of bearing offspring who become schizophrenic as are those with overly high vitamin D (McGrath et al., 2010a).

What might be the relative impact of phenotypic plasticity vs. genetic traits of risk? For schizophrenia, the calculations show that the impact of the season-of-birth effect is not minor but rather roughly equivalent to that of family history of disease (Mortensen et al., 1999). For the northern hemisphere, the population attributable risk caused by a late winter/spring birth is on average 3.3% (Davies et al., 2003) but ranges to 10.5% in some locales (Mortensen et al., 1999), whereas the population-attributable risk if a parent or sibling was schizophrenic was 5.5% in the Mortensen et al. study (1999).

The season-of-birth effect could also be viewed as an example of gene-environment interaction because the effect is not uniform across the population; rather, it is reported to be greatest in those without a family history of the disease (O'Callaghan et al., 1991). Similarly, gene-environment interactions may underlie the remarkably increased risk of schizophrenia for immigrant populations from Afro-Caribbean countries who have relocated to the U.K. (McGovern and Cope, 1987; Wessely et al., 1991; Harrison et al., 1997; Sharpley et al., 2001; Coid et al., 2008), as compared to the incidence of schizophrenia in their native lands (Hickling and Rodgers-Johnson, 1995; Bhugra et al., 1996; Mahy et al., 1999) and as compared to immigrants from other countries (Coid et al., 2008). Barring the unlikely possibility of preferential migration of the most genetically at-risk individuals, their increased predisposition to schizophrenia in the U.K. must be triggered by some factor in the environment interacting with particular aspects of their genetic background. The model put forth in this paper would presume that the culpable environmental factor is related to lower light levels in northerly climates, although

the effect of the stress of immigrating to a different culture cannot easily be discounted, as discussed by Coid et al. (2008). In addition, rates of usage of illicit drugs may be higher amongst Afro-Caribbeans immigrants, particularly of concern if the drug of choice is cannabis (McGuire et al., 1995; Moore et al., 2007; Arendt et al., 2008; Di Forti et al., 2009; McGrath et al., 2010b). The rate of cannabis use in the Afro-Caribbean immigrant population may be somewhat higher vs. long term residents of the U.K. (Harvey et al., 1990) but may not be higher than rates in their home countries which have been reported to be already quite high (reviewed by Sugarman and Craufurd, 1994; Maharajh and Konings, 2005). However, other investigators found no elevation in drug use in Afro-Caribbeans in the U.K. as compared to non-immigrants (Cochrane and Bal, 1989; McGuire et al., 1995; reviewed by Coid et al., 2008).

Obviously, two of the major light-responsive physiological systems that would be strongly affected in Afro-Caribbean immigrants to the U.K. would be UV-induced vitamin D and the melanotropins. UV-induced vitamin-D would be expected to be particularly low for these individuals (Ford et al., 2006; Chen et al., 2007), as would the stimulation of the light-responsive melanotropin system in the climatic regime found in the U.K., since skin pigment would be expected to lessen the responsiveness of *alpha-MSH* levels to the relatively low level of UV radiation found there (Holzmann et al., 1983; Altmeyer et al., 1986; Chakraborty et al., 1996). It is noteworthy that the schizophrenia risk is higher for the second generation than the first (McGovern and Cope, 1987; Coid et al., 2008), suggestive of epigenetic effects during growth and development.

There is reason to believe that gene-environment interactions may also underlie certain of the genetic associations with schizophrenia identified in the melanotropin genes (Severinsen et al., 2006; Miller et al., 2009; Demontis et al., 2012). When evaluating genetic association studies, it must be kept in mind that they occur in particular environments. Thus, the resulting associations can be for genetic polymorphisms which exhibit strong interactions with that environment as well as those that don't. Based on the "flexible stem" hypothesis, the expectation would be that if an association was found for an ancestral polymorphism of relatively lower prevalence in the study environment than in the ancestral environment, this outcome might be indicative of a gene-environment interaction causing disease in the study environment. The ancestral polymorphism would represent the "flexible stem" form of the gene, a form which eventually was selected against. Such may be the case for the association between schizophrenia and a coding change in the *MC5R* gene identified in a temperate-zone genetic association study (Miller et al., 2009). Against a background of other risk genes (*TDO2* and *MCHR2*), the *MC5R* polymorphism of risk (rs2236700) was unexpectedly found to be the ancestral allele, an allele roughly twice as prevalent in the Yoruba peoples of Nigeria as in Caucasians represented by the CEPH collection (HapMap, www.hapmap.org). Based on the fact that meta-analyses showed a higher incidence of schizophrenia with higher latitude (Saha et al., 2006), it would not be expected that an allele more prevalent in Nigeria would be associated with a greater risk of disease. But the key fact to remember is that in the genetic association study of interest (Miller et al., 2009) the disease was diagnosed in people living in another climate, i.e., in temperate zone latitudes.

Thus, the association of MC5R with schizophrenia could theoretically represent a gene-environment interaction relevant to the outcome seen for Afro-Caribbean peoples migrating to the U.K. *MC5R* is a receptor for *alpha-MSH* which, as described above, is one of the light-responsive hormones elevated during long-day photoperiods and when light levels are more intense. The function of its *MC5R* receptor is diverse, ranging from stimulation of sebaceous glands (Eisinger et al., 2011) to immunoregulation (Taherzadeh et al., 1999; Taylor and Namba, 2001), to behavioral effects that include modulation of aggression (Morgan et al., 2004). Because *MC5R*'s association with schizophrenia was identified against the genetic background of a risk allele for the immunomodulatory kynurenine pathway enzyme *TDO2* (Miller et al., 2009), it is most likely that the key action in this case would be the reported inhibition of *IFNγ* expression by *MC5R* (Taylor and Namba, 2001). *IFNγ* stimulates the expression of IDO (Taylor and Feng, 1991), one of the other enzymes responsible for activating the immunomodulatory, and pigment-generating kynurenine pathway. Kynurenine pathway activation has been demonstrated in several studies of schizophrenia (reviewed by Schwarcz et al., 2012). Although the necessary studies have not yet been done to determine the functional effect of the risk allele of *MC5R*, if it were to be the case that it coded for a less sensitive version of the *MC5R* receptor, the result would be increased activation of the kynurenine pathway, further augmenting pathway flux in low light environments where the *MC5R* agonist *alpha-MSH* is already low.

### OTHER EVIDENCE FOR THE INVOLVEMENT OF THE PHYSIOLOGY OF PHOTIC RESPONSE IN THE EXPRESSION OF SCHIZOPHRENIA

Additional evidence for light-responsive melanotropin involvement can be found in an alternative mechanism of action proposed for antipsychotic drugs (Miller, 2013), based on the observed reaction between antipsychotic drugs and a neurotoxic catecholamine breakdown product to form the more innocuous pigment polymer, melanin. Consistent with this outcome, the melanotropin *alpha-MSH*, which enhances melanin formation and its sequestration, has been shown to normalize sensory gating in an auditory model of a schizophrenia endophenotype (Miller et al., 1993). In contrast, the melanotropin that inhibits the formation of melanin and its subsequent sequestration (*MCH*), inhibits effective sensory gating (Miller et al., 1993; Chung et al., 2011). Furthermore, nutritional imbalances that perturb melanogenesis can also elicit symptoms of psychosis (reviewed by Miller, 2011).

## CONCLUSIONS

The correlations between epidemiological data and light levels are strong for schizophrenia, and should not be ignored in the search for means of lowering the incidence of this major mental disorder. The need for vitamin D may have affected not only gene frequencies of relevance to schizophrenia but may also have modulated gene-environment interactions that can occur in differing light regimes. We modern humans tend to downplay the effect of

environment in controlling our health and well-being, particularly in regards to an environmental force such as light that can be replaced by an artificial source. Yet it would be unwise to discount the importance of natural light, particularly when our reliance on it was so high during our recent evolutionary past.

## LIMITATIONS OF THE STUDY

The methods of ascertainment of cases in the data set for the former Yugoslavia cannot be effectively validated. Much criticism of diagnostic methodology has been directed toward many studies of schizophrenia, and the current study is particularly vulnerable to such critiques. Despite the fact that the methods employed in Ireland have been overseen and well-supervised by the Health Research Board of Ireland, their methodology undoubtedly changed over time. Furthermore, no correction for potentially confounding variables such as demographics of the local population, drug use or obstetrical complications was possible.

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