January 2014

Role Of The α1A-Adrenergic Receptor In Synaptic Plasticity, Cognition & Neurogenesis

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ROLE OF THE $\alpha_{1A}$-ADRENERGIC RECEPTOR IN SYNAPTIC PLASTICITY, COGNITION & NEUROGENESIS

by

Brianna Lynn Goldenstein
Bachelor of Science, University of North Dakota, 2007

A Dissertation
Submitted to the Graduate Faculty
of the
University of North Dakota
In partial fulfillment of the requirements

for the degree of
Doctor of Philosophy

Grand Forks, North Dakota
May
2014
This dissertation, submitted by Brianna Lynn Goldenstein in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done, and is hereby approved.

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This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.

Dr. Wayne Swisher

April 25, 2014

Date
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Department: Pharmacology, Physiology & Therapeutics

Degree: Doctor of Philosophy

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Brianna Lynn Goldenstein
April 17th, 2014
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... viii

LIST OF TABLES ............................................................................................................. xi

ACKNOWLEDGEMENTS ................................................................................................. xii

ABSTRACT .................................................................................................................... xiv

CHAPTER

I. ADRENERGIC RECEPTORS ......................................................................................... 1

1.1 Discovery, history, and classification ................................................................. 1

1.2 Structure and second messenger pathway ......................................................... 6

1.3 Pharmacological characterization ....................................................................... 10

1.4 Localization and physiological function ............................................................ 12

II. LEARNING, MEMORY & THE $\alpha_1$-ADRENERGIC RECEPTOR ....................... 16

2.1 Learning and memory ....................................................................................... 16

2.2 History of $\alpha_1$-adrenergic receptors, learning & memory ............................... 18

2.3 Synaptic plasticity and the $\alpha_1$-adrenergic receptor ....................................... 20

2.4 Learning & memory testing paradigms ............................................................... 25

III. HIPPOCAMPAL NEUROGENESIS, LEARNING, MEMORY, 
& THE $\alpha_1$-ADRENERGIC RECEPTOR .................................................................. 29

3.1 Adult neurogenesis ............................................................................................ 29

3.2 Detecting and modifying adult neurogenesis .................................................... 33
3.3 Learning, memory and adult neurogenesis .................................................. 39
3.4 Adult neurogenesis and the α1-adrenergic receptor ................................... 41

IV. NEUROGENESIS, METABOLISM AND EXERCISE .................................. 44
4.1 The role of adrenergic receptors in metabolism ........................................... 44
4.2 Neurogenesis and metabolism ................................................................. 45
4.3 Norepinephrine, adrenergic receptors and exercise .................................... 47
4.4 Exercise-induced neurogenesis ................................................................. 48

V. LONG TERM α1A-ADRENERGIC RECEPTOR STIMULATION
ENHANCES COGNITIVE FUNCTION AND SYNAPTIC PLASTICITY ............... 51
5.1 Introduction ............................................................................................ 51
5.2 Methods .................................................................................................... 51
5.3 Results ...................................................................................................... 58

VI. ACTIVATING THE α1A-ADRENERGIC RECEPTOR RESCUES THE
NEGATIVE EFFECTS THAT INHIBITING NEUROGENESIS HAS ON LEARNING AND MEMORY IN MICE ............................................................ 67
6.1 Introduction ............................................................................................ 67
6.2 Methods .................................................................................................... 68
6.3 Results ...................................................................................................... 84

VII. CYTOSINE ARABINOSIDE DISRUPTS HYPOTHALAMIC
NEUROGENESIS AND INDUCES OBESITY IN MICE ............................... 92
7.1 Introduction ............................................................................................ 92
7.2 Methods .................................................................................................. 92
7.3 Results ................................................................................................................................. 95

VIII. ROLE OF THE $\alpha_{1A}$-ADRENERGIC RECEPTOR IN EXERCISE-INDUCED NEUROGENESIS ............................................................................................................ 100

8.1 Introduction ..................................................................................................................... 100
8.2 Methods .......................................................................................................................... 100
8.3 Results ............................................................................................................................ 104

IX. DISCUSSION ................................................................................................................... 109

9.1 Neurogenesis, the $\alpha_{1A}$-AR, synaptic plasticity and cognition ......................................... 109
9.2 Hypothalamic neurogenesis and metabolism .................................................................. 119
9.3 Exercise-induced neurogenesis and the $\alpha_{1A}$-adrenergic receptor .................................... 124

APPENDIX ............................................................................................................................... 130

REFERENCES .......................................................................................................................... 133
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diagram of the historical categorization of adrenergic receptors</td>
<td>2</td>
</tr>
<tr>
<td>2. Complete categorization of the adrenergic receptors and their subtypes</td>
<td>3</td>
</tr>
<tr>
<td>3. The adrenergic receptor family and corresponding G-proteins</td>
<td>6</td>
</tr>
<tr>
<td>4. Adrenergic receptor binding epinephrine</td>
<td>7</td>
</tr>
<tr>
<td>5. Second-messenger pathway for $\alpha_1$-AR activation</td>
<td>9</td>
</tr>
<tr>
<td>6. Full and partial agonists for the $\alpha_1$-AR after external calcium removal</td>
<td>12</td>
</tr>
<tr>
<td>7. Types of memory</td>
<td>17</td>
</tr>
<tr>
<td>8. Induction of long-term potentiation</td>
<td>23</td>
</tr>
<tr>
<td>9. The progressive discovery of neurogenic regions in the brain</td>
<td>31</td>
</tr>
<tr>
<td>10. The five stages of neurogenesis</td>
<td>32</td>
</tr>
<tr>
<td>11. The stages of neurogenesis with coinciding markers</td>
<td>34</td>
</tr>
<tr>
<td>12. Expression of DCX, NeuN and BrdU in the adult female rat dentate gyrus of the hippocampus</td>
<td>35</td>
</tr>
<tr>
<td>13. Diagram of the hypothalamus</td>
<td>46</td>
</tr>
<tr>
<td>14. The satiety and reward circuits of the hypothalamus</td>
<td>47</td>
</tr>
<tr>
<td>15. Barnes maze apparatus</td>
<td>54</td>
</tr>
<tr>
<td>16. Stimulating LTP in the CA1 region of the hippocampus</td>
<td>57</td>
</tr>
<tr>
<td>17. Chronic $\alpha_{1A}$-AR stimulation improves cognitive performance in the Barnes maze</td>
<td>59</td>
</tr>
</tbody>
</table>
18. Hippocampal synaptic plasticity is enhanced with chronic α1AAR activation ................................................................. 62

19. Chronic treatment with an α1A-AR selective agonist improves cognitive function .............................................................. 64

20. Cognitive performance in the Barnes maze is reduced in mice lacking the α1A-ARs ............................................................... 66

21. Increasing concentrations of anti-mitotic agent Ara-C induces neuronal toxicity at 3-4% ...................................................... 70

22. Complete pump apparatus .................................................................................................................................................. 72

23. Stereotaxic surgery showing bregma on a mouse .............................................................................................................. 74

24. Novel object recognition test ................................................................................................................................. 77

25. Morris water maze ....................................................................................................................................................... 79

26. Open field apparatus ..................................................................................................................................................... 80

27. Timeline of experiments ............................................................................................................................................... 81

28. Outline of dentate gyrus and visible neurogenesis ........................................................................................................ 83

29. Treatment with Ara-C significantly decreases the number of immature and dividing neurons in the hippocampus .......... 85

30. Motor activity of mice in each experiment group ......................................................................................................... 86

31. Novel object recognition test results .......................................................................................................................... 87

32. Morris water maze learning results .................................................................................................................................. 89

33. Morris water maze test results ....................................................................................................................................... 91

34. Exposure to antimitotic agent Ara-C caused significant increase in weight and food intake .................................................. 96

35. Ara-C-treatment significantly increased fat deposits in mice ....................................................................................... 97
36. Ara-C significantly reduced neurogenesis in the hypothalamus ............... 98
37. Ara-C did not affect hypothalamic neuronal activity ................................ 99
38. Running cage set-up .............................................................................. 102
39. WT running mice consumed significantly more food than their sedentary counterparts .......................................................... 104
40. Running $\alpha_{1A}$-AR KO mice exhibit signs of anxiety as zero maze progresses ........................................................................... 105
41. Running mice without the $\alpha_{1A}$-AR have a lower total cell population and significantly less neurogenesis ........................................... 106
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Progress in Understanding Adrenergic Receptors by Decade of the 20th Century</td>
<td>5</td>
</tr>
<tr>
<td>2. Pharmacological profile of the cloned human $\alpha_1$-adrenoceptor subtypes</td>
<td>11</td>
</tr>
<tr>
<td>3. Distribution of Adrenergic Receptor Subtypes in the Brain Via In Situ or Autoradiography</td>
<td>13</td>
</tr>
<tr>
<td>4. Distribution of the $\alpha_{1A}$- and $\alpha_{1B}$-adrenergic receptors in the brain</td>
<td>14</td>
</tr>
<tr>
<td>5. Markers of Stages in Adult Neurogenesis</td>
<td>36</td>
</tr>
<tr>
<td>6. Effects of KO mutations on adult neurogenesis</td>
<td>38</td>
</tr>
<tr>
<td>7. Neural stem cell-promotor tamoxifen-inducible and tetracycline-regulated mice</td>
<td>39</td>
</tr>
</tbody>
</table>
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ABSTRACT

Norepinephrine (NE) is a neurotransmitter involved in learning and memory. NE activates adrenergic receptors (ARs) and stimulating the \( \alpha_{1A} \)-AR subtype has been known to increase adult neurogenesis (ANG). We hypothesized that \( \alpha_{1A} \)-AR-induced ANG would enhance learning and memory. Constitutively active mutant (CAM) \( \alpha_{1A} \)-AR, \( \alpha_{1A} \)-AR knock-out (KO), normal wild type (WT) mice, and mice treated with the \( \alpha_{1A} \)-AR selective agonist cirazoline (CRZ) were tested on the Barnes maze. CAM \( \alpha_{1A} \)-AR and CRZ-treated mice performed better and \( \alpha_{1A} \)-AR KO mice performed poorer than WT. Long-term potentiation (LTP) experiments on aged CAM \( \alpha_{1A} \)-AR mice revealed enhanced LTP in CAM \( \alpha_{1A} \)-AR mice versus WT. Therefore, we hypothesized that \( \alpha_{1A} \)-AR-induced ANG underlies enhanced learning, memory and synaptic function. We used CRZ to activate \( \alpha_{1A} \)-ARs and the anti-mitotic agent cytosine arabinoside (Ara-C) to impair ANG in CRZ-treated and WT mice, and tested mice on novel object recognition (NOR), Morris water maze (MWM), and open field (OF). No difference was found in NOR and OF. MWM revealed that CRZ-treated mice were protected from Ara-C-induced learning and memory impairments, and surgery-induced learning impairments.

We observed that Ara-C treatment was causing weight gain and hypothesized that Ara-C inhibits cellular proliferation in the hypothalamus, the metabolic center of the brain. Fat deposition analysis and hypothalamic
stereological investigation revealed that Ara-C treated mice gained significantly more weight and had significantly fewer dividing cells and immature neurons than WT mice. We concluded stem cells and immature neurons in the hypothalamus are important in metabolism and normal weight gain. We launched a pilot study investigating the α_{1A}-AR in exercise-induced neurogenesis using WT and α_{1A}-AR KO mice and running wheels. We measured anxiety-like behavior and neurogenesis and found enhanced anxiety and less neurogenesis in running α_{1A}-AR KO mice. Results from this study are ambiguous; therefore we cannot dismiss α_{1A}-AR involvement in exercise-induced neurogenesis. In conclusion, activating α_{1A}-ARs increases neurogenesis, enhances learning and memory, and has neuroprotective effects against brain injury. These insights may lead to therapeutic interventions for patients suffering from chemotherapy’s negative effects on memory and other neurodegenerative diseases, as Ara-C (also known as cytarabine) is a common leukemia treatment in humans.
CHAPTER I
ADRENERGIC RECEPTORS

1.1 Discovery, history, and categorization

Adrenalin (a.k.a. epinephrin) was discovered by Oliver and Schafer in 1894-1895, though it would not receive this name until two other scientists, Jokichi Takamine and John Jacob Abel, tried and successfully purified the compound in 1899-1900 (Davenport, 1982). Adrenergic “receptors” were a hypothetical concept, thought to exist somewhere near or inside a cell, and responsive only to epinephrine (EPI) or adrenaline in the body, and norepinephrine (NE) or noradrenaline in the brain, thus the term “adrenergic” (Ahlquist, 1948). Then in 1948, Raymond Ahlquist defined two categories for adrenergic receptors (ARs), the $\alpha$- and $\beta$-ARs. Ahlquist based this categorization on a series of experiments using varying concentrations of epinephrine and several other adrenergic receptor agonists in the blood vessels, uterus, heart, pupils and gut. His results showed striking contrast in the agonists potency and effect (constriction or dilation) depending on the tissue in question. Then, an excellent study clarifying the role of EPI (also known as “sympathin” at the time) in the body was published shortly after Ahlquist’s discovery (Von Euler, 1951). Von Euler and Ahlquist were beginning the characterization and classification of adrenergic receptors familiar to scientists today.
After Ahlquist’s landmark study, Lands reported that having only a single set of receptors in each α- and β-AR group was too simplified. Using a similar approach with various adrenergic agonists and making observations in several different tissues, Lands’ results showed an even more diverse response, and he concluded that at least two receptor types existed within the β-AR category (Lands, Arnold, McAuliff, Luduena, & Brown, 1967). Shortly after this discovery, Langer (1974) hypothesized that there may be an α-adrenergic auto-receptor, which works by causing “presynaptic inhibition of transmitter release through a negative feedback mechanism which is mediated through the neurotransmitter itself.” Finally, based on mounting evidence from these experiments, it was proposed that the α-AR family be split into two groups: the α₁- and the α₂-AR (Berthelsen & Pettinger, 1977). As a result, the categorization of ARs was almost complete by the late 1970’s (Fig. 1). And twenty years after Langer proposed the auto-receptor hypothesis, the α₂-AR would be classified into one of the main three categories of ARs: the α₂- (auto-receptor), the α₁-, and the β (Bylund, 1985).

![Figure 1. Diagram of the historical categorization of adrenergic receptors. Scientists responsible for each category are above. All subclasses of receptors (except the β3-AR) were classified before the 1980’s. This figure modified from David B Bylund, 2007. AR, adrenergic receptor.](image-url)
A third subtype of the β-AR class, the β₃, was added in the mid to late 1980’s (Arch et al., 1984; Emorine et al., 1989). The α₂-AR was discovered to have several different subtypes of its own by the late 1980’s – the α₂A, α₂B, and α₂C (Bylund, 1988; Kobilka et al., 1987; Minneman, Han, & Abel, 1988; Regan et al., 1988; Zeng et al., 1990). Finally, the α₁-AR was thought to have 4 subtypes – the α₁A, α₁B, (Minneman et al., 1988; Morrow & Creese, 1986) α₁C, and the α₁D (Ford, Williams, Blue, & Clarke, 1994; Lomasney et al., 1991; Perez, Piascik, & Graham, 1991). Then in 1994, investigators studied the binding profiles of both the α₁C-AR and α₁A-AR and found them the same. Furthermore, in situ hybridization showed the α₁C-AR receptor localized to α₁A-AR rich tissue (hippocampus, vas deferens, aorta, and the submaxillary gland). It was concluded that the α₁C-AR was actually the same as the α₁A-AR subtype (Perez, Piascik, Malik, Gaivin, & Graham, 1994). Thus, the α₁C-AR was removed from the classification chart, leaving a curious gap in the naming convention (Fig. 2).

Figure 2. Complete categorization of the adrenergic receptors and their subtypes. Scientists that discovered each subtype with corresponding year can be seen above. As noted in figure 1, Lands discovered the β₁- and β₂-AR subtypes in the late 1960’s and Bylund discovered all three of the α₂-AR subtypes in the 1980’s. This figure is a modified version of the diagram appearing in David B Bylund, 2007. Adrenergic receptor, AR.
Then again in the late 1990’s, a tentative fourth receptor subtype was again identified for the $\alpha_1$-AR class, the $\alpha_{1L}$-AR (Ford et al., 1997; Kava, Blue, Vimont, Clarke, & Ford, 1998). However, it is not included in traditional classification charts because it has properties that are very similar to the $\alpha_{1A}$-AR, and it is only located in the bladder, prostate and urethra.

Highlighted here are the historical points relevant to the classification of ARs. A more detailed summary of work in the AR field during the 20th century can be found in Table 1. However, the focus of this introduction will be on the $\alpha_1$-AR family and the $\alpha_{1A}$-AR in particular.
<table>
<thead>
<tr>
<th>Decade</th>
<th>Key Events</th>
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<tbody>
<tr>
<td>1901—1910</td>
<td>• Langley proposes that cells have &quot;receptive substances&quot;</td>
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<td>• Dale refers to &quot;receptive mechanisms for adrenalin&quot;</td>
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<td>• Abel isolates epinephrine from the adrenal medulla, the first hormone</td>
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<td>1911—1920</td>
<td>• Ahlquist defines α— and β-types of adrenergic receptors</td>
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<td>1921—1930</td>
<td>• von Euler demonstrates that norepinephrine is the sympathetic</td>
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<td>neurotransmitter</td>
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<td>1931—1940</td>
<td>• Sutherland discovers cyclic AMP, leading to the second messenger</td>
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<td>• Sir James Black develops propranolol, the first clinically useful beta-</td>
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<td>• Lands defines β₁- and β₂-subtypes</td>
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<td>1941—1950</td>
<td>• Snyder and Lefkowitz develop radioligand binding assays for the most</td>
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<td>adrenergic receptors</td>
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<td>• Lefkowitz develops the ternary complex model for G protein-coupled</td>
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<td>receptors</td>
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<td>1951—1960</td>
<td>• Bylund defines α₁, α₂, and β as the three types of adrenergic receptors</td>
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<td>• Dixon, Strader, and Lefkowitz clone the β₂-adrenergic receptor</td>
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<td>• Creese proposes α₁₄⁻ and α₁₈⁻-subtypes based on radioligand binding</td>
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<td>1961—1970</td>
<td>• Lefkowitz works out desensitization mechanism involving β-adrenergic</td>
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<td>receptor kinase and beta-arrestin</td>
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<td>1971—1980</td>
<td>• Bylund defines α₁₄⁻, α₂¹⁻, α₂³⁻, α₁₄⁺, and α₁₈⁻-receptors</td>
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<td>• Strosberg clones the β₂-receptor</td>
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<td>• Bylund’s laboratory and other laboratories use site-directed</td>
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<td>mutagenesis to define ligand-binding site and signaling mechanisms</td>
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<td>1981—1990</td>
<td>• Graham and Perez clone α₁D</td>
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<tr>
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<td>• Nathans and Hogness clone rhodopsin, the first of the G protein-</td>
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<td>• Arch defines the β₃-receptor using pharmacological criteria</td>
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<td>• Touchstone generates β₁⁻, β₂⁻, α₂¹⁻, α₂³⁻, and α₁₈⁻-knockout mice</td>
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<tr>
<td></td>
<td>• Cotecha generates α₁₁⁻knockout mice</td>
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<td>• Crystal structure of rhodopsin, a G protein-coupled receptor,</td>
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</tbody>
</table>

Table courtesy of Perez, 2006, p. 5.
1.2. Structure and second messenger pathway

Adrenergic receptors belong to one of the largest classes of receptors called G protein-coupled receptors. Structurally, ARs are heptahelical transmembrane receptors coupled to a specific G protein. These G proteins have an α subunit with intrinsic enzymatic activity. The difference in α subunits between G proteins confers specificity to the G protein function and ultimate pharmacological response. Each AR subtype is coupled to a different G protein. The β-ARs are couple to the Gαs; the α2-ARs are coupled to the Gαi/o; and the α1-ARs are coupled to the Gαq/11 (Wess, 1998). In turn, these G proteins lead to different second messenger pathways and, therefore, a different physiological response (Fig. 3).

Figure 3. The adrenergic receptor family and corresponding G-proteins. Also seen above is the second messenger effects, and the physiological effects of receptor activation. Adapted from figure 7 in Perez, 2006, p. 54.
The G protein-coupled AR binds EPI in the peripheral nervous system (PNS) and NE in the central nervous system (CNS). EPI binds to the AR through four key interactions during the binding process (Perez, 2006). First, ionic bonding occurs between the catechol and the third helical transmembrane of the receptor. Second, hydrogen bonding occurs between the hydroxyl groups on the catechol and the serine residues in the fifth helical transmembrane. Third, the aromatic residues in the sixth helical transmembrane interact with the aromatic catechol of NE. Fourth, and finally, hydrogen bonding occurs between the chiral β-hydroxyl group and another residue in the sixth helical transmembrane (Fig. 4, left). Structural differences between agonists (e.g., EPI vs. NE; Fig 4, right) cause slightly different binding in the receptor’s binding pocket, and therefore different responses. For example, the methyl group present on the amine in EPI may interfere with binding slightly more than when methyl is not present on the amine, as in NE.

Figure 4. Adrenergic receptor binding epinephrine. Shown here is the binding of epinephrine to the adrenergic receptor, showing the key interactions of the agonist with each transmembrane (left). Norepinephrine (right), showing the absence of a methyl group on the amine, in contrast to epinephrine. Adapted from figure 2 in Perez, 2006, p. 27.
Each receptor subtype (β, α₁ or α₂) activates a different second messenger pathway. The α₁-AR second messenger pathway activates several different proteins, enzymes, and receptors within the cell. All of the α₁-AR subtypes activate pathways that lead to increases in calcium via the Gα₉/₁₁ subunit and the activation of phospholipase C (PLC) and protein kinase C (PKC), leading to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) increases (Offermanns & Simon, 1998). However, activation of the α₁-AR has also been shown to lead to a number of different outcomes such as increases in arachidonic acid (Burch et al., 1986; Insel et al., 1991; Perez, DeYoung, & Graham, 1993), which is thought to occur because of the activation of phospholipase A₂ (PLA₂; Xing & Insel, 1996). Additionally, activation of the α₁-AR (Fig. 5) leads to growth factor regulation (Zhong & Minneman, 1999), such as the mitogen-activated protein kinase (MAPK) pathway (Gutkind, 1998). MAPKs are serine/threonine kinases and for the α₁-AR include the subfamilies extracellular signal-regulated kinase, ERK (Liu et al., 2011), c-Jun N-terminal Kinase, JNK, and p38 kinase. There is speculation as to how specificity for the subtypes of each main class of ARs is gained (Dianqing Wu, Katz, Lee, & Simon, 1992). One hypothesis is that specificity is at least somewhat dependent on the intracellular loops of the G protein coupling – specifically the i₃ loop (Wess, 1998). For example, the Gα₉/₁₁ family of α-subunits (which includes α-subunits q, 11, 14, 15 and 16), are shown to interact with a specific sequence of residues in the i₃ segment for coupling, yet the Gα₁₄ subunit of this family, which couples with
the $\alpha_{1A}$- and $\alpha_{1B}$-AR, does not need this segment to function (Wu, Jiang, & Simon, 1995).

Figure 5. Second-messenger pathway for $\alpha_{1}$-AR activation. The above diagram shows PIP$_2$, DAG, IP$_3$, PKC and ERK activation followed by increases in [Ca$^{2+}$]. PIP$_2$, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP$_3$, inositol 1,4,5-trisphosphate; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; [Ca$^{2+}$], internal calcium concentration. Adapted from figure 8 in Perez, 2006, p. 55.
1.3 Pharmacological characterization

The natural, endogenous agonists of the ARs system are EPI and NE. As mentioned, NE is the main AR agonist in the brain and is released from the locus coeruleus. However, each AR subtype has a different potency (the amount of ligand compared to the intensity of cellular response), affinity (how well the ligand binds to the receptor), and efficacy (or efficiency of cellular response). Affinity measures how well a ligand binds to the receptor and efficacy measures how well the ligand elicits a response after receptor activation. Just like endogenous and natural agonists, synthetic agonists and antagonists have been produced for each AR class and subtypes within each class. For the sake of brevity, the $\alpha_1$-AR class affinity (Table 2) alone and efficacy of the agonist cirazoline (CRZ, Fig. 6) alone will be highlighted here, as they are most relevant to these studies (Horie, Obika, Foglar, & Tsujimoto, 1995). This study used human receptors transfected into Chinese hamster ovary cells. Paired with radioligand binding and tracking $[Ca^{2+}]_i$ levels, agonist potency and efficacy were determined. Additional information about the pharmacological characterization of the other AR classes and subtypes can be found in the International Union of Basic Clinical Pharmacology (IUPHAR database, 2014, Adrenoceptors: Introduction).
Table 2*
Pharmacological profile of the cloned human α1-adrenergic receptor subtypes

<table>
<thead>
<tr>
<th>Drugs</th>
<th>$\alpha_{1A}$-AR</th>
<th>$\alpha_{1B}$-AR</th>
<th>$\alpha_{1D}$-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Adrenaline</td>
<td>600 ± 250</td>
<td>400 ± 35</td>
<td>56 ± 12</td>
</tr>
<tr>
<td>(+)-Adrenaline</td>
<td>8,100 ± 560</td>
<td>7,600 ± 600</td>
<td>920 ± 90</td>
</tr>
<tr>
<td>(-)-Noradrenaline</td>
<td>990 ± 100</td>
<td>680 ± 90</td>
<td>42 ± 8.8</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>4,400 ± 200</td>
<td>110,000 ± 6,000</td>
<td>11,000 ± 1,300</td>
</tr>
<tr>
<td>Cirazoline</td>
<td>120 ± 18</td>
<td>960 ± 130</td>
<td>660 ± 160</td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>6.0 ± 0.60</td>
<td>320 ± 15</td>
<td>390 ± 100</td>
</tr>
<tr>
<td>Antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.17 ± 0.020</td>
<td>0.26 ± 0.032</td>
<td>0.070 ± 0.0010</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>2.5 ± 0.10</td>
<td>30 ± 3.0</td>
<td>7.0 ± 0.83</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>400 ± 50</td>
<td>520 ± 5.0</td>
<td>240 ± 25</td>
</tr>
<tr>
<td>5-Methylurapidil</td>
<td>0.89 ± 0.081</td>
<td>39 ± 3.1</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td>WB-4101</td>
<td>0.20 ± 0.030</td>
<td>3.4 ± 0.30</td>
<td>0.25 ± 0.012</td>
</tr>
</tbody>
</table>

Dose response curves determined the -log EC$_{50}$ values, and $E_{\text{max}}$ (the maximum response produced) was determined. Table adapted from Horie et al., 1995.
1.4. Localization and physiological function

Adrenergic receptors are located throughout the body, in the PNS as well as the CNS. In the PNS, ARs are located in the heart, blood vessels, lung, liver, gut, pancreatic islet cells, adipocytes, leukocytes, platelets, skeletal muscles, kidney, uterus, prostate, bladder, penis and pineal gland (Perez, 2006). In the
CNS, NE is involved in sleep, arousal, attention, mood, cardiovascular regulation, appetite, pain, motor output, and learning and memory (Pupo & Minneman, 2001). NE is supplied to the CNS through efferent output from the locus coeruleus (Berridge & Waterhouse, 2003). ARs receiving this output from the locus coeruleus are present in the cerebral cortex, hippocampus, basal ganglia, cerebellum, olfactory, amygdaloid (amygdala), thalamus, hypothalamus, midbrain, brain stem, pons, pineal gland, spinal cord and pituitary gland (Papay et al., 2006). It stands to reason, then, that these receptors will influence the particular function of each organ or system they are expressed in, be it in the PNS or the CNS (table 3).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α₁A</th>
<th>α₁B</th>
<th>α₁D</th>
<th>α₂A</th>
<th>α₂B</th>
<th>α₂C</th>
<th>β₁</th>
<th>β₂</th>
<th>β₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>++r</td>
<td>+++r</td>
<td>++r</td>
<td>++m,r</td>
<td>+h</td>
<td>++m,r</td>
<td>++r</td>
<td>++r</td>
<td>ND</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>++r</td>
<td>+h</td>
<td>++r</td>
<td>+h</td>
<td>+m,h</td>
<td>++r,h</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Dentate Gyrus</td>
<td>--</td>
<td>+r</td>
<td>--</td>
<td>--</td>
<td>+m</td>
<td>+m,m,r</td>
<td>++r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Basal Ganglia</td>
<td>--</td>
<td>ND</td>
<td>ND</td>
<td>+++m,h</td>
<td>++m,r</td>
<td>++m,r</td>
<td>++r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>--</td>
<td>ND</td>
<td>ND</td>
<td>++m,r</td>
<td>++r</td>
<td>+r</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Granule cell layer</td>
<td>--</td>
<td>--</td>
<td>+r</td>
<td>+m</td>
<td>+m</td>
<td>+r</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+r</td>
<td>ND</td>
<td>ND</td>
<td>++m,r</td>
<td>++m,r</td>
<td>++m</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Anterior olfactory nuclei</td>
<td>++r</td>
<td>+r</td>
<td>++r</td>
<td>+m</td>
<td>+m</td>
<td>+m</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Amygdaloid</td>
<td>++r</td>
<td>ND</td>
<td>ND</td>
<td>++m,r</td>
<td>++m,r</td>
<td>++m</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Lateral</td>
<td>++r</td>
<td>ND</td>
<td>ND</td>
<td>++m,r</td>
<td>++m,r</td>
<td>++m</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Dorsal raphe nuclei</td>
<td>++r</td>
<td>+r</td>
<td>++r</td>
<td>--</td>
<td>+m,r</td>
<td>--</td>
<td>+r</td>
<td>--</td>
<td>ND</td>
</tr>
<tr>
<td>Brain stem and pons</td>
<td>++r</td>
<td>ND</td>
<td>ND</td>
<td>++m,r</td>
<td>++m,r</td>
<td>++m</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Lateral reticular nuclei</td>
<td>++r</td>
<td>+r</td>
<td>++r</td>
<td>+m</td>
<td>+r</td>
<td>+r</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Pineal gland</td>
<td>++r</td>
<td>+r</td>
<td>++r</td>
<td>--</td>
<td>+r</td>
<td>+r</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>++r</td>
<td>++r</td>
<td>++r</td>
<td>++r</td>
<td>--</td>
<td>+r</td>
<td>+r</td>
<td>+r</td>
<td>ND</td>
</tr>
<tr>
<td>Pituitary</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+r,h</td>
<td>+r,h</td>
<td>ND</td>
</tr>
</tbody>
</table>

gp, guinea pig; h, human; m, mouse; ND, not determined; r, rat; rb, rabbit; --, not detected; +, low expression; ++medium expression; ++++, high expression. *Table adapted from table 1 in Perez, 2006, p. 176-177.

The AR subtypes are found in varying amounts throughout the aforementioned organ systems and brain regions, but the α₁-AR has specifically
been shown to be involved in memory formation (Sirviö & MacDonald, 1999), and is present in high concentrations in one of the main areas for learning and memory, the hippocampus. In fact, a few studies have gone on to show the specific localization of the $\alpha_{1A}$- and $\alpha_{1B}$-AR in the brain using a more accurate technique using transgenic mice expressing enhanced green fluorescent protein with each of these receptor subtypes. Results from this study can be found in Table 4 below (Papay et al., 2006).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$\alpha_{1A}$-AR</th>
<th>$\alpha_{1B}$-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdaloid</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Granule cell layer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hindbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal raphe nuclei</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CA1 field</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CA3 field</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamic nuclei</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Hypothalamic area</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raphe cap</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Olfactory</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Pituitary</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Relative levels of expression based on intensity of label within each mouse model. *Adaptation of table 3 in Papay et al., 2006.

On a cellular level, each main category of ARs are located either pre- or post-synaptically. In the CNS, $\alpha$-ARs are mainly post-synaptic. However, $\alpha_{2}$-
ARs are autoreceptors and are the exception as they are located pre-synaptically; Hein, Altman, & Kobilka, 1999), and α₁-ARs are also mostly post-synaptic (Perez, 2006). Additionally, the α₂-AR may be extrasynaptic while the α₁-AR may be intrasynaptic (Curet & de Montigny, 1988). The β₁- and β₃-ARs are located post-synaptically, and the β₂-ARs located pre-synaptically (Lakhani, Amenta, Napoleone, Felici, & Eikenburg, 1994).

The AR has been studied for nearly 100 years. Each subtype, and sub-subtype, has been classified and characterized with regards to its natural activator, NE, as well as a variety of synthetic agonists and antagonists that have further helped to characterize each receptors function. Each receptor is unique in its physiological effects in the body. The following chapter highlights the effects of the α₁-AR in particular, and its involvement with learning and memory, specifically.
CHAPTER II

LEARNING, MEMORY & THE $\alpha_1$-ADRENERGIC RECEPTOR

2.1 Learning and memory

Learning and memory take place in several different brain regions depending on the type of learning and memory. For instance, fear-based and emotional learning and memory is associated with the amygdala – one of the emotional centers of the brain (Cahill & McGaugh, 1995; Costafreda, Brammer, David, & Fu, 2008). Other areas of learning and memory in the brain include the prefrontal cortex (PFC), or “neocortex” (Goldman-Rakic, 1987; Jacobsen & Nissen, 1937), the striatum, the cerebellum, and the hippocampus in the medial temporal lobe (Pergola & Suchan, 2013). Each area is responsible for a different type of learning and memory (Fig. 7).

Interestingly, the prefrontal cortex is directly and indirectly connected to several other structures that also play important roles in learning and memory – most notably, the hippocampus – through the anterior and posterior cingulate cortices (Goldman-Rakic, 1987). This area also receives input from the parietal lobe of the brain. As previously mentioned, these areas of the brain process different types of learning and memory (Fig. 7) and spatial memory tasks, in particular, require an interaction between the PFC and the
ventral striatum – with the processing of these memories done in the hippocampus (Floresco, Seamans, & Phillips, 1997).

![Diagram of memory types](image)

**Figure 7.** Types of memory. The hippocampus (in the medial temporal lobe) is involved in explicit or declarative memory (facts and events). The prefrontal cortex/neocortex, the striatum, amygdala, cerebellum and other reflex pathways are involved in implicit (non-declarative) memory. Under the implicit memory category, the prefrontal cortex controls memory priming while the striatum controls skills and habits (procedural learning). The amygdala controls emotional memory, and the cerebellum controls skeletal musculature memory. Adapted from figure 62-4, Kandel, Schwartz, Jessell (2000), p. 1231.

It has been known for quite some time that the hippocampus is important for learning and memory, evident from a patient in the 1950's known as H.M. (Neylan, Scoville, & Milner, 2000) who's hippocampus was removed to alleviate the severe seizures the patient was suffering. To what extent the hippocampus was important, however, was unclear. Since H.M., experiments have discovered that other brain regions such as the caudate nucleus and parts of the cortex have a much smaller role in spatial learning and memory than the hippocampus (Olton & Papas, 1979). Additionally, the hippocampus has been implicated in certain types of memory (i.e.
topographical memory) over other important memory structures, like the amygdala (Sutherland & McDonald, 1990). The type of memory processed in the medial temporal lobe and hippocampus is declarative memory, which includes facts (semantic) and events (episodic) (Squire, 1992). Because the hippocampus is also an important region for neurogenesis (discussed in chapter 3.1), this research described herein focused mainly on the involvement of the hippocampus in memory and its relationship to the $\alpha_1$-AR (specifically, the $\alpha_{1A}$-AR).

### 2.2 History of the $\alpha_1$-adrenergic receptors, learning & memory

Several neuromodulatory systems have been implicated in learning and memory such as the opioid peptidergic, $\gamma$-aminobutyric acid (GABA)-ergic, and cholinergic systems. In fact, a synergistic interaction exists between the cholinergic system and adrenergic system in enhancing learning and memory performance in rodents – though mainly mediated through the $\beta$-adrenergic receptor system with a minor to negligible role for the $\alpha_1$-AR (Puumala, Sirviö, Ruotsalainen, & Riekkinen, 1996). Individually, NE and EPI have been shown to be important in retention and enhancement of memory – both of these ligands are released when animals are learning (McGaugh & Cahill, 1997). Furthermore, there is a large number of noradrenergic inputs into the mammalian hippocampus from the locus coeruleus (Blackstad, Fuxe, & Hökfelt, 1967) and a very high expression of $\alpha_{1A}$- and $\alpha_{1B}$-ARs in this same region (Tables 3 and 4). However, it is not clear which AR is mediating these enhancements. The $\alpha_1$-AR is highly expressed in key learning and memory
areas of the brain. For instance, if we look at Table 4 (section 1.4), the gray rows are areas in the brain with high \( \alpha_{1A} \) and \( \alpha_{1B} \)-AR expression, and are also key brain areas for learning and memory, namely the basal ganglia, cerebral cortex, hippocampus and amygdala. There are also high levels of all three of the \( \alpha_1 \)-ARs in the cerebral cortex, despite the use of less optimal expression techniques (Table 3, section 1.4).

High hippocampal input from the locus coeruleus hints at the importance of the \( \alpha_1 \)-AR in this key learning and memory area of the brain. However, what role the \( \alpha_1 \)-AR plays in learning and memory is still controversial. For instance, some studies show that \( \alpha_1 \)-AR stimulation inhibits memory consolidation in chicks (Gibbs & Summers, 2001) and impairs spatial working memory in monkeys (Arnsten & Jentsch, 1997; Mao, Arnsten, & Li, 1999) and rats (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999). Additionally, another study shows that the AR system is altered after traumatic brain injuries (TBIs) and contributes to PFC-controlled working memory dysfunction. Here, blocking the \( \alpha_1 \)-ARs after TBI improves working memory, and expression of the \( \alpha_1 \)-AR is down-regulated in the medial-PFC of brain-injured rats (Kobori, Hu, & Dash, 2011). Yet other studies have suggested that activating the \( \alpha_1 \)-AR facilitates spatial learning and memory in rodents (Pussinen et al., 1997; Puumala et al., 1998) – and this facilitation, especially in aged animals, may be through a synergistic relationship with the \( N \)-methyl-\( D \)-aspartate (NMDA) receptor (Riekkinen, Kemppainen, & Riekkinen, 1997; Riekkinen, Stefanski, Kuitunen, & Riekkinen, 1996).
2.3 Synaptic plasticity and the α₁-adrenergic receptor

Long term potentiation (LTP) is a form of synaptic plasticity that is thought to be the underlying mechanism of learning and memory (Deupree, Turner, & Watters, 1991; Squire, 1992). LTP is defined as potentiation of the synapse that is both NMDA and receptor-dependent and lasts for longer than one hour (Bliss & Collingridge, 1993). This definition is based on hippocampal LTP, and is measured by an increase in the amplitude of the evoked excitatory post-synaptic potential (EPSP) from a single neuron or a population, which is typically induced by quick injections of current, also called a tetanus. The tetanus varies from study to study, but generally involves several short bursts of 50-100 stimuli at about 100 Hz. Theta-bursts are a popular technique to induce LTP and includes 4 current injections, separated by 200 ms each (called interburst intervals) at a rate of 100 Hz. This sequence of bursts mimics the frequency of input during the actual process of learning (Otto, Eichenbaum, Wiener, & Wible, 1991). Inducing LTP is dependent on the intensity of these bursts as well as the frequency. If the intensity is not high enough or the frequency not right, short-term potentiation (STP) or even post-tetanic potentiation (PTP) can be induced – each lasting for a shorter amount of time than LTP, with PTP lasting the shortest amount of time (Lovinger, Routtenberg, Lovinger, & Routtenberg, 1988; Malenka, 1991).

The induction of LTP involves a few key components on a molecular level (Fig. 8). First, the depolarization of the neuron or group of neurons
needs to be large enough to eject the Mg\(^{2+}\)-blocks of NMDA receptors while in the presence of glutamate. If the depolarization is too weak or stimulus too infrequent, not all of the Mg\(^{2+}\)-blocks will be ejected, resulting in either STP or PTP. The NMDA receptors have been shown to be essential in the induction of LTP, although their activation alone is not usually enough to induce LTP (Bashir, Tam, & Collingridge, 1990) and may sometimes inhibit LTP (Coan, Irving, & Collingridge, 1989; Izumi, Clifford, & Zorumski, 1992). Second, Ca\(^{2+}\) plays a very important and critical role in the induction of LTP; this is evident when the use of a Ca\(^{2+}\) chelator, like ethylene glycol tetraacetic acid (EGTA), inhibits the induction of LTP (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983). However, NMDA receptors are permeable to Ca\(^{2+}\). This feature of NMDA receptors, as well as the intracellular release of Ca\(^{2+}\), is thought to synergistically induce LTP (Bliss & Collingridge, 1993) since LTP is inhibited when intracellular stores of Ca\(^{2+}\) are blocked or depleted (Bortolotto & Collingridge, 1993; Harvey & Collingridge, 1992; Obenaus, Mody, & Baimbridge, 1989). Ca\(^{2+}\) is also thought to play an important role in the recruitment of \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors – the second key component in LTP (Malinow & Malenka, 2002). Recruitment of AMPA receptors to the synapse after LTP induction is thought to be involved in the maintenance of LTP – as blocking exocytosis of these receptors to the synapse inhibits LTP (Maletic-Savatic, Koothan, & Malinow, 1998). Interestingly, blocking the NMDA receptor alone does not necessarily block LTP as long as metabolic glutamate receptors (mGluRs) have activated
enough IP$_3$ to increase internal concentrations of Ca$^{2+}$ (Bortolotto & Collingridge, 1993). The NMDA receptor function is also known to be enhanced by PKC activation (Lovinger et al., 1988), and one of the ways to activate PKC is through $\alpha_1$-AR stimulation (Offermanns & Simon, 1998).
Figure 8. Induction of long-term potentiation. Normal synaptic transmission, which is low-frequency, involves several types of glutamate receptors, including NMDA and non-NMDA (metabotropic glutamate receptors, and AMPA) receptors. Glutamate activates non-NMDA receptors during normal transmission (and Na⁺ and K⁺ flow through) as NMDA-receptor have an additional Mg²⁺ block, requiring significant depolarization of the membrane to remove (top). When long-term potentiation is induced, the membrane is depolarized by non-NMDA receptors, removing the Mg²⁺ block, causing Ca²⁺ to flow through NMDA-receptors, increasing intercellular calcium concentrations and further depolarizing the cell. This action of the NMDA-receptor and the subsequent actions of calcium, and recruitment of AMPA receptors to the synapse is linked to LTP maintenance (bottom). Adapted from figure 63-10, Kandel et al. (2000), p. 1261. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca²⁺, calcium; Glu, glutamate; K⁺, potassium; Mg²⁺, magnesium; Na⁺, sodium; NO, nitric oxide; NMDA, N-methyl-D-aspartate; PKC, protein kinase C.
The $\alpha_1$-AR is important in promoting LTP and long-term depression (LTD) in the rat hippocampus – modulators of synaptic plasticity in the adult rodent (Scheiderer, Dobrunz, & McMahon, 2004). Compared to other neurotransmitters like serotonin (5-HT) depleting NE reduces LTP (Stanton & Sarvey, 1985), and it is thought $\beta$-ARs mediate the induction and early maintenance of LTP (Pussinen & Sirviö, 1998). It is unclear what role the $\alpha_1$-AR plays in either LTP or LTD. Several studies showed that NE mediates inhibition of excitatory transmission through the $\alpha_1$-AR, whereas the $\beta$-AR increases excitation (Mynlieff & Dunwiddie, 1988; Scanziani, Gähwiler, & Thompson, 1993); another found that activating the $\alpha_1$-AR suppressed endogenous LC output of NE onto pyramidal cells of the hippocampus, while NE activation of $\beta$-ARs excited pyramidal cells (Curet & de Montigny, 1988; Pang & Rose, 1987). Despite there being a lack of evidence for an important role of $\alpha_1$-ARs in the initial induction or maintenance of LTP, a study by Izumi et al. (1992) showed that when LTP was inhibited, applying NE overcame this block. Additionally, this study showed that using $\alpha_1$-AR agonists specifically were responsible for overcoming the LTP inhibition and that using an $\alpha_1$-AR antagonist blocked the effect of NE ($\beta$- and $\alpha_2$-AR agonists and antagonists had no effect). And as previously mentioned, the $\alpha_1$-AR pathway activates PKC and directly affects internal $Ca^{2+}$ levels in the cell – which are both key components for LTP (Lovinger et al., 1988; Lynch et al., 1983).
2.4 Learning & memory testing paradigms

Multiple methods exist for testing cognition in animals, specifically learning and memory. These methods can be adjusted for the species in question, for instance, mazes for rats are larger than those that accommodate a smaller rodent such as a mouse. Careful attention is usually paid to the ratio of the maze to the size of the animal being tested so that this ratio remains the same between species. One of the first behavioral tests to be designed for learning and memory was the Barnes maze (Barnes, 1979). The Barnes maze is a large circular platform with holes around the perimeter and spatial cues within the environment (section 5.2.2). Beneath one of these holes is an escape box that the animal will eventually find and disappear into—based on an evolutionary instinct to escape open, bright areas and avoid predation. Additional aversive stimuli besides bright lights, such as sounds and fans, can also be added to the test to increase the animal’s drive to escape the open area. This test relies on hippocampal-dependent spatial reference memory and the animals natural instinct to escape open areas (Harrison, Reiserer, Tomarken, & McDonald, 2006). It was originally made for rats but has since been converted into a maze for mice as well. Over time, mice learn the location of an escape box based on spatial cues set in the surrounding environment. Thus after several days of training, they are able to remember where the escape box is and solve the maze more efficiently (with fewer errors) and quickly. Barnes maze assesses errors made – the number of incorrect holes approached and investigated – and
assesses the amount of time taken to solve the maze, generally stopping at 300 s or 5 min. Training times can vary anywhere from 4-5 days, with testing occurring once or multiple times after training. For more details concerning this maze, see section 5.2.2.

The Morris water maze (MWM) (Morris, 1984) is another behavioral test for hippocampal-dependent spatial learning and memory, though it is more stressful for animals than the Barnes maze (Harrison, Hosseini, & McDonald, 2009). The MWM uses a large tub filled with water, visual cues, and a hidden platform to test memory (section 6.2.5). This test may induce hypothermia because of the water component (Iivonen, Nurminen, Harri, Tanila, & Puoliväli, 2003) that can be offset with heat lamps and/or slightly longer inter-trial intervals (the time in between each training trial for each animal). The advantage of using this maze is that it is faster to conduct experiments, as each training trial takes 1-2 min (depending on the protocol used) versus 5 min in the Barnes maze. The MWM test is also thought to be neurogenesis-dependent (Dupret et al., 2008), making this test ideal for use in this research. Procedures for this test can vary, but typically, it consists of 3-5 days of training, with 4-6 trials per day per animal. Each trial consists of 1-2 min of swimming in the maze and searching for the hidden platform. After the time is up, animals that did not successfully find the platform are placed on the platform for 15-30 s and allowed to observe the spatial cues in the area to better associate the location of the platform with the cues. Probe tests are conducted usually the day after the last learning trial, and the
platform is removed. Then the animal is allowed to explore the maze for 1-2 min. Additionally, experimenters may add a reversal day, where 24 h after the probe test, the platform is placed in the opposite quadrant of where it was for the probe test. How long it takes the animal to learn the new position is measured. Typical data analyzed include distance travelled to platform, latency to the platform, and swim speed, but several other parameters may be assessed depending on the study (Vorhees & Williams, 2006; Wenk, 2004). For more information on the testing paradigm used in this dissertation, see section 6.2.5.

The novel object recognition (NOR) task was first developed to assess working memory (Ennaceur & Delacour, 1988). In this task, animals are shown two identical objects and, a designated amount of time later, are shown one of the old objects and a new object (section 6.2.4). Animals that remember the old object will spend more time exploring the new object. The amount of time in between showing the animals the old objects and then adding the new object will assess different aspects of memory. For instance, it was later found that NOR task also assesses spatial and non-spatial memory (Ennaceur & Meliani, 1992) and is hippocampal-dependent (Clark, Zola, & Squire, 2000). NOR task can also be used to assess shorter retention periods, or short-term memory, if necessary (Hammond, Tull, & Stackman, 2004). In fact, rats had intact retention times (i.e. remembering the old object and exploring the new more) up to 60 min after being shown the old object, but did not distinguish between the new and the old object after
24 h (Ennaceur & Meliani, 1992). Therefore, depending on the procedural set-up, NOR task can be used to assess diverse aspects of memory. Typically, NOR task data is analyzed as the amount of time spent with the novel object over the familiar object, and preference of the novel object over the familiar object (percent time spent with the novel object versus the familiar object). For more information about the procedural set-up used in this dissertation, see section 6.2.4.
CHAPTER III

HIPPOCAMPAL NEUROGENESIS, LEARNING, MEMORY, & THE $\alpha_1$-ADRENERGIC RECEPTOR

3.1 Adult neurogenesis

In 1965, Joseph Altman and Gopal Das showed evidence of dividing neurons in the post-natal hippocampus – a process known as neurogenesis (Altman & Das, 1965). They also unknowingly identified neural stem cells in the brain – those cells that could continually divide and give rise to new neurons. Neurogenesis was only thought to occur during prenatal development at the time. Adult neurogenesis was undiscovered: the general consensus, up until the mid-twentieth century, was that mammals were born with a fixed number of neurons. This perspective began to waiver as more evidence became available to support Joseph Altman’s initial evidence of postnatal neurogenesis (Eckenhoff & Rakic, 1988; Reynolds & Weiss, 1992). Eventually, by the late 1990’s, the “fixed-neuron” dogma finally came to an end. When a landmark study done with humans showed neurogenesis occurring in adults as old as 72 (Eriksson et al., 1998), the paradigm shift supporting adult neurogenesis came shortly after (Gross, 2000).
Neurogenesis has been discovered in many areas of the brain since the initial discovery in the mid-twentieth century. They include, but are not limited to, the striatum (Eckenhoff & Rakic, 1988), the olfactory bulb (Bédard & Parent, 2004), the amygdala and cortex (Bernier, Bedard, Vinet, Levesque, & Parent, 2002), the substantia nigra (Zhao et al., 2003), the hypothalamus (Kokoeva, Yin, & Flier, 2005), and the spinal cord (Fernández, Radmilovich, & Trujillo-Cenóz, 2002). Since then, several areas of the brain have been identified as neurogenic niches, or areas where neurogenesis is occurring at a higher rate than other areas. These neurogenic niches also have neural stem cells, or constitutively dividing cells that give rise to progenitor cells with limited self-renewal properties. A controversial area, the subcallosal zone (Seri et al., 2006) and possibly another area in the hypothalamus (Kokoeva, Yin, & Flier, 2007) may contain these neural stem cells. The two widely accepted main areas are known as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG). Newborn cells from the SVZ travel via the rostral migratory stream to the olfactory bulb (Alvarez-Buylla & Garcia-Verdugo, 2002; Lois & Alvarez-Buylla, 1994). Newborn cells from the SGZ populate the hippocampus (Fig. 9) (Cameron & McKay, 2001; Lledo, Alonso, & Grubb, 2006; Ming & Song, 2005).
As previously discussed, the hippocampus is an important region for learning, memory, and neurogenesis. Thus, hippocampal neurogenesis in the SGZ will be the focus and main example of neurogenesis referred to throughout the rest of this dissertation. Hippocampal neurogenesis has five key stages of development, and six different cell types are expressed during those stages. These are based on morphology, specific proteins expressed during development, and whether cells are mitotic or post mitotic as follows (Kempermann, Jessberger, Steiner, & Kronenberg, 2004; von Bohlen und Halbach, 2007):

- **Stage 1, mitotic** – type-1 cell: dividing stem cells
- **Stage 2, mitotic** – type-2a: dividing progenitor cell without certain determined lineage, some migration occurring; type-2b: progenitor cell
with limited-proliferative capacity, determined lineage, some migration occurring

- **Stage 3, mitotic** – *type-3*: progenitor cell with limited-proliferative capacity, determined lineage, migration occurring as cell prepares to exit mitotic stage

- **Stage 4, post-mitotic** – *immature granule cell*: no proliferative capacity, still migrating and preparing for synaptic integration

- **Stage 5, post-mitotic** – *mature granule cell*: no proliferative capacity, migration has ceased and synaptic integration is complete.

In each of these stages, the cell is producing different proteins and potential markers. These markers will help to identify the type and stage of the developing newborn cells in the hippocampus (discussed in the next section).

![Figure 10. The five stages of neurogenesis. Cells progress through the mitotic stages to the postmitotic stages. Types 1 – 3 are capable of self-renewal and proliferation while immature and mature cells are not. Figure from von Bohlen Und Halbach, 2011.](image-url)
3.2 Detecting and modifying adult neurogenesis

One of the most important immunohistological discoveries in detecting newborn cells was 5-bromo-2'-deoxyuridine (BrdU), a synthetic nucleotide that is incorporated into any dividing cell (Miller & Nowakowski, 1988) during the S-phase of the cell cycle. BrdU is typically delivered by an intraperitoneal injection. The age of the animal at the time of injection and the amount injected can greatly affect the number of dividing neurons that are labeled (Cameron & McKay, 2001). Depending on the amount injected, BrdU can also label events such as DNA repair, abortive cell cycle re-entry, and gene duplication (Bauer & Patterson, 2005; Nowakowski & Hayes, 2000; Taupin, 2007). In general, concentrations of BrdU given in neurogenesis studies are typically low enough to only mark mitotic cells in the S-phase (Bauer & Patterson, 2005; Cooper-Kuhn & Kuhn, 2002) – this includes cell types 1 – 3 (Fig 11).
Another important marker for detecting neurogenesis is a microtubule binding protein called doublecortin (DCX). DCX is an important protein that aids the newborn cell in migrating (Gleeson, Lin, Flanagan, & Walsh, 1999) and is heavily expressed in the dendrites of new neurons, making it useful to study dendrite growth as well (Rao & Shetty, 2004). Because DCX coexpresses with nearly 90% of BrdU-positive cells (Fig. 12, Brown et al., 2003), it has been suggested that DCX detection alone is enough to determine levels of neurogenesis (Couillard-Despres et al., 2005). Using DCX alone would bypass the need for intraperitoneal injections of BrdU and the extra complication that comes with BrdU labeling and detection (Taupin, 2007).
Figure 12. Expression of DCX, NeuN and BrdU in the adult female rat dentate gyrus of the hippocampus. In the above diagram from Brown et al., 2003, up to 90% of BrdU-positive cells (y-axis) are also colabeled with DCX-positive labeling. This peak occurs from day 4 - 7 after BrdU injection (x-axis). DCX- and BrdU-positive cells drop sharply after 14 days, disappearing almost entirely at 30 days. Other markers, like NeuN, show a sharp increase after 14 days as this marker typically denotes mature neurons (n = 4 mice per group, mean ± SEM). DCX, doublecortin; NeuN, neuron-specific neuronal peptide; BrdU, 5-bromo-2'-deoxyuridine.

Other important markers of neurogenesis include nestin, polysialylated-neural cell adhesion molecule (PSA-NCAM), and neuron differentiation protein (NeuroD). Nestin is an intermediate filament protein expressed through stages 1 and 2 of neurogenesis in stem and progenitor cells (Messam, Hou, & Major, 2000). PSA-NCAM is an important molecule for neuroblast migration and is expressed mainly in stage 2 of neurogenesis (Seki, 2002). NeuroD is a basic helix-loop-helix protein that is expressed during differentiation from progenitor to immature neuron (Lee et al., 1995).

An excellent review by von Bohlen und Halbach (2007) summarizes in detail several markers for all of the adult neurogenesis stages, and includes markers for cell division, early stage neurogenesis, immature neurons, mature
neurons and even glial cells. Some of this marker information is summarized in Table 5.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Markers of Stages in Adult Neurogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Division</td>
<td>Progenitor</td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen (PCNA)</td>
<td>SRY-related HMG-box gene 2</td>
</tr>
<tr>
<td>Kiel 67 (Ki-67)</td>
<td>Musashi-1</td>
</tr>
<tr>
<td>Phosphohistone 3 (PhH3)</td>
<td>Paired box gene 6</td>
</tr>
<tr>
<td>Minichromosome maintenance protein 2 (MCM2)</td>
<td>Nestin</td>
</tr>
</tbody>
</table>

These common markers of neurogenesis help scientists determine the stage of neurogenesis a neuron is in, and also helps determine levels of neurogenesis. Normal physiological levels of neurogenesis can be influenced by environmental, pharmacological, and genetic manipulations. Beneficial environmental influences that increase neurogenesis in the brain include estrogen, (Fowler, Liu, & Wang, 2008; Tanapat, Hastings, Reeves, & Gould, 1999), voluntary exercise (van Praag, Christie, Sejnowski, & Gage, 1999), environmental enrichment (Bekinschtein, Oomen, Saksida, & Bussey, 2011), and electroconvulsive therapy (Madsen et al., 2000) which all increase cell proliferation in the dentate gyrus. Detrimental environmental influences that decrease neurogenesis include factors such as the adrenal hormone corticosterone (Gould, Cameron, Daniels, Woolley, & McEwen, 1992), stress (Gould, Tanapat, McEwen, Flügge, & Fuchs, 1998), and opiates (Eisch,
Barrot, Schad, Self, & Nestler, 2000), all of which decrease cell proliferation in the dentate gyrus.

In order to assess what purpose neurogenesis serves in the brain, scientists can deplete or even knock out neurogenesis and then observe the subsequent behavioral, cellular, or molecular result. Several pharmacological approaches to depleting neurogenesis in the hippocampus exist. Two of the most common are by using methylaoxymethanol acetate (MAM) and cytosine arabinoside (Ara-C), which are both anti-mitotic agents (Ko et al., 2009; van Pelt, de Haan, Vellenga, & Daenen, 2005). However, one of the advantages to using Ara-C is that it is a common chemotherapy agent and therefore has more translational relevance (Li et al., 2008). Ara-C is thought to work by interfering with cell cycle kinetics, causing cell cycle arrest just 2 h after administration of BrdU (van Pelt et al., 2005). After Ara-C treatment ceases, however, the cell cycle resumes and regular incorporation of BrdU occurs in 6-12 h (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1999). Several mechanisms of action have been proposed to explain how Ara-C works to stop cellular proliferation by interrupting the cell cycle. Among them include a Bax-dependent apoptosis hypothesis (Besirli, Deckwerth, Crowder, Freeman, & Johnson, 2003), incorporation into DNA and RNA causing inhibition of polymerase α and β, and impairment of DNA repair mechanisms (Braess et al., 1999). The exact mechanism of action of Ara-C is controversial and remains under investigation (Sreenivasan, Sarkar, & Manna, 2003).
Genetic manipulations of neurogenesis enable scientists to more specifically examine the purpose of neurogenesis and how it works, without having to interrupt other cellular processes. Therefore, multiple genetic manipulations have been produced to influence adult neurogenesis expression. A few of these are listed in table 6 (Filipkowski, Kiryk, Kowalczyk, & Kaczmarek, 2005) below.

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Name/Function</th>
<th>Effects on ANGE</th>
<th>Site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D2</td>
<td>Cell cycle reg. protein</td>
<td>Complete reduction</td>
<td>DG, OB</td>
<td>Kowalczyk et al., 1994</td>
</tr>
<tr>
<td>Tlx (tailless)/Tlx</td>
<td>Transcription factor</td>
<td>Complete reduction</td>
<td>DG, SVZ</td>
<td>Shi et al., 2004</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal NO synthase</td>
<td>Enhancement</td>
<td>SVZ, OB, DG</td>
<td>Packer et al., 2003</td>
</tr>
<tr>
<td>BDNF</td>
<td>Growth factor</td>
<td>Reduction</td>
<td>DG</td>
<td>Lee et al., 2002</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor</td>
<td>Enhancement</td>
<td>DG</td>
<td>Cheng et al., 2001</td>
</tr>
<tr>
<td>CB1R</td>
<td>Cannabinoid receptor</td>
<td>Reduction</td>
<td>DG, SVZ</td>
<td>Jin et al., 2004</td>
</tr>
<tr>
<td>VR1</td>
<td>Vanilloid receptor</td>
<td>Enhancement</td>
<td>DG, SVZ</td>
<td>Jin et al., 2004</td>
</tr>
<tr>
<td>GiPR</td>
<td>GiP receptor</td>
<td>Reduction</td>
<td>DG</td>
<td>Nyberg et al., 2005</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
<td>Reduction</td>
<td>DG</td>
<td>Gass et al., 2000</td>
</tr>
<tr>
<td>NK1R</td>
<td>Neurokinin-1 receptor</td>
<td>Enhancement</td>
<td>DG</td>
<td>Morcuende et al., 2003</td>
</tr>
<tr>
<td>mCD24</td>
<td>Membrane-assoc. molecule</td>
<td>Enhancement</td>
<td>DG, SVZ</td>
<td>Belvindrah et al., 2002</td>
</tr>
<tr>
<td>Sox2</td>
<td>Transcription factor</td>
<td>Reduction</td>
<td>DG, SVZ</td>
<td>Ferri et al., 2004</td>
</tr>
<tr>
<td>Naglu</td>
<td>α-N-acetylglucosaminidase</td>
<td>Reduction</td>
<td>DG, SVZ</td>
<td>Li et al., 2002</td>
</tr>
<tr>
<td>Cystatin</td>
<td>Cysteine protease inhibitor</td>
<td>Reduction</td>
<td>DG</td>
<td>Pirttila et al., 2004</td>
</tr>
<tr>
<td>Bax</td>
<td>Proapoptotic Bcl-2</td>
<td>Enhancement</td>
<td>DG</td>
<td>Sun et al., 2004</td>
</tr>
</tbody>
</table>

*This table is adapted from table 1 in Filipkowski et al., 2005, and includes all manipulations that affect the DG. Note that references listed in this table do not necessarily appear in the “References” section. For further information on each manipulation, please refer to Filipkowski et al., 2005. DG, dentate gyrus; SVZ, subventricular zone; OB, olfactory bulb.

Other conditional genetic manipulations – meaning the manipulation can be turned on and off – have since been developed (table 7). These genetic manipulations are often referred to as “Cre/loxP” and “Flp” recombinase systems. Developed in the early 2000’s, these manipulations are helpful in ascertaining a neuron’s current developmental stage (also known as fate-mapping). They work by using a promoter common only to neural stem cells to drive Cre-estrogen receptor recombination. When a
specific ligand, usually tamoxifen and sometimes tetracycline, is introduced, cells recombine their genes, or mutate, so that the promoter that usually signals cell proliferation is skipped (Feil et al., 1996).

<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter</th>
<th>Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tamoxifen-inducible</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRAP-CreERT2</td>
<td>huGFAP</td>
<td>Cre-ERT2</td>
<td>Hirrlinger et al., 2006</td>
</tr>
<tr>
<td>GLAST::CreERT2</td>
<td>Knockin to endogenous GLAST locus</td>
<td>Cre-ERT2</td>
<td>Mori et al., 2006</td>
</tr>
<tr>
<td>Nestin-CreER</td>
<td>Nestin second intron/Hsp-68 minimal</td>
<td>Cre-ERT1</td>
<td>Burns et al., 2007</td>
</tr>
<tr>
<td>Nes-CreER(T2)</td>
<td>Nestin/Nestin second intron</td>
<td>Cre-ERT2</td>
<td>Imayoshi et al., 2006</td>
</tr>
<tr>
<td>Nestin-creERtm</td>
<td>Nestin promoter and second intron</td>
<td>Cre-ERT1</td>
<td>Kuo et al., 2006</td>
</tr>
<tr>
<td>Ngn2-CreERTM</td>
<td>Knockin to endogenous Ngn2 locus</td>
<td>Cre-ERT1</td>
<td>Raineteau et al., 2006</td>
</tr>
<tr>
<td><strong>Tetracycline-regulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nestin-rtTA-M2</td>
<td>Nestin/Nestin second intron</td>
<td>rTA-M2</td>
<td>Yu et al., 2005</td>
</tr>
<tr>
<td>Nestin-tTA</td>
<td>Xh5 plasmid (large fragment including Nestin promoter/introns/exons)</td>
<td>tTA</td>
<td>Beech et al., 2005</td>
</tr>
</tbody>
</table>

*Table adapted from table 2 in Gage, Kempermann, Song (2008), p. 62. Note that references within this table do not necessarily appear in the “Reference” section of this dissertation.

### 3.3 Learning, memory and adult neurogenesis

Though adult neurogenesis was discovered nearly 50 years ago, controversy still exists over whether or not adult neurogenesis is important to cognition – specifically learning and memory. Several studies have shown that neurogenesis is important for hippocampal-dependent learning and memory tasks (Bruel-Jungerman, Laroche, & Rampon, 2005; Dupret et al., 2008; Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Snyder, Hong, McDonald, & Wojtowicz, 2005) while another shows that neurogenesis may only be important for some hippocampal-dependent learning tasks (Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Additionally, a recent review on the topic suggests that newborn hippocampal neurons, during different
times in their development, may contribute to different aspects of learning and memory – such as pattern separation (Deng, Aimone, & Gage, 2010).

It has been shown that higher levels of cell proliferation and numbers of new neurons correlated with better spatial memory, whereas lower levels of neurogenesis correlated with poorer memory in aged rats (Drapeau et al., 2003). In addition, inhibiting neurogenesis negatively affects learning and memory. For instance, mice that underwent low-dose x-irradiation to focally ablate hippocampal neurogenesis performed poorly in a behavioral test for spatial discrimination, compared to those with intact neurogenesis (Clelland et al., 2009). Neurogenesis is important for the formation of hippocampal-dependent memories and when neurogenesis is knocked-down with a toxin specific to proliferating cells (methylazoxymethanol acetate) this formation is impaired (Shors et al., 2001). Additionally, prenatal stress-induced learning deficits are also associated with inhibition of neurogenesis in the hippocampus (Lemaire, Koehl, Le Moal, & Abrous, 2000).

Adult neurogenesis is also implicated in the enhancement of hippocampal synaptic plasticity – the underlying mechanism for learning and memory (see chapter 2.3). Adult born hippocampal granule cells have a lower threshold to induce LTP (Ge, Yang, Hsu, Ming, & Song, 2007) and are more sensitive to excitatory input (Couillard-Despres et al., 2006; Schmidt-Hieber, Jonas, & Bischofberger, 2004). Furthermore, young neurons generated in the adult mouse have been shown to play a significant role in the generation of synaptic plasticity in the DG (Snyder, Kee, & Wojtowicz,
NMDA receptors have a significant role in the induction of LTP (see chapter 2.3) and have also been shown to be important in the process of neurogenesis (Nacher & McEwen, 2006). Blocking NMDA receptors increases neurogenesis in the DG and also the density of neurons in the granule cell layer of the DG – showing the regulatory effects of excitatory amino acids on neurogenesis (Cameron, McEwen, & Gould, 1995). Additionally, NMDA receptor levels have been shown to positively correlate with hippocampal dependent learning abilities (Adams et al., 2001), further strengthening the connection between neurogenesis, learning and memory, and synaptic plasticity.

3.4 Adult neurogenesis and the $\alpha_1$-adrenergic receptor

As previously mentioned, a substantial amount of efferent NE projections from the locus coeruleus synapse onto the mammalian hippocampus (Blackstad et al., 1967). The SGZ of the DG has more of these NE-innervations than other regions of the hippocampus (Loy, Koziell, Lindsey, & Moore, 1980). It is easy to see that the AR system may have an important role in adult neurogenesis, especially in the SGZ of the DG. In fact, depleting catecholamines in the brain, such as NE, with reserpine caused drastic decreases in neurogenesis (Lewis, Patel, Béndek, & Balázs, 1977). Furthermore, selectively depleting NE with a noradrenergic neurotoxin ($N$-(2-chloroethyl)-$N$-ethyl-2-bromo-benzylamine hydrochloride) reduced neural stem cell proliferation, specifically, but did not affect survival and differentiation (Kulkarni, Jha, & Vaidya, 2002). Increasing NE, but not
serotonin, activated precursor cell populations from the adult hippocampus (Jhaveri et al., 2010). However, the AR subtype mediating this response is controversial.

The $\alpha_1$-, $\alpha_2$-, $\beta_1$- and $\beta_2$-ARs are all present in the DG of the hippocampus (see table 2, section 1.4) in varying amounts. One study found that directly activating the $\beta_2$-ARs led to an increase in type-2A progenitor neurons, and increases neurogenesis both in cell culture and in the hippocampus (Masuda et al., 2012). Surprisingly, the $\beta_3$-AR was not detected in the DG (see table 2), but another study showed that proliferation of neural precursors in the DG was specifically mediated by the $\beta_3$-AR (Jhaveri et al., 2010). The opposite was found with agonizing $\alpha_2$-AR, where activating this subtype caused a decrease (Yanpallewar et al., 2010) in hippocampal neurogenesis and blocking it caused an increase (Veyrac, Didier, Colpaert, Jourdan, & Marien, 2005; Yanpallewar et al., 2010). However, there is still controversy over whether blocking the $\alpha_2$-AR causes an increase in neurogenesis by a selective effect that increases proliferation via $\alpha_2$-AR directly located on neural stem and progenitor cells (Yanpallewar et al., 2010) or by increasing the long-term survival by reducing apoptosis in newborn neurons (Rizk et al., 2006).

There are significant concentrations of the $\alpha_{1D}$-AR in the DG, but interestingly, this receptor may mediate a stress response in the hippocampus (Campeau et al., 2010), and has not been shown to be linked to neurogenesis. The $\alpha_{1A}$-AR is found in very high concentrations in the DG and
the $\alpha_{1B}$-AR has a slightly lower concentration in this area (see table 3, section 1.4), comparable to $\alpha_{1D}$-AR levels. The $\alpha_{1A}$- and $\alpha_{1B}$-AR have both been shown to regulate gliogenesis and neurogenesis in the subventricular zone of the brain – the other highly neurogenic region besides the SGZ (Gupta et al., 2009). This study also found $\alpha_{1A}$-ARs present on stem cells and early progenitor cells. Therefore, it is highly possible that $\alpha_{1A}$-AR activation in the hippocampus causes an increase in neurogenesis.
CHAPTER IV

NEUROGENESIS, METABOLISM, & EXERCISE

4.1 The role of adrenergic receptors in metabolism

Norepinephrine plays an important role in metabolism via the hypothalamus. It is known that noradrenergic projections into the ventral medial hypothalamus (VMH) convey important information to the CNS regarding the tissue in the PNS, and also serve as part of the communication pathway between the brain and the periphery for fatty acid oxidation during exercise (Miyaki et al., 2011). Several subtypes of ARs are expressed in the hypothalamus of the brain (tables 2 and 3, sect 1.4). Research shows a high density of $\alpha_{1A}$-ARs, a moderate density of $\alpha_{1B}$-ARs and $\alpha_{2A}$-ARs, and low amounts of $\alpha_{2C}$-ARs in the mouse hypothalamus (Papay et al., 2006). And according to table 2, no $\beta$-AR subtypes were detected in the hypothalamus, but the $\beta_1$- and $\beta_2$-AR seem to play a role in conjunction with the $\alpha_1$-AR as their activation causes inhibition of food intake (Racotta & Soto-Mora, 1993). In contrast, activating the $\alpha_2$-AR causes an increase in food intake (Goldman, Marino, & Leibowitz, 1985; Leibowitz, 1988). Taking a closer look at the $\alpha_1$-AR, and specifically the $\alpha_{1A}$-AR, this receptor may play a more significant role in regulating weight.
The $\alpha_1$-AR agonist, cirazoline (CRZ), causes acute activation of these receptors in the paraventricular hypothalamic nucleus (PVN) affecting feeding behavior by decreasing food intake and appetite (Davies & Wellman, 1992; Rossi & Scharrer, 1994; Wellman & Davies, 1992). Though several subtypes of the AR family are implicated in metabolism, the $\alpha_1$-AR is most relevant to these studies and therefore will be emphasized throughout this chapter.

### 4.2 Neurogenesis and metabolism

Typically, studies have connected the brain to obesity and metabolism by focusing on hormonal regulators of appetite and satiation, such as appetite-suppressing leptin and appetite-stimulating ghrelin (Elmquist, Elias, & Saper, 1999; Nakazato et al., 2001). Both leptin and ghrelin have receptors located on neurons in the hypothalamus – a metabolic center – in the central nervous system (Guan et al., 1997; Håkansson, Brown, Ghilardi, Skoda, & Meister, 1998). More recently, neurogenesis in the hypothalamus became the new connection between the brain and obesity (Park, Baum, Paredes, & Tobet, 1996; Pencea, Bingaman, Wiegand, & Luskin, 2001). Within the last decade, studies of neurogenesis in the hypothalamus revealed that new hypothalamic neurons play a role in energy balance and weight maintenance (Kokoeva et al., 2005; Pierce & Xu, 2010).
Figure 13. Diagram of the hypothalamus. Hypothalamic nuclei, the neurogenic region of the hypothalamus, and the third ventricle (A; figure from Morton, Cummings, Baskin, Barsh, & Schwartz, 2006); Median eminence revealing hypothalamic neurogenesis (B; figure from Bennett, Yang, Enikolopov, & Iacovitti, 2009). DMN, dorsal medial nucleus; VMC, ventral medial nucleus; ARC, arcuate nucleus; ME, median eminence; PFA, parafornical area; FX, fornix.

Hypothalamic neurogenesis appears to influence normal weight maintenance, metabolism, and energy balance (Lee & Blackshaw, 2012). Hypothalamic neurons that seem to play a role include the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and the anorexigenic proopiomelanocortin (POMC) neuron. NPY/AgRP neurons in newborn mice have no impact on feeding, yet in adult mice they are essential (Luquet, Perez, Hnasko, & Palmiter, 2005). A sudden loss of AgRP neurons causes severe anorexia in adult mice (Gropp et al., 2005). However, a gradual loss of AgRP neurons had no effect on weight or fat deposits until a proliferation-blocking drug was added (cytosine arabinoside, Ara-C), which induced significant fat loss (Pierce & Xu, 2010). These results suggest that neurogenesis in the hypothalamus is necessary for energy balance and weight maintenance.
4.3 Norepinephrine, adrenergic receptors and exercise

The fight or flight response of the sympathetic nervous system activates our EPI and NE systems in the body. Naturally, it would be logical to assume that when running, or exercising, EPI levels rise in the periphery, and NE levels rise in the brain. Experimentally, this was confirmed, as NE brain levels in running rats were compared to their sedentary counterparts, and the levels in the running group were significantly higher than the sedentary group (Brown & Huss, 1973; Brown, Payne, & Kim, 1979). This result was confirmed again nearly 20 years later with a more sophisticated test for NE metabolites in the brain after exercise, again finding that exercise increased NE metabolites in areas of the brain containing NE cell bodies and NE terminals (Dunn & Reigle, 1996). Exercise was also shown to increase levels of PI3 kinase expression in the
hippocampus (Chen & Russo-Neustadt, 2005). And as previously mentioned in section 1.2, PI₃ is part of the α₁-AR second-messenger pathway (Fig. 5). Therefore, it is possible that the α₁-AR is a key mediating receptor between increased NE levels and exercise.

4.4 Exercise-induced neurogenesis

As adult neurogenesis became a burgeoning field in the late 1990’s, studies began to surface observing increases in neurogenesis correlated with running (van Praag, Kempermann, & Gage, 1999), while physical activity appeared to have the opposite effect on microglia cell populations (Kohman, DeYoung, Bhattacharya, Peterson, & Rhodes, 2012). Upon further investigation, exercise-induced neurogenesis was shown to enhance learning and memory in mice (van Praag, Christie, et al., 1999). Other studies echoed these results (Snyder, Glover, Sanzone, Kamhi, & Cameron, 2009), and even showed that the usual learning and memory impairments accompanying aging can be offset by voluntary exercise (van Praag, Shubert, Zhao, & Gage, 2005). However, whether or not neurogenesis is important for enhancing learning and memory is still controversial (Rhodes, Gammie, & Garland, 2005).

As investigations into exercise-induced neurogenesis increased, complications arose. For instance, different strains of mice respond much differently to exercise-induced neurogenesis (Clark et al., 2011). For instance, the C57BL/6J mice showed the least difference in running-mice versus sedentary-mice in neurogenesis levels when compared to the AKR/J strain,
which showed the greatest difference. Another confounding factor was that many exercise-induced neurogenesis studies were not tracking how far mice were running, which generally correlates with the amount of neurogenesis induced (Castilla-Ortega et al., 2013). The type of running can also affect the amount of neurogenesis induced from exercise. For instance, one study found that voluntary resistance running induced significantly higher levels of neurogenesis in mice compared to regular “free-load” running (Lee et al., 2013). And running in wild rodents induces little to no effect on neurogenesis levels (Klaus & Amrein, 2012) further complicating the question of what the relationship is between exercise and neurogenesis, if any. This nascent field has many questions left to address, especially regarding which receptors may be mediating exercise-induced neurogenesis. Many studies show links between exercise induced neurogenesis and several different neurotransmitters (e.g., NE, serotonin, acetylcholine, and γ-aminobutyric acid), as well as brain-derived neurotrophic factor (Ma, 2008), but receptors mediating this response remain elusive.

There are many similarities between the consequences of exercising and the consequences of activating the α1A-AR. Exercising increases NE levels in the brain (Brown & Huss, 1973), increases heart mass (Brown & Huss, 1973), increases neurogenesis (van Praag, Kempermann, et al., 1999), and decreases anxiety-like behavior (Duman & Schlesinger, 2008). Activating the α1A-AR with NE increases heart mass (when chronically activated) (Papay, Shi, Piascik, Naga Prasad, & Perez, 2013), increases neurogenesis (Gupta et al., 2009), and
decreases anxiety-like behavior (Doze et al., 2011). These similarities lead to the question of whether or not exercise-induced neurogenesis, along with the benefits of exercising, is mediated by the $\alpha_{1A}$-AR.
CHAPTER V

LONG TERM $\alpha_{1A}$-ADRENERGIC RECEPTOR STIMULATION ENHANCES COGNITIVE FUNCTION AND SYNAPTIC PLASTICITY

5.1. Introduction

In this study, transgenic mice engineered to express a constitutively activated mutant-$\alpha_{1A}$AR and normal mice treated with an $\alpha_{1A}$-AR-selective agonist, cirazoline (CRZ), were used to determine the effects of chronic $\alpha_{1A}$-AR stimulation on learning, memory and synaptic plasticity. We used the Barnes maze to test cognitive function and electrophysiology to test for enhanced synaptic plasticity to determine whether chronically or transgenically activating or knocking out the $\alpha_{1A}$-AR enhances these functions. Our results may afford a potential new strategy for treating the decline in cognitive function with aging and other neurological disorders.

5.2. Methods

5.2.1. Transgenic mice

This study utilized 26 male and female transgenic CAM-$\alpha_{1A}$AR mice (on a B6/CBA background) and 34 wild type (WT) littermate controls, along with 12 male and female $\alpha_{1A}$-AR KO mice. CAM-$\alpha_{1A}$adrenergic receptors were mutated on the sixth transmembrane of the receptor (methionen 292 to leucine) causing
the G-protein inside of the cell to continually signal (Hwa, Graham, & Perez, 1996). For $\alpha_{1A}$-AR KO mice, the $\alpha_{1A}$-AR exon was replaced with the LacZ exon (Papay et al., 2006). Mice were bred and genotyped at the Cleveland Clinic Foundation and then transferred to the University of North Dakota’s AAALAC-accredited animal care facility. Mice were maintained on a 12 h light/dark cycle (lights on 0500) and housed in 17 x 28 x 13-cm translucent, polycarbonate boxes attached to an automatic watering system (Edstrom Industries, Inc, Waterford, WI, USA), and were provided ad libitum access to pelleted food with 5% fat (Teklad 22/5 Rodent Diet (W) 8640, Harlan, Indianapolis, IN, USA). Room air was 100% exchanged 12-40 times per h with no recirculation, the temperature was 22°C, and the humidity was 23-27%. Mice were identified by ear tags placed at the Cleveland Clinic. The experimental protocols employed in this study conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Animal Care and Use Committees at both institutions.

5.2.2. Barnes maze

Mice were aged 3-6 mo, except for mice treated long-term with cirazoline, which were aged 6-11 mo. Mice were acclimated in separate cages in the testing room for 30 min prior to Barnes maze testing, and deprived of food and water for the duration of the test. The Barnes maze (Fig. 15) was performed between 0800 and 1200 h. The Barnes maze was used to assess spatial learning and memory in mice with a modified protocol (Harrison et al., 2006). The Barnes maze consisted of a white, flat, circular platform (120 cm in diameter)
elevated 140 cm above ground (Med Associates, St. Albans, VT, USA). An escape box (21 x 5.5 x 5 cm), not visible from the top of the maze, was located under one of 40 holes (5 cm in diameter) evenly spaced, 3.5 cm apart, around the perimeter. Three visual cues were placed on a black curtain surrounding the maze, and their locations in relation to the escape box remained the same throughout the experiment. Two flood lights (1700 total lux) and four evenly spaced fans, above the maze, provided aversion (Hong et al., 2007). Between trials, the apparatus was cleaned with alcohol. All testing was video captured, performed, and analyzed blind to mouse type.

The first four days consisted of four learning trials with 30 min intervals between trials. At the start of each trial, a mouse was placed in the center of the maze under a holding chamber for 30 s. When the chamber was lifted, timing of the trial began and the mouse was allowed up to 300 s to enter the escape box. If a mouse failed to enter after the allowed time, it was gently placed into the hole containing the escape box for 30 s. Memory trials were conducted on days 1, 4, 5, and 8 (transgenic mice) and days 1, 4, and 6 (cirazoline-treated mice) after the four days of training. The procedure for the memory trials was the same as learning, except the mice were allowed only one attempt to solve the maze each day. Later analysis included the time to solve, number of errors made, and distance traveled on the maze. Errors were defined as when a mouse poked more than three-quarters of its head into any hole other than the appropriate escape hole. Distance traveled was measured using ANY-maze software (Version 4.73; Stoelting Co., Wood Dale, IL, USA).
Figure 15. Barnes maze apparatus. (Top left) Mouse navigating the Barnes maze based on spatial cues shown in bright pink and black. (Top right) Aversive stimulus was produced with bright flood lights and fans. (Bottom left) Barnes maze on raised pedestal, showing a large black curtain draped around the apparatus to block distractions. (Bottom right) Mouse in red mouse house moments after being placed on the maze (house will be removed once mouse exits the house).

5.2.3. Hippocampal slice preparation

The effects of long-term chronic $\alpha_{1a}$AR stimulation on synaptic transmission and plasticity were examined in aged mice. Hippocampal slices were prepared from 22-24 mo old mice as follows. Mice were weighed, deeply anesthetized with isoflurane, and then immediately decapitated. The brain was removed quickly and placed in ice cold oxygenated choline chloride solution (110 mM C$_5$H$_{14}$CINO, 25 mM NaHCO$_3$, 25 mM dextrose, 11.6 mM NaC$_6$H$_7$O$_6$, 3.10
mM C₃H₃NaO₃, 2.50 mM KCl, 1.25 mM NaH₂PO₄, 0.50 mM CaCl₂). While submersed, hippocampi were removed and placed on a tissue chopper. Coronal brain slices were cut 400 μm thick and immediately transferred to an oxygenated holding chamber filled with aCSF (119 mM NaCl, 5.0 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃) warmed to 32 ± 1°C in a water bath for approximately 30 min. Slices were then removed from the water bath and allowed to cool to room temperature (22 ± 1°C). After 15 min, the entorhinal cortex and CA3 region of each slice were quickly removed. Slices were then returned to the holding chamber and allowed an acclimation period of approximately 2 h. Slices were transferred to recording chambers in preparation for electrophysiological recordings, where they were constantly perfused with oxygenated aCSF at a rate of 1.5 mL/min at 22 ± 1°C.

5.2.4. Electrophysiology recordings

Recording electrodes were made from glass micropipettes backfilled with 3 M NaCl solution. The electrodes were placed in the stratum radiatum of the hippocampal cornu ammonis (CA) 1 region. Evoked field excitatory postsynaptic potentials (fEPSPs) were recorded and measured using a BVC-700A Cornerstone amplifier (Dagan Corporation, Minneapolis, MN, USA) in current clamp mode with 100X gain. An ISO-flex stimulator (A.M.P.I., Jerusalem, Israel) paired with a 7.5 cm bipolar tungsten stimulating electrode (World Precision Instruments, Sarasota, FL, USA) was used for presynaptic stimulation of the Schaffer collateral-commissural fibers in stratum radiatum, between the CA3
region and the recording electrode (Fig. 16). Signals were converted from analog to digital using Axon Digidata1440A Data Acquisition System (Molecular Devices Inc., Sunnyvale, CA, USA), and electronic cycling and noise was filtered using a HumBug 50/60 Hz noise eliminator (Quest Scientific, Vancouver, BC, Canada). Recordings were made using Axon Instruments Clampex v10.2. Basal synaptic transmission was assessed using input-output (I/O) curves, generated by applying a stepwise increase in stimulation intensity with a range of 5-80 μA, using increments of 5 μA. Responses were elicited every 20 s with a duration of 100 μs per pulse. For subsequent experiments, the stimulus was set to approximately 50% of the maximal response. Short-term plasticity was investigated by assessing paired-pulse facilitation (PPF) by applying two pulses with inter-pulse intervals of 35, 50, 75, 100, 150, 200, and 300 ms. A baseline response of 30 min was recorded immediately after PPF, which was followed by theta burst stimulation (TBS = 10 trains, each train of 4 pulses at 100 Hz, inter-train interval of 200 ms, total train duration of 40 ms) given at 80% maximal response to induce LTP. fEPSPs were then recorded at 50% maximal response every 20 s for 90 min.
Figure 16. Stimulating LTP in the CA1 region of the hippocampus. The stimulating electrode (left) injects a stimulus to the Schaffer collaterals that synapse on neurons in the CA1 region of the hippocampus. The recording electrode (right) records the response of the neurons there. CA, cornu ammonis; LTP, long-term potentiation.

5.2.5. Cirazoline treatment

Normal, non-transgenic WT mice received bottled water containing cirazoline at 10 mg/L for 2 - 9 mo. This concentration was chosen based on the binding values of the \( \alpha_{1A} \)-AR versus the \( \alpha_{1B} \)-AR (Hwa et al., 1996). The water was changed weekly or biweekly as needed. Food was provided \textit{ad libitum}. No adverse side effects were observed during treatment, or in CAM-\( \alpha_{1A} \)-AR mice.

5.2.6. Statistical analysis

All results were analyzed using GraphPad Prism 5.0 (La Jolla, CA, USA). Statistical comparisons were performed between both male and female CAM \( \alpha_{1A} \)-AR mice and their WT littermate controls using a Student's unpaired \( t \)-test. Electrophysiological data was analyzed using Clampfit v10.2 (Molecular Devices, Sunnyvale, CA, USA) and Prism 5.0 (GraphPad, La Jolla, CA, USA). Analysis of I/O curves was performed by finding the slope of each fEPSP from 5-80 \( \mu \)A, at 5
μA increments. PPF analysis compared the slope of the second elicited fEPSP to the first elicited fEPSP. Fiber volley amplitude was also analyzed to assess basal synaptic transmission. Pre- and post-TBS baselines were analyzed by measuring fEPSP slope every 20 s, and comparing the average pre-TBS baseline slope to the average post-TBS baseline slope for both CAM α1A-AR and WT mice. fEPSP slopes were expressed as a ratio of the pre-TBS baseline and normalized to the pre-TBS baseline. Data are presented as mean ± SEM. Significance levels were taken as * p < .05, ** p < .01, or *** p < .001.

5.3. Results

5.3.1. Chronic stimulation of the α1AAR enhances learning and memory

Learning and memory were assessed using the Barnes mazes – widely accepted as a hippocampal-dependent task of spatial learning and memory. The time to solve, number of errors, and distance traveled are inversely correlated with learning and memory. As shown in Fig. 17, CAM α1A-AR mice (n = 15) showed enhanced cognition when compared to WT mice (n = 17). During learning trials, CAM α1A-AR mice took less time to solve the maze (56 ± 8.9 s) (inset, Fig. 17A1) when compared to WT mice (87 ± 9.8 s) [t(30) = 2.26, p < .05]. CAM α1A-AR mice also made fewer errors during learning trials (9.7 ± 1.1) (inset, Fig. 17A2) than the WT mice (18.0 ± 2.0) [t(30) = 3.46, p < .001]. During memory trials, the CAM α1A-AR mice remembered the location of the escape box better than the WT mice, shown by a decreased mean solve time (21 ± 3.5 s) (inset, Fig. 17B1) when compared to WT mice (38 ± 5.8 s) [t(30) = 2.43, p < .05].
CAM α1A-AR mice also made fewer errors (5.2 ± 0.6) (inset, Fig. 2B2) than the WT mice (10.7 ± 1.3) [t(30) = 3.56, p < .001].

Figure 17. Chronic α1A-AR stimulation improves cognitive performance in the Barnes maze. During learning trials, CAM α1A-AR mice (n = 17) took less time to solve the maze (A1) and made fewer errors (A2) when compared to the WT mice (n = 15). During memory trials, CAM α1A-AR mice took less time to solve the maze (B1) and made fewer errors (B2) compared to WT mice. Schematic drawings represent paths traveled during learning (C1) and memory (C2) trials of the WT and CAM α1A-AR mice. The bar graph insets show the mean solve time and errors during learning and memory trials. Statistically significant at * p < .05, ** p < .01 or *** p < .001. CAM, constitutively active mutant; WT, wild type.
5.3.2. Aged CAM $\alpha_{1A}$-AR mice have enhanced synaptic plasticity

To assess whether the cognitive behavioral gains seen in CAM $\alpha_{1A}$-AR mice correlated with enhanced hippocampal plasticity, several cellular properties were investigated using electrophysiology, including basal synaptic transmission, short-term plasticity as assessed by PPF, and long-term plasticity (i.e., LTP).

Basal synaptic transmission was investigated by analyzing the fEPSP slope at various stimulus intensity intervals (10-80 $\mu$A) and plotting it against fiber volley amplitude. As illustrated in Fig. 18A, a significant difference was observed in the mean I/O slopes of basal synaptic transmission between CAM $\alpha_{1A}$-AR mice (2.92 ± 0.48, $n = 8$) and WT mice (1.11 ± 0.07, $n = 9$; $p < 0.001$) (Fig. 3A, inset). These findings suggest that basal synaptic transmission is enhanced in CAM $\alpha_{1A}$-AR mice compared to WT.

The slope ratio of fEPSP was calculated by taking the slope of the second elicited fEPSP and dividing it by the first elicited fEPSP in PPF experiments. Inter-pulse intervals were then set at 35, 50, 75, 100, 150, 200, or 300 ms. As shown in Fig. 18B, significant difference was found between CAM $\alpha_{1A}$-AR and WT mice at 35 ms (1.28 ± 0.037, $n = 23$ vs. 1.59 ± 0.052, $n = 21$, $p < 0.001$), 50 ms (1.27 ± 0.035, $n = 23$ vs. 1.56 ± 0.049, $n = 21$, $p < 0.001$), 75 ms (1.28 ± 0.043, $n = 13$ vs. 1.51 ± 0.047, $n = 17$, $p < 0.001$), 100 ms (1.23 ± 0.027, $n = 23$ vs. 1.45 ± 0.037, $n = 21$, $p < 0.001$), 150 ms (1.17 ± 0.039, $n = 13$ vs. 1.37 ± 0.032, $n = 19$, $p < 0.001$) and 200 ms (1.14 ± 0.027, $n = 10$ vs. 1.30 ± 0.079, $n =$...
These results indicate that short-term plasticity is enhanced in CAM α1A-AR mice compared to WT.

Synaptic plasticity, particularly LTP, is thought to underlie learning and memory. LTP was measured in the apical dendrites of the hippocampal CA1 region of very old (age 22 to 24-mo) CAM α1A-AR and WT mice induced by TBS (10 trains of 4 pulses at 100 Hz) of the Schaeffer collateral pathway (Fig. 18C1). These recordings showed a significant enhancement of normalized LTP in CAM α1A-AR compared to WT mice at 15 min (1.45 ± 0.073 vs. 1.22 ± 0.036, p < 0.01), 30 min (1.37 ± 0.053 vs. 1.17 ± 0.036, p < 0.01) and 60 min (1.31 ± 0.052 vs. 1.11 ± 0.041, p < 0.01) after TBS (Fig. 3C2) (n = 9 animals for each comparison). In a finding consistent with the observations of enhanced basal synaptic transmission and PPF in CAM α1A-AR mice compared to WT mice, the above results demonstrate that CAM α1A-AR mice have increased LTP relative to WT mice, suggesting that chronic CAM α1A-AR stimulation enhances LTP.
Figure 18. Hippocampal synaptic plasticity is enhanced with chronic α1A-AR activation. (A) Basal synaptic transmission, as determined by the I/O relation between fiber volley amplitude and fEPSP slope, is increased in CAM α1A-AR (n = 11 slices from 3 mice) compared to WT mice (n = 23 slices from 7 animals). The bar graph inset shows the mean I/O slopes. (B) PPF is enhanced in the CAM α1A-AR mice (n = 21 slices from 3 mice) compared to the WT mice (n = 23 slices from 7 mice). The facilitation was plotted as a function of interpulse interval of 35, 50, 75, 100, 150, 200, and 300 ms. Superimposed representative fEPSPs were recorded at 150 ms intervals. (C1) Chronic α1A-AR activation enhances LTP in the hippocampal CA1 region, shown by cumulative data of the normalized changes in field potential slope in CAM α1A-AR mice (n = 23 slices from 9 mice) and WT mice (n = 27 slices from 9 mice). Superimposed representative fEPSPs were recorded 15 min before and 60 min after LTP induction. (C2) Multiple LTP recordings for each mouse were grouped and averaged, giving a single fEPSP slope ratio per animal at different time points before or after TBS (-15, 15, 30, 60 min). CAM α1A-AR mice (n = 9) showed enhanced mean LTP when compared to WT mice (n = 9) at each post-TBS time point. Statistically significant at * p < .05, ** p < .01 or *** p < .001. CAM, constitutively active mutant; fEPSP, field excitatory post-synaptic potential; I/O, input/output; LTP, long-term potentiation; TBS, theta-burst stimulation.
5.3.3. Chronic treatment with an $\alpha_{1A}$-AR selective agonist improves cognitive function

To determine whether exogenous $\alpha_{1A}$-AR stimulation mimics the cognitive effects observed in CAM $\alpha_{1A}$-AR mice, we treated normal WT mice with the $\alpha_{1A}$-AR selective agonist, cirazoline. We assessed learning and memory using the Barnes (Fig. 19). During learning, cirazoline-treated mice solved the Barnes maze in less time ($16 \pm 3.4$ s, $n = 14$) (Fig. 19A1) than control mice ($49 \pm 13$ s, $n = 11$) [$t(23) = 2.1$, $p < .05$] while making fewer errors ($2.9 \pm 1.0$) (Fig. 19A2) than control ($7.1 \pm 2.0$) [$t(23) = 1.7$, $p < .05$]. During memory trials for the Barnes maze (Fig. 19B1), cirazoline-treated mice solved the maze in less time ($24.5 \pm 3.6$ s) (inset, Fig. 19B1) than control mice ($43.7 \pm 7.3$ s) [$t(23) = 2.2$, $p < .05$], while making fewer errors ($3.0 \pm 0.7$) (inset, Fig. 19B2) than control ($7.3 \pm 1.7$) [$t(23) = 2.1$, $p < .05$]. The improved performance observed in the CRZ-treated mice suggests that activating the $\alpha_{1A}$-AR by a subtype selective agonist improves cognitive function in normal mice.
Figure 19. Chronic treatment with an $\alpha_{1A}$-AR-selective agonist improves cognitive function. Normal WT mice treated for 9 mo with the $\alpha_{1A}$-AR selective agonist cirazoline ($n = 11$) solved the Barnes maze in less time ($A_1$) and made fewer errors ($A_2$) than the control WT mice ($n = 14$) on the last day (day 4) of learning trials. During memory trials, cirazoline-treated WT mice required less time to solve ($B_1$) and made fewer errors ($B_2$) than the control WT mice in the Barnes maze. The bar graph insets show the mean solve time and errors during memory testing. Statistically significant at * $p < 0.05$ or ** $p < 0.01$. AR, adrenergic receptor; WT, wild type.

5.3.4. $\alpha_{1A}$-AR KO mice display poor cognitive function

To further define the role of $\alpha_{1A}$-ARs in learning and memory, we next examined the effects of blocking $\alpha_{1A}$-ARs on cognitive function. Because $\alpha_1$-AR antagonists can cause sedation, which would affect behavior testing, we studied $\alpha_{1A}$-AR KO mice using the Barnes maze. The Barnes maze was chosen because it creates a less stressful environment than the water maze and is safer for the $\alpha_{1A}$-AR KO mice which are prone to seizures under stressful conditions. As shown in Fig. 20, $\alpha_{1A}$-AR KO mice ($n=12$) displayed impaired learning and memory compared with WT mice ($n = 10$). During learning trials, $\alpha_{1A}$-AR KO mice took more time to solve the maze ($120 \pm 27$ s) (Fig. 20A$_1$, inset) than control mice ($54 \pm 9.6$ s) [$t(20) = 1.9$, $p < 0.05$]. $\alpha_{1A}$-AR KO mice also made more errors ($36 \pm 9.8$) (Fig. 20A$_2$, inset) than control mice ($16 \pm 1.8$) [$t(20) = 1.8$, $p < 0.05$]. During memory trials, the $\alpha_{1A}$-AR KO mice displayed a poorer recollection of the escape box’s location, indicated by an increased mean solve time ($95 \pm 36$ s) (Fig. 20B$_1$, $p < 0.05$).
inset) compared with WT mice (19 ± 4.8 s) \( [t(20) = 1.9, p < 0.05] \). \( \alpha_{1A}-\text{AR KO} \) mice also made more errors (35 ± 15) (Fig. 20B2, inset) than the WT mice (6.3 ± 1.0) \( [t(20) = 3.6, p < 0.001] \). The poor performance of \( \alpha_{1A}-\text{AR KO} \) mice in learning and memory tasks indicates that this particular transgenic mouse has poor cognitive abilities, and that the \( \alpha_{1A}-\text{AR} \) is directly involved in affecting cognitive function.
Figure 20. Cognitive performance in the Barnes maze is reduced in mice lacking the $\alpha_{1A}$-ARs. During learning trials, $\alpha_{1A}$-AR KO mice (n = 12) took more time to solve the maze ($A_1$) and made more errors ($A_2$) compared with the WT mice (n = 10). During memory trials, $\alpha_{1A}$-AR KO mice took more time to solve the maze ($B_1$) and made more errors ($B_2$) compared with WT mice. Schematic drawings represent paths traveled during learning ($C_1$) and memory ($C_2$) trials of the WT and $\alpha_{1A}$-AR KO mice. The bar graph insets show the mean solve time and errors during learning and memory trials. Statistically significant at *, $p < 0.05$. AR, adrenergic receptor; KO, knock-out; WT, wild type.
CHAPTER VI

ACTIVATING THE $\alpha_{1A}$-AR RESCUES THE NEGATIVE EFFECTS THAT INHIBITING NEUROGENESIS HAS ON LEARNING AND MEMORY IN MICE

6.1. Introduction

Activating the $\alpha_{1A}$-AR subtype increases neurogenesis in the SGZ of the hippocampus (Gupta et al., 2009) and enhances learning, memory, and synaptic plasticity in mice (Doze et al., 2011) as was previously discussed. However, it is unclear whether activating the $\alpha_{1A}$ receptor enhances cognition directly or whether it enhances cognition by increasing neurogenesis. In this study, it was hypothesized that $\alpha_{1A}$-AR induced neurogenesis is responsible for the learning and memory improvements shown previously, but that activating the $\alpha_{1A}$-AR is not solely responsible for this improvement.

The aforementioned hypothesis was tested by first using a common anti-mitotic agent, cytosine arabinoside (Ara-C) along with the $\alpha_{1A}$-AR agonist, cirazoline (Horie et al., 1995; Ruffolo & Waddell, 1982) delivered via osmotic pump (Azlet, Cupertino, CA, USA). Second, learning and memory were tested with novel object recognition and Morris water maze during Ara-C and cirazoline treatment. This novel approach, one of activating the $\alpha_{1A}$-AR while inhibiting neurogenesis, helped to reveal how the $\alpha_{1A}$-AR enhances learning and memory. The results of this study may elucidate the mechanism behind $\alpha_{1A}$-AR enhanced
learning and memory. Investigation of the $\alpha_{1A}$-AR mechanism may lead to new therapeutic strategies targeting the $\alpha_{1A}$-AR and improving learning and memory deficits, along with other cognitive impairments.

6.2. Methods

6.2.1. Mouse husbandry

4-wk old B6/CBA strain mice of both sexes (32 male and 45 female) were transferred to the University of North Dakota’s Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal care facility from Dr. Dianne Perez of the Cleveland Clinic Foundation. Mice were housed in groups of 3-4 until one day prior to surgery (see 6.2.3.), then singly in 17 x 28 x 13-cm transparent, polycarbonate boxes containing one red mouse house, an individual water bottle filled with pH-adjusted water (Edstrom Industries, Inc., Waterford, WI), or water containing the treatment drug Cirazoline (see “Cirazoline Treatment” below for further details). Pelleted food with 5% fat was provided ad libitum (Teklad 22/5 Rodent Diet (W) 8640, Harlan, Indianapolis, IN). Mice were maintained on a 12-h light/dark cycle (lights on at 0600) and room air was 100% exchanged 12 to 40 times per hour with no recirculation and a constant temperature of 22°C and humidity of 23% to 27%. Mice were ear tagged and identified in this way at the Cleveland Clinic. The experimental protocol employed in this study conform to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and were approved by the
Animal Care and Use Committee at the University of North Dakota (protocol no. 1012-1).

6.2.2. **Cirazoline treatment**

4-wk old mice received bottled pH-adjusted water with (16 male, 14 female) or without (8 male, 16 female) 10 mg/L of the $\alpha_1A$adrenergic receptor agonist, cirazoline hydrochloride (CRZ, Tocris Bioscience, Bristol, UK). Treatment duration was 8.5 wks and water was changed weekly.

6.2.3. **Stereotaxic surgery**

For this study, a separate cohort of mice ($n = 6$) were also treated with increasing concentrations of 2%, 3% and 4% Ara-C or vehicle alone to serve as a short pilot study. Qualitative results were analyzed to assess the effect of Ara-C (Fig. 21).
Figure 21. Increasing concentrations of anti-mitotic agent Ara-C induces neuronal toxicity at 3-4%. (A) Mice treated with aCSF (n=2, vehicle-control) or (B) 2% Ara-C (n=2) showed no neuronal toxicity as opposed to mice treated with (C) 3% Ara-C (n=2) and (D) 4% Ara-C (n=2) that show significant losses, particularly in the CA3 region of the hippocampus. (E-H) 10x magnification of CA1 and (I-L) of CA3 in each treatment group. Sections stained with cresyl violet for qualitative analysis. Ara-C, cytosine arabinoside; aCSF, artificial cerebral science fluid; CA, cornu ammonis.

10-wk old mice were prepared for stereotaxic implantation of cannulas (PlasticsOne, Roanoke, VA, USA) and micro-osmotic pumps (flow rate of 0.25 µl/h for 14 d; ALZET, model 1002) with a fill capacity of 100 µL. One day prior to surgery, mice were separated into individual cages, one mouse per cage (to ensure no suture-tampering by other mice), with one red mouse house, pelleted food ad libitum, and pH-adjusted water containing 0.2 mg/mL oral suspension of ibuprofen as an analgesic (Children’s Ibuprofen Oral Suspension, Grape, 100mg/5mL, Target Corp, Minneapolis, MN, USA). Mice were allowed the oral
suspension of ibuprofen for 24 h pre- and post-surgery (total of 48 h), after which the oral suspension was replaced with either plain pH-adjusted water, or water containing CRZ.

The micro-osmotic pumps were prepared according to the manufacturer’s instructions. Briefly, each pump was weighed with its plastic flange (“flow moderator”) and steel tube, and recorded. This base weight helped determine the total amount of fluid inside the pump after filling was complete. Then, a sterile 3 mL syringe (Beckton Dickinson & Co, Franklin Lakes, NJ, USA), a sterile 0.22 µm Millipore Express PES membrane filter unit (Millipore, Billerica, MA, USA) and a filling tube attachment was assembled and inserted into the pump, keeping the pump perpendicular to the bench. A syringe was attached to a sterile filter with a micro-osmotic pump, and either aCSF (123 mM NaCl, 25 mM NaHCO₃, 15 mM C₆H₁₂O₆, 2.5 mM KCl, 1.5 mM CaCl₂, 2.0 mM MgSO₄, and 1.2 mM NaH₂PO₄), or aCSF with 2% Cytosine β-D-arabinofuranoside hydrochloride, Ara-C (20µg Ara-C/1µl aCSF; Fisher Bioreagents, Loughborough, UK) was filtered into the pump. The flow moderator (small, steel tube) was inserted into the micro-osmotic pump. The filled pump was weighed and post-filling weight was recorded to deduce total fill amount. If the fill amount was less than 90% of the reservoir volume (indicative of air bubbles), the pump was refilled. If the fill amount was greater than 90% of the reservoir volume, the plastic flange was removed from the steel tube using a small scissors. Approximately 35 mm of polyethylene tubing (OD: 1.22 mm; ID: 0.72mm) was cut and attached to the cannula (Length: 3.0 mm below pedestal, Plastics One, Roanoke, VA, USA) and
super glue (Loctite) was used to secure the tubing to the cannula. A 20 µL pipette tip, syringe, and filter was used to fill cannula with aCSF or aCSF + 2% AraC. The tubing was attached to the flow moderator, and secured with a small amount of adhesive. The whole apparatus (Fig. 22) was weighed and recorded in order to subtract pump weight from the total weight of the mouse (for accurate BrdU injections and weight monitoring). After weighing, the apparatus was placed in a 15 mL scintillation vial filled with sterile saline, and placed in an incubator at 40°C for approximately 60 min or until needed for surgery.

Figure 22. Complete pump apparatus. (Left) Alzet micro-osmotic pump with flow moderator and attached to polyethylene tubing (i.e. catheter tube in the above diagram). (Right) The cannula attached via catheter tubing to the micro-osmotic pump. The removable tab served to attached the entire pump apparatus to the stereotaxic equipment. Once inserted into the mouse, the tab was removed with a small cauterizing tool. Spacers were not used in this study design but serve to correct the depth of the cannula into the brain.

Mice were brought into a sterile surgical room and weighed. A surgical mask, gloves and a laboratory coat were worn at all times during surgery, and gloves were changed between mice. Mice were given an injection of ketamine HCl (Butler, Dublin, OH, USA) and xylazine (Sigma-Aldrich, St. Louis, MO, USA),
an anesthetic/analgesic and paralytic, respectively (0.775 mL sterile saline, 0.125
mL 100mg/mL ketamine, 0.10 mL 20mg/1mL xylazine) at 0.09 mL cocktail per
gram of body weight. After the mouse was no longer responding to a toe pinch,
the mouse's head was shaved for the incision area and cleaned thoroughly with
alcohol and iodine. Lidocaine was applied with a swab and allowed to soak into
the skin for proper numbing. The mouse was mounted onto the stereotaxic
equipment and the head secured with ear bars and a nose clamp. Ophthalmic
ointment was applied to both eyes to prevent drying of the corneas (I-Drop Vet

Using a scalpel, an incision was made down the middle of the skull, from just
posterior to the eyes to just posterior to the ears (about 1.5 cm). A pocket was
created for the pump using a small surgical scissors, reaching to the anterior side
of the left, hind hip (to insure minimal interfere with movement and respiration).
After the pump pocket was made, the incision on the skull was fitted with, and
held open by, a skin retractor. The surface of the skull was then cleaned with a
sterile Q-tip (Kendall, Mansfield, MA) to remove the superficial fascia, and the
surface was allowed to dry, briefly. Bregma was then located and marked with a
permanent marker (Fig. 23). The stereotaxic instrument (Stoelting, Wood Dale,
IL, USA) was fitted with the cannula and pump apparatus, and the cannula was
aligned with bregma. The instrument was zeroed and positioned to the following
coordinates, corresponding to the right lateral ventricle: Anterior/posterior -0.30,
medial/lateral +1.00, dorsal/ventral -2.20 (Breton-Provencher, Lemasson,
Peralta, & Saghatelyan, 2009; Pierce & Xu, 2010). Once located, a mark was
made on the skull with a permanent marker. A hole was drilled (diameter 0.37 mm) where the mark was made, and a sterile Q-tip was placed over the hole to clean any fluid released from the brain.

![Figure 23. Stereotaxic surgery showing bregma on a mouse. Mouse mounted on stereotaxic equipment with arrow pointing to a drilled hole near bregma, denoted with B. The coronal suture just left of bregma is also visible, along with lambda, located posterior to bregma (stereotaxic coordinates: Anterior/posterior -0.30, medial/lateral +1.00, dorsal/ventral -2.20). B, bregma; CS, coronal suture; L, lambda.](image)

A small hole was drilled lateral to lambda (near the posterior portion of the brain) for a surgical screw. A sterile Q-tip was used to remove fluid and the surgical screw was positioned. The screw served to secure the cannula to the skull once cement was applied. The skin retractor was removed and the pump placed into the pocket. The cannula was then positioned over the hole and the tip of the cannula aligned with the top of the skull. The stereotaxic coordinates were zeroed to ensure the correct position of the dorsal/ventral coordinates. The
cannula was partially inserted into the brain and dental cement (Stoelting, Wood Dale, IL, USA) was mixed, drawn into a small 1 mL syringe, and placed between the cannula holder and the skull. The cannula was lowered completely into the brain, and cement was placed around the top of the cannula and around the surgical screw. The cement dried for 5-6 min, the connection between the cannula and the stereotaxic instrument was severed with a cauterizer (Bovie, Melville, NY, USA), and the mouse dismounted from the ear bars and nose clamp. The mouse was placed on a 40°C warming pad and covered with a paper towel to maintain heat while suturing. Suturing was done with a small surgical needle (Roboz Surgical Instrument Co, Gaithersburg, MD, USA) and suturing thread (Harvard Apparatus, Holliston, MA, USA). A total of 7-8 sutures was applied to the incision to ensure proper healing of the wound. New skin liquid band-aid (Medtech Products, Inc, Irvington, NY, USA) was applied over the top of the sutures along the incision to disinfect and further seal the incision. The mouse was placed in a paper-towel-lined, polycarbonate cage with no bedding. The cage was placed on top of a pet bed warmer (K&H Manufacturing, Colorado Springs, CO, USA) to ensure the core temperature would remain elevated (as anesthesia can cause a drop in core temperature). After each surgery, the stereotaxic equipment and surgical instruments were thoroughly cleaned with Clorox disinfectant wipes (The Clorox Company, Oakland, CA, USA), and new sterile Q-tips were made available. The ibuprofen water was made available, but no food, until the mouse was fully conscious, or about 1-2 h after surgery. Once fully conscious, the mouse was transferred to a cage with a red mouse hut,
bedding, food, and the ibuprofen water. Ibuprofen water was removed the next day (approximately 24-h after surgery) and replaced with plain pH-adjusted water or pH-adjusted water containing CRZ. Mice were allowed 7 days to recover from surgery before beginning behavioral testing. 10 μL of methylene blue was injected into cannulas and pump volumes were measured post-mortem to verify cannula placement and pump function, respectively.

6.2.4. Novel object recognition

The novel object recognition task (Fig. 24, Ennaceur & Delacour, 1988) measures the ability of a mouse to remember old objects and recognize new objects, a behavior thought to be dependent on the medial temporal lobe, or hippocampus (Squire & Zola, 1996). Novel object recognition task was used on day 8 of the testing protocol (Fig. 27). Between 800 h – 1200 h, mice were allowed to acclimate in acclimation cages for 30 min. Then, each mouse was placed in the 41.3 x 41.3 x 34.3 cm empty experiment box (Stoelting, Wood Dale, IL, USA) for 5 min, the acclimation phase. After an hour, the mouse was placed in the box with two identical objects (black spheres or white cylinders with cone-tops, Stoelting/Custom-made) for 5 min, the familiarization phase. After another hour, the mouse was place in the box with one old object (used during the familiarization phase) and one new object for 5 min, the testing phase. After, mice were returned to their home cages, and objects and the experiment box were thoroughly cleaned with 70% ethyl alcohol before the next mouse was placed in the experiment box. Experiments were recorded using a computer camera (Logitech, Newark, CA, USA) and the testing phase data was later
analyzed for the amount of time spent with the old object, compared to the new object (data presented in seconds) using Excel (Microsoft, Redmond, WA, USA) and Prism (see “statistical analysis”).

6.2.5. Morris water maze

The Morris water maze is a hippocampal- and neurogenesis-dependent assay that tests learning and long-term spatial memory (Fig. 25, Morris, 1984). The maze uses water as the aversive stimuli that motivates the mouse to solve the maze by finding the non-visible platform based on spatial cues (Vorhees & Williams, 2006). Between 800 h – 1200 h, each mouse was placed in an acclimation cage with a heat lamp (Petco, San Diego, CA) suspended above for the first learning trial. After 30 min, mice were placed in the water maze (121.9
cm diameter and 76.2 cm high) divided into four quadrants, surrounded by a tent. An invisible platform (32.4 cm high, Stoelting Co., Wood Dale, IL, USA) was placed in one of the quadrants (each quadrant had the platform for 25% of the trials and 25% of the total mouse population) and covered by 1-1.5 cm of water. Visual cues were placed on the tent walls inside and outside of the maze to help mice navigate. Mice were allowed to swim in the maze for 1 min or until they found the hidden platform. Mice were removed from the maze with a dry towel and allowed to completely dry off under the heat lamp for a minimum of 5 min to prevent hypothermia. Mice had a total of six learning trials each day for four learning days (Fig. 27, days 9 – 12). Mice were then tested with a probe trial (Fig. 27, day 13). The platform was removed from the maze and mice were placed in the quadrant 180° opposite of where the platform used to be. Pathways for learning and probe trials were tracked using ANY Maze Software (Stoelting Co., Wood Dale, IL, USA) and later analyzed for latency to platform, total distance travelled, and swim speed.
Figure 25. Morris water maze. View from the camera suspended over the maze. Visual cues are on both the tent walls and on the walls of the maze. Outlined in orange is the area of ANY Maze tracking potential platform positions in each quadrant.

6.2.5. Open field

Open field is a test of motor movement in mice (Jähkel, Rilke, Koch, & Oehler, 2000; Wilson, Vacek, Lanier, & Dewsbury, 1976) and is now commonly performed in a specialized apparatus that measures movement in real-time based on laser-beam breaks. Mice motor movement was tested for 20 min in the open field (Fig. 26, 43.2 x 43.2 cm, MedAssociates, St. Albans, VT, USA), between 1100 h and 1400 h (Fig. 27, day 13). Data was automatically collected by Activity Monitor software (MedAssociates, St. Albans, VT, USA).
Figure 26. Open field apparatus. The mouse, visible in the box above, is allowed to explore the box (measuring motor movement) freely for 20 min and then removed and returned to its home cage.

6.2.6. BrdU injection & cardiac perfusion

Mice were intraperitoneally injected (Fig. 27, day 11) once with 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). Three days after BrdU injections (Fig. 27, day 14), mice were lethally injected with pentobarbital (150 mg/kg, Euthasol, Virbac Animal Health, Fortworth, TX, USA) and perfused transcardially with heparinized saline for approximately 20 min, or until 30 mL of saline had perfused through the mouse. Brains were removed and cut in half along the central fissure (into left and right hemispheres) and were fixed with 4% paraformaldehyde in normal buffered PBS. After 48 h, the right hemisphere was serially sectioned (in coronal sections) at 40 μm and each
section placed in cryoprotectant solution of a 96-well plate. Sections were labeled and placed in -20 °C until immunohistochemical staining was conducted.

Figure 27. Timeline of experiments. Day 0, stereotaxic surgery was conducted osmotic pumps were given 24 h to begin working. Days 1-7, recovery. Day 8, novel object recognition (NOR). Days 9-12, Morris water maze (MWM) training. Day 11, BrdU injections. Day 13, MWM testing and open field testing. Day 14, cardiac perfusions and removal of brain for immunohistochemical analysis. BrdU, 5-bromo-2’-deoxyuridine; OF, open field; MWM, Morris water maze; NOR, novel object recognition.

6.2.7. Immunohistochemistry

For BrdU-DCX labeling, PBS was used to wash sections 3 times for 5 min in between the following incubations: (1) 0.3% H₂O₂ was used for 5 min; (2) 2 N HCl, 30 min at 37° C; (3) 0.1M borate buffer, 10 min; (4) 5% goat serum (Jackson ImmunoResearch, West Grove, PA, USA) and 10% BSA in PBS block, 1 h at room temperature (RT); (5) rat monoclonal to BrdU antibody (Abcam, Cambridge, England; 6326, 1:10,000) at 4°C, 24 h; (6) biotinylated goat anti-rat (1:2,000), 1 h at RT; (7) avidin/ biotin enzyme complex conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA), 1 h at RT; (8) Diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) 10 min. TBS was used for washes between the following incubations – (9) 0.2 N HCl for 10 min – strip step; (10) block, 1 h at RT; (11) rabbit polyclonal to DCX (Abcam,
Cambridge, England; 77450, 1:500) at 4°C, 24 h; (12) biotinylated goat anti-rabbit at 1:1500 dilution, 1 h at RT; (13) avidin/biotin enzyme complex conjugated to alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) for 1 h at RT; (14) chromagen solution (Vector Laboratories, Burlingame, CA, USA) for 15 min. BrdU/DCX sections were mounted and coverslipped using polyvinyl alcohol mounting medium (Evans, Janson, & Nyengaard, 2004).

6.2.8. Stereology

DCX⁺ and BrdU⁺/DCX⁺ cells were counted in the DG at a selected 1 in 12 series. The optical fractionator method (West, Slomianka, & Gundersen, 1991) was used in combination with StereoInvestigator 9.0 (Microbrightfield Bioscience, Williston, VT, USA) to quantify cells, and every other site (or counting frame) was counted. First, using Stereoinvestigator, the microscope stage and slide was synchronized with the program. After locating the DG, a picture was taken at 10x magnification. Second, the region of interest was traced (Fig. 28, left panel). Next, the high magnification was set to 60x to identify the labeled cells (Fig. 28, right panel). Mounted thickness was variable depending on each mouse (sections were cut at 40 μm but individually assessed for thickness, usually ranging from 28-34 μm). The counting frame was set at 100μm x 100μm. Grid size, determining sites counted, was set at 100μm x 200μm for this study. Parameters were optimized to achieve the lowest mean coefficient of error (CE) possible (with less than 10% being ideal). The experimenter was blinded to treatment groups and used an Olympus BX51WI microscope to analyze sections.
and count cells. StereoInvestigator software was then able to estimate counts for each cell marker delineated based on the following:

Equation 1

\[ N = \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{hsf} \cdot \sum Q \]

Equation 1: ssf, section sampling fraction; asf, area sampling fraction; hsf, height sampling fraction; \( \sum Q \), total count of particles sampled.

Figure 28. Outline of dentate gyrus and visible neurogenesis. (Left) The dentate gyrus of the hippocampus, shown here outlined with a red line. (Right) High magnification of the subgranular zone in the dentate gyrus, stained with BrdU (brown) and DCX (blue) in order to identify dividing neurons, or neurogenesis. BrdU, 5-bromo-2'-deoxyuridine; DCX, doublecortin.

6.2.9. Statistical Analysis

Prism Software (ver. 5.01; GraphPad, La Jolla, CA, USA) was used to employ two-way analysis of variance (ANOVA) and determine interactions between groups of three or more. If significance was found, one-way ANOVA was used with a Tukey post hoc test when analyzing groups of three or more. If variance was not normal, Kruskal-Wallis non-parametric test was used for groups of three or more. Unpaired two-tailed t-test, with or without Welch’s correction for unequal variance, was used as required. Sex differences were not investigated. Data are presented as mean ± S.E.M and significance was set at \( p < 0.05 \).
6.3. Results

6.3.1. Ara-C treatment is toxic to neurons at increasing concentrations

A short pilot study was conducted to test increasing concentrations of Ara-C and used qualitative analysis to assess the results (Fig. 21). Mice that received 4% Ara-C (n = 2) showed neuronal toxicity (Fig. 21D) in both the CA1 (Fig. 21H) and (more visibly) in the CA3 (Fig. 21L) when compared with the vehicle control (n = 2, Fig. 21A, E, I). However, mice that received 2% (n = 2, Fig. 21B, F, J) or 3% (n = 2, Fig. 21C, G, K) Ara-C showed little neuronal toxicity. Treatment with 2% Ara-C was chosen for the study because it was non-toxic at this concentration and was the most common concentration reportedly used in the literature.

6.3.2. Ara-C significantly reduced immature and dividing neuron populations in the hippocampus

To determine whether treatment with the anti-mitotic agent, Ara-C, was successful in disrupting neurogenesis, stereology was used to count the number of immature (DCX⁺) and dividing neurons (DCX⁺/BrdU⁺). Mice treated with Ara-C showed significantly reduced populations of immature neurons regardless of CRZ pretreatment (No CRZ, n = 8; CRZ, n = 12, F (5, 45) = 32.96, p < 0.001) when compared with vehicle (No CRZ, n = 9; CRZ, n = 12). Dividing neurons were also significantly reduced in mice treated with Ara-C (n =20, F (5, 45) = 18.18, p < 0.001) when compared to vehicle. Representative images of the DG are shown in both vehicle (Fig. 29C, E) and Ara-C treated (Fig. 29D, F) mice.
85

Figure 29. Treatment with Ara-C significantly decreases the number of immature and dividing neurons in the hippocampus. (A) Doublecortin\(^+\) (DCX\(^+\)) cells, labeling immature neurons, are significantly decreased in mice treated with Ara-C (No CRZ, \(n = 8\); CRZ, \(n = 12\)) compared to vehicle-control mice (No CRZ, \(n = 9\); CRZ, \(n = 12\)). (B) Ara-C treatment significantly inhibited dividing neurons (labeled with both BrdU and DCX) compared with vehicle. Representative figures of vehicle-control mice at 10x (C) and 60x (D) and Ara-C treated mice (D, F). Error bars represent SEM; *** \(p < 0.001\). Ara-C, cytosine arabinoside; BrdU, 5-bromo-2'-deoxyuridine; CRZ, cirazoline; DCX, doublecortin; SEM, standard error of the mean.

6.3.3. Motor activity in CRZ-Ara-C treated mice is significantly increased compared to CRZ-vehicle-controls

To determine if motor activity was influenced by CRZ or Ara-C treatment, mice were tested in an open field test (Fig. 30). CRZ-Ara-C treated mice (\(n = 14\)) moved significantly more than their CRZ-vehicle-controls (\(n = 16\), \(F(5, 69) = \ldots\))
Mice that had no surgery and no CRZ treatment did not show a difference in movement when compared to their control. Mice treated with CRZ in combination with Ara-C have increased motor movement.

**Figure 30.** Motor activity of mice in each experiment group. Total distance travelled (cm) was measured for each mouse in no surgery, vehicle, and 2% Ara-C treated group and the average distance travelled was measured. Motor activity is significantly increased in CRZ-Ara-C treated mice compared to their CRZ-vehicle-controls. There was no difference between any groups except CRZ-vehicle-control (n=16) and CRZ-Ara-C treated (n=14) mice. Error bars represent SEM; ** p < 0.01. Ara-C, cytosine arabinoside; CRZ, cirazoline; SEM, standard error of the mean.

### 6.3.4. All mice recognized and spent more time with the novel object

Mice were tested in novel object recognition to determine whether short-term memory was interrupted or enhanced. All groups, regardless of treatment, spent more time with the novel object (Fig. 31A-C). The percentage of time spent with the novel object was considered and whether this time was greater than chance (50%). Findings indicate that all groups, regardless of treatment, spent more time with the novel object at a percent greater than chance (Fig.
All mice tested in the novel object recognition test spent more time with the novel object over the old object, showing that short-term, hippocampal dependent memory was still intact in all groups.

Figure 31. Novel object recognition test results. All groups of mice successfully identified the new (novel) object over the old object at greater than chance. (A) No surgery mice, both no CRZ (n=12) and CRZ-treated groups (n=13), (B) no CRZ-vehicle (n=12) and CRZ-vehicle groups (n=16), and (C) no CRZ-Ara-C treated (n=11) and CRZ-Ara-C treated (n=15) groups, spent significantly more time with the new object over the old object. All groups spent more time (D) with the novel object over the old object at greater than chance (50% of the time). Error bars represent SEM; ** p < 0.01, *** p < 0.001. Ara-C, cytosine arabinoside; CRZ, cirazoline.

6.3.5. Performance was similar for all groups during training in the Morris water maze

Mice were trained in Morris water maze to determine the effects of CRZ and Ara-C treatment on long-term learning and memory. All groups of mice solved the maze faster (Fig. 32A1-C1), travelled less (Fig. 32A2-C2, with the exception of CRZ-Ara-C-treated mice) and swam slower (Fig. 32A3-C3) by day 2 or 3 of training when compared to day 1. There was a significant difference in
speed on day 2 of learning between no surgery mice treated with CRZ and those without CRZ treatment. This is likely due to the slight difference in distance and latency to solve, which compounded when speed was calculated.
Figure 32. Morris water maze learning results. All groups of mice solved the maze faster, travelled less and swam slower by day 2 or 3 of the Morris water maze. (A1) No surgery (n=12) and CRZ-no surgery (n=13); (B1) vehicle (n=12) and CRZ-vehicle (n=16); and (C1) Ara-C (n=10) and CRZ-Ara-C treated (n=14) groups all solved the maze significantly faster by day 2 of MWM learning. (A2) No surgery and CRZ-no surgery mice travelled less by day 2; (B2) vehicle mice travelled less by day 3 and CRZ-vehicle mice by day 2; (C2) Ara-C mice travelled less by day 4 and CRZ-Ara-C treated mice travelled less by day 2. (A3) No surgery mice were slower by day 3 and CRZ-no surgery treated were slower by day 3; (B3) vehicle and CRZ-vehicle treated were slower by day 3; (C3) Ara-C and CRZ-Ara-C treated mice were slower by day 2. Error bars represent SEM; * p < 0.05, ** p < 0.01, *** p < 0.001. Ara-C, cytosine arabinoside; CRZ, cirazoline; MWM, Morris water maze; SEM, standard error of the mean.
6.3.6. Mice treated with CRZ solved the maze faster during training; CRZ-Ara-C-treated mice solved the maze significantly faster than Ara-C treated mice that did not receive CRZ

To better understand the potential progression of memory loss induced by Ara-C, the average time to solve for only the first trial of each training day (9-12) for Morris water maze was measured (Fig. 33 A-B). Mice treated with CRZ, regardless of surgery (vehicle, Fig. 33 B2) or Ara-C treatment (Fig. 33 B3) remembered where the hidden platform was and were solving the maze significantly faster on day 2 compared to day 1. Mice without CRZ treatment that had surgery, either with vehicle (Fig. 33A2) or Ara-C (Fig. 33A3) treatment, did not remember where the platform was until day 4 of training (compared to day 1 of training). Mice were also probe tested in the Morris water maze on day 13 post-surgery to determine if long term memory was intact. Mice that were treated with CRZ before receiving Ara-C treatment (n = 12, 10) solved the maze significantly faster (Fig. 33A) and swam a shorter distance (Fig. 33B) than Ara-C-treated mice that did not receive CRZ (n = 11, 10). To ensure treated mice were not swimming slower (resulting in the difference observed), their swim speed was determined. No significant difference in swim speed between any of the groups was observed (Fig. 33C). These results indicate that CRZ-treatment, and activation of the \( \alpha_{1A} \)-AR, is neuroprotective for brain injuries (such as that induced by surgery) and also rescues the affect of ablating neurogenesis on learning and memory.
Figure 33. Morris water maze test results. Ara-C treated mice with CRZ treatment solved (■) the maze significantly faster than Ara-C treated mice without CRZ treatment (□) and vehicle mice. (A₁) No surgery mice without CRZ treatment were learning the maze significantly faster on by day 2 of MWM. Both vehicle (A₂) and Ara-C mice (A₃) without CRZ treatment did not solve the maze faster until day 4 of training. (B₁, B₂, B₃) No-surgery, vehicle and Ara-C mice treated with CRZ all solved the maze significantly faster by day 2 of training. (C₁) No surgery (n=12) and CRZ-no surgery (n=12) mice, vehicle (n=10) and CRZ-vehicle (n=14) mice solved the maze in approximately 3 seconds. Vehicle and CRZ-Ara-C treated (n=12) mice solved the maze faster than mice that received Ara-C alone (n=11). (C₂) Similar to C₁, mice with Ara-C alone travelled the longest distance, significantly more than vehicle and CRZ-Ara-C treated mice. (C₃) There was no difference in speed between any of the groups, or within groups. Error bars represent SEM; * p < 0.05, ** p < 0.01, *** p < 0.001. Ara-C, cytosine arabinoside; CRZ, cirazoline; MWM, Morris water maze; SEM, standard error of the mean.
CHAPTER VII
CYTOSINE ARABINOSIDE DISRUPTS HYPOTHALAMIC NEUROGENESIS AND INDUCES OBESITY IN MICE

7.1. Introduction

The different stages of adult neurogenesis (see review by Ming and Song, 2011), and the influence of each neuron type on weight maintenance during those stages, remains unclear. In this study, the aim was to clarify the effect of hypothalamic neurogenesis and the $\alpha_{1A}$ adrenergic receptor on weight maintenance in mice. To do this, cellular proliferation was disrupted while simultaneously activating the $\alpha_{1A}$-adrenergic receptor with cirazoline. To clarify the role of hypothalamic neurogenesis, motor activity was tested, fat deposition was measured, and neurons were counted in the hypothalamus of both treated and control groups. The approach taken in this study specifically addresses the role of $\alpha_{1A}$ adrenergic receptor activation, neuronal stem cells and immature neurons in weight maintenance. The results of this study help clarify how the various stages of neuronal development affect weight maintenance.

7.2. Methods

7.2.1. Mouse husbandry

Cohorts of 4-wk old B6/CBA strain mice (24 male and 30 female) were received from Dr. Dianne Perez (Cleveland Clinic Foundation) and were
transferred to the University of North Dakota’s animal care facility (protocol no. 1012-1). For further details on mouse husbandry, see section 6.2.1.

7.2.2. Cirazoline Treatment.

See section 6.2.2 for further details.

7.2.3. Microinfusion Pumps & Stereotaxic Surgery.

See section 6.2.3. for further details.

7.2.4. Open field.

The open field test (43.2 x 43.2 cm, MedAssociates) was used to measure motor activity on day 13 post-surgery. Mice were tested for 20 min between 1100 h and 1400 h. Data was automatically collected and analyzed each min by Activity Monitor software (MedAssociates).

7.2.5. Weight, Food Intake, & Fat Deposition Analysis.

Mice were weighed on days 0, 11 and 14, and food intake was measured by weighing food given on day 0, the day of surgery, weighing food eaten on day 7 and replenishing the food supply (again weighing the initial food given), and then weighing food left on day 11 post-surgery, and day 14 post-surgery. Fat deposits from gonadal (surrounding the testes/ovaries in the corresponding male/female), perirenal (behind the kidneys), subcutaneous (the abdomen and hind), and intestinal areas (surrounding the small and large intestine) were dissected using standard dissection tools. Fat was placed in tared weigh boats, weighed using an analytical scale (Mettler Toledo), and then recorded for later statistical analysis.
7.2.6. BrdU Injection & Cardiac Perfusion.

To label dividing cells, mice were intraperitoneally injected once with 5-bromo-2'-deoxyuridine (BrdU, 10 mg/mL dissolved in saline, Sigma-Aldrich) at 50 mg/kg on day 11 post-surgery. After three days, mice were given a lethal dose of pentobarbital (150 mg/kg, Euthasil, Virbac Animal Health) and perfused transcardially with heparinized saline. Brains were removed and fixed with 4% paraformaldehyde in normal buffered PBS. After 48 h, the right hemisphere was serially sectioned at 40 µm. For more procedural details, see section 6.2.6.

7.2.7. Immunohistochemistry.

For BrdU-DCX details, see section 6.2.7. For c-Fos sections, PBS was used to wash sections 3 times for 5 min in between the following incubations: (1) 5% goat serum (Jackson ImmunoResearch) and 10% BSA in PBS block, 1 h at room temperature (RT); (2) rabbit polyclonal to c-Fos antibody (Abcam 7963, 1:250) at 4°C, 24 h; (3) biotinylated goat anti-rabbit (1:2,000) 2 h at RT; (4) avidin/ biotin enzyme complex conjugated to horseradish peroxidase (Vector Laboratories), 1 h at RT; (8) Diaminobenzidine (DAB, Vector Laboratories) 3 min. C-fos sections were mounted and coverslipped using polyvinyl alcohol mounting medium (Evans et al., 2004).

7.2.8. Stereology.

For BrdU+, DCX+ or c-Fos+ cell counting, a 1 in 6 series between bregma -1.34 and -2.06 (Franklin and Paxinos, 2001) was selected. The dorsal medial
hypothalamus, ventral medial hypothalamus (VMH) and the arcuate hypothalamus were identified and outlined. c-Fos+ cells were investigated in the VMH only. The observer was blinded for analysis. Unbiased quantification of BrdU+/ DCX+ or c-Fos+ cells was performed by the optical fractionator method (West et al., 1991) using Stereoinvestigator 9.0 (Microbrightfield Inc) and an Olympus BX51WI microscope. Parameters were set the same as those mentioned in section 6.2.8, except for grid size (sites counted) which was set at 100μm x 100μm for this study. For more parameter details, see section 6.2.8.

7.2.9. Statistical Analysis.

Physical (weight, food intake, and fat deposits), behavioral, and stereological data were analyzed using Prism (ver. 5.01; GraphPad, La Jolla, CA, USA). Unpaired two-tailed t-test with or without Welch’s correction for unequal variance was used as required. One-way analysis of variance was used with a Tukey post hoc test when analyzing groups of three or more. Data are presented as mean ± S.E.M and significance was set at p < 0.05.

7.3. Results

7.3.1. Ara-C treatment significantly increased weight and food intake; cirazoline treatment had no effect.

Mice were divided into four groups (untreated, vehicle-control, CRZ-vehicle, CRZ-Ara-C-treated) to determine how activating the α₁Aadrenergic receptor effects neurogenesis and weight. Weight and food intake was unchanged between CRZ-vehicle (n = 12), vehicle-control (n = 12) and untreated mice (n = 16, Fig. 34A, B). However, CRZ-vehicle mice gained significantly less
weight ($F(3, 50) = 32.11, p < 0.0001$) and consumed less food ($n = 7, F(3, 45) = 21.79, p < 0.0001$) compared with CRZ-Ara-C-treated mice ($n = 14$, Fig. 34A, B).

Neither CRZ nor Ara-C treatment affected mouse motor activity when examined in the open field test (Fig. 34C). These findings suggest that inhibiting cellular proliferation significantly increases weight and food intake, and activating the $\alpha_{1A}$-AR has no ameliorating effect on these factors.

Figure 34. Exposure to antimitotic agent Ara-C caused significant increase in weight and food intake. (A) Non-CRZ treated (□) mice in both untreated (n = 12) or vehicle-control groups (n = 12), and CRZ-treated (■) vehicle mice (n = 16) showed no significant difference in weight gain between groups. Ara-C treated mice (n = 14) gained significantly more weight after 2 wks of Ara-C when compared with vehicle-control mice. (B) CRZ-treated Ara-C mice had significant increases in food intake compared with CRZ-treated vehicle-controls. (C) There was no significant difference in motor activity between any of the groups, as measured in the open field. Error bars represent SEM; *** $p < 0.001$. Ara-C, cytosine arabinoside; CRZ, cirazoline; SEM, standard error of the mean.

7.3.2. Mice treated with Ara-C had significantly greater fat deposits.

Fat deposits were analyzed to determine whether weight-gain in the Ara-C-treated mice was due to increases in fat. Significantly greater fat deposits were observed in Ara-C treated mice compared to vehicle-control mice in the gonadal (Fig. 35A, $1.215 \pm 0.155$ n = 14 vs. $0.407 \pm 0.040$, n = 16; $p < 0.001$), perirenal (Fig. 35A, $0.247 \pm 0.029$ vs. $0.074 \pm 0.008$; $p < 0.001$), subcutaneous (Fig. 35A, $0.752 \pm 0.091$ vs. $0.226 \pm 0.017$; $p < 0.001$), and intestinal deposits (Fig. 35A, $0.647 \pm 0.086$ vs. $0.231 \pm 0.030$; $p < 0.001$). It was also observed that
fat deposits increased uniformly and were proportionate with weight gained (Fig. 35B).

Figure 35. Ara-C-treatment significantly increased fat deposits in mice. When Ara-C-treated mice were compared to CRZ-vehicle control mice (A) gonadal, perirenal, subcutaneous, and intestinal fat deposits were increased, but no difference was found in the distribution of fat to these regions between groups (B). CRZ-vehicle (n = 16), CRZ-Ara-C-treated (n=14); Error bars represent SEM; *** p < 0.001. Ara-C, cytosine arabinoside; CRZ, cirazoline; SEM, standard error of the mean.

7.3.3. Treatment with Ara-C depleted neuronal and dividing cell numbers in the hypothalamus.

To determine whether 2-wk Ara-C treatment effectively disrupted neurogenesis (20µg/µl at a flow rate of 0.25 µl/h for 14 d) and decreased cell populations, the number of dividing cells (BrdU) and immature neurons (DCX) were quantified in the hypothalamus. After 2 wks, there was significantly reduced numbers of DCX⁺ (Fig. 36 B, C; p < 0.05) and BrdU⁺ cells (Fig. 36 E, F; p < 0.01) in CRZ-Ara-C treated mice when compared to CRZ-vehicle-controls (Fig. 36 A, D). Double-labeled cells could not be counted confidently. However, reduced numbers of immature neurons and dividing cells suggests that effective disruption of neurogenesis was achieved in the hypothalamus of Ara-C-treated mice.
Figure 36. Ara-C significantly reduced neurogenesis in the hypothalamus. Representative fields of (A) DCX⁺ and (D) BrdU⁺ cells are shown in vehicle mice (n = 7) but are both absent in CRZ-Ara-C-treated mice (n = 7) (B, E). The number of DCX⁺ (C) and BrdU⁺ cells (F) were significantly fewer in CRZ-Ara-C-treated mice when compared with CRZ-vehicle-control mice. Error bars represent SEM; * p < 0.05, ** p < 0.01. Ara-C, cytosine arabinoside; BrdU, 5-bromo-2'-deoxyuridine; CRZ, cirazoline; DCX, doublecortin; SEM, standard error of the mean.

7.3.4. There was no difference in neuronal activity in the VMH of Ara-C-treated compared to non-treated mice.

Neuronal activity (c-Fos, Dragunow & Faull, 1989) was investigated in the VMH (an important hypothalamic nucleus within the satiety circuit) to determine whether activity changes correlated with the increased food intake resulting from Ara-C treatments. No difference (p = 0.8617) between c-Fos labeled cell numbers in Ara-C-treated mice (Fig. 37 B, C) and vehicle-control mice (Fig. 37 A, C) was observed. This data suggests that neuronal activity in the VMH is independent of the changes in appetite and weight we observed in Ara-C-treated mice.
Figure 37. Ara-C did not affect hypothalamic neuronal activity. There was no difference in neuronal activity between CRZ-Ara-C-treated mice and CRZ-vehicle-control mice in the ventral medial hypothalamus. Representative fields showing (A) c-Fos+ cells (brown) in CRZ-vehicle-control mice (n = 7), and (B) c-Fos+ cells in CRZ-Ara-C-treated mice (n = 5). No statistical difference was seen between the CRZ-vehicle-control and CRZ-Ara-C-treated mice (C). Error bars represent SEM. Ara-C, cytosine arabinoside; CRZ, cirazoline; SEM, standard error of the mean.
CHAPTER VIII

ROLE OF THE ALPHA1A-ADRENERGIC RECEPTOR IN EXERCISE INDUCED NEUROGENESIS

8.1. Introduction

The influence of the $\alpha_{1A}$-AR on exercise-induced neurogenesis has not been investigated yet. Therefore, the goal of this study was to clarify the role of the $\alpha_{1A}$-AR in exercise-induced neurogenesis. A small pilot study was launched to investigate this question by using transgenic $\alpha_{1A}$-AR knock out ($\alpha_{1A}$-AR KO) and normal C57/B6 mice and splitting these groups into cages with free-spinning running wheels and cages with fixed-wheels. After 3 wks, changes in anxiety were tested with the zero maze and changes in neurogenesis, using immunohistochemistry and stereology for unbiased quantification. The results of this study will help determine whether the $\alpha_{1A}$-AR plays a role in exercise-induced neurogenesis, and whether further investigation is needed.

8.2. Methods

8.2.1. Mouse Husbandry

6-wk old B6/CBA strain and $\alpha_{1A}$-AR KO mice were received from Dr. Dianne Perez (Cleveland Clinic Foundation) and were transferred to the University of North Dakota’s Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal care facility. 14 male and 10 female
mice were housed in pairs (sorted by sex) in 41.9 x 21.6-cm transparent, polycarbonate boxes containing one red mouse house, an individual water bottle filled with pH-adjusted water (Edstrom Industries, Inc., Waterford, WI), and a running wheel that was either free-moving ("running" group) or fixed and unmoving ("sedentary" group). For further details about food and room conditions, please see section 6.2.1. The experimental protocol employed in this study conformed to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and were approved by the Animal Care and Use Committee at the University of North Dakota (protocol #1109-2).

8.2.2. Running Apparatus

Running wheels (Mini Silent Spinners, 11.4 cm, Super Pet, Elk Grove Village, IL, USA) were secured with gorilla tape at roughly 2 cm from the cage wall (to allow free movement). Each wheel was equipped with a rare earth-metal magnet (1.9 g each) that was secured to the side of the wheel with gorilla tape (Fig. 38). A bike computer (Schwinn 20-function bike computer, Vancouver WA, USA) was programmed as instructed and the sensor was taped to the cage wall about 7-8 cm from the bottom of the cage. The sensor was placed such that if a mouse was running, the magnet would pass close enough to the sensor to trigger it. The sensor and all wiring were covered with duct tape to ensure no mouse tampering. Bedding was placed over the wheel stand to cover the tape and evenly spread throughout the cage. Food was place in a suet basket (C & S EZ Fill Suet Basket, Duncraft, Concord, NH, USA) to allow easy access, and
taped to the side of the cage wall with duct tape. Water bottles (Petco) were also secured in this way. For sedentary cages with fixed-wheels, the wheel was disassembled and a piece of duct tape was secured to the ball bearing in the wheel. The wheel was reassembled and rendered stationary. The bike sensor was not in the sedentary cages, but the tape was used to mimic a sensor in the cage. Cages were cleaned once a week. Food and water was monitored, weighed and recorded once a week. Mouse weights were recorded once a week and mouse miles were recorded daily (via odometer readings from the bike computer).

8.2.3. Elevated Zero Maze

The zero maze (diameter: 50 cm, height: 40 cm, Stoelting, Wood Dale, IL, USA) was used to test for anxiety. Mice were placed in acclimation cages for 1 hour before beginning the zero maze. Then, each mouse was placed on the
maze for 10 min and video recorded (Logitech, Newark, CA, USA). After, mice were removed from the maze and placed back into their home cages. The maze was cleaned with 70% ethyl alcohol in between each session. Videos were analyzed for time spent in the open area (all four paws in the open) versus time spent in the close areas, and number of entries into the open areas versus closed area.

8.2.4. BrdU Injections & Cardiac Perfusions

On day 20 of running, mice were given two injections of 5-bromo-2'-deoxyuridine (BrdU, 10 mg/mL dissolved in saline, Sigma-Aldrich, St. Louis, MO, USA) at 50 mg/kg about 10 h apart, with the last injection occurring around 76 h before sacrifice. BrdU was made immediately before the first injection to insure potency and integrity. See section 6.2.6. for more procedural details.

8.2.5. Immunohistochemistry

See section 6.2.7. for procedural details.

8.2.6. Stereology

See section 6.2.8. for procedural details.

8.2.7. Statistics

Prism Software (ver. 5.01; GraphPad, La Jolla, CA, USA) was used to one-way analysis of variance followed by a Tukey post hoc test when analyzing groups of three or more. Unpaired two-tailed t-test with or without Welch’s
correction for unequal variance was used as required. Data are presented as mean ± S.E.M and significance was set at $p < 0.05$.

### 8.3. Results

#### 8.3.1. Running WT mice ate more than their sedentary counterparts; there was no difference among $\alpha_{1A}$-AR KO mice

To determine differences food intake, running, and weight gain, all three were tracked for the duration of the pilot study. There was no observed difference between any groups in any of these areas except for food consumed (Fig. 39A). Among WT mice, the running group consumed significantly more food than the sedentary group ($p < 0.01$). No difference was observed in the $\alpha_{1A}$AR-KO mice; this may indicate that the $\alpha_{1A}$-AR is important for appetite regulation during times of high activity.

![Figure 39. WT running mice consumed significantly more food than their sedentary counterparts.](image)

(A) Among WT mice groups, the running group (n = 6) ate significantly more than their sedentary counterparts (n = 6), while no difference was seen among the $\alpha_{1A}$-AR KO mice between sedentary group (n = 6) and the running group (n = 6). (B) No difference was observed between WT and $\alpha_{1A}$-AR KO mice in running. (C) There was no difference in weight gain between any of the groups. Error bars represent SEM; ** $p < 0.01$. AR, adrenergic receptor; Ara-C, cytosine arabinoside; CRZ, cirazoline; KO, knock-out; SEM, standard error of the mean; WT, wild type.

#### 8.3.2. Running $\alpha_{1A}$-AR KO mice exhibit signs of anxiety in the last 5 min of zero maze

The zero maze test was used to determine whether exercising relieved anxiety in any of our experiment groups. No significant difference was observed
between any of the groups in number of entries for the maze (Fig. 40, B1-3).

However, when the second 5 min of time the mice spent in the open parts of the zero maze was examined, a significant difference between the running WT mice and the running $\alpha_{1A}$-AR KO mice and running $\alpha_{1A}$-AR KO mice and their sedentary controls was observed (Fig. 40, A2; $p < 0.01$). Thus, it was concluded that running mice lacking the $\alpha_{1A}$-AR are more likely to exhibit signs of anxiety the longer they stay in the maze, whereas their sedentary and WT running controls are more likely to explore the maze as the test progresses.

Figure 40. Running $\alpha_{1A}$-AR KO mice exhibit signs of anxiety as zero maze progresses. (A1) There was no difference in time spent in the open area of the maze between WT (■) sedentary (n = 6), WT running (n = 6), $\alpha_{1A}$-AR KO (■) sedentary (n = 6), or $\alpha_{1A}$-AR KO running (n = 6) groups during the first 5 min of the zero maze. (A2) $\alpha_{1A}$-AR KO running mice spent significantly more time in the closed parts of the maze than either WT running mice or sedentary $\alpha_{1A}$-AR KO mice. (A3) Overall time spent in open areas on the zero maze showed no difference between groups. (B1-3) There was no difference between any of the groups in entries into open areas of the zero maze. Error bars represent SEM; ** $p < 0.01$. AR, adrenergic receptor; Ara-C, cytosine arabinoside; KO, knock-out; SEM, standard error of the mean; WT, wild type.
8.3.3. Wild type running mice had significantly more dividing neurons than α1A-AR KO running mice

To determine whether running increased neurogenesis in WT and α1A-AR KO mice, immunohistochemistry was used to label new neurons (DCX⁺) and dividing cells (BrdU⁺), and dividing neurons (BrdU⁺/DCX⁺). No difference was observed between WT sedentary mice (n = 6) and WT running mice (n = 6) for any of these cell markers. However, a significant difference was found between WT running mice and α1A-AR KO running mice in both total population of cells marked (Fig. 41A) and dividing cells (Fig. 41C). It was concluded that mice lacking the α1A-AR do not have the significant increases in neurogenesis that typically accompany aerobic exercise.

Figure 41. Running mice without the α1A-AR have a lower total cell population and significantly less neurogenesis. (A) There was a significant difference in total population of cells between the running WT mice (☐, n = 6) and both sedentary (n = 6) and running α1A-AR KO mice (■, n = 6) groups. (B) There was no difference between any of the groups in the number of immature neurons (DCX⁺). (C) There were significantly fewer cells in the running α1A-AR KO group when compared to the running WT group. Error bars represent SEM; ** p < 0.01. AR, adrenergic receptor; Ara-C, cytosine arabinoside; KO, knock-out; SEM, standard error of the mean; WT, wild type.
9.1 Neurogenesis, the α₁A-AR, synaptic plasticity and cognition

9.1.1 Cognition and synaptic plasticity

In this study, we used two transgenic α₁A-AR mouse models, the α₁A-AR selective agonist, CRZ, and Barnes maze. We found that chronic α₁A-AR stimulation improved learning and memory (Figs. 17-20) as well as synaptic plasticity in mice. In contrast, knocking out the α₁A-AR had a negative effect on learning and memory. Specifically, we found that chronic α₁A-AR stimulation improves cognitive performance in the Barnes maze (Fig. 17). To assess whether the cognitive behavioral gains seen in CAM α₁A-AR mice correlated with enhanced hippocampal plasticity, we looked at several different indicators of synaptic plasticity. We found enhanced basal synaptic transmission, PPF (short-term synaptic plasticity), and LTP in hippocampal slices prepared from aged CAM α₁A-AR mice when compared to WT mice (Fig. 18). Then, we treated normal WT mice with CRZ to determine whether we could pharmacologically mimic the effects observed in the CAM α₁A-AR mice. Indeed, CRZ-treated mice had improved cognitive function compared with normal mice (Fig. 19). Finally, we next examined the effects of blocking α₁A-ARs on cognitive function using a transgenic α₁A-AR KO model. We found that cognitive performance in the
Barnes maze was reduced in α1A-AR KO mice (Fig. 20). Taken together, these results indicate that the α1A-AR plays an essential role in learning and memory – though it remains to be seen how the α1A-AR is mediating this effect.

Synaptic plasticity is widely held as an essential component of learning and memory. The hippocampus is a critical structure with respect to learning, memory, and synaptic plasticity. Furthermore, it is well established that young neurons near the proliferative zone in the DG have a lower threshold for LTP than mature neurons (Ge et al., 2007; Schmidt-Hieber et al., 2004) and that reduction of DG stem cell proliferation selectively inhibits LTP (Snyder et al., 2001), suggesting a relationship between the birth of new neurons and LTP. Within the adult hippocampus, synaptic plasticity occurs primarily in two areas: the perforant path and the Schaffer collaterals. Both areas show susceptibility to age-related declines in LTP (Burke & Barnes, 2006; Diana, Domenici, Loizzo, Scotti de Carolis, & Sagratella, 1994; Froc et al., 2003; Landfield & Lynch, 1977). Conversely, LTD occurs more readily in aged mice, suggestive of an age-related increase in the susceptibility to depression in synaptic strength (Landfield & Lynch, 1977). Moreover, hippocampal CA1 pyramidal neurons show age-related deficits in PPF (Landfield & Lynch, 1977), a form of short-term plasticity related to the amplitude of synaptic responses. Each of these alterations in synaptic plasticity (LTP, LTD, PPF) correlates to age-related deficits in cognitive performance in the murine brain (Bach et al., 1999; Foster, 1999). Alzheimer’s disease (AD) mouse models also show similar reductions to aged mice in long-term potentiation (Bach et al., 1999). The AD phenotype is not expressed until
later in life, coinciding with considerable neuronal death. Conversely, the present study shows that aged CAM $\alpha_{1A}$-AR mice possess markedly improved basal synaptic transmission, PPF, and LTP at the CA3-CA1 synapses (Fig. 18A-C). Taken together, these results may suggest a role for the $\alpha_{1A}$-AR in improving synaptic efficiency, possibly throughout senescence.

The cognitive enhancements of CAM $\alpha_{1A}$-AR mice may be due to $\alpha_{1A}$-AR activation increasing neurogenesis (Gupta et al., 2009) or the survival of neurons. An important kinase in the $\alpha_{1A}$-AR second messenger pathway is ERK (Fig. 5; Liu et al., 2011). ERK has neuroprotective effects in the brain (Emery et al., 2010) and may be involved in regulating hippocampal neurogenesis (Aberg et al., 2003). One study linked ERK to cognition and found that reducing ERK levels in hippocampal neurons led to memory deficits (Yan, Hou, Wu, Liu, & Zhou, 2007).

In summary, using a constitutively activate mutant $\alpha_{1A}$-AR mouse model and long-term administration of an $\alpha_{1A}$-AR agonist in normal mice, we demonstrated that stimulating the $\alpha_{1A}$-AR subtype enhances learning and memory, and synaptic plasticity in aged CAM $\alpha_{1A}$-AR mice. Therefore, $\alpha_{1A}$-AR agonists may offer a potential new strategy for treating the decline in cognition associated with aging and many neurological disorders.
9.1.2 Neurogenesis, the $\alpha_{1A}$-AR and cognition

We hypothesized that $\alpha_{1A}$-AR induced neurogenesis underlies the enhanced learning, memory and synaptic function we previously observed (Doze et al., 2011). To test this hypothesis, we activated the $\alpha_{1A}$-AR with CRZ and simultaneously inhibited neurogenesis with the anti-mitotic agent, Ara-C. Then, we measured the cognitive function of these mice with behavioral tests. We found that all treatment groups remembered the location of the platform by day 2 of Morris water maze (MWM), and mice receiving CRZ (despite Ara-C treatment or surgery) learned as quickly as mice that did not have Ara-C treatment or surgery. Finally, mice treated with both CRZ and Ara-C performed the same as vehicle and no surgery mice, and significantly better than mice treated with Ara-C alone. Additionally, we found that increasing concentrations of Ara-C killed hippocampal neurons and a lower concentration of Ara-C effectively inhibited hippocampal neurogenesis and depleted immature neurons (DCX$^+$) and dividing neurons (BrdU$^+$/DCX$^+$) populations. Ara-C treatment did not negatively affect motor movement and there was no difference between treatment groups in novel object recognition. These data support the hypothesis that activating the $\alpha_{1A}$-AR enhances learning and memory—in the absence of hippocampal neurogenesis—protects against brain injury (induced by surgery). This is evident in our Morris water maze test results (comparing the CRZ-vehicle mice, Fig. 7A$_2$, to the untreated vehicle mice, Fig. 7B$_2$) which are suggestive of a neuroprotective action of $\alpha_{1A}$-AR activation. We think this novel observation may clarify the benefits of activating the $\alpha_{1A}$-AR to brain function.
Several studies have found that hippocampal neurogenesis is crucial component of learning (Dupret et al., 2008). Moreover, others have shown that increasing hippocampal neurogenesis benefited cognition (Wong-Goodrich et al., 2010), and decreasing or eliminating hippocampal neurogenesis was detrimental to cognitive function (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Our results support and extend these findings as mice treated with Ara-C alone performed worse in the probe trial of the MWM than mice that received vehicle or CRZ (Fig. 7C). Therefore, the presence of intact hippocampal neurogenesis in the brain is important for learning and memory. However, there are several regions in the brain where neurogenesis occurs (albeit at lower rates) that may be involved in learning and memory. Because the anti-mitotic agent we chose to inhibit neurogenesis (Ara-C) was delivered into the lateral ventricles, it was able to act on all dividing cells in the brain including other important, neurogenic learning and memory areas such as the neocortex, the striatum, and the amygdala (Gould, 2007). We previously showed that chronically activating the $\alpha_{1A}$-AR increases hippocampal neurogenesis (Gupta et al., 2009). The neocortex, striatum, and amygdala, in addition to the hippocampus, also have a high expression of $\alpha_{1A}$-AR (Papay et al., 2006). Thus, neurogenesis may have also been enhanced in these regions through the activation of the $\alpha_{1A}$-AR, which may also explain the cognitive enhancements seen in our previous study. We tested the hypothesis that $\alpha_{1A}$-AR induced neurogenesis underlies the enhanced learning, memory and synaptic function. However, we found that learning and memory was still improved in CRZ-Ara-C mice, suggesting that another
mechanism is responsible for enhanced learning and memory and synaptic function we previously observed. Therefore, it is not the neurogenesis aspect but the $\alpha_{1A}$-AR activation that we will focus on here.

To explain the mechanism behind the $\alpha_{1A}$-AR behavioral enhancements, and in the absence of neurogenesis, observed in this study, we hypothesize that NE through the actions of the $\alpha_{1A}$-AR, modulates the intrinsic properties of postsynaptic neurons in the CA1, thus making these neurons more responsive to potentiation. Subsequently, appropriate input such as theta-burst stimulation or the actual learning-process induces synaptic plasticity more easily and learning and memory improvements are the result.

We previously showed LTP was enhanced in mice with chronically activated $\alpha_{1A}$-ARs (Doze et al., 2011). Others have shown that activating the $\alpha_{1}$-AR enhances LTP (Izumi & Zorumski, 1999). However, the mechanism underlying this connection is not clear. $\alpha_{1A}$-AR activation is coupled to the $G_q$ protein, which increases IP$_3$ and [Ca$^{2+}$]$_i$ levels (Hwa et al., 1996). Further, blocking the IP$_3$ receptor inhibits LTP in hippocampal CA1 neurons (Fujii, Matsumoto, Igarashi, Kato, & Mikoshiba, 2000), and in the presence of a Ca$^{2+}$ chelator like EGTA, LTP is blocked (Lynch et al., 1983). Other studies echo that increasing postsynaptic Ca$^{2+}$ concentrations is an important step for induction of LTP (Rose & Konnerth, 2001), and required for mossy fiber-CA3 LTP through Ca$^{2+}$ from internal stores (Kwon & Castillo, 2008). Gibbs and Bowser (2010) went one step further and showed that increases in free cytosolic Ca$^{2+}$ from internal stores enhances learning and memory. However, there may be other
important receptors that may be affected by the changes in cellular activity after activation of the \( \alpha_{1A}-\text{AR} \), include the glutamate activated N-methyl-D-aspartate receptor (NMDAR) and the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR).

Hippocampal-dependent memory is reliant on the activation of the NMDAR (Gilbert & Mack, 1990; Morris, 1989). The behavioral effects of blocking NMDARs mimic that of a damaged hippocampus in rats (Robinson Jr., Crooks Jr., Shinkman, & Gallagher, 1989). Theta-burst stimulation (TBS) is a common technique that induces hippocampal CA1-LTP based on the electrical signals that occur during the actual learning process in the rat (Otto et al., 1991). This particular type of LTP is prevented when NMDARs are blocked (Bashir et al., 1990; Coan, Saywood, & Collingridge, 1987; Collingridge, Kehl, & McLennan, 1983). Furthermore, LTP can be blocked if depolarization of the post-synaptic cell is limited (Kelso, Ganong, & Brown, 1986; Malenka, 1991). These studies reveal that the temporal component of synaptic plasticity (whether post-tetanic, short-term or long-term) is dependent on the level of depolarization mediated by internal calcium [Ca\(^{2+}\)] levels.

AMPARs are thought to be a key component to the induction and maintenance of LTP (Malinow & Malenka, 2002). In the hippocampus, specifically, post-synaptic AMPARs increase after LTP is induced (Maren, Tocco, Standley, Baudry, & Thompson, 1993). Additionally, the exocytosis of AMPARs to the synapse is critical in the induction of LTP as when exocytosis is blocked, LTP induction does not occur (Lledo, Zhang, Südhof, Malenka, & Nicoll, 1998; Lu
et al., 2001). This exocytosis occurring in the post-synaptic dendrites of hippocampal neurons is also thought to be dependent on \([\text{Ca}^{2+}]_i\) levels (Maletic-Savatic et al., 1998). The AMPAR is composed of four common subunits (GluR1-4) and in the hippocampus, the GluR1, 2 and 3 are most prevalent in GluR1/2 or GluR2/3 complexes (Wenthold, Petralia, Blahos J, & Niedzielski, 1996). And, after high frequency stimulation such as TBS, GluR1 subunits rapidly move to the synapse and spines of dendrites, and was NMDAR-dependent (Shi et al., 1999). Finally, knocking out the GluR1 subunit inhibits the induction of LTP (Mack et al., 2001; Zamanillo et al., 1999). The \(\alpha_{1A}\)-AR increases \([\text{Ca}^{2+}]_i\) levels, important for depolarization of the post-synaptic cell and dendritic exocytosis, and also activates phospholipase D (PLD; Parmentier et al., 2004), known to play an important role in cellular exocytosis and endocytosis (Foster & Xu, 2003). These studies support our hypothesis that NE through the actions of the \(\alpha_{1A}\)-AR, modulates the intrinsic properties of post-synaptic neurons in the CA1, thus making these neurons more responsive to potentiation. However, this does not explain how activating the \(\alpha_{1A}\)-AR could be neuroprotective.

As previously mentioned, CRZ is not a specific activator of just one receptor. It fully activates the \(\alpha_{1A}\)-AR, partially-activates the \(\alpha_{1B}\)- and \(\alpha_{1D}\)-AR, and blocks \(\alpha_2\)-ARs (Horie et al., 1995). What effects do these other receptors have on learning and memory? Previous studies show that stimulating the \(\alpha_{1A}\)-AR specifically enhances cognition and neurogenesis (Doze et al., 2011; Gupta et al., 2009), while chronic \(\alpha_{1B}\)-AR activation causes a neurodegenerative response.
(Yun et al., 2003; Zuscik et al., 2000). Blocking $\alpha_2$-ARs, however, may also play a role in enhanced cognition. One study showed that blocking $\alpha_2$-ARs promoted memory consolidation while activating it promoted memory loss (Gibbs, Hutchinson, & Summers, 2010). But another using Morris water maze, showed that younger rats showed no deficits in cognition when blocking $\alpha_2$-ARs, but in older rats it did (Sirviö et al., 1992). It seems that $\alpha_2$-ARs may be involved in learning and memory functions, but how $\alpha_2$-ARs have a role in cognition remains unclear.

In other areas of the brain such as the prefrontal cortex, $\alpha_2$-ARs and $\alpha_1$-ARs appear to play a role in cognition. When $\alpha_1$-ARs are activated during attention-switching tasks, performance is improved (Lapiz & Morilak, 2006), but impairs short term spatial working memory (Arnsten et al., 1999; Birnbaum, Gobeske, Auerbach, Taylor, & Arnsten, 1999). One study used CRZ and tested spatial working memory in monkeys but found that CRZ impaired memory, and speculated that activating $\alpha_1$-ARs impairs spatial working memory while activating $\alpha_2$-ARs improves memory (Arnsten & Jentsch, 1997; Mao et al., 1999). These findings support the hypothesis that activating $\alpha_{1\alpha}$-AR in the PFC is not responsible for the learning and memory improvements we saw in our mice.

The amygdala is another area of the brain shown to be involved in fear-associated memory tasks. One study showed that when $\beta$-ARs (not $\alpha_1$-ARs) were activated by NE, learning and memory in the basolateral amygdala were enhanced over untreated controls. But another study showed that blocking $\alpha_1$-ARs in the lateral amygdala enhances fear-conditioning and LTP (Lazzaro, Hou,
Cunha, LeDoux, & Cain, 2010). And a study done by Ferry, Roozendaal, & McGaugh (1999) showed that activating $\alpha_1$-ARs enhances memory formation even while in the presence of an $\alpha_2$-AR blocker. Additionally, this study showed that $\alpha_1$-ARs need to interact with $\beta$-ARs in order to see the beneficial effects to memory storage. The interaction of these receptors and the effect on cognition is a subject that needs further exploration.

Another consideration is the type of cells that may be mediating the responses we observed. Both neurons and astrocytes express $\alpha_1$- and $\alpha_2$-ARs. We have only considered neurons because of the increased neurogenesis we saw with this response, but gliogenesis has also been shown to be regulated by the $\alpha_{1\mathrm{A}}$-AR (Gupta et al., 2009). Memory consolidation was enhanced when activating $\alpha_1$-ARs on astrocytes, specifically (Gibbs & Bowser, 2010). Several studies have linked the $\mathrm{Ca}^{2+}$ levels in astrocytes to ARs and to $\mathrm{Ca}^{2+}$ levels in neurons. In hippocampal astrocytes from rats, activating $\alpha_1$-ARs inhibited the spread of $\mathrm{Ca}^{2+}$ waves, whereas activation of $\beta$-ARs or $\alpha_2$-ARs did not alter the waves (Muyderman et al., 1998). But another study found that activating $\alpha_1$-ARs increases astrocyte internal $\mathrm{Ca}^{2+}$ concentrations within an intact rat hippocampal slice, but only 5% of astrocytes responded when isolated from the slice (Duffy & MacVicar, 1995). Another study showed that when $\alpha_2$-ARs are blocked on the astrocyte, $\mathrm{Ca}^{2+}$ levels increased and the astrocytes are potentiated (Bekar, He, & Nedergaard, 2008). This observation is supported by a few other studies showing that astrocytes and neurons directly modulate $\mathrm{Ca}^{2+}$ levels of neurons, either through gap junctions (Nedergaard, 1994) or through the $\mathrm{Ca}^{2+}$-dependent
release of glutamate (Parpura et al., 1994). All of these studies support the idea that astrocytes may be just as important for information processes as neurons, and clearly modulate and influence neuronal activity.

Finally, we discovered that activating the $\alpha_{1A}$-AR with CRZ protected the brain from surgical injury. While CRZ is a potent $\alpha_1$-AR activator (a full agonist at the $\alpha_{1A}$-AR and only partial agonist at the $\alpha_{1B}$-AR), it is also an $\alpha_2$-AR blocker (Ruffolo & Waddell, 1982). It has been shown that microglia express $\alpha_{2A}$- and $\beta_2$-ARs, and NE acts on the $\alpha_{2A}$-AR on activated microglia and suppresses microglial reactivity and motility (Gyoneva & Traynelis, 2013). And the loss of NE that occurs during neurodegeneration (Mann, Lincoln, Yates, Stamp, & Toper, 1980) would disinhibit microglia, allowing them to react and be more motile in the event of injury. Therefore, the presence of CRZ blocking the $\alpha_{2A}$-AR would have a similar effect. We think that it is the action of CRZ through the $\alpha_2$-AR on microglia that may be mediating the neuroprotective effects in the present study. As a result, this study is the first to discover the behavioral and neuroprotective effects of CRZ.

Several additional confirmatory studies could be conducted to further support and extend our initial findings. First, specific memory tests that target the PFC, amygdala or hippocampus need to be used in conjunction with cell-specific ablation of neurons or astrocytes. Activation and inhibition of the $\alpha_1$-AR should be tested in this paradigm, along with activation and inhibition of the $\alpha_2$-AR. Investigation of the role of the $\alpha_1$- and $\alpha_2$-AR in brain injury and the role these receptors play in microglia is necessary to help us understand the
neuroprotective effects of CRZ treatment. Mechanistically, Ca$^{2+}$ levels and NMDAR function need to be examined while acutely and chronically activating the $\alpha_{1A}$-AR in vivo and in vitro. Additionally, AMPA receptor trafficking, number and subunit composition could also be investigated under acute and chronic $\alpha_{1A}$-AR activation. Results from these experiments would further elucidate the extent of the involvement of the $\alpha_{1A}$-AR in synaptic plasticity.

The results of our study showed that activating the $\alpha_{1A}$-AR protects mice from the negative effects of inhibiting neurogenesis on learning and memory, as well as from brain injury. The anti-mitotic agent, Ara-C, also widely known as cytarabine, is a common treatment for certain types of cancer, such as leukemia (Ogbomo, Michaelis, Klassert, Doerr, & Cinatl, 2008; Wang et al., 1997). Up to 50% of patients treated with chemotherapy suffer from post-chemotherapy cognitive impairment, also known as chemo-brain or chemo-fog (Tannock, Ahles, Ganz, & Van Dam, 2004). This results in cognitive impairments such as trouble learning and remembering. Therefore, activating the $\alpha_{1A}$-AR prior to and during treatment may help to protect patients from chemo-brain. Furthermore, blocking the $\alpha_{2}$-AR may also help those who have suffered from traumatic brain injuries. The results of our study may help elucidate the mechanism behind learning and memory and help those with learning and memory disorders and other neurodegenerative diseases affecting cognition.
9.2 Hypothalamic neurogenesis and metabolism

Our results suggest that disrupting cellular proliferation in the hypothalamus induces obesity in mice. This finding is supported by data showing that Ara-C-treated mice had significantly (1) increased weight and increased food intake (Fig. 34), (2) increased fat deposits (Fig. 35), and (3) fewer dividing cells (BrdU) and immature neurons (DCX) in the hypothalamus (Fig. 36). In addition, neuronal activation was not isolated to or increased in a specific region of the hypothalamus in either Ara-C-treated or vehicle-control mice. These findings suggest that cellular proliferation in the adult hypothalamus plays an important role in weight maintenance.

We found Ara-C-treated mice showed significantly fewer dividing cells and immature neurons in the hypothalamus. Since neurogenesis in the hypothalamus leads to the production of neurons important to appetite and satiety in the brain, we deduced that it is the disruption of neurogenesis specifically that plays an important role in weight maintenance. This result is consistent with other studies using Ara-C to disrupt neurogenesis in the hypothalamus (Kokoeva et al., 2005; Pierce & Xu, 2010). Interestingly, obesity caused by diet or a leptin-deficiency disrupts hypothalamic neurogenesis and depletes neuronal stem cell populations (McNay, Briançon, Kokoeva, Maratos-Flier, & Flier, 2012). Disrupting cellular proliferation (i.e., neurogenesis) in our mice resulted in obesity; in contrast, inducing obesity in mice resulted in disrupted neurogenesis. Together, these results further support the idea that neurogenesis, specifically, and weight gain are mechanistically connected.
Several possible mechanisms exist for hypothalamic neurogenesis-related weight disturbances. To explore these mechanisms, it is necessary to compare our results with a key study by Pierce and Xu (2010). This study showed AgRP neuron-deficient mice had significantly reduced body fat mass compared to control mice when treated with Ara-C for 4 wks—suggesting that newly generated cells in the hypothalamus are required for maintaining normal appetite and body fat. It is possible that a large percent of proliferating and newborn neurons are orexigenic AgRP neurons versus anorexigenic POMC neurons. AgRP neurons release GABA onto POMC neurons (Cowley et al., 2001). Thus, knocking out the immature and proliferating AgRP neurons (which normally inhibit POMC neurons) would allow POMC neurons to fire, suppress appetite, and reduce body fat. But because Ara-C is non-specific and affects all dividing cells in the brain—though disrupting hypothalamic neurogenesis is more likely the reason for weight disturbances—disrupting dividing cells in other neurogenic brain regions (e.g., amygdala or substantia nigra, Bernier et al., 2002; Zhao et al., 2003), may have contributed to the weight phenomena observed.

Our results were similar to the Pierce and Xu study results (2010): newly generated cells are required for maintenance of normal body fat. Our study—which used 20 times more Ara-C (2%) than Pierce and Xu’s study (0.1%)—found the opposite effect using Ara-C. Whereas chronic Ara-C treatment depleted proliferating, early-, and late-stage immature neuron populations in the hypothalamus, our acute and high-concentration of Ara-C-treatment targeted only proliferating cells and early-stage immature neurons. It is possible that
immature hypothalamic neurons are strongly orexigenic, producing excessive quantities of AgRP (relative to mature and proliferating neurons) and this excess AgRP causes obesity in mice (Ollmann et al., 1997) – similar to our study. Alternatively, some newborn neurons are leptin responsive (Pierce & Xu, 2010), and if there are no proliferating or newborn neurons in the hypothalamus to receive leptin, then there is nothing to suppress appetite; weight gain results. One study suggests leptin acts on receptors primarily at the stem cell level yet does not regulate neuronal cell division or the fate of newborn neurons (McNay et al., 2012). Conversely, a different study shows that leptin influences hypothalamic feeding circuits in newborn mice and may have long-lasting effects on metabolism in adults (Bouret & Simerly, 2006). Finally, another study shows that newborn hypothalamic cells are adaptive and express different proteins and receptors depending on their environment (Pierce & Xu, 2010). This adaptive hypothesis may explain why such contrasting results are observed across hypothalamic neurogenesis and weight studies.

The adaptive hypothesis of newborn hypothalamic neurons may parallel with hippocampal newborn neurons. Immature hippocampal neurons play a significantly different role in pattern separation (an important process in creating memories) compared with mature, integrated neurons (Aimone, Deng, & Gage, 2011). Even the strategic addition and removal of specific new hippocampal neurons influences spatial learning (Dupret et al., 2007). Furthermore, critical periods (windows in development when the neuron is especially sensitive to outside input) of immature neurons in the hippocampus determine the survival,
population response, and types of neurons in the DG once neurons mature (Tashiro, Makino, & Gage, 2007). Therefore, hypothalamic neurons may have a critical period of development similar to hippocampal neurons; depending on environmental influences, hypothalamic neurons may contribute to opposite energy balance outcomes (obesity versus anorexia) when in proliferating, immature, and mature stages of neurogenesis.

To understand more about the underlying mechanisms in our study, we investigated the neuronal activity marker c-Fos, in the ventromedial nucleus – just one of the important hypothalamic nuclei involved in the satiety circuit of the hypothalamus. We found no difference in c-Fos+ cells in the VMH of either Ara-C-treated or vehicle-control mice. Our results were comparable to another study investigating c-Fos activity in diet-induced obesity mice and found c-Fos activity was also unchanged in the VMH (Lin & Huang, 1999). The varying levels of neuronal activity (c-Fos) may be explained by a contrast in techniques: we used Ara-C to broadly deplete hypothalamic neurons whereas others used genetic methods to specifically target different types of hypothalamic neurons (i.e., AgRP vs. POMC).

Neurogenesis in the hypothalamus is more difficult to detect than in strong neurogenic regions (e.g., the SGZ in the DG of the hippocampus) because hypothalamic neuronal stem cells tend to divide more slowly (Lee & Blackshaw, 2012). Exposure to BrdU, the marker for dividing cells, is higher in regions like the hippocampus because it is surrounded by cerebrospinal fluid (Bennett, Yang, Enikolopov, & Iacovitti, 2009). The hypothalamus, however, only has a small
region exposed to cerebrospinal fluid – the arcuate nucleus and the median eminence, where neurogenesis is strongly detected. Because this investigation of the hypothalamus was only after we observed weight gain in our mice, we did not design the study with hypothalamic neurogenesis in mind. Therefore, we gave a single injection of BrdU to our mice and this may be the reason for our inability to detect dividing neurons (double labeled with both BrdU and DCX). It should be noted that neurogenesis can be reliably detected by using DCX alone (Brown et al., 2003), as there is a high expression of DCX with BrdU only 3-4 days after BrdU injection. However, because hypothalamic neurogenesis is still a controversial neurogenic region, BrdU administration may be advisable – and multiple injections or central administration of BrdU would address the problem of low incorporation rate due to slower proliferation in this area.

In the future, it will be important to investigate levels of hormones such as leptin and ghrelin, and cellular markers, such as pSTAT3 (part of the leptin signaling pathway), AgRP and POMC. We agree with the authors of a recent review on hypothalamic neurogenesis and feeding regulation (Sousa-Ferreira, de Almeida, & Cavadas, 2013), and feel fate mapping of developing hypothalamic neurons (from birth to maturation) in the various regions of the hypothalamus (arcuate, ventral medial, dorsomedial nuclei, and the median eminence) is an essential experiment to perform. Further, fate-mapping these neurons while tracking food intake and weight changes may elucidate how each phase of hypothalamic neurogenesis is affecting energy balance and weight maintenance.
Our findings show that $\alpha_{1A}$-AR activation had no effect on weight or food intake, but acutely disrupting cellular proliferation (i.e. neurogenesis) in the hypothalamus induced obesity in mice. These findings contribute to our knowledge of the hypothalamic neurons and their role in weight, metabolism and energy balance. Continued research in this area is important since determining how and when these neurons influence weight and metabolism may lead to new therapeutic strategies for obesity and other weight-related disorders.

### 9.3 Exercise-induced neurogenesis and the $\alpha_{1A}$-adrenergic receptor

We hypothesized that exercise-induced neurogenesis is mediated by the $\alpha_{1A}$-adrenergic receptor. To test this hypothesis, we used $\alpha_{1A}$-AR KO mice along with their normal WT counterparts, and split each strain into two groups: sedentary or running. Then mice were allowed to run freely for 3 weeks. Food consumed and miles run were tracked throughout the duration of the experiment. Mice were also tested using the zero maze after 3 weeks of running to determine whether exercise decreased anxiety, as is commonly found in exercising mice (Duman & Schlesinger, 2008; Salam et al., 2009). Finally, brains were analyzed for levels of neurogenesis in the DG of the hippocampus (using BrdU and DCX as markers). We found that: 1) sedentary WT mice ate less than their running counterparts – but there was no difference among $\alpha_{1A}$-AR KO mice groups (Fig. 39); 2) Running $\alpha_{1A}$-AR KO mice had increased anxiety compared to running WT mice during the last 5 min of zero maze (Fig. 40); 3) there was not an increase in neurogenesis between running WT and sedentary WT mice, but there was a significant decrease found between running WT and running $\alpha_{1A}$-AR KO mice.
Though this was a pilot study, these results indicate there may be a connection between the $\alpha_{1A}$-AR and the anxiolytic effects of exercise, as well as between the $\alpha_{1A}$-AR and exercise-induced neurogenesis.

The first result from this study is perplexing: running WT mice ate more than sedentary WT mice but there was no difference among the $\alpha_{1A}$-AR KO groups. Of course, it is obvious in the WT mice that running mice burn more calories and therefore consume more calories – explaining the difference found. However, this difference was not found among the $\alpha_{1A}$-AR KO mice. This may be explained simply, in that perhaps $\alpha_{1A}$-AR KO mice ran less than the WT mice. Even though this was tracked and no difference was found between WT and $\alpha_{1A}$-AR KO running groups, it is possible that there was a slight enough difference to affect food intake. Another explanation may be that the absence of the $\alpha_{1A}$-AR may affect appetite or metabolism. Though activating the $\alpha_{1A}$-AR usually suppresses appetite (Davies & Wellman, 1992; Morien, McMahon, & Wellman, 1993), perhaps the absence of the $\alpha_{1A}$-AR has the opposite effect on appetite.

Results from the zero maze showed that running $\alpha_{1A}$-AR KO mice were found to be more anxious during the last 5 min of the test when compared to running WT mice. Since we know that exercise increases NE in the brain (Brown & Huss, 1973; B. Brown et al., 1979) as well as NE metabolites (Dunn & Reigle, 1996), the adrenergic receptor system was being activated as the mice were running. It is possible then that the $\alpha_{1A}$-AR may mediate, at least in part, the anxiolytic effects of exercise, as $\alpha_{1A}$-AR KO mice became more anxious as the test progressed. Though one study found that the $\alpha_{1A}$-AR does not affect anxiety-
related behavior in mice, as measured with the elevated-plus maze (Doze et al., 2009). Another found that the chronic activation of the $\alpha_{1A}$-AR reduces obsessive compulsive-type anxiety (as measured with the marble burying test) and anxiety-like behavior (as measured in light-dark exploration) (Doze et al., 2011). Experimental evidence supports the activation of the $\alpha_{1A}$-AR as an anxiolytic and possibly as a mediator in the anxiolytic effects of exercise, though to what extent remains controversial.

A surprising result from this pilot study was that running WT mice did not have higher levels of neurogenesis than sedentary WT mice. The strain we used in this study was C57BL/6. We were aware that this strain shows the least amount of reaction to exercise-induced neurogenesis when compared to other strains (Clark et al., 2011), however we needed to match the background of our $\alpha_{1A}$-AR KO mice, which were constructed on the C57BL/6 strain. There was also no difference found between any groups when examining DCX$^+$-cells, but there was a difference found between the running WT group and the running $\alpha_{1A}$-AR KO group when we examined BrdU$^+$/DCX$^+$-double labeled cells. We found a significant decrease in double labeled cells in the running $\alpha_{1A}$-AR KO group. This suggests that running decreases neurogenesis when the $\alpha_{1A}$-AR is not present – implying that the $\alpha_{1A}$-AR is offsetting a separate pathway that decreases neurogenesis during exercise. This ambiguous result does not rule out the potential for the $\alpha_{1A}$-AR to mediate exercise-induced neurogenesis.

There are several ways to address the ambiguity of the results in this study. First, subsequent studies on this topic should have one mouse per wheel,
each in separate cages. This would make measuring total miles run for each mouse easier. Mice running significantly less or more than other mice should be excluded from the study. Mice should be tested in the open field test (motor movement) as well to ensure they are not physically impaired. The strain problem may be difficult to address as constructing transgenic mice is sometimes reliant on strain behavior during offspring rearing, one of the reasons the C57BL/6 background was chosen. However, it may be beneficial to investigate whether an $\alpha_{1A}$-AR KO transgenic mouse can be constructed on a strain that is more responsive to exercise-induced neurogenesis, such as the AKR/J strain (Clark et al., 2011). A final possibility would be to use osmotic pumps and cannulate mice to deliver an affective $\alpha_{1A}$-AR selective antagonist such as 5-methylurapidil (table 2; Horie et al., 1995) during exercise. This would also isolate the antagonism of the $\alpha_{1A}$-AR to the CNS, as a transgenic manipulation is global and affects the PNS as well. A pharmacological approach would also give more flexibility to the experiment – as one could block the $\alpha_{1A}$-AR during exercise, and then unblock the $\alpha_{1A}$-AR to determine if the expected response would return.

Discovering the mediating receptors in exercise-induced neurogenesis has important therapeutic implications. For instance, if we are able to pinpoint the receptor subtype mediating this response, such as the $\alpha_{1A}$-AR, we may be able to target this receptor and induce neurogenesis in physically impaired patients and patients with neurodegenerative disorders. Additionally, as mentioned in 9.1, activating the $\alpha_{1A}$-AR has neuroprotective affects during brain injury, rescues learning and memory deficits seen when blocking neurogenesis, and enhances
learning and memory in normal mice. Activation of the α1A-AR during chemotherapy may protect patients from chemo-brain, and may help them recover the lost brain cells during chemotherapy treatment by stimulating neurogenesis. Though there is much left to investigate and discover with the α1A-AR, the possibilities of a multitude of positive therapeutic interventions are endless.
APPENDIX
Abbreviations used in text

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>[Ca^{2+}]_i</td>
<td>Internal calcium concentration</td>
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<td>3V</td>
<td>Third ventricle</td>
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<tr>
<td>AAALAC</td>
<td>American Association for Accreditation of Laboratory Animal Care</td>
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<td>aCSF</td>
<td>Artificial cerebral spinal fluid</td>
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<td>AgRP</td>
<td>Agouti-related peptide</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANG</td>
<td>Adult neurogenesis</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Adrenergic receptor</td>
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<td>Cytosine arabinoside</td>
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<td>Arcuate nucleus</td>
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<td>5-bromo-2'-deoxyuridine</td>
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<td>CA</td>
<td>Cornu ammonis</td>
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<td>CaCl_2</td>
<td>Calcium chloride</td>
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<td>CAM</td>
<td>Constitutively activated mutant</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Carbon dioxide</td>
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<td>Cirazoline</td>
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<td>Coronal suture</td>
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<td>Diacylglycerol</td>
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<td>DCX</td>
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<td>DMN</td>
<td>Dorsal medial nucleus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>Effective concentration (50% maximal effect)</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>E_{max}</td>
<td>Maximum effect, potency</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory post-synaptic potential</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Field excitatory post-synaptic potential</td>
</tr>
<tr>
<td>FX</td>
<td>Fornix</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IUPHAR</td>
<td>International Union of Basic Clinical Pharmacology</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram (10^3 gram)</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
</tbody>
</table>

130
L  Lambda
LHA  Lateral hypothalamic area
LTD  Long-term depression
LTP  Long-term potentiation
MAPK  Mitogen activated protein kinase
ME  Median eminence
MgSO4  Magnesium sulfate
min  minute(s)
mL  milliliter (10^(-3) liter)
mm  millimeter (10^(-3) meter)
MWM  Morris water maze
n  number
NaCl  Sodium chloride
NaH2PO4  Sodium phosphate
NaHCO3  Sodium bicarbonate
NE  Norepinephrine
NeuN  Neuron-specific neuronal peptide
NeuroD  Neuron differentiation protein
NIH  National Institute of Health
NMDA  N-methyl-D-aspartate
NOR  Novel object recognition
NPY  Neuropeptide Y
NTS  Nucleus of the solitary tract
PFA  Paraformical area
PIP2  Phosphatidylinositol 4,5-bisphosphate
PKC  Protein kinase C
PLA2  Phospholipase A2
PLC  Phospholipase C
PNS  Peripheral nervous system
POMC  Proopiomelanocortin
PPF  Paired-pulse facilitation
PSA-NCAM  Polysialylated-neural cell adhesion molecule
PTP  Post-tetanic potentiation
PVN  Paraventricular nucleus
RNA  Ribonucleic acid
s  second
SEM  Standard error of the mean
SGZ  Subgranular zone
STP  Short-term potentiation
SVZ  Subventricular zone
TBS  Theta-burst stimulation
VMH  Ventral medial hypothalamus
WT  Wildtype
μA  micro amp
μg  microgram (10^-6 gram)
μm  micrometer (10^-6 meter)
μM micromolar (10⁻⁶ mole/liter)
REFERENCES


