Brain-Derived Neurotrophic Factor Signaling in the Amygdala: A Role in Anxiety and Alcoholism

BY

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B.S. (Biomedical Engineering), Jinan University, Guangzhou, China, 2004

THESIS

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Chicago, Illinois

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>1.1 Alcohol Addiction</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Historical Aspect of Alcoholism</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Definition and negative effect of alcohol abuse and alcoholism</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Alcoholism and Anxiety</td>
<td>7</td>
</tr>
<tr>
<td>1.2.1 Positive and Negative Affective States of Alcohol</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2 Neuroanatomy of Alcoholism with the focus on limbic system</td>
<td>10</td>
</tr>
<tr>
<td>1.2.3 Amygdala</td>
<td>11</td>
</tr>
<tr>
<td>1.3 Pharmacology of alcohol</td>
<td>17</td>
</tr>
<tr>
<td>1.4 BDNF signaling transduction pathway</td>
<td>23</td>
</tr>
<tr>
<td>1.4.1 BDNF</td>
<td>23</td>
</tr>
<tr>
<td>1.4.2 TrkB</td>
<td>27</td>
</tr>
<tr>
<td>1.4.3 Erk</td>
<td>29</td>
</tr>
<tr>
<td>1.4.4 BDNF-Arc signaling in synaptic plasticity, anxiety and alcoholism</td>
<td>30</td>
</tr>
<tr>
<td>1.5 Epigenetics</td>
<td>34</td>
</tr>
<tr>
<td>1.5.1 Overview of Epigenetics</td>
<td>34</td>
</tr>
<tr>
<td>1.5.2 Mechanisms of Epigenetics</td>
<td>36</td>
</tr>
<tr>
<td>1.5.2.1 DNA methylation</td>
<td>36</td>
</tr>
<tr>
<td>1.5.2.2 Histone modification</td>
<td>38</td>
</tr>
<tr>
<td>1.5.2.2.1 Histone Acetylation</td>
<td>39</td>
</tr>
<tr>
<td>1.5.2.2.2 Histone Methylation</td>
<td>44</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>PAGE</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.5.2.3 Alcoholism and chromatin remodeling</td>
<td>46</td>
</tr>
<tr>
<td>1.6 Animal Models for Anxiety and Alcoholism</td>
<td>49</td>
</tr>
<tr>
<td>1.6.1 HAD/LAD rats</td>
<td>49</td>
</tr>
<tr>
<td>1.6.2 Anxiety in HAD/LAD rats</td>
<td>52</td>
</tr>
<tr>
<td>1.7 Summary and Rational</td>
<td>54</td>
</tr>
<tr>
<td>1.8 Specific Aims</td>
<td>57</td>
</tr>
<tr>
<td>2. THE ROLE OF CENTRAL AMYGDALOID BDNF-ERK1/2 SIGNALING AND ARC EXPRESSION IN ANXIETY-LIKE AND ALCOHOL-DRINKING BEHAVIORS</td>
<td></td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>59</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>61</td>
</tr>
<tr>
<td>2.3 Materials and Methods</td>
<td>64</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>71</td>
</tr>
<tr>
<td>2.5. Discussion</td>
<td>86</td>
</tr>
<tr>
<td>3. THE EFFECT OF HDAC INHIBITOR TREATMENT ON AMYGDALOID BDNF AND ARC EXPRESSION, DENDRITIC SPINE DENSITY, AND ANXIETY-LIKE BEHAVIORS DURING ETHANOL WITHDRAWAL AFTER CHRONIC EXPOSURE</td>
<td></td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>91</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>93</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 Materials and Methods</td>
<td>96</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>102</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>111</td>
</tr>
</tbody>
</table>

## 4. INNATE DIFFERENCES IN ANXIETY LEVELS, AS WELL AS IN THE EXPRESSION OF BDNF AND CREB IN THE AMYGDALA OF HIGH ALCOHOL DRINKING (HAD) AND LOW ALCOHOL DRINKING (LAD) RATS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Abstract</td>
<td>116</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>117</td>
</tr>
<tr>
<td>4.3 Materials and Methods</td>
<td>121</td>
</tr>
<tr>
<td>4.4 Results</td>
<td>126</td>
</tr>
<tr>
<td>4.5 Discussion</td>
<td>134</td>
</tr>
</tbody>
</table>

## 5. CONCLUSIONS AND FUTURE DIRECTIONS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Conclusions</td>
<td>137</td>
</tr>
<tr>
<td>5.2 Future Directions</td>
<td>153</td>
</tr>
</tbody>
</table>

CITED LITERATURE

<table>
<thead>
<tr>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>157</td>
</tr>
</tbody>
</table>

VITA

<table>
<thead>
<tr>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>197</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Intra-CeA infusion of U0126 produced anxiety-like behavior while BDNF infusion produced anxiolytic effects in rats.</td>
<td>72</td>
</tr>
<tr>
<td>2. Effect of central amygdaloid BDNF/U0126 infusion into CeA on mRNA and protein levels of Arc and protein levels of pErk1/2 and pCREB in amygdaloid brain regions of rats</td>
<td>74</td>
</tr>
<tr>
<td>A, Representative photomicrographs</td>
<td>74</td>
</tr>
<tr>
<td>B, Bar diagram showing quantification of the data</td>
<td>75</td>
</tr>
<tr>
<td>3. Effect of intra-CeA infusion of Arc sense/antisense ODNs (with and without BDNF co-infusion) on open- and closed-arm activities of the EPM</td>
<td>77</td>
</tr>
<tr>
<td>4. Effect of intra CeA Arc sense/antisense ODNs infusion with or without BDNF co-infusion on the levels of Arc mRNA and protein.</td>
<td>79</td>
</tr>
<tr>
<td>A, Representative photomicrographs</td>
<td>79</td>
</tr>
<tr>
<td>B, Bar diagram showing quantification of the data</td>
<td>80</td>
</tr>
<tr>
<td>5. Effect of intra-CeA infusion of U0126 with or without BDNF co-infusions on alcohol preference as measured by the two-bottle free-choice paradigm.</td>
<td>83</td>
</tr>
<tr>
<td>A, Total fluid intake</td>
<td>83</td>
</tr>
<tr>
<td>B, Percentage of 7% v/v ethanol intake</td>
<td>83</td>
</tr>
<tr>
<td>C, percentage of water intake</td>
<td>83</td>
</tr>
<tr>
<td>6. Effect of intra-CeA infusion of U0126 on 2% sucrose preference as measured by the two-bottle free-choice paradigm</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Effect of TSA treatment in rats during ethanol withdrawal after chronic ethanol exposure on anxiety-like behaviors, as measured by elevated plus maze (EPM)</td>
<td>104</td>
</tr>
<tr>
<td>8. Effect of TSA treatment in rats during ethanol withdrawal after chronic ethanol exposure on anxiety-like behavior, as measured by light dark box (LDB)</td>
<td>105</td>
</tr>
</tbody>
</table>
| 9. Effect of TSA treatment on BDNF and Arc expression in amygdala during ethanol withdrawal after chronic ethanol exposure  
  A, Representative photomicrographs | 107 |
|  | B, Bar diagram showing quantification of the data | 108 |
| 10. Effect of TSA treatment on dendritic spine density measured by Golgi-Cox staining in the amygdala of rats during ethanol withdrawal after chronic exposure  
  A, Representative photomicrographs | 110 |
|  | B, Bar diagram showing quantification of the data | 110 |
| 11. Anxiety levels of HAD1/LAD1 and HAD2/LAD2 rats measured by Light-dark-box (LDB) test.  
  A, HAD1 rats display heightened anxiety level compared to LAD1 rats | 127 |
<p>|  | B, HAD2 rats display heightened anxiety level compared to LAD2 rats | 127 |</p>
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12. Differences in BDNF protein and mRNA levels in the amygdala of HAD/LAD rats.</td>
<td></td>
</tr>
<tr>
<td>A, Representative photomicrographs</td>
<td>129</td>
</tr>
<tr>
<td>B, Bar diagram showing quantification of the data</td>
<td>130</td>
</tr>
<tr>
<td>13. Innate differences in CREB protein levels and phosphorylated CREB protein levels in amygdaloid brain regions of HAD and LAD rats.</td>
<td></td>
</tr>
<tr>
<td>A, Representative photomicrographs</td>
<td>132</td>
</tr>
<tr>
<td>B, Bar diagram showing quantification of the data</td>
<td>133</td>
</tr>
<tr>
<td>14. Schematic summary of Model 1</td>
<td>138</td>
</tr>
<tr>
<td>15. Schematic summary of Model 2</td>
<td>140</td>
</tr>
<tr>
<td>16. Schematic summary of Model 3</td>
<td>142</td>
</tr>
<tr>
<td>17. Schematic summary of the role of BDNF signaling in anxiety and alcoholism</td>
<td>150</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+/+)</td>
<td>Wide type</td>
</tr>
<tr>
<td>(+/-)</td>
<td>Haplodeficient</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>AUD</td>
<td>Alcohol Use Disorder</td>
</tr>
<tr>
<td>B.C.</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Nucleus of Amygdala</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus of the Stria Terminalis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>C57</td>
<td>C57BL/6J Mouse</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>CaMKs</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; calmodulin-dependent protein kinases</td>
</tr>
<tr>
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<td>CREB-Binding Protein</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Nucleus of Amygdala</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>Cyclic-AMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP Responsive Element</td>
</tr>
<tr>
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<td>Cyclic AMP Responsive Element-Binding Protein</td>
</tr>
<tr>
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<td>Corticotrophin-Releasing Factor</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>DNMT</td>
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</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>EPM</td>
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</tr>
<tr>
<td>Erk 1/2</td>
<td>Extracellular Signal-Regulated Kinases 1/2</td>
</tr>
<tr>
<td>Fig(s)</td>
<td>Figure(s)</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>g</td>
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</tr>
<tr>
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</tr>
<tr>
<td>H1</td>
<td>Histone Linker Protein H1</td>
</tr>
<tr>
<td>H2A</td>
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</tr>
<tr>
<td>H2B</td>
<td>Histone Protein H2B</td>
</tr>
<tr>
<td>H3</td>
<td>Histone Protein H3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
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<td>Histone Protein H4</td>
</tr>
<tr>
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<td>High Alcohol Drinking rats</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MAPK</td>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>PNDs</td>
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</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PTB</td>
<td>Phosphotyrosine Binding</td>
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<tr>
<td>P-value</td>
<td>Probability Value</td>
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<tr>
<td>R</td>
<td>Arginine</td>
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<tr>
<td>Ras</td>
<td>Rat Sarcoma</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide Hydroxamic Acid</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SARE</td>
<td>Synaptic Activity-Responsive Element</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SH2</td>
<td>Src homology2</td>
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<tr>
<td>SHC</td>
<td>Src homology 2 domain-containing</td>
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<tr>
<td>T</td>
<td>Thymine</td>
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**LIST OF ABBREVIATIONS (continued)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin-related kinase receptor</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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<tr>
<td>w/v</td>
<td>Weight/Volume</td>
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<tr>
<td>XCI</td>
<td>X chromosome inactivation</td>
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SUMMARY

Alcohol addiction is a chronic psychiatric disease that negatively impacts a massive population spanning across the country, and the world, costing a tremendous loss in lives, productivity and resources. The development of better treatment strategies for the prevention and cure of alcoholism is needed. Ethanol withdrawal symptoms, especially anxiety, are the crucial factors promoting and maintaining alcohol consumption. The high co-morbidity of anxiety and alcoholism therefore emphasize the importance of research providing better understanding of the neurobiological basis of anxiety and alcoholism. Brain-derived neurotrophic factor (BDNF) has been shown to play an important role in the regulation of synaptic plasticity and has been implicated in anxiety-like and alcohol-drinking behaviors. However, the specific mechanism by which BDNF and related signaling regulates anxiety and alcohol drinking behaviors, is still not clear. In the current thesis, three animal models of anxiety were used to address this critical question. The anxiety-like behaviors in these animals were induced by 1) pharmacological manipulation of BDNF signaling, 2) withdrawal from chronic alcohol administration, and 3) genetic predisposition, respectively.

We found that when exogenous BDNF was infused into the central nucleus of amygdala (CeA) it produced an anxiolytic effect in rats. On the other hand, infusion of the Erk1/2 signaling inhibitor U0126 into the CeA produced anxiety-like behavior and increased alcohol preference in rats. The behavioral effects of BDNF were found to be reversible by U0126 co-infusion, indicating the
regulatory role of BDNF on anxiety-like and alcohol-drinking behavior may occur through the Erk1/2 signaling pathway. Molecular changes, including decreased Erk1/2 and cAMP response element-binding (CREB) phosphorylation and reduced Arc mRNA and protein expression, were also observed in the amygdala following U0126 infusion. BDNF infusion produced opposite effects. U0126 also attenuated BDNF induced molecular changes. These observations suggest that CREB and Arc might be associated with the BDNF-Erk1/2 signaling pathway and downstream regulation of anxiety-like and drinking behaviors. To further address the possible involvement of Arc in BDNF regulated anxiety, we infused Arc antisense oligodeoxynucleotides (ODNs) into the CeA. This treatment provoked similar anxiogenic effects as observed in U0126 infused rats and significantly reduced Arc mRNA and protein expression. On the contrary, BDNF CeA infusion alone increased Arc expression and decreased anxiety-like behaviors in rats. BDNF co-infusion with Arc antisense ODNs again normalized the anxiety levels, and Arc expression, implicating Arc as the mediator by which BDNF signaling regulates anxiety-like behaviors. The data we obtained from the first animal model of anxiety collectively suggested a regulatory role of BDNF-Erk1/2–CREB signaling and Arc expression in anxiety and alcohol drinking.

As we observed in our second model of anxiety, animals undergoing withdrawal from chronic ethanol exposure displayed increased anxiety levels. These behavioral changes were accompanied by decreased BDNF and Arc expression in central (CeA), medial (MeA), but not basolateral (BLA) nuclei of
amygdala, confirming the involvement of BDNF and Arc in anxiety-like behaviors. Because of the close association between BDNF and Arc with synaptic plasticity, we measured the dendritic spine densities (DSD) in the amygdaloid brain regions in order to determine whether DSD modification is involved in the BDNF and Arc regulated behavioral changes. We indeed observed a decrease of DSD in the CeA, MeA, but not BLA, of ethanol-withdrawal rats. Interestingly, these deficits in BDNF and Arc expression, as well as DSD are reversible by treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), suggesting a crucial role of histone acetylation in transcriptional modification and regulation of BDNF and Arc genes, which, in turn regulate DSD and eventually anxiety-like behaviors.

We next employed the use of a well established animal model with high alcohol drinking behavior for further exploration of the involvement of BDNF signaling in genetic factor induced anxiety. The high alcohol-drinking and low alcohol-drinking (HAD/LAD) rats provide us with two replicates (HAD1/LAD1 and HAD2/LAD2) of paired strains, selectively bred from heterogeneous stock to have high and low alcohol-drinking behaviors. We used the light dark box (LDB) exploration paradigm to examine the anxiety-like behaviors in these rats and found that both replicates of HAD rats have innately higher anxiety levels than their counterpart LAD rats strains. Accompanied with heightened anxiety were decreases in BDNF and CREB expression, and phosphorylated CREB in CeA, MeA, but not BLA, of HAD rats compared with LAD rats. These data again
confirmed the hypothesis that deficits in BDNF signaling may be involved in anxiety-like and alcohol-drinking behaviors. In addition to the evidence from pharmacological manipulation and ethanol withdrawal-induced anxiety models, here the important role of BDNF signaling has been suggested in genetic basis of anxiety and alcoholism.

Collectively, the data from the current thesis indicates that the deficits in BDNF signaling may be involved in high anxiety levels and alcohol preference. These behavioral changes may be regulated by the BDNF downstream Erk1/2 signaling cascade and its target CREB, which, in turn, controls the expression of synaptic plasticity related genes including BDNF and Arc. Chromatin remodeling mechanisms, in particular, histone acetylation may contribute to the regulation of gene transcription during alcohol dependence because the anxiety-like behaviors and deficits in BDNF and Arc expression and in DSD were reversed by treatment of an HDAC inhibitor.
1. LITERATURE REVIEW

1.1 Alcohol Addiction

1.1.1 Historical Aspect of Alcoholism

The earliest evidence of the development of alcohol beverages came from China around the year 7000 BC (McGovern 2003). However, the use of alcohol was also documented in ancient history of many other countries including India (Peele and Grant 1999), Persia (World Health Organization. Dept. of Mental Health and Substance Abuse. 2004), Egypt (Bard and Shubert 1999), Greece, and Rome. Low to moderate alcohol use has protective properties on heart disease (Piano 2005; Lakshman et al. 2009), diabetes (Wannamethee et al. 2003; van de Wiel 2004), dementia (Ruitenberge et al. 2002), and can also increase cognition (Espeland et al. 2005); yet, detrimental effects of alcohol abuse were also recognized. Alcohol abstinence was promoted as a moral value in many religions. On the other hand, these attempts to prevent alcohol use and abuse were always unsuccessful as laws against making wine were enacted and repealed for many times in many countries even the United States (Cherrington 1925). Just as the famous Chinese commentator once wrote in the 650 B.C. that "To prohibit it (Alcohol) and secure total abstinence from it is beyond the power even of sages." people simply "will not do without beer".

Historically, alcoholism, and addictive behaviors related to other drugs of abuse was stigmatized as possessed by individuals with poor self-control, willful misconduct, or immorality. The use of alcohol was once banned by the 18th amendment of the United States until the year 1932. Since then the
understanding of alcoholism as an illness instead of “weak self control” was gradually recognized among clinicians and the public. Although documented descriptions of alcohol intoxication as a disease and addiction occurred as early as 1785 (Katcher 1993), and the term “alcoholism” was used to describe excessive alcohol consumption (Lader 1983), American Medical Association declared alcoholism as an illness in 1956, and alcoholism for the first time gained recognition in the medical field as a primary diagnosis with morbidity and mortality (Report of the Board of Trustees. JAMA 1956; 162:750.). The research on alcoholism was facilitated by the National Institute of Alcohol Abuse and Alcoholism since its inception in 1970.

1.1.2 Definition and negative effects of alcohol abuse and alcoholism.

According to the Diagnostic and Statistical Manual 4TH Edition (DSM-IV) of Mental Disorders published by APA (1994), alcohol use disorders (AUDs) are in parallel with other substance abuses. AUD are categorized into alcohol abuse and alcohol dependence. The diagnosis of alcohol abuse is based on the pattern of and the result of the alcohol use. Identification of one or more of the following criteria during past 12 month indicates an individual as an alcohol abuser clinically:

1. Repeated alcohol consumption that causes lost of working and social ability
2. Drinking despite awareness of negative impact on health
3. Drinking despite legal and social consequences
More severe than alcohol abuse, the American Psychiatric Association (APA) currently defines alcohol dependence, to be equated with alcoholism, as a drinking pattern that results in personal harm affecting one’s health, interpersonal relationships, or ability to work. According to the DSM IV, diagnostic criteria for alcoholism that take into account physical symptoms, as displayed no less than three of the following seven categories, occurring at any time in a 12-month period:

1. **Tolerance**, that an individual
   - Needs significantly increased amounts of alcohol to achieve intoxication or desired effect.
   - Has significantly reduced effect with continued use of the same amount of alcohol.

2. **Withdrawal symptoms**, that an individual
   - Has the characteristic withdrawal syndrome for alcohol.
   - Consumes alcohol (or a closely related substance) to relieve or avoid withdrawal symptoms.

3. The individual usually takes in larger amounts or over a longer period than was intended.

4. There is a persistent wish or unsuccessful attempts to cut down or control use of alcohol.

5. The individual spent a great deal of time in activities to obtain and consume alcohol or recover from its effects.

6. The individual give up or reduce important social, occupational or recreational activities due to alcohol use.
7. Continued used of alcohol despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by drinking.

Alcohol use can progresses to alcohol abuse or alcoholism. AUD has a 70-80% relapse rate by 12 months post-treatment (Bradizza et al. 2006). There is an estimated 76.3 million people globally with diagnosable AUDs (World Health Organization. Dept. of Mental Health and Substance Abuse. 2004), and over 17.6 million AUDs in the US alone (Grant et al. 2004). The United States alone bears an annual alcohol related cost of $184.6 billion according to the estimate by National Institute on Alcohol Abuse and Alcoholism (Rehm et al. 2009). Alcohol abuse-related loss of workplace productivity, traffic accidents, domestic violence and many other crimes add a substantial social and economical burden on society (Rehm et al. 2009). Responsible for about 85,000 deaths per year, alcohol abuse has become the third-leading avoidable death in the United States (Mokdad et al. 2004) and, worldwide, alcohol accounts for nearly 2.5 million annual death including 320,000 people ages between 15 and 29 according to the World Health Organization (World Health Organization. Management of Substance Abuse Team. 2011).

Alcoholism raises many health concerns including heart disease (Urbano-Marquez and Fernandez-Sola 2004; George and Figueredo 2010), malnutrition, Alzheimer’s disease (Marinho et al. 2006), stroke (Ohkubo et al. 2009), liver disease (Stewart et al. 2001; Mills and Harrison 2005; Cederbaum et al. 2009), cancer (Seitz and Becker 2007), chronic respiratory disease (Morris 1990),
diabetes (Baliunas et al. 2009), bone diseases (Chen et al. 2009), necrotic neurodegeneration (Obernier et al. 2002) and impaired neurogenesis and synaptic plasticity (Silvers et al. 2003) was also observed in alcoholism and is believed to be involved in cognitive dysfunction during alcoholism. Ethanol produces alteration in multiple neurotransmitter systems in the brain, including opiates, gamma-Aminobutyric acid (GABA), glutamate, serotonin, and dopamine (Diamond and Gordon 1997). Alcohol use disorder has been clinically indicated to be co-morbid with psychiatric disorders such as anxiety disorders (Morris et al. 2005; Bradizza et al. 2006; Vukovic et al. 2008), schizophrenia (Jones et al. 2011; Ribbe et al. 2011), panic disorders (Bystritsky et al. 2010), major depressive disorders (Grant and Harford 1995; Cornelius et al. 2003), post-traumatic stress disorder and bipolar disorder (Goldstein et al. 2006).

The unique metabolic characteristic of alcohol defines its ability to damage specific organs and tissues. The absorption of alcohol begins with 20% of the total alcohol oral administration directly diffused through the wall of stomach, and then 80% remaining alcohol absorbed through duodenum and small intestine (Norberg et al. 2003). The metabolism of alcohol occurs mainly in the liver, through oxidation by alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), cytochrome P4502E1 (CYP2E1) and catalase enzymes (Agarwal 2001; Zakhari 2006).

There are three Food and Drug Administration (FDA) approved medications for alcohol dependence: disulfiram, approved in 1949; naltrexone, oral form approved in 1994 and injectable form in 2006; and acamprosate,
approved in 2004 (Johnson et al. 2007). Disulfiram works by blocking the enzyme acetaldehyde dehydrogenase, preventing formation of acetic acid from acetaldehyde, which causes severely aversive and sometimes lethal effects in patients (Heilig and Egli 2006). Naltrexone uses a different mechanism than disulfiram, instead blocking the mu opioid receptors associated with the rewarding aspect of alcohol (Bouza et al. 2004). Unfortunately, naltrexone, however, not only blocks the reward produced by alcohol, but universally blocks all brain reward circuitry and only is effective in a select population of alcoholics (Davies et al. 2012). Acamprosate functions by decreasing glutamate activity thereby decreasing the negative effect of alcohol withdrawal. Unfortunately, although approved by the FDA, clinical studies in the U.S. have yet to provide evidence of efficacy (Davies et al. 2012). Given that alcohol produces tremendous social and interpersonal negative impacts on people’s life style, it is crucial to understand the neurobiological base of alcoholism to develop better treatment strategies to prevent and cure this brain disorder.
1.2 Alcoholism and Anxiety

1.2.1 Positive and Negative Affective States of Alcohol

Reinforcement is a process of consolidation of certain set of behaviors defined by prior experience (Gilpin and Koob 2008). Positive reinforcement is the state when reward is expected, based on prior experience, in these instances the chance of alcohol intake is increased. On the other hand, negative reinforcement occurs when the presumption is made that certain behaviors will lead to an decrease in the aversive state (Gilpin and Koob 2008). Addiction, as a psychiatric disorder, comprises both aspects of the impulse control disorder as well as the compulsive disorder (Koob 2003). The loss of impulse control attributes to the positive reinforcement, while the negative reinforcement drives the compulsive aspect of addiction. Anticipation of positive outcomes, including improved mood and a relaxed state, promote the positive reinforcement of alcohol intake; while the hope of reduction of negative states such anxiety, depression and pain, stimulates the negative reinforcing properties of alcohol drinking (Koob 2003; Grusser et al. 2006). Thus, both positive and negative states of addiction have great importance for the development of alcoholism, although the roles plays by these two states of addiction differs during the development and maintenance of alcoholism (Pandey 2004; Gilpin and Koob 2008). Clinical studies indicate that the impulse to drink for the pleasant feelings is known as enhancement motives (EM), while the need to drink to allay unpleasant feelings is known as coping motives (CM) (Cooper et al. 1995; Grant and Stewart 2007). EM drinkers self-reported higher frequencies of drinking behavior with associated positive
emotions (Carrigan et al. 1998), whereas CM drinkers described increased cognition during alcohol use (Birch et al. 2004). Researchers used music to induce positive or negative mood states on both EM and CM drinkers and found that EM drinkers respond to positive mood induction while CM drinkers respond to the negative mood induction (Birch et al. 2004). It is suggested that 25~30 percent of the alcoholics would drink primarily for one of the motives, either for the rewarding effects of alcohol or to suppress the aversive effects of alcohol withdrawal; while the many alcoholics would drink for both motives (Cooper et al. 1995; Lewis 1996; Koob 2003).

Positive reinforcement plays a critical role in the initiation of alcohol use (Gilpin and Koob 2008). The mesolimbic dopaminergic system, which is believed to regulate the reward aspect of behaviors such as food and sex, is involved in the positive affective state of alcoholism (Gonzales et al. 2004; Spanagel 2009; Moonat et al. 2010). This system involves the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC), as well as the extended amygdala and hippocampus (Gonzales et al. 2004; Spanagel 2009). The dopaminergic projection from the VTA to the NAc appears to be a key neuroanatomical pathway that may be involved in the association between reward experience and further reward seeking behaviors (Koob 2003; Pandey 2004; Wise 2004).

In the early stage of alcohol use, negative reinforcement mainly affects only individuals with preexisting affective disorders (Koob 2003; Gilpin and Koob 2008). During the later stages of alcoholism, after chronic alcohol exposure,
neuroadaptations including alcohol sensitization, tolerance, and withdrawal take place (Gilpin and Koob 2008). The positive state of addiction, at this stage, reflects the increased need of alcohol to elicit same euphoric effect. Conversely, the desire for relief from alcohol withdrawal symptoms triggers factors eliciting alcohol craving, thus lead to the negative affect state of alcohol use. Both positive and negative states of alcohol can trigger relapse in abstinent alcoholics (Connors et al. 1996), but negative affect of alcohol addiction is believed to be the key factor for the maintenance of alcoholism (Koob 2003). Gradually increasing alcohol dose could produce tolerance to the hedonic effects of alcohol, while more severe withdrawal symptom makes quitting drinking even harder, signaling the transition between alcohol use to alcohol abuse and dependence. As early as in the 1950, Dr. Williams noticed the comorbidity of anxiety-disorders in alcoholism. In the course of chronic alcoholism treatment, a few common symptoms were observed among which the anxiety symptoms were the most significant (Williams 1950). Since then, many clinical and preclinical studies reported close association between anxiety disorder and alcoholism (Bowen et al. 1984; Brady and Lydiard 1993; Kushner and Sher 1993; Merikangas et al. 1998; Kushner et al. 2001; Kushner et al. 2005). Anxiety-like emotional states are often present during alcohol withdrawal and are believed to contribute to alcohol relapse in alcoholics and promote drinking (Willinger et al. 2002). Anti-anxiety drugs were used in the attempt to either prevent or to treat the withdrawal syndromes in order to treat the alcoholism (Malka 1988). The amygdala brain regions have been implicated in the regulation of anxiety and
fear, and its role in negative affective state of alcohol addiction (dark side of addiction) has been intensively studies (Koob 2003; Pandey 2003; Pandey 2004).

1.2.2 Neuroanatomy of Alcoholism with the focus on limbic system

The neuroanatomy of alcoholism comprises a daunting amount of information, therefore the focus of the discussion below is the major brain structures and circuits that particularly involved in the positive and negative states of alcohol addiction: the limbic system including extended amygdala (Koob 2003; Koob 2004; Moonat et al. 2010). The extended amygdala is an anatomical system that is composed of the bed nucleus of stria terminalis (BNST), the central medial amygdala (CeA & MeA), the medial part of the nucleus accumbens (NAc shell), and sublenticular substantia innominata (Alheid and Heimer 1988; Koob 1999).

The attempt to identify the center of emotion has a long history of research. The early hypothesis was that the hypothalamus may mediate the responses to emotional stimuli (LeDoux 2003). In 1878, Broca first discovered the limbic lobe, which exists in all mammals. He suggested that the limbic lobe might be related to the olfactory system and referred to it as the “smell brain” (Haines 2006). Later the concept of the limbic system was formed under the light of the “Papez circuit” (Papez 1995) and has since been referred as comprising all emotion-related areas of the brain and the subsequent pathways. In 1952, Paul MacLean added the amygdala and the hypothalamus into the circuit of Papez to define the limbic system as we now know (Maclean 1952) as the set of brain...
structures that forms the inner border of the cortex, including the structure on the most medial edge of the hemisphere, the limbic lobe, plus subcortical nuclei such as amygdala, nucleus accumbens (NAc), hippocampus, etc., which seemingly support a variety of behavioral functions including emotion, motivation, long-term memory, and olfaction (Haines 2006). Structurally the limbic system generally has fewer layers than the classical 6-layered neocortex, and these layers are usually classified as the allocortex (Haines 2006).

It is now generally believed that alternation of the limbic system is associated with many psychiatric disorders, such as addiction (Robbins and Everitt 2002; Koob 2006; Li and Sinha 2008), anxiety (Stathis et al. 2007; Yalug et al. 2009; Lanzenberger et al. 2010; Dzirasa et al. 2011), autism (Sparks et al. 2002; Schumann and Amaral 2006; Amaral et al. 2008), and schizophrenia (Csernansky and Bardgett 1998; Grace 2000; Tamminga et al. 2000; White et al. 2008). Amygdala as the key structure in this emotional circuitry plays specific roles in fear and anxiety (LeDoux 2003; Sah et al. 2003).

### 1.2.3 Amygdala

Amygdala is believed to function as both the modulator of autonomic responses (Sah et al. 2003), and the emotional processor for fear and anxiety (Pessoa and Adolphs 2010). Both of these two functions play crucial roles in the development of alcoholism. Environmental cues such as sounds, smell, and sights related to drinking can act as a trigger to the compulsive craving of alcohol, which contribute to the relapse to drinking. The correlation between these external signs and cravings is suggested to be linked by autonomic
responses such as increased heart rate, sweating and respiration, which in this case is activated by the previous experience of drinking (Tiffany and Conklin 2000). Over time, this linkage becomes a learned (conditioned) response (Tiffany and Conklin 2000; Koob 2003). Anxiety developed during withdrawal after chronic alcohol exposure also plays a crucial role in promoting drinking (Koob 2003; Pandey 2004).

The amygdala is a complex structure composed of 13 nuclei with diverse function and structure (Sah et al. 2003). It is located in the rostromedial part of the temporal lobe, immediately rostral to the hippocampal formation (Haines 2006). There are various ways to group the 13 nuclei of amygdala (LeDoux 2003; Sah et al. 2003; Davis et al. 2010). Amygdala is mainly divided into three sub-complexes; namely the basolateral, centromedial and cortical-like complex (Sah et al. 2003; LeDoux 2007). The basolateral complex includes the accessory basal nuclei (AB), plus the basolateral amygdala (BLA), which can be further divided into lateral amygdala (LA), the basal amygdala (BA) (Sah et al. 2003; LeDoux 2007). The BLA receives inputs from the dorsal thalamus, the prefrontal cortex, the cingulate and parahippocampal gyri, the temporal lobe and insular cortex (Haines 2006). Thus the BLA acts as a gateway and supplier of various information of somatosensory, visual, and visceral input into the remaining amygdala nuclei. It is suggested that sensory information arrives at the amygdala through the BLA, is processed and then passed to the centromedial nuclei which act as an output station to other brain regions (Pitkanen et al. 1997; Sah et al. 2003). The centromedial amygdala complex is composed of the central nucleus
(CeA) and medial nucleus (MeA) of amygdala, and the bed nucleus of stria terminalis (BNST) (Sah et al. 2003). In addition to the inputs from the BLA, the centromedial complex of amygdala also receive afferent inputs from olfactory nuclei, hypothalamus, and dorsal thalamus, while the CeA specifically receives ascending inputs from the brainstem (Haines 2006). However, the major role of the complex is as the output center of amygdala. There are two major efferent pathways for amygdala: the stria terminalis and the ventral amygdalofugal pathway (Haines 2006). The first pathway distributes information to hypothalamus, caudate and putamen, and the NAc (Haines 2006) while the second pathway is the major efferent pathway of amygdala. It is composed of two sub-pathways. One pathway starts from the BLA and CeA, passes through the substantia innominata, which then sends cholinergic projections to the cerebral cortex. This pathway is suggested to be involved in response to behavioral stimuli. The other pathway begins at the CeA and then descends diffusely into nuclei of the brainstem (Haines 2006). The cortical-like complex of amygdala is within the medial temporal lobe and is structurally similar with layered cortical regions (Sah et al. 2003). This complex is closely associated with the olfactory system (Sah et al. 2003; LeDoux 2007), therefore has a relatively smaller contribution to anxiety and alcoholism, however it may be involved in appetitive responses (Brennan and Kendrick 2006).

The function of amygdala nuclei can be described by complex afferent and efferent connections. Inputs to the amygdala can be divided into polymodal, sensory and memory information. The polymodal information derived from the
cortical regions such as the PFC and insular cortices (Turner et al. 1980). The sensory inputs mostly project to the BLA except the olfactory inputs that project to the cortical-like complex (McDonald and Mascagni 1996; Davis 1997). Somatosensory information is primarily from the posterior insular cortex and projects to BLA and CeA (See et al. 2003). The gustatory and visceral information from the anterior insular cortex projects to BLA, CeA and MeA of amygdala (McDonald 1998). Auditory information on the other hand originates from the auditory cortex and thalamus and projects to the LA, BLA and CeA (McDonald 1998). The temporal lobe gives higher order visual input into the LA. The connections between amygdala and hippocampus may explain the close association between emotion and memory. Besides the hippocampus, the perirhinal cortex (PRC), the entorhinal cortex (ERC), and the parahippocampal cortex (PHC) also involves in memory formation and recall, and have heavy connections with the amygdala (McDonald 1998; Sah et al. 2003). The outputs of the amygdala complex are primarily from the centromedial complex and project into the hypothalamus and brain stem (Davis 1997; Sah et al. 2003). The targets within the hypothalamus include the lateral hypothalamus (LH), the ventromedial hypothalamus (VMH), and the paraventricular nucleus of hypothalamus (PVN) (Sah et al. 2003; Haines 2006). The defensive response to emotional stimuli is carried out through the amygdaloid projection to the brain stem nuclei (Misslin 2003), including PAG, parabrachial nucleus, NTS, and dorsal nucleus of the vagus (Davis 1997; Lang et al. 2000; Sah et al. 2003). The parasympathetic nervous system is controlled by these structures and is responsible for the “fight
or flight” response to emotional stimulation (Lang et al. 2000). Ascending noradrenergic, dopaminergic, serotonergic and cholinergic pathways of amygdala to the VTA, raphe nuclei (RN) and nucleus basalis (NB) may be involved the controlling of attention, arousal and motivational aspect of emotion (Lang et al. 2000). Studies have revealed that amygdala nuclei also have widespread intranuclear and internuclear connections (Sah et al. 2003). The major interconnection within the amygdala is from lateral to medial (McDonald 1998; Sah et al. 2003). The projection from the basolateral complex to the centromedial complex of amygdala is glutamatergic (Davis et al. 2010), which is processed by spiny neurons resemble cortical pyramidal neurons (Millhouse and DeOlmos 1983). There are also spiny stellate cells that are GABAergic tonic regulator of excitatory projections (Sah et al. 2003). Interconnections within the centromedial complex are GABAergic (Alheid 2003), and mediated by primarily medium size spiny neurons (Sah et al. 2003).

The amygdaloid nuclei in general all play important roles in anxiety and fear emotion, especially, the involvement of basolateral and centromedial amygdala complex have been indicated in many studies (Campeau and Davis 1995; Davis et al. 2010). However, the anatomical locations of these two complexes define their differential roles in fear and anxiety. As we mentioned before, the basolateral nucleus of amygdala functions as a gate to the amygdaloid complex. Informational inputs from various parts of the brain including the thalamus, hippocampus and sensory, limbic, insular cortices converge at the BLA (Romanski et al. 1993). The LA neurons are responsive to both auditory and
somatosensory stimulations during fear conditioning (LeDoux et al. 1990; LeDoux 2007). The contextual fear conditioning, on the other hand, involves hippocampal projection into the BA and AB (LeDoux 2000). Therefore the BLA may serve as a learning and conditioning site, which initiates the association between memory, sensory, and emotion. Early studies have shown that lesions of BLA can block fear conditioning (Campeau and Davis 1995; Maren et al. 1996). Similarly, BLA infusion of the GABA(A) agonist muscimol also disrupts fear conditioning (Holland 2007). Therefore, the BLA is particularly crucial for studies of conditioned and cue-induced cocaine (Fuchs et al. 2009) and alcohol (Gass et al. 2011; Sinclair et al. 2012) seeking behaviors. The centromedial complex of the amygdala, on the other hand, may serve as executor of emotional stimuli. The projections from centromedial amygdala into the various brain regions regulate the hormonal, autonomic responses of emotional stimuli (Davis 1997; Lang et al. 2000; Sah et al. 2003), therefore is responsible for the physical symptoms of fear and anxiety such as increased heart rate, sweating, vomiting, attention and arousal. A recent study using an optogenetic approach further defined the important role of CeA in anxiety: optogenetic stimulation of the BLA terminals in CeA, but not the BLA somata, produced anxiolytic effect, while optogenetic inhibition of the BLA terminals in CeA induced anxiogenic effect (Tye et al. 2011). These results indicated that the BLA-CeA projection is particularly crucial in anxiety behavior as compared to other output projections of BLA, and that the CeA may be the executive center of anxiety behavior.
1.3 Pharmacology of alcohol

Despite decades of research, the pharmacology of alcohol in the central nervous system remains poorly understood. The exact mechanism underlying the development of alcoholism is still less known, however several neurotransmitter and neuromodulator systems have been implicated in this process. It has long been established that ethanol modulates the function of gamma-aminobutyric acid (GABA) and the N-methyl-D-aspartic acid (NMDA) receptor (Nestoros 1980; Lovinger et al. 1989). In the earlier years of research on alcohol, it was hypothesized that ethanol affected cells through a nonspecific mechanism by causing lipid bilayer fluidization and interfering with the protein-lipid interactions (Johnson et al. 1980). However, recent findings indicate possible pharmacological interactions between ethanol and specific enzymes, neurotransmitter receptors and ion channels (Narahashi et al. 2001; Harris et al. 2008).

The two primary enzymes identified as having specific ethanol interactions are alcohol dehydrogenase (ADH) and adenylyl cyclase (AC) (Harris et al. 2008). Although equally important in regulation of ethanol affect, ADH primarily facilitates the metabolic aspect of ethanol, while AC mediates cellular signaling in the CNS and therefore will be the focus of this discussion. Upon stimulation by G proteins, AC converts adenosine triphosphate (ATP) into second messenger adenosine 3’ ,5’ -monophosphate (cAMP). There are nine known isoforms of AC, all of which can be found in the CNS. The AC isoforms share the similar structural components of two hydrophobic domains, six transmembrane spans,
and two cytoplasmic domains (Sunahara et al. 1996), however, the isoforms vary in sensitivity to ethanol exposure. (Yoshimura and Tabakoff 1995; Harris et al. 2008). Acute ethanol exposure has been shown to increase cAMP production via activation of AC, while chronic ethanol administration results in desensitization of AC to ethanol (Rabbani and Tabakoff 2001). Synthesis of cAMP is directly linked to activation of protein kinase A (PKA), which can, in turn, lead to phosphorylation and activation of CREB further binding to promoter regions of DNA, causing increased expression of specific genes; therefore interaction between ethanol and AC may be involved in the mechanism by which ethanol induces gene expression (Purves 2008).

Although there are several ligand-gated ion channels that interact with ethanol (Mascia et al. 1996; Harris et al. 2008), the NMDA receptor (NMDAR) is among the most important. NMDAR is an iontropic glutamate receptor, which exists as a heterotetramer formed by two NR1 and two NR2 subunits. The NR1 subunits bind the co-agonist glycine and the NR2 subunits bind the neurotransmitter glutamate. Activation of the NMDAR leads to opening of a nonselective cation channel, which allows for the passage of Ca\(^{2+}\) and Na\(^{+}\) into the cell and K\(^{+}\) out of the cell. Calcium flux through NMDAR functions as a second messenger in various signaling pathways, including CaMK, PKA, and MAPK. The NMDAR-ethanol interaction was indicated in an early electrophysiological study, which reported that ethanol exposure inhibits NMDAR mediated current in hippocampal neurons (Lovinger et al. 1989), a phenomenon later replicated in CeA neurons (Roberto et al. 2004). Chronic exposure of
ethanol, on the other hand, increases NMDAR induced calcium current in cortical cultured neurons (Hu and Ticku 1997). During alcohol withdrawal, NMDAR is over-activated leading to a robust increase of current in the CeA (Roberto et al. 2004). Over-activation of NMDAR can cause symptoms such as agitation and in extreme case, seizures, providing a physiological explanation for Delirium tremens.

As noted above, both the AC and NMDAR can regulate function of CREB. The AC regulates activation PKA, while NMDAR induced calcium influx serves as second messenger, triggering activation of CaMK, PKA, and MAPK. These signal transduction pathways are closely related to phosphorylation and activation of CREB (Impey and Goodman 2001; Pandey et al. 2001; Pandey et al. 2003). CREB is phosphorylated at Ser133 in sympathetic and sensory neurons in vivo (Lonze et al. 2002) and this phosphorylation of CREB leads to the recruitment of CREB-binding protein (CBP) that assists the interaction with cAMP-responsive element (CRE) on specific gene promoter regions eventually promoting gene transcription. Using a combination of bioinformatic approaches, CREB has been identified as a potential mediator of ethanol effects on gene expression, (Uddin and Singh 2007). Acute ethanol exposure increased CRE-mediated gene transcription in multiple brain regions including PFC, BNST, BLA, hippocampus, VTA, and hypothalamic nuclei in mice (Asyyed et al. 2006). Chronic alcohol administration decreased the phosphorylation of CREB (Yang et al. 1998), however, withdrawal from chronic ethanol administration resulted in decrease CREB phosphorylation specifically within the amygdala (Pandey et al. 2003;
Pandey et al. 2008), and decreased CRE-DNA binding and expression of BDNF in rat cortex (Pandey et al. 1999). Reduced CREB and phosphorylated CREB in NAc shell of C57 mice has been correlated to their innate high alcohol preference as compared with DBA mice (Misra and Pandey 2003). Similar reduction of CREB phosphorylation has also been observed in the NAc of rats after voluntary ethanol intake (Misra et al. 2001). Decreased levels of CREB and phosphorylated CREB in the CeA and MeA were also found to be associated with anxiety and increased alcohol preference in P rats compared with NP rats (Pandey et al. 1999; Pandey et al. 2005). Interestingly, heightened anxiety and higher alcohol consumption was also observed in CREB deficient mice compared to wild-type littermate mice (Pandey et al. 2004). Withdrawal from chronic ethanol exposure induced anxiety-like behavior and decreased phosphorylation of CREB and Ca$^{2+}$/calmodulin-dependent protein kinase IV in the CeA and MeA in SD rats (Pandey et al. 2003). As transcription factor, CREB activation can mediate the expression of genes that are key factors in synaptic plasticity, further explaining the importance of CREB in the development of anxiety and alcoholism. Brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) are CREB target genes and have been implicated in neuroadaptive changes during development of alcoholism. In addition to the involvement of BDNF signaling and Arc expression in anxiety and alcoholism, BDNF and Arc have been implicated in many other neurological and psychiatric disorders (Davis 2008; Pandey et al. 2008; Moonat et al. 2011).
Before the discussion on BDNF and Arc, a point should be made that besides these two genes, there are many other CREB targets genes that are also associated with anxiety and alcoholism. Examples of such genes are neuropeptide Y (NPY) and corticotropin-releasing factor (CRF). It is suggested that within the CeA NPY modulation is a vital mediator of alcohol dependence (Gilpin and Roberto 2012). The use of animal models of alcoholism have suggested that reduced neuropeptide Y (NPY) levels in the CeA may contribute to the behavioral differences observed in these animals. Although the mechanism has yet to be identified, consistent findings in the CeA of both alcohol-preferring (P) rats and high alcohol drinking (HAD) rats indicate that lower NPY levels in the CeA of P and HAD rats may be operative in the regulation of alcohol drinking behavior (Hwang et al. 1999). NPY mRNA expression was found to be significantly lower in the CeA and MeA of P rats than NP rats, whereas no differences were found in the BLA (Suzuki et al. 2004; Pandey et al. 2005). Intra CeA infusion of NPY decreased anxiety-like behavior of alcohol dependent unselected stock of rats and abolished alcohol-drinking behavior (Gilpin et al. 2008). Studies in our lab also revealed that the possible mechanism, that NPY may regulate anxiety and alcohol drinking behaviors in P rats through modulation of CREB phosphorylation (Pandey et al. 2005; Zhang et al. 2010). In addition, changes in NPY levels in the CeA and MeA of SD rats has been shown to be involved in alcohol tolerance and dependence (Sakharkar et al. 2012). Comparison of P and non-preferring (NP) rats indicated significantly lower CRF mRNA levels in CeA of the P rats, which was correlated with higher anxiety
(Hwang et al. 2004). Similarly, basal extracellular levels of CRF in the CeA were higher in the Sardinian alcohol-preferring (sP) and nonpreferring (sNP) rats and stress significantly increased the extracellular levels of CRF in the CeA of both lines (Richter et al. 2000). These data indicate a widespread effect of ethanol on gene expression and the interplay between these CREB target genes with alcohol drinking and anxiety-like behaviors.
1.4 BDNF signaling transduction pathway

1.4.1 BDNF

BDNF is a member of the neurotrophin family of growth factors (Leibrock et al. 1989). BDNF is essential for several aspects of the nervous system including neural development (Ernfors et al. 1995), survival (Kalcheim et al. 1987; Klein et al. 1993), growth (Pencea et al. 2001), synaptic plasticity (Pang and Lu 2004; Pang et al. 2004), and learning and memory (Bekinschtein et al. 2007; Bekinschtein et al. 2008). Deletion of BDNF, selectively during development, leads to impaired hippocampal plasticity, hyperactivity and hyperphagia (Kernie et al. 2000; Zorner et al. 2003; Glorioso et al. 2006). Cortex-specific deletion of BDNF also causes degeneration of striatal medium spiny neurons (Baquet et al. 2004; Strand et al. 2007). Abnormal BDNF signaling is also believed to be involved in psychiatric disorders such as depression (Zhou et al. 2011), cocaine addiction (Lu et al. 2010), schizophrenia (Ho et al. 2007; Ikeda et al. 2008; Thompson Ray et al. 2011), heroin abuse (Cheng et al. 2005), bipolar disorder (Sklar et al. 2002; Lohoff et al. 2005) and ADHD (Kent et al. 2005); as well as many neurological diseases including Parkinson's disease, Alzheimer's disease, epilepsy, chronic pain (Pezet and Malcangio 2004), and Huntington's disease (Strand et al. 2007).

Spatial and temporal changes of BDNF expression has been observed in relation to ethanol exposure. BDNF levels were found to be decreased in the plasma of alcoholic patients (Joe et al. 2007), ethanol self-administration was able to increase BDNF expression in dorsal striatum (Jeanblanc et al. 2009).
BDNF expression in the hippocampus was decreased during withdrawal, but not during chronic ethanol exposure (Tapia-Arancibia et al. 2001). Ethanol withdrawal after chronic ethanol exposure also reduced BDNF expression in CeA and MeA, but not BLA, and produced anxiety-like behavior in rats; while BDNF infusion normalized both BDNF expression and attenuated development of anxiety-like behavior (Pandey et al. 2008). On the other hand, acute ethanol exposure increased the expression of BDNF, which is believed to be a compensatory mechanism for homeostasis or repair (McGough et al. 2004; Pandey et al. 2008). A vapor ethanol inhalation binge model produced a decrease in BDNF mRNA and protein levels in the prefrontal cortex (Melendez et al. 2006). There are compelling evidence to suggest the involvement of reduced BDNF function in anxiety and alcoholism. BDNF(+/−) mice have higher preference for ethanol measured by two-bottle choice paradigm, while infusion of BDNF into the dorsal striatum and hippocampus attenuated alcohol preference (McGough et al. 2004). CREB haplodeficient mice also exhibit innately high anxiety-like behavior, increased voluntary ethanol consumption and deficits in BDNF expression (Pandey et al. 2004). Compared to their non-preferring (NP) counterparts, alcohol preferring (P) rats have innately lower BDNF mRNA and protein levels in amygdala, later (IBNST), medial (mBNST), and ventral bed nucleus of stria terminalis (vBNST), but not in the NAc shell and core (Prakash et al. 2008). BDNF antisense oligodeoxynucleotides (ODNs) infusion to the CeA and MeA, but not BLA, induced anxiety and alcohol-drinking behaviors in rats, and reduced Erk and CREB phosphorylation (Pandey et al. 2006). A dinucleotide
repeat in an intron 5’ to the first coding exon in the BDNF gene has been implicated with increased vulnerability for alcohol abuse (Uhl et al. 2001). A BDNF single nucleotide polymorphism, Met66Met, results in decreased BDNF secretion and localization (Egan et al. 2003; Chen et al. 2004), and is associated with high anxiety levels in both human (Lang et al. 2005) and mouse models (Chen et al. 2006) of high alcohol consumption (Colzato et al. 2011), early onset of alcoholism (Matsushita et al. 2004), and higher risk of relapse (Wojnar et al. 2009).

The BDNF gene contains one coding exon and eight non-coding exons in rodents and humans. Thus splicing of BDNF gene can produce nine transcript variances (Aid et al. 2007; Pruunsild et al. 2007). The 3’ coding exon (exon IX) encodes the common pro-BDNF protein, while the eight 5’ exons with independent promoters provide different BDNF splice variants (Aid et al. 2007; Pruunsild et al. 2007). The complex structure of the BDNF gene permits both signaling-specific (Dias et al. 2003; Tian et al. 2010; Wong et al. 2010) and region-specific (Timmusk et al. 1993) regulations of its transcriptosome. For example, in hippocampus, stress-induced changes in BDNF mRNA expression have been shown to depend on the timing, type, duration and frequency of the stressors (Nair et al. 2007). Different expression of BDNF exons has been observed across different phases of prefrontal cortex development (Wong et al. 2009). Exon IV of BDNF is mainly responsive to neuronal BDNF expression (Tao et al. 1998). It was found that expression of exon IV of BDNF is significantly up-regulated in the amygdala and hippocampus during fear-conditioning paradigm.
(Ou and Gean 2007; Lubin et al. 2008). On the other hand, context exposure leads to increased expression of BDNF exons I and VI (Lubin et al. 2008) and electroconvulsive seizures triggers increased expression of BDNF exons II and VI (Tsankova et al. 2004). Intriguingly, despite the diversity of BDNF at mRNA level, when translated, all BDNF exons give rise to a common BDNF protein (Greenberg et al. 2009).

Another interesting observation of rodent BDNF promoter IV shows that it contains a binding site for CREB (Tao et al. 2002), a transcription factor which binds and recruits co-factor CREB binding protein (CBP), which itself has intrinsic HAT activity. Therefore BDNF expression might be regulated by histone acetylation. BDNF protein is seen in both the soma and the fibers of neurons (Altar et al. 1997; Conner et al. 1997), possibly because the BDNF protein is transported to (Altar et al. 1997), or translated at the dendrites (Kang and Schuman 1996; Tongiorgi et al. 2004; An et al. 2008). The expression of the BDNF gene is controlled not only by regulation of promoters and transcription splicing, but also at the post-transcriptional level. After the BDNF gene is translated to pro-BDNF, it must be cleaved to become mature BDNF (Barker 2009), which then exerts its effects through binding to the tropomyosin-related kinase receptor B (TrkB), an essential compartment of long-term-potentiation and cell survival (Lu et al. 2005; Jia et al. 2010). The pro-BDNF binds to p75, which is a non-specific receptor of all neurotrophins (DeFreitas et al. 2001) and is destined to trigger cell death and long-term-depression (Teng et al. 2005; Woo et al. 2005), however, pro-BDNF has also been indicated to be involved in the
regulation of BDNF secretion (Lou et al. 2005) and anxiety behavior (Chen et al. 2006).

1.4.2 TrkB

Tropomyosin-related kinase receptor B (TrkB) is appropriately named because it is a catalytic receptor with kinase function. It belongs to the Trk receptor family which is composed of TrkA, TrkB and TrkC. TrkA is the receptor for nerve growth factor (NGF), TrkB binds BDNF and neurotrophin 4 (NT4) and TrkC is the receptor for neurotrophin 3 (NT3). Trk receptors share basic structures and functions. Here I will focus on TrkB for the purpose of current thesis. TrkB is located in the cellular membrane, with a single hydrophobic transmembrane-spanning domain, an extracellular N-terminal region, and an intracellular C-terminal region (Lodish 2003). The N-terminal region contains a ligand-binding site, while the intracellular C-terminal region includes catalytic domains responsible for the kinase activity (Zwick et al. 2001). In addition to the full-length TrkB receptor (TrkB^{TK+}, which will be referred to as TrkB), there are two truncated TrkB (TrkB-T1 and -T2) receptors which lack tyrosine kinase activity (Minichiello 2009). Little is known about the truncated form of TrkB receptors, however, recent studies have indicated a regulatory role of the TrkB-T1 and TrkB-T2 on BDNF-TrkB binding (Davis 2008; Luberg et al. 2010). Ligand such as BDNF binding to the extracellular domain of TrkB induces the formation of receptor dimers. Dimerization leads to activation of the TrkB's C-terminal catalytic domains. Then, TrkB autophosphorylates itself first before catalyzing substrate proteins. Phosphate groups are transferred from high-energy donor
molecules such as ATP to the substrate. In the case of TrkB, the phosphate groups are added specifically to tyrosine amino acids of the substrates. Autophosphorylation of TrkB creates binding sites on the receptor for proteins with the Src homology2 (SH2) domain and the phosphotyrosine binding (PTB) domain (Pawson 1995). The Shc adaptor protein binds to TrkB and leads to recruitment of Grb2 and SOS, which results in the binding of Ras protein. Consequent phosphorylation of this small GTPase leads to the activation of two signaling pathways: the mitogen-activated protein kinase (MAPK) pathway which involves in neuronal differentiation and branching (Cohen-Cory and Fraser 1995; McAllister et al. 1999); and the phosphoinositide-3 kinase (PI3K) pathway which is primarily involved in cell survival. There is an additional phospholipase C (PLC) pathway, which is activated by direct interaction between PLC and the TrkB SH2 domain. This pathway mainly facilitates neurotransmission (Qian et al. 1998; Atwal et al. 2000; Minichiello et al. 2002; Jeanneteau et al. 2010).

TrkB has been shown to be involved in anxiety and alcoholism. Acute ethanol administration produced anxiolytic effects and has been associated with increased TrkB in the CeA, MeA, but not BLA, of SD rats (Pandey et al. 2008). On the other hand, ethanol withdrawal-induced anxiety was closely related with decreased TrkB in these brain regions (Pandey et al. 2008). Genetic variations of the TrkB gene is believed to be involved in alcohol dependence (Xu et al. 2007). Furthermore, increased alcohol consumption and preference in mice has been observed following TrkB inhibition (Jeanblanc et al. 2006).
1.4.3 Erk

The Erk pathway of BDNF is activated by a cascade involving activation of the small G protein, Ras, which activates the protein kinase Raf; Raf phosphorylates and activates MEK, which then phosphorylates and activates Erk. Erk1 (MAPK3) and Erk2 (MAPK1) have 85% sequence identity (Boulton and Cobb 1991), and both play critical roles in axon growth. Erk1/2 is widely expressed in limbic brain regions including mesolimbic dopaminergic system, amygdala and prefrontal cortex (Lein et al. 2007). Besides BDNF regulated TrkB activation, Erk can also be activated through the Ras-Raf-MEK signaling pathway activated by other receptor tyrosine kinase, or by calcium influx through NMDAR and voltage-gated calcium channels (Ron and Messing 2011). Erk has shown its great potential as a mediator of BDNF in alcoholism: Ethanol-induced increases in BDNF mRNA lead to an increase of BDNF synthesis and Erk activation in primary striatal neurons (Logrip et al. 2008). It was also found that ethanol-produced Erk phosphorylation is dependent on BDNF concentration (Ohrtman et al. 2006). Erk activation has been shown to be elevated by chronic ethanol administration (Ku et al. 2007), yet inhibited by acute exposure in primary cortical culture cells (Chandler and Sutton 2005), hippocampus (Davis et al. 1999) and cerebellar granule cells (Ohrtman et al. 2006).

In vitro study using striatal neurons revealed activation of the TrkB and MAPK signaling pathway after exposure to ethanol (Logrip et al. 2008). Systematic administration of the MEK inhibitor SL327 increased operant self-administration of ethanol in C57BL/6J mice (Faccidomo et al. 2009). Involvement
of Erk was also long been identified in many other drugs of addiction. For example, chronic morphine or cocaine caused a continuous increase in ERK activity, while intra-VTA infusions of BDNF blocked this effect (Ortiz et al. 1995; Berhow et al. 1996). MEK/Erk pathway inhibitors infused into the VTA decreased behavioral sensitization initially induced by cocaine (Pierce et al. 1999), whereas systemic administration of similar inhibitors blocked cocaine-induced locomotor hyperactivity and place conditioning (Pierce et al. 1999; Valjent et al. 2000). Interestingly, the discussion on alcoholism related molecules again converges on CREB, which not only can induce expression of BDNF, but also can be activated by the Erk pathway downstream of BDNF signaling. Evidence for a direct correlation between Erk and CREB has been suggested in alcoholism studies (Chandler and Sutton 2005; Pandey et al. 2008).

1.4.4 BDNF-Arc signaling in synaptic plasticity, anxiety and alcoholism

BDNF signaling plays a critical role in regulation of Arc gene expression through regulation of CREB functioning. Activity-regulated cytoskeleton-associated protein (Arc, also known as Arg3.1) (Link et al. 1995; Lyford et al. 1995), is a member of the immediate-early gene (IEG) family. A major synaptic activity-responsive element (SARE) was found upstream of the Arc/Arg-3.1 transcription initiation site, which was found to be a cluster of binding sites for CREB, further indicating the regulatory role of these transcription factors on Arc expression (Kawashima et al. 2009). Additionally, Arc transcription has been shown to be dependent on activation of MAPK cascade (Waltereit et al. 2001).
Erk phosphorylation is required for Arc mRNA to further transported (Huang et al. 2007). Activity regulated localization of Arc mRNA to dendrites where synaptic activities take place (Wallace et al. 1998), thereby regulating structural and functional modulation of dendritic spines (Bramham et al. 2008), thus making Arc an important marker for plastic changes in the nervous systems. This dendritic transport of Arc mRNA has been closely associated with BDNF-induced LTP (Link et al. 1995; Lyford et al. 1995; Ying et al. 2002).

Evidence for the important roles that BDNF and Arc play in synaptic plasticity are abundant: BDNF is released from glutamatergic synapses after high-frequency stimulation and act as a major regulator of high-frequency stimulation induced LTP (Bramham and Messaoudi 2005). Indication that BDNF signaling at excitatory synapses promotes the development of transcription-dependent late-phase LTP has also been found (Messaoudi et al. 2002; Minichiello et al. 2002). BDNF hippocampal infusion induced Erk-dependent transcription of Arc (Messaoudi et al. 2002), which in term provoked late-phase LTP (Ying et al. 2002). Deficits in Arc, induced either by Arc antisense oligodeoxynucleotides (ONDs) (Messaoudi et al. 2007; Ploski et al. 2008) or genetic knockout (Plath et al. 2006), both caused decreased LTP and learning. Changes in Arc expression in the brain has been associated with many behavioral models including fear conditioning (Huff et al. 2006; Monti et al. 2006), memory (Guzowski et al. 2000; Plath et al. 2006), and operant conditioning (Kelly and Deadwyler 2003). The expression of Arc mRNA and protein following BDNF induced LTP were both shown to be significantly up-regulated in the
hippocampus (Ying et al. 2002). These changes appear to require Erk signaling since infusion of MEK inhibitors, which blocked Erk activity, completely abolished BDNF-LTP induction (Ying et al. 2002). These data suggest a BDNF-induced signaling pathway through MEK-Erk activation, triggering synaptic plasticity through up-regulation of Arc. BDNF signaling and Arc regulated synaptic plasticity may be able to explain the involvement of BDNF and Arc in anxiety and alcoholism: Acute ethanol increases Arc expression in the amygdala, while withdrawal from chronic ethanol exposure had the opposite effect on Arc expression (Pandey et al. 2008). Interestingly, BDNF infusion into the amygdaloid brain region was able to normalize this withdrawal-induced decrease of Arc expression (Pandey et al. 2008). Arc antisense ODNs produced anxiogenic effects in rats, promoted alcohol drinking, and decreased amygdaloid dendritic spine density (Pandey et al. 2008). Deficits in BDNF and Arc expression and associated deficits in dendritic spines have also been associated with innately high anxiety behavior and alcohol preference observed in P rats (Moonat et al. 2011).

BDNF signaling and Arc expression may regulate LTP by regulating dendritic spine morphology and function. Changes in dendritic spines structure have been found to accompany induction of LTP (Bramham 2008). BDNF promotes synaptic strength and neuronal signaling (Poo 2001), as well as enhances dendrite formation and axonal branching (McFarlane 2000; Danzer et al. 2002; Horch and Katz 2002). The regulatory role of BDNF on dendritic spine density may also require cAMP (Ji et al. 2005) and Erk1/2 signaling (Alonso et al.
While the exact role of BDNF in the modulation of DSD is not clear, Arc seems to play a direct role in the morphology and functioning of dendritic spines: Arc has been found to stabilize F-actin, which functions as a modulator of dendritic spine size (Huang et al. 2007). F-actin’s function is further enhanced by cofilin, which is accompanied by increased Arc expression following LTP induction (Messaoudi et al. 2007). On the other hand, when Arc was knocked down by antisense ODNs, dendritic spines showed significant retraction, which was blocked by administration of an F-actin stabilizer (Messaoudi et al. 2007). These data suggest the extensive involvement of both BDNF and Arc in dendritic spine morphology and function, which may serve as the mechanism by which BDNF and Arc regulate synaptic plasticity during development of anxiety and alcohol drinking behaviors.
1.5 Epigenetics

1.5.1 Overview of Epigenetics

“Epi” is a Greek word meaning over, above, outer. Therefore the term “Epigenetics” represents the functional modifications to the genome that do not involve a change in the DNA sequence and was introduced by Conrad Waddington in 1937. Epigenetics was derived from the word “epigenesis”. Contrasting to the “preformation” theory of development, the “epigenesist” theory of development proposed that the early embryo was undifferentiated. As development proceeds, increasing levels of complexity give rise to the adult organism (Holliday 1994). Thus the first definition of epigenetics was the study of the changes in gene expression that occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression (Holliday 1994). A supplementary definition stated along with this was nuclear inheritance, which is not based on differences in DNA sequence (Holliday 1994). Epigenetics was later broadly defined as the sum of all the mechanisms necessary for the unfolding of the genetic program for development (Holliday 2006). Epigenetic marks were thought to be completely erased and then re-established in each generation. Maynard Smith introduced the inheritability of epigenetic changes within his “dual inheritance theory” (Maynard Smith 1990). This theory includes a classic inheritance of DNA sequence changes, and the epigenetic inheritance of non-DNA-sequence changes (Maynard Smith 1990). The latter is now called “transgenerational epigenetic inheritance” (Horsthemke 2007; Daxinger and Whitelaw 2010).
Compared to genetic changes, which are usually stable and rarely reversed, epigenetic changes are often reversed by environmental influences. When aberrant epigenetic changes happen because of the environmental challenges, expression of certain genes would be altered, and thus causing serious effects and might eventually result in the development of disease. Evidence of this link among environmental factors, epigenetic changes, and diseases were shown in many cancers (Esteller 2007; Jones and Baylin 2007; Claes et al. 2010; Sandoval and Esteller 2012), developmental diseases (Dennis Lo 2006; Mathers and McKay 2009; Joss-Moore et al. 2010; Woroniecki et al. 2011), and noncancerous diseases like immunological, cardiovascular, developmental and neurological/psychiatric diseases (Petronis et al. 2000; Abdolmaleky et al. 2005; Bredy 2007; Stahl 2010; Toyokawa et al. 2012).

How do the epigenetic modifications regulate neuronal activities? Synaptic transmission is a process of receptor mediated neuronal response to neurotransmitters, initiating cascades of signaling transductions and eventually activating or inhibiting transcription. Transcriptional regulation occurs through interactions with transcription factors and their co-factors, as well as modifications of chromatin structure. Thus chromatin remodeling is important in neuronal survival, synaptic activity, morphology and eventually the integrated regulation of complex behavior (Tsankova et al. 2007). The involvement of epigenetic mechanisms in mental retardation has been suggested (Franklin and Mansuy 2011), as has epigenetics role in memory formation in general (Trollope et al. 2012) and risk of developing neurodegenerative disorders such as
Alzheimer's disease or Parkinson's disease (Iraola-Guzman et al. 2011). A growing body of literature also suggests that epigenetic processes play a critical role in psychiatric disorders such as schizophrenia, post-traumatic stress disorder (PTSD), anorexia nervosa, depression (Uher 2011), and substance dependence (Tsankova et al. 2007; McGowan and Szyf 2010; Ptak and Petronis 2010; Boulle et al. 2011; Galea et al. 2011; Toyokawa et al. 2012). The following sections will give brief introductions on specific mechanisms of epigenetics and their roles in alcoholism.

1.5.2 Mechanisms of Epigenetics

In the cell nucleus, DNA is not alone, but rather exists in a complex called chromatin, which is composed of DNA, RNA, histones and other proteins (Black and Whetstine 2011). A condensed state of chromatin, namely heterochromatin, is considered inactive and does not allow transcription of genes. When activated (opened), now called euchromatin, is accessible to the transcriptional machinery thus allows individual genes to be transcribed (Tsankova et al. 2007; Starkman et al. 2012). The switches between this two states of chromatin and many stages in between, is made by a dynamic process called chromatin remodeling, which improves or prevents accessibility of specific promoter regions for the transcriptional machinery.

1.5.2.1 DNA methylation

DNA methylation refers to the catalytic addition of a methyl group S-adenosyl methionine (SAM) to the fifth carbon position of a cytosine residue that is followed by guanine, also known as a CpG dinucleotide. The CpG
dinucleotides cluster in concentrations called CpG-islands, which is located in the
promoter region of greater than 50% of all human genes (Issa 2004; Christensen
and Marsit 2011). In normal conditions, most of these CpG-islands are
unmethylated, while methylated CpG-islands in the gene promoter would cause
silencing of the gene leading to decreased gene transcription (Issa 2004). DNA
methylation is believed to regulate gene transcription through blocking binding of
transcription machinery, with the assistant of repressor proteins include methyl-
CpG-binding domain proteins (MBD) (Fan and Hutnick 2005). DNA
methyltransferases (DNMTs) are the enzymes responsible for this modification
(Lachner and Jenuwein 2002). There is no known enzyme for DNA demethylate
yet, however, recent evidence suggested that DNMT3a and DNMT3b may
contribute in the process (Sharma et al. 2010). Interestingly, both the NDMTs
(Woodcock et al. 2006) and MBD (Kimura and Shiota 2003) can recruit HDACs,
which condense chromatin structure, leading to further decrease of gene
transcription.

Griffith and Mahler in 1969 suggested the involvement of DNA methylation
in long term memory (Griffith and Mahler 1969). It was later proposed that DNA
methylation could attribute to gene expression and explain the switching on and
off of genes during X chromosome inactivation (Riggs 1975) and genetic
imprinting. X chromosome inactivation (XCI) is an epigenetic phenomenon that
happens in females when one of the X chromosomes is silenced in order to
compensate for the difference in the number of X chromosomes between
females (XX) and males (XY) (Kubota et al. 2002). Genomic imprinting is a
unique phenomenon in which only one of two parental alleles is expressed, while the other allele is suppressed. There is believed to be an epigenetic mark for suppression in one of the parent alleles. This mark is considered a reversible mechanism, because the suppressed allele should be reactivated during gametogenesis when it is passed to the next generation (Glenn et al. 1993; Kubota et al. 1997). Since then, research has demonstrated important roles of DNA methylation in many cancers (Giovannucci et al. 1995; Esteller 2007; Christensen et al. 2008; Schernhammer et al. 2010; Sandoval and Esteller 2012), neurodegenerative (Franklin and Mansuy 2011; Iraola-Guzman et al. 2011) and psychiatric disorders. Consequently, many psychopharmacological medications have been shown to have epigenetic effects; for example antipsychotics for schizophrenia and bipolar disorder, valporate, L-methionine, clozapine and sulpiride, decreased DNA methylation at the reelin and the 67kDa glutamic acid decarboxylase (GAD) promoters (Dong et al. 2008; Dong et al. 2010).

1.5.2.2 Histone modification

As we mentioned before, histones are components of chromatin, which function as a core around which DNA coils. This structure of about 147 base pairs of DNA wrapped around a core histone octamer is named nucleosome, and is the fundamental unit of chromatin. Each octamer contains two copies each of the histones H2A, H2B, H3 and H4 (Horn and Peterson 2002). With additional H1 histone excluded in the octamer, five isoforms of histone are all thought to be important in chromatin remodeling (Woodcock et al. 2006). The histone
modifications, occur on the amino (N)-terminal tail of a histone, which is mainly composed of basic amino acids that interact with negatively charged DNA. Modification of histone tails playing a crucial role in switching between open and closed forms of chromatin structure, leading to changes in accessibility of genes for transcriptional machinery, thus causing alterations in gene transcription (Cosgrove and Wolberger 2005; Tsankova et al. 2007). The histone modification mechanisms include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Tsankova et al. 2007). The opening of chromatin is usually associated with acetylation of histones, however, histone methylation can result in either activation or repression of gene expression, depending on the residue of modification (Lachner and Jenuwein 2002).

1.5.2.2.1 Histone Acetylation

Histone acetyltransferases (HATs) add acetyl groups to the N terminal tails of histone to relax chromatin structure allowing transcriptional machinery to bind and transcription to occur (Jenuwein and Allis 2001). On the other hand, histone deacetylases (HDACs) remove acetyl groups and condense chromatin (Narlikar et al. 2002), which in turn reduces gene transcription. Despite the exception of H4 Lys12 acetylation which is coincident with silencing (Jasencakova et al. 2000), the acetylation of histones usually represents transcriptionally active chromatin, while deacetylation is generally linked with transcriptional silencing (Kouzarides 2007; Gelato and Fischle 2008). Enzymatic acetylation on lysine residues in N-terminal tails of histones decreases their overall positive charge. Because histone tails are basic, it is proposed that
acetylation decreases their affinity for the DNA (negatively charged) thus enabling the binding of transcriptional machinery (Lee et al. 1993; Vettese-Dadey et al. 1996). HATs are evolutionarily conserved among species (Kouzarides 2007; Lee and Workman 2007). HATs, upon recruitment by transcription factors, can locally modify histones, thus chromatin structure, to specifically regulate gene expression. Interestingly, several transcriptional activators themselves contain intrinsic HAT activity, and CREB binding protein (CBP). CREB when phosphorylated triggers the recruitment of CBP, causing loosening of the chromatin, leading to activation of transcription. CBP is suggested to be critical for normal learning and memory, and also alcohol addiction (Polesskaya et al. 2001; Victor et al. 2002; Bardag-Gorce et al. 2007; Bazan 2008; Guo et al. 2011; Miller 2011). HDACs have more diversity in terms of structure compared to HATs. Unique accessory domains of each subtype of enzymes offer diverse and specific targeting of regulation. There are four classes (class I-IV) of HDACs (Dokmanovic et al. 2007). Some of the isoforms of the class I (HDAC 1,2,3,8) and class II (HDAC 4,5,6,7,9,10) are called the classic HDACs. The classic HDACs plus class IV (HDAC11) HDACs are zinc-independent, while class III HDACs (7 sirtuins) are zinc-independent, instead depend on NAD$^+$ (Harting and Knoll 2010). The class I HDACs, HDAC 4,5,9 from class II, and HDAC 11 of class IV (Dokmanovic et al. 2007), and Sirt 1 and 2 from the classs III (Harting and Knoll 2010) are found in the brain, therefore are the most widely studies within the neuroscience field because they are potential therapeutic targets for
the treatment of neurodegenerative, neurodevelopmental, and psychiatric disorders.

The involvement of histone acetylation in psychiatric diseases has been implied in many studies. It has been shown that social defeat stress-induced decreased H3 acetylation and increased H3-K4 dimethylation at brain derived neurotrophic factor (BDNF) promoters in the hippocampus of mice (Tsankova et al. 2006). Increase of histone H3 acetylation was found following fear conditioning task training, suggesting the role of histone acetylation in learning and memory (Levenson et al. 2004). Both cocaine and antipsychotic drugs cause changes in the acetylation of H4 and phosphoacetylation of H3 in the striatum (Li et al. 2004; Kumar et al. 2005). A drug used to treat bipolar disorder, Lithium, induces the down-regulation of H3-K4 histone demethylase (Wang et al. 2011). It has also been shown in cells in hippocampus (Chawla et al. 2003) and cerebellum (Linseman et al. 2003) that the Ca\textsuperscript{2+} pathway triggers activation of calcium/calmodulin-activated protein kinases (CaMKs), which can phosphorylate class II HDACs thus resulting in increased histone acetylation. Deficits in CBP functioning led to impairment of memory formation in transgenic mice model (Alarcon et al. 2004), while the HDAC inhibitor trichostatin A (TSA) blocked these behavior impairment (Korzus et al. 2004). Recent studies from our lab have also revealed a close association between histone acetylation and alcoholism. Acute ethanol exposure inhibited HDAC activity and increased H3 and H4 acetylation in the amygdala of rats, however, opposite effects were observed following ethanol withdrawal after chronic ethanol exposure (Pandey et al. 2008). Interestingly,
treatment with the TSA was able to block these effects of withdrawal in the amygdala of rats (Pandey et al. 2008).

The HDAC inhibitors have recently emerged as therapeutic agents for several diseases, with the initial interest in these inhibitors arised from cancer research (Minucci and Pelicci 2006; Shankar and Srivastava 2008). HDACs inhibitors are classified into four families: the blood brain barrier permeable short-chain fatty acids family, which include the sodium butyrate and phenylbutyrate; the hydroxamic acids, which include the trichostatin A and suberoylanilide hydroxamic acid (SAHA); the epoxyketones family, and the benzamides family (Carey and La Thangue 2006). The potent anti-cancer properties of HDAC inhibitors are that they can block cell growth, provoke differentiation and initiate apoptosis (Carey and La Thangue 2006; Minucci and Pelicci 2006; Shankar and Srivastava 2008). Some HDAC inhibitors are already in phase I/II clinical trials for cancer prevention and treatment, while SAHA (also called vorinostat) is the FDA approved medication for T-Cell lymphoma (Kavanaugh et al. 2010). Increasing evidence has shown that HDAC inhibitors have the potential to serve as therapeutic agents for brain disorders (Kazantsev and Thompson 2008). For example, HDAC inhibitors attenuated neuronal death in both mice (Ferrante et al. 2003; Hockly et al. 2003) and drosophila (Steffan et al. 2001) Huntington models. The toxicity of the alpha-synuclein can be rescued by treatment of HDAC inhibitors in both cell culture and transgenic drosophila Parkinson’s disease model (Kontopoulous et al. 2006). HDAC inhibitors also restored deficits in histone acetylation and regained learning and memory function in a neurodegeneration
mouse model (Fischer et al. 2007). Especially, HDAC inhibitors TSA and butyrate were found to assist the initiation of LTP in the fear conditioning behavior (Levenson et al. 2004). The neuroprotective effect of the HDAC inhibitor SAHA has been shown by its ability to prevent H3 deacetylation as well as increase the expression of neuroprotective protein expression of mice suffered with ischemic stroke (Faraco et al. 2006). The HDAC inhibitors have been used to develop therapeutic targets for psychiatric disorders (Abel and Zukin 2008). It has been shown that tricyclic antidepressants block HDAC 5 activity and increases in histone acetylation at BDNF promoters in the hippocampus of socially defeated mice (Tsankova et al. 2007), indicating that HDAC inhibitor activity may be involved in the action of antidepressant. The HDAC inhibitor TSA may modulate anxiety behavior through increased histone acetylation, transcription factor binding, glucocorticoid receptor (GR) expression, and hypothalamic-pituitary-adrenal (HPA) responses following stress responses in rats who received low maternal care (Weaver et al. 2004; Weaver et al. 2006; Weaver 2007), suggesting that HDAC inhibitors can potentially reverse the epigenetic modifications and related gene expression caused environmental stimuli. The loss of learning and memory in the HDAC overexpressed mice can be rescued by treatment with HDAC inhibitors (Fischer et al. 2007; Guan et al. 2009). The dendritic spines and number of synapses were also increased following the HDAC inhibitor treatment, which were decreased in the hippocampus due to overexpression of HDAC2 (Guan et al. 2009). The neuroprotective effects of
HDACs inhibitors may be related to their ability to improve loss of dendritic morphology and function in HDAC2 overexpressed mice (Guan et al. 2009).

1.5.2.2.2 Histone Methylation

In contrast to histone acetylation, early studies on histone methylation found little turnover of methyl groups, and therefore methylation was thought to be a static modification (Shepherd et al. 1971; Borun et al. 1972). Many years later, subsequent studies revealed the dynamic nature of histone methylations mechanisms (Chen et al. 1999; Strahl et al. 1999). Histone methylation occurs on lysine and arginine residues of histone tails, facilitated by histone methyltransferases (HMTs), while histone demethylases (HDMs) act to reverse the methylation (Shi et al. 2004). Unlike acetylation of histone as previously discussed, histone methylation does not modify the total charge of the histone tails, however, addition of methyl groups (mono, di or tri methylation) does increase its basicity and hydrophobicity (Rice and Allis 2001). Therefore, the levels of histone methylation can also affect the transcription. For example on the H3 Lysine 27 residue, a monomethylation modification is usually associated with increased gene transcription, however, a trimethylation usually leads to gene silencing (Rosenfeld et al. 2009). The most common areas of histone methylation research occur in lysine residues. The various lysine methylation sites include 4, 9, 27 and 36 in H3, and on position 20 in H4, and also at the H1 amino terminus (Jenuwein and Allis 2001). The complexity of histone lysine methylation is that it can occur several times (mono-, di- or trimethylation) on same lysine side chain, and each level of modification can lead to different outcomes. Depending on the
specific modification site, histone methylation can induce different effects. For example, trimethylation of histone H3 on its lysine 4 residue results in increased gene expression while when the same modification occurs on the H3 lysine 9 residue, it is believed to suppress gene expression (Berger 2007). The arginine residues on H3 and H4 tails can also be mono- or di-methylated, and is suggested to activate gene transcription (Berger 2007). Histone methylation has been implicated in learning and memory. A recent study found that mice deficient in H3 lysine (K) 4-specific methyltransferase, Mll, showed deficits in long-term memory (Gupta et al. 2010). In normal mice, contextual fear conditioning resulted in immediate increases in H3K4 trimethylation and H3K9 dimethylation in the hippocampus.

Chromatin remodeling is a complex system, and with the introduction above, we have only peeked at the tip of the iceberg. There are even more layers of complexity among DNA methylation and histone modification, for example, histone lysine methylation has been shown to trigger DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002). The interactions between histone acetylation and methylation are also indicated by studies of histone acetyltransferase CBP/p300 (CREB-binding protein) that the CBP co-immunoprecipitates with an HMT (Vandel and Trouche 2001). It has also been shown that acetylated H3 and H4 are the preferential targets of histone methylation (Annunziato et al. 1995). The HDAC inhibitor sodium butyrate (NaB) increased trimethylation of H3K4 and decreased dimethylation of H3K9 in the hippocampus during consolidation of fear-conditioned memories (Gupta et al.
suggesting that histone acetylation and methylation are closely associated in the regulation of synaptic plasticity. Another interesting finding is that DNA methylation of cytosines in CpG islands is associated with the transcriptional silencing of human tumor suppressor genes via the recruitment of methyl-binding proteins (MBPs) and their associated repressive complexes, including Sin3A and NuRD, which also contain HDACs (Ahringer 2000; El-Osta and Wolfe 2000). Other modifications of histones, although not discussed here in detail, are also instrumental in chromatin remodeling. For example, Ser10 phosphorylation is often associated with inhibition of Lys9 methylation (Rea et al. 2000) but during mitogenic and hormonal stimulation in mammalian cells, Ser 10 phosphorylation is paired with Lys9 or Lys14 acetylation (Cheung et al. 2000). On the other hand, Lys9 methylation blocks Ser10 phosphorylation, resulting in mitotic chromosome dysfunction (Rea et al. 2000). One prerequisite of Lys9 methylation however, requires deacetylation of H3 Lys14 (Nakayama et al. 2001). These findings suggest that histone modifications and DNA methylation could influence one another in either a synergistic or an antagonistic way and therefore regulate gene transcription.

1.5.2.3 Alcoholism and chromatin remodeling

Alcohol exposure is associated with chromatin changes (Mahadev and Vemuri 1998; Bonsch et al. 2005; Kim and Shukla 2006), for example, alcohol administration induced an open chromatin structure in rat brain (Mahadev and Vemuri 1998). Preclinical studies revealed that alcohol exposure selectively acetylates H3 at lysine 9 (H3K9) in a primary culture of rat hepatocytes (Park et
al. 2003) as well as rats in vivo (Kim and Shukla 2006). Acute alcohol exposure also caused inhibition of amygdaloid HDAC activity and increases acetylation of H3 and H4, consequently increasing CBP and NPY gene expression in the CeA and MeA of rats (Pandey et al. 2008). Cellular tolerance at the level of HDAC and NPY in the amygdala may be involved in the process of alcohol tolerance after chronic exposure (Sakharkar et al. 2012). Alcohol withdrawal increase HDAC activity and decrease H3 and H4 acetylation in the rat amygdala, and attenuated expression of NPY (Pandey et al. 2008), however, TSA rescued the withdrawal-induced anxiogenic effect and deficits of CBP and NPY expression (Pandey et al. 2008), indicating the possible reversible modulation of chromatin structure during ethanol withdrawal.

Interestingly, BDNF is one of the most commonly studied genes regulated by epigenetic modifications. Histone 3 methylation on lysine 27 has been shown to decrease transcription while histone H3 and H4 acetylation is associated with increased transcription of BDNF (Tsankova et al. 2007). BDNF gene transcription is suggested to be down-regulated by the methyl-CpG binding protein 2 (MeCP2), which forms a complex with HDAC1 and the co-repressor Sin3a at the BDNF promoter IV region (Im et al. 2010). BDNF exon VI expression is associated with dissociation of MeCP2 from the promoter region, preventing HDACs recruitment, eventually leading to acetylation of histone H4 on exon VI (He et al. 2010). On the other hand, phosphorylation and dissociation of MeCP2 from the BDNF promoter is observed after membrane depolarization and increased BDNF expression (Chen et al. 2003). Therefore, chromatin
remodeling, especially histone acetylation may be involved in the regulatory mechanism of BDNF in alcoholism.
1.6 Animal Models for Anxiety and Alcoholism

1.6.1 HAD/LAD rats:

Alcoholism is a very complex disorder and can be caused by both environmental and genetic factors. Clinical studies of twin, family, and adoption cases indicated alcoholism is a heritable disease with over 50% of estimated heritability (Heath et al. 1997; Froehlich et al. 2000). To provide neurobiological basic of alcoholism, several animal models have been developed. Animals that were selectively bred to exhibit alcohol preference have similar phenotypic traits with human conditions, thus have served as powerful tools in alcoholism research. One example of these lines of rats is the high alcohol drinking (HAD) and low alcohol drinking (LAD) rats. The HAD/LAD rats were generated from the N/Nih heterogeneous stock rats, which were developed by crossing eight inbred strains ACI/N, WKY/N, F344/N, BUF/N, BN/Ssn, WN/N, M520/N, and Mr/N (Li et al. 1987; Li et al. 1993). These HAD/LAD rats were selectively bred for high and low alcohol preference through systematic mating of rats from the same level of alcohol preference for over several generations to obtain lines exhibiting different alcohol drinking behavior (Li et al. 1993; Murphy et al. 2002). Within-family selection and a rotational breeding design were used to discourage inbreeding (Li et al. 1993). The selection criteria for the lines are that HAD rats voluntarily consume 10% (v/v) alcohol of more than 5g/kg/day and have an ethanol to water ratio of higher than 2:1, whereas LAD rats drink less than 1.5g/kg/day, and their ethanol to water consumption ratio is less than 0.5:1 (Murphy et al. 2002). The alcohol preference differs tremendously between the counterparts in this pair of
lines. After generations of selectively breeding, the HAD lines now have 9.5 g/kg/day average voluntary ethanol consumption while LAD rats now consume approximately 0.5 g/kg/day (Murphy et al. 2002). Given the difference of ethanol elimination rate in rats and humans, we can compare the average consumption of HAD rats as 8 standard-size alcoholic drinks by a 150–160 pound person per day. This selectively breeding method is designed to obtain lines of rats with consistent trait-relevant genes, while eliminate fixation of trait-nonrelevant genes (Murphy et al. 2002). These characteristics of the HAD/LAD lines made them good animal models for studying genetic factors involved in high alcohol drinking behavior.

Although sharing the same N/Nih heterogeneous stock rats (Li et al. 1993), HAD1/LAD1 and HAD2/LAD2 were independently derived. Therefore despite the fact that these two pairs of lines share the name and many properties, they also exhibit variations. Take drinking pattern for instance, although HAD1 and HAD2 rats have similar total voluntary consumption of alcohol per day, HAD1 have fewer episodes but larger volumes than HAD2 (Files et al. 1998; Samson et al. 1998). However, the two replicates of HAD1/LAD1 and HAD2/LAD2 rats give them advantage in terms of confidence in association between alcoholism with traits found in both replicates of HAD/LAD.

Similar to HAD/LAD, there is another pair of animal models that were selectively bred for high and low alcohol preference from an outbred Wistar rat stock. They are alcohol preferring (P)/ and -nonpreferring (NP) rats. Both HAD and P rats show early onset of alcohol preference, with HAD rats during
adolescence, and P rats as early as postnatal days (PNDs) 22–25 (McKinzie et al. 1998). P/NP and HAD/LAD rats also share some behavior phenotypes. Righting reflex tests (when an animal is flipped onto their back and time is recorded until they are able to “right” themselves onto their stomach) show that P (Kurtz et al. 1996) and HAD1 (Froehlich and Wand 1997) rats are slower in losing but faster in regaining the righting reflex, indicating P and HAD rats have less sensitivity to alcohol than NP and LAD rats. P and HAD rats also show greater novelty seeking behavior than NP/LAD rats (Nowak et al. 2000). HAD rats also express many similarities in neurobiological and neurochemical phenotypes with P rats, compared with their low alcohol drinking counterparts. For example, it was found that compared with LAD rats, HAD rats have lower serotonin (5-HT) levels in cerebral cortex, striatum, nucleus accumbens, hippocampus and hypothalamus (Gongwer et al. 1989). Similarly P rats also show lower 5-HT levels in these brain regions compared with NP rats (Hwang et al. 1999). Studies on brain dopamine (DA) contents indicate that P and HAD rats have lower levels of DA in nucleus accumbens compared with their nonpreferring counterparts (NP and LAD rats) (Murphy et al. 1982; Murphy et al. 1987; Gongwer et al. 1989). Higher densities of GABAergic terminals were also identified in nucleus accumbens of naïve P and HAD rats compared with low alcohol drinking NP and LAD counterparts (Hwang et al. 1990). More interestingly, HAD rats have lower innate NPY expression in CeA (Hwang et al. 1999), a phenomenon often observed in P rats (Hwang et al. 1999; Pandey et al. 2005; Zhang et al. 2010) and other animal models with high anxiety level such as
rats withdrawn from chronic ethanol drinking (Roy and Pandey 2002). Intracerebroventricular or CeA infusions of NPY significantly decreased ethanol intake in P rats (Badia-Elder et al. 2001; Pandey et al. 2005; Zhang et al. 2010) and HAD1 (Thiele and Badia-Elder 2003), but not in NP or LAD1 rats.

Although the P/NP and HAD/LAD strains share some ethanol drinking phenotypes, P/NP rats show more consistent phenotypic differences than between HAD and LAD rats. HAD/LAD rats lines were from a more heterogeneous foundation stock than P/NP strains, therefore HAD/LAD strains can exhibit greater genetic diversity than P/NP rats. However, HAD/LAD rats have an advantage over P/NP rats that there are two pairs of lines: replicate 1 and 2 of HAD/LAD. Therefore, a more confident conclusion might be obtained if both replicates exhibit similar results.

1.6.2 Anxiety in HAD/LAD rats:

Compared with NP rats, P rats have been shown to have higher innate anxiety levels as indicated in many studies (Stewart et al. 1993; Hwang et al. 1999; Pandey et al. 2005; Moonat et al. 2011). Less is known about HAD/LAD rats compared with P/NP rats (Rodd et al. 2004). However, a working hypothesis is that when similar differences are found in both line pair, it is more likely that the alcohol drinking characteristics between the lines are resulted from those differences. Therefore similarities in behavioral and neurobiological/neurochemical characteristics of HAD/LAD rats with P/NP rats suggested the possibility that HAD rats are more anxious than LAD rats. A recent study on selectively bred animal models for alcoholism gave a comprehensive
behavioral profiling of HAD/LAD rats. Although the authors observed only minor overall behavioral differences between HAD and LAD rats, the EPM test indicated that HAD1 rats spent more time on the closed arms and less time in the center compared with the LAD1 rats, while HAD2 rats spent less time in total and shorter time per visit on the open arms compared with the LAD2 rats (Roman et al. 2012). In addition, an interesting observation revealed that HAD rats have higher ultrasonic vocalization in response to stress than their counterpart rats (Overstreet et al. 1997). Since rate of vocalization can be reduced by anxiolytic drugs (Kehne et al. 2000), this behavior appears to be associated with anxiety levels. Due to excessive cue-elicited fear and anxiety, both HAD1 and HAD2 rats exhibit excessive freezing to the cue used to signal a fearful stimuli (Rorick et al. 2003), which interfered with their ability to learn avoidance response (Rorick et al. 2003). These avoidance learning deficits could be reversed by anxiolytic effect of ethanol (Rorick et al. 2003), suggesting that HAD rats serve as a reliable model of the anxiety vulnerability to alcohol abuse. We hypothesis that there are innate difference of anxiety levels between HAD and LAD rats. Only one anxiety behavioral test method (EPM) was used in Badia-Elder’s study to measure baseline anxiety of female HAD1/LAD1, which is not enough to conclude innate anxiety-like behavior of HAD rats. Therefore, in the current thesis, we measured the anxiety levels using an additional behavior test the light/dark box exploration test (LDB) to determine the baseline anxiety level of HAD/LAD rats.
1.7 Summary and Rationale

Anxiety is one of the major factors driving strong alcohol cravings through the negative reinforcement leading to alcohol drinking relapse (Koob 2003; Pandey et al. 2003), therefore anxiety contributes greatly to the development of alcoholism. Three models of anxiety behavior are used in the current thesis:

The first model of anxiety is induced by pharmacological blockage of Erk1/2 signaling in the central amygdala (CeA) of normal rats. The protective role of BDNF in anxiety and alcoholism is well documented (Pandey et al. 2006; Moonat et al. 2011), and Erk1/2 signaling is one of the downstream pathways activated by BDNF and has been shown to be involved in anxiety and alcohol addiction (Thoenen 1995; Impey et al. 1999; Ying et al. 2002; Einat et al. 2003; Pandey 2004). Arc is a target gene of BDNF located downstream of the Erk1/2 signaling pathway, and is believed to be involved in synaptic plasticity. Although there is evidence that all three points of this signaling cascade are involved in anxiety and alcoholism, it is not known whether the same cascade is regulating these functions within the amygdala. Infusion of BDNF into the dentate gyrus results in activation of Erk and up-regulated Arc mRNA and protein expression (Ying et al. 2002). MEK inhibitor U0126 infusion blocked the above effect of BDNF, indicating that BDNF prompts Arc expression through Erk signaling (Ying et al. 2002). Therefore, we manipulated this signaling cascade by systematic infusions of BDNF, MEK inhibitor U0126, and Arc antisense ODNs. By monitoring the anxiety-like and alcohol drinking behavior, and measuring the cellular changes induced by these pharmacological infusions, we will be able to address the role
of central amygdaloid extracellular signal-regulated kinase (Erk1/2) signaling and Arc expression in BDNF regulated anxiety-like and alcohol-drinking behaviors.

The second model of anxiety is provoked by withdrawal from chronic ethanol exposure. Anxiety is one of the major alcohol withdrawal syndromes which act as a triggering factor for the reinitiation of drinking, thus drinking relapse (Koob 2003). Alcohol withdrawal induced anxiety is correlated to increased HDAC activity and decreased histone acetylation (H3-K9 & H4-K8) in amygdala (Pandey et al. 2008). Treatment with the HDAC inhibitor trichostatin A (TSA) during ethanol withdrawal rescued these deficits (Pandey et al. 2008), indicating a possible role of chromatin remodeling in the process of alcohol addiction. In addition, decreased BDNF, activity-regulated cytoskeleton-associated protein (Arc) expression and dendritic spines in the CeA and MeA have been shown to be involved in ethanol withdrawal-related anxiety and alcohol drinking behaviors in rats (Pandey et al. 2008; Moonat et al. 2011). Therefore, we examined the role of chromatin remodeling in the regulation of BDNF and Arc expression and associative changes in dendritic spines in the amygdaloid brain regions during ethanol withdrawal after chronic exposure by treatment with TSA.

The third model of anxiety is an innate anxiety-like behavior in alcohol drinking (HAD) and low alcohol drinking (LAD) rats, selectively bred to have high-alcohol-drinking and low-alcohol-drinking phenotypes, respectively. The observed anxiety levels of HAD/LAD rats have been less than defined in previous literatures. An early study indicated that HAD rats do not exhibit significant differences in anxiety-like behavior compared with LAD rats (Badia-Elder et al.
However, behavioral and neurobiological characteristics of HAD/LAD and their similarities with alcohol preferring/ non-preferring (P/NP rats suggested a possibility that the alcohol drinking characteristics between the rats strains are result from those differences. Additional evidence is that, under stress, P and HAD rats have higher ultrasonic vocalization (Overstreet et al. 1997), a phenomenon that could be reduced by anxiolytic drugs (Kehne et al. 2000). We hypothesized that HAD rats may show higher innate anxiety levels as compared to LAD rats using the light-dark-box paradigm (LDB). A previous study in our lab indicated that P rats, compared to NP rats, have innate deficits in BDNF and Arc levels, suggesting the involvement of this signaling pathway on anxiety-like and alcohol-drinking behavior in these rats (Moonat et al. 2011). Therefore to study whether BDNF-Erk1/2 signaling is involved in the genetic predisposition to co-morbidity of anxiety and alcohol drinking behaviors, we measured baseline levels of anxiety and amygdaloid BDNF-Erk1/2 signaling activity in HAD/LAD rats.
1.8 Specific Aims

The overall objectives of this study are to identify and characterize the molecular mechanism(s) by which amygdaloid BDNF-Erk1/2 signaling pathway may regulate anxiety-like and alcohol-drinking behaviors. We hypothesize that BDNF-Erk1/2 signaling in the amygdala may be critical in regulating both innate and induced anxiety-like behavior through regulation of Arc expression. BDNF Erk1/2 signaling may regulate Arc expression through chromatin remodeling and thereby regulate synaptic plasticity. These objectives and hypothesis will be addressed by following specific aims:

**Specific Aim 1:** To examine the role of central amygdaloid BDNF-Erk1/2 signaling and Arc expression in anxiety-like and alcohol-drinking behaviors.

- **a.** To determine the effect on anxiety-like and alcohol-drinking behaviors by activation and inhibition of BDNF-Erk1/2 signaling
- **b.** To determine the status of Erk1/2 phosphorylation, CREB phosphorylation and Arc expression during activation and inhibition of BDNF-Erk1/2 signaling
- **c.** To determine if the Arc downstream pathway is involved in the BDNF regulated anxiety-like behavior by blocking Arc expression.

**Specific Aim 2:** To examine The effect of HDAC inhibitor treatment on amygdaloid BDNF and Arc expression, dendritic spine density, and anxiety-like behavior during ethanol withdrawal after chronic exposure.
a. To determine whether TSA has effect on anxiety-like behavior in ethanol withdrawal rats.

b. To determine the effect of TSA on BDNF and Arc expression in amygdaloid brain regions.

c. To determine the effect of TSA treatment on dendritic density during ethanol withdrawal.

**Specific Aim 3:** To examine the innate differences in anxiety levels, as well as in the expression of BDNF and CREB in the amygdala of high alcohol drinking (HAD) and low alcohol drinking (LAD) rats.

a. To determine the baseline anxiety levels in HAD1/LAD1 and HAD2/LAD2 rats.

b. To determine the levels of BDNF, CREB, phosphorylated CREB in amygdala of HAD1/LAD1 and HAD2/LAD2 rats.
2. THE ROLE OF CENTRAL AMYGDALOID ERK1/2 SIGNALING AND ARC EXPRESSION IN BDNF REGULATED ANXIETY-LIKE AND ALCOHOL-DRINKING BEHAVIORS.

2.1 Abstract

Alcohol addiction is a chronic disease with tremendous social impacts. The anxiolytic effect of alcohol self-medication is a major contributor that drives strong alcohol cravings. Studies have shown a possible involvement of amygdaloid brain-derived neurotrophic factor (BDNF) signaling in anxiety and alcohol drinking. BDNF may regulate immediate early gene, activity-regulated cytoskeleton-associated protein (Arc) expression via activation of extracellular signal-regulated kinase (Erk1/2). However, it is not known if amygdaloid BDNF-linked Erk1/2 signaling and Arc expression act as regulator of anxiety-like behavior and alcohol preference. Therefore, we investigated the effects of pharmacological manipulations of the BDNF-Erk1/2 signaling and Arc expression in the central amygdala (CeA) on anxiety-like and alcohol-drinking behaviors in rats.

We found that infusion of an Erk kinase inhibitor (U0126) into the CeA provoked anxiety-like behavior in rats, and decreased phosphorylation of Erk1/2, phosphorylation of CREB, expressions of Arc mRNA and protein. BDNF infusion into the CeA produced anxiolytic effects in rats. It also increased Erk1/2 and CREB phosphorylation, Arc mRNA and protein expressions. Interestingly, co-infusion of U0126 with BDNF blocked the behavioral and molecular effects of
both agents. We also found that Arc antisense oligodeoxynucleotides (ODNs) co-infusion with BDNF also attenuated the anxiolytic effect of BDNF. It was found that U0126 infusion into the CeA was able to increase alcohol preference, as measured by two-bottle free-choice paradigm. This increase was also diminished by CeA co-infusion of BDNF with U0126. These data suggest that the decreased functioning of the BDNF-Erk1/2 signaling pathway and decreased downstream Arc expression in the CeA may be involved in the molecular mechanisms of anxiety-like and alcohol-drinking behaviors.
2.2 Introduction

Because of the social and economical impacts of the alcoholic population, it is very important to understand the cause of alcoholism and to develop methods of prevention and treatment of this disorder. One major factor of alcohol addiction, which drives strong alcohol craving, is the anxiety induced by either genetic predisposition or environmental stimulus (Koob 2003; Castle 2008). Anxiety plays a crucial role in the initiation of alcohol drinking and maintenance of drinking through the negative reinforcement of alcohol. Clinically, this is characterized as one of the five subtypes of alcoholism: anxiopathic subtype (Cardoso et al. 2006). Studies using animal models have also shown that innate anxiety plays a critical part in the initiation of alcohol drinking (Pandey 2003; Thorsell 2008). Therefore, the cellular mechanisms of anxiety-associated alcohol drinking behavior is an important piece in the puzzle of the pathogenesis of alcoholism, and thus is critical for identifying the possibilities for target drug developments.

Amygdaloid brain regions are responsible for autonomic and neuroendocrine responses to stress and anxiety (Davis 1997; Sajdyk et al. 1999; de la Mora et al. 2012). The central nucleus of amygdala (CeA) in particular, has been shown to play an essential role in anxiety disorders (Davis 1997), and initiation and maintenance of alcohol addiction (Koob et al. 1998; McBride 2002; Koob 2003; Pandey 2003; Pandey 2004).
Studies have suggested that brain derived neurotrophic factor (BDNF) may play a crucial role in regulating anxiety-like and alcohol-drinking behaviors (Koob et al. 1998; Hensler et al. 2003; McGough et al. 2004; Pandey et al. 2008; Colzato et al. 2011; Moonat et al. 2011). BDNF is a member of the nerve growth factor family of neurotrophins and is highly involved in synaptic plasticity (Thoenen 1995; Thoenen 2000; Poo 2001; Carter et al. 2002; Monteggia et al. 2004). Among three well known downstream signaling pathways of BDNF, the MAPK pathway leading to phosphorylation of Erk1/2 is closely associated with neuronal plasticity (Impey et al. 1999), anxiety-like (Einat et al. 2003; Di Benedetto et al. 2009) and alcohol-drinking behaviors (Pandey 2004). BDNF-Erk1/2 signaling induces phosphorylation of CREB, leading to expression of downstream genes. One of the genes regulated by this pathway is the effector immediate-early gene, activity-regulated cytoskeleton-associated protein (Arc, also known as Arg 3.1). Arc has been shown to be rapidly localized to dendritic spines during synaptic stimulation (Link et al. 1995; Lyford et al. 1995; Steward and Worley 2001) and is critical for synaptic plasticity (Rodriguez et al. 2005; Beique et al. 2011; Shepherd and Bear 2011). BDNF can induce LTP in the hippocampus through activation of Erk1/2 and via increased expression of Arc (Ying et al. 2002). In vivo study has also revealed that BDNF provoked transcription of Arc through MEK1/2 dependent anterograde signaling (Cohen et al. 2011). The anxiolytic effect of acute ethanol has been shown to accompany increases of BDNF and Arc expression, and Erk1/2 phosphorylation, while decreased BDNF-Erk1/2 signaling and Arc expression were associated with
anxiety-like behavior during ethanol withdrawal in rats (Pandey et al. 2008). Therefore, we hypothesize that the regulatory mechanisms of BDNF on anxiety-like and alcohol-drinking behaviors might involve in the Erk1/2 signaling pathway, and subsequently Arc gene expression. To address this hypothesis, we manipulated BDNF levels, Erk1/2 signaling activities, and Arc expression in the CeA to determine their roles in anxiety-like and alcohol-drinking behaviors in rats.
2.3 Materials and Methods

**Animals and implantation of cannulas:**

All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (approximately 2 months old) were housed individually in a temperature-controlled room with a 12/12-hr light/dark cycle, with food and water provided *ad libitum*. Rats were anesthetized using sodium pentobarbital [50mg/kg, intraperitoneal (I.P.)], placed in a stereotaxic apparatus. CMA/11 guide cannulas (CMA Microdialysis, North Chelmsford, MA, USA) were implanted bilaterally targeting 3 mm above the CeA. Cannulas were covered with guided caps (CMA Microdialysis) and then secured to the skull using dental cement and screws. The coordinates for CeA are: 2.5 mm posterior and ± 4.2 mm lateral to the bregma and 5.1 mm ventral from the point of entry at the skull surface, as previously published by our lab (Pandey et al. 2006; Pandey et al. 2008). Total of 106 rats were used in this experiment.

**BDNF/U0126/Arc sense/antisense infusion:**

After a recovery period of 1 week, rats were subjected to intra-CeA infusions, using a microdialysis probe attached to an automatic pump. The microdialysis probe extended 3 mm beyond the guide cannula into the CeA to deliver pharmacological agents.
In BDNF/U0126 infusion experiment, rats were randomly separated into five groups: (1) aCSF + 1% DMSO; (2) BDNF + 1% DMSO; (3) aCSF + 100ng U0126; (4) aCSF + 250ng U0126; and (5) BDNF + 250ng U0126. Paired infusions were given 15 minutes apart and once daily for three days. The behavior tests were performed 1 hour after the last infusion. BDNF (dose: 50ng/0.5µl/rats, Sigma, B3795-5µg) was dissolved in aCSF, U0126 (two doses: 250ng/0.5µl/rats or 100ng/0.5µl/rats, Calbiochem, 662005) was dissolved in DMSO and then diluted to 1% DMSO by aCSF.

In BDNF/Arc sense/antisense experiment, the following groups were used for infusions: (1) aCSF + aCSF; (2) aCSF + Arc antisense; (3) BDNF + aCSF; (4) BDNF + Arc antisense; and (5) aCSF + Arc sense. BDNF (dose: 50ng/0.5µl/rats) infusions were given at 48 hours, 24 hours, and 1 hour before behavior as described previously. Arc antisense (dose: 50pmole/0.5µl/rats, sequence: 5'-GTCCAGCTCCATCTGCTCGC-3', Integrated DNA technologies. Inc) and sense (dose: 50pmole/0.5µl/rats, sequence: 5'-GCGAGCAGATGGAGCTGGAC-3', Integrated DNA technologies. Inc) were dissolved in aCSF, and were given 12 hours before behavior tests. This sequence is derived from bases 209–228 of the Arc gene sequence based on previous literature (Lyford et al. 1995), and was used in our previous study (Pandey et al. 2008).

**Measurement of Anxiety-Like Behavior by Elevated Plus Maze (EPM) Test.**
The EPM test procedure was the same as previously described by us (Pandey et al. 2008). The EPM apparatus has 2 open arms and 2 closed arms arranged directly opposite of each other and connected to a central platform. After a 5-minute habituation period, the subject was placed on the central platform facing an open arm. The observation period for each rat’s exploration abilities to both open and closed arms was 5 minutes. The number of entries made to open or closed arm was recorded. EPM results were expressed as the mean ± SEM of the percent of open-arm entries and the mean percent of time spent on the open arms (open-arm activity). The total number of the arm entries on the EPM represented the general activity of the each rat.

Immediately after behavioral testing, rats were perfused, and brains were collected and used for gold-immunolabeling and in situ RT-PCR studies.

**Gold-Immunolabeling Procedure for phosphorylated Erk1/2, phosphorylated CREB, Arc Protein and neuron-specific neuronal proteins in rat brain:**

After EPM tests, rats were anesthetized [50mg/kg, intraperitoneal (I.P.)], and perfused intracardially with 200ml of n-saline, followed by 300ml of 4% ice-cold paraformaldehyde (PFA) fixative prepared in 0.1M phosphate buffer (pH 7.4). After perfusion, brains were dissected out and post-fixed overnight in PFA at 4 °C, then cryoprotected in a sucrose gradient (10%, 20%, and 30%) prepared in 0.1M phosphate buffer (pH 7.4). Brains were later frozen and 20 mm coronal sections were collected using a cryostat. We used the gold-immunolabeling
histochemical procedure, as previously described by us (Pandey et al. 2008; Moonat et al. 2011), to measure cellular expression of phosphorylated Erk1/2, phosphorylated CREB, Arc protein and neuronal marker (NeuN). The brain sections were washed (two times for 10 min) with 0.01 M PBS and then blocked with RPMI 1640 (with L-glutamine) medium (Invitrogen, Auckland, New Zealand) for 30 min, followed by 10% normal goat serum (diluted in PBS containing 0.25% Triton X-100) for 30 min then 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were further incubated with following antibodies: anti-pCREB (Ser133, Millipore, 06-519, 1:500 dilution), anti-pErk1/2(Thr202/Tyr204, Cell Signaling, 9101, 1:200 dilution), anti-Arc (H-300, Santa Cruz, SC-15325, 1:200 dilution), anti-NeuN (Millipore, NG1715199, 1:200 dilution) in 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 18 h at room temperature. After two washes for 10 min each with PBS and two washes for 10 min each with 1% BSA in PBS, sections were incubated with gold particle (1.4 nm) conjugated anti-rabbit or anti-mouse secondary antibody (Nanoprobes, Yaphank, NY; 1:200 dilution in 1% BSA in PBS) for 1 h at room temperature. Sections were further rinsed several times in 1% BSA in PBS followed by rinsing in double-distilled water. The gold immunolabeling was developed using Silver Enhancement Solution (Ted Pella, Redding, CA) for 15–30 min, and then sections were washed several times using tap water before mounted on slides. Gold-immunolabeled proteins were quantified using the Loats Image Analysis System (Loats Associates Inc., Westminster, MD) connected to a light microscope that calculates the number of gold particles/100 µm² at high
magnification (100x). The threshold of each image was set up to ensure that an area without staining should give zero counts. Under this condition, gold particles in the defined areas (3 randomly chosen fields within each brain structure in each brain section) of 3 brain sections (bregma: 2.12-2.56 mm) of each rat were counted and values averaged for each rat. Total of 9 fields were counted for each brain structures for each rat. The results were represented as the number of immunogold particles/100 μm² area of a defined amygdaloid structure (CeA, MeA, and BLA).

**In situ RT-PCR for Arc mRNA Measurement.**

We used *in situ* RT-PCR procedure as we have previously reported (Pandey et al. 2008; Moonat et al. 2011) to measure Arc mRNA expression in the amygdala. Briefly, 40 μm free-floating brain sections were treated with proteinase K (1µg/ml in 1X DEPC-PBS containing 0.05% Triton X-100) for 15 minutes at 37°C, followed by washing with 1X DEPC-PBS, the sections then were subjected to DNase digestion. After another washing with 1X DEPC-PBS, sections were transferred to PCR tubes containing 100 μl of reverse transcription reaction mixture (Applied Biosystems, Foster City, CA) and were reverse transcribed with reverse transcriptase enzyme in the presence of oligo(dt). For negative sections there were no reverse transcriptase enzyme. Then PCR was performed with Taq DNA polymerase enzyme and primers. The primer sequences we used are based on previous studies (Pandey et al. 2008; Moonat et al. 2011): 5′-ACAGAGGATGAGACTGAGGCAC 3′ and 5′ TATTCAAGGCTGGGTGCTGTCAC 3′. PCR conditions for Arc mRNA are: 94°C for 5 min; 95°C for 15 sec; 55°C for
30 sec; 72 °C for 30 sec; total of 30 cycles and then 72 °C for 3 min. After PCR, sections were mounted on slides. Arc-positive cell bodies were detected using an alkaline phosphatase-conjugated anti-DIG antibody and subsequent staining of the complex with the specific substrate, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics, Indianapolis, IN). The optical densities (OD) of positive cell bodies were calculated by the Loats Image Analysis System (Loats Associates Inc., Westminster, MD). The ODs of negative brain sections were subtracted from the ODs of positive brain sections. The mean OD of Arc-positive cell bodies in the central, medial and basolateral amygdala were calculated. Values were averaged for each rat. The results were represented as mean OD/100 pixels of area for Arc mRNA levels.

**Ethanol/Sucrose preference measurement:**

Ethanol and sucrose preference were measured by the two-bottle free-choice paradigm used previously by us (Pandey et al. 2006). Rats were bilaterally implanted with cannulas targeted 3mm above the CeA as described above. After a recovery period of 1 week, rats were habituated to drinking water from two bottles. Once they started drinking water equally from both bottles, rats were randomly assigned to four groups: (1) aCSF + 1% DMSO; (2) BDNF + 1% DMSO; (3) aCSF + U0126 and (4) BDNF + U0126. BDNF (50ng/0.5µl/rats) and U0126 (250ng/0.5µl/rats) were infused once daily for 3 days; 1 hour after last infusion, rats were subjected to 7% (v/v) ethanol or 2% sucrose drinking. For the sucrose intake experiment, there were only two groups: 1) 1% DMSO and 2) U0126. Ethanol or sucrose was given in one bottle and water in the other bottle.
Sides of the ethanol or sucrose bottles were switched with water bottles every day until drinking was back to normal. Consumptions of ethanol/sucrose and water were measured daily at 5:00 P.M., and fresh water and 7% (v/v) ethanol solution were provided every day. The mean percentage of ethanol/sucrose intake and percentage of water intake were calculated from their total fluid intake (in milliliters) for each day. The rats were then perfused, and brains were processed for cresyl violet staining to check the position of cannulas or tissue damage.

**Analysis of Results:**

The differences between the various groups for anxiety-like behavior and neurochemical data were evaluated by one-way ANOVA tests. The alcohol intake data was evaluated by repeated-measure ANOVA with day as the within-subject factor and experimental groups as the between-subject factor. *Post hoc* comparisons were performed using Tukey’s test.
2.4 Results

**Effect of intra CeA BDNF/U0126 infusion on anxiety-like behavior:**

Erk1/2 signaling pathway is one of the major pathways downstream of BDNF and has been implicated in anxiety disorders (Einat et al. 2003; Pandey et al. 2008; Di Benedetto et al. 2009). Therefore, to study the relationship between Erk1/2 signaling pathway activities in the CeA and anxiety-like behavior, we pharmacologically manipulated Erk1/2 signaling activities in the CeA by infusions of Erk kinase (MEK) inhibitor U0126, and compared the effects on anxiety-like behavior with BDNF and vehicle infusions. EPM tests were performed to measure anxiety-like behavior (Figure 1). We found that intra-CeA infusion of BDNF increased both percentage of open-arm entries (p <0.05) and percentage of time (p <0.001) spent on open arms, compared with vehicle infused rats. On the other hand, both doses (100ng and 250ng) of U0126 intra CeA infusion produced significant reduction of percentage of open-arm entries (p <0.001) and percentage of time (p <0.05) spent on open arms in rats. Co-infusion of BDNF with U0126 prevented the reduction in percentage of open-arm entries and time spent on open arms induced by U0126. As an indication of general activity, the total number of entries (closed and open arms) was not significantly different among the groups. In summary, U0126 infusion into the CeA provoked anxiety-like behavior and diminished the anxiolytic effect of BDNF in rats. These results indicate that BDNF in CeA via Erk1/2 signaling pathway might be involved in the regulation of anxiety-like behavior.
**Figure 1.** Intra-CeA infusion of U0126 produced anxiety-like behavior while BDNF infusion produced anxiolytic effects in rats. BDNF/U0126 co-infusion neutralized each other’s effect on anxiety-like behavior in rats. The effect of U0126 (100ng or 250ng/0.5µl/rat) and BDNF (50ng/0.5µl/rat) infusions on open arm and closed arm activities in the EPM test. Total number of entries represents the total numbers of closed- and open-arm entries in EPM. Values are the mean ± SEM of eight rats in each group. For % open arm entries F(4,39)=26.08, p<0.001; for % time spent in open arm F(4,39)= 27.38, p<0.001, significantly different from control (vehicle infused) rats (one way ANOVA followed by Tukey’s test).
Cellular changes in the CeA after BDNF/U0126 infusion.

To study the underlying molecular mechanisms of U0126 and BDNF induced changes of anxiety-like behavior in rats, brains were collected immediately after behavioral tests to measure protein levels of phosphorylated Erk1/2, phosphorylated CREB, NeuN, Arc protein and mRNA levels in amygdaloid structures of rats. As shown in Figure 2, U0126 infusion into CeA decreased phosphorylation of Erk1/2 (p<0.01) and CREB in the CeA of rats (P<0.001). It also resulted in a significantly decrease in the protein (P<0.001) and mRNA (P<0.001) levels of Arc in the CeA. On the other hand, BDNF infusions into CeA caused increases in the phosphorylation of Erk1/2 (P<0.001) and CREB (P=0.001) and increased Arc protein (P<0.001) and mRNA (P<0.001) levels in the CeA. These BDNF induced increases in signaling molecules were normalized by co-infusion with U0126 into CeA. In medial amygdala (MeA) and basal lateral amygdala (BLA), there were no significant differences among all groups on phosphorylation of Erk1/2, phosphorylation of CREB, and Arc mRNA and protein expression, suggesting that infused pharmacological agents were restricted to CeA. The visual observation of neuronal marker (NeuN) labeling indicate that there was no neuronal toxicity due to the infusions of 1% DMSO or U0126 or BDNF.

Taken together, these results raise the possibility that the anxiolytic effect of BDNF might be associated with increased phosphorylation of Erk1/2 and CREB, and increased expression of Arc in the CeA. U0126 promoted anxiogenic effects in rats that might be related to decreased activity of Erk1/2-CREB.
signaling and Arc expression. U0126 co-infusion with BDNF blocked the BDNF induced behavioral and molecular changes, suggesting that BDNF might regulate anxiety-like behavior through Erk1/2 signaling.

**Figure 2A**, Effect of central amygdaloid BDNF/U0126 infusion into CeA on mRNA and protein levels of Arc and protein levels of pErk1/2 and pCREB in amygdaloid brain regions of rats. Representative photomicrographs of phosphorylated Erk1/2, phosphorylated CREB, Arc protein gold-immunolabeling and Arc mRNA in situ RT-PCR in CeA structures of rats infused with U0126 or BDNF. Scale bar = 40 µm.
Figure 2B, Effect of BDNF/U0126 infusion into CeA on mRNA and protein levels of Arc and protein levels of pErk1/2 and pCREB in amygdaloid brain regions of rats. Bar diagram showing quantification of pErk1/2, pCREB, Arc mRNA and protein levels in CeA, MeA and BLA of rats. Values are mean ± SEM of eight rats in each group. For pErk1/2, F(3,31)= 51.71, p <0.001; For pCREB, F(3,31)= 25.01, p <0.001; For Arc, F(3,31)= 70.31, p <0.001; For Arc mRNA, F(3,31)= 31.13, p <0.001; significantly different from the control rats (one way ANOVA followed by Tukey’s test). Intra-CeA BDNF infusion increased phosphorylation of Erk1/2 and CREB, and increased the Arc expression on mRNA and protein levels in CeA of rats. Co-infusion of U0126 with BDNF reversed these effects. U0126 infusion caused decreases of phosphorylation of Erk1/2, phosphorylation of CREB, and Arc mRNA and protein expression. No significant difference of was observed in MeA and BLA of rats among groups.
*Arc antisense ODNs infusion into CeA provoked anxiety-like behavior: reversed by BDNF co-infusion.*

Both Arc mRNA and protein expression were altered by BDNF and U0126 infusion as we discussed above. It has been shown that Arc expression in the CeA was highly associated with anxiety-like behavior in rats (Pandey et al. 2008). This might indicate that BDNF-Erk1/2 signaling regulates anxiety-like behavior via modulating the downstream Arc gene expression. In order to determine the relationship between central amygdaloid BDNF and Arc expression, and their roles in the anxiety-like behavior, we infused Arc antisense/sense ODNs with or without BDNF co-infusion into CeA of rats, and measured the anxiety-like behavior of these rats using elevated plus maze (EPM) test.

The results are shown in Figure 3. Arc antisense ODNs infused rats had a decreased percentage of open-arm entries (p <0.05) and percentage of time spent on open arms (p <0.005), compared with control rats. BDNF infusions produced reciprocal effects (p <0.05) to that of Arc antisense infusion, indicated by the increased percentage of both open-arm entries and time spent on open arms. Interestingly, anxiety-like behavior of rats co-infused with Arc antisense ODNs and BDNF did not differ from that of aCSF infused rats. Arc sense ODNs infusion had no significant effect in anxiety-like behavior of rats. The total numbers of entries which indicating general activity, were not significantly different among the groups, suggesting that the treatment has no effect on the visual and movement behavioral aspects of the rats.
These data indicate that Arc antisense infusion into the CeA provoked anxiogenic effects in rats, whereas BDNF infusion decreased anxiety-like behavior. Co-infusion of Arc antisense with BDNF attenuated the anxiolytic effect of BDNF, consistent with the involvement of Arc in BDNF signaling regulated anxiety-like behavior.

* significantly increased p<0.05, n=5
# significantly decreased p<0.05, n=5
## significantly decreased p<0.005, n=5

Figure 3, Effect of intra-CeA infusion of Arc sense/antisense ODNs (with and without BDNF co-infusion) on open- and closed-arm activities of the EPM. Total number of entries represents the total numbers of closed- and open-arm entries in EPM. Values are the mean ± SEM of five rats in each group. For % open arm entries, F (4, 24)= 13.70,  p < 0.001; for % time spent in open arm, F(4,24)= 14.50, P<0.001, significantly different from control rats. (one way ANOVA followed by Tukey’s test)
Cellular changes by BDNF/Arc sense/antisense infusion into CeA:

Immediately following behavioral testing, rats brains were collected for gold-immunolabeling and in situ PCR to measure the Arc protein and mRNA levels, respectively. As shown in Figure 4, Arc antisense ODNs infusion resulted in a significant decrease in the expression of Arc protein (p <0.001) and mRNA (p <0.001) in the CeA. BDNF infusion increased expression of Arc protein (p<0.001) and mRNA (p<0.001) in the CeA, When co-infused with BDNF, Arc antisense ODNs blocked the BDNF-induced increases in Arc protein and mRNA levels. Infusion of Arc sense alone did not alter levels of Arc mRNA or protein. No significant difference of Arc expression was observed in MeA or BLA, suggesting that CeA-targeted infusions produced CeA-specific effects. These data, together with the anxiety-like behavior data, indicate that Arc synthesis is necessary for BDNF induced anxiolytic effects, thus suggesting Arc as a molecular effector for BDNF in synaptic plasticity.
Figure 4A, Effect of intra CeA Arc sense/antisense ODNs infusion with or without BDNF co-infusion on the levels of Arc mRNA and protein. Representative photomicrographs of Arc protein gold-immunolabeling and Arc mRNA in situ RT-PCR in CeA structures of rats infused with BDNF or Arc antisense/sense ODNs. Scale bar = 40 µm.
Figure 4B, Effect of intra CeA Arc sense/antisense ODNs infusion with or without BDNF co-infusion on the levels of Arc mRNA and protein. Bar diagram showing quantification of the effect of intra CeA Arc sense/antisense/BDNF infusion on Arc mRNA and protein levels. Values are mean ± SEM of five rats in each group.

For Arc protein, $F(4,24)= 137.76, p<0.001$; for Arc mRNA, $F (4,24)= 69.83, P<0.001$, significantly different from the control rats (one way ANOVA followed by Tukey’s test). Intra-CeA Arc antisense infusion decreased Arc mRNA and protein levels in CeA of rats. Co-infusion of BDNF reversed these effects.
Changes of BDNF-Erk1/2 signaling activity in the CeA altered alcohol preference of rats:

Anxiety is believed to be one of the factors of promoting drinking behavior, and is closely associated with development of alcoholism (Koob 2003; Pandey 2003; Cardoso et al. 2006; Castle 2008; Thorsell 2008; Moonat et al. 2011). Based on our observation that decreased activities of Erk1/2 signaling pathway induced anxiety-like behavior in rats, we hypothesized that there might be a close relationship between decreased BDNF-Erk1/2 signaling activities in CeA and alcohol preference in rats. Therefore, we tested the above hypothesis. After rats were cannulated and habituated with two bottle choice and were taking equal amount of water from both bottles, we gave these rats paired infusions of BDNF or/and U0126 for three consecutive days as described in the previous anxiety experiment, and subjects were given access to 7% ethanol intake 1 hour after the last infusion. Because both doses (250ng and 100ng) of U0126 yield the same anxiety-like behavioral effect in rats, we therefore used the higher dose (250ng) of U0126 in this experiment. The mean percentage of ethanol or water intake of their total fluid intake (in milliliters) for each day was calculated. A group x day repeated-measure ANOVA was performed on changes in alcohol intake that yielded a significant group effect (p < 0.001), a significant day effect (p < 0.001), and a significant interaction (p < 0.001) of these variables in rats. A group x day repeated-measure ANOVA was performed on changes in water intake that yielded a significant group effect (p < 0.001), a significant day effect (p < 0.001), and a significant interaction (p <0.001) of these variables in rats. The results are...
shown in Figure 5, it was found that U0126 (250ng) infused rats consumed significantly higher amounts of ethanol (figure 5B) and less water (figure 5C) in the first day post-infusion compared with control group (p<0.001). These differences decreased gradually, and disappeared by the end of the 4th day post-infusion. Although a decrease of ethanol consumption on the first day post-infusion was found between BDNF (50ng) infused and control rats, the difference did not reach statistical significance (p=0.170). Interestingly, BDNF and U0126 co-infused rats’ ethanol preferences were similar to that of control rats, indicating that BDNF does play a regulatory role in alcohol preference. The total fluid intake (in milliliters per day) was similar in all groups at each post-infusion day (Figure 5A). No significant body weight difference was found between all groups [mean ± SEM (n=7-8): Control = 343.71g ± 4.76, BDNF = 342.43g ± 2.81, U0126 = 338.13g ± 2.24, BDNF+U0126= 331.57g ± 5.35].
**Figure 5A.** Effect of intra-CeA infusion of U0126 with or without BDNF co-infusions on alcohol preference as measured by the two-bottle free-choice paradigm. Paired infusions were given once daily for three days and 1 hour after last infusion, 7% ethanol were introduced. No significant difference was observed in the total fluid intake (ml/day) of rats from different groups. **B,** Percentage of 7% v/v (ethanol in water solution) ethanol intake and **C,** percentage of water intake of total fluid intake (milliliters per day). Values are the mean ± SEM of seven to eight rats in each group, and alcohol intake was measured for 5 days in the same group of rats. * p <0.001 significantly different from control rats. (Repeated-measure ANOVA followed by Tukey’s test)
In order to determine whether increased ethanol preference is due to taste preference, we measured sucrose preference (figure 6) of rats by two-bottle-choice paradigm. After the same cannulation procedure as described above for alcohol preference, rats were infused with U0126 (250ng) or vehicle. 2% sucrose was offered in one bottle one hour after last infusion. Consumption of 2% sucrose and water were recorded. For 3 days post-infusion, there was no significant difference in 2% sucrose and water consumption and no significant difference in total fluid consumption between U0126 and vehicle infused rats on any post-infusion day. This data suggesting that effect of U0126 CeA infusion on ethanol preference is independent from taste preference.

![Figure 6](image_url)

**Figure 6** Effect of intra-CeA infusion of U0126 on 2% sucrose preference as measured by the two-bottle free-choice paradigm. No significant difference was observed in sucrose intake in percentage of total fluid intake between U0126 and vehicle infused rats.
Taking together, blocking Erk1/2 function by U0126 produced a time dependent effect on alcohol preference of rats, indicating that decreased Erk1/2 signaling function in the CeA of rats may be involved in promoting alcohol-drinking behavior. Although BDNF infusion alone did not induce a significant decrease of alcohol preference, co-infusion of BDNF attenuated the increased alcohol preference induced by U0126 infusion, indicating that effects of U0126 are reversible, and that Erk1/2 signaling may play a regulatory role in alcohol drinking behavior.
2.6 Discussion

The self-medication hypothesis of alcoholism development suggests that individuals with high anxiety may drink to self medicate themselves as ethanol produces anxiolytic effects (Swendsen et al. 2000; Carrigan and Randall 2003; Robinson et al. 2009). Clinical studies have shown that an individual with anxiety disorder has a significantly greater chance to be diagnosed with alcohol use disorder (Cardoso et al. 2006; Castle 2008). Our study provided evidence to suggest that BDNF-Erk1/2 signaling pathway in the CeA may be involved in anxiety-like and alcohol-drinking behaviors. Here, we found that increased anxiety levels in rats by intra CeA U0126 infusions were associated with increased alcohol drinking behavior. Similar correlations between innate anxiety levels and alcohol drinking behavior were also found in many animal models such as P/NP rats (Suzuki et al. 2004; Pandey et al. 2005; Moonat et al. 2011), CREB deficient mice (Pandey et al. 2004), Fawn-Hooded rats (Overstreet et al. 2009), and Long–Evans rats (Hayton et al. 2011). Other studies have also shown that knockdown of Arc and BDNF in the CeA using Arc antisense ODNs (Pandey et al. 2008) and BDNF antisense ODNs (Pandey et al. 2006) provoked anxiety-like behavior and produced higher alcohol preference in rats.

Several pieces of evidence indicate that BDNF involved in the development of alcohol addiction: for example, BDNF deficient mice display higher sensitivity to ethanol after ethanol withdrawal compared to wild type (McGough et al. 2004). When exposed chronically to ethanol, wild-type mice also show increased BDNF mRNA level in the dorsal striatum (McGough et al. 2004).
One early study in our lab had shown that CREB-haplodeficient (+/-) mice also have lower BDNF mRNA and protein levels in several brain regions including central amygdala. These CREB-haplodeficient (+/-) mice display higher anxiety level and voluntarily consume higher amounts of alcohol compared with wild-type (+/+ ) littermates (Pandey et al. 2004). Alcohol preferring (P) rats also have lower BDNF in CeA and MeA, and display higher innate anxiety levels compared to non-preferring (NP) rats (Prakash et al. 2008; Moonat et al. 2011). When P rats were exposed to ethanol acutely, the BDNF expression in CeA and MeA were increased, along with decreased anxiety level (Moonat et al. 2011). Similar associations were found in the anxiolytic effect of acute ethanol treatment with higher BDNF expression in CeA and MeA in SD rats (Pandey et al. 2008). On the other hand, alcohol withdrawal treatment provoked anxiety-like behavior in rats and decreased BDNF expression (Pandey et al. 2008). BDNF intra CeA infusion prevented development of anxiety-like behavior during ethanol withdrawal within CeA and MeA of rats (Pandey et al. 2008). In the current study we demonstrated that BDNF not only blocked U0126 induced anxiety-like behavior but also decreased anxiety from baseline level when infused into CeA of alcohol naïve rats.

Despite the anxiolytic effect of BDNF, we found it very interesting that BDNF infusion alone did not induce significant changes in alcohol intake. However, when co-infused with U0126, BDNF blocked the alcohol preference induced by U0126 infusion into CeA. There are two possible explanations for this observation. First, the alcohol-drinking behavioral effect of BDNF infusion might
be undetectable due to the low baseline alcohol drinking behavior of SD rats. Using other animal models, such as P/NP rats and HAD/LAD rats, which have much higher baseline alcohol consumptions, this behavioral change might be evident. Secondly, BDNF might act as a protector against anxiety and alcohol intake, while excessive BDNF in the CeA doesn’t affect normal drinking behavior. A previous study showed BDNF antisense ODNs infusion into CeA and MeA increased anxiety levels and alcohol preference of rats, and these behaviors were attenuated by co-infusion with BDNF (Pandey et al. 2006). BDNF (-/+ ) mice also have higher alcohol preference than wild type (Hensler et al. 2003), while BDNF infusion into dorsal striatum and hippocampus attenuated alcohol drinking in these mice (McGough et al. 2004). Therefore, the lack of regulatory effect of BDNF infusion alone on alcohol preference might indicate that BDNF functions to maintain the normal drinking behavior. Nevertheless, in the present study, BDNF attenuated the effects of U0126 on alcohol preference in rats. Taken together this research suggests a regulatory role of BDNF in alcohol-drinking behavior.

Study in rats with acute ethanol treatment and withdrawal from chronic ethanol exposure have suggested a strong link between BDNF expression and its downstream Erk1/2 signaling pathway, as well as immediate early gene Arc with alcoholism (Pandey et al. 2008). Here, we address the hypothesis that BDNF may regulate anxiety-like and alcohol drinking behaviors through the Erk1/2 signaling pathway and downstream Arc gene expression in the CeA.

In current study, infusion of BDNF into the CeA increased phosphorylation of Erk1/2 and CREB, and increased expression the levels of Arc mRNA and
protein. These molecular changes in the CeA produced anxiolytic effects in rats. On the other hand, Erk kinase inhibitor U0126 infusion induced opposite behavioral and molecular changes. Interestingly, BDNF and U126 co-infusion canceled out the changes induced by each other alone. These results indicate that the Erk1/2 signaling pathway may be one of the downstream pathways through which BDNF regulate anxiety-like and alcohol drinking behaviors. Additionally, Arc antisense ODNs infusion produced an anxiogenic effect similar to U0126 infusion in rats. BDNF infusion was also able to attenuate this anxiogenic effect and normalized the reduction in Arc expression in the CeA and MeA of rats. A recent study indicated that inducible BDNF knockout in mice induced significantly decrease in baseline Arc mRNA expression in the neocortex (Benekareddy et al. 2012). Knocking down BDNF expression by BDNF antisense also decreased phosphorylation of CREB (Pandey et al. 2006), which is a transcription factor that regulates Arc expression. These data together suggested Arc as a downstream mediator of BDNF signaling, through BDNF’S regulation of CREB. Collectively, our data demonstrate that decreased functioning of the Erk1/2 signaling pathway produces reduction in Arc expression in the CeA and thus may be an underlying molecular mechanism of anxiety-like and alcohol-drinking behaviors.

Arc has been shown to undergo transport to dendritic processes by synaptic activation (Link et al. 1995; Lyford et al. 1995; Steward and Worley 2001; Moga et al. 2004). The observations that increase of Arc expression by BDNF infusion, and a decrease of Arc expression by U0126 infusion in the CeA
suggest the possibility that BDNF-Arc signaling may regulate synaptic plasticity during anxiety and alcoholism. Arc expression has been closely associated with dendritic spine morphology (Peebles et al. 2010), and density (Pandey et al. 2008; Moonat et al. 2011). It has been shown that increased BDNF levels lead to expression of Arc, which in turn provokes synaptic spine morphology changes (Ji et al. 2010). Taken together the results presented here suggest that BDNF might regulate anxiety-like and alcohol-drinking behaviors by modulating the dendritic spine density and synaptic plasticity through Arc in the central amygdaloid circuitry in rats.
3. THE EFFECT OF HDAC INHIBITOR TREATMENT ON AMYGDALOID BDNF AND ARC EXPRESSION, DENDRITIC SPINE DENSITY, AND ANXIETY-LIKE BEHAVIOR DURING ETHANOL WITHDRAWAL AFTER CHRONIC EXPOSURE.

3.1 Abstract

Development of anxiety-like behavior during ethanol withdrawal has been correlated with increased histone deacetylase (HDAC) activity and decreased brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) gene expression in the amygdala. Furthermore, HDAC-mediated histone modifications play a role in synaptic plasticity. Here, we used HDAC inhibitor trichostatin A (TSA) to determine whether inhibition of HDAC could reverse ethanol withdrawal-induced deficits in dendritic spine density (DSD), BDNF and Arc expression in the amygdala of rats. Our results were in conjunction with a previous report that TSA treatment was able to attenuate anxiety-like behavior during ethanol withdrawal: however, we extended these findings and found that decreased BDNF and Arc expression in the central (CeA) and medial nucleus of amygdala (MeA) observed during withdrawal were normalized following TSA treatment in rats. The observed decrease in DSD in the CeA and MeA of ethanol-withdrawn rats was also corrected by TSA treatment. Taken together, these findings demonstrate that correcting the deficits in histone acetylation by TSA treatment also correct the deficits in synaptic plasticity-related events (BDNF, Arc expression and DSD) in the CeA and MeA.
as well as attenuated anxiety-like behavior in rats during withdrawal after chronic ethanol exposure.
3.2 Introduction

The development of alcohol dependence occurs following repeated ethanol exposures, which is mediated by both positive and negative effects of ethanol (Koob 2003; Gilpin et al. 2008). It is believed that the anticipation of the euphoric effect of alcohol promotes the positive reinforcement of alcohol intake, while relief from alcohol withdrawal symptoms fosters the negative reinforcing properties of alcohol drinking (Koob 2003; Pandey 2004; Grusser et al. 2006). Chronic ethanol exposure induced neuroadaptations in the specific neurocircuitry (Gilpin and Koob 2008; Moonat et al. 2010), produces withdrawal symptoms upon the cessation of alcohol consumption, which makes quitting drinking a great emotional and physical challenge. As one of the major alcohol withdrawal symptoms, anxiety is considered to be the key element in continuous consumption of alcohol, thus playing a critical role in the maintenance of alcohol addiction (Koob 2003; Pandey 2003).

The amygdala is a structure located in the rostromedial part of the temporal lobe (Haines 2006) which functions as the modulator of the autonomic system (Sah et al. 2003) and the emotional responses to fear and anxiety (Pessoa and Adolphs 2010). The central and medial nucleus of amygdala (CeA and MeA) serve as an output station for distributing the sensory information (Pitkanen et al. 1997) throughout other limbic regions (Haines 2006). Due to the large number of efferent and afferent connections to and from the amygdala, it plays a crucial role in alcohol addiction. Therefore, it is important to investigate the molecular mechanism of neuroadaptive changes in the amygdala due to
ethanol exposure to better understand the pathophysiology of alcoholism. Emerging epigenetic research provides us with a new mechanism for the molecular import of synaptic plasticity underlying alcoholism.

Chromatin remodeling, either due to histone modifications or changes in DNA methylation, is essential for neuronal survival, activity, and morphology, and is subsequently involved in the regulation of psychiatric disorders (Tsankova et al. 2007). Histone modifications occur on the amino (N)-terminal tail of histones and play a crucial role in switching between open and closed forms of chromatin structure and changing gene promoter accessibility for transcriptional machinery, thus causing alterations in gene transcription (Cosgrove and Wolberger 2005; Tsankova et al. 2007; Starkman et al. 2012). We have shown that development of anxiety-like behavior during alcohol withdrawal is correlated with increased histone deacetylase (HDAC) activity, decreased histone acetylation (H3-K9 & H4-K8), and neuropeptide Y expression in the amygdala, while treatment with the HDAC inhibitor trichostatin A (TSA) during ethanol withdrawal rescued these deficits (Pandey et al. 2008). In addition, decreased BDNF and activity-regulated cytoskeleton-associated protein (Arc) expression and dendritic spines in the CeA and MeA have been shown to be involved in ethanol withdrawal-related anxiety and alcohol drinking behaviors in rats (Pandey et al. 2008; Moonat et al. 2011). However, it is unknown if the correction of deficits in histone acetylation by TSA treatment can also correct deficits in synaptic plasticity related events produced during ethanol withdrawal after chronic ethanol exposure. We, therefore,
examined the effects of HDAC inhibition on amygdaloid BDNF, Arc expression and dendritic spines during alcohol dependence.
3.3 Materials and Methods

**Chronic ethanol treatment and TSA injection:**

All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats were housed individually in a temperature-controlled room with a 12/12-hr light/dark cycle, with food and water provided *ad libitum*. Rats were randomly separated into five groups: (1) Control-diet fed + Vehicle injection, (2) Ethanol-diet fed + Vehicle injection, (3) Withdrawal + Vehicle injection, (4) Withdrawal + TSA injection, (5) Control-diet fed + TSA injection. As described previously by us (Pandey et al. 2008), rats were offered 80 ml/day of the nutritionally complete Lieber-DeCarli liquid control diet (Lieber-DeCarli Diet 82; Bio-Serv, Frenchtown, NJ) for 3 days. Control groups continued with control diet, while ethanol groups gradually (1.8% through 9%) were introduced to ethanol diets, and then maintained on 9% v/v ethanol diet for 15 days. These rats were pair fed and their liquid diet intake and body weights were measured on weekly basis. Ethanol-diet fed groups (2, 3 & 4) were withdrawn from ethanol for 0 and 24 hr. The rats were intraperitoneally (i.p.) injected 2 hr before measuring anxiety-like behavior with either vehicle (DMSO, 1:5 dilution with PBS) or trichostatin A (TSA) [2 mg/kg; dissolved in DMSO and then diluted (1:5 dilution) with PBS] to assigned groups as reported earlier by us (Pandey et al., 2008a). Anxiety-like behavior of rats were measured by elevated plus maze (EPM) or light-dark-box exploration (LDB) tests. Immediately after behavioral measurements, rats were anesthetized using
pentobarbital (50 mg/kg) and brains were collected for gold immunolabeling, in situ RT-PCR, or Golgi-Cox staining, as described below. Blood was also collected to measure blood ethanol levels using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA). Total of 63 rats were used in this experiment.

**Elevated plus maze test (EPM):**

The test procedure was the same as previously described by us (Pandey et al. 2008; Pandey et al. 2008). After a 5-minute habituation period in test room, a test rat was placed on the central platform facing an open arm. The exploratory behavior to both open and closed arms of EPM was observed for 5 min. The number of entries made to and the time spent in open or closed arm was recorded. EPM test results were expressed as the percent of open-arm entries and the mean percent of time spent on the open arms (open-arm activity). The total number of closed arm and open arm entries on the EPM represented the general activity of the each rat.

**Light/dark-box (LDB) exploration test:**

The LDB exploration test procedure was used, as published by us previously (Pandey et al. 2008; Sakharkar et al. 2012). The light/dark box is located in a dark room and consisted of a dark compartment without illumination and a light compartment with illumination. Both compartments are connected through an opening. Each rat was allowed a 5 minutes pretest habituation period in the room before testing. Then the rat was placed in the dark compartment with
its head facing away from the opening. The rat was observed for a 5 min test period, and the time spent in each compartment of the LDB was recorded. The compartments of the LDB are connected to an infrared beam and exploratory behavior of rat was monitored by computer. The percentage of time spent in either the dark compartment or light compartment was calculated for each rat. The total ambulations for each rat were also calculated.

**Gold-Immunolabeling Procedure for BDNF and Arc proteins in rat brain:**

After behavior tests, rats were anesthetized (50mg/kg, IP), and perfused with 200ml of n-saline, followed by 300ml of 4% ice-cold paraformaldehyde (PFA) fixative prepared in 0.1M phosphate buffer (PB; pH 7.4). After perfusion, brains were removed and post-fixed overnight in PFA at 4°C. After post-fixation, brains were cryoprotected using a sucrose gradient (10%, 20%, and 30%) prepared in 0.1M PB. Brains were then frozen until used for gold-immunolabeling histochemical procedure, as previously described by us (Pandey et al. 2008; Moonat et al. 2011), to measure protein levels of BDNF and Arc. The coronal brain sections (20 µm) were washed (two times for 10 min) with 0.01 M phosphate buffered saline (PBS) and then blocked with RPMI 1640 (with L-glutamine) medium (Life Technologies, Grand Island, NY) for 30 min, followed by 10% normal goat serum [diluted in PBS containing 0.25% Triton X-100 (PBST)] for 30 min then 1% BSA (prepared in PBST) for 30 min at room temperature. Sections were then incubated with following antibodies: anti-BDNF antibody (H-117, Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 dilution) and anti-Arc antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 dilution) in
1% BSA (prepared in PBST) for 18 h at room temperature. After two washes for 10 min each with PBS and two washes for 10 min each with 1% BSA in PBS, sections were incubated with gold particle (1.4 nm) conjugated anti-rabbit secondary antibody (Nanoprobes, Yaphank, NY; 1:200 dilution in 1% BSA in PBS) for 1 h at room temperature. Sections were further rinsed several times in 1% BSA in PBS followed by rinsing with water. The gold immunolabeling was developed using Silver Enhancement Solution (Ted Pella, Redding, CA) for 15–30 min, and sections were washed several times with tap water and then mounted on slides. Gold-immunolabeled proteins were quantified at high magnification (100x) using Image Analysis System connected to a light microscope. The threshold of each image was set up to ensure that an area without staining should give zero counts. Under this condition, gold particles in the defined areas (3 fields in each section) of 3 adjacent brain sections and total of nine fields for each rat (bregma: 2.12-2.56 mm) of each rat were counted and values averaged for each rat. The results were represented as the number of immunogold particles/100 µm² area of a defined amygdaloid structure (CeA, MeA, or BLA).

**In situ RT-PCR for Arc mRNA Measurement.**

We used an *in situ* RT-PCR procedure similar to one we have previously reported (Pandey et al. 2008; Moonat et al. 2011) to measure Arc mRNA levels. Briefly, 40 µm free-floating brain sections were treated with proteinase K (1µg/ml in 1X PBST prepared in DEPC treated water) for 15 minutes at 37°C, followed by washing with 1X PBS, the sections then were subjected to DNase digestion.
After they were washed again using 1X PBS, sections were transferred to PCR tubes containing 100 µl of reverse transcription reaction mixture (Applied Biosystems, Foster City, CA) and be reverse transcribed with reverse transcriptase enzyme in the presence of oligo (dt). For negative sections no reverse transcriptase enzyme was added. PCR was performed with Taq DNA polymerase enzyme and primers. The primer sequences we used are based on our previous study (Pandey et al. 2008; Moonat et al. 2011): 5'-ACAGAGGATGAGACTGAGGCAC-3' and 5'-TATTCAGGCTGGGTCTGTCAC-3'. PCR conditions for Arc mRNA are: 94 °C for 5 min; 95 °C for 15 sec; 55 °C for 30 sec; 72 °C for 30 sec; total of 30 cycles and then 72 °C for 3 min. After PCR, sections were mounted on slides. Arc-positive cell bodies were detected using an alkaline phosphatase-conjugated anti-DIG antibody and subsequent staining of the complex with the specific substrate, nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics, Indianapolis, IN). The optical density (OD) of positive cell bodies was calculated by the Image Analysis System. The ODs from negative brain sections were subtracted from the OD of positive brain sections. The mean OD of Arc-positive cell bodies in the areas of CeA, MeA, and BLA were calculated, and then values were averaged for each rat for each amygdaloid nuclei. The results were represented as mean OD/100 pixels of area.

**Golgi-Cox staining procedure:**

Dendritic spine density (DSD) in the amygdaloid brain regions were measured using the Golgi-Cox staining procedure (FD Neuro Technologies,
As we described before (Pandey et al. 2008; Moonat et al. 2011), brains were rapidly immersed in impregnation solution and allowed to remain in this solution for at least 1 week. Then 200 µm brain sections were cut and then mounted and stained according to the protocol provided by FD Neuro Technologies. After staining, sections were dehydrated and cleared in xylene solution and then coverslipped using mounting medium. Sections were observed under a light microscope at high magnification (100x), and the DSD were counted by Sholl analysis using the Neurolucida Neuroexplorer program (MicroBrightField, Williston, VT). We only counted spines in those neurons that have complete impregnation as observed by the labeling of dendrites connected to the soma.

**Analysis of Results:**

The differences between the various groups for anxiety-like behavior and neurochemical data were evaluated by a one-way ANOVA test. *Post hoc* comparisons were performed using Tukey’s test and p<0.05 was considered to be significant.
3.4 Results

**Effect of TSA treatment on anxiety-like behavior during ethanol withdrawal in rats:**

In conjunction with previous results from our lab (Pandey et al. 2008), TSA treatment was found to attenuate anxiety-like behavior in rats withdrawn from chronic ethanol. Two well established behavioral tests, EPM and LDB exploration tests were used to determine anxiety-like behavior in rats. In EPM test (Fig 7), we found rats withdrawn from chronic ethanol exposure showed both decreased percentage of open-arm entries (p<0.001) and percentage of time (p<0.001) spent on open arms, compared with control-diet and ethanol-diet fed rats. TSA treatment during ethanol withdrawal rescued anxiety-like behavior by increasing both the percentage of open-arm entries and percentage of time spent on open arms in ethanol-withdrawn rats. Control-diet fed rats did not differ in anxiety measures regardless of whether they received vehicle or TSA injections.

In LDB exploration (Fig 8) test, withdrawal rats spent significantly (p<0.001) more time in the dark compartment and significantly (p<0.001) less time in the light compartment. On the contrary, withdrawal rats that received TSA injections spent significantly (p<0.001) more time in the light and significantly (p<0.001) less time in dark, compared to control-diet fed and ethanol-withdrawn rats injected with vehicle. The anxiety measures of chronic ethanol-diet fed rats not undergoing withdrawal or rats fed with control-diet treated with TSA did not differ from their respective control groups. As reported by us earlier (Pandey et al. 2008), both behavior tests demonstrated development of anxiety-like behavior
during ethanol withdrawal in rats, an effect that was blocked by treatment with TSA. The general activities of the rats doesn’t differ between groups in EPM and LDB tests, suggesting that the treatment has no effect on the visual and movement behavioral aspect of the rats. The blood ethanol levels (mean ± SEM) in ethanol fed rats were 172.12 ± 8.60 mg/dl, and in ethanol-withdrawal rats were 0 mg/dl. There were no significant differences in body weight of rats of various groups at the end of experiment [mean ± SEM (n=12-14): Control+Vehicle = 330.08g ± 2.54, Ethanol+Vehicle = 330.23g ± 2.74, Withdrawal+Vehicle = 327.17g ± 2.09, Withdrawal+TSA= 324.93g ± 2.89, Control+TSA = 331.33g ± 2.49].
Figure 7. Effect of TSA treatment in rats during ethanol withdrawal after chronic ethanol exposure on anxiety-like behavior, as measured by elevated plus maze (EPM). EPM shows increased anxiety levels in ethanol withdrawal rats, while TSA treatment reversed the anxiogenic effect. No difference in anxiety-like behavior was observed between vehicle and TSA treatment in control fed rats. Chronic ethanol exposure did not affect the anxiety-like behavior in rats. The anxiety-like behavior are represented by open arm and closed arm activities in the EPM test. Values are the mean ± SEM of 6~8 rats in each group. For % open arm entries, F(4,31)= 21.25, p < 0.001; for % time spent in open arm, F(4,31)= 17.14, p < 0.001. * Significantly different from control diet fed, vehicle injected rats. (One way ANOVA followed by Tukey’s post hoc test).
Figure 8, Effect of TSA treatment in rats during ethanol withdrawal after chronic ethanol exposure on anxiety-like behavior, as measured by light dark box (LDB). Similar to the results indicated in elevated plus maze data, ethanol withdrawal treatment provoked anxiety-like behavior in rats. TSA treatment blocked the withdrawal induced anxiogenic effect. TSA treatment has no effect on anxiety levels in rats fed with control diet. Chronic ethanol administration did not change anxiety levels. Anxiety-like behavior is represented in the data as time spent in light and dark chamber respectively. Values are the mean ± SEM of 6~7 rats in each group. For time spent in dark compartment, \( F(4,30)= 43.71, p < 0.001; \) for time spent in light compartment, \( F(4,30)= 43.71, p < 0.001 \) * Significantly different from control diet fed, vehicle injected rats. (One way ANOVA followed by Tukey’s post hoc test).
In order to examine the effect of TSA treatment on BDNF and Arc levels during ethanol withdrawal, we measured BDNF protein, Arc protein and mRNA levels in amygdaloid brain regions of rats withdrawn from chronic ethanol exposure with or without TSA treatment. We observed significant (p<0.001) decreases (Fig 9) in protein levels of BDNF and Arc and Arc mRNA level (p<0.001) in CeA and MeA, but not BLA of ethanol withdrawn rats compared with control-diet fed or ethanol-fed rats. These changes were not seen in the ethanol fed TSA treatment group. On the other hand, control-diet fed rats treated with TSA showed no significant difference in BDNF and Arc levels in all three amygdaloid brain regions. These data suggested that TSA normalized the withdrawal-induced decreases in BDNF and Arc expression. Since HDAC activity was increased and histone acetylation was decreased in the amygdala during ethanol withdrawal (Pandey et al. 2008), collectively, the results indicate that decreased histone acetylation may be involved in decreased BDNF and Arc expression, as these deficits were corrected by TSA treatment.
Figure 9A, Effect of TSA treatment on BDNF and Arc expression in amygdala during ethanol withdrawal after chronic ethanol exposure. Representative photomicrographs of Arc and BDNF protein gold-immunolabeling and Arc mRNA in situ RT-PCR in CeA of control-diet-fed, ethanol-diet-fed, and ethanol-withdrawn rats with or without TSA treatment. Scale bar = 40 µm.
Figure 9B, Effect of TSA treatment on BDNF and Arc expression in amygdala during ethanol withdrawal after chronic ethanol exposure. Bar diagram showing quantification of BDNF protein, Arc protein and mRNA levels in CeA, MeA and BLA of rats. Values are mean ± SEM of 6 rats in each group. For BDNF in CeA, F(4, 29)= 109.11, p <0.001; for BDNF in MeA, F(4, 29)= 100.51, p <0.001. For Arc in CeA, F(4, 29)= 60.23, p <0.001; for Arc in MeA, F(4, 29)= 29.93, p <0.001. For Arc mRNA in CeA, F(4, 29)= 25.59, p <0.001; for Arc mRNA in MeA, F(4, 29)= 16.24, p <0.001. * Significantly different from the control diet + vehicle injection group (one way ANOVA followed by Tukey’s post hoc test). Ethanol withdrawal treatment decreased Arc and BDNF expression in CeA, MeA, but not BLA. TSA treatment during alcohol withdrawal corrected the deficits in Arc and BDNF expression in these brain regions. TSA treatment did not alter Arc and BDNF levels in control diet fed rats. Ethanol diet fed rats also did not show any changes in Arc and BDNF expression in amygdaloid brain regions of rats.
Effects of TSA treatment on dendritic spine density (DSD) in the amygdala during ethanol withdrawal:

We observed that TSA treatment was able to normalize the effects of ethanol withdrawal-induced reduction in BDNF and Arc expression. We tested the possibility that TSA treatment might also rescued deficits in DSD in CeA and MeA during withdrawal after chronic ethanol exposure. We used Golgi-Cox staining to determine changes in DSD in amygdaloid brain regions. The representative photomicrographs of Golgi-Cox staining in various groups were shown in figure 10A and quantifications of dendritic spines were shown in figure 10B. As reported earlier (Pandey et al. 2008), here again we observed a significant decrease (p<0.001) in DSD in the CeA and MeA, but not BLA of ethanol-withdrawn rats as compared with control-diet fed or ethanol-diet fed rats. We extended these findings and found that reductions in dendritic spines in the CeA and MeA were attenuated by TSA treatment. Similar to BDNF and Arc, DSD in the amygdaloid brain regions of control-diet fed rats was not altered by TSA treatment. These results indicate that TSA treatment not only attenuated anxiety-like behavior but also corrected the deficits in BDNF, Arc, and DSD in the CeA and MeA of rats during withdrawal after chronic ethanol exposure.
Figure 10 A, Effect of TSA treatment on dendritic spine density measured by Golgi-Cox staining in the amygdala of rats during ethanol withdrawal after chronic ethanol exposure. Representative photomicrographs of dendrite and spines in CeA structures of rats. Top panel scale bar = 10 µm. Bottom panel scale bar = 50 µm. B, Bar diagram showing quantification of dendritic spine density in amygdaloid brain regions. Represented as dendritic spine density/10 mm of dendritic length. Values are mean ± SEM of 6 rats in each group. For CeA, F(4,29)= 14.89, P<0.001; for MeA, F(4,29)= 13.51, P<0.001 *Significantly different from the control diet + vehicle injection group (one way ANOVA followed by Tukey’s post hoc test). Dendritic spine density in CeA, MeA but not BLA is decreased during ethanol withdrawal. The DSDs in amygdala of rats with chronic ethanol exposure or TSA treatment following withdrawal treatment did not show difference compared with that in control-diet-fed rats. TSA treatment had no effect on amygdaloid DSD in control fed rats.
3.5 Discussion

The current study demonstrated a possible role of HDAC-induced histone modifications in the regulation of synaptic plasticity related events in the amygdala during withdrawal from chronic ethanol exposure in rats. We have previously reported that the anxiogenic effects of ethanol withdrawal are attenuated by the HDAC inhibition, which in turn is associated with correcting the deficits in histone acetylation (H3-K9 and H4-K8) in the CeA and MeA in rats (Pandey et al. 2008). Additionally, BDNF and Arc gene expression and DSD were found to be reduced in CeA and MeA of ethanol withdrawn rats and BDNF signaling along with dendritic spines in the CeA has been shown to be involved in anxiety-like behavior during ethanol withdrawal in rats (Pandey et al. 2008). Here, we extended these studies and found for the first time that, TSA treatment is able to restore the deficits in BDNF, Arc, and DSD, indicating an association between HDAC-induced histone modifications and synaptic plasticity in the development of anxiety-like behavior during withdrawal from chronic ethanol exposure in rats.

Histone acetylation appears to play an important role in the adaptive changes produced by ethanol exposure. For example, in vitro study revealed an ethanol-induced dose-dependent increase of HDAC2 expression, which was corrected by TSA treatment (Agudelo et al. 2011). In rats, acute ethanol exposure produced anxiolytic effects, decreased HDAC activity, and increased histone H3-K9 and H4-K8 acetylation in amygdala (Pandey et al. 2008), whereas withdrawal from chronic ethanol exposure decreased histone H3-K9 and H4-K8 acetylation, and increased HDAC activity (Pandey et al. 2008). Inhibition of
HDAC by TSA treatment not only attenuated development of anxiety-like behavior but also corrected the reduction of histone H3 and H4 acetylation in the CeA and MeA (Pandey et al. 2008). In addition to the acute ethanol and ethanol withdrawal, evidence of the involvement of histone acetylation in alcohol tolerance has been provided by Wang et al (2007) who found that inhalation of benzyl alcohol regulates potassium channels leading to alcohol tolerance by H4 acetylation (Wang et al. 2007). Further evidence suggesting epigenetic mechanisms via histone modifications play a role in the development of rapid ethanol tolerance was recently provided by our lab (Sakharkar et al. 2012). We found that acute ethanol exposure inhibits amygdaloid HDAC activity and increases histone acetylation in the CeA and MeA of rats, a phenomenon that could not be observed by administration of the same dose of ethanol in rats. Because of the multiple roles that HDAC plays in different stages of alcoholism, the development of HDAC inhibitors might have therapeutic implications for the treatment of alcoholism.

As described in the first part of thesis, involvement of the BDNF system in anxiety and alcoholism is well established. Deficit in BDNF expression promotes anxiety and alcohol drinking behaviors (Pandey et al. 2004; Colzato et al. 2011). BDNF and Arc expression in the CeA and MeA is decreased by ethanol withdrawal but increased by acute ethanol exposure (Pandey et al. 2008). Rats selectively bred to prefer alcohol have been found to exhibit innately higher anxiety levels as well as lower Arc and BDNF expression in the CeA and MeA (Prakash et al. 2008; Moonat et al. 2011). BDNF antisense
oligodeoxynucleotides (ODNs) infused into the CeA and MeA induced anxiety-like behavior and increased alcohol intake in rats (Pandey et al. 2006). Conversely, BDNF infusion was able to rescue withdrawal-induced anxiety and normalize decreased Arc expression (Pandey et al. 2008). Despite compelling evidence showing an involvement of BDNF and Arc expression in anxiety and alcoholism, the mechanism by which they may be interacting remains less known. We have previously shown decreased histone H3-K9 and H4-K8 acetylation and NPY expression in amygdaloid brain regions as the result of increased HDAC activity after ethanol withdrawal, and inhibition of HDAC reversed the withdrawal-induced deficits in histone acetylation and gene expression (Pandey et al. 2008). Our current data suggest that BDNF and Arc are most likely regulated by HDAC, suggesting the possible involvement of histone acetylation in the regulation of BDNF and Arc expression during ethanol withdrawal-induced anxiety. Several studies in the field indicate that histone acetylation regulates the expression of BDNF in the hippocampus during depression-like behavior and learning and memory (Tsankova et al. 2006; Bredy et al. 2007; Lubin et al. 2008; Takei et al. 2011). Further study is needed to determine the direct role of histone acetylation in the regulation of BDNF and Arc expression in the amygdala or other brain regions during alcohol dependence.

In the current study, we demonstrated a close association between expression of BDNF and Arc expression with dendritic spine density (DSD) in the CeA and MeA during alcohol dependence. HDAC inhibition not only reversed deficits of BDNF and Arc expression, but also normalized DSD in the CeA and
MeA indicating a mechanism by which histone modification underlies synaptic plasticity during alcohol dependence. These results are consistent with several recent studies that identify critical roles of histone acetylation in synaptic plasticity. For example, overexpression of HDAC2 decreased DSD and impaired long-term potentiation (LTP) in the hippocampus of mice; conversely, HDAC2 knock out mice were found to have increased DSD in the hippocampus and increased LTP (Guan et al. 2009). Histone acetylation was increased specifically within the BDNF promoter II in HDAC2 knock out mice and decreased in mice overexpressing HDAC2 over expressed mice (Guan et al. 2009). Chronic treatment with the HDAC inhibitor SAHA enhanced memory formation and reversed decreased DSD caused by over expression of HDAC2 (Guan et al. 2009). Furthermore, aging-related cognitive declines as well as a coupled loss of synaptic plasticity and dendritic spines in the hippocampus have been associated with increased HDAC2 and decreased histone acetylation at specific promoters of BDNF and treatment with TSA was able to restore BDNF expression and reverse age-dependent reductions in dendritic spines (Zeng et al. 2011).

Based on our previous and current research, we propose a mechanism by which withdrawal from chronic ethanol exposure leads to deficits in synaptic plasticity associated events. Acute ethanol exposure was suggested to decrease HDAC activity, increase H3 and H4 acetylation (Pandey et al. 2008). However, after chronic ethanol exposure, neuroadaptations may occur to balance against constant stimulation of ethanol. These changes might include increased HDAC expression or activity. It is not known what mechanisms may be involved in this
adaptation. However, genomic analysis has indicated a correlation between HDAC11 in NAc with average ethanol drinking in mice (Wolstenholme et al. 2011). A recent study also showed EtOH treatment induced a dose-dependent increase in HDAC2 expression (Agudelo et al. 2011). When suddenly withdrawn from ethanol, the once balanced system again destroyed by removing stimulation of ethanol. The adapted (increased) expression or activity of HDAC, on the other hand, may not disappear immediately. Therefore, during ethanol withdrawal, the HDAC hyper-activity may cause condenses chromatin structure, which, in turn, decreases downstream gene expression (Pandey et al. 2008). BDNF and Arc gene expression would therefore be decreased, resulting in decreased dendritic spine density and possibly an overall regulation of synaptic plasticity. These epigenetic mediated deficits in the CeA and MeA may be involved in the development of anxiety-like behavior, as they are normalized by treatment with TSA. These results collectively suggest that HDAC inhibitors may serve as potentially important therapeutic agents for the treatment of alcoholism.
4. INNATE DIFFERENCES IN ANXIETY LEVELS, AS WELL AS IN THE EXPRESSION OF BDNF AND CREB IN THE AMYGDALA OF HIGH ALCOHOL DRINKING (HAD) AND LOW ALCOHOL DRINKING (LAD) RATS.

4.1 Abstract

Studies have estimated an over 50% heritability of alcoholism. The high alcohol drinking (HAD) and low alcohol drinking (LAD) rats are selectively bred from the same level of alcohol preference over generations to obtain strains exhibiting different alcohol drinking behaviors, therefore are powerful tools in alcoholism research. The co-morbidity of alcoholism with anxiety is well established. However, the anxiety levels in HAD/LAD rats are controversial and remain to be elucidated. Therefore we used both replicates of HAD/LAD (HAD1/LAD1 and HAD2/LAD2) alcohol naïve male rats, and measured their baseline anxiety-like behavior using light/dark box exploration test, along with innate differences in the BDNF and CREB expression in amygdala of these genetic rats. We found in both replicates that HAD rats have innate anxiety-like behavior than LAD rats. The anxiety-like behavior in HAD rats were associated with lower BDNF (mRNA and protein) and CREB levels, as well as lower levels of phosphorylated CREB in the central (CeA) and medial (MeA) but not basolateral (BLA) amygdala, compared with that of LAD rats. These data suggest that the innate anxiety in HAD rats may contribute to their alcohol preference and the innate deficits of BDNF expression and CREB function in HAD rats may be responsible for the behavioral differences between HAD and LAD rats.
4. 2 Introduction

In clinical research of twin, family, and adoption studies indicated that alcoholism as a heritable disease with over 50% of estimated heritability (Heath et al. 1997; Froehlich et al. 2000). Animals that were selectively bred to exhibit alcohol preference have similar phenotypic traits with human conditions, thus have been powerful tools in alcoholism investigate neurobiological basis of alcoholism. The HAD/LAD rats were selectively bred for high and low alcohol preference through systematic mating of rats from the same level of alcohol preference for over several generations to obtain strains exhibiting different alcohol drinking behaviors (Li et al. 1993; Murphy et al. 2002). This selectively breeding method is designed to obtain lines of rats with consistent trait-relevant genes, while eliminate fixation of trait-nonrelevant genes (Murphy et al. 2002). These characteristics of the HAD/LAD rats made them good animal models for studying genetic factors affecting high alcohol drinking behavior.

The co-morbidity of alcoholism with anxiety and anxiety related disorder has been demonstrated in many studies (Morris et al. 2005; Bradizza et al. 2006; Vukovic et al. 2008). Anxiety-like emotional states are often present during alcohol withdrawal and are believed to contribute to alcohol relapse in alcoholics (Willinger et al. 2002; Koob 2003). Because of their highly heterogeneous background, and unique selective methods by which they were bred (Li et al. 1993; Murphy et al. 2002), HAD/LAD rats appear to have some comparatively less consistent phenotypes than some other animal models for alcoholism. Anxiety levels of HAD/LAD rats for instance had been inconsistently reported in
Badia-Elder reported in a study in 2003 that female HAD2/LAD2 rats don’t differ with each other in terms of anxiety levels (Badia-Elder et al. 2003). However, other research do indicate the possibility of difference in innate anxiety-levels between HAD and LAD rats (Rorick et al. 2003; Roman et al. 2012). A working hypothesis is that when similar differences are found in both line pairs, it is more likely that the alcohol drinking characteristics between the strains are result from those differences. Alcohol preferring (P) and non-preferring (NP) rats were also generated using similar selective breeding system with HAD/LAD rats (Murphy et al. 2002). P rats have higher innate anxiety levels than NP rats (Stewart et al. 1993; Hwang et al. 1999; Pandey et al. 2005; Moonat et al. 2011). It has been found that compared with LAD rats, HAD rats have lower serotonin levels in cerebral cortex, striatum, nucleus accumbens, hippocampus and hypothalamus (Gongwer et al. 1989), and in these brain regions P rats also show lower 5-HT levels compare with NP rats (Hwang et al. 1999). Studies on brain dopamine (DA) contents indicate that P and HAD rats have lower levels of DA in nucleus accumbens compared with their nonpreferring counterparts (NP and LAD rats) (Murphy et al. 1982; Murphy et al. 1987; Gongwer et al. 1989). Higher densities of GABAergic terminals were also identified in nucleus accumbens of naïve P and HAD rats compared with low alcohol drinking NP and LAD counterparts (Hwang et al. 1990) More interestingly, HAD rats have less innate NPY expression in CeA (Hwang et al. 1999), which is often seen in P rats (Hwang et al. 1999; Pandey et al. 2005), and other alcohol dependence models such as rats undergoing alcohol withdrawal (Roy and Pandey 2002).
Microinjections of NPY into the central nucleus of the amygdala (CeA) produce anxiolytic effects (Heilig et al. 1993; Pandey et al. 2005). Because HAD/LAD and P/NP rats shares many neurochemical and behavioral differences within their own pair, it is possible that the alcohol drinking characteristics between the strains are resulted from those differences, including anxiety levels. Therefore, here we used both replicates of HAD1/LAD1 and HAD2/LAD2 alcohol naïve male rats, and measured their baseline anxiety-like behavior using the light/dark box exploration test.

Brain-derived neurotrophic factor (BDNF) expression is closely associated with anxiety level and alcohol drinking in both clinical (Joe et al. 2007) and pre-clinical studies (McGough et al. 2004; Pandey et al. 2006; Pandey et al. 2008; Moonat et al. 2011). A dinucleotide repeat an intron 5′ to the first coding exon in the BDNF gene has been implicated with vulnerability for alcohol abuse (Uhl et al. 2001). BDNF single nucleotide polymorphism Met66Met decreased BDNF secretion and localization (Egan et al. 2003; Chen et al. 2004), and has been shown to be involved in alcoholism in violent alcoholics (Matsushita et al. 2004). Deficits of BDNF in the gene level such as haploid deficiency (BDNF-/-) (McGough et al. 2004) and single nucleotide polymorphism Met66Met (Chen et al. 2006) have been associated with high anxiety levels in mice. Study from our lab also found lower BDNF levels in amygdala in P rats, and acute ethanol increased BDNF expression in the amygdala of P rats, but not in NP rats. (Moonat et al. 2011).
cAMP response element-binding (CREB) protein is a gene transcription factor. Using a combination of bioinformatic approaches, CREB was identified as a potential mediator of ethanol effect on gene expression (Uddin and Singh 2007). Acute ethanol exposure increased CRE-mediated gene transcription in multiple brain regions including NAc, PFC, BNST, BLA, hippocampus, VTA, and hypothalamic nuclei in mice brains (Asyyed et al. 2006). Chronic alcohol administration decreased the phosphorylation of CREB (Yang et al. 1998). Reduced CREB expression and function were associated with increased alcohol preference in animal models such as SD rats undergoing ethanol withdrawal after chronic ethanol exposure (Pandey et al. 2003), CREB deficient mice (Pandey et al. 2004), C57 mice (Misra and Pandey 2003), and P rats (Pandey et al. 2005). However, whether HAD and LAD rats also have innate differences in BDNF and CREB expression and function is currently unknown. We hypothesize that there are innate difference in anxiety level between HAD and LAD rats, and this might be related to innate BDNF and CREB deficits in amygdaloid brain regions.
4.3 Materials and Methods

Animals:

All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Adult male HAD1/LAD1 (56th generation) and HAD2/LAD2 rats (54th generation) were provided by Indiana University, Indianapolis, IN. Animals were housed individually in a temperature-controlled room with a 12/12-hr light/dark cycle, with food and water provided ad libitum. Age-matched HAD and LAD rats were used and there were no differences in body weight among the groups [mean ± SEM (n=6): HAD1 = 289g± 3.75, LAD1 = 315g ± 8.47, HAD2 = 275g ± 6.75, LAD2 = 316g ± 4.76]. Total of 24 rats were used in this experiment.

Light-dark-box test (LDB):

The LDB exploration test procedure was used by us in our prior studies (Pandey et al. 2008; Moonat et al. 2011). The light/dark box is located in a dark room and consisted of a dark compartment without illumination and a light compartment with illumination (0.25 Amp; light-emitting diode light). Both compartments are connected through an opening. Each rat was allowed a 5 minute pretest habituation period in the room before testing. Then the rat was placed in the dark compartment with its head facing away from the opening. The rat was observed for a 5 min test period, and the time spent in each compartment of the LDB was recorded. The compartments of the LDB are connected to an
infrared beam and animal activity was computer monitored. The percentage of time spent in either the dark compartment or light compartment was calculated for each rat. The box was wiped with a wet paper towel and dried after each rat testing.

Immediately after the behavior tests, rats were anesthetized [50mg/kg, intraperitoneal (I.P.)], and perfused intracardially with 200ml of n-saline, followed by 300ml of 4% ice-cold paraformaldehyde (PFA) fixative prepared in 0.1M phosphate buffer (pH 7.4). After perfusion, brains were dissected out and post-fixed overnight in PFA at 4 °C, and were used for gold-immunolabeling and in situ RT-PCR studies to determine expression of BDNF and CREB as described below.

**Gold-Immunolabeling Procedure for BDNF, CREB and phosphorylated CREB in rat brain:**

Perfused brains were frozen and 20 mm coronal sections were collected using a cryostat. We used the gold-immunolabeling histochemical procedure, as previously described by us (Pandey et al. 2008; Moonat et al. 2011), to measure cellular expression of BDNF, CREB and P-CREB. The brain sections were washed (two times for 10 min) with 0.01 M PBS and then blocked with RPMI 1640 (with L-glutamine) medium (Invitrogen, Auckland, New Zealand) for 30 min, followed by 10% normal goat serum (diluted in PBS containing 0.25% Triton X-100) for 30 min then 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were further incubated with following
antibodies: anti-BDNF antibody (H-117, Santa Cruz Biotechnology, 1:200 dilution), anti-CREB antibody (Millipore, 1:500 dilution), anti-pCREB antibody (Ser133, Millipore, 1:500 dilution) in 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 18 h at room temperature. After two washes for 10 min each with PBS and two washes for 10 min each with 1% BSA in PBS, sections were incubated with gold particle (1.4 nm) conjugated anti-rabbit secondary antibody (Nanoprobes, Yaphank, NY; 1:200 dilution in 1% BSA in PBS) for 1 h at room temperature. Sections were further rinsed several times in 1% BSA in PBS followed by rinsing in double-distilled water. The gold immunolabeling was developed using Silver Enhancement Solution (Ted Pella, Redding, CA) for 15–30 min, and then sections were washed several times using tap water before mounted on slides. Gold-immunolabeled proteins were quantified using the Loats Image Analysis System (Loats Associates Inc., Westminster, MD) connected to a light microscope that calculate the number of gold particles/100 µm² at high magnification (100x). The threshold of each image was set up to ensure that an area without staining should give zero counts. Under this condition, gold particles in the defined areas (3 fields in each section) of 3 adjacent brain sections (bregma: 2.12-2.56 mm) of each rat were counted and values averaged for each rat. The results were represented as the number of immunogold particles/100 µm² area of a defined amygdaloid structure.

**In situ RT-PCR for BDNF mRNA Measurement.**

We used in situ RT-PCR procedure as we have previously reported (Pandey et al. 2008; Moonat et al. 2011) to measure BDNF mRNA expression in
the amygdala. Briefly, 40 µm free-floating brain sections were treated with proteinase K (1µg/ml in 1X DEPC-PBS containing 0.05% Triton X-100) for 15 minutes at 37 °C, followed by washing with 1X DEPC-PBS, the sections then were subjected to DNase digestion. After been washed again using 1X DEPC-PBS sections were transferred to PCR tubes containing 100 µl of reverse transcription reaction mixture (Applied Biosystems, Foster City, CA) and be reverse transcribed with reverse transcriptase enzyme in the presence of oligo (dt). For negative sections there were no reverse transcriptase enzyme. Then PCR was performed with Taq DNA polymerase enzyme and primers. The primer sequences we used are based on previous study: 5′-TAACGGGCCGACACAAAAAGACT-3’ and 5′-GTGTCTATCCTTATGAAATCGCCAGCAA- 3’. PCR conditions for BDNF mRNA are: 94 °C for 2 min; 94 °C for 30 sec; 57 °C for 30 sec; 72 °C for 90 sec; total of 30 cycles and then 72 °C for 10 min. After PCR, sections were mounted on slides. BDNF-positive cell bodies were detected using an alkaline phosphatase-conjugated anti-DIG antibody and subsequent staining of the complex with the specific substrate, nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics, Indianapolis, IN). The optical density (OD) of positive cell bodies were calculated by the Loats Image Analysis System (Loats Associates Inc., Westminster, MD). The ODs from negative brain sections were subtracted from the OD of positive brain sections. The mean OD of BDNF-positive cell bodies in the central, medial and basolateral amygdala were
calculated, and then values were averaged for each rat. The results were represented as mean OD/100 pixels of area for BDNF mRNA levels.

**Analysis of Results:**

The differences between the various groups for anxiety-like behavior and neurochemical data were evaluated by student t test.
4.4 Results

**Innate differences in anxiety level of HAD1/LAD1 and HAD2/LAD2 rats:**

We used alcohol naïve adult male HAD1/LAD1 and HAD2/LAD2 rats with matching ages and body weights, and measured their innate anxiety-like behavior using light-dark-box exploration test (LDB). We observed that HAD1 rats spent significantly more time \((p<0.001, n=6)\) in the dark compartment and significantly less time \((p<0.001, n=6)\) in the light compartment, compared to LAD1 rats (figure 11A). Meanwhile, the time that HAD2 rats spent in the dark compartment were also significantly longer \((p<0.005, n=6)\) compared to LAD2 rats, and that in light compartment were significantly shorter \((p<0.005, n=6)\) (figure 11B). These data indicate that both HAD1 and HAD2 rats displayed anxiety-like behavior as compared with LAD1 and LAD2 rats, respectively.

Because HAD1/LAD1 and HAD2/LAD2 rats are two independently developed replicates from the same stock and by the same breeding method, they are more convincing animal models of alcoholism than many others. The advantage of two replicates of these models is that if both replicates of HAD/LAD rats exhibit same behavioral or neuronal characteristics, then it is more likely to be highly relevant to mechanism of alcohol drinking behavior. Here, both HAD1 and HAD2 rats displayed higher anxiety levels than their counterparts, indicating that innate high anxiety in these rats may have contributed to the high alcohol drinking behavior of HAD rats.
Figure 11. Anxiety levels of HAD1/LAD1 and HAD2/LAD2 rats measured by Light-dark-box (LDB) test. A, HAD1 rats display heightened anxiety level compared to LAD1 rats, as indicated by significantly increased time spent in dark chamber and decreased time in light chamber. Values are the mean ± SEM of 6 rats in each group. p =< 0.001. B, HAD2 rats also spent more time in dark compartment but less time in light compartment compared to LAD2 rats, indicating a higher anxiety-like behavior than its counterpart. Values are the mean ± SEM of 6 rats in each group. p < 0.005, significantly different from control diet fed, vehicle injected rats (Student's t test).
Innate differences in BDNF mRNA and protein levels in amygdala of HAD1/LAD1 and HAD2/LAD2 rats:

Next, we investigated baseline differences in the BDNF expression in the amygdala of HAD and LAD rats. As discussed above, BDNF has been shown to be associated with anxiety and alcohol drinking in both clinical (Joe et al. 2007) and pre-clinical studies (McGough et al. 2004; Pandey et al. 2005; Pandey et al. 2008; Moonat et al. 2011). We used gold immuno labeling and in situ RT-PCR to measure the BDNF protein and mRNA levels in both pairs of HAD/LAD rat strains. The results are shown in figure 12: protein (p<0.001, n=6) and mRNA (p<0.05, n=6) levels of BDNF are significantly lower in CeA, MeA but not BLA of HAD1 rats compared with LAD1 rats. Similarly, there are also a significant lower BDNF protein levels in CeA (n=5, p<0.001) and MeA (n=5, p<0.05) but not BLA of HAD2 rats than LAD2 rats. The mRNA levels of BDNF in CeA (n=5, p<0.001) and MeA (n=5, p<0.05) of HAD2 rats were also significantly lower compared with LAD2 rats. Both replicates of HAD/LAD rats exhibit similar differences in BDNF expression within the pair of strains, indicating a possibility that phenotypes of anxiety-like and alcohol-drinking behaviors of HAD rats might be associated with innate deficit in BDNF expression in amygdala of the rats.
Figure 12 A, Differences in BDNF protein and mRNA levels in the amygdala of HAD/LAD rats. Representative photomicrographs of BDNF protein gold-immunolabeling and BDNF mRNA in situ RT-PCR in CeA structures of HAD1/LAD1 and HAD2/LAD2 rats. Scale bar = 40 µm.
Figure 12B, Innate differences in BDNF protein and mRNA levels in the amygdala of HAD/LAD rats. Bar diagram showing quantification of BDNF protein and mRNA expression in CeA, MeA and BLA of rats. Compare HAD1/LAD1 strains, the HAD1 have lower expression of both BDNF protein (n=6, p<0.001) and mRNA (n=6, p<0.05) in CeA and MeA but not BLA. HAD2 rats have significantly decreased expression of BDNF protein in CeA (n=5, p<0.001) and MeA (n=5, p<0.05) but not BLA of amygdala, compared with LAD2 rats. BDNF mRNA expression in HAD2 rats were also lower in CeA (n=5, p<0.001) and MeA (n=5, p<0.05) than that of LAD2 rats. Values are mean ± SEM of (HAD1/LAD1, N=6; HAD2/LAD2, N=5) in each group. (Student T test)
Innate differences in expression and phosphorylation of CREB in the amygdala of HAD1/LAD1 and HAD2/LAD2 rats:

CAMP response element-binding (CREB) is a gene transcription factor that has been associated with excessive alcohol drinking behavior and heightened anxiety levels in P rats (Pandey et al. 2005). Since BDNF is one of the CREB target genes, we examined whether lower BDNF expression in the amygdala of HAD rats is related to lower CREB expression and phosphorylation. We used gold-immunolabeling to measure the CREB protein levels and phosphorylated CREB protein levels in the amygdaloid brain regions of HAD and LAD rats. As shown in figure 13, the CREB protein levels in CeA (p<0.001, n=6) and MeA (p=0.002, n=6) of HAD1 rats were significantly lower than that of LAD1 rats. We also found less phosphorylation of CREB in CeA (p<0.001, n=6) and MeA (p=0.005, n=6) of HAD1 rats, suggesting a reduced functioning of CREB. HAD2 rats compared to LAD2 rats also showed lower CREB protein levels in CeA (p<0.005, n=5) and MeA (p<0.05, n=5), as well as lower CREB phosphorylation in CeA (p<0.001, n=5) and MeA (p<0.02, n=5). CREB expression and phosphorylation in the BLA of amygdala remained unchanged between these strains of rats. These data suggested that the heightened anxiety levels and high alcohol drinking behavior in HAD1 and HAD2 rats might be related to their innate deficiency in CREB expression and functioning and related target gene, BDNF.
Figure 13 A, Innate differences in CREB protein levels and phosphorylated CREB protein levels in amygdaloid brain regions of HAD and LAD rats. Representative photomicrographs of CREB protein and phosphorylated CREB gold-immunolabeling in CeA structures of rats. Scale bar = 40 µm.
**Figure 13 B.** Innate differences in CREB protein levels and phosphorylated CREB protein levels in amygdaloid brain regions of HAD and LAD rats. Bar diagram showing quantification of CREB protein and phosphorylated CREB expression in CeA, MeA and BLA of rats. There was a significant decrease of CREB protein level in CeA (p<0.001, n=6) and MeA (p=0.002, n=6), and decrease of phosphorylated CREB labeling in CeA (p<0.001, n=6) and MeA (p=0.005, n=6) of HAD1 rats compared to HAD2 rats. HAD2 rats also have significantly lower CREB protein in CeA (p<0.005, n=5) and MeA (p<0.05, n=5), as well as decreased CREB phosphorylation in CeA (p<=0.001, n=5) and MeA (p<0.02, n=5), compared to LAD2 rats. Values are mean ± SEM of (HAD1/LAD1, N=6; HAD2/LAD2, N=5) in each group. (Student T test)
4.5 Discussion

HAD/LAD rats have been used in alcohol research field to investigate the biological basis of genetic alcohol preference. The alcohol preference differs tremendously between the HAD and LAD rats. HAD rats have 9.5 g /kg/day average voluntary ethanol consumption while LAD rats consuming approximately 0.5 g/kg/day (Murphy et al. 2002). Given the difference of ethanol elimination rate in rats and humans, we can compare the average consumption of HAD rats as 8 standard-size alcoholic drinks by a 150–160 pound person per day. The selectively breeding method which is used to generate HAD/LAD rats can eliminate fixation of trait-nonrelevant genes while contain trait-relevant genes through generations of selections, making these rats suitable animal models for studying alcoholism related genetic factors (Murphy et al. 2002). However, compared with P/NP rats that are also generated with similar breeding method, HAD/LAD rats show less consistency in the differences between the pair of rats in some phenotypes. The reason might be that HAD/LAD rats were generated from a more heterogeneous foundation stock (from eight strains of rats) (Li et al. 1993) than P/NP strains (from Wistar stock) (Murphy et al. 2002). Therefore HAD/LAD strains can exhibit greater genetic diversity than P/NP rats. The genetic diversity of HAD/LAD’s origin can serve as a two-headed arrow for research. The inconsistency might discourage some research from adopting this animal model. But on the other hand, the heterogeneous genetic background of HAD/LAD is more analogous to that of human alcoholics. Additionally, HAD/LAD rats have another advantage over P/NP rats and other animal models of
alcoholism in that there are two pairs of strains: replicate 1 and 2 of HAD/LAD. Therefore, if both replicates exhibit similar phenotypes, they make a strong and more confident argument on the involvement of these phenotypes in alcoholism.

Our data suggested that both HAD1 and HAD2 rats have significantly higher anxiety levels compared with LAD1 and LAD2 rats, indicating the involvement of innate anxiety in high alcohol drinking behavior in HAD rats. This data is in contrary to what was indicated in another study, in which female HAD1/LAD1 rats didn’t display difference in their anxiety-like behavior (Badia-Elder et al. 2003). The reason for this controversy might due to the sex difference of the animal that was used in the studies. Another explanation could be the different behavioral paradigms used in the two studies. Badia-Elder used elevated plus maze test (EPM), while we used light-dark box (LDB). Both tests are widely used and are believed to adequately reflect anxiety-like behavior in rodents. Due to the diverse genetic background of HAD/LAD rats, innate anxiety levels in these rats might not be significant in one method, but in another. In addition, an interesting observation revealed that both P and HAD rats have higher ultrasonic vocalization in response to stress than their counterpart rats (Overstreet et al. 1997). Since rate of vocalization can be reduced by anxiolytic drugs (Kehne et al. 2000), this behavior appears to be associated with anxiety levels. Due to excessive cue-elicited fear and anxiety, both HAD1 and HAD2 rats exhibit excessive freezing to the cue used to signal (Rorick et al. 2003), which interfered with their ability to learn avoidance response (Rorick et al. 2003). These results support our observation that HAD rats are innately more anxious...
than LAD rats, and that HAD/LAD rats are reliable models of the anxiety vulnerability to alcohol abuse.

We also found that HAD rats have innate deficiency in BDNF and CREB expression, as well as phosphorylation of CREB. These deficits were correlated with higher anxiety levels in HAD rats compared with LAD rats. The literatures has emphasized the importance of these two molecules and their close association with anxiety and alcoholism. BDNF deficits in other animal models have also been shown to attribute to alcohol addiction and anxiety. BDNF deficient (+/-) mice have higher preference for ethanol as measured by two-bottle choice paradigm, while induction of BDNF into the dorsal striatum and hippocampus attenuated alcohol preference (McGough et al. 2004). P rats also show innate lower BDNF levels in amygdala compared with NP rats (Prakash et al. 2008; Moonat et al. 2011) During ethanol withdrawal, the phosphorylation of CREB in CeA and MeA was decreased while anxiety levels were increased compared to control rats (Pandey et al. 2003). Increased alcohol consumption was also observed in CREB deficient (+/-) mice (Pandey et al. 2004). Innate deficits of CREB function is also related to excessive alcohol drinking behavior of C57 mice compared with DBA mice (Misra and Pandey 2003), and that of P rats compared with NP rats (Pandey et al. 2005). Therefore, both BDNF and CREB are critical mediators of anxiety and alcohol drinking behaviors. Our data suggest that deficits in BDNF and CREB might be operative in regulating anxiety-like and alcohol-drinking behaviors in HAD rats compare to LAD rats.
5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Anxiety disorder and alcoholism are highly co-morbid (Bowen et al. 1984; Brady and Lydiard 1993; Kushner et al. 2005; Kushner and Sher 1993; Kushner et al. 2001). Anxiety is a major component in the negative effective states of alcohol addiction. Both positive and negative affective states of alcohol play critical roles in the development of alcoholism, however, the negative affective state of alcohol has more dramatic effects in promoting alcohol intake. Therefore it is the main cause of relapse. In the current thesis, we used three animal models of anxiety and alcoholism, in the hope of understanding the molecular mechanisms underlying the co-morbidity of anxiety and alcohol drinking. Each model represented one scenario that is replicated in the human alcoholic population.

Model 1: The first model of anxiety was produced by pharmacological blockage of the BDNF-Erk1/2 signaling pathway in the central amygdala of normal SD rats. Deficits in BDNF have been shown to be closely associated with anxiety and alcohol drinking behaviors in both clinical (Uhl et al. 2001; Matsushita et al. 2004; Joe et al. 2007) and pre-clinical (McGough et al. 2004; Chen et al. 2006; Pandey et al. 2006) studies. In agreement, increased BDNF levels are usually accompanied with decreased anxiety and alcohol drinking (McGough et al. 2004; Pandey et al. 2008). However, it is not clear which signaling pathway downstream of BDNF is responsible for the regulation of anxiety and alcohol drinking, nor is it known by
what mechanisms BDNF and its downstream gene targets regulate anxiety and alcohol drinking. We hypothesized that BDNF may regulate anxiety and alcohol drinking through manipulation of Arc gene expression downstream of the Erk1/2 signaling pathway.

To address these questions, we manipulated the function of the BDNF-Erk1/2 signaling pathway by infusion of BDNF (activator of Erk1/2) and the inhibitor (U0126) of Erk1/2 signaling pathway into the CeA of rats. Using the elevated plus maze test (EPM), two-bottle free-choice paradigm, gold-immunolabeling, and in situ PCR, we determined the effect of alterations of BDNF-Erk1/2-Arc signaling on anxiety-like and alcohol-drinking behaviors. As illustrated in Model 1, BDNF infusion into the CeA decreased anxiety levels,
increased phosphorylation of Erk1/2 and CREB, and expression of Arc protein and mRNA. U0126 CeA infusion, on the contrary, provoked anxiety-like behaviors in rats and decreased phosphorylated Erk1/2, phosphorylated CREB, and Arc mRNA and protein levels. When BDNF and U0126 were co-infused, we observed normal anxiety levels and the phosphorylation of Erk1/2 and CREB, and Arc expression were normalized. We also found an increase in alcohol drinking behavior when rats were infused into CeA with U0126, while BDNF co-infusion blocked these behavior changes. When amygdaloid Arc expression was blocked by Arc antisense ODNs, we observed anxiety-like behavior similar to that of rats treated with U0126. However when rats were co-infused with BDNF and Arc antisense ODNs, observed anxiogenic effect of Arc antisense ODNs was reversed.

**Model 2: The second model of anxiety was produced via withdrawal from chronic ethanol exposure.** Chronic alcohol administration induces neuroadaptations, which changes the threshold of an individual’s sustainability to the absence of alcohol. The negative effective state of alcoholism is defined by the enormous physical and emotional pain that alcoholics experience during alcohol withdrawal. Anxiety is a major withdrawal symptom that is believed to be greatly accountable for the relapse of alcohol drinking, therefore, we used ethanol withdrawal-induced anxiety as our second animal model. We hypothesized that the neuroadaptations occurred during chronic alcohol drinking involves epigenetic modifications, and when suddenly withdrawal from alcohol, the alcoholic individual still carries these epigenetic modifications. Without the
compensation of constant stimulation of ethanol, adaptive epigenetic changes, such as histone acetylation, lead to changes in gene expression, alterations in dendritic spine morphology and function, which eventually resulting in behavioral changes. If that is the case, we hypothesized that these cascades of events might be reversible by inhibiting histone deacetylase (HDAC) activity using the HDAC inhibitor TSA.

As demonstrated in **Model 2**, we found that rats undergoing withdrawal from chronic ethanol administration display significantly higher anxiety levels measured by both the EPM and the light dark box (LDB) paradigm. These rats also have decreased expression of BDNF and Arc, as well as decreased dendritic spine density (DSD) in CeA and MeA, as measured by gold-immuno labeling, *in situ* PCR, and Golgi-Cox staining, respectively. TSA treatment
reversed withdrawal-induced anxiety-like behavior in rats, prevented the
decrease in BDNF and Arc expression, and attenuated the reduction in DSD.
These protective effects of TSA only occur when rats are undergoing withdrawal.
We did not observe any change in control-fed rats treated with TSA. Chronic
ethanol administration itself did not have any significant effect on rat's anxiety
behavior, BDNF and Arc expression, nor DSD in the amygdala.

**Model 3:** The third animal model was generated through generations
of selectively breeding to obtain an innate preference for alcohol drinking.
Alcoholism has a heritability of over 50% (Heath et al. 1997; Froehlich et al.
2000), therefore genetic models of alcoholism become valuable tools for
understanding the genetic factors of alcoholism. Although many genetic models
are available in the field, we chose to study the high alcohol drinking/ low alcohol
drinking (HAD/LAD) rats for three major reasons: First, HAD/LAD rats were
generated by a unique breeding method, which conserves consistent trait-
relevant genes, while significantly eliminating the fixation of trait-nonrelevant
genes in these rats. Second, HAD/LAD rats were derived from N/Nih
heterogeneous stock, which was developed by crossing eight inbred strains.
Compared with other animal models, HAD/LAD rats' genetic diversity best
mimics the human alcoholic population. Third, HAD/LAD rats have two
independent replicates HAD1/LAD1 and HAD2/LAD2. This provides further
confidence to our results on these rats because when both replicates display the
same phenotype, it is more likely that observed phenotypes are relevant to their
distinctive drinking behavior.
We addressed the hypothesis that HAD/LAD rats have innate differences in anxiety-like behavior and that BDNF expression and CREB expression and phosphorylation are associated with innate anxiety levels and alcohol preference. We found that both replicates of HAD rats innately displayed more anxiety-like behavior than LAD rats as measured by the LDB exploration test. Gold-immunolabeling measurements revealed that BDNF and CREB protein levels are lower in HAD1 and HAD2 rats compared to that of LAD1 and LAD2 rats, respectively. Phosphorylated CREB protein levels, which indicate CREB function, were also lower in the amygdala of both replicates of HAD rats compared with that of LAD rats, as shown in the schematic diagram named Model 3.
These three models gave us valuable tools to investigate the molecular mechanism underneath anxiety and alcohol drinking behaviors. The data we collected here from all three models consistently suggesting **BDNF, Erk1/2, CREB, and Arc** as important molecular elements that are involved in anxiety-like and alcohol-drinking behaviors. Our data also suggested possible involvement of epigenetic mechanism in the regulation of dendritic spine density (**DSD**) during alcoholism.

**BDNF** played significant roles in all three animal models of anxiety and alcohol drinking in current thesis. The levels of BDNF in the amygdaloid brain regions were consistently found to be negatively related to anxiety levels and alcohol preference in rats, indicating a protective effect of BDNF on anxiety and alcoholism. Moreover, BDNF infusion into the CeA produced anxiolytic effects in rats. When the BDNF downstream Erk1/2 signaling pathway was blocked by infusion of the inhibitor U0126, it provoked anxiety-like and promoted alcohol-drinking behaviors in rats, a phenomenon that was reversed by co-infusion of BDNF. In rats undergoing ethanol withdrawal, we also observed a decrease in BDNF protein levels in the CeA and MeA but not BLA of the rats, accompanied with anxiety-like behavior. When ethanol-withdrawn rats were treated with the HDAC inhibitor TSA, the decreased amygdaloid BDNF levels were reversed along with the anxiogenic effect of withdrawal. In the CeA and MeA of genetically predisposed HAD rats, BDNF protein and mRNA were both significantly lower compared with LAD1 rats. Similar replication of this BDNF expression pattern was also observed between HAD2/LAD2 rats. HAD rats' deficit in BDNF was also
correlated with innately high anxiety levels. These data are consistent with observations from previous studies that BDNF expression and anxiety levels in rats are inversely related. Acute ethanol exposure produces anxiolytic effects in rats, and increases BDNF expression in CeA and MeA (Pandey et al. 2008). In innately anxious P rats (Prakash et al. 2008; Moonat et al. 2011) and CREB-haplodeficient (+/-) mice (Pandey et al. 2004), BDNF expression is lower than their non-anxious counterparts. BDNF antisense ODNs infusion into the CeA and MeA has anxiogenic effects in rats (Pandey et al. 2006). A single nucleotide polymorphism in the BDNF gene, Met66Met, results in high anxiety levels (Chen et al. 2006) and alcoholism (Matsushita et al. 2004). Levels of BDNF in the amygdala have been consistently found to give an inverse relationship to anxiety levels and alcohol preference, implicating the regulatory role of BDNF in anxiety and alcoholism.

The **Erk1/2** signaling pathway may be involved in the mechanism by which BDNF regulates anxiety and alcohol drinking. We found that inhibition of amygdaloid Erk1/2 signaling by U0126 CeA infusion had significant anxiogenic effects in rats, and also increased alcohol preference in rats. A previous study in our lab showed increased phosphorylation of Erk1/2 in CeA and MeA of rats after acute ethanol exposure, and decreased phospho-Erk1/2 during withdrawal after chronic ethanol exposure (Pandey et al. 2008). These data indicate the involvement of Erk1/2 signaling in different stages of alcohol addiction. Interestingly, when BDNF is co-infused with U0126, the effects of U0126 on rats’ anxiety-like and alcohol-drinking behaviors, as well as decreased phosphorylated
Erk1/2 protein levels were reversed. It has been shown that BDNF infusion can also be normalized in both anxiety levels and phospho-Erk1/2 in ethanol-withdrawn rats (Pandey et al. 2008). When rats were infused with BDNF antisense ODNs, there was also a decrease in phosphorylated Erk1/2 in CeA or MeA, respectively, and increases in anxiety levels and alcohol intake (Pandey et al. 2006). These data indicate that BDNF regulates anxiety and drinking through regulation of the downstream Erk1/2 signaling pathway. This mediator role of Erk observed in the current thesis is consistent with studies of other drugs of abuse. MEK/ERK pathway inhibitor infusion into the VTA decreases behavioral sensitization induced by cocaine (Pierce et al. 1999), whereas systemic administration of similar inhibitors blocks cocaine-provoked locomotor hyperactivity, and place conditioning (Pierce et al. 1999; Valjent et al. 2000).

As a critical gene transcription factor, CREB was identified as a potential mediator of ethanol’s effect on gene expression (Uddin and Singh 2007). CREB is phosphorylated at Ser133 and activated by multiple kinases including BDNF-Erk1/2 signaling. Therefore, it is not surprising that CREB function shows a consistent inverse relationship to anxiety and alcohol drinking in our animal models. We observed a decrease of phosphorylated CREB co-existing with a rise in anxiogenic behavior induced by inhibition on Erk1/2 signaling, while BDNF co-infusion rescued this decrease of CREB function. We also determined baseline CREB protein and phosphorylated CREB protein levels in amygdala of HAD/LAD rats, which exhibited an innate difference in anxiety levels and alcohol preference. We found that both replicates of HAD1 and HAD2 rats have
significantly lower CREB protein levels than those of LAD1 and LAD2 rats, in CeA and MeA. Phosphorylated CREB was also lower in the CeA and MeA of both replicates of HAD rats compared with LAD rats. These observations indicated that the heightened anxiety level and alcohol preference HAD rats may be related to deficits in CREB function in the amygdala. This result is consistent with that observed in CREB deficient mice, which have decreased phosphorylated CREB, increased anxiety-like behavior and alcohol consumption as compared to wild-type controls (Pandey et al. 2004). The reduced levels of CREB and phosphorylated CREB in the CeA and MeA were also shown to be associated with innate levels of anxiety and high alcohol preference in P rats compared to NP rats (Pandey et al. 2005).

**Arc** has a synaptic activity response element (SARE) sequence that contains binding sites for CREB (Kawashima et al. 2009). It has also been shown that Arc transcription depends on activation of the MAPK cascade (Waltereit et al. 2001). Erk phosphorylation is required for Arc mRNA to be further transported to the activated synaptic sites (Huang et al. 2007). Therefore, Arc is a possible target gene of BDNF-Erk1/2-CREB signaling, and might be involved in anxiety and alcohol addiction. We found that Arc expression at both mRNA and protein levels in the CeA and MeA of ethanol-withdrawn rats are decreased significantly. Similar decreases of Arc expression were observed when U0126 was infused into the CeA of normal rats. When BDNF was co-infused with U0126, however, the decreased Arc expression was not observed. When Arc expression was blocked directly by infusion of Arc antisense ODNs,
rats also display heightened anxiety levels, a phenomenon again reversed by 
BDNF co-infusion. Rats undergoing ethanol withdrawal, or that were infused with 
U0126 or Arc antisense all displayed anxiety-like behavior, indicating the 
involvement of Arc-BDNF signaling in the regulation of anxiety-like and alcohol-
drinking behaviors. On the other hand, Arc expression was increased after acute 
ethanol exposure, in conjunction with decreased anxiety levels (Pandey et al. 
2008). BDNF’s regulatory role in Arc expression and anxiety was also indicated 
in previous studies, which found that BDNF normalizes Arc expression and 
anxiety levels of rats after ethanol withdrawal (Pandey et al. 2008). Arc 
expression is also related to Erk1/2 and CREB function, as evidenced by the 
observation that expression of Arc mRNA and protein following BDNF induced 
LTP were both significantly up-regulated in the hippocampus (Ying et al. 2002). 
These changes appear to be required activities of Erk signaling, because MEK 
inhibitors blocked Erk activity and completely abolished BDNF-LTP induction 
(Ying et al. 2002).

We also observed a significant decrease in the DSD in the CeA and MeA 
of ethanol-withdrawn rats, which display high anxiety levels. Changes in DSD 
have been associated with anxiety and alcohol drinking in previous studies: 
Acute ethanol administration increased DSD while Arc antisense decreased DSD 
in rats (Pandey et al. 2008). Lower DSD was also observed in P rats compared 
with NP rats, while acute ethanol only increased DSD in P rats (Moonat et al. 
2011). The close associations we observed between DSD, BDNF and Arc are 
also consistent with previous literature: BDNF promotes synaptic strength and
neuronal signaling (Poo 2001) and it is known to enhance dendrite formation and axonal branching (McFarlane 2000; Danzer et al. 2002; Horch and Katz 2002). BDNF-induced LTP is associated with the induction and dendritic transport of Arc mRNA (Link et al. 1995; Lyford et al. 1995; Ying et al. 2002). Aging-related cognitive declines, coupled with loss of synaptic plasticity and dendritic spines, in the hippocampus have been associated with increased HDAC2 and decreased histone acetylation in specific BDNF promoters. Furthermore, TSA restored BDNF expression and reversed age-dependent reductions in the spine number and density (Zeng et al. 2011).

The emerging epigenetics field provides us with new aspects and tools to study anxiety and alcoholism. The current thesis focused on one important mechanism of epigenetic modification, histone acetylation. As discussed above, behavioral tests indicated a significant increase in anxiety in rats undergoing ethanol withdrawal. Withdrawal-induced anxiety is also associated with decreased DSD within the amygdaloid brain regions. Interestingly, although also undergoing ethanol withdrawal, rats that received TSA-treatment two hours before the behavioral tests displayed remarkably decreased anxiety, and increased DSD in amygdala. Particularly in LDB test, anxiety-like behaviors exhibited by these TSA treated ethanol-withdrawn rats are even lower than control rats. These data suggest that histone acetylation may be involved in withdrawal-induced anxiety and synaptic plasticity, and that inhibiting HDAC activity by TSA can block these effects. TSA treatment did not have an effect on anxiety-like behavior in alcohol naïve rats, indicating the protective effect of TSA
acts through inhibiting excessive HDAC activity. Our current data is consistent with recent studies that suggested critical roles of histone acetylation in synaptic plasticity: Overexpression of HDAC2 decreased dendritic spine density and impaired LTP in the hippocampus of mice (Guan et al. 2009). Additionally, HDAC2 knock out mice were found to have increased synaptophysin in the hippocampus and amygdala and increased LTP (Guan et al. 2009). Histone acetylation was increased within the BDNF promoter II in HDAC2 knock out mice, but decreased in HDAC2 over-expressed mice (Guan et al. 2009). Chronic treatment with HDAC inhibitor SAHA enhanced memory formation and reversed decreased dendritic spine density caused by over expression of HDAC2 (Guan et al. 2009). A previous study in our lab also revealed that BDNF infusion into CeA of ethanol-withdrawn rats can compensate the decrease in BDNF expression during withdrawal and normalize anxiety levels in these rats (Pandey et al. 2008). Here we showed that TSA treatment restored the BDNF level, and corrected the anxiety-like behavior in withdrawal rats. These results suggest decreased BDNF expression and anxiety-like behavior in rats during ethanol withdrawal may be regulated by histone acetylation.

A summary of results we obtained from three models in the current thesis is as below:
The BDNF signaling pathway is activated by binding of BDNF with its receptor TrkB, which further phosphorylate and activate downstream effector cascades, such as the MAPK pathway. During the signaling cascade transduction, Erk1/2 is phosphorylated, which in term phosphorylate and activate transcription factor CREB. Phosphorylated CREB assists the expression of genes including BDNF and Arc. Both BDNF and Arc are associated with synaptic plasticity and dendritic spine density, and are possible impact factors on behaviors such as anxiety and alcohol drinking. The three anxious animal models used in the thesis are induced by pharmacological manipulation (U0126 infusion), environmental factor (chronic ethanol withdrawal), and genetic factors (selectively bred HAD rats).
All three models showed deficits in the BDNF signaling in the CeA. For example, when Erk1/2 functioning was blocked by U0126, we found decreased phosphorylated CREB and reduced Arc expression. Decreased BDNF and Arc expression was also found in the CeA, MeA, but not BLA of rats withdrawn from chronic ethanol intake. In high alcohol drinking (HAD) rats, we observed significantly decreased BDNF and CREB levels and functioning. On the other hand, when CeA BDNF levels were increased by exogenous BDNF infusion, phosphorylation of Erk1/2 and CREB, and Arc expression were increased. Higher anxiety levels were also observed in all three animal models to accompany the deficits in BDNF signaling. In particular, the anxiogenic effect of U0126 intra CeA infusion was associated with increased alcohol preference in rats. We further explored the possible involvement of dendritic spine changes in BDNF signaling regulated anxiety behavior. In ethanol-withdrawal rats, we found decreased DSD in the CeA, MeA, but not BLA, indicating that decreased DSD may associate with ethanol withdrawal-induced anxiety. However, correcting the deficits in this signaling pathway can reverse these behavioral changes. BDNF was able to compensate deficits caused by blockage of the signaling pathway by U0126 or Arc antisense, and reversed anxiety and alcohol preference. Ethanol withdrawal-induced anxiety and decrease of BDNF, Arc expression, and DSD can also be overturned by TSA treatment, indicating involvement of histone acetylation in regulation of anxiety and alcohol drinking behaviors. Taken together, these data indicated that the deficits in BDNF-Erk1/2 signaling, CREB
functioning, Arc expression, and DSD might be responsible for reversible higher anxiety-levels and alcohol preference.
5.2 Future Directions

While our understanding of the role of BDNF signaling in anxiety and alcoholism is advanced further through the results discussed in this thesis, many questions remain to be answered.

In the current studies, histone acetylation appeared to be involved in the transcriptional regulation of the BDNF and Arc genes during alcohol-withdrawal. However, rather as a sole player, histone acetylation is only one component within the complex epigenetic modification network. The eventual outcome of transcriptional regulation combines the effects of all the epigenetic modification mechanisms, which interplays and counter-plays between each other. Therefore, further exploration into other possible epigenetic involvement in anxiety and alcohol drinking is necessary. Data from our first animal model suggested the involvement of the ERK (ERK/MAPK) signaling cascade in BDNF regulated anxiety-like and alcohol-drinking behaviors. The decreased phosphorylation of CREB and Arc expression induced by an inhibitor of this pathway indicated that CREB may be the mediator between the reduced signaling and gene expression. On the other hand, CREB is not the only target of ERK signaling which regulates gene transcription. ERK/MAPK signaling has been implicated in the regulation of histone phosphorylation following contextual fear conditioning (Chwang et al. 2006). Specifically, stimulation of the MAPK pathway results in phosphorylation of H3 at Ser 10 and 28, which also induces H3 K14 acetylation and promoting gene expression in fibroblasts (Dunn and Davie 2005). Activation of both the extracellular signal-regulated kinase (ERK) and p38 MAPK signaling pathways
increased differentiation of embryonic stem (ES) cells coupled with H3 phosphorylation (Ser-10)-acetylation (Lys-14) while inhibition of these pathways produced delayed ES cell differentiation (Lee et al. 2006). Therefore, reduced activation of the Erk1/2 signaling pathway may also suppress histone phosphorylation, contributing to the decreased CeA Arc expression observed in U0126 infused rats. Further understanding of the role the BDNF-Erk1/2 signaling pathway in anxiety and alcoholism may start with the determination of whether histone phosphorylation is involved in the Erk1/2 inhibitor induced gene expression and behavior changes.

Our data indicated that ethanol withdrawal induced anxiety is associated with decreased Arc and BDNF gene expression that is similar with our precious findings (Pandey et al. 2008). We extended these studies and found that administration of the HDAC inhibitor TSA was able to reverse the withdrawal-induced deficits in Arc and BDNF expression, as well as to normalized anxiety levels in rats, indicating the involvement of histone acetylation in the regulation of Arc and BDNF expression during anxiety. However, more evidence is needed to demonstrate the occupancy of histone acetylation on promoters of BDNF exons and Arc genes during ethanol withdrawal. Chromatin immunoprecipitation (ChIP) is a powerful tool to probe for specific protein-DNA interactions in vivo, and therefore it may be used to determine which genes are undergoing histone acetylation during the development of anxiety and alcoholism. DNA methylation, with the assistant of repressor proteins include methyl-CpG-binding domain proteins (MBD), is believed to regulate gene transcription through blockage of
transcriptional machinery, (Fan and Hutnick 2005). Evidence has been provided for DNA methylations involvement in long term memory (Griffith and Mahler 1969), cancers (Giovannucci et al. 1995; Esteller 2007; Christensen et al. 2008; Schernhammer et al. 2010; Sandoval and Esteller 2012), neurodegenerative (Franklin and Mansuy 2011; Iraola-Guzman et al. 2011) and psychiatric disorders (Dong et al. 2008; Dong et al. 2010). Therefore, future studies may use methylated DNA immunoprecipitation (MeDIP) assay to determine roles of DNA methylation in the regulation of gene expression during anxiety-like and alcohol-drinking behaviors.

We found that HAD rats have an innate deficiency in BDNF and CREB expression, as well as decreased phosphorylation of CREB. These deficits were correlated with higher anxiety levels in HAD rats compared with LAD rats. Therefore it would be interesting to further investigate whether correcting the deficits in BDNF by exogenous BDNF infusion into the CeA of HAD rats can normalize their anxiety and alcohol preference. BDNF, Erk1/2 signaling, CREB and Arc are indicated to be involved in the regulation of anxiety and alcoholism, however, further investigation into the mechanisms underlying the observed associations between these major players is needed to correctly interpret their role in alcoholism and anxiety. Whether molecules such as Erk1/2 and Arc, that have been studied in other animal models of anxiety, also account for the heightened anxiety and high alcohol preference in HAD rats will be another interesting question to explore. Furthermore, it was found that in comparison with NP rats, P rats have lower DSD in CeA and MeA, but not BLA, while acute
ethanol treatment increased DSD in these brain regions (Moonat et al. 2011). Therefore, golgi-cox staining may be employed to measure the DSD in amygdaloid brain region of the HAD/LAD rats in order to determine if deficits of BDNF and CREB function in HAD rats amygdala results in deficits in downstream DSD, and if heightened anxiety and alcohol preference is related to the changes in the dendritic spines.


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**PRESENTATIONS**


