Antidepressant treatment reverses the influence of maternal care on fearful behaviour and hippocampal neuronal survival in adult rat offspring

Naghmeh Rastegar

Department of Neurology & Neurosurgery

Integrated Program in Neuroscience

Supervisor: Dr. Michael J. Meaney

McGill University, Montreal QC

October 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of M.Sc. in Neurological Science.

© Naghmeh Rastegar
Acknowledgments

I would like to extend my gratitude first and foremost to my supervisor Dr. Michael Meaney for allowing me the opportunity and freedom to pursue this line of research. It was Dr. Meaney’s big picture vision and support that made this project possible. I would also like to thank the members of my advisory committee, Dr. Cecila Flores and Dr. Anne McKinney, for their unique insight and guidance throughout the years.

Of course, as many of my colleagues can attest, research in the Meaney lab would not be possible without the supervision and sound advice of Josie Diorio. Along with Dr. Tie Yuan Zhang, their pragmatic insight into cellular and molecular biology helped me to realize what is experimentally possible and to fully express the concepts behind this research.

I would also like to extend my appreciation to all the past and present members of the Meaney lab whose help, support and general collegial attitude created the best environment to pursue academic research. In particular, I would like to thank the biochemistry guru, Dr. Anita Thambirajah, who was not only a source of invaluable insight in the lab, but made exploring Montreal and Quebec a delight, while making us all, envious of the scenic West Coast. I would also like to thank Dr. Carine Parent, for sharing her impeccable knowledge of animal behaviour and especially for her support during the revision process of this thesis.

Finally, I would like to extend my deepest gratitude to my parents and sister for allowing me to see the brighter side of life. Without whose love, support and understanding I could never have completed this degree.
# Table of Contents

Abstract 2

Sommaire 3

## Chapter 1. Introduction

1.1. Maternal care model 7
1.2. Variations in maternal care mediate fearfulness in the adult offspring 9
1.3. Adult hippocampal neurogenesis 11
1.4. Neurogenic hypothesis of fearful behaviour 15
1.5. Pattern separation in the dentate gyrus and the development of fearfulness 16
1.6. Early life maternal care influences neuronal survival in adult offspring 20
1.7. Early life maternal care influences the expression of the pro-apoptotic gene BAX 22
1.8. Statement of the problem 23

## Chapter 2. Materials and Methods

2.1. Animals 24
2.2. Maternal observations 25
2.3. Fluoxetine hydrochloride treatment 25
2.4. Behavioural tests 26
   2.4.1. Forced swim test 26
   2.4.2. Novelty suppressed feeding task 27
2.5. Analysis of adult hippocampal neurogenesis 28
   2.5.1. Bromodeoxyuridine (BrdU) injections 28
   2.5.2. Transcardial perfusion and brain slice preparation 28
   2.5.3. Immunohistochemistry 29
2.6. Gene expression analysis 30
   2.6.1. Tissue preparation 30
   2.6.2. RNA-extraction and cDNA conversion 31
   2.6.3. Quantitative real time PCR 32
### 2.7. Statistical analysis

### Chapter 3. Results

3.1. The effect of chronic fluoxetine treatment on fearfulness and depressive-like behaviour in the offspring of high and low LG mothers.

- 3.1.1. Forced swim test
- 3.1.2. Novelty suppressed feeding test (NSF)

3.2. The effect of chronic fluoxetine treatment on hippocampal neurogenesis.

- 3.2.1. Proliferating cells following fluoxetine treatment
- 3.2.2. Immature neurons following fluoxetine treatment
- 3.2.3. Adult-born mature neurons following fluoxetine treatment
- 3.2.4. Number of dividing cells expressing the transcription factor NeuroD1 following fluoxetine treatment

3.3. Fluoxetine treatment and the expression of pro- and anti-apoptotic genes

### Chapter 4. Discussion

4.1. Fluoxetine treatment reduces behavioural despair in the forced swim test

4.2. Chronic fluoxetine treatment eliminates differences in fearfulness between the offspring of high and low LG mothers

4.3. Reduced fearfulness is associated with an increase in neuronal survival in the ventral hippocampus

4.4. Chronic fluoxetine treatment may eliminate differences in fearfulness between high and low LG offspring by decreasing pro-apoptotic signalling in the ventral DG

4.5. The elimination of differences in fearfulness between high and low LG offspring is associated with an increase in the pro-survival transcription factor NeuroD1

4.6. Implications: How does maternal licking and grooming influence neuronal survival in adulthood?

### Concluding Remarks

### Bibliography
List of Tables and Figures

Chapter 1.

Figure 1. Frequency distribution of cumulative licking/grooming (LG) observed during the first 6 days of life in Long Evans rat dams. 8

Figure 2. The number of newborn dentate granule neurons in the hippocampus of adult offspring of high and low LG dams. 21

Figure 3. Proliferation (i.e. the number of dividing cells) in the hippocampus of adult offspring of high and low LG dams. 22

Chapter 2.

Table 1. List of antibodies used in the immunohistochemical studies. 29

Table 2. List of designed qRT-PCR primer sequences. 33

Chapter 3.

Figure 4. Forced swim test following chronic fluoxetine treatment in the offspring of high and low LG dams. 35

Figure 5. Latency to feed (s) in the novelty suppressed feeding task following chronic fluoxetine treatment. 37

Figure 6. Estimated number of proliferating cells in the dorsal and ventral hippocampal DG region of the offspring of high and low LG dams. 39

Figure 7. Proliferating cells in the hippocampal DG region of high and low LG offspring. 40

Figure 8. Estimated number of doublecortin (DCX) stained cells in the dorsal and ventral hippocampal DG of the offspring of high and low LG mothers. 41

Figure 9. Doublecortin positive cells in the hippocampal DG region of high and low LG offspring. 42

Figure 10. Regression analysis of the latency to feed in the novelty suppressed feeding task and the number of DCX+ cells in the high and low LG offspring. 44

Figure 11. Doublecortin gene expression profile (DCX) in the dorsal and ventral hippocampal DG of high and low LG offspring. 45

Figure 12. Estimated number of mature adult-born neurons in the dorsal and ventral hippocampal DG of the offspring of high and low LG mothers. 47

Figure 13. Adult-born mature neurons in the DG region of high and low LG offspring. 48
Figure 14. Estimated number of BrdU+/NeuroD1+ cells in the dorsal and ventral DG region of the offspring of high and low LG dams. 51

Figure 15. BrdU+/NeuroD1+ double-stained cells in the hippocampal DG region in the offspring of high and low LG mothers. 52

Figure 16. Apoptotic pathway genes following chronic fluoxetine treatment in the offspring of high and low LG mothers. 53
Abstract

Early life abuse and neglect is associated with an increased risk for anxiety disorders and major depression in humans, which suggests a sustained influence of social adversity on brain function. Naturally occurring variations in maternal licking/grooming (LG) in the rat are associated with the level of fearfulness and neuronal survival in the hippocampus of adult male offspring. In rodents, increased fearfulness is associated with a reduced number of adult-born neurons in the hippocampus. Chronic treatment with the selective serotonin reuptake inhibitor, fluoxetine is associated with both reduced fearfulness and enhanced hippocampal neuronal survival in rodents.

We found that the enhanced level of fearfulness observed in offspring of low LG mothers compared to high LG offspring is associated with reduced neuronal survival in the dorsal and ventral hippocampal dentate gyrus of low LG compared to high LG adult male offspring. Chronic treatment with fluoxetine eliminated the differences in fearfulness between offspring from high and low LG mothers. Fluoxetine-treated low LG offspring were less fearful than low LG controls and showed comparable levels of neuronal survival in the ventral dentate gyrus compared to fluoxetine-treated and control high LG offspring. Fluoxetine-treated offspring of low LG mothers also showed reduced ratio of expression of the pro-apoptotic gene BAX to the anti-apoptotic gene BCL2 and an increase in the expression of the transcription factor NeuroD1 in the ventral dentate gyrus. Fluoxetine treatment thus increased neuronal survival in the offspring of low LG mothers and this effect was associated with reduced pro-apoptotic signalling and increased expression of NeuroD1, a transcription factor that is essential for survival of adult-born neurons within the ventral dentate gyrus. These results show that the effects of lower frequency of maternal LG in early life can be reversed using antidepressant treatment in
adulthood. Further, the mechanism by which fluoxetine exerts its effects on reducing fearfulness may involve enhanced neuronal survival in the ventral dentate gyrus.

Sommaire

La négligence et l’abus au début de la vie sont associés à un risque accru pour le développement des troubles de l'anxiété et de la dépression majeure chez l'humain. Les variations de léchage maternelle / toilettage (LG) d'origine naturelle chez la mère rat, sont associées avec le niveau de peur et aussi le nombre de neurones qui survivent dans l'hippocampe chez les rejetons adultes. La peur accrue chez les rongeurs est associée à une réduction du nombre des nouveaux neurones dans l’hippocampe chez l’adulte. Le traitement chronique avec l’inhibiteur de la recapture de sérotonine sélective, la fluoxétine est associée à la fois avec une réduction de la peur et une augmentation de la survie de nouvelles neurones dans l’hippocampe des rongeurs.

Nous avons trouvé que les rejetons venant de mères à bas LG démontrent plus de peur et ces rejetons on moins de neurones qui survivent dans l’hippocampe dorsale et ventrale à l’âge adulte en comparaison avec des rejetons venant de mères haut LG. Le traitement chronique avec la fluoxétine a éliminé les différences au niveau de la peur entre les rejetons de mères haut et bas LG. Le traitement avec la fluoxétine a rendu les rejetons de mères bas LG moins craintifs et a augmenté la survie neuronale dans l’hippocampe ventrale à des niveaux comparables à la survie de neurones chez les rejetons de mères haut LG. Les rejetons de mères bas LG ont également montré une expression réduite du gène pro-apoptotique BAX en comparaison au gène anti-apoptotique BCL2 et aussi une augmentation de l'expression du facteur de transcription NeuroD1 dans le gyrus denté ventrale de l’hippocampe. Le traitement avec la fluoxétine a donc augmenté
la survie neuronale en réduisant la signalisation apoptotique et en augmentant l'expression de NeuroD1, un facteur de transcription qui régule l'expression de gènes impliqués dans la neurogenèse chez les rejetons de mères bas LG. Ces résultats démontrent que les effets des soins maternels réduits en début de vie peuvent être inversés en utilisant un traitement antidépresseur à l'âge adulte. De plus, le mécanisme par lequel la fluoxétine exerce ses effets sur la régulation de la peur est probablement en améliorant la survie des neurones dans le gyrus denté ventrale de l’hippocampe.
Chapter 1. Introduction

The early environment, and especially parental signals, influence offspring development in many species including all mammals (Mousseau & Fox, 1998; de Haas et al., 2014). Since the environment at birth is often the same environment the organism will reside in as an adult, Hinde et al. (1987) among others suggest that slight variations in parental behaviour serve as an indicator of the environmental conditions that the offspring will face in adulthood. In consequence, the ability of the offspring to develop a physiological and behavioural phenotype based on early life parental signals might be adaptive to their survival in adulthood (Hinde et al., 1987, Belsky et al. 1991). These “maternal effects” have been observed in many species of plants and animals. For example in a species of the scincid lizard (Pseudomoia pagenstecheri), individuals that are larger and have longer tails are more successful in avoiding snake predation. Shine & Downes (1999) exposed gestating female scincid lizards to the odour of a predator exclusively during pregnancy and observed that the offspring exhibited increased weight, longer tails and enhanced sensitivity to predator odour. The mother’s exposure to predator odour thus led to the offspring developing a phenotype that was protective against enhanced predation.

Similar patterns are observed in non-human primates (Suomi, 1997; Rosenblum et al., 1994). In a population of Bonnet macaques, mother-infant interactions changed significantly upon prolonged exposure to different foraging conditions. Mother-infant pairs were exposed to three foraging paradigms: Low foraging demand (LFD) where food was readily available, High foraging demand (HFD) where access to food required a great degree of exploratory behaviour and a variable condition where the foraging conditions were unpredictable (VFD). This last condition proved to be the most disruptive and stressful. The offspring raised in VFD paradigm displayed a greater degree of submissive, depressive-like and fearful behaviour during...
adolescence and adulthood. In this highly stressful VFD environment, where unpredictable access to food and greater foraging demand is required, the perceived risk of predation is also enhanced. Therefore, organisms that develop a highly vigilant and fearful behavioural response to the perceived risks are at an evolutionary advantage. These behavioural traits along with reduced social activity have also been observed in offspring that were separated from their mothers in infancy (Suomi, 1997).

In humans, the early environment has a significant influence on brain development and function (Greenough et al, 1987; Fox et al, 2010; Dawson et al, 2000). The quality and quantity of early life parental care is a fundamental component of the early life environment. Studies consistently show that the quality of mother-child interactions influences the offspring’s development and overall health in adulthood (Meaney, 2001; Bowlby, 1988). In particular, maternal care can mediate the negative influence associated with an adverse early life environment. Psychiatric disorders such as anxiety disorders and depression are more prevalent in individuals that experienced early life abuse and neglect (Rutter et al., 2004; Nugent et al., 2011; Heim & Binder, 2012). Adverse conditions during childhood also enhance the risk for internalizing (i.e. depression or anxiety-like) and externalizing (i.e. aggressive and impulsive) behaviours (Hackman et al., 2010). The length of exposure to such adverse conditions greatly increases the propensity for both internalizing and externalizing behaviours (Korenman et al., 1995). A greater quality of parental-child interactions can buffer children from adverse environmental conditions leading to greater stress-resilience in children (Masten et al., 1990). Similarly, clinical intervention programs that improve parental care dynamics improve cognitive and social outcomes in children (Hackman et al, 2010; Olds et al., 1998; Fisher et al., 2000). Despite the relative success of these early childhood intervention programs, the correlational
nature of clinical studies is a major hurdle towards understanding the causal relationship of maternal care on susceptibility to developing psychopathology. To overcome this problem, studying non-human primates and rodents is highly advantageous.

1.1. Maternal care model

We focus on naturally occurring variations in maternal care as the primary feature of the early environment in the rat (Champagne et al., 2003). The first week of life represents a sensitive period in postnatal development, as the pup is very responsive to environmental signals, especially those emanating from the mother. Maternal licking and grooming (LG) is of particular importance during this period as stimulation of the pups not only facilitates growth and development, but also greatly enhances basic physiological functions including urination and defecation (Rosenblatt & Lehrman, 1963; Schanberg et al., 1984).

There are considerable and highly stable individual differences in the frequency of pup LG across lactating female rats. In a population of out-bred Long Evans rats, the frequency of pup licking and grooming by a lactating female follows a normal distribution. We use the frequency of licking/grooming (LG) behaviour of the dams to quantify variations in maternal care during the first 6 days of life. In each cohort, rat mothers are designated as high, mid or low LG based on their frequency of pup LG over the first 6 days post-partum. Low LG dams have LG frequency scores one or more standard deviations below the mean for the breeding cohort, while high LG dams have LG frequency scores one or more standard deviations above the mean (Figure. 1).

The frequency of maternal LG remains stable across multiple litters. The weight and number of pups as well as the ratio of male to female offspring in each litter is similar among the
high and low LG dams. There is also no difference in the number of pups that survive to adulthood among the offspring of high and low LG mothers. Moreover, the degree of nourishment that the pups receive is comparable as there is no difference in the amount of contact time that high and low LG mothers spend with their pups. Cross fostering studies in our lab show that morphological and phenotypic outcomes in adolescent and adult rat offspring are a result of naturally occurring variations in active maternal care behaviour (i.e. LG) that the rat mother engages in during her offspring’s early development (Champagne et al, 2003).

![Figure 1. Frequency distribution of cumulative licking/grooming (LG) observed during the first 6 days of life in Long Evans rat dams.](image)

The amount of time the dams spend licking and grooming follows a normal distribution. The population Mean and Standard Deviation (SD) is calculated. Dams with LG scores greater than 1 SD above the mean are designated as high LG (white bars), while those exhibiting scores lower than 1 SD below the mean are characterized as low LG mothers (black bars) (Adapted from Champagne et al, 2003).

Individual differences in maternal LG have a profound influence on cognitive and behavioural outcomes in the offspring. In a measure of hippocampal-dependent learning, the male offspring of high LG mothers, are able to learn the location of a hidden platform in the
Morris water maze with significantly reduced latency and display greater ability to recall the location in subsequent trials compared to the offspring of low LG mothers (Liu et al., 2000). In a non spatially dependent learning test, the object recognition task, the male offspring of high LG dams show significantly enhanced recall of the previously explored object compared to the offspring from low LG mothers (Bredy et al., 2003b).

Early life experience also influences the level of behavioural despair that the offspring exhibit as adults. In a measure of behavioural despair, developed by Porsolt et al. (1977), the animals are exposed to a highly stressful swimming environment. The amount of time that the animal spends immobile (stops struggling to exit the stressful situation) is taken. This task is classically used to measure the efficacy of anti-depressant treatment in rodents. Following treatment with anti-depressants such as fluoxetine, the amount of time that the animal spends immobile is significantly reduced. In the model of maternal care, the offspring from high LG mothers display reduced immobility compared to low LG offspring (Weaver et al., 2005). In the forced swim test, offspring from high LG mothers spend more time in motion compared to offspring from low LG mothers. However, the outcome of anti-depressant treatment on reversing the influence of maternal care on forced-swim immobility in adult offspring is unknown.

1.2. Variations in maternal care mediate fearfulness in the adult offspring

Naturally occurring variations in maternal LG also influence fearful and defensive responses in adult offspring. Fearfulness is defined as “a motivational state aroused by specific stimuli that gives rise to defensive or escape behaviour” (McFarland, 1987). Anxiety-like behaviour is believed to be a more complex form of emotional response requiring knowledge of an internal cognitive and decision-making process (Craig et al., 1997; Steimer et al., 2002).
Offspring from low LG mothers show enhanced fearfulness when exposed to a novel environment compared to the offspring from high LG mothers (Caldji et al., 1998). When exposed to the anxiogenic environment of a novel, open field, the offspring of low LG mothers displayed greater fear behaviour by spending less time at the centre of the open field. In the novelty-suppression feeding task, food-deprived offspring from low LG mothers show an increased latency to approach and eat food in the centre of a novel, open field compared to offspring from high LG mothers (Caldji et al., 1998). This hesitation to approach the food source derives from the conflict between the need to satiate their hunger, as exhibited by rapid feeding upon return to their home environment, and the neophobia associated with a novel brightly lit environment. In the shock-probe burying test, adult offspring of low LG dams displayed higher levels of fear reactivity compared to offspring from high LG mothers. The offspring of low LG mothers showed increased freezing behaviour and spent more time burying the electrified probe in response to shock compared to offspring from high LG mothers (Menard et al., 2004).

Postnatal manipulations occurring during early development also influence fearfulness in adult offspring. Postnatal handling of pups leads to an increase in maternal LG of the pups by the mothers (Barnett & Burn, 1967; Smotherman & Bell, 1980). The handled offspring are less fearful as adults in the novelty-suppression feeding task compared to pups from non-handled litters (Caldji et al., 2000; Bodnoff et al., 1987). The female offspring from handled litters also show increased maternal LG behaviour as adults compared to females from non-handled litters (Francis et al., 1999). Similarly, a maternal separation paradigm that involves varying the amount of time a rat mother spends with her offspring is associated with the level of fearfulness displayed in novel settings. Maternally-separated offspring showed increased levels of fear as non-handled animals in novel environments (Caldji et al., 2000). These results are not exclusive
to rodent models. In a cross-fostering study, anxious rhesus monkey infants that were housed with mothers displaying high levels of maternal care showed reduced sensitivity to novel environments and less inhibited behaviour. In contrast, increased levels of maternal care did not affect less anxious infants (Suomi, 1997).

1.3. Adult hippocampal neurogenesis

New neurons continue to develop in the adolescent and adult mammalian brain. These new neurons develop in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) adjacent to the lateral ventricles (Altman & Das, 1965; Cameron & McKay, 1999; Cameron et al., 1993; Kaplan & Hinds, 1977; Kuhn et al, 1996). Rats and mice that display enhanced fearfulness show a decreased number of newborn neurons in the subgranular zone (SGZ) of the hippocampus during postnatal development and adulthood (Santarelli et al., 2003; Revest et al., 2009). The dentate gyrus (DG) of the hippocampus is one of the brain regions that has garnered much attention in explaining the association between the development of new neurons and the risk for anxiety disorders (Revest et al, 2009, Kempermann et al, 2003; Petrik et al, 2012).

Immature neurons of the SGZ integrate into the granule cell layer of the DG (Overstreet-Wadiche & Westbrook, 2006). Approximately 1 week after birth, adult-born neurons receive GABAergic input. At 2-3 weeks of age these new neurons receive glutamatergic input and begin to extend their dendrites and form spines (Ming & Song, 2011). At 4 weeks of age, the new neurons form functional outputs to hilar cells and the CA3 region of the hippocampus (Jessberger & Kempermann, 2003; Ge et al, 2007). During this critical three-week period the survival of adult-born dentate granule cells (DGCs) is activity-dependent. Signals that are
associated with neuronal activity such as the expression of immediate early genes regulate the rate of survival of newborn neurons (Veyrac et al., 2013). The majority of adult-born DGCs will fail to integrate into the hippocampal neuronal circuitry and undergo apoptosis (Veyrac et al., 2013).

Bromodeoxyuridine (BrdU) labeling analysis is the gold standard in examining the number of newborn and proliferating neurons (Gratzner, 1982). The endogenous DNA base, thymidine, is substituted with the BrdU analogue during mitosis and thus specifically labels dividing cells. The analogue is then passed on to the progeny of the dividing cells, making BrdU labeling a very useful method for deciphering the lineage and fate of newborn cells. Typically BrdU is injected intraperitoneally (IP) and animals are perfused and sacrificed at variable time points depending on the nature of the experiment to examine proliferation, cell fate and survival of newborn cells. The dividing cells are easily detected using fluorescent or confocal microscopy.

Different markers used to differentiate cell types can denote the fate of the dividing cell. One such cell type marker is known as NeuN and is commonly used to identify neurons (Mullen et al., 1992). This nuclear protein is present in most neuronal cell types throughout the vertebrate nervous system. Mitral cells of the olfactory bulb, purkinje cells and photoreceptor cells in the retina, however, do not express the NeuN protein (Mullen et al., 1992). In neurogenic studies, adult-born new neurons are present mainly in the dentate gyrus and the olfactory bulb. Therefore, NeuN in combination with the cell division marker BrdU, has become a gold standard to determine whether the dividing cell born in adulthood matures into a neuron.
Another marker that identifies a dividing cell is the proliferation marker Ki-67. Traditionally, the Ki-67 antibody is used in clinical settings to determine the progression of carcinomas (Scholzen & Gerdes, 2000). The antibody recognizes a nuclear structure present only in the dividing cells. The protein is present in the nuclei of the cells in active phases of the cell division cycle (G1, S and G2), but is not present in the nuclei of cells during the resting phase (G0) (Gerdes et al., 1984). This unique pattern of expression makes it the ideal candidate in studying all proliferating cells. In studies of newborn cells in the adult brain, Ki-67 is used in combination with other cell fate makers to denote proliferation. This antibody is especially useful in studies where multiple BrdU injections and animal sacrifice at different time points is not feasible within the timeline of the experiment.

Doublecortin (DCX), another neuronal marker, is a protein that binds to microtubules in immature neurons throughout the nervous system. This protein is associated with cell migration in neuroblasts. In the adult brain, the expression of doublecortin is restricted to the dentate gyrus, the rostral migratory stream and the olfactory bulb where the new neurons are mainly generated (Brown et al, 2003). As the newborn neurons enter the adult stage and start expressing mature neuronal markers, the expression of doublecortin is decreased to levels where it cannot be detected using immunohistochemical methods. Similar to findings using BrdU labelling, the number of DCX stained cells also decreases throughout the lifespan of rodents in accordance with the notion that the number of newborn neurons decline with age (Kuhn et al, 1996, Kempermann et al, 1998, Brown et al, 2003). The transient nature of doublecortin expression makes it a good marker for measuring the number of immature neurons without the need to sacrifice multiple cohorts of animals at different time points.
The precise mechanisms regulating the differentiation and survival of new adult-born neurons remain elusive. Gao et al. (2009) show that the expression of the proneural basic helix-loop-helix (bHLH) transcription factor NeuroD1 is essential in the differentiation and survival of the adult born hippocampal neurons. The bHLH family of transcription factors is involved in regulating cell fate and differentiation throughout development in many organisms. NeuroD1 in particular, is required for granule cell differentiation in the hippocampus and the cerebellum (Miyata et al., 1999). When NeuroD1 is specifically knocked-out in neuronal stem cells, the number of adult-born neurons is significantly reduced in both the hippocampus and the olfactory bulb (Gao et al, 2009). The promoter sequence of NeuroD1 possesses a T-cell factor/lymphoid enhancer factor (TCF/LEF) regulatory element (Kuwabara et al, 2009). TCF/LEF is the major transducer of the canonical Wnt/beta-catenin pathway that is essential in adult hippocampal neurogenesis (Wu & Hen, 2013). Recent studies show that astrocytes that induce production of new neurons also induce an increase in levels of NeuroD1 expression in the neurogenic niche (Kuwabara et al, 2009). Gao et al posit that environmental manipulations such as environmental enrichment lead to an increase in the proliferation and survival of newborn neurons in the hippocampus by upregulating the activity of downstream target genes of the NeuroD1 transcription factor. Seo et al. (2007) showed that NeuroD1 induces the expression of genes that regulate transcription, signal transduction, and cytoskeletal rearrangement, all processes involved in neuronal differentiation and migration. Among the predicted downstream targets of NeuroD1 are genes involved in the regulation of neurogenesis including members of the wingless (Wnt) family that play a major role in development (Seo et al., 2007). NeuroD1 targets the expression of genes that regulate neurogenesis. Here we aim to examine whether environmental manipulations such as anti-depressant treatment can influence the expression of NeuroD1 in newborn neurons.
1.4. Neurogenic hypothesis of fearful behaviour

Fear in animals is characterized by a set of avoidance, arousal and defensive responses to a specific object or situation such as a novel environment (Perusini & Fanselow, 2015). For example, heights, light and open spaces produce fear in rodents and are therefore used in behavioural tasks to reflect fearfulness (Bailey & Crawley, 2009). The open-field test (OF) and the novelty suppressed feeding test (NSF) are commonly used tasks to assess fearfulness in rats (Britton & Britton, 1981; Walsh & Cummins, 1976). The amount of time animals spend at the centre of the brightly lit open field box is used as a measure of fearfulness (Walsh & Cummins, 1976); more fearful animals show reduced time spent in the centre area. In the novelty suppressed feeding task (NSF) task a food-deprived rat is presented with food at the centre of a brightly lit arena. The latency to begin eating is used as a measure of fearfulness. The latency to begin feeding in the NSF test can be reduced using anxiolytic and antidepressant drugs including fluoxetine (Britton & Britton, 1981, Malberg et al, 2000; Santarelli et al, 2003).

The neurogenic hypothesis of fear behaviour proposes that a decrease in the number of adult-born neurons is associated with an increased risk for the development of increased fearfulness in rodent models and anxiety disorders in humans. Effective pharmacological and psychological treatments for fearful behaviour such as selective serotonin reuptake inhibitor treatment or cognitive behavioural therapy are associated with increased SGZ neurogenesis (Petrik et al, 2012; Gross & Hen, 2004). Anxiety disorders, such as post-traumatic stress disorder (PTSD), are characterized by a heightened fear response in the presence of neutral stimuli that are reminiscent of the traumatic experience. Patients seem unable to distinguish between the different contexts in which the neutral stimuli are presented and thus elicit fearful behaviour. Kheirbek et al. (2012) suggest this excessive overgeneralization stems from the inability of the
individual to distinguish the new environment from previously stored memories. The authors posit that pattern separation, a function of the dentate gyrus, is essential in the ability to distinguish distinct memory traces of similar experiences.

1.5. Pattern separation in the dentate gyrus and the development of fearfulness

Perhaps the most intriguing evidence for the role of DG granule neurons in pattern separation comes from ablation studies. In a test of spatial pattern separation, DG-lesioned rats, were unable to discriminate between distinct objects that are placed spatially close together (Gilbert et al., 2001). Similarly, the lesioned rats were unable to distinguish small changes in the location of the object. Since the DG is also one of the few regions of the brain where birth of new neurons continues throughout adulthood, studies examined the role of adult-born neurons in pattern separation (Aimone et al., 2011; Sahay et al., 2011). This is a particularly intriguing idea since the newborn neurons comprise around 10% of the dentate granule cells and can functionally integrate into the adult dentate gyrus. In a series of experiments, mice with x-ray irradiation ablated DG neurogenesis showed deficits in spatial discrimination tasks such as the radial arm-maze and the touch screen test. In the radial arm-maze, x-irradiated mice were more error-prone when the arms were placed close together compared to controls. Similarly, in the touch screen task, x-irradiated mice were unable to correctly distinguish visual cues that were placed close together (Clelland et al., 2009). In a contextual fear-conditioning task, x-ray irradiated mice did not change their freezing behaviour in similar contexts, but were able to distinguish between highly different contexts (Kheirbek et al., 2012; Nakashiba et al., 2012). Moreover, Sahay et al (2011) used a genetic gain-of-function strategy to selectively knockout the expression of the pro-apoptotic gene BAX in neuronal stem cells in the adult mouse brain. They
show that this enhanced survival of newborn neurons in mice leads to increased ability to discriminate between similar contexts.

In studies of fearfulness, rodents that were exposed to x-ray irradiation to ablate hippocampal neurogenesis are more fearful than non-irradiated rats (Santarelli et al., 2003; Revest et al, 2009). For example, inhibition of DG neurogenesis leads to increased latency to feed in the NSF task. Treatment with the antidepressant fluoxetine, reduces the latency to feed in the NSF test revealing an important anxiolytic effect of this antidepressant drug. Hippocampal irradiation eliminates the anxiolytic effects of fluoxetine on the latency to feed in the NSF test suggesting that the mechanism for the anxiolytic effects of fluoxetine is due to the effect of the drug on hippocampal neurogenesis (Santarelli et al, 2003).

Ablation of neurogenesis (new adult-born granule cells) in the DG also reduced inhibition of the mature granule cells. Singer et al, (2011) conclude that these young neurons have an inhibitory influence and directly modulate the excitability of the dentate gyrus. Kheirbek et al. (2011) suggest that this influence will be spatial or emotional in nature depending on the location of the input along the dorso-ventral axis of the DG. An increasing number of studies suggest differential roles for the dorsal and ventral regions of the hippocampus (Fanselow & Dong , 2010). Lesion experiments supported by anatomical connectivity and gene expression profiles show that the dorsal hippocampus regulates spatial learning and memory whereas the ventral hippocampus mediates emotional behaviours (Kheirbek & Hen, 2011). Spatial learning and memory mainly occurs via dorsal hippocampal outputs that connect to mammillary nuclei, the anterior cingulate and the retrosplenial cortex. Projections from the ventral hippocampus extend to the prefrontal cortex (PFC), the amygdala, the nucleus accumbens shell, the bed nuclei of the stria terminalis and the hypothalamus (Fanselow and Dong, 2010). This network coordinates the
neuroendocrine and autonomic responses to emotional stimuli. Recent studies show a particular role of the ventral hippocampus in fearful behaviour in rodents. Activation of the basolateral amygdala using optogenetic stimulation is associated with increased fearfulness as evidenced by less time in exploration of the centre of the open field and the open arms of the elevated plus maze as well as an increased latency to feed in the NSF task (Felix-Ortiz & Tye, 2013). Conversely, inhibition of basolateral amygdala input to the ventral CA3 pyramidal cells had an anxiolytic effect. The mice spent an increased amount of time at the open arms of the elevated plus maze as well as in the centre of the open field. Consistent with the inhibitory hypothesis of the newborn granule cells, Kheirbek et al (2013) showed that stimulation of the ventral dentate gyrus decreased the anxiety-like behaviour of mice resulting in increased exploration of the open field and the elevated plus maze. These studies to date suggest a prominent role for an influence of newborn DG neurons in pattern separation. Dysfunction of the dentate gyrus due to reduced number of adult-born new neurons could contribute to the overgeneralization of fearful behaviour observed in many anxiety disorders (Kheirbek et al., 2012). Using treatments to increase adult-born neurons in the ventral axis of the DG may improve pattern separation thus reducing the risk of overgeneralization of anxious behaviour in similar contexts. Indeed, chronic, but not acute treatment with anti-depressants including selective serotonin reuptake inhibitors such as fluoxetine, increase adult hippocampal neurogenesis and survival (Malberg et al., 2000; Duman et al, 2001). Chronic fluoxetine treatment also leads to early maturation and increased plasticity of new neurons (Wang et al, 2008). This effect is not limited to fluoxetine, but also includes tricyclic anti-depressants. Environmental enrichment, exercise running and electroconvulsive shock therapy are also accompanied by an increase in neurogenesis (Santarelli et al., 2003; Malberg et al., 2000; van Praag et al., 2000; Madsen et al, 2000; Manev et al., 2001).
In patients with major depressive disorder, antidepressants increased populations of newborn neurons mainly in the ventral (anterior) portion of the hippocampus (Boldrini et al., 2009).

The evidence to support the neurogenesis hypothesis in the etiology and neurobiology of affective disorders is less conclusive. Initially, the overlap of the timeline of integration and survival of the newborn hippocampal neurons with the course of effectiveness of anti-depressant treatment suggested a potential causal role for neurogenesis in depression (Schoenfeld and Cameron, 2015). However, more recent results with the fast acting anti-depressant ketamine as well as a meta-analysis showing that common antidepressants can become effective faster than previously thought, cast doubt on this timeline (Browne and Lucke, 2013; Lam, 2012). Moreover, several studies show conflicting results as to the correlation between increased depressive symptoms and decreased adult-born neurons in the hippocampus (Petrik et al., 2012; Eisch & Petrik, 2012; Hanson et al, 2011). These discrepant results could stem from the different methods used to inhibit adult neurogenesis including x-ray irradiation in some studies and the use of the mitotic blocker methylazoxymethanol acetate (MAM) in other studies. Additionally, tests used to assess “depressive-like” behaviours in rodents such as the forced swim test (FST), can contribute to the differential results observed. For instance, several studies show that ablating adult neurogenesis using either MAM or x-ray irradiation did not change the time spent immobile (a measure of behavioural despair) in the FST (Airan et al, 2007; Bessa et al, 2009; Holick et al, 2008). On the other hand, Snyder et al (2009) showed that using MAM to inhibit adult neurogenesis increased immobility in the FST. They posit that since the FST is very stressful compared to the NSF test, the results highlight the potential role of newborn neurons in mediating the response to stress.
Antidepressant treatment and environmental enrichment both increase neurogenesis in the SGZ and reduce fearfulness in rodent models (Malberg et al, 2000, Duman et al, 2001; van Praag et al, 2000). Anti-depressant treatment reduces fearful behaviour in rodents by enhancing adult hippocampal neurogenesis. Similarly, ablation of adult-born SGZ neurons eliminates the anxiolytic effects of chronic antidepressant treatment (Santarelli et al, 2003). Control mice treated with the antidepressant fluoxetine showed decreased latency to feed indicating reduced fearfulness. Mice exposed to X-irradiation in the hippocampus showed no effect of chronic fluoxetine treatment in the NSF test. This suggests that survival of newborn neurons is necessary to elicit the anxiolytic effects associated with chronic antidepressant treatment.

1.6. Maternal care influences neuronal survival in adult offspring

Individual differences in maternal care influence neuronal survival in adult offspring. Over the course of development, the offspring of high LG mothers show greater survival of newborn hippocampal neurons compared to offspring from low LG mothers (Bredy et al, 2003a). Bredy et al (2003a) analysed proliferation and survival of new neurons during postnatal development (P7, P21) and adulthood (P90). The offspring of high and low LG mothers were injected with the cell division marker BrdU at postnatal day 6. To measure proliferation, one group of offspring were sacrificed 24 hours post-injection at P7. BrdU+ cells were also examined at P21 and P90 to measure the number of dividing cells at each stage. The neuronal marker NeuN was used to examine the fate of the dividing cells. Measurements of the number of BrdU/NeuN-positive labelled cells were taken as a measure of adult-born neurons. There was no difference in the number of dividing cells (i.e. proliferation) at P7. During adolescence (P21) and in adulthood (P90), there were significantly greater numbers of BrdU+/NeuN+ cells across the whole hippocampus in the male offspring of high LG mothers compared to the offspring of low
LG mothers. An assessment of neuronal proliferation and survival along the dorso-ventral axis of the hippocampus in adulthood also showed that high LG offspring display greater survival of new neurons in both the dorsal and ventral dentate gyrus compared to low LG offspring (Figure 2). In a measure of proliferation, the offspring of high and low LG mothers, did not display a significantly different number of Ki-67-positive cells across the dorso-ventral axis (Figure 3). The influence of maternal care on neuronal survival is consistent across the dorso-ventral axis. However, it is not known whether the heightened fear response of the offspring of low LG mothers is associated with a decrease in hippocampal neurogenesis including the survival of newborn neurons.

Figure 2. The number of newborn dentate granule neurons in the hippocampus of adult offspring of high and low LG dams. A) In the dorsal DG, there is a significantly increased number of new neurons in the offspring of high LG mothers. (n=8, t-test, p < 0.005). B) In the ventral DG, the offspring of high LG dams also show significantly greater numbers of adult born new neurons. (n=8, t-test, p < 0.005) (Danik, M. 2011; unpublished data).
1.7. Early life maternal care influences the expression of the pro-apoptotic gene BAX

One of the most crucial pathways that regulates the cell death or apoptosis of neurons involves the Bcl-2 family of proteins. The Bcl-2 family includes both pro-apoptotic and anti-apoptotic signalling genes that share one or more Bcl-2 homology (BH) domains (Merry & Korsmeyer, 1997). One of the main pro-apoptotic genes is the Bcl-2-associated X (BAX) gene (Deckwerth et al, 1996). BAX is present in the outer membrane of the mitochondria, endoplasmic reticulum and perinuclear membrane. As an organism ages, there is a significant decrease in the number of immature neurons. BAX-deficient mice show an increase in the number of neurons (particularly in superior cervical ganglia and facial nuclei) and resistance to cell death following nerve growth factor withdrawal (Deckwerth et al, 1996). As the organism advances in age and the neurotrophic factor levels decline, or following an injury, the BAX gene plays a crucial role in neuronal cell death (Yuan & Yankner, 2000).

The anti-apoptotic proteins in the Bcl-2 family are crucial for cell maintenance and neuronal survival. As the gene name implies, their function in enhancing neuronal survival was
first identified in cancer cells where the apoptotic mechanism is downregulated. The BCL2 gene is highly expressed in the central nervous system throughout development to promote neuronal survival and decreases as the organism advances in age. In mice that overexpress the BCL2 gene, neurons overcome the apoptotic signals that follow a reduction in levels of nerve growth factor (Garcia et al, 1992). In contrast, BCL2 knockout mice develop normally, but lose a significant portion of sympathetic, motor and sensory neurons after birth (Veis et al, 1993; Michaelidis et al, 1996).

Previous studies from the lab indicate that maternal care influences the expression of the pro-apoptotic signalling gene Bcl-2-associated X (BAX). Weaver et al (2002) extracted protein from whole hippocampus in the male offspring of high and low LG mothers. Using western blot analysis they showed that the offspring from low LG dams have increased levels of the BAX protein in the whole hippocampus compared to high LG offspring. For the anti-apoptotic protein BCL2, the offspring of high LG and low LG dams showed similar BCL2 protein levels. Furthermore, the offspring of high LG mothers display lower numbers of pyknotic (i.e. dying) cells compared to low LG offspring. Weaver et al (2002) suggest that during the life of the cell, the ratio of pro-apoptotic to anti-apoptotic signals is what decides the final fate of the cell (Yuan & Yankner, 2000).

1.8. Statement of the problem

The main hypothesis of this thesis is that variations in early life maternal care influence hippocampal neurogenesis in adult offspring and thus affect levels of fearfulness in adult offspring. Further, antidepressant treatment in adulthood may reverse the effects of early life maternal care on both hippocampal neurogenesis and fearfulness in adult rat offspring.
Specifically, we hypothesize that a reduced frequency of maternal licking and grooming results in a reduced number of new hippocampal neurons that could be the mechanism for enhanced fearfulness in offspring from low LG mothers. We hypothesize that treatment with the SSRI, fluoxetine, will enhance neuronal survival thus reducing fearfulness in the offspring of low LG mothers. We propose that reduced fearfulness following antidepressant treatment will associate with an increase in hippocampal neuronal survival along the dorso-ventral DG axis. We also hypothesize that an increase in the number of adult newborn neurons will associate with increased expression of the transcription factor, NeuroD1. In summary, we propose that chronic antidepressant treatment with fluoxetine will reverse the effects of early life maternal care on adult fearfulness through enhanced hippocampal neuronal survival.

Chapter 2. Materials and Methods

2.1. Animals

Long-Evans Hooded rats from Charles River Canada (St.-Constant QC) were bred in house at McGill University (Douglas Mental Health University Institute Animal Facility). Pregnant female rats were pair-housed in 18 x 30 x 46 cm cages until a week before birth in a room with a 10:14 light:dark cycle with lights on at 09:00 and ad libitum access to food and water. All animals used in this study were the adult male offspring of dams that were observed at the Douglas animal facility and weaned at postnatal day 21 (P21). Upon weaning, the male offspring were pair-housed under a 12:12 light-dark cycle with lights on at 8 AM. Except for regular cage maintenance, the pups were left undisturbed until the start of the experiment. All
procedures conformed to the guidelines established by the Canadian Council on Animal Care (CCAC).

2.2. Maternal observations

Maternal observations were performed during the first 6 days of life according to the previously published protocol (Champagne et al., 2003). Maternal behaviour was scored in five sessions each day during postpartum days 1-6, with two sessions (07:00h and 20:00h) in the dark and three sessions (10:00h, 13:00h and 17:00h) under the light condition. In each 72-min session, the behaviour of each dam was scored once every 3 minutes for a total of 750 observations per litter over the first six days of life. Behaviours scored included: licking and grooming (LG) of pups, nursing posture, passive or active nursing and contact with pups or being away from pups (Myers et al., 1989; Champagne et al., 2003). For the purposes of the following studies, the frequency of LG was calculated for cohorts of 30-40 dams. For each cohort this frequency follows a normal distribution (Champagne et al 2003). The mean LG score for the cohort was then calculated and dams with LG scores greater than one standard deviation above the mean were designated as “high LG” mothers and those with scores one standard deviation below the mean and lower were designated as “low LG” dams. The adult (P90) male offspring from these litters were used in the following experiments.

2.3. Fluoxetine hydrochloride treatment

Fluoxetine (13mg/kg/day) was administered to adult rats (P90-100, n=10-12/group) ad-libitum in drinking water for the duration of the experiment until the date of sacrifice. This dose is shown to be effective in improving fearful behaviour in rats without inducing adverse physiological effects (Thompson et al., 2004). Male offspring of high LG and low LG mothers
were weighed and their water consumption over a 24-hr period was measured to determine the required concentration of drug in the drinking water (e.g. assuming a 500g rat consumes 30ml of water in 24hrs, a dose of 13mg/kg=6.5mg/30ml= [0.3g/L] solution was required). The bottles were protected from light to prevent degradation and the solution was replaced and freshly made every 48 hours. Control rats were given regular drinking water during this time. Water consumption among the experimental litters remained constant. Despite weight loss early on in the experimental fluoxetine treatment group, both the experimental and control rat groups had similar body weights by the end of the study.

2.4. Behavioural tests

Adult male offspring of high LG and low LG mothers (10-12 animals/group) were treated with fluoxetine solution or drinking water alone as detailed above for the entire duration of the experiment. Twenty-eight days following the start of the treatment, the forced swim test (FST) and the novelty suppressed feeding task (NSF) were used to measure immobility and fearful behaviour respectively as detailed below.

2.4.1. Forced swim test

The forced swim test (Porsolt et al., 1977) was used to examine the degree of behavioural despair among the offspring of high LG and low LG mothers following chronic fluoxetine treatment. During day 1 (training or acclimatization session) rats were placed individually into a clear plexiglass cylinder (25cm diameter, 65cm height) with water at a depth of 35-40 cm (23-25°C) for a period of 15 min. This session was recorded using a video camera and the total time the rat spent immobile, swimming or climbing was measured. The cylinder was emptied of water and cleaned using Peroxigard (Hydrogen Peroxide) solution and rinsed after each rat was tested.
Day 2 (test day, 24-hr post-training session): The training phase and the testing phase of the experiment were completed at the same time of day for each rat. The test session lasted 10 min. Both training and test sessions were recorded using a digital camera kept at water level to observe the entire body length of the animal and assist in better coding the activity. Time spent immobile was defined as the period of time the animal spent motionless or floating, or only taking the minimal actions required to keep its head above water level. Climbing was considered as the time the animal spent in thrashing motions on the walls of the container. Swimming was measured as any coordinated motion of the animal in the container away from the walls. To ensure accuracy, immobility was calculated as follows: $T_{\text{immobile}} = 600s - (\text{Time Climbing} + \text{Time Swimming})$. The time spent immobile in the FST was used as a measure of “behavioural despair”. Classically, the forced swim paradigm has been used to test the efficacy of anti-depressant treatment. Acute and chronic administration of anti-depressants such as fluoxetine has been shown to reduce the time that the animal spends immobile (Thompson et al., 2004; Dulawa et al., 2004).

2.4.2. Novelty suppressed feeding task

Experimental animals were deprived of food overnight (approximately 16 hours) with ad libitum access to water prior to the test. Rats were placed into an open field arena (100 x 100 x 50 cm). Two light sources (~40-50 lux) were used to illuminate the arena. Eight food pellets (in a large clump) were placed at the centre of the arena. The latency to feed was measured as the time taken for the animal to begin eating (defined as when the rat first bit the food and continued eating). Immediately after the completion of this 10 min test, the animal was transferred to its home cage, and the latency to feed in its home environment was measured. A single pellet was placed in the home cage at the same location in each cage and the time taken for the animal to
first bite the pellet was measured. The test was recorded by placing a digital camera above the open field.

2.5. Analysis of adult hippocampal neurogenesis

2.5.1. Bromodeoxyuridine (BrdU) injections. BrdU (20 mg/ml) stock solution was prepared as follows: 1g BrdU (B5002, Sigma-Aldrich) was dissolved in a 50 ml solution of 0.9% saline + 47 ul NaOH 10N. Following the last day of the behavioural tasks outlined above, two intraperitoneal injections of 100 mg/kg (100 mg/kg; for a 500 g rat = 2.5 ml of stock solution) were administered 24-hrs apart. Twenty-eight days following the last injection the animals were sacrificed via transcardial perfusion.

2.5.2. Transcardial perfusion and brain slice preparation. Twenty-eight days following the last BrdU injection, the animals received a single 1.5 ml intraperitoneal injection of sodium pentobarbital (dose 200 mg per kg). The rats were transcardially perfused following the standard protocol (Bredy et al, 2003; Gage et al, 2012). The animals were then decapitated and the whole brain was extracted, and stored in 4% paraformaldehyde overnight followed by 24 hours in a 30 % sucrose solution, until the brain reached the bottom of the tube. The brains were then flash frozen in 2-methylbutane on dry ice and stored at -80°C until sectioning. Using a cryostat (Microm, HM500) 40 µm serial sections (6 x 10-12 slices per well) of dorsal hippocampus (coronal, Bregma -2.6 to -4.6, Paxinos and Watson, 1986) and ventral hippocampus (horizontal, Bregma -4.8 to -8.4, Paxinos and Watson, 1986) were prepared. The slices were kept in cryoprotectant solution and stored at -20°C.
2.5.3. Immunohistochemistry. Free-floating sections from the above serial sections were used in the following immunohistochemical experiments with approximately 8-10 dorsal hippocampus and 8-10 ventral hippocampus sections selected per animal for each experiment.

Table 1. List of antibodies used in the immunohistochemical studies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
</table>
| BrdU/NeuN Labelling      | 1) Anti-BrdU antibody: rat monoclonal, Abcam (ab6326) (1:400).  
| BrdU/NeuroD1 Labelling   | 1) BrdU antibody: rat monoclonal, Abcam (ab6326) (1:400).  

All immunohistochemical procedures followed the standard protocol for assessing new, proliferating and adult neurons (Wojtowicz and Kee, 2006). Briefly, sections were treated with 1 N HCl (30 min at 45°C) and washed in PBS (pH 7.2-7.4). Sections were then incubated in 0.3% Triton X 100 in PBS for 30 min and then blocked in a solution of 3% normal donkey serum + 0.1% Triton-PBS for 1 hour at room temperature. Primary anti-body incubation took place under agitation for 48 h at 4°C. Sections were incubated in secondary antibody at room temperature for 2 hours followed by washes in PBS.

For double-labelling experiments, sections were incubated in the first primary antibody for 48 h at 4°C followed by incubation in the second primary antibody under the same
conditions. Both secondary antibody incubations took place at room temperature simultaneously for 2h. Sections were then rinsed in PBS and mounted on gelatin-coated slides and fixed with Vectashield mounting medium (Vector laboratories, Burlingame, CA). Serial sections from all animals were processed in parallel.

Labeled cells were counted in the subgranular zone (SGZ) and granule cell layer (GCL) of every sixth section (300 µm apart) throughout the dorsal and ventral hippocampus (8-10 sections per animal) under 20X objective magnification using a fluorescent microscope (Olympus, BX63). For each section, three images of equivalent scale and surface area were analyzed comprising the entire SGZ and GCL regions. The total number of labeled cells per dentate gyrus was calculated by multiplying the average number of labeled cells in each section (i.e. numerical density per volume) by the thickness of each section and the number of sections per animal (Bredy et al, 2003a). Double-labeled cells were imaged using the fluorescence microscope and confirmed by confocal microscopy.

2.6. Gene expression analysis

2.6.1. Tissue preparation. A second cohort of male offspring of high and low LG mothers was chronically treated with fluoxetine (13 mg/kg, ad libitum, n=12/group). Twenty-eight days following the start of treatment, the latency to feed was measured in the NSF task as described above. Animals were sacrificed using live decapitation and the whole brain was harvested and flash-frozen in 2-methylbutane on dry ice and stored at -80°C until sectioning. Dorsal (coronal, Bregma -2.6 to -4.6, Paxinos and Watson, 1986) and ventral (horizontal, Bregma -4.8 to -8.4, Paxinos and Watson, 1986) hippocampal sections were sliced using a cryostat at a thickness of 200 µm. Using a 0.5 µm micro-punch, the dorsal and ventral dentate
gyrus (DG) were excised and gene expression was quantified using quantitative-real time polymerase chain reaction (PCR).

2.6.2. RNA-extraction and cDNA conversion. Excised micro-punched dentate gyrus tissue were kept in -80°C until the tissue was used for RNA extraction using a Zymo direct-zol RNA miniprep kit (Zymo Research, Irvine, CA). Briefly, tissue was homogenized by trituration through a syringe and a 22-gauge needle by suspending the tissue in 0.35 ml Trizol. Samples were then centrifuged at 12 000g for 1 min. The supernatant was transferred to a new tube containing 350 µl 100% Ethanol. The mixture was then loaded onto a Zymo-spin IIC column and centrifuged at 12 000g for 1 min. The column was then rinsed with wash buffer according to the Zymo protocol and treated with DNAse (QIAGEN, Germantown MA), washed, and centrifuged at 12 000g for 1 min. To elute the RNA, 15 µl of DNAse/RNase free water was added to the column and the RNA was stored at -80°C.

RNA was quantified by absorbance at the 260 nm wavelength using a nanophotometer (MIDSCI, St. Louis, MO) and the ratio of absorbances at 260 and 280 nm wavelengths were used to estimate the quality and concentration of extracted RNA. Based on the sample concentration, the volume of RNA required for a final total RNA amount of 300 ng was calculated. 1µg of RNA was diluted into a consistent volume of water and combined with a master-mix consisting of deoxynucleotide triphosphates (dNTPs) (1 mM, Fermentas R0191; Burlington ON), random hexamer primers (Fermentas S0141, 10 µg/ml), RNase inhibitor (Fermentas EO0381, 0.5 µl stock per 20 µl reaction), AMV reverse transcriptase (RT; Fermentas EP061, 0.5 µl stock per 20 µl reaction) and the appropriate volume of enzyme buffer. Negative controls were generated by using a second aliquot of RNA and the same reaction mix with the exclusion of the reverse transcriptase enzyme (RT-) and also by using an aliquot of water alone.
in the cDNA conversion reaction (NTC). The conversion was performed in a thermal cycler using the following program: 10 min at 25°C, 60 min at 50°C, 5 min at 85°C. cDNA was then quantified by quantitative real-time PCR (qRT-PCR).

2.6.3. Quantitative real time PCR. All qRT-PCR experiments were performed using the LightCycler 480 cycler platform (Roche). A standard curve for measuring cDNA concentration was generated by mixing 5 µl of cDNA from each sample. This was taken as 1:1 standard, which was then diluted 3 times, each by a factor of 10 for a standard curve consisting of concentrations ranging from 1 to 0.001. An aliquot of each sample was then diluted to “working concentration” of 1:10 for use in quantification. qRT-PCR reactions were performed in 10 µl volumes using 96-well plates in triplicate. Each reaction consisted of 2 µl DNA, 5 µl SYBR green master mix (Roche), and 10 µM primer pairs. Primers were designed utilizing the Primer3 web software at idtdna.com and were referenced against the rat genome using BLASTn application from the NCBI website. The designed primer sequences are outlined in Table 2.

For each plate, the running template was as follows: 10 min at 95°C (denatures DNA and activates HotStart polymerase) followed by 45 cycles at 95°C for 20s (denaturation), 55°C for 15s (primer annealing), and 72°C for 15s (cDNA elongation). After each elongation step, the intensity of SYBR green fluorescence (i.e. existence of double-stranded RNA) was measured. The software then analysed the generated amplification curve and calculated a cycle number where amplification was at a linear maximum. DNA concentration was determined by comparing this cycle number to those from the standard curve. The mean value for each triplicate measure was counted as a single independent measure for statistical analysis. Samples from the standard curve were also used to calculate efficiency and error for each reaction; reactions were
rejected if the efficiency was more than ± 0.2 from the ideal 2.0, and if error values exceeded 0.1.

Specificity of amplification was confirmed by melting peak analysis. Samples showing non-specific amplification, broad peaks or multiple peaks were excluded from analysis. The number of RT+ cycles was compared to the number of RT- cycles to check for possible genomic contamination. Samples with RT+ cycles less than 3 cycles away from the RT- were excluded from analysis due to potential contamination. The reference gene, Beta-2-microglobulin (B2M) was used as an internal (loading) control.

**Table 2. List of designed qRT-PCR primer sequences.**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX Forward Primer</td>
<td>GAACCATCATGGGCTGGACA</td>
</tr>
<tr>
<td>BAX Reverse Primer</td>
<td>GAAGCCTCAGCCCATCTTCT</td>
</tr>
<tr>
<td>BCL2 Forward Primer</td>
<td>CTGCACCTGACGCCCTTCACC</td>
</tr>
<tr>
<td>BCL2 Reverse Primer</td>
<td>CACATGACCCCAACGAATCAAAGA</td>
</tr>
<tr>
<td>DCX Forward Primer</td>
<td>CATCACCAGGTCTCCATCAATAT</td>
</tr>
<tr>
<td>DCX Reverse Primer</td>
<td>GGGCTTAGTCCTTCTCTGTCT</td>
</tr>
<tr>
<td>B2M Forward Primer</td>
<td>CCGTGATTTTCTGGTGCTT</td>
</tr>
<tr>
<td>B2M Reverse Primer</td>
<td>AAGTTGGGCTTCCATTCTC</td>
</tr>
</tbody>
</table>

**2.7. Statistical analysis.** All the experiments consisted of four groups (High LG Control, High LG Fluoxetine Treatment, Low LG Control and Low LG Fluoxetine Treatment), therefore results were analysed using a two-way ANOVA (Maternal Care X Fluoxetine Treatment). To
assess statistical significance, main effects, interaction analysis and Bonferroni’s post-hoc analysis were performed where appropriate. All analyses were performed using Prism 4 (GraphPad Software, La Jolla, CA). \( p=0.05 \) or less denoted statistical significance.

Chapter 3. Results

3.1. The effect of chronic fluoxetine treatment on fearfulness and depressive-like behaviour in the offspring of high and low LG mothers

3.1.1. Forced swim test. The effect of fluoxetine on behavioural “despair” in the offspring of high and low LG mothers was measured using Porsolt’s forced swimming task (Porsolt, 1977). In the forced swim test, there was a significant reduction in immobility (the amount of time the animal spent motionless in the water) following chronic anti-depressant treatment in both the offspring of high and low LG mothers (\( n=10-12 \), 2-way ANOVA, \( df=41, F=6.705, p=0.013 \), Figure 4a). However, in the two-way ANOVA, no significant main effect of maternal care was observed (\( p=0.363 \)). Similarly, there was no significant interaction effect observed (\( p=0.843 \)). Moreover, there were no significant differences observed in the amount of time the animals spent climbing and swimming during the 10-minute test (\( p>0.1 \), Figure 4b, c).

3.1.2. Novelty suppressed feeding test (NSF). The effect of chronic fluoxetine treatment on fearful behaviour in the NSF test was assessed in the offspring of high and low LG mothers. In the two-way ANOVA, there was a main effect of maternal care on fearful behaviour (\( df=46, F=4.657, p=0.036 \)). Similarly, there was a main effect of fluoxetine treatment observed on the latency to feed in the offspring of high and low LG mothers (\( df=46, F=8.189, p=0.006 \), Figure 5a). In the two-way ANOVA there was also a significant interaction effect between the effect of maternal care and fluoxetine treatment (\( df=46, F=5.416, p=0.02 \), Figure 5a).
A) In a measure of immobility there is a main effect of 13mg/kg chronic fluoxetine treatment (n=10, 2-way ANOVA, \( p = 0.013 \)) in both the offspring of high and low LG mothers. However, a main effect of maternal LG was not observed (2-way ANOVA, \( p = 0.363 \)). The time spent swimming (B) or climbing (C) did not significantly differ within the four groups (2-way ANOVA, \( p > 0.1 \)).

**Figure 4.** Forced swim test following chronic fluoxetine treatment in the offspring of high and low LG dams.
The offspring of high LG mothers displayed a decreased latency to begin feeding in the NSF compared to the offspring of low LG mothers (Figure 5). The differences in fearful behaviour between high LG and low LG offspring were eliminated following chronic fluoxetine treatment (Bonferroni post-hoc test, $p<0.01$). Fluoxetine-treated offspring of low LG mothers displayed a significantly decreased latency to feed compared to low LG controls. The latency to begin feeding in fluoxetine-treated low LG offspring was similar to the latency to begin feeding in fluoxetine-treated and control high LG offspring.

To assess whether the differences in fearful behaviour in the NSF test were independent of the animals’ state of hunger, latency to feed in the home cage immediately following the NSF test was examined. There were no significant differences in latency to feed in the home environment observed among the four groups. In a 2-way ANOVA analysis of variance there were no significant main effect of maternal care ($df=44$, $F=0.047$, $p=0.82$, Figure 5b) or fluoxetine treatment observed ($df=44$, $F=0.032$, $p=0.86$). Similarly, no significant interaction effect of maternal care X fluoxetine treatment was observed on the latency to feed in the home cage (2-way ANOVA, $df=44$, $F=0.35$, $p=0.57$, Figure 5b). All animals began to feed within the first 25 seconds of being presented with food pellets in their home environments.

3.2. The effect of chronic fluoxetine treatment on hippocampal neurogenesis

Two intraperitoneal BrdU (100 mg/kg) injections were administered 24-hours apart following the completion of the behavioural tests. Animals were sacrificed and perfused 28 days after the last injection. The number of proliferating (Ki-67+), immature (DCX+) and mature adult-born (BrdU+/NeuN+) neurons in the dorsal and ventral hippocampus were analysed using immunohistochemistry.
Figure 5. Latency to feed (s) in the novelty suppressed feeding task following chronic fluoxetine treatment.
There is a main effect of maternal LG (n=10 animals per group, 2-way ANOVA, \( p = 0.036 \)) as well as fluoxetine treatment (2-way ANOVA, \( p = 0.006 \)) on the latency to feed. The fearful behaviour of offspring of low LG mothers is decreased following fluoxetine treatment to similar levels as offspring of high LG dams (Bonferroni post-hoc test \( p < 0.01 \)). B) The latency to feed in the home cage following the test did not significantly differ among the four groups (2-way ANOVA, \( p = 0.857 \)).
3.2.1. Proliferating cells following fluoxetine treatment. The number of proliferating cells in the dorsal and ventral hippocampal DG in the offspring of high and low LG mothers were examined using the Ki-67 marker. Similar to previous findings (Bredy et al., 2003a), no significant differences were found in neuronal proliferation in either the dorsal or ventral DG between the offspring of high LG and low LG mothers (Figures 6 & 7). Following chronic fluoxetine treatment, a significant main effect of maternal care was found in both the dorsal (df=21, F= 24.11, p= 0.0002, Figures 6a & 7a) and ventral DG (df= 21, F= 4.96, p= 0.041; Figures. 6b & 7b). Moreover, there were significant interaction effects of maternal care and fluoxetine treatment on the number of proliferating cells in both the dorsal and ventral DG. Chronic fluoxetine treatment led to an increase in the number of proliferating cells in the offspring of high LG mothers in the dorsal DG (df=21, F= 13.95, p= 0.002; Bonferroni post-hoc test p<0.01). Similarly, in the ventral DG, chronic fluoxetine treatment led to a decrease in the number of proliferating cells in the offspring of low LG mothers (df=21, F= 11.93, p= 0.003; Bonferroni post-hoc test p<0.05).

3.2.2. Immature neurons following fluoxetine treatment. Doublecortin (DCX) was used to mark immature neuronal cells. Again similar to previous findings (Bredy et al, 2003a, Fig. 3) the offspring of high LG mothers displayed a significant increase in the number of adult-born immature neurons in both the dorsal and ventral DG compared to the offspring of low LG mothers (df= 21, F= 4.748, p= 0.04, Figures. 8 & 9). Fluoxetine treatment did not significantly increase the number of immature neurons in the dorsal DG (p>0.1, Figures 8a & 9a). Fluoxetine treatment did increase the number of immature neurons in the ventral DG of offspring from low LG mothers. In Tukey’s pair-wise comparison test, there was a significant difference between the offspring of low LG mothers following fluoxetine treatment and low LG control (p<0.05).
contrast, there was no significant difference among the high and low LG offspring following fluoxetine treatment ($p>0.05$). In the two-way ANOVA analysis, there was a main effect of anti-depressant treatment observed in the ventral DG. Fluoxetine increased the number of immature neurons in the ventral DG of offspring from low LG mothers to levels that were comparable to fluoxetine-treated and control high LG offspring ($df=21$, $F=5.13$, $p=0.04$, Figures. 8b & 9b).

**Figure 6.** Estimated number of proliferating cells in the dorsal and ventral hippocampal DG region of the offspring of high and low LG dams. A) In the dorsal DG there is a significant interaction effect observed following fluoxetine treatment in the high and low LG offspring ($n=5$ animals per group, 2-way ANOVA, $p=0.002$). The offspring of high LG mothers show an increase in Ki-67+ cells compared to controls (Bonferroni post-hoc test $p<0.01$). B) In the ventral DG there is also a significant interaction between the number of proliferating cells following fluoxetine treatment in the high and low LG offspring ($n=5$ animals per group, 2-way ANOVA, $p=0.003$). There is no significant difference in the number of proliferating cells in the offspring of high and low LG dams under control conditions.
Figure 7. Proliferating cells in the hippocampal DG region of high and low LG offspring. Representative Ki-67+ cells in the dorsal (A) and ventral (B) DG of the offspring of high and low LG mothers (Scale bar=100µm).
A) There is a main effect of maternal LG in the dorsal DG (n= 5-6 animals per group, 2-way ANOVA, $p= 0.04$) on the number of DCX+ cells. 

B) In the ventral DG, there is a main effect of fluoxetine treatment (n= 5-6 animals per group, 2-way ANOVA, $p= 0.04$) as well as maternal LG (2-way, ANOVA, $p= 0.03$) on the number of immature neurons. In the untreated animals, offspring of low LG dams showed a decreased number of immature neurons. In the ventral DG, fluoxetine treatment increased the number of newborn neurons to similar levels compared to the offspring of high LG mothers (Bonferroni post-hoc test $p<0.05$).
Figure 9. Doublecortin positive cells in the hippocampal DG region of high and low LG offspring.
Representative doublecortin stained (DCX, immature neurons) cells in the dorsal (A) and ventral (B) hippocampal dentate gyrus of male offspring of high and low LG mothers. (Scale bar = 100µm)
Regression analysis was used to assess the association between the number of DCX+ cells and performance in the NSF task. There was a significant negative correlation between the number of DCX+ cells and the latency to feed in the NSF test ($r = 0.681$, $p = 0.002$; Figure. 10b). These strong correlations were not observed in the dorsal DG ($r = 0.420$, $p = 0.0647$; Figure. 10a). However, there is a trend towards a negative correlation between the number of immature neurons and the latency to feed in the dorsal DG.

Quantitative real-time PCR was performed on micro-punches of the DG in fluoxetine-treated and control high and low LG offspring. There were no significant differences in DCX gene expression between fluoxetine-treated and control high and low LG offspring in the dorsal DG (two-way ANOVA, $p = 0.52$; Figure. 11a). The gene expression levels of DCX in the ventral DG between fluoxetine-treated and control high and low LG offspring were comparable to DCX protein levels found between the four groups using immunohistochemistry. There was a significant interaction effect observed in the offspring of high and low LG mothers following chronic fluoxetine treatment. In the ventral DG, fluoxetine treatment decreased the expression of the DCX gene in the offspring of high LG mothers, while increasing the expression of the DCX gene in the offspring of low LG mothers (df = 47, $F = 10.85$, $p = 0.002$, Bonferroni post-hoc test $p < 0.05$; Figure. 11b).
Figure 10. Regression analysis of the latency to feed in the novelty suppressed feeding task and the number of DCX+ cells in the high and low LG offspring. A) In the dorsal DG there is a trend towards a negative correlation between the number of DCX+ cells and latency to feed in the NSF task ($r = 0.420, p = 0.0647$). B) In the ventral DG, latency to feed in the NSF is negatively correlated with the number of newborn neurons regardless of treatment conditions ($r = 0.681, p = 0.0018$).
Figure 11. Doublecortin (DCX) gene expression profile in the dorsal and ventral hippocampal DG of high and low LG offspring. A) In the dorsal DG, there is no significant difference observed in the offspring of high and low LG mothers following fluoxetine treatment compared to controls (n=12 animals per group, 2-way ANOVA, $p=0.523$). B) In the ventral DG, there is a significant interaction effect following chronic fluoxetine treatment (n=12 animals per group, 2-way ANOVA, $p=0.002$).
3.2.3. Adult-born mature neurons following fluoxetine treatment. BrdU/NeuN double staining analysis was performed to assess the cell fate and survival of adult-born neurons. BrdU was used to identify dividing cells. NeuN was used as a biomarker for neurons. Therefore the cells positively stained with both BrdU and NeuN antibodies were identified as adult born cells that resulted in mature neurons. The number of adult born neurons in the dorsal DG was significantly greater in the offspring of high LG offspring compared to low LG offspring (2-way ANOVA, df= 19, F=22.15, p= 0.0005; Figures. 12a & 13a). Fluoxetine treatment did not have a significant effect on the number of adult-born mature neurons in the dorsal DG of either high LG or low LG offspring. Similar to the immature neuron (DCX) analysis, there was a significant effect of fluoxetine treatment on neuronal survival in the ventral DG (2-way ANOVA, p= 0.016; Figures. 12b & 13b). There was a significant interaction effect in response to fluoxetine treatment in the ventral DG. The offspring of low LG mothers showed an increase in the number of mature neurons following fluoxetine treatment compared to the low LG offspring control group and to similar levels as the control and fluoxetine treated offspring of high LG mothers (2-way ANOVA, df=19, F=24.37, p= 0.0003). The number of surviving neurons in the offspring of high LG mothers following chronic fluoxetine treatment remained unchanged.

3.2.4. Number of dividing cells expressing the transcription factor NeuroD1 following fluoxetine treatment. The transcription factor NeuroD1 is an essential mediator of survival of adult-born neurons (Gao et al, 2009). Further immunohistochemical analysis was used to examine whether the difference in the number of adult-born neurons that reach maturity is related to differences in NeuroD1 expression. The co-expression of NeuroD1 and BrdU+ was examined in the dorsal and ventral DG of fluoxetine-treated and control high and low LG offspring.
Figure 12. Estimated number of mature adult-born neurons in the dorsal and ventral hippocampal DG of the offspring of high and low LG mothers. A) There is a main effect of maternal LG in the dorsal DG (n=5-6 animals per group, 2-way ANOVA, $p = 0.0005$) on the number of new mature neurons. B) In the ventral DG, there is a main effect of fluoxetine treatment (n=5-6 animals per group, 2-way ANOVA, $p = 0.0161$) as well as maternal LG (2-way ANOVA, $p = 0.0003$). In the ventral DG fluoxetine treatment presents a significant interaction effect in the offspring of low LG and high LG dams (2-way ANOVA, $p = 0.0110$). In the untreated animals, offspring of low LG dams showed decreased number of mature neurons. In the ventral DG, fluoxetine treatment increased the number of mature adult-born neurons to similar levels as offspring of high LG mothers (Bonferroni posthoc test, $p < 0.01$).
Figure 13. Adult-born mature neurons in the DG region of high and low LG offspring. Representative BrdU+/NeuN+ stained (mature neurons) cells in the dorsal (a) and ventral (b) dentate gyrus of the male offspring of high and low LG mothers. (Scale bar= 100µm)
A significant main effect of maternal care was observed in the dorsal DG (2-way ANOVA, df=21, F= 6.499, p= 0.0214; Figures. 14a & 15a). There was an increased number of BrdU+/NeuroD1+ positive cells in the dorsal DG of high LG offspring compared to low LG offspring. There was a main effect of fluoxetine treatment observed in the ventral DG (df= 21, F= 15.91, p= 0.0011; Figures. 14b & 15b). Fluoxetine treatment resulted in an increase in the number of BrdU+/ NeuroD1+ double-stained cells in the ventral DG of both high LG and low LG offspring.

3.3. Fluoxetine treatment and expression of pro- and anti-apoptotic genes. Micro-punches of the dorsal and ventral DG were excised and gene expression was quantified using quantitative-real time PCR. Apoptotic signalling genes were examined since they influence neuronal survival. The pro-apoptotic Bcl-2-associated X protein (BAX) gene and the anti-apoptotic B-cell lymphoma 2 (BCL-2) gene were examined. BAX or BCL-2 expression did not differ significantly in the dorsal DG of fluoxetine-treated or control high and low LG offspring (n=12 animals per group, 2-way ANOVA, p>0.1; Figures. 16a & c). There was a significant interaction effect for both BAX and BCL-2 gene expression in the ventral DG. BAX and BCL-2 gene expression increased in the ventral DG of low LG offspring (df= 46, F= 5.037, p= 0.029; Fig. 13b & df= 46, F= 7.365, p= 0.009; Figure. 16d).

The ratio of pro-apoptosis to anti-apoptosis signals is critical in determining the apoptotic fate of the cell (Benn & Woolf, 2004). We therefore examined the ratio of BAX to BCL-2 gene expression in the dorsal and ventral DG of fluoxetine-treated and control high and low LG offspring. There were no significant differences in the BAX/BCL-2 ratio between fluoxetine-treated and control high and low LG offspring in the dorsal DG. (2-way ANOVA, p= 0.471; Figure. 16e). In a two-way ANOVA, there was a significant interaction effect observed in the
ventral DG. Chronic treatment with fluoxetine decreased the pro/anti-apoptotic (BAX/BCL-2) mRNA ratio in the ventral dentate gyrus of offspring from low LG mothers (df= 47, F= 4.078, p= 0.048; Figure 16f). The BAX/BCL-2 mRNA ratio was decreased in the fluoxetine-treated low LG offspring compared to the other groups. This suggests that fluoxetine treatment can rescue neuronal survival in the ventral DG of low LG offspring by decreasing the level of pro-apoptotic signalling in this brain region.
A) In the dorsal DG there is a significant main effect of maternal care observed in the BrdU+/NeuroD1+ cells in high and low LG offspring (n=5-6 animals per group, 2-way ANOVA, p=0.021). Following fluoxetine treatment there are no significant differences among the offspring of high and low LG mothers (Bonferroni post-hoc test).

B) In the ventral DG there is a significant main effect of fluoxetine treatment in the offspring of high and low LG mother (n=5-6 animals per group, 2-way ANOVA, p=0.001, Bonferroni post-hoc test).

Figure 14. Estimated number of BrdU+/NeuroD1+ cells in the dorsal and ventral DG region of the offspring of high and low LG dams. A) In the dorsal DG there is a significant main effect of maternal care observed in the BrdU+/NeuroD1+ cells in high and low LG offspring (n=5-6 animals per group, 2-way ANOVA, p=0.021). Following fluoxetine treatment there are no significant differences among the offspring of high and low LG mothers (Bonferroni post-hoc test). B) In the ventral DG there is a significant main effect of fluoxetine treatment in the offspring of high and low LG mother (n=5-6 animals per group, 2-way ANOVA, p=0.001, Bonferroni post-hoc test).
Figure 15. BrdU+/NeuroD1+ double-stained cells in the hippocampal DG region in the offspring of high and low LG mothers. Representative images of BrdU+/NeuroD1+ cells in the dorsal (A) and ventral (B) dentate gyrus region of the hippocampus in the fluoxetine treated and control offspring of high and low LG mothers (Scale bar=100µm).
Figure 16. Apoptotic pathway genes following chronic fluoxetine treatment in the offspring of high and low LG mothers. Pro-apoptotic BCL2-Associated X Protein (BAX) expression in the dorsal (A) and ventral (B) DG of high and low LG mothers. There is a significant interaction effect in the ventral DG following fluoxetine treatment (n=12 animals per group, 2-way ANOVA, p = 0.029). C, D) Anti-apoptotic B-Cell CLL/Lymphoma 2 (BCL2) expression in the dorsal and ventral DG. In the ventral DG, there is a significant interaction effect following fluoxetine treatment (n=12 animals per group, 2-way ANOVA, p = 0.009). E, F) There is no significant difference in BAX/BCL2 transcript ratio observed in the dorsal DG (2-way ANOVA, p=0.471). In the ventral DG, there is a significant BAX/BCL2 ratio interaction in the offspring of high and low LG mothers (2-way ANOVA, p=0.048).
Chapter 4. Discussion

The early life environment influences offspring development in many species including all mammals (Mousseau & Fox, 1998; de Haas et al, 2014). One of the main components of early life experience is parental care. Animals present naturally varying levels of parental care in the wild, leading to differing behavioural phenotypes in the offspring (Rossiter, 1998; Gottlieb G. 1998; Maestripieri & Mateo, 2009). One such adaptive behavioural trait is increased fearfulness, particularly in environments where there is a greater risk of predation. Little is known about the mechanisms through which parental care influences the fear response of the offspring. How are these differences in fearfulness sustained in adulthood? These issues appear relevant for the human condition, where variations in parental care predict the risk for anxiety disorders over the lifespan (Wanner et al, 2012; McLaughlin et al, 2010).

The experiments outlined in this thesis aimed to examine whether variations in maternal care during early life have an influence on the degree of fearful behaviour in adult rat offspring. Specifically, are the differences in fearfulness observed between the offspring of high and low LG mothers mediated by differences in neuronal survival in the hippocampal SGZ? Furthermore, would treatment with anti-depressants that have been shown to increase neuronal survival eliminate the differences in fearfulness between the offspring of high and low LG mothers?

We show that reduced frequency of maternal LG results in a reduced number of adult-born hippocampal neurons. This decrease in number of newborn neurons in the adult hippocampus is associated with enhanced fearfulness in the NSF test in the offspring of low LG dams. We also demonstrate that treatment with the SSRI fluoxetine hydrochloride eliminates the difference in fearfulness in the NSF test between the offspring of high and low LG mothers.
Fluoxetine-treated low LG offspring show reduced fearfulness in the NSF test that is associated with an increase in adult-born neuronal survival in the ventral hippocampus. The increase in neuronal survival in the ventral DG following fluoxetine treatment is also associated with an increase in the expression of the transcription factor NeuroD1. Our results demonstrate that chronic treatment with the antidepressant fluoxetine eliminates differences in fearfulness between the offspring of high and low LG mothers and reduced fearfulness is associated with enhanced neuronal survival in the ventral hippocampus.

4.1. Fluoxetine treatment reduces behavioural despair in the forced swim test. In the current study we find no differences in behavioural despair between the offspring of high and low LG mothers. Both high and low LG offspring spend a similar amount of time immobile in the forced swim test. Following chronic fluoxetine treatment, both high and low LG offspring show a significant reduction in immobility time. The forced swim task is classically used to show the effectiveness of anti-depressants. Multiple studies using the standard protocol outlined in Porsolt et al. (1977), demonstrate that following chronic treatment with antidepressants including fluoxetine, there is a reduction in immobility in the experimental animals. Similarly, we exhibit the efficacy of 13 mg/kg chronic fluoxetine treatment in reducing behavioural despair in both offspring of high and low LG mothers.

The degree of fearfulness measured in the NSF test is associated with the number of adult-born new neurons in the hippocampus. Santarelli et al (2003) show that rodents lacking newborn neurons in adulthood due to x-ray ablation exhibit greater fearfulness in the NSF test as measured by an increased latency to begin feeding compared to rodents with a fully intact ability to generate adult-born neurons. In contrast, in the forced swim task, x-ray ablation does not have an effect on the amount of time the animals spent immobile (Santarelli et al., 2003). In the
experiments described in this thesis, we also focus mainly on the NSF test as a measure of fearfulness that is associated with an increase in neuronal survival in the offspring of high and low LG mothers.

4.2. Chronic fluoxetine treatment eliminates differences in fearfulness between the offspring of high and low LG mothers. Individual differences in maternal LG in the rat are associated with individual differences in behavioural phenotypes within the offspring. Previous studies demonstrate that the adult offspring of low LG mothers display increased fearfulness in the open field (Caldji, 1998). In the NSF test, food-deprived offspring of low LG mothers display an increased latency to begin feeding at the centre of the open field compared to food-deprived high LG offspring (Caldji, 1998). Previous studies show that chronic anti-depressant treatment reduces rodent fearful behaviour in the NSF test. Animals treated with antidepressants including fluoxetine show a decreased latency to feed compared to control animals (Malberg et al, 2000; Duman et al, 2009; Snyder et al, 2011).

We examined whether chronic treatment with fluoxetine would eliminate the differences observed in the latency to feed between the offspring of high and low LG mothers. The fluoxetine-treated offspring of low LG mothers display a reduced latency to begin feeding that is similar to the latency to begin feeding in fluoxetine and control-treated high LG offspring. There were no differences observed in the latency to begin feeding between high LG control and fluoxetine-treated offspring. The differences in fearfulness in the NSF test between the groups is not due to differences in the level of hunger since all four groups were deprived of food overnight (approximately 16 hours) prior to the start of the test. When tested in the home cage where the level of neophobic stress is minimal, both fluoxetine and control treated high and low LG offspring display a similar latency to begin feeding. The similarity in the latency to feed in
the home cage between fluoxetine and control-treated high and low LG offspring suggests that
the latency to feed in the bright open arena is directly related to the level of fearfulness that each
rat experiences. The reduction in the latency to feed in the anti-depressant treated offspring of
low LG mothers shows that chronic fluoxetine treatment is an effective treatment to reduce
fearfulness in adulthood. In clinical settings, in the human population, fluoxetine is also used to
treat generalized anxiety and social phobia disorders. Birmaher et al. (2003) show that in a
randomized trial, patients treated with fluoxetine showed reduced anxiety and phobia symptoms
compared to the placebo-treated group. In patients who experienced childhood trauma and
exhibit PTSD symptoms, fluoxetine treatment is shown to reduce the frequency of relapse in
adulthood compared to the placebo group (Martenyi et al., 2002).

4.3. Reduced fearfulness is associated with an increase in neuronal survival in the
ventral hippocampus. Enhanced fearfulness in rodents is associated with a decreased number
of newborn neurons in the subgranular zone (SGZ) of the hippocampus during postnatal
development and in adulthood (Santarelli et al, 2003; Revest et al, 2009). Treatments with anti-
depressants and other interventions that alleviate fearful behaviour are associated with an
increase in the number of newborn neurons in the SGZ (Malberg et al, 2000; Duman et al, 2001;
van Praag et al, 2000). Reduced fearfulness following chronic treatment with anti-depressants is
also associated with increased levels of adult-born hippocampal neurons. Further, ablation of
adult-born SGZ neurons results in a diminished response to anti-depressants (Santarelli et al,
2003).

Individual differences in early life maternal care also influence the rate of neuronal
survival in the offspring. Over the course of development (P21) and in adulthood (P90), the
offspring of high LG mothers show greater survival of newborn hippocampal neurons compared
to offspring of low LG mothers (Bredy et al, 2003a). This difference in neuronal survival is not associated with a difference in the number of proliferating cells between the offspring of high and low LG mothers (Bredy et al, 2003a; Figure 3). We examined whether the reduction in fearfulness observed following chronic fluoxetine treatment in the offspring of low LG mothers is associated with an increase in neuronal survival in the hippocampus.

The dorsal and ventral regions of the hippocampus are often regarded as functionally distinct structures due to important differences in connectivity between the dorsal and ventral hippocampus and other brain regions (Fanselow & Dong, 2010). The dorsal hippocampus has connections to the mammillary nuclei, the anterior cingulate and the retrosplenial cortex. This dorsal region of the hippocampus mediates spatial learning and memory. The ventral hippocampus extends connections to the hypothalamus and the amygdala. This ventral region of the hippocampus mediates emotional responses and motivated behaviour. Our lab has recently shown that maternal care has distinct effects on the dorsal and ventral hippocampus (Nguyen et al., 2015). Offspring of low LG mothers show enhanced long-term potentiation (LTP) and increased neuronal excitability in the ventral hippocampus compared to the offspring of high LG mothers. Meanwhile, in the dorsal hippocampus high LG offspring exhibit enhanced synaptic plasticity compared to offspring from low LG mothers under resting conditions (Champagne et al., 2008; Bagot et al 2009; Nguyen et al 2015).

We examined whether the number of adult-born newborn neurons is also influenced by maternal care in a region specific manner along the dorsal-ventral axis of the hippocampus. Previous findings from our lab demonstrate that the offspring of high LG dams have a greater number of adult-born neurons in both the dorsal and ventral hippocampus compared to low LG offspring (Figure 2). We report here that fluoxetine treatment affects the offspring of high and
low LG mothers in a region-specific manner based on the frequency of LG they experienced in early life (Figures 12 & 13). BrdU/NeuN double-labelling results demonstrate that in the dorsal hippocampal DG, consistent with previous findings, high LG offspring present a greater number of adult-born mature neurons compared to offspring of low LG mothers.

Chronic fluoxetine treatment enhances the number of mature adult-born neurons only in the ventral hippocampus of low LG offspring. Chronic antidepressant treatment has been shown to enhance hippocampal adult born neurons (Malberg et al, 2000; Duman et al, 2001; Wang et al, 2008). However, these studies focus on the hippocampus as a whole, assessing the number of newborn neurons in coronal sections of the entire hippocampus. Tanti and Belzung (2013) show using a meta-analysis that depending on the region of the hippocampus selected for study, the outcome of anti-depressant treatment is highly variable. For the studies outlined in this thesis, the brain coordinates used in the present analysis are consistent with regions analysed by Fanselow and Dong (2010) and represent two functionally distinct structures (i.e. the dorsal and ventral DG of the hippocampus).

We next examined the effect of chronic fluoxetine treatment on the number of proliferating and immature neurons using Ki-67 and doublecortin markers respectively. Consistent with previous studies, the number of proliferating cells does not differ in either the dorsal or ventral DG between the control offspring of high and low LG mothers (Figure 6). Chronic fluoxetine treatment increases the number of proliferating cells in the dorsal DG only of high LG offspring. Meanwhile, chronic fluoxetine treatment leads to a decrease in the number of proliferating cells in the ventral DG of low LG offspring. As Ki-67 is a measure of proliferating stem cells that can give rise to a multitude of cellular phenotypes, the apparent discrepancies observed following chronic fluoxetine treatment could point to the differential number of
astrocytes and other glial cells in addition to neurons being generated between adult high and low LG offspring (Scholzen & Gerdes, 2000). Further, we performed Ki-67 labelling on the same serial sections as all other immune-labelling studies and thereby measured the number of proliferating cells at the time of perfusion (P140). Previous studies indicated not only a general decline in the proliferative activity of hippocampal stem cells, but also a reduction in effectiveness of anti-depressant treatment in inducing new cell birth in older rodents (de Guzman et al., 2015). In order to parse the fate of the proliferating stem cells following fluoxetine treatment, multiple double-labelling experiments at different time points are required with markers for each cellular phenotype and as such lies outside the scope of the present study.

We further assessed the effect of fluoxetine treatment on the number of immature neurons in the offspring of high and low LG mothers along the dorsal-ventral axis of the hippocampus. High LG offspring have a higher number of immature adult-born neurons compared to low LG offspring in both the dorsal and ventral hippocampus. Chronic fluoxetine treatment increased the number of immature neurons in the ventral hippocampus of low LG offspring (Figure 11). Quantitative PCR analysis of doublecortin gene expression confirms the immunohistochemical results (Figure 14). We also examined the correlation between the number of immature neurons and fearful behaviour in the NSF test. We report a significant negative correlation between the number of doublecortin positive cells in the ventral hippocampus and the latency to feed. The rats with greater latencies to feed (i.e. most fearful) in the NSF test possess fewer numbers of immature neurons in the ventral hippocampus.

Adult-born new neurons in the ventral dentate gyrus can potentially have a mediating influence on the level of fearfulness in the NSF test. Previous optogenetic studies show that stimulation of ventral DG neurons resulted in reduced fearfulness in the NSF test (Kheirbek et al,
Whereas, activation of the basolateral amygdala enhanced fearful behaviour (Felix-Ortiz & Tye., 2013). Ablation studies show that new adult-born neurons in the dentate gyrus inhibit mature granule cells thereby modulating the excitability of the DG. Low LG offspring show enhanced ventral hippocampal excitability in the CA1 region (Nguyen et al., 2015). The ventral CA1 region of the hippocampus is an important output region to the amygdala (Amaral et al, 2007). Chronic fluoxetine treatment increases the number of immature neurons in the ventral hippocampal DG of low LG offspring. An increase in the number of newborn neurons in the ventral DG of fluoxetine–treated low LG offspring could lead to reduced excitability of the mature granule cells in this region that extend connections to the basolateral amygdala thereby reducing the level of fearfulness in these rats.

The differences in the number of immature neurons suggest differences in the development of the neuronal niche between high and low LG offspring. The “recruitment hypothesis” of adult-born neurons suggests that the recruitment, maturation and survival of newborn neurons is an activity-dependent process (Wojtowicz et al, 2012; Veyrac et al, 2013). Our results indicate that the offspring of high and low LG mothers exhibit similar levels of cell proliferation in the adult DG. The fate of these proliferative cells depends on cues from the surrounding local environment (e.g. astroglia), the expression of activity-dependent genes and cellular apoptosis pathways (Song et al, 2002; Veyrac et al, 2013). Our lab showed that the expression of GFAP (a marker for glial cells) is enhanced in the offspring of low LG mothers compared to those from high LG dams (Bredy et al, 2003a). Transcriptome analysis on distinct cell populations isolated from the dorsal and ventral hippocampus could lead to better insight into the neurogenic niche and the signalling pathways that lead to differential neuronal survival between high and low LG offspring.
4.4. Chronic fluoxetine treatment may eliminate differences in fearfulness between high and low LG offspring by decreasing pro-apoptotic signalling in the ventral DG. There are significantly enhanced levels of hippocampal neuronal survival in the offspring of high LG mothers compared to the offspring of low LG mothers. There are also a greater number of pyknotic cells (in a state of dying) in the offspring of low LG mothers compared to the offspring of high LG mothers (Weaver et al 2002). Weaver et al (2002) analysed protein levels of pro-apoptotic BAX (Bcl-2-associated X) and anti-apoptotic protein BCL2 (B-cell lymphoma 2) in whole hippocampal extracts in high and low LG offspring. The offspring of low LG mothers show greater BAX expression in whole hippocampus extracts with no differences in BCL2 levels between them and high LG offspring. This suggests a greater level of hippocampal apoptotic cell death in low LG offspring compared to high LG offspring. Low LG offspring also show a greater number of hippocampal cells exhibiting non-random DNA fragmentation and greater apoptotic morphology compared to high LG offspring.

We examined the transcript levels of BAX and BCL2 in the dorsal and ventral DG in the offspring of high and low LG mothers following chronic fluoxetine treatment. The transcript levels of BAX and BCL2 are equivalent in fluoxetine-treated and control high and low LG offspring in the dorsal DG (Figure 16). In the ventral DG, chronic fluoxetine treatment enhanced BAX and BCL2 mRNA levels in the offspring of low LG mothers compared to low LG controls as well as the control and fluoxetine-treated offspring of high LG dams. There is no difference in BAX or BCL2 mRNA levels between high and low LG offspring in the dorsal or ventral DG. However, the current result is not necessarily in contrast to previous findings by Weaver et al (2002). The adult hippocampus is a complex structure comprising myriad cellular phenotypes such as astrocytes and other glial cells that contribute to the neurogenic niche. In the current
study, we chose hippocampal subregions directly associated with the generation of adult-born new neurons (i.e. the DG). Differential protein levels at the remaining hippocampal subregions not used in our present study, taken together as a whole could contribute to the differences previously observed in BAX expression between high and low LG offspring.

BAX and BCL2 proteins play opposing roles in the cellular apoptotic process. Therefore, as suggested by Weaver et al (2002), examining the BAX:BCL2 ratio will provide better insight into the degree of apoptotic signalling occurring within a given region. There are no differences in the BAX:BCL2 transcript ratio between fluoxetine-treated or control high and low LG offspring in the dorsal DG (Figure 16 E). Chronic fluoxetine treatment reduces the pro-apoptotic to anti-apoptotic signalling ratio in the ventral DG of offspring from low LG mothers only. This reduction in the pro:anti-apoptotic transcript ratio suggests that chronic fluoxetine treatment increases the survival of DG neurons by enhancing anti-apoptotic signalling in the offspring of low LG mothers.

4.5. The elimination of differences in fearfulness between high and low LG offspring is associated with an increase in the pro-survival transcription factor NeuroD1. Our results indicate that chronic fluoxetine treatment can eliminate differences in fearfulness between the offspring of high and low LG mothers by enhancing neuronal survival in the ventral DG of low LG offspring. The increase in the number of adult-born new neurons in fluoxetine-treated low LG offspring could be due to a reduced ratio of pro-apoptotic to anti-apoptotic signalling in the ventral DG of low LG offspring following chronic fluoxetine treatment. Examining pro and anti-apoptotic markers indicates if a cell is undergoing cell death or is prevented from entering an apoptotic state. We next examined an upstream factor that could influence neuronal survival, the proneural basic helix-loop-helix (bHLH) transcription factor NeuroD1 (Gao et al, 2009). There
exists a T-cell factor/lymphoid enhancer factor (TCF/LEF) regulatory element in the promoter sequence of NeuroD1 (Kuwabara et al, 2009). TCF/LEF is the major transducer of the canonical Wnt/beta-catenin pathway that is essential in adult hippocampal neurogenesis (Wu & Hen, 2013). Kuwabara et al (2009) show that astrocytes that induce production of new neurons also induce an increase in levels of NeuroD1 expression in the neurogenic niche. In a knockout mouse model, Gao et al (2009), further show that NeuroD1 is essential in the maintenance and survival of adult born neurons. Moreover, in a recent collaborative effort with the Genome Institute of Singapore, transcriptome analysis show that chronic fluoxetine treatment results in a 3-fold increase in NeuroD1 mRNA levels in the hippocampal DG (unpublished results).

We used immunohistological analysis to demonstrate that in the dorsal DG, the offspring of high LG mothers show enhanced levels of mitotic cells (BrdU+) that also express NeuroD1 compared to low LG offspring. Fluoxetine treatment does not have a significant effect on the number of co-labelled BrdU+/NeuroD1+ expressing adult-born cells in the dorsal DG. In the ventral DG, the number of BrdU+/NeuroD1+ cells increases following chronic treatment with fluoxetine in both the offspring of high and low LG mothers. Similar to previous immunohistological experiments, the effect of fluoxetine treatment on NeuroD1 expression is also regionally specific and restricted to the ventral hippocampus. Interestingly, the influence of early life maternal care on the expression of NeuroD1 in the dividing cells is specific to the dorsal DG. Previous studies suggest that the newborn neurons in the dorsal and ventral hippocampus mature at different rates due to distinct connective circuitry and rates of recruitment by the surrounding local environment (Piatti et al, 2011). It is then likely that the influence of maternal care on NeuroD1 expression could be observed by examining the ventral DG at different stages throughout development. In the current study, we showed that fluoxetine
treatment leads to an increase in NeuroD1 expression in the dividing cells of the ventral DG. This enhanced transcription factor expression does not further increase the survival of newborn neurons in the offspring of high LG mothers. However, in the offspring of low LG dams, enhanced NeuroD1 expression may promote the maturation and survival of newborn neurons through a Wnt/beta-catenin dependent pathway (Kuwabara et al, 2009). Future siRNA studies using a NeuroD1 knockdown could be used to study NeuroD1’s role in promoting differences in hippocampal neuronal survival between high and low LG offspring in the dorsal and ventral DG. It would also be interesting to examine epigenetic regulation of the NeuroD1 promoter sequence as a means to explain differences in hippocampal neuronal survival between high and low LG offspring.

4.6. Implications: How does maternal licking and grooming influence neuronal survival in adulthood? Previous studies in our lab have shown that increased LG during the first week of life leads to an increase in serum thyroid hormone (T3) levels in developing offspring. Increased levels of T3 stimulate serotonin (5-HT) signalling in the hippocampus leading to epigenetic regulation of downstream genes through a cAMP/PKA-dependent signalling pathway (Hellstrom et al, 2012). Low LG mothers, lick and groom their offspring much less frequently than high LG mothers. This reduction in LG elicits a reduced increase in serum T3 levels and hippocampal serotonin levels in offspring from low LG mothers.

Serotonin signalling is also involved in regulation of adult hippocampal neurogenesis. There are seven identified subclasses (5-HT1-7) of serotonin receptors, a majority of which are members of the G protein-coupled transmembrane receptor family, and are the target of several anti-depressant drugs. Agonists of 5-HT1A, for instance, show anxiolytic properties, whereas 5-HT1A receptor knockouts result in anxiogenic effects (Ansorge et al, 2004). Stimulation of 5-
HT1A receptors increases the number of adult-born neurons in the hippocampus (Banasr et al, 2004). Santarelli et al (2003) also show that the pro-neurogenic and anxiolytic effects of the selective serotonin inhibitor fluoxetine in the NSF test are dependent on 5-HT1A. In mice that lack the 5-HT1A receptor, anti-depressant treatment did not result in an increase in the number of newborn neurons. The increase in the number of newborn neurons following anti-depressant treatment is dependent on the Wnt/beta-catenin pathway (Jang et al, 2013; Seib et al, 2013).

We hypothesize that treatment with fluoxetine eliminates differences in fearfulness between high and low LG offspring by enhancing the number of surviving adult-born neurons in the ventral dentate gyrus of low LG offspring. This increase in neuronal survival following fluoxetine treatment is potentially mediated by increased NeuroD1 activity in the TCF/LEF complex and Wnt/beta-catenin pathways. Fluoxetine treatment increases neuronal survival in the ventral dentate gyrus of low LG offspring and the increase in new adult born neurons in this region would allow fluoxetine-treated low LG offspring greater capacity for pattern separation (Kheirbek et al, 2012). Enhanced ability to distinguish between contexts allows for reduction in generalization and improved ability to distinguish between fearful and similar unrelated stimuli. The inhibitory function of these surviving newborn neurons in the DG can allow for greater ability to distinguish between similar contexts. In the highly stressful NSF task, improved pattern separation along with the inhibitory influence of ventral DG on the hippocampal downstream connections to the basolateral amygdala perhaps allows the rodent to display reduced fearful behaviour.

Interestingly, fluoxetine treatment does not enhance the number of newborn neurons or fearfulness in the offspring of high LG dams. The early life conditions that the offspring of high LG mothers experience is comparable to an enriched environment. In fact, previous findings in
our lab have shown that a reduced capacity for spatial and non-spatial learning and memory in the offspring of low LG mothers can be eliminated by exposing low LG offspring to an enriched environment from weaning to adulthood (Bredy, 2003b). It is therefore likely that the number of adult-born neurons have reached a structural threshold, where fluoxetine treatment does not result in enhancement effects in the number of new neurons in the offspring of high LG mothers. Ablating neurogenesis in the dorsal and ventral DG of offspring from high and low LG mothers could further our understanding of the mediating role of hippocampal neuronal survival in explaining differences in fearfulness between the offspring of high and low LG mothers.

**Concluding Remarks**

Variations in the early life environment profoundly influence susceptibility to developing affective disorders such as anxiety and depression in adulthood. In Canada alone, anxiety disorders affect 20% of the population at least once during their lifetime (Statistics Canada, 2012). One major source of variation during early childhood is the quality and quantity of parental care. In the developing rat pup, the most salient feature of the early environment is the quality and quantity of maternal care.

Decreased levels of maternal care are associated with increased fearfulness later in life. Variation in maternal care also influences survival of newborn neurons throughout development and in adulthood. Decreased levels of hippocampal adult-born neurons are also associated with anxiety-like behaviour and fearfulness. However, the mechanisms through which maternal care influences the fear response of the offspring is not fully understood.

Here we show that lower levels of maternal care is associated with reduced number of new hippocampal neurons leading to increased display of fearful behaviour. We report that
treatment with the anti-depressant fluoxetine that increases neuronal survival reduces fearful behaviour in the offspring of low LG dams. This decrease in fear response is associated with increased number of immature and mature neurons in the ventral SGZ following fluoxetine treatment. The reduced fearfulness in the fluoxetine-treated low LG offspring is also associated with an increase in the transcription factor NeuroD1 in the ventral SGZ.

We have previously shown that early life maternal care influences the expression of stress response genes through epigenetic mechanisms (Liu et al, 1997; Francis & Meaney, 1999; Weaver et al, 2004; Fish et al, 2004). Regulation of downstream pathway genes particularly those involved in mediating fearfulness and neuronal survival could potentially be a novel mechanism to explain how antidepressant treatment eliminates differences in fearfulness between high and low LG offspring.

Bibliography


Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. *Nat Neurosci*, 12(9), 1097-1105.


Neuropsychopharmacology, 29(4), 694-704.


