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Prenatal nicotine exposure and maternal separation alter the nicotinic acetylcholine receptors in mice

By

Muhamed Waseem Khan

2019

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Submitted in fulfilment of the requirements for the degree of Master of Medical Science (MSc) in the Department of Human Physiology, in the Faculty of Health Sciences at the University of KwaZulu-Natal – Westville Campus

Supervisor: Prof MV Mabandla _____

Date Submitted: _____

Preface and Declaration

The experimental work described in this thesis was conducted at the Westville Campus of UKZN under the supervision of Prof M.V. Mabandla.

I, Muhamed Waseem Khan, declare that:

i. The research reported in this thesis, except where otherwise indicated, is my original work.
ii. This thesis has not been submitted for any degree or examination at any other university.
iii. This thesis does not contain person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
iv. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written, but the general information kept the same.

Muhamed Waseem Khan _____

Date _____

Acknowledgements

Prof MV Mabandla

Dr GT Ngoupaye

Staff at the BRU and Department of Human Physiology

The UKZN Neuroscience Team

My family and friends

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Abbreviations

α –	alpha
µ -	micro
11-HSD –	11 hydroxysteroid dehydrogenase
Ach –	acetylcholine
AChE –	acetylcholinesterase
ACTH –	adrenocorticotropic hormone
CRH –	corticotrophin releasing hormone
DA –	dopamine
DAT –	dopamine transporter
EDTA –	ethylenediamine tetraacetic acid
EPM –	elevated plus maze
GABA –	gamma aminobutyric acid
GC –	glucocorticoid
GR –	glucocorticoid receptor
GRE –	glucocorticoid response element
HPA –	hypothalamic pituatry adrenal
MR –	mineralocorticoids
nAChR –	nicotinic acetylcholine receptors
NE –	norepinephrine
OD –	optical density
OF –	open field
PND –	postnatal day
PNE –	prenatal nicotine exposure
SEM –	standard error of the mean
VTA –	ventral tegmental area

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General abstract of the thesis

Maternal cigarette smoking during pregnancy has been associated with long term cognitive dysfunction. The harmful behavioural effects of cigarette smoking have been shown to be primarily due to nicotine. While the mechanism of nicotine's harmful actions remain unclear, studies have shown a link to the hypothalamus–pituitary–adrenal (HPA) axis. HPA axis dysfunction as a consequence of exposure to perinatal stressors such as maternal separation results in major long-term systemic and neurological disruptions and malfunction. Using a mouse model we showed that prenatal nicotine exposure (PNE) resulted in hyperlocomotivity. There are also long-term increases of the α7 nicotinic acetylcholine receptor (n-AChR) expression while the α4 n-AChR expression was decreased. Glucocorticoid receptor (GR) expression varied in the PNE groups by brain location, while no changes were found in dopamine concentration in the hippocampus or striatum. Maternally separated animals exhibited anxiety-like behaviour in the open field test. There were also significant changes in the hippocampal expression of n-AChRs, specifically decreased α4 expression and increased α7 expression of the maternally separated animals suggesting a link between HPA dysfunction and cholinergic signalling. Animals exposed to both stress and nicotine insults however show no significant difference in α7 nAChR, GR or dopamine levels when compared to the control. However, α4 nAChR expression was significantly different in the hippocampus but not the striatum of animals who experienced both insults when compared to the control. This suggests that this may be due to competitive inhibition as a result of the link between nicotine exposure in utero and HPA axis dysfunction.

Chapter 1: Introduction

The harmful effects of cigarette smoking during pregnancy for both the mother and foetus are common knowledge (England et al., 2017). However approximately 10% of women continue smoking during pregnancy and that number is estimated to be higher in third world countries (Tong et al., 2013). Maternal tobacco smoking has been established to have negative effects on birth weight, cognitive development and behaviour, and increases the risks of foetal morbidity and mortality (Wickstrom, 2007, Huizink and Mulder, 2006, Sexton and Hebel, 1984) . While cigarettes contain up to 400 toxic ingredients, the harmful behavioural effects seen in cigarette smoking are considered to be primarily due to nicotine's actions on both the mature and developing brain (Aoyama et al., 2016, Slotkin et al., 1990,). Prenatal nicotine exposure (PNE) has been linked to multiple neurodevelopmental disorders such as anxiety, ADHD, depression, addiction, conduct disorder and schizophrenia (Tiesler and Heinrich, 2014, Brown et al., 2000).

Nicotine exerts its effects via the cholinergic signalling pathway in the brain. It binds to acetylcholine receptors, specifically the nicotinic acetylcholine receptors (nAChR). Cholinergic signalling has effects on the regulation of neurogenesis, neuronal differentiation and migration through the other neurotransmitters, including dopamine (Bryden et al., 2016, Zhu et al., 2012). Therefore, nicotine exposure to the foetus results in more than just cholinergic dysfunction, specifically, it alters dopaminergic (DA) and other neurotransmitter signalling that is responsible for brain development in the foetus (Bryden et al., 2016, Zhu et al., 2012). Alterations of the DA system during foetal development have also been shown to change the responsiveness of the hypothalamus–pituitary–adrenal (HPA) axis during stress (Uban et al., 2013).

Stress during critical developmental stage of the brain in early life may cause long-lasting alterations that result in neurological disorders such as anxiety, addiction and depression (Lundberg et al., 2017, Popoli et al., 2012). Stress results in increased glucocorticoids in the general circulation as an effect of continuous stimulation of the HPA axis from the hypothalamus (Turecki and Meaney, 2016). The paraventricular nucleus in the hippocampus secretes CRH (corticotropin releasing hormone) which stimulates the anterior pituitary to release Adrenocorticotropic hormone (ACTH). This promotes cortisol release from the adrenal cortex which binds to glucocorticoid receptors to exert its effects (Lundberg et al., 2017, Popoli et al., 2012) . Cortisol is a steroid glucocorticoid hormone in humans with the

equivalent in rodents being corticosterone (Kanatsou et al., 2016). Animals exposed to developmental stress, such as maternal separation, show altered neurobehavioural effects such as anxiety-like behaviour and are more prone to addiction (Kanatsou et al., 2016). Exposure of an offspring to maternal separation results in excessively increased ACTH and corticosterone levels causing anxiety-like behaviour as well as cognitive impairment and structural changes in neuronal dendrites and spines in the hippocampus and prefrontal regions of the brain involved in emotion and behaviour control (Kanatsou et al., 2016).

Conversely, nAChRs themselves have also shown involvement in the stress response in the mature brain. A non-selective nAChR antagonist, mecamylamine, prevents CRH-induced rises in plasma corticosterone (Okada et al., 2008) and nicotine-induced increases in urinary corticosterone (Loomis and Gilmour, 2010). However, it remains unknown which types of nAChRs and how their location within the brain controls this process (Holgate and Bartlett, 2015). The α4 nAChR subunit plays a prominent role in alcohol consumption driven by stress and genetic studies in humans have pointed to mutations in gene encoding the α4 subunit being responsible for an increased susceptibility to depression (Reuter et al., 2012). A developmental stressor in the form of prenatal stress has also been shown to alter both α4β2 and α7 nAChR expression in the hippocampus (Baier et al., 2015, Schulz et al., 2013).

Therefore, one of the possible deleterious effects of PNE is a dysregulation of the foetal HPA-axis (Huizink et al., 2004). Glucocorticoid receptors (GR) control the organism's response to stress and control the programming of the HPA axis (Slone and Redei, 2002). It has been shown that PNE results in foetal rats being over-exposed to maternal GCs, which can inhibit the development of the foetal HPA axis (Zhang et al., 2014). The expression level of foetal GR was increased due to PNE while the activity of the foetal HPA axis was inhibited (Xu et al., 2012, Chen et al., 2007).

Multiple animal models of developmental nicotine exposure exist, however it is essential to consider the administration route and dose of nicotine consumed (Alkam et al., 2013). Injections of nicotine at doses mimicking human consumption induce maternal stress and high plasma concentrations of nicotine immediately post injection (Slotkin et al., 1987). These transient elevated concentrations of nicotine cause hypoxia in the foetus (Slotkin et al., 1987). The implantation of a mini-osmotic pump, while getting rid of plasma concentration differences, changes nicotine concentration in plasma during non-active parts of the day and adds a stress.

Nicotine in the drinking water eliminates the variables of stress and hypoxia and mimics the periodic consumption of nicotine in smoking during active hours of the day by the general human population (Alkam et al., 2013). This model also allows the animal to have three weeks of prior consumption to nicotine for them to acclimatise to its taste. It also mimics the maternal plasma concentrations of nicotine seen in human maternal smoking prior to pregnancy i.e. women who smoke, are trying nicotine replacement therapy or are exposed to second hand smoke during pregnancy do not begin exposure when they fall pregnant (Alkam et al., 2013). The exact timing of when nicotine exposure begins to alter development has also not been fully clarified therefore making maternal nicotine plasma concentration an important variable.

1.1 Prenatal nicotine exposure (PNE)

Nicotine acts as an exogenous agonist of Ach, with the ability to bind to nAChRs. These receptors are pentameric ligand-gate cation channels consisting of homomeric or heteromeric subunit combinations, and are widely present in the mature and developing nervous system. The agonism of nAChRs is extended by nicotine in comparison to ACh as there are differences in concentrations and clearance mechanisms between the two compounds (Tiesler and Heinrich, 2014, Moylan et al., 2013). Overstimulation of nAChRs by nicotine may have varied developmental influences that are dependent on the pharmacological properties and localisation of the receptors. Nicotine has been known to cross the placental barrier, concentrate in foetal blood and amniotic fluid, and is present in breast milk when taken by pregnant women (Archer et al., 2014). Nicotine is usually present in high concentrations in utero which consequentially allows enhanced activation of nAChRs, allowing modifiable effects such as receptor desensitisation (Moylan et al., 2013). As neurotransmitters act as trophic factors and play a role in both the architectural and cellular development within the developing central nervous system, activation of their receptors exert different effects than it would in a fully developed nervous system (Wickstrom, 2007). It has been suggested that through its receptors, specifically nAChRs, ACh plays a role in brain maturation in both foetuses and infants up to adolescence (Tiesler and Heinrich, 2014).

Nicotinic receptors are considered neuroprotective particularly in circumstances contesting neuronal survival (Winzer-Serhan, 2007), the heteromeric α4/β2 nAChR functions to increase neuronal survival. While the homomeric α7 receptor can stimulate the increase of cell death in developing neurons. As nAChR are area specific in the foetal brain, the particular effects

could result in decreased or increased neuronal vulnerability depending on the subtype expression (Winzer-Serhan, 2007). Nicotine could therefore have neurotoxic effects, to which immature neurons are particularly vulnerable.

Nicotine has an additive effect of alteration of neurotransmitters and their systems, possibly via a modulatory effect of presynaptic nAChR (Figure 1). Studies have shown elevated presynaptic release of ACh, DA, glutamate and GABA (Tiesler and Heinrich, 2014). Receptor mediated signalling pathways have similarly been shown to be altered postsynaptically. This cellular development disruption, by means of neurotransmission, would likely further alter synaptic function (Tiesler and Heinrich, 2014). Catecholamines have exhibited this synaptic hypoactivity in the postnatal period and into adolescence (Chen et al., 2017). Consequently, as a result of both neurotransmitter dysfunction and developmental disruption, nicotine exposure during the developing period has been shown to elicit long lasting abnormal behaviours such as impairment of emotion, cognitive function, attention, anxiety and hyperlocomotion (Tiesler and Heinrich, 2014).

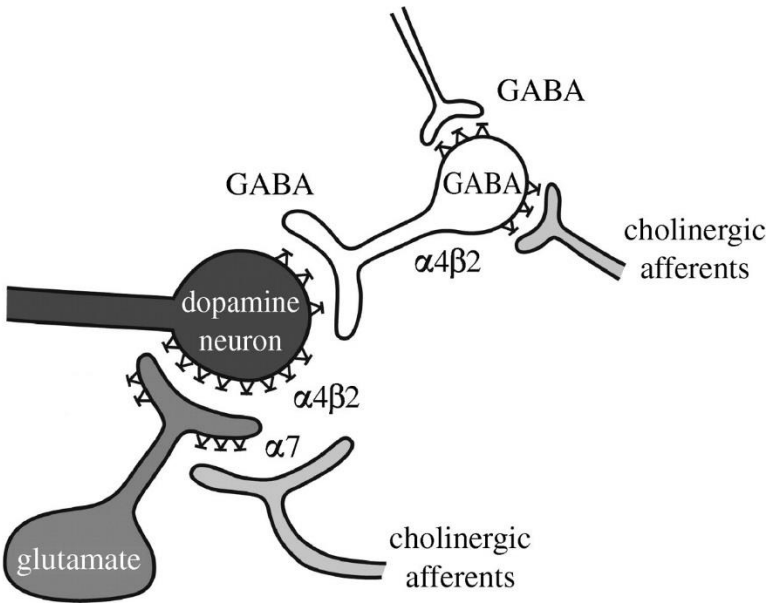

Figure 1: Cholinergic signalling altering the dopaminergic system via glutamatergic and GABA-ergic neurons (Adapted from Markou (2008))

1.2 Stress

Stress can be defined as exposure to any environmental condition that disturbs the homeostasis of an organism (Kagias et al., 2012, Charmandari et al., 2005). This exposure stimulates the brain to initiate a chain of adaptive physiological responses that results in the

release of neurotransmitters, peptides, and hormones throughout the body as the homeostatic control of stressful situations (Joëls and Baram, 2009). Early life stress exposure has been shown to be a contributing factor in cognitive impairment (Maccari and Morley-Fletcher, 2007; Ruiz and Avant, 2005) . The period of the stress experience is also of significance as a determining factor both structurally and functionally in the severity of cognitive impairment.

1.2.1 Postnatal stress exposure

Postnatal or postpartum stress is exposure of an infant to stressful or adverse life events after birth, which can lead to a number of neurological and behavioural disorders such as learning deficits, ADHD and anxiety disorders (Zalosnik et al., 2014). Exposure to postnatal stress results in increased glucocorticoids in the general circulation as an effect of continuous stimulation of the HPA axis from the hypothalamus (Popoli et al., 2012).

Stressful experiences during the developmental stage of the brain in early life can induce numerous changes in the HPA axis, neurotransmitter and neurotrophin levels (Zalosnik et al., 2014). This causes long-lasting alterations in structure and function of the hippocampus as well as synaptic plasticity resulting in deficits of learning and memory in an offspring (Lupien et al., 2009).

Immediately after birth, offspring have elevated resting corticosterone levels which progressively decline and remain low from postnatal day 4 (P4) until P14 which is called the stress-hyporesponsive period (Mabandla and Russell, 2010, Meaney and Aitken, 1985). The period is characterised by low circulating corticosterone levels due to the insensitivity of adrenal glands to the low levels of circulating ACTH and a low glucocorticoid receptor concentration in the hippocampus as well as the inability of mild stressors to induce a corticosterone response (Mabandla and Russell, 2010, Schmidt, 2010,). The stress-hyporesponsive period is critical in protecting the developing brain from the impaired neural and behavioural development that accompanies excessively high glucocorticoid levels (Sapolsky and Meaney, 1986). Offspring exposed to postnatal stress in the form of maternal separation results in markedly increased ACTH and corticosterone levels causing anxiety-like behaviour (Daniels et al., 2004). This increase also results in cognitive impairment and structural changes in neuronal dendrites and spines in the striatum which is involved in emotion, behaviour control and movement (Zalosnik et al., 2014, Aisa et al., 2009, Matthews et al., 1996) .

1.3 Effect of nicotine on HPA axis

Studies conducted in rats illustrate the relationship as nicotine and smoking stimulate the HPA axis and continue via a central mechanism stimulating CRH which results in the release of ACTH from the anterior pituitary (Tweed et al., 2012). Smoking cigarettes containing higher nicotine content also leads to increased peak nicotine levels followed by an increase of HPA axis hormones (ACTH and cortisol).

Interestingly, the use of nicotine replacement reduced affective withdrawal symptoms in dependent smokers who recently quit (Tweed et al., 2012). And dependent smokers who report that smoking helps cope with stress found that smoking cessation is accompanied with reduction of stress (Tweed et al., 2012). Wong et al. (2014) also found decreased levels of cortisol in regular smokers that abstained from smoking. Therefore, despite the acute effects smoking may have on perceived stress, it appears the long-term effect of smoking may worsen stress levels (Hajek et al., 2010). This elevated stress in smokers, reflects a dysregulated HPA axis.

1.3.1 Prenatal Nicotine Exposure and HPA Axis

One possible mechanism that PNE could harm development is a dysregulation of the HPA-axis (Huizink and Mulder, 2006). The characteristics and specific mechanisms regarding the effects on the HPA axis during different periods and the long-term detrimental effects have not been fully elucidated (Zhang et al., 2014). However, GC and their receptors are the final effectors of the HPA axis and participate in the control of whole body homeostasis and the organism's response to stress as well as the programming of the HPA axis (Joëls and Baram, 2009).

As such, PNE has resulted in foetal rats being over-exposed to maternal GCs, which can inhibit the development of the foetal HPA axis (Zhang et al., 2014). It has also been indicated that the dysfunction of the HPA axis developmentally is caused by GCs along with alterations of GCs receptor density in regions including the prefrontal cortex and hippocampus. It has also been demonstrated that prenatal nicotine exposure resulted in over-exposure to maternal GC, and the expression levels of foetal hippocampal 11-hydroxysteroid dehydrogenase-1 (11-HSD-1) and glucocorticoid receptor (GR) were increased while the activity of the foetal HPA axis was inhibited (Figure 2) (Liu et al., 2012, Xu et al., 2012,Chen et al., 2007).

Furthermore, increased stress-induced GC release has been shown to stimulate the dopamine pathway (Zhang et al., 2014).

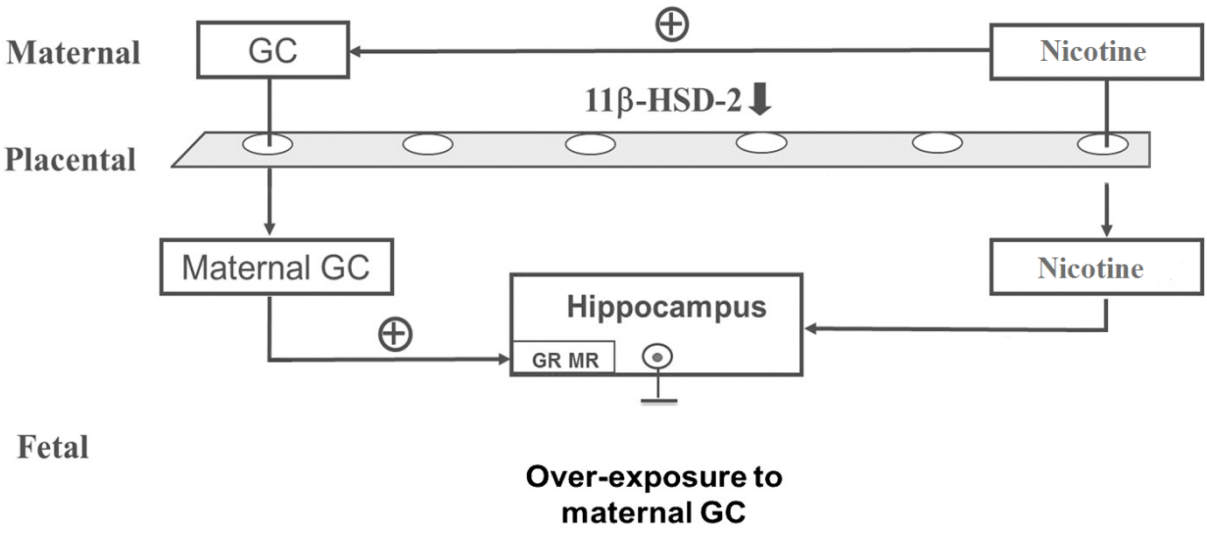

Figure 2: Nicotine results in overexposure of the foetus to maternal glucocorticoids (Adapted from Zhang et al. (2014))

1.4 Stress and dopamine

Although cortisol acts at GR and mineralocorticoid receptors (MR), it has been hypothesised that GC/GR could affect the synthesis and metabolism of DA and NE which are systems that are disturbed by PNE (Chen et al., 2017). Previous studies suggested that GC/GR could affect not only the synthesis and metabolism of DA and NE but also the function of DA system (Chen et al., 2017).

To further illustrate the relationship, Chen et al. (2017) showed a GR agonist can reduce the expression of dopamine transporter (DAT) in the brain of ADHD rats, while the GR inhibitor did the opposite. The GR agonist also led to the levels of DA and NE increasing. It has been shown that the activated GR bounded to glucocorticoid response element (GRE) affects the production and survival of DA neurons and regulates the activity and secretion of DA which disrupts the DA system's function to maintain the normal functioning of the body (Yang et al., 2007).

With regard to the MR receptor, de Oliviera et al. (2017) recently showed that the ventral tegmental area (VTA) upregulates the dopaminergic system. This points to regional specific actions of the HPA receptors being influenced and further altering the DA systems, summarised by Figure 3.

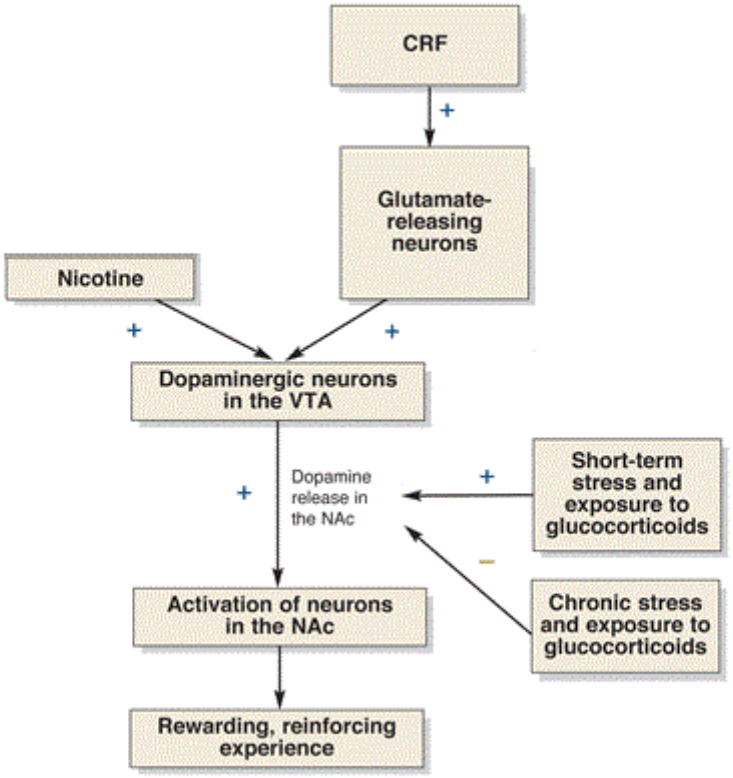

Figure 3: Relationship between nicotine and HPA axis via the dopaminergic system (Adapted from Wand (2008))

While there have been many studies exploring the effects of PNE on the HPA axis, the results have not yet fully elucidated the relationship. Additionally, many studies have explored the effect of stress exposure in adulthood on nAChRs, but few have looked at developmental stress.

1.5 Aims of the present study:

Our study aims to further elucidate the relationship between:

i. Early stress exposure, in the form of maternal separation, and the cholinergic system

ii. a double insult induced by the prenatal nicotinic exposure and a subsequent exposure to maternal separation in the brain. Specifically, to understand how this double insult may affect the nicotinic and glucocorticoid receptors

Chapter 1 reviewed the literature regarding the cholinergic system and perinatal stressors and how these affects are long lasting and affect behaviour later in life. Chapter 2 is a study investigating the effect of postnatal stress in the form of maternal separation on the receptors of the cholinergic system.

Chapter 2 has been submitted to *'Behavioural and Brain Functions'* and uses the Vancouver style of formatting.

Early postnatal stress exposure alters nicotinic acetylcholine receptor expression in the hippocampus leading to anxiety-like behaviour later in life

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Early postnatal stress exposure alters nicotinic acetylcholine receptor expression in the hippocampus leading to anxiety-like behaviour later in life

Abstract

Exposure to early developmental stressors have been shown to lead to neurobehavioural disorders such as depression and anxiety as a consequence of long term hypothalamus–pituitary–adrenal (HPA) axis dysfunction. This dysfunction often leads to major systemic and neurological malfunctions and disruptions of neurotransmitter signalling pathways such as the cholinergic system. Disruption of cholinergic innervation and receptors has been linked to cognitive impairment, and often plays a role in both depression and anxiety disorders. While the effect of prenatal stressors on the cholinergic receptors has been studied, the effect of postnatal stress has not. Using a mouse model of maternal separation, a model of postnatal stress, we assessed anxiety-like behaviour in the elevated plus maze and the open field apparatus. We also measured hippocampal expression of the nicotinic acetylcholine receptors (n-AChRs), α7 and α4. Glucocorticoid receptor expression and dopamine concentration were also measured. Exposure to maternal seperation resulted in increased anxiety-like behaviour in both the elevated plus maze and open field test. The expression of α7 nicotinic acetylcholine receptors (n-AChRs) was increased in the maternally separated group while the α4 n-AChRs was decreased. No changes were observed in glucocorticoid receptor and dopamine concentration in the groups. The result suggests a long term connection between HPA axis and cholinergic signalling as well as species specific responses to stress. These receptors have never been observed before in postnatal stress exposure and supports further research into cholinergic networks in relations to both anxiety and depressive disorders as well as developmental cognitive functioning.

Key words: postnatal stress, maternal separation, n-AChR, dopamine, glucocorticoid receptors, HPA axis

1. Introduction

Early life stress exposure has been shown to be a contributing factor in cognitive impairment (1, 2). Perinatal stress is exposure of the foetus or infant to stressful or adverse life events before or after birth and can lead to a number of neurological and behavioural disorders such as anxiety disorders, learning deficits, schizophrenia and depression (3). The aetiology of these disorders is also linked to a dysfunction in the cholinergic system, specifically the nicotinic acetylcholine receptors (n-AChR) (4). The cholinergic network in the central nervous system modulates plasticity, neuroprotection and neurodegeneration (5).

The most abundant n-AChRs in the brain are the α7 n-AChR and α4β2 n-AChR subtypes. Loss of cholinergic innervation and n-AChRs is a normal result of aging and has been widely linked to the development of cognitive impairments (6). It has been shown that hippocampal concentration of the acetylcholine precursor, choline, increases with corresponding alleviation of a patient's depression symptoms (7). Furthermore, in animal studies, drugs targeting either n-AChR subtype modulate depressive-like symptoms (8, 9). In addition, activation of hippocampal α7 n-AChR changes anxiety-like behaviour in a social anxiety paradigm (10). Interestingly having both anxiogenic and anxiolytic effects depending on dose and route of nicotine administered (10).

The link between developmental stressors and the n-AChRs has been studied using prenatal restraint stress which has shown to increase α4β2 n-AChR in the hippocampus, while the hippocampal α7 n-AChR increases were gender dependant (11). Baier et al. (12) found that α7 n-AChR was increased in the hippocampus of rats restrained prenatally. While these studies tested the effect of prenatal stress, the influence of postnatal stress on the n-AChRs remain unexplored.

Established by a similar link in neurobehavioural disorder aetiology and strengthened by the results of prenatal restraint stressors on the n-AChRs, we aimed to explore whether exposure to postnatal stress in the form of maternal separation will change the expression of the most abundant n-AChRs in the hippocampus and whether this can be linked to behavioural changes in these mice.

2. Materials and methods

2.1 Animals

Five male and ten female C57BL/6 mice were acquired from the Biomedical Resource Centre (BRC) of the University of KwaZulu- Natal. The animals were housed at room temperature (± 22˚C) and 70 % humidity (standard BRC conditions). A 12hr light/dark cycle (lights on at 06h00) was maintained and food and water were available ad libitum. All experimental procedures were approved by the Animals Ethics Research Committee of the University of KwaZulu-Natal in accordance with the guidelines of the National Institute of Health, USA (Ethics clearance number AREC/076/015D).

2.1.1 Prenatal handling and mating

The female mice were allowed to synchronize there oestrus cycles by placing them in pairs before mating commenced.

2.1.2. Postnatal Handling

Following birth, the litters were divided into a stressed and non-stressed groups. Birth was termed postnatal day 0 (PND 0) and on PND 2 the stressed group underwent maternal separation until PND 14. The pups stayed in the home cage until PND 21 when they were weaned.

2.1.3. Maternal separation

The dams were removed from their pups and taken to a separate room for 3 hours once a day from 09h00 to 12h00.

2.2. Grouping and study design

2.2.1 Grouping

Sixteen C57BL/6 male mice were used for this study and were grouped (n=8) as follows: Group one: control not stressed (W); Group 2: antenatal stress in the form of maternal separation (S).

2.2.2. Study design

After the animals were obtained, the female mice were allowed to synchronise their oestrus cycle before a male was placed in the cage for mating. After birth, the pups were divided into stressed and non-stressed groups. Those that were stressed were separated from their dams as described above. After weaning on PND 21, only males were kept for this study.Following weaning, mice were housed eight per cage and had access to food and water ad libitum. On PND 35 the elevated plus maze test was carried out. This was followed three weeks later (PND 58) by assessment using the open field after which the animals were sacrificed on PND 59.

2.3. Behavioural tests

2.3.1. Elevated plus maze

The elevated plus maze is used to measure anxiety-like as well as locomotor activity (Elliot, Faraday 2006). It involves placing the animal in a cross shaped apparatus, with two open and two closed arms, for five minutes. The arms' measurements are width: 10 cm, length: 50 cm, height: 30 cm, elevation height from floor: 55 cm. The animal's aversion to exploration (anxiety-like behaviour) and movement are measured by counting arm entries and the amount of time spent in the closed arm.

2.3.2. Open field test

The open field apparatus was used to further assess the activity and exploratory behaviour of the animals. Each animal was placed in a 40× 60 × 50 cm arena, which had 12 identical rectangular grids. The total time spent in the inner zone of the field was recorded for a period of 5 minutes. Both behavioural tests were video recorded.

2.4. Decapitation

A day following the open field test, the animals were sacrificed by decapitation. The striatum and hippocampus were dissected out for neurochemical analysis. The samples were stored in a biofreezer at -80°C until neurochemical analysis was performed.

2.5. Western blot

The western blot was used to quantify the expression of α4 n-AChR and α7 n-AChR expression in the hippocampus.

Hippocampal and striatal tissue were thawed on ice and the homogenate was prepared in a ratio 10% w/v for protein determination using the Bradford Reagent (Bradford, 1976). After measuring protein concentration, the samples were diluted with B-mercaptoethanol to the required amount of protein, and topped with sample buffer to have equal volume. Samples were then boiled at 95 ºC for 5 min and stored.

Ten percent resolving gel and 4% stacking gel was prepared prior to loading samples for the western blot. Samples where then subjected to electrophoresis before being electro-transferred to a polyvinylidene diflouride (PVDF) membrane. Phosphate-buffered saline (PBS) blocking buffer (Li-Cor, Nebraska, USA) was used to block the membrane for 1 hour after which the membrane was left overnight in blocking buffer containing the primary antibodies (α7 n-AChR 1:3000; α4 n-AChR 1:800, Elabscience, Johannesburg, South Africa). After the overnight incubation, the membrane was washed 3 times for 5 minutes using phosphate-buffered saline containing tween 20 (PBS-T) then left to incubate again for 1 hour in secondary antibody (goat anti rabbit 1:10 000; Bio-rad, Johannesburg, South Africa). This was followed by washing 3 more times for a further 5 minutes using PBS-T after which the membrane was immediately read using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected using the Chemi-doc XRS gel documentation system and analysed with quantity one software (BioRad, Johannesburg, South Africa). Samples were then normalised using the loading standards (β-actin 1:5 000).

2.6. ELISA

Hippocampal and striatal tissue were thawed on ice and then homogenised in a solution containing HCl (0.01 N, Sigma, South Africa) and 1mM EDTA (Sigma, South Africa) solution using a sonicator. After tissue preparation, the concentration of DA and GR in hippocampal tissue was evaluated using Dopamine (eLabScience, Texas, USA) and Glucocorticoid receptor (eLabScience, Texas, USA) ELISA kits according to the manufacturer's instructions.

2.6.1. Glucocorticoid receptors

Standard or sample (50 µL) was loaded in duplicate to each well of the 96 well ELISA plate. The liquid was then removed before adding Biotinylated Detection Ab (100 µL). This was followed by incubation for 1 hour at 37℃. The plate was then aspirated and washed 3 times

in wash buffer. HRP Conjugate (100 µL) was added before further incubation for 30 minutes at 37℃. The plate was then aspirated and washed 5 times in wash buffer. Substrate Reagent (90 µL) was added before incubation for 15 minutes at 37℃. Lastly, Stop Solution (50 µL) was added before using a plate reader (Optical Density at 450 nm) immediately thereafter.

2.6.2. Dopamine receptors

The instructions for the Dopamine ELISA kit were as follows. Firstly, 50 µL of standard or sample was added in duplicate to each well of the 96 well ELISA plate. Immediately after, 50 µL Biotinylated Detection Ab was added to each well. This was followed by incubation for 45 minutes at 37℃. The plate was then aspirated and washed 3 times in wash buffer. HRP Conjugate (100 µL) was then added to each well before further incubation for 30 min at 37℃. The plate was then aspirated and washed 5 times in wash buffer. A further 90 µL of Substrate Reagent was added before incubation for 15 min at 37℃. Lastly, 50 µL of Stop Solution was added before using a plate reader (OD at 450 nm) immediately thereafter.

2.7. Statistical Analysis

Data was analysed using the software program GraphPad Prism 5. The values were expressed as mean ± SEM. After normality was tested, unpaired t-tests with Welch's correction were used. A probability of $p<0.050$ was considered statistically significant.

3. Results

3.1 Time spent and activity in the closed arm of the elevated plus maze (EPM)

The time spent and number of entries into the closed arms by the non-stresssed (W) and maternally separated (S) groups are depicted in Figure 1. Maternally separated animals spent significantly more time in the closed arm of the EPM *(W V S, p=0.0430, Figure 1A).

Figure 1B showed no significant differences in number of entries into the closed arms between the groups.

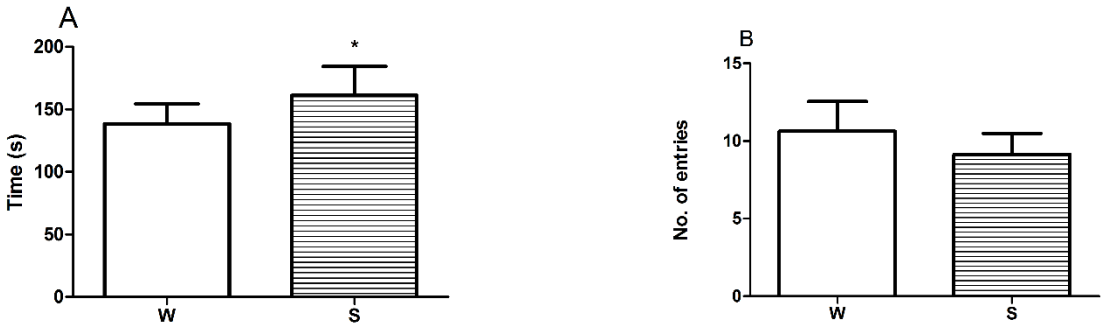

Figure 1(A). Time spent in the closed arm of the elevated plus maze and (B) number of entries into the closed arms by non-stressed (W) and stressed (S) animals (n=8/group). All data presented as mean ± SEM.*p<0.05.

3.2. Effect of maternal separation on the activity in the open field apparatus

Figure 2 depicts the number of lines crossed and time spent in the inner zone by the two groups. Figure 2A showed no significant differences in number of lines crossed in the open field between the groups.

Animals exposed to stress preferred the outer zone than the inner zone when compared to the control group *(W V S, p<0.0001, Figure 2B).

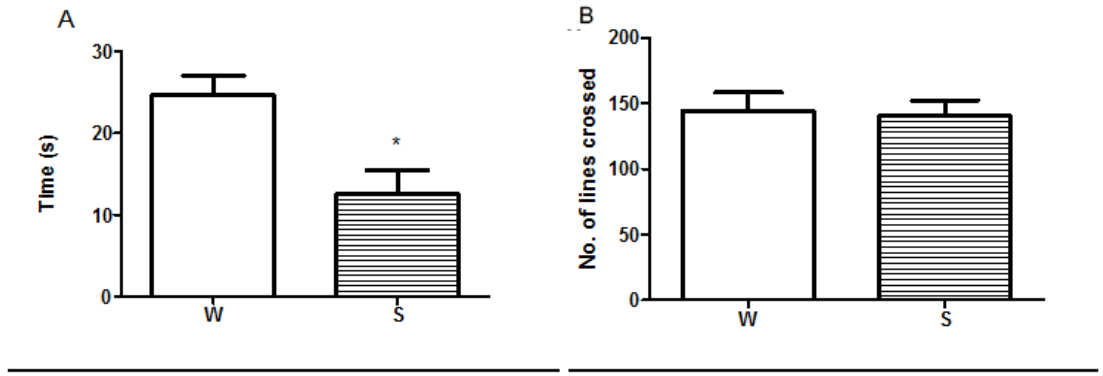

Figure 2(A). Time spent in the closed arm of the elevated plus maze and (B) number of entries into the inner zone by non-stressed (W) and stressed (S) animals (n=8/group). All data presented as mean ± SEM.*p<0.05.

3.3 Hippocampal and striatal expression of α7 nicotinic-Acetylcholine Receptor (n-AChR)

Figure 3 shows the effects of maternal separation on the expression of α7 n-AChR in the hippocampus and striatum of stressed and non-stressed mice. The hippocampal expression of α7 n-AChR was increased in the stressed group *(W V S, p<0.05, Figure 3A).

Figure 3B shows no significant differences in expression were found in the striatum.

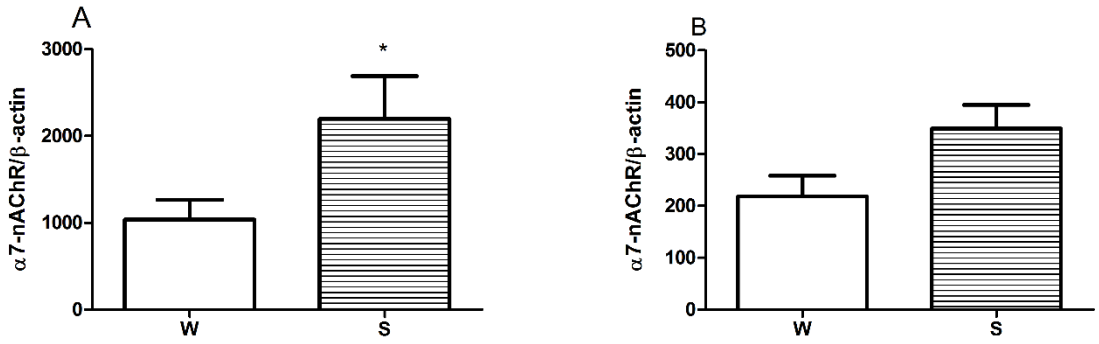

Figure 3. (A) Hippocampal and (B) striatal expression of α7 n-AChR in non-stressed (W) and stressed (S) animals. (n=4/group). All data presented as mean ± SEM. *p<0.05. Unpaired t-test with Welch's correction.

3.4 Hippocampal and striatal expression of α4 nicotinic-Acetylcholine Receptor (n-AChR)

Figure 4 illustrates the changes in hippocampal expression of α4 n-AChR following maternal separation. The expression of α4 n-AChR was decreased in the stressed group *(W V S, p=0.0081, Figure 4A).

α4 n-AChR expression was not significantly different in the striatum (figure 4b).

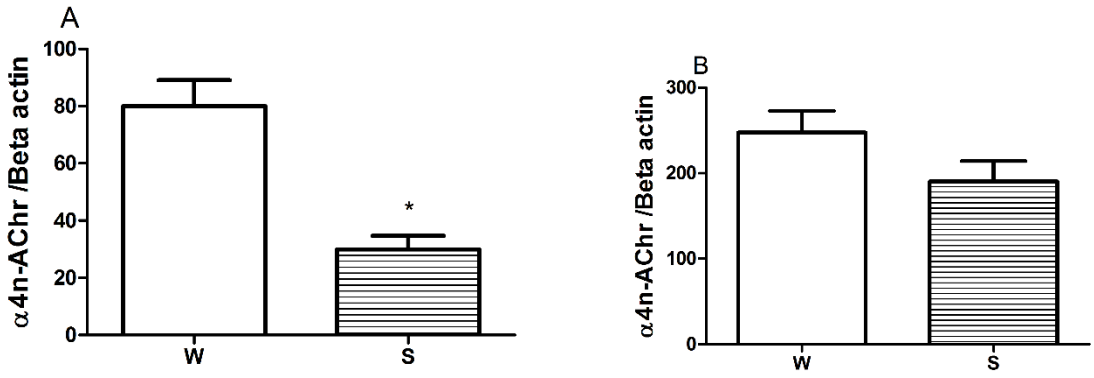

Figure 4. (A) Hippocampal and (B) striatal expression of α4 n-AChR in non-stressed (W) and stressed (S) animals. (n=4/group). All data presented as mean ± SEM. *p<0.05. Unpaired t-test with Welch's correction.

3.5. Glucocorticoid receptor concentration in the hippocampus and striatum

The changes in glucocorticoid receptor concentration were not affected by maternal separation (Figure 5a and b).

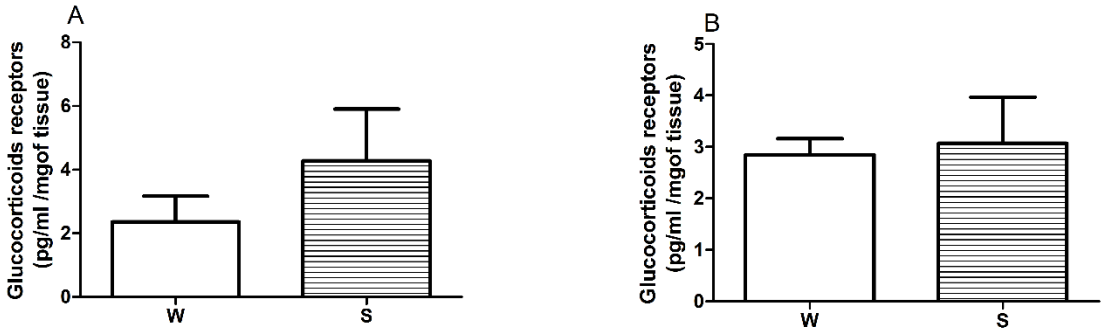

Figure 5. (A) Hippocampal and (B) striatal concentration of glucocorticoid receptor in non-stressed (W) and stressed (S) animals. (n=4/group). All data presented as mean ± SEM. Unpaired t-test with Welch's correction.

3.6. Hippocampal and striatal dopamine concentration

The changes in dopamine concentration were not affected by maternal separation (Figure 6a and b).

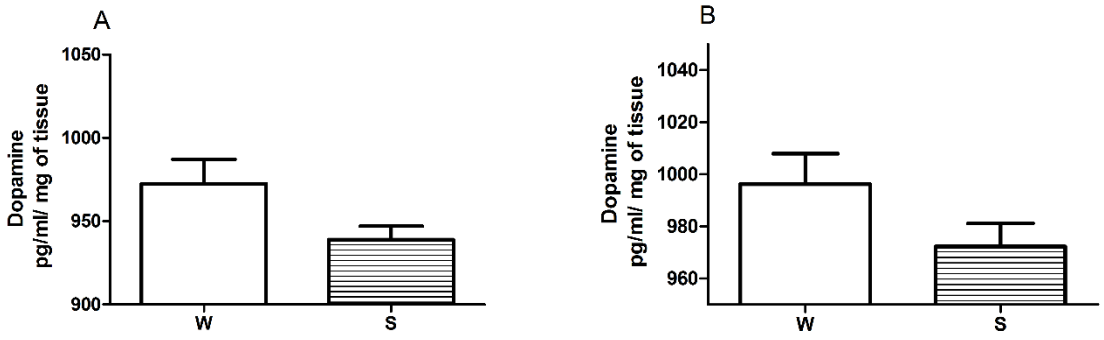

Figure 6. (A) Hippocampal and (B) striatal concentration of dopamine in non-stressed (W) and stressed (S) animals. (n=4/group). All data presented as mean ± SEM. Unpaired t-test with Welch's correction.

Discussion

Exposure to early life maternal separation has been shown to result in the development of anxiety-like and depression-like behaviour later in life (13-15). While prenatal stress models have looked at the cholinergic receptors, the effects of postnatal stress exposure has not been catalogued. Our study aimed to assess the effects of maternal separation (MS) on anxiety-like behaviour in mice and assess the involvement of cholinergic receptors (α7 n-AChR and α4 n-AChR) as well as glucocorticoid receptor and dopamine concentration in the hippocampus which showed no significant changes.

As expected, our results showed that maternally separated (MS) animals preferred spending time in the closed arms of the elevated plus maze suggesting anxiety-like behaviour (16, 17). However, there was no significant difference in the number of entries into the closed arms between the two groups.

Similarly in the open field test, the MS group generally avoided spending time in the inner zone. It has been suggested that avoidance of the inner zone is a sign of fearfulness and anxiety-like behaviour (18). Immediately after birth, the offspring have elevated resting corticosterone concentration which progressively declines and remains low between postnatal day 4 (P4) and P14; this period is called the stress-hyporesponsive period (19, 20). Stressful experiences during neuronal pathway development in early life can induce changes in the HPA axis as well as in the concentration of neurotransmitters and neurotrophin factors (3). This causes long-lasting alterations in the structure and function of the hippocampus as well as synaptic plasticity resulting in deficits in learning and memory in the offspring (21).

The expression of the α7 n-AChR in the hippocampus was increased in the MS group. This is a novel finding as studies have only focused on cholinergic receptor expression following exposure to prenatal stress where it has been shown that α7 receptor expression in the hippocampus was reduced in male but not female rats (12). In our study, we used male mice. It has been suggested that exposure to MS may lead to network malformation that could increase acetylcholinesterase (AChE) activity which may then affect receptor expression (22). Benetti, Mello (23) found that maternal deprivation resulted in increased AChE activity with resultant neurobehavioural deficits. Administration of AChE inhibitors resulted in the reversal of the neurobehavioural deficits. The α7 n-AChR can stimulate increase in cell death and points to a mechanism by which stress can use n-AChRs in effecting neurodegeneration (10). The mechanism could either be due to oxidative stress or excitotoxicity (24). Increased

levels of the receptor have been linked to increased oxidative stress following chemical exposure specifically in the hippocampus (24).

On the other hand, α4 n-AChR expression was decreased in the stressed animals. It has been previously shown that exposure to prenatal stress leads to an increase α4 n-AChR expression in rats while the changes in the α7 receptor are gender dependant, impairing α7 expression in male rats (12). Our results show that in the MS group, an increase in the α7 n-AChR expression was linked to a decrease in α4 n-AChR. The α4 n-AChR expression result is consistent with Schulz et al. (11) who hypothesised that an increase in α4 expression contributes to anxiety and depression-like behaviour.

While there is an MS effect on cholinergic receptor expression, we did not find changes in glucocorticoid receptor (GR) concentration in both the hippocampus and striatum. Studies have shown that developmental stressors lead to a decrease in hippocampal GR concentration (21, 25). This decrease has been linked to a decrease in GR messenger RNA (26). The C57BL/6 mouse strain has had inconsistent results in multiple studies of neurobehavioural disorders in comparison to rat models (27). It has been suggested that conventional strategies have to be altered for anxiety-like behaviour to be displayed in mice due to the literature being largely rat based (17). Veenema, Bredewold (28) also noted that in a maternal separation model, aggressive behaviour of the C57BL/6 mouse conflicted with the behaviour and hypothalamic vasopressin and oxytocin levels expected in terms of the literature. Paylor, Tracy (29) found that behaviour was species dependant even between mice when comparing the DBA/2 and C57BL/6 strains.

Dopamine concentration was not significantly different between the groups in the hippocampus and striatum. It has been shown that in early developmental stress models, there needs to be a further insult for there to be any changes in dopamine concentration (30). Therefore we suggest that in our study, there needed to be a further insult/stressor to the brain for significant changes to be observed in DA neurotransmission.

In conclusion, our results show that MS results in long term changes to the α7 and α4 nicotinic acetylcholine receptors in mice. This supports the hypothesis that HPA axis disturbances results in dysfunction of the cholinergic system, specifically the nAChRs. These receptors have never been observed before in postnatal stress exposure and supports cholinergic network malformation in relation to both anxiety and depressive disorders and

possibly developmental cognitive functioning. Our results also point to species and strain specific responses that further the understanding of the response to stress.

Acknowledgements

We would like to thank the Biomedical Resource Centre for their assistance in this study as well the University of KwaZulu-Natal and the National Research Foundation (NRF) of South Africa for financial support.

Conflict of Interest

The authors declare that there is no conflict of interest

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Chapter 2 found changes of the cholinergic receptors in a maternal separation model with increased anxiety and depression-like behaviour. Chapter 3 investigates in what way a double insult of prenatal nicotine exposure and maternal separation alters the systems of stress and neurotransmission to determine the outcomes behaviour later in life. Chapter 3 will be submitted to a journal once chapter 2 has been accepted.

Prenatal nicotine exposure and maternal separation alter nicotinic acetylcholine and glucocorticoid receptors in mice

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Abstract

Maternal smoking during pregnancy is associated with long term cognitive dysfunction. Harmful behavioural effects of smoking have been shown to be primarily due to exposure to nicotine. While the mechanism of nicotine's harmful actions remain unclear, studies have shown a link to the hypothalamus–pituitary–adrenal (HPA) axis. HPA axis dysfunction due to exposure to perinatal stressors such as maternal separation (MS) results in major long-term systemic and neurological disruptions and malfunction. Using a mouse model we showed that prenatal exposure to nicotine (PNE) resulted in hyperlocomotivity, chronic increase in hippocampal α7 nicotinic acetylcholine receptor (n-AChR) expression and a decrease in α4 n-AChR expression. Glucocorticoid receptor (GR) expression varied in the PNE groups by brain location while no changes were found in dopamine concentration in the hippocampus or striatum. Maternal separation resulted in anxiety-like behaviour in the open field test, decreased α4 n-AChR and increased α7 expression in the hippocampus, suggesting a link between HPA dysfunction and cholinergic signalling. Exposure to both stress and nicotine attenuated changes in hippocampal α7 n-AChR and GR expression but resulted in elevated dopamine concentration. In this study, we were able to show that the PNE effects on nicotinic receptors were similar to those found in MS animals which have been shown to be due to HPA dysregulation. Therefore, our results suggest that cognitive dysfunction following PNE may be due to dysregulation in the cholinergic system that affects expression of α4 and α7 n-AChR and this effect may affect HPA axis function.

Key words: prenatal nicotine exposure, maternal separation, nicotinic acetylcholine receptors, glucocorticoid receptors, HPA axis, hippocampus

Introduction

The harmful effects of cigarette smoking during pregnancy for both the mother and foetus are common knowledge (England et al., 2017). Approximately 10% of women continue smoking during pregnancy and that number is estimated to be higher in third world countries (Tong et al., 2013). Maternal tobacco smoking has been shown to have negative effects on birth weight, cognitive development and behaviour as well as increased risk in foetal morbidity and mortality (Wickstrom, 2007, Huizink and Mulder, 2006, Sexton and Hebel, 1984).While cigarettes contain up to 400 toxic ingredients, the harmful behavioural effects associated with cigarette smoking are considered to be primarily due to the effect of nicotine in both the mature and developing brain (Aoyama et al., 2016, Slotkin et al., 1990). Prenatal nicotine exposure (PNE) has been linked to multiple neurodevelopmental disorders such as anxiety, ADHD, depression, addiction, conduct disorder and schizophrenia (Tiesler and Heinrich, 2014, Brown et al., 2000). While there are considerable variations in tobacco use in countries around the world, nicotine's effect is likely the highest adjustable neuropharmacological exposure to the foetus and an increasingly crucial issue (Aoyama et al., 2016, Niemelä et al., 2016, Wickstrom, 2007).

Nicotine exerts its effects via the cholinergic signalling pathway in the brain. It binds to acetylcholine receptors, specifically the nicotinic acetylcholine receptors (n-AChR) (Bryden et al., 2016). Cholinergic signalling has effects on the regulation of neurogenesis, neuronal differentiation and migration which also involves neurotransmitters such as dopamine (Bryden et al., 2016, Zhu et al., 2012). Therefore, foetal exposure to nicotine results in more than just cholinergic dysfunction as it also alters dopaminergic and other neurotransmitter signalling involved in brain development (Bryden et al., 2016, Zhu et al., 2012). Alterations of the dopaminergic system during foetal development have also been shown to change the responsiveness of the hypothalamus–pituitary–adrenal (HPA) axis during stress (Uban et al., 2013).

Stress during the critical stage of neuronal pathway formation in the brain in early life may cause long-lasting alterations that result in neurological disorders such as anxiety, addiction and depression among others (Lundberg et al., 2017, Popoli et al., 2012). Stress results in increased glucocorticoid concentration in circulation as an effect of continuous stimulation of the hypothalamo-pituitary-adrenal (HPA) axis (Turecki and Meaney, 2016). Animals exposed to developmental stress such as maternal separation, show altered neurobehavioural effects

such as anxiety-like behaviour and are more prone to addiction (Kanatsou et al., 2016). Exposure of an offspring to maternal separation results in excessively increased corticosterone concentration causing anxiety-like behaviour as well as cognitive impairment and structural changes in neuronal dendrites and spines in the hippocampus and prefrontal regions of the brain involved in emotion and behaviour control (Kanatsou et al., 2016).

Conversely, n-AChRs have also shown involvement in the stress response in the mature brain (Holgate and Bartlett, 2015). However, it remains unknown which types of n-AChRs and how their location within the brain controls this process (Holgate and Bartlett, 2015). The α4 n-AChR subunit has been shown to play a role in stress driven addiction (Kim et al., 2004) and depression (Reuter et al., 2012). A developmental stressor in the form of prenatal stress has also been shown to alter both α4β2 and α7 nAChR expression in the hippocampus (Baier et al., 2015, Schulz et al., 2013).

Therefore, one of the possible deleterious effects of prenatal nicotine exposure (PNE) is a dysregulation of the foetal HPA-axis (Huizink et al., 2004). Glucocorticoid receptors are the final effectors of the HPA axis and participate in the control of whole body homeostasis and the organism's response to stress as well as the programming of the HPA axis (Slone and Redei, 2002). It has been shown that PNE results the over-exposure of foetal rats to maternal glucocorticoids, which can inhibit the development of the foetal HPA axis (Zhang et al., 2014). It has also been indicated that the dysfunction of the HPA axis developmentally is caused by the high concentration of glucocorticoids as well as alterations in glucocorticoid receptor density in regions of the brain such as the prefrontal cortex, hippocampus, amygdala and pituitary (Zhang et al., 2014). These areas are important for the negative feedback regulation and activation of the HPA axis. The expression of foetal hippocampal 11-hydroxysteroid dehydrogenase-1 (11-HSD-1) and glucocorticoid receptors (GR) have been shown to be increased following PNE while the activity of the foetal HPA axis is inhibited (Chen et al., 2017, Xu et al., 2012).

Multiple animal models of developmental nicotine exposure exist with the two most common being nicotine administration via injection and implantation of mini-osmotic pumps. These models have resulted in functional changes of the n-AChR, cholinergic, dopaminergic and serotonergic signalling disruptions and increased behavioural stimulation (Muneoka et al., 1997, Olale et al., 1997, Ksir et al., 1987).

Nicotine in drinking water eliminates the stress and hypoxia variables associated with the models above and mimics the periodic consumption of nicotine through smoking by the general human population (Alkam et al., 2013).

While there have been many studies exploring the effects of PNE on the HPA axis, the results have not yet fully elucidated the relationship. Additionally, many studies have explored the effect of stress exposure in adulthood on nAChRs, but few have looked at developmental stress.

Therefore, our aim was to further elucidate the mechanism behind a double insult induced by prenatal nicotinic exposure and a subsequent exposure to maternal separation in the brain. We aimed to investigate the effect this double insult may have on nicotinic and glucocorticoid receptor expression in the hippocampus as well as effects on dopamine concentration in the hippocampus and striatum.

2. Materials and methods

2.1 Animals

Ten female and five male C57BL/6 mice were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were housed at room temperature (± 22˚C) and 70 % humidity (standard BRC conditions). A 12hr light/dark cycle (lights on at 06h00) was maintained and food and water were available ad libitum. All experimental procedures were approved by the Animals Ethics Research Committee of the University of KwaZulu-Natal in accordance with the guidelines of the National Institute of Health, USA (Ethics clearance number AREC/076/015D).

2.1.1 Prenatal handling and mating

Three weeks prior to mating, female mice were divided into 2 groups: a non- nicotine and a nicotine exposed group. The nicotine was dissolved in the drinking water. The non-nicotine mice were given normal drinking water. Mating occurred 3 weeks after exposure to nicotine.

2.1.2. Postnatal Handling

Following birth, the litters were further subdivided into stressed and non-stressed groups. Birth was termed postnatal day 0 (PND 0) and on PND 2 the stressed group underwent maternal separation until PND 14.

2.1.3. Maternal separation

The dams were removed from their pups and taken to a separate room for 3 hours once a day from 09h00 to 12h00 until PND 14. Following exposure to the maternal separation protocol, the pups stayed with their dams until PND 21 when the pups were weaned.

2.2. Grouping and study design

2.2.1 Grouping

Forty-eight C57BL/6 male mice were used for this study and were grouped (n=8, PER GROUP) as follows: Group one: control exposed to normal water and not stressed (W); Group 2: exposed to nicotine prenatally (N); Group 3: antenatal stress in the form of maternal separation and exposed to normal water (S); Group 4: prenatally exposed to nicotine and antenatal stress in the form of maternal separation (NS).

2.2.2. Study design

After the animals were obtained, the female mice were divided into nicotine and non-nicotine exposed groups. The mice in the nicotine exposed group were GIVEN a dose of 0.1 mg/ml nicotine ((-)-Nicotine ditartrate, Alomone Labs, Jerusalem, Israel) dissolved in the drinking water (Zhu et al., 2012) for 3 weeks. The water for both groups was measured and changed every 3 days (Seckar et al., 2008). After 3 weeks of exposure to nicotine or water, these animals were mated with non-nicotine exposed males. The nicotine exposed animals continued to be exposed to nicotine in their drinking water throughout pregnancy. After birth of the pups, all dams were exposed to normal drinking water.

The pups were then sub divided into stressed and non-stressed groups. Those in the stressed group were separated from their dams as described above. After weaning on PND 21, only males were kept for the study. On PND 35, the elevated plus maze test to measure anxiety and locomotivity was carried out. This was followed three-weeks later (PND 58) by an assessment of both anxiety and locomotivity using the open field after which the animals were sacrificed on PND 59.

2.3. Behavioural tests

2.3.1. Elevated plus maze

The elevated plus maze assesses the animal's state of anxiety and locomotivity (Elliott et al. 2004). It consists of placing the animal in a plus shaped apparatus, with two open and two closed arms, for five minutes. The arms' measurements are width: 10 cm, length: 50 cm, height: 30 cm, elevation height from floor: 55 cm. The animal's aversion to exploration and activity are assessed using the entries and amount of time spent in the closed arm.

2.3.2. Open field

The open field was used to measure the activity and exploratory behaviour of the animals. The animal was placed in a 40 × 60 × 50 cm arena, which was divided into 12 identical rectangles with grids. Animals were video recorded and the number of grid lines crossed and amount of time spent in the centre of the field were recorded for a period of 5 min.

Both behavioural tests were video recorded.

2.4. Decapitation

A day following the behavioural tests, the animals were sacrificed by decapitation. The striatum and hippocampus were dissected out for neurochemical analysis. The samples were stored in a biofreezer at -80°C until neurochemical analysis.

2.5. Western blot

Hippocampal and striatal tissue were thawed on ice and homogenate was then prepared in a ratio 10% w/v and used for protein determination using the Bradford Reagent (Bradford, 1976). After measuring protein concentration, the samples were diluted to the required amount of protein and topped with sample buffer and B-mercaptoethanol to have equal protein concentration and volume. Samples were then boiled at 95 ºC for 5 min and stored.

The 10% resolving gel and 4% stacking gel WERE prepared for the western blot subsequent to loading samples. Samples were subjected to electrophoresis before being electro-transferred to polyvinylidene diflouride (PVDF) membrane. The membrane then exposed for 1 hour in phosphate-buffered saline (PBS) blocking buffer (Li-Cor). This was followed by exposing the membrane overnight in blocking buffer containing the primary antibodies (α7 nAChR 1:3000; α4 nAChR 1:800, Elabscience, USA). After the overnight incubation, the membrane was washed 3 times for 5 minutes using PBS containing tween 20 (PBS-T) then incubate again for one hour with the secondary anti-body (goat anti rabbit 1:10 000; Bio-Rad). After incubation, the membrane was washed 3 times for a further 5 minutes using PBS-T after which it was immediately prepared using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected using the Chemi-doc XRS gel documentation system and analysed with quantity one software (BioRad, Johannesburg, South Africa). Samples were then normalised using the loading standards (β-actin 1:5 000).

2.6. ELISA

Hippocampal and striatal tissue were thawed on ice and then homogenised in HCl (0.01 N), 1mM EDTA solution using a sonicator. After tissue preparation, the concentration of DA and GR in the hippocampus and striatal tissue was evaluated using dopamine (eLabScience,

USA) and glucocorticoid receptor (eLabScience, USA) ELISA kits according to the manufacturer's instructions.

2.6.1. Glucocorticoid receptor concentration

As per the instructions of the GR ELISA kit, 50 µL of standard or sample was added in duplicate to each well of the 96 well plate. The liquid was then aspirated before adding 100 µL Biotinylated Detection Antibody. This was followed by incubation for 1 hour at 37℃. The plate was then aspirated and washed 3 times in wash buffer. HRP Conjugate (100 µL) was added before further incubation for 30 min at 37℃. Following aspiration, the plate was washed 5 times in wash buffer. Substrate Reagent (90 µL) was added before incubation for 15 min at 37℃. This was followed by the addition of a Stop Solution (50 µl). The plate was then read using a plate reader at an optical density (OD) of wavelength 450 nm.

2.6.2. Dopamine concentration

The instructions for the Dopamine ELISA kit were as follows. Firstly, 50 µL of standard or sample was added to each well in duplicate. Immediately thereafter, Biotinylated Detection Antibody (50 µL) was added to each well. This was followed by incubation for 45 minutes at 37℃. The plate was then aspirated and washed 3 times in wash buffer. HRP Conjugate (100 µL) was added to each well before further incubation for 30 min at 37℃. The plate was then aspirated and washed 5 times in wash buffer. Substrate Reagent (90 µL) was added before incubation for 15 min at 37℃. Lastly, 50 µL of Stop Solution was added before using a plate reader (OD at 450 nm) immediately thereafter.

2.7. Statistical Analysis

Data was analysed using the software program GraphPad Prism 5. The values were expressed as mean ± SEM. After normality was tested, Two-way ANOVA followed by Newman-Keuls post-hoc test was used. A probability of $p<0.05$ was considered statistically significant.

3. Results

3.1 Effect of prenatal nicotine exposure and maternal separation on the time spent and number of entries in the closed arms of the elevated plus maze (EPM)

The time spent and number of entries into the closed arms are depicted in Figure 1. The four groups being assessed are: the control (W), animals exposed to nicotine prenatally (N), animals exposed to maternal separation (S) and those that experienced both prenatal nicotine and postnatal stress (NS) exposure. Exposure to a combined nicotine and stress insult, attenuated the stress effect on time spent in the closed arms *(S VS NS, [$F_{(3,31)}$ = 5.350; p=0.0049], Figure 1A).

Exposure to nicotine resulted in an increase in the number of entries into the closed arms ###(W vs N, [$F_{(2,23)}$ = 25.96; p<0.0001], Figure 1B). Similarly, nicotine attenuated the stress effect on the number of entries into the closed arms ***(S VS NS, [$F_{(2,23)}$ = 40.87; p<0.0001], Figure 1B).

I

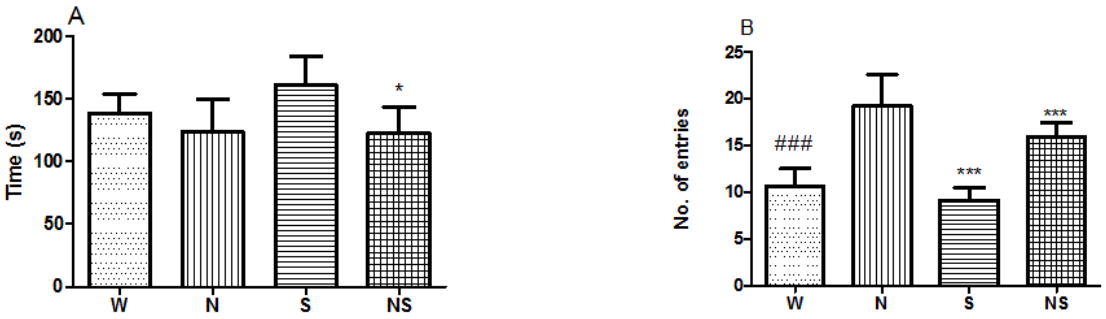

Figure 1. Effect of prenatal nicotine exposure and maternal separation on behaviour in the elevated plus maze. (A) Time spent in the closed arms. (B). Number of entries into the closed arms (n=8/group). All data presented as mean ± SEM. *p<0.01; ***p<0.001; ###p<0.001 as compared to N. Two-way ANOVA followed by Newman-Keuls post hoc analysis.

3.2. Effect of prenatal nicotine exposure and maternal separation in the open field

Figure 2 depicts the effect of prenatal exposure to nicotine and postnatal maternal separation on the number of line crossings (hyperlocomotivity) and time spent in the inner zone by the different groups assessed in the open field. There was a nicotine effect on hyperlocomotivity

***(W vs N, [$F_{(2,23)}$ = 10.98; p=0.0005], Figure 2A) and ###(S vs NS, [$F_{(2,23)}$ = 15.27; p<0.0001], Figure 2A).

Figure 2B depicts the time spent in the inner zone by the different groups. The was a stress only effect on time spent in the inner zone #(W vs S, [$F_{(2,23)}$ = 38.24; p<0.0001], Figure 2B). We also found a nicotine effect for both nicotine groups on time spent in the inner zone ***(W vs N, [$F_{(2,23)}$ = 28.78; p<0.0001], Figure 2B) and *** (S vs NS, [$F_{(2,23)}$ = 117.2; p<0.0001], Figure 2B).

II

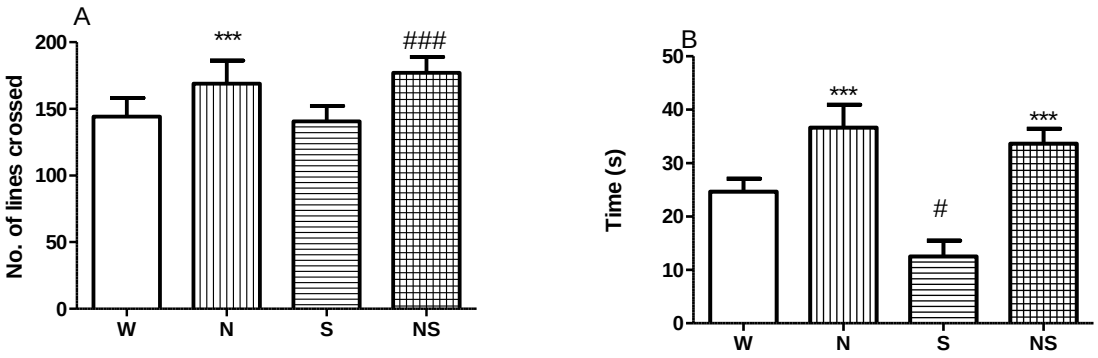

Figure 2. Effect of prenatal nicotine exposure and maternal separation on anxiety and locomotor behaviour in the open field test. (A) Represents the number of lines crossed. (B) Time spent in the inner zone. (n=8/group). All data presented as mean ± SEM. ***p<0.01; ***p<0.001 compare to S; #p<0.001 compare to W. Two-way ANOVA followed by Newman-Keuls post hoc test.

3.3 Effect of prenatal nicotine exposure and maternal separation on hippocampal and striatal expression of α7 nicotinic-Acetylcholine Receptor (n-AChR)

Figure 3 shows the effects of PNE and maternal separation on the expression of α7 n-AChR in the hippocampus and striatum. Figure 3A illustrates hippocampal expression of α7 n-AChR among the groups. There was both a prenatal (N) as well as a postnatal (S) stress effect on hippocampal α7 n-AChR expression a(W vs N, [$F_{(2,11)}$ =6.299; (p=0.0195)], Figure 3A) and #(W vs S, [$F_{(3,14)}$ =4.726; (p=0.0236)], Figure 3A). A combined nicotine and stress

exposure attenuated the effects of prenatal stress *(N vs NS, [$F_{(3,12)}$ =4.952; (p=0.0207)], Figure 3A).

Figure 3B illustrates the striatal expression of α7 n-AChR among the groups. There was a nicotine effect on the expression of α7 n-AChR #(W vs N, [$F_{(3,14)}$ =5.417; (p=0.0137)], Figure 3B). In the nicotine exposed animals, co-exposure with stress attenuated increase in α7 n-AChR expression a(N vs NS, [$F_{(3,11)}$ =5.274; (p=0.0305)], Figure 3B).

III

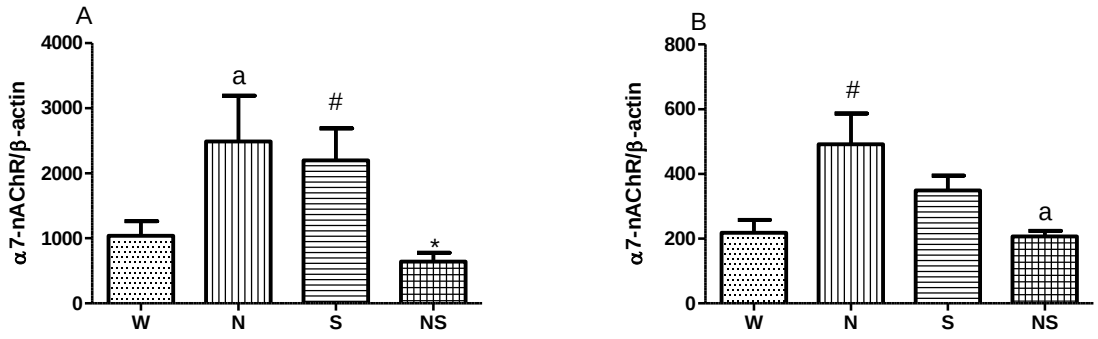

Figure 3. Effect of prenatal nicotine exposure and maternal separation on the concentration of α7 nicotinic-Acetylcholine Receptor (n-AChR). (A) Hippocampal concentration of α7 n-AChR. (B) Striatal concentration of α7 n-AChR. (n=4/group). All data presented as mean ± SEM.#p<0.05 compare to W; *p<0.05 compare to S; a p<0.05 compare to NS. Two-way ANOVA followed by Newman-Keuls post hoc analysis.

W= Prenatal exposure to Water; N: Prenatal exposure to Nicotine; S= Postnatal stress exposure (maternal separation); NS: exposure to nicotine prenatally and maternal separation postnatally.

3.4 Effect of prenatal nicotine exposure and maternal separation on hippocampal and striatal expression of α4 nicotinic-Acetylcholine Receptor (n-AChR)

Figure 4 illustrates the changes in hippocampal and striatal expression of α4 n-AChR following exposure to PNE and maternal separation. Figure 4A illustrates hippocampal expression of α4 n-AChR among the groups. Exposure to prenatal as well as postnatal stress individually and combined had an effect on α4 n-AChR expression #(W vs N; S; NS, [$F_{(3,15)}$ = 7.179; p=0.0051, Figure 4A). Figure 4B depicts the α4 n-AChR striatal expression in the

different groups. There was a nicotine effect on striatal α4 n-AChR #(W vs N, [$F_{(2,11)}$ = 4.433; p=0.0457], Figure 4B).

IV

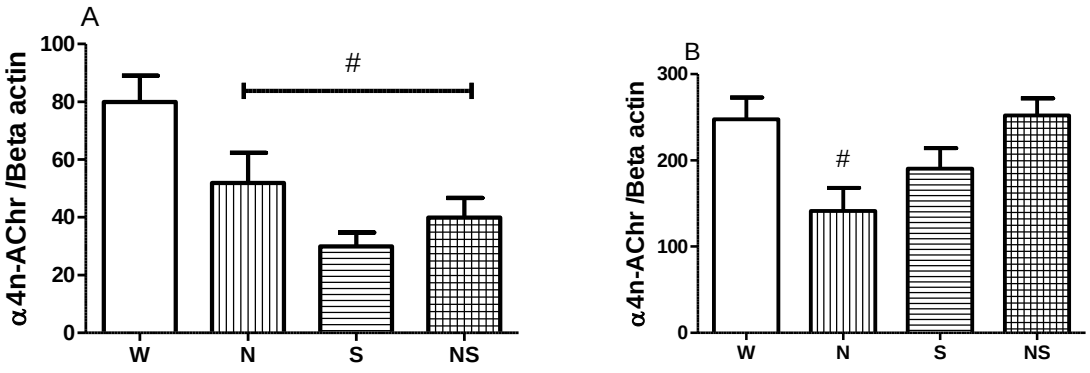

Figure 4. Effect of prenatal nicotine exposure and maternal separation on the concentration of α4 nicotinic-Acetylcholine Receptor (n-AChR). (A) Hippocampal concentration of α4 n-AChR. (B) Striatal concentration of α4 n-AChR. (n=4/group). All data presented as mean ± SEM. #p<0.05 compare to W; #p<0.05 compare to N. Two-way ANOVA followed by Newman-Keuls test.

W= Prenatal exposure to Water; N: Prenatal exposure to Nicotine; S= Postnatal stress exposure (maternal separation); NS: exposure to nicotine prenatally and maternal separation postnatally.

3.5. Effect of prenatal nicotine exposure and maternal separation on glucocorticoid receptor (GR) expression in the hippocampus and striatum

The changes in GR expression due to PNE and maternal separation are shown in Figure 5. Figure 5A illustrates hippocampal expression of GR among the groups while figure 5B depicts striatal expression.

V

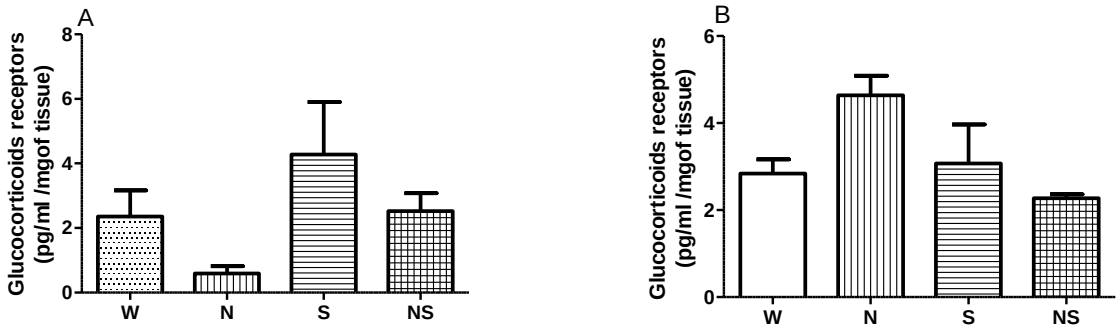

Figure 5. Effect of prenatal nicotine exposure and maternal separation on the concentration of Glucocorticoid receptor (GR). (A) Hippocampal concentration of GR. (B) Striatal concentration of GR. (n=4/group). All data presented as mean ± SEM.

W= Prenatal exposure to Water; N: Prenatal exposure to Nicotine; S= Postnatal stress exposure (maternal separation); NS: exposure to nicotine prenatally and maternal separation postnatally.

3.6. Effect of prenatal nicotine exposure and maternal separation on striatal and hippocampal dopamine concentration

The differences in dopamine concentration due to PNE and maternal separation are shown in Figure 6. Figure 6A illustrates hippocampal dopamine concentration. Nicotine and stress co-exposure exacerbated dopamine concentration *(N vs NS [$F_{(2,11)}$ = 5.675; p=0.0254], Figure 6A).

There were no changes in dopamine concentration in the striatum figure 6B.

V1

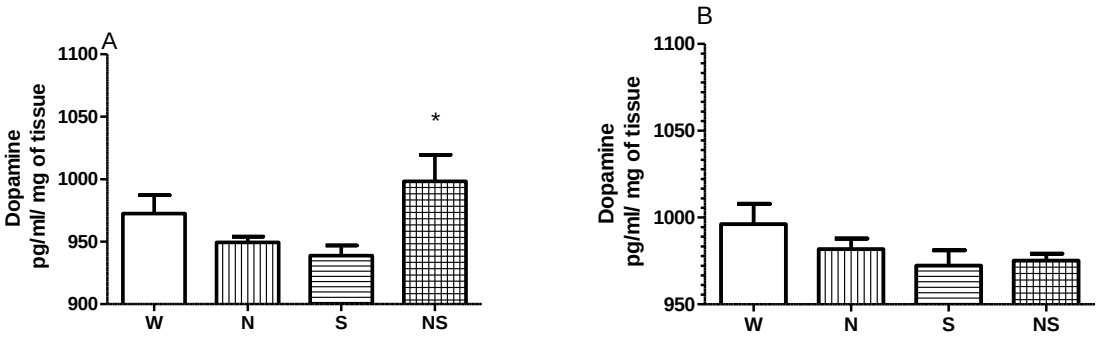

Figure 6. Effect of prenatal nicotine exposure and maternal separation on dopamine concentration. (A) Hippocampal dopamine concentration. (B) Striatal dopamine concentration. (n=4/group). All data presented as mean ± SEM. *$p<0.05$ compare to S. Two-way ANOVA followed by the Newman Keuls post hoc test.

W= Prenatal exposure to Water; N: Prenatal exposure to Nicotine; S= Postnatal stress exposure (maternal separation); NS: exposure to nicotine prenatally and maternal separation postnatally.

Discussion

Prenatal nicotine exposure (PNE) has been linked to multiple neurodevelopmental disorders such as anxiety, depression, ADHD and schizophrenia; while maternal separation (MS) has been shown to increase anxiety and depression-like behaviour as well as hyperactivity (Aya-Ramos et al., 2017, Kwak et al., 2009, Tiesler and Heinrich, 2014). Our study aimed to investigate the effects of PNE on cholinergic receptors and whether PNE may have the same behavioural effects as another perinatal stressor, MS. To this effect, we assessed the effects of exposure to nicotine prenatally or in combination with MS, a postnatal stress, on locomotor activity as well as anxiety-like behaviour in mice. We further assessed the involvement of the nicotinic acetylcholine receptors (n-AChRs) such as the α7 n-AChR and α4 n-AChR, as well as glucocorticoid receptor and dopamine concentration on the behavioural changes observed.

We found that animals exposed to NS (combined nicotine and maternal separation exposure) showed decreased anxiety-like behaviour as they spent less time in the closed arm. This result suggests that exposure to MS in these mice attenuated anxiety-like behaviour.

The number of entries into the arms of the EPM was used as a measure of locomotor activity. The relative immobility displayed by MS animals was attenuated in animal exposed to both MS and PNE. This suggests that NS animals showed less anxiety-like behaviour. This decrease in anxiety-like behaviour supports previous studies that have shown a similar effect in both rats and mice that were exposed to nicotine in utero (Zhu et al., 2012, Tizabi et al., 1997). Animals exposed prenatally to nicotine showed increased activity in the elevated plus maze. This effect has been previously reported as hyperactivity (Tizabi et al., 1997). Nicotine acts as a stimulant and exposure in utero has shown to result in altered neurotransmission that continues following birth (McCarthy et al., 2018, Pauly and Slotkin, 2008). While exposure to MS may have resulted in increased anxiety-like behaviour, exposure to both stress and nicotine attenuated this effect suggesting that the two stresses cancel each other out hence limiting the associated disruption in HPA axis function.

Nicotine exposure in utero has been shown to alter the HPA axis thereby allowing for multiple pathways to possibly be affected by way of the disrupted cholinergic network. Cholinergic signalling has effects on the regulation of neurogenesis, neuronal differentiation and migration through other neurotransmitters, including dopamine (Bryden et al., 2016, Zhu et al., 2012). Therefore, foetal exposure to nicotine results in more than just cholinergic dysfunction, specifically, it alters dopaminergic and other neurotransmitter signalling that is

responsible for brain development in the foetus (Zhu et al., 2012, Bryden et al., 2016). Alterations of the DA system during foetal development have also been shown to change the responsiveness of the HPA axis during stress (Uban et al., 2013). Additional parameters investigating the exploratory behaviour of the animals were measured by the amount of time spent in the centre of the open field apparatus. Exposure to maternal separation, as seen in our study, results in animals avoiding the inner zone of the open field apparatus and this has been deemed as an indication of fearful or anxiety-like behaviour (Kulesskaya and Voikar, 2014). The PNE groups however, spent more time in the centre of the open field suggestive of increased exploratory behaviour and lack of anxiety which were comparable to behaviour observed in the EPM test. PNE has been shown to cause alterations in neuroendocrine programming resulting in HPA-axis hypersensitivity (He et al., 2017, Zhang et al., 2014). He et al. (2017) found that not only was HPA-axis hypersensitivity present, but imbalanced afferent outputs of glutamate and GABA, with enhanced expression of glutamic acid decarboxylase 67 (GAD67) in the hippocampus was related to PNE. However, the possible involvement of nicotinic receptors in that alteration in relation to PNE was not explored.

We assessed nicotinic receptor function in the hippocampus and striatum. PNE resulted in an increased α7 n-AChR expression in the hippocampus and striatum. Tizaby et al. (1997) hypothesised that an initial upregulation of nicotinic receptors, due to PNE, caused hypersensitivity of the receptor which resulted in a functional downregulation of the receptor. Interestingly, this increased receptor expression is attenuated in PNE animals exposed to MS. This decrease may be due to the fact that MS as a second stressor following nicotine exposure induced a desensitisation of the receptors leading to down-regulation.

It has been suggested that the increase in α7 n-AChR expression following exposure to nicotine is responsible for the increased activity of these animals in the EPM and OFT (Zhu et al., 2012). The stimulatory effects of nicotine on the cholinergic system during development seem to be chronic resulting in altered receptor activity later in life. It has also been postulated that cholinergic ligands of the n-AChR may be used to treat psychotic and neurodegenerative diseases (Feuerbach et al., 2010). The increased activity in a novel environment could also be translated as maldevelopment of the cholinergic system.

Hippocampal and striatal expression of α4 n-AChRS was lower in the stressed animals than in the non-stressed animals. Avraam et al. (2016) has postulated that the decrease in α4 n-AChR expression is either due to disinhibition or a change in expression of other n-AChR

subunits. In our study, we found an increase in α7 n-AChR and a decreased α4 n-AChR expression thus supporting Avraam et al. (2016). Schulz et al., (2013) has shown that exposure to prenatal restraint stress resulted in decreased α4 n-AChR expression. The difference between Schulz et al. (2013) and our study was the period of stress which is a vital variable. Another study investigating the α4 n-AChR in a prenatal stress model showed no difference in the protein levels of the receptor, but changes in the mRNA levels in the prefrontal cortex without exploring the changes of the receptor in the hippocampus (Baier et al., 2015).

Glucocorticoid receptor (GR) expression was also evaluated in the hippocampus and striatum. No changes were seen in GR expression between the groups. While early life stress effects in rats are prominent, a systematic review found that in mice, findings are more inclined to non-significant results which they attributed to species specificities during development and behavioural maternal care (Tractenberg et al., 2016). Zhang et al. (2014) showed that prenatal exposure to nicotine in rats leads to overexposure to maternal glucocorticoids which can inhibit the development of the HPA axis. This over exposure during development could be due to long term downregulation of GR, in favour of mineralocorticoid receptors (MR) as is the case with developmental stressors (Lupien et al., 2009). It has been shown that exposure to MS in rats results in decreased cholinergic function (Aisa et al., 2009). It has also been suggested that the HPA axis changes that follow PNE are epigenetic thus resulting in HPA axis dysfunction (Zhang et al., 2014). Interestingly, GR expression in the striatum of the PNE group was higher than in other groups; however this was attenuated by exposure to MS. This could be due to normal functioning of the HPA axis whereby a shift to MR has not been made (Aisa et al., 2009).

DA concentration in the hippocampus of the PNE mice exposed to NS was higher than in the other mice. This effect was not present in the striatum. Studies have shown a notable change in DA concentration in early developmental stress models only following an insult e.g. a lesion (Mpofana et al., 2016). Exposure to NS resulted in an increase in DA concentration when compared to animals exposed to a single stressor. This may suggest that the dual stress exposure has an effect of cancelling out the deleterious effects of exposure to either prenatal or postnatal stress. It must be noted that exposure to both PNE and MS in this study coincided with the period of rapid neural pathway formation in the brain (Chocyk et al., 2010). It therefore could stand to reason that if the co-exposure to PNE and MS stress cancels the stress effect, neural pathway formation will be less compromised.

Our results show that PNE results in long term changes to the α7 and α4 nicotinic acetylcholine receptors. We have also shown that MS alone affects α7 and α4 n-AChR expression, which supports the hypothesis that PNE results in dysfunction of the HPA axis, and that the dysfunction caused by early life stress also results in changes of the cholinergic system, specifically the n-AChRs. Animals exposed to the double insult of prenatal (PNE) and postnatal (MS) stress exhibited behaviour suggesting a cancellation of the effects of perinatal stress (when the stressors are applied individually) on the HPA axis.

Conclusion

Our results have shown that PNE alters neurodevelopment resulting in changes to functional regions of the brain, neurotransmission and behaviour into adulthood. With regard to MS, our results display that species specificities play a role in determining the outcomes of exposure to early life stressors. Our results show that exposure to perinatal stress affects n-AChR expression. This suggests that cholinergic signalling could be targeted for therapeutic intervention in anxiety- and depression-like disorders.

Acknowledgement

We would like to thank the Biomedical Resource Centre for their assistance in this study as well the University of KwaZulu-Natal and the National Research Foundation (NRF) of South Africa for financial support.

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4 Summary and Recommendations

The results of both our studies have shown a link in developmental cholinergic signalling and the HPA axis. Whether the HPA axis is influenced by or influences this signalling has not been fully elucidated. Therefore, recommendations for studying the relationship further would be to introduce another prenatal stressor or have the nicotine exposure at a different timepoint of development. Other neurotransmitters such as glutamate and GABA could also be tested as they may have possible linkage in the connection of the HPA axis and cholinergic signalling.

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