



Increased anxiety-like phenotype in female guinea pigs following reduced neurosteroid exposure *in utero*



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ABSTRACT

Neurosteroids are essential for aiding proper fetal neurodevelopment. Pregnancy compromises such as preterm birth, prenatal stress and intrauterine growth restriction are associated with an increased risk of developing behavioural and mood disorders, particularly during adolescence. These pathologies involve the premature loss or alteration of trophic steroid hormones reaching the fetus leading to impaired neurodevelopment. While the specific programming mechanisms are yet to be fully elucidated, in adult life, dysfunctions of allopregnanolone action are prevalent in individuals with depression, post-traumatic stress disorder and anxiety disorders. The objective of this study was to assess if changes in concentrations of the neurosteroid, allopregnanolone, may be a fetal programming factor in priming the brain towards a negative behavioural phenotype during the childhood to adolescent period using a guinea pig model. Pregnant guinea pigs received either vehicle (45% (2-hydroxypropyl)- β -cyclodextrin) or the 5 α -reductase inhibitor, finasteride (25 mg/kg maternal weight) from gestational age 60 until spontaneous delivery (~71 days gestation). Male and female offspring from vehicle and finasteride treated dams were tested at postnatal day 20 (juvenile-equivalence) in an open field arena, and hippocampus and amygdala subsequently assessed for neurological changes in markers of development and GABA production pathways 24 h later. Females with reduced allopregnanolone exposure in utero displayed increased neophobic-like responses to a change in their environment compared to female controls. There were no differences in the neurodevelopmental markers assessed; MAP2, NeuN, MBP, GFAP or GAD67 between intrauterine finasteride or vehicle exposure, in either the hippocampus or amygdala whereas GAT1 staining was decreased. This study indicates that an intrauterine reduction in the supply of allopregnanolone programs vulnerability of female offspring to anxiety-like disorders in juvenility without impacting long term allopregnanolone concentrations.

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1. Introduction

Behavioural and mood disorders remain a major clinical problem in today's society. Mood disorders can incorporate anxiety disorders, bipolar disorder and depression, whilst behavioural disorders include attention deficit hyperactivity disorder (ADHD) and conduct disorder among others (Costello et al., 2003). The prevalence of these disorders differs between the sexes, with

women more often developing mood disorders whilst males tend to develop behavioural disorders (Beesdo et al., 2009). The childhood and adolescent periods are times of vulnerability for onset of many of these problems, and adverse events during fetal life are increasingly being linked to an elevated risk of developing these disorders (Kofman, 2002; Moster et al., 2008; Pallotto and Kilbride, 2006).

Neurosteroids, particularly the 5 α -, 3 α -reduced progesterone metabolite allopregnanolone, are important endogenous neuroprotective and neurotrophic hormones (Purdy et al., 1990a). Allopregnanolone acts to enhance γ -aminobutyric acid (GABA) activity by increasing tonic conductance at the GABA type-A receptor (GABA_AR) (Purdy et al., 1990b; Hosie et al., 2006). The greatest concentrations of allopregnanolone are seen during fetal life when

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the placenta provides the majority of both allopregnanolone and precursors for its production (Gilbert Evans et al., 2005). Inhibition of allopregnanolone synthesis during late gestation using the 5 α -reductase (5 α R) enzyme inhibitor, finasteride, produces neurodegenerative effects within the fetal brain, including marked cell death within the hippocampus (Yawno et al., 2009). Finasteride treatment has also been shown to reduce expression of mature myelination and increase astrocyte activation within the fetal guinea pig hippocampus (Kelleher et al., 2011). These deficits are similar to those seen in animal models of placental insufficiency, highlighting the importance of neurosteroids in neurodevelopment (Yawno et al., 2011).

In postnatal life, allopregnanolone is increased during acute stress events, to attenuate the release of glucocorticoids and remodel the stress response (Patchev et al., 1996; Purdy et al., 1991). Furthermore, reductions in allopregnanolone levels have been found in mood disorders such as post-traumatic stress disorder (Rasmusson et al., 2006), premenstrual dysphoric disorder (PMDD) (Lombardi et al., 2004; Monteleone et al., 2000) and major depressive disorder (Ströhle et al., 1999; Uzunova et al., 2003; Evans et al., 2012). The limbic system controls the emotional responses and memory acquisition to external stimuli, and abnormal activation of this system underpins many anxiety and mood disorders (Gale et al., 2004; Etkin and Wager, 2007; Bannerman et al., 2004; Carlini et al., 2004; Andrews et al., 1994; Zola-Morgan et al., 1986). Inhibition of allopregnanolone production within the adult rat amygdala and hippocampus individually has been shown to increase anxiety-like behaviours (Walf et al., 2006; Frye and Walf, 2002), whilst administration of allopregnanolone into these two regions produces anxiolytic affects (Akwa et al., 1999; Mödöl et al., 2011). Considering the reciprocal connections between these two areas (Kim et al., 2001), it is important to consider the neurosteroidogenic influence on both regions in relation to behavioural output.

The actions of allopregnanolone are dependent on the presence of GABA in the extrasynaptic space (Bitran et al., 1995; Wang, 2011). The production and transport of GABA is controlled by glutamic acid decarboxylases 65 and 67 (GAD65 and GAD67) and GABA transporters 1 and 3 (GAT1 and GAT3) proteins, respectively. Changes in the levels of these enzymes impact the anxiogenic and anxiolytic behavioural output by the modulation of GABA tonic inhibition (Heldt et al., 2012; Liu et al., 2006; Jensen et al., 2003). The expression of GAD67 has been shown to co-localise with enzymes in the allopregnanolone synthesis pathway (Magnaghi et al., 2010), and GAT1 had been shown to co-localise with GAD67 (Yasumi et al., 1997). However, little is known regarding on the effects of allopregnanolone inhibition on these regulatory pathways, particularly in gestational programming of susceptibility to psychiatric disorders.

Allopregnanolone levels in the adult brain have been shown to be important in hyperactivity disorders, however it remains unclear if alterations in allopregnanolone exposure during pregnancy can affect vulnerability to these disorders. The objective of this study was to determine if a reduction in allopregnanolone levels during late gestation alters long term brain development and behaviour in juvenile guinea pigs.

2. Materials and methods

2.1. Animals and finasteride administration

Time-mated, outbred guinea pigs were acquired from the University of Newcastle Research Support Unit, and housed in individual cages within visual and vocal distance to each other in a home room for the duration of the protocol. All animals were acclimatised to researchers prior to commencement of protocol

to allow guinea pigs to familiarise themselves with researchers and reduce stress. Guinea pigs were randomly allocated to either vehicle (45% (2-hydroxypropyl)- β -cyclodextrin, 400 μ L/kg, Sigma Aldrich, Castle Hill, NSW, Australia) or finasteride (25 mg/400 μ L/kg (Kelleher et al., 2011), orally, Sternaloids, Newport, RI, United States) once daily, commencing at gestational age 60 until delivery (term \sim 71 days). The vehicle (2-hydroxypropyl)- β -cyclodextrin was used due to its capacity for dissolving and transporting hydrophobic drugs and its reported absence of side effects make it the most appropriate vehicle for these agents (Usayapant et al., 1991). This gestational age was chosen as allopregnanolone concentrations peak and begin to plateau at this age (Bennett et al., 2013; Bennett et al., 2016; Kelleher et al., 2013). Dams were allowed to spontaneously deliver, with sex, birth weight and nose-rump length recorded. Pups were maintained with dams in normal housing conditions until testing. On postnatal day (PND) 21 all juveniles (prenatal vehicle treatment n = 18, males = 9, females = 9; prenatal finasteride treatment, n = 15, males = 8, females = 7) were weighed and measured before euthanasia via carbon dioxide inhalation, with plasma and brain collection for analysis as previously described (Shaw et al., 2015; Bennett et al., 2015).

2.2. Behavioural testing

On postnatal day (PND) 20 all offspring underwent behavioural testing in open field and environment exploration as previously published (Bennett et al., 2015). Briefly, guinea pigs had pre-test saliva collected before being placed in the open field arena along the bottom wall and allowed to explore the arena for 10 min. Total distance travelled, number of grid crossings, and entries into the inner zone as well as time spent in the inner zone were recorded by the tracking software using whole body positioning of the animal. These parameters were used as measures of exploratory and motor behaviour. Neophobia-like responses to changes in the environment were assessed by willingness of offspring to interact with unknown objects. Immediately after the open field test offspring were placed in the arena with two fixed objects placed on the inner zone boundary of the open field testing. Guinea pigs were allowed to explore the objects for 10 min. The total amount of time spent investigating objects, as measured by time the head was within contact or within a 10% distance of the object's boundary, was used as a measure of neophobic-like behaviour. Following the test, post-test saliva was collected as above. The arena and objects were thoroughly cleaned between each test to remove olfactory cues within the arena.

2.3. Brain immunohistochemistry

Fixed, paraffin-embedded brains (prenatal vehicle n = 10; females n = 5, males n = 5; prenatal finasteride n = 11; females n = 6, males n = 5) were sectioned at an 8 μ m thickness in serials of 3, and immunostained as previously described (Bennett et al., 2013). Briefly, tissues were dewaxed and incubated in 10mM Citrate buffer, pH6.0 solution for antigen retrieval. Sections were blocked for 1 h (2% normal goat serum, 0.4% BSA, 0.3% Triton X-100), at room temperature, before overnight incubation in primary antibodies glial fibrillary protein (GFAP, G3893, 1:1000, Sigma; St. Louis, USA; (Poesen et al., 2008)), neuronal nuclei (NeuN, MAB377, 1:500, Millipore-Chemicon; Darmstadt, Germany; (Tsutsui-Kimura et al., 2015)), microtubule-associated protein 2 (MAP2, M9942, 1:20000, Sigma; St. Louis, USA; (Teng et al., 2001)), myelin basic protein (MBP, M9434, 1:1000, Sigma; St. Louis, USA; (Lampe et al., 1983)) or glutamic acid decarboxylase 67 (GAD67, ab26116, 1:1000, Abcam; Cambridge UK; (Cheng et al., 2016)), at room temperature. Primary antibody for GABA Transporter 1 (GAT1, ab426, 1:500, Abcam; Cambridge UK; (Aligny et al., 2014))

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