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Maternal separation stress reduced prenatal-ethanol-induced increase in exploratory behaviour and extracellular signal-regulated kinase activity

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ABSTRACT

In an attempt to better represent the aetiology of fetal alcohol spectrum disorder (FASD) and the associated psychological deficits, prenatal-ethanol exposure was followed by maternal separation in a rat model in order to account for the effects of early-life adversities in addition to *in utero* alcohol exposure. Extracellular signal-regulated kinase 1/2 (ERK1/2) and glycogen synthase kinase 3-β (GSK3β) are converging points for many signalling cascades and have been implicated in models of FASD and models of early-life stress. Therefore, these kinases may also contribute to the behavioural changes observed after the combination of both developmental insults. In this study, ethanol-dams voluntarily consumed a 0.066% saccharin-sweetened 10% ethanol (EtOH) solution for 10 days prior to pregnancy and throughout gestation while control-dams had *ad libitum*access to a 0.066% saccharin (sacc) solution. Whole litters were randomly assigned to undergo maternal separation (MS) for 3 h/day from P2 to P14 while the remaining litters were left undisturbed (nMS). This resulted in 4 experimental groups: *control* (sacc + nMS), *MS* (sacc + MS), *EtOH* (EtOH + nMS) and *EtOH + MS*. Throughout development, EtOH-rats weighed less than control rats. However, subsequent maternal separation stress caused EtOH + MS-rats to weigh more than EtOH-rats. In adulthood both MS- and EtOH-rats were hyperactive but the combination produced activity levels similar to that of control rats. All treated animals (MS-, EtOH- and EtOH + MS-rats) demonstrated a negative affective state shown by increased number and duration of 22 kHz ultrasonic vocalizations compared to control rats. Prenatal-ethanol exposure increased the P-GSK3β/GSK3β ratio in the prefrontal cortex (PFC) and maternal separation decreased the P-GSK3β/GSK3β ratio in the dorsal hippocampus (DH) of adult rats. However, maternal separation stress decreased the effect of prenatal-ethanol exposure on the P-ERK/ERK ratio in the PFC and DH and reduced prenatal-ethanol-induced hyperactivity. Therefore, indicating a significant interaction between prenatal-ethanol exposure and early-life stress on behaviour and the brain and may implicate P-ERK1/2 signalling in exploratory behaviour.

1. Introduction

Children born with foetal alcohol spectrum disorder (FASD) may also be subjected to adverse childhood experiences when living with alcohol-abusing parents [1]. It is well known that exposure to adversity in childhood can lead to the development of psychological disorders later in adulthood [2–5]. However, early-life adversity is a factor which has not yet been fully characterized in animal models of FASD. Recent studies have combined prenatal-ethanol exposure and early-life stress in an attempt to better understand the combination of these developmental insults on the brain and the long-term behavioural outcomes [6,7]. However, the molecular mechanisms have yet to be elucidated. Therefore, this study aimed to further investigate the combined effects of prenatal-ethanol exposure and early-life adversity on the brain and behaviour in a rat model.

Rodents exposed to ethanol have been shown to develop abnormal behavioural traits characteristic of FASD-associated psychological disorders [8]. For example, adult Sprague-Dawley (SD) rats exposed to ethanol throughout gestation spent more time in the closed arms of the elevated plus maze (EPM) compared to non-exposed control rats [9] which is indicative of heightened anxiety-like behaviour. Further, adult SD rats exposed to prenatal-ethanol spent more time immobile in the forced swim test (FST) indicating depression-like behaviour [10]. Ethanol-induced behavioural outcomes depend on several factors such as the timing, amount and pattern of ethanol exposure during development [8]. However, the effects of additional early-stress are not clearly understood. The maternal separation model of early-life stress [11,12] has been used to model early-life adversities after prenatal-

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ethanol exposure [6,7]. Maternal separation is usually performed during the early-postnatal period (P2 - P14), when glucocorticoid levels are low. Daniels and others have shown that maternal separation results in impaired feedback regulation of the hypothalamic-pituitary-adrenal axis which occurs via the hippocampus and prefrontal cortex (PFC) [13]. This leads to an imbalance in stress regulation and can have long lasting structural and functional consequences on the brain which may contribute to the development of behavioural abnormalities. [14–16]. Rats exposed to 3 h of daily maternal separation from P2 - P14 exhibited anxiety- and depression-like behaviour in the EPM and FST [12,17,18]. Further, maternally separated rats displayed increased number and duration of 22 kHz ultrasonic vocalizations [18] which is indicative of a depressive-phenotype [19]. Studies that have combined prenatal-ethanol exposure with maternal separation have shown modest interactions between prenatal-ethanol and maternal separation on exploratory activity [7]; and enhanced anxiety-like behaviour in the EPM compared to maternal separation or ethanol exposure alone [6]; as well as reduced ultrasonic vocalizations made by Long-Evans rat pups [20]. Therefore, the varying behavioural outcomes highlight the need to further investigate the combination of prenatal-ethanol exposure and maternal separation and more importantly explain the underlying neurochemistry.

At the neuronal level, extracellular signal-regulated kinase 1/2 (ERK1/2) and glycogen synthase kinase 3-β (GSK3β) are converging points for many signalling cascades that are involved in neurogenesis, differentiation, migration and survival [21]. For example, the mitogen-activated protein kinase (MAPK) signalling cascade consists of a series of serine/threonine kinases, including Ras, Raf and MEK, that result in the phosphorylation of ERK1/2. Activity of the MAPK pathway is mediated by several upstream activators and growth factors such as nerve growth factor, neurotrophins 3–6 and brain derived neurotrophic factor (BDNF) which bind to the family of tyrosine receptor kinases [22]. In addition, the phosphatidylinositol 3-kinase (PI3K) / Akt (also known as protein kinase B) pathway results in the phosphorylation and *inactivation* of GSK3β [23]. Significant components of these signalling cascades are vulnerable to ethanol exposure and dysregulation of these kinases may underlie the observed deficits in neural plasticity in FASD [24–28]. In addition, the HPA-axis hypo-responsive responsive period (P2 - P14 in rats) coincides with the brain growth spurt [29], characterized by a rapid increase in synaptogenesis, dendritic complexity and spine formation. ERK1/2 and GSK3β have a significant role during this period of rapid brain maturation and are susceptible to the effects of stress during this period due to crosstalk between glucocorticoid- and neurotrophin-signalling [30]. Changes in ERK1/2 and GSK3β signalling have also been implicated in maternal separation models of early-life stress in which animals presented with anxiety- and depression-like behaviour [15,17,31,32]. Since, the ERK1/2- and GSK3β-signalling cascades have been identified in models of prenatal-ethanol as well as models of early-life stress, they may also contribute to the behavioural changes observed after the combination of both developmental insults. Importantly, changes in ERK1/2 and GSK3β would influence the functionality of their downstream substrates such as cAMP response element binding protein (CREB), a transcription factor downstream of both ERK1/2 and GSK3β signalling [33–35]. Therefore, disruption of ERK1/2 and GSK3β signalling may lead to the irregular expression of plasticity-related proteins. Synaptophysin, a synaptic vesicle-related protein can then be used to indirectly measure synaptic density and identify deficits in neural plasticity [36] as a consequence of prenatal-ethanol exposure and maternal separation.

By coupling prenatal-ethanol with maternal separation stress we hoped to provide further insight into maternal separation as a factor representing early-life adversity in animal models of FASD and the associated-psychological disorders. We measured behavioural outcomes in adult rats using a battery of behavioural tests. These tests included the recording of ultrasonic vocalizations [19] and the EPM [37] to assess anxiety-like behaviour, the OFT to measure exploratory activity and the FST to measure depression-like behaviour [31,38,39]. We aimed to investigate the long-term prenatal-ethanol and early-life stress-induced changes in the ERK1/2- and GSK3β-signalling cascades in order to provide insight into the molecular mechanisms that underlie the behavioural abnormalities observed after exposure to these developmental insults. We hypothesized that adult rats exposed to prenatal-ethanol would show anxiety- and depression-like behaviour, as well as changes in neuroplasticity-related proteins compared to controls and that further additional maternal separation would enhance prenatal-ethanol-induced behavioural deficits and changes in neuroplasticity-related proteins.

2. Methods

2.1. Animals

Adult male and female Sprague-Dawley (SD) rats were obtained from the University of Cape Town, Faculty of Health Sciences Animal Unit and housed in a nearby Satellite Animal Facility. Animals were housed in a 12 h: 12 h light/dark cycle (lights on at 06:00) with *ad libitum* access to food and water. The temperature was maintained at 23 ± 1 °C and the light intensity at 120–150 lux. Ethics approval was obtained from The University of Cape Town Animal Ethics Committee [015/004]. All experimental procedures aimed to minimize the pain and suffering of the animals used and adhered to the guidelines set out in the South African National Standard: The Care and Use of Animals for Scientific Purpose (2008).

2.1.1. Prenatal-ethanol exposure

Adult female SD rats (4 months of age, weighing approximately 300 g) were introduced to a 0.066% saccharin solution with the choice of water for 24 h to introduce the dams to the voluntary drinking paradigm [40]. The following day the water bottle was removed. Saccharin-control dams (n = 13) continued to have *ad libitum* access to 0.066% saccharin solution whereas ethanol-treated dams (n = 11) were introduced to a 10% ethanol saccharin-sweetened solution in a stepwise manner (0%, 2%, 5%, 10%) every second day in order for the dams to become accustomed to the high ethanol concentration [40]. Ethanol-treated dams then had *ad libitum* access to 10% ethanol for 10 days prior to mating to establish a stable drinking pattern [40]. In previous studies, the blood alcohol levels were measured and found to be 80–90 mg/dl [41,42]. These are considered to be moderate levels of intoxication [43]. Once a stable drinking pattern was established, vaginal smears were performed [44] before male rats were paired with the adult females for breeding. Males and females were paired for the duration of the estrus cycle or until the presence of a vaginal plug, after which the male rat was removed. This was considered gestational day (GD) 1. A red plastic tube and nesting material (tissue paper and shredded paper) were placed in the cage with the dam throughout gestation. The 0.066% saccharin and 10% ethanol bottles were weighed daily throughout gestation to determine how much alcohol was consumed during pregnancy. Around the expected date of birth (DOB), females were monitored twice daily for the presence of pups. The DOB was designated as P0. On P2, litters were sexed and culled to a maximum of 10 pups with a minimum of 2 female pups per litter in order to promote equal nourishment and nurturing of pups [45,46]. Further on P2, the red tube and nesting material were removed from the cage to avoid any effect of environmental enrichment on the pups. The ethanol concentration was then decreased in a stepwise manner (10%, 5%, 2%, 0%) every 2 days in order to minimize the effects of ethanol withdrawal. Therefore, by P6 ethanol-treated dams had *ad libitum* access to 0.066% saccharin.

2.1.2. Maternal separation

Whole-litters were chosen at random to undergo maternal separation (MS, n = 13 litters) from P2 to P14 for 3 h/day (09:00–12:00)

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