Restorative effect of estrogen on basal forebrain cholinergic neurons

Zsombor Kőszegi

A thesis submitted for the degree of
Doctor of Philosophy
at the University of Otago
Dunedin, New Zealand

April 2011
Abstract

The basal forebrain cholinergic (BFC) system is one of the most important neurotransmitter systems in the brain. It has received much attention in the past two decades, primarily for its role in learning, memory, attention and behavior. The BFC system has also been reported to be particularly vulnerable in neurodegenerative diseases, such as in Alzheimer’s disease (AD). The gonadal steroid, estrogen, is an essential contributor in controlling the vulnerability of the BFC system. Besides its classical or genomic mechanism, estrogen is known to have non-classical actions on intracellular signaling pathways. In this study, we investigated the ameliorative effects of estrogen treatment and the role of non-classical estrogen actions on BFC neurons in a neurodegenerative mouse model, *in vivo*.

N-methyl-D-aspartate (NMDA) was injected unilaterally into the substantia innominata - nucleus basalis magnocellularis (SI-NBM) complex of the basal forebrain to elicit cholinergic cell death in the injected area and thus fiber loss in the ipsilateral cortex. An acute treatment of 17β-estradiol (E2) after the NMDA-induced lesion restored the ipsilateral cholinergic fiber density in the cortex in a time- and dose-dependent manner. Conversely, it did not have any effect on the cholinergic cell loss in the SI-NBM. The ameliorative action of E2 on cholinergic fiber loss was detected in both intact and gonadectomized young male and female mice, but not in aged animals. The E2-induced cholinergic fiber density restoration was also absent in neuron-specific estrogen receptor α (ERα) knockout mice. Selective blockade of the mitogen activated protein kinase (MAPK) and protein kinase A (PKA) pathways prevented E2’s ability to restore the cholinergic fiber density. Furthermore, activation of non-classical estrogen signaling by a non-classical pathway activator (estren) induced E2-like fiber restoration.

Our findings demonstrate that estrogen restores the cholinergic fiber density in the cortex through a non-classical signaling mechanism after the loss of subcortical cholinergic input.
Similar restorative effects were observed in young animals, irrespective of sex or endogenous estrogen levels. These observations reveal a critical role for non-classical estrogen signaling via ERα and MAPK-PKA pathways in BFC neurons, \textit{in vivo}. Taken together, our study discloses important aspects relating to the vulnerability of the BFC system in neurodegenerative processes, such as AD or traumatic brain injury and might shed light on future medical treatments through the use of non-classical estrogen pathway activators.
Acknowledgement

It would not have been possible to finish this doctoral study without the help and support from the people around me.

Firstly, I would like to thank my supervisor, Dr. István Ábrahám, for offering an opportunity in his lab. Thanks to his encouragement, advice, guidance, and personal support from the preliminary to the concluding level enabled me to develop an understanding of the subject. Working in the Ábrahám lab has been an enjoyable experience for me due to his friendly and easy-going nature.

I am extremely thankful to my Ph.D. Committee Members, Prof. Allan Herbison and Dr. Colin Brown whose good advice and support has also been invaluable. In addition, I appreciate Mr. Rob Porteous’s technical support in the generation of knockout mice for this study.

I am most grateful to my fellow Ph.D. colleague, Rachel Cheong, for her kindness, friendship, and scientific assistance. I should also note that without her insightful comments and numerous corrections of my English documents my life would have been much more difficult. I would like to thank her for all she is, as her presence has always been cheerful.

I would like to express my appreciation to my co-workers, Zsuzsanna Barad and Emeline Tolod-Kemp for their kind support throughout my studies. Not forgetting, other members of the Ábrahám lab, Marion (Toni) Turnbull, Soohyun Kim, Daniil Potapov, and Andrea Kwakowsky who have also been very helpful, and creating a friendly and entertaining environment.

This thesis is completed with the financial, academic, and technical support of the Department of Physiology, Centre for Neuroendocrinology, and staff. I also thank the University of Otago for awarding me the Doctoral Scholarship.
# Table of content

## CHAPTER 1  1

### Introduction  1

1.1 The basal forebrain cholinergic system  1  
1.1.1 Anatomy and nomenclature  1
1.1.2 BF projections  3
1.1.3 Other neuronal phenotypes  6
1.1.4 Physiological role  6
1.1.5 BFC neurodegenerative models  9

1.2 Actions of estrogen on BFC neurons  12  
1.2.1 Mechanism of estrogenic actions  13
1.2.2 Estrogen-induced amelioration of BFC neurons  17

1.3 Rationale  26

1.4 Aims  27

## CHAPTER 2  28

### Methods  28

2.1 Animals  28
2.2 Gonadectomy  29
2.3 Intracerebral injections and treatments  30
2.4 Brain tissue collection  33
2.5 Methods to study the uterotrophic effects of estren  34
2.6 Methods to study the ERα distribution in cholinergic neurons  34
2.7 Histochemistry and immunohistochemistry  35
2.7.1 AChE histochemistry  35
2.7.2 ChAT, VACHT, and ERα peroxidase immunohistochemistry  35
2.7.3 ERα and ChAT double-labeling fluorescence immunohistochemistry  37
2.7.4 Haematoxylin-eosin staining  37

2.8 Analysis and statistics  38
2.8.1 Cholinergic cell counting  38
2.8.2 Cholinergic fiber densitometry  38
2.8.3 Analysis of ERα distribution in cholinergic neurons  41
2.8.4 Statistical analysis  42
### CHAPTER 3

The neurodegenerative mouse model

#### Introduction

#### Results

3.1 Effects of NMDA on cholinergic fibers

3.1.1 Different survival times following NMDA lesion

3.1.2 Different NMDA concentrations

3.1.3 Fiber loss detection using different histological approaches

3.2 Effects of NMDA on cholinergic cell bodies

#### Discussion

3.3 Visualization of cholinergic fibers and cell bodies

3.4 NMDA-induced neurodegeneration

3.5 Summary

### CHAPTER 4

Restorative effect of E2 in OVX mice

#### Introduction

#### Results

4.1 Effects of E2 on cholinergic fibers following NMDA lesion

4.1.1 Time dependence

4.1.2 Dose dependence

4.1.3 Effects of E2 on cholinergic fibers in non-lesioned cortical areas

4.2 Effects of E2 on cholinergic cell bodies following NMDA lesion

#### Discussion

4.3 Estrogen treatment model

4.4 Cholinergic fiber restoration

4.5 Summary

### CHAPTER 5

Restorative effect of E2 under different physiological conditions

#### Introduction

#### Results

5.1 Effects of E2 on cholinergic fibers in intact female mice

5.1.1 Effects of E2 on cholinergic fibers in non-lesioned cortical areas

5.2 Effects of E2 treatments in male animals
5.2.1 Effects of E2 on cholinergic fibers following NMDA lesion 77
5.2.2 Effects of E2 on cholinergic cells bodies following NMDA lesion 79
5.3 Effects of E2 on cholinergic fibers in aged animals 80

Discussion 83
5.4 Influence of endogenous estrogen levels 83
5.5 Sex differences 85
5.6 Effects of aging 86
5.7 Summary 88

CHAPTER 6 89
Mechanism of the E2-induced cholinergic fiber restoration

Introduction 89

Results 91
6.1 Effects of E2 on cholinergic fibers in ERα KO mice 91
6.2 Subcellular distribution of ERα in cholinergic neurons 93
6.3 Effects of signaling inhibitors on the E2-induced restoration 96
6.4 Effects of estren 98
  6.4.1 Effects of estren on the uterus 98
  6.4.2 Effects of estren on cholinergic fibers following NMDA lesion 100

Discussion 102
6.5 Involvement of the ERα 102
6.6 Participation of intracellular signaling pathways 103
6.7 Estren as a non-classical pathway activator 105
6.8 Summary 108

CONCLUSION 109

REFERENCES 116

APPENDIX 133
List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BFC groups</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>BFC projections</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Classical versus non-classical estrogen pathway</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Membrane-linked estrogenic actions</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Estrogen-induced ameliorative actions on cholinergic neurons</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Non-classical estrogen pathway activators</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Cre – loxP technique</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>Intracerebral injections</td>
<td>33</td>
</tr>
<tr>
<td>9</td>
<td>Time-line of the investigation of estrogenic actions</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>AChE-positive fiber densitometry</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>NMDA lesioned cholinergic fibers in the cortex</td>
<td>46</td>
</tr>
<tr>
<td>12</td>
<td>Effects of different survival times on NMDA-induced cholinergic fiber loss</td>
<td>47</td>
</tr>
<tr>
<td>13</td>
<td>Effects of different NMDA concentrations on cholinergic fibers</td>
<td>48</td>
</tr>
<tr>
<td>14</td>
<td>AChE- and VACht-positive fibers in the cortex</td>
<td>49</td>
</tr>
<tr>
<td>15</td>
<td>AChE and VACht fiber loss comparison</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>Cholinergic cell bodies in the SI-NBM</td>
<td>51</td>
</tr>
<tr>
<td>17</td>
<td>Effects of NMDA lesion on cholinergic cell bodies in the SI-NBM</td>
<td>52</td>
</tr>
<tr>
<td>18</td>
<td>Effects of E2 treatment on cholinergic fibers following NMDA lesion in OVX</td>
<td>62</td>
</tr>
<tr>
<td>19</td>
<td>Effects of different E2 treatment timings on cholinergic fibers following NMDA</td>
<td>63</td>
</tr>
<tr>
<td>20</td>
<td>Effects of different E2 doses on cholinergic fibers following NMDA lesion in OVX</td>
<td>64</td>
</tr>
<tr>
<td>21</td>
<td>Effects of E2 treatment on non-lesioned cholinergic fibers in OVX mice</td>
<td>65</td>
</tr>
<tr>
<td>22</td>
<td>Effects of E2 treatment on cholinergic cell bodies following NMDA lesion in OVX</td>
<td>66</td>
</tr>
<tr>
<td>23</td>
<td>Model of the E2-induced fiber density restoration</td>
<td>71</td>
</tr>
<tr>
<td>24</td>
<td>Effects of E2 treatment on cholinergic fibers following NMDA lesion in intact female mice</td>
<td>76</td>
</tr>
<tr>
<td>25</td>
<td>Effects of E2 treatment on non-lesioned cholinergic fibers in intact female mice</td>
<td>77</td>
</tr>
<tr>
<td>26</td>
<td>Effects of E2 treatment on cholinergic fibers following NMDA lesion in ORX mice</td>
<td>78</td>
</tr>
</tbody>
</table>
Figure 27: Effects of E2 treatment on cholinergic cell bodies following NMDA lesion in ORX mice 80
Figure 28: Effects of E2 treatment on cholinergic fibers following NMDA lesion in aged male and female mice 82
Figure 29: ERα in a cholinergic neuron in wild-type and ERα KO mice 92
Figure 30: Effects of E2 treatment on cholinergic fibers following NMDA lesion in ERα KO mice 93
Figure 31: ERα distribution in cholinergic neurons 95
Figure 32: ERα distribution in cholinergic neurons following NMDA lesion 96
Figure 33: Effects of E2 treatment on cholinergic fibers following NMDA lesion in the presence of signaling inhibitors in OVX mice 97
Figure 34: Estren and E2 treated uteri 99
Figure 35: Effects of estren and E2 on the uterus 100
Figure 36: Effects of different estren doses on cholinergic fibers following NMDA lesion in OVX mice 101
Figure 37: Proposed dose dependent action of estren 107
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AF64A</td>
<td>ethylcholine aziridinium ion</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid-beta</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BF</td>
<td>basal forebrain</td>
</tr>
<tr>
<td>BFC</td>
<td>basal forebrain cholinergic</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CamKIIα</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II α</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ch1-4</td>
<td>cholinergic cell group 1-4</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>extracellular-signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>ERT</td>
<td>estrogen replacement therapy</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino-butyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GPR30</td>
<td>G protein coupled receptor 30</td>
</tr>
<tr>
<td>HACU</td>
<td>high affinity choline uptake</td>
</tr>
<tr>
<td>HDB</td>
<td>horizontal limb of the diagonal band of Broca</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK 1/2</td>
<td>MAP kinase kinase 1/2</td>
</tr>
<tr>
<td>MS</td>
<td>medial septum</td>
</tr>
<tr>
<td>NBM</td>
<td>nucleus basalis magnocellularis</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>ORX</td>
<td>orchidectomy or orchidectomized</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomy or ovariectomized</td>
</tr>
<tr>
<td>P75NTR</td>
<td>low-affinity nerve growth factor receptor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>S1 and S2</td>
<td>somatosensory cortex 1 and 2</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SI</td>
<td>substantia innominata</td>
</tr>
<tr>
<td>SI-NBM</td>
<td>substantia innominata–nucleus basalis magnocellularis</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TrkA</td>
<td>high-affinity nerve growth factor receptor or neurotrophic tyrosine kinase A</td>
</tr>
<tr>
<td>TrkB</td>
<td>neurotrophic tyrosine kinase B or tropomyosin-related kinase B</td>
</tr>
<tr>
<td>VACHT</td>
<td>vesicular acetylcholine transferase</td>
</tr>
<tr>
<td>VDB</td>
<td>vertical limb of the diagonal band of Broca</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

1.1 The basal forebrain cholinergic system

1.1.1 Anatomy and nomenclature

The cholinergic system is one of the most important neurotransmitter systems in the brain. A major population of cholinergic neurons is located in the basal forebrain (BF) (Lewis and Shute, 1967). The BF has received much attention in the past two decades, primarily because of the role of cholinergic neurons in learning, memory, attention, behavior as well as their involvement in the pathophysiology of Alzheimer’s disease (AD). The BF is located at the base of the forebrain, anterior to the hypothalamus. Laterally, it extends towards the amygdala and the piriform cortex, while the olfactory bulb and nucleus accumbens form its rostral border. Numerous nomenclatures can be found in the literature about the basal forebrain cholinergic (BFC) system. Although various cell clusters can be distinguished, there is no clear anatomical border among the different BFC groups. The most common classification divides these neuron populations into four anatomical regions: medial septum (MS), vertical and horizontal limbs of the diagonal band of Broca (VDB and HDB, respectively), and nucleus basalis magnocellularis (NBM) (Mesulam et al., 1983a). Most of the cholinergic neurons can be found in the MS and HDB. The NBM has also been reported to have a great number of cholinergic neurons. Meynert discovered the NBM neurons in 1872 in humans and described the structure as the nucleus of the ansa lenticularis (Meynert, 1872). Later, Kolliker changed the name to nucleus basalis (Kolliker, 1896). The NBM region is a complex nucleus and is commonly labeled as subcommisural grey, sublenticular region, preoptic magnocellular nucleus or substantia innominata (SI), depending on the classification (Mesulam and Mufson, 1984). Luiten and colleagues (1987) have defined this region as a complex of the SI and
ventral pallidum. Heimer classified the anterior portion of the SI as the ventral extension of the pallidum while he associated the posterior portion with the extended amygdala (Heimer, 2000). Despite these various names, a study has shown that the SI and NBM areas are rich in choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) containing neurons (Poirier et al., 1977). These two key enzymes are responsible for acetylcholine (ACh) synthesis (CHAT) and cleavage (AChE) (Ravin et al., 1953; Tucek, 1985). Moreover, the SI and NBM neurons provide widespread projections to the cortex, differentiating them from other BFC populations. To avoid the confusion and to be able to apply classification to the BFC system, Mesulam proposed a new nomenclature. This classification allows us to use simple terms for complex anatomical structures. The different BFC groups were named as “cholinergic cell groups”: Ch1 (MS), Ch2 (VDB), Ch3 (HDB), and Ch4 (NBM) (Mesulam et al., 1983b). The NBM (Ch4) was further subdivided into six sectors: anteromedial (Ch4am), anterolateral (Ch4al), anterointermediate (Ch4ai), intermediodorsal (CH4id), intermedioventral (Ch4iv) and posterior (CH4p) regions based on their preferred projection targets (Mesulam et al., 1983a).

In this study, the term “SI-NBM complex” refers to the Ch4 (SI, NBM, preoptic magnocellular nucleus, and ansa peduncularis), without further differentiating smaller anatomical subpopulations inside this area.
Figure 1 demonstrates the four different BFC structures in a rodent brain.

Figure 1: BFC groups

Figure showing the different cholinergic groups (MS, VDB, HDB, SI-NBM) in the rodent BF. H: hippocampus, OB: olfactory bulb. Figure adapted from Mesulam et al., 1983b.

1.1.2 BF projections

Several pathways run through the BF, which contains diverse neuronal populations that add to the anatomical complexity of this structure. The organization of cholinergic projections from the BF was investigated using retrograde horseradish peroxidase (Hreib et al., 1988; Parent et al., 1988; Barstad and Bear, 1990) and anterograde Phaseolus vulgaris tracing techniques (Luiten et al., 1987). AChE histochemistry and ChAT immunohistochemistry were also widely used to map cholinergic innervations in the brain (Johnston et al., 1979; Wenk et al., 1980; Ribak and Kramer, 1982; Houser et al., 1983; Levey et al., 1983; Woolf et al., 1983). Studies show that the MS innervates the hippocampus (Kitt et al., 1987; Zaborszky et al., 1999). The VDB projects to the hippocampus and hypothalamus. The MS and VDB projections form the “cholinergic limbic system”. This term applies to those cholinergic neuronal populations that are connected to hippocampal formation (Lewis and Shute, 1967). Retrograde labeling studies revealed that cholinergic neurons in the HDB project to the olfactory bulb (Woolf et al., 1984; Zaborszky et al., 1999). The SI-NBM complex has a
widespread projection area in the cortex and amygdala (Mesulam and Van Hoesen, 1976; Lehmann et al., 1980; Boegman et al., 1992). In fact, the SI-NBM area is the only major source of cholinergic innervation in the cortex. Studies in several species including rodent (Jacobowitz and Palkovits, 1974; Kimura et al., 1980), cat (Kimura et al., 1981) and monkey (Struble et al., 1986; Kitt et al., 1987) have shown that many projecting neurons in the SI-NBM are positive for ChAT and AChE. Lesion studies also demonstrated correlation between the loss of cholinergic neurons in the SI-NBM and the decline in cortical function using AChE histochemistry or memory performance tests (el-Defrawy et al., 1985; Knowlton et al., 1985; Stewart et al., 1985; Struble et al., 1986). Track tracing studies have shown that cortical areas receive unilateral innervations from the SI-NBM (Pearson et al., 1983; Walker et al., 1985; Kitt et al., 1987). Thus, unilateral lesion of the SI-NBM in rats showed a decrease in the AChE-positive fiber density in the ipsilateral cortex (Wenk et al., 1980). Houser and colleagues (1985) demonstrated that all layers of the cortex receive ChAT-positive fiber innervations with relatively dense terminal fields in both motor and sensory areas. It has also been reported that silver intensified ChAT and AChE fiber patterns in the cortex are identical in the mouse (Kitt et al., 1994). Although cholinergic fibers are found throughout the cortex, denser areas can be located depending on the cortical area (Kitt et al., 1994). The visual cortex has thicker cholinergic bands in layers I-IV. The somatosensory cortex has the majority of the projections in layer IV. The frontal cortex has increased AChE-positive densities in layers I, IV, and lower V, upper VI (Kitt et al., 1994).
Figure 2 demonstrates the most important BFC projections.

Figure 2: BFC projections

Figure showing cholinergic projections from the BF to other brain areas. The MS and VDB provide innervations to the hippocampus, whereas the HDB projects axons mainly to the olfactory bulb. The SI-NBM complex is the major cholinergic source for the cortex. H: hippocampus, OB: olfactory bulb. Figure adapted from Mesulam et al., 1983b.

Although the BF represents significant inputs to other brain areas, afferent projections from other regions to the BF are also present. Several prefrontal regions contribute to the innervation of the BF (Gaykema et al., 1991). The amygdaloid innervations to the SI-NBM are the most prominent among the forebrain afferents, whereas the prefrontal cortex also projects to this area (Russchen et al., 1985). The hippocampus is also known to be an important source of projections to the MS and diagonal bands (Mesulam and Mufson, 1984). Studies using light and electron microscopy have demonstrated that the BF receives numerous inputs, favouring gamma-amino-butyric acid (GABA)ergic, somatostatin, substance P, cholinergic, galanin and neuropeptide Y axon terminals (Zaborszky et al., 1999). Together, these different innervations contribute to the complexity of the BF.
1.1.3 Other neuronal phenotypes

Studies on the BF focus mainly on cholinergic neurons and their physiological relevance. The well-described BFC projections and the availability of the selective cholinergic toxin (192 IgG-saporin) provide an effective basis for functional studies. However, the BF (including the SI-NBM) contains several other neuronal phenotypes, such as GABAergic neurons (Gritti et al., 1993). To label GABAergic neurons, glutamic acid decarboxylase (GAD)-immunoreactivity (the biosynthetic enzyme of GABA) is a commonly used method (Oertel and Mugnaini, 1984; Veenman and Reiner, 1994). The Ca$^{2+}$ binding protein parvalbumin is also exclusively expressed by GABAergic neurons in the BF (Brauer et al., 1993). Although GABAergic neurons are smaller than the magnocellular cholinergic neurons, they outnumber them, indicating their importance in different BF functions (Gritti et al., 1993). It has also been shown that none or very few neurons in the BF (less than 1%) are positive for ChAT and GAD together (Kosaka et al., 1988; Gritti et al., 1993). GABAergic neurons in the BF also provide cortical projections (Zaborszky et al., 1999; Henny and Jones, 2008). Moreover, cholinergic neurons directly connect to GABAergic interneurons providing complex excitatory and inhibitory effects on cortical cells (Sarter and Bruno, 2002). Apart from the GABAergic neurons, the BF contains other neuronal phenotypes that express glutamate and several types of neuropeptides (substance P, somatostatin, neuropeptide Y, neurotensin) (Semba, 2000); however, their function is less understood.

1.1.4 Physiological role

Influence of cortical functions

The major source of cholinergic projections to the neocortex is the SI-NBM (Mesulam et al., 1983b; Semba, 2000). Therefore, neurons located in the SI-NBM play an important role in cortical brain functions, influencing them from early stages of development (Hohmann and Ebner, 1985; Kostovic, 1986). ACh is essential in the structural and functional remodeling of
cortical circuits, particularly in the establishment of synaptic contacts (Berger-Sweeney, 2003). Various studies have implicated the role of the BF in higher brain functions. The first study to report that the BFC system is involved in learning and memory was done by Deutsch (1971). Behavioral studies showed that BFC lesions evoke cognitive impairments, especially in processes related to attention (Sarter and Bruno, 1997). BF lesions by ibotenic acid in rats and monkeys also showed that cholinergic neurons and their projections are important in specific aspects of attention (Dunnett et al., 1991; Voytko et al., 1994). BFC projections to the cortex are vital factors in learning-induced synaptic plasticity as well (Zaborszky et al., 1999). Conner and colleagues (2005) with the use of a selective cholinotoxin (192 IgG-saporin) have demonstrated that the BFC system is essential in enabling plasticity mechanisms and cortical reorganization for functional recovery following brain injury. An *in vivo* microdialysis study demonstrated that visual and somatosensory stimulations evoke ACh release in a modality and region specific manner (Fournier et al., 2004). BFC neurons consist of different neuronal populations suggesting that these groups of cells can be activated by different sensory inputs, leading to region specific responses (Fournier et al., 2004). The BFC system also participates in cognition-linked sensory processes and memory. ACh levels in the cortex are critical for incoming sensory stimuli and necessary for adaptive responses that require memory (Gray, 1999). It is worth noting that many of the functional consequences of non-selective lesions (e.g. by ibotenic acid) on memory and other performance related tasks can be attributed to the disruption of other neuronal phenotypes. Although studies using saporin-induced selective cholinergic lesion have shown that BFC neurons influence spatial memory and other learning processes (Dashniani et al., 2009a, 2009b), no robust effects were found. Results from various studies are mixed, which could be due to different experimental parameters. Non-selective lesions showed robust changes in EEG recordings (Vanderwolf et al., 1993), whereas selective cholinergic lesions had minor or no effects on the spontaneous EEG activity or sleep-wake states (Bassant et al., 1995; Berntson et al., 2002). It is likely that complex
learning and memory behaviors are results of a synchronized activity of various neuronal networks, including the BFC system, rather than a clearly defined cholinergic region. The neurochemically heterogeneous BF projections have been proposed to modulate cognitive, emotional, and regulatory functions (Semba, 2000). Furthermore, non-cholinergic neurons in the BF are known to be able to modulate prefrontal cortical activity in a rapid manner (Lin et al., 2006). Nevertheless, it is still accepted that ACh from the BF plays a pivotal role in the prefrontal, parietal, and somatosensory regions of the cortex (Klinkenberg et al., 2011).

Involvement in pathological conditions

During aging, cholinergic neurons undergo degenerative changes resulting in a down-regulation of ChAT activity, which eventually leads to a decreased activity of progressing memory (Nyakas et al., 2010). Furthermore, several research groups reported that the BFC system is also affected in neurodegenerative diseases. These studies showed a severe deficiency of cholinergic markers in the cortex of AD patients (Perry et al., 1978; Gilmor et al., 1999; Gibbs, 2010; Nyakas et al., 2010). It has been known for years that cholinergic blockade with scopolamine (cholinergic receptor antagonist) results in cognitive impairments and creates symptoms of mild dementia, indicative that cholinergic dysfunction is one of the major contributors in the cognitive decline of AD (Delwaide et al., 1980; Caine et al., 1981). Studies demonstrated a reduction in the number of cholinergic neurons, ChAT activity, high affinity choline uptake (HACU), and ACh release in brain tissues of AD patients (Whitehouse et al., 1982; Rylett et al., 1983; Nilsson et al., 1986; Lehericy et al., 1993). Moreover, cholinergic deficits correlate with cognitive impairments (Perry et al., 1978; DeKosky et al., 1992). Treatment with drugs targeting cholinergic functions has proven to be effective in AD, although with limited success (Gauthier, 2002). The two major hallmarks of the disease, amyloid-beta (Aβ) plaques and neurofibrillary tangles, have been shown to be related to cholinergic degeneration. However, the molecular mechanism of the degeneration of
cholinergic neurons in AD is still not fully understood. Aβ plaques co-localize with AChE-positive fibers (Mesulam, 1986) and it has been hypothesized that Aβ peptides cause neurodegeneration at cholinergic terminals (Selkoe, 2002; Dolezal and Kasparova, 2003). Aβ depresses ACh synthesis and release, increases Ca²⁺ influx via N-methyl-D-aspartic acid (NMDA) receptors and interacts with both nicotinic and muscarinic ACh receptors (Harkany et al., 2000a; Auld et al., 2002). Degeneration of cholinergic neurons has been observed not only in AD but also in Parkinson’s disease (Whitehouse et al., 1983; Bohnen et al., 2003), Jakob-Creutzfeld disease (Arendt et al., 1984) or traumatic brain injury (Salmond et al., 2005).

1.1.5 BFC neurodegenerative models

Dysfunction of the cholinergic projections from the SI-NBM is thought to be responsible for some of the cholinergic impairments in AD (Saper et al., 1985). Observations of degenerated cholinergic neurons in the SI-NBM and their projections in the cortex of human AD patients served as a basis for the development of animal models, in which cholinergic neurons were lesioned. SI-NBM lesioned animals represent a model of cholinergic hypofunction. This model has been widely used in understanding the cholinergic system and for identifying pharmacological treatments on cholinergic neurons. Lesioning of the SI-NBM has been considered a valuable model, mimicking memory deficits in many studies (Dekker et al., 1991; Boegman et al., 1992).

Among the chemical BFC lesion models, there are different strategies to induce cholinergic dysfunction. The most prevalent approach is the injection of different excitatory amino acids, such as glutamate, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA), quisqualic acid, kainic acid, NMDA or Aβ₁-₄₂ (Dekker et al., 1991; Abraham et al., 2000; Horvath et al., 2000; Harkany et al., 2000c). Glutamate exposure (or its analogs) has been reported to induce AD associated antigens in neuron cultures (De Boni and McLachlan,
1985). The NMDA subclass of the excitatory glutamate receptors is known to be involved in mediating neuronal death following acute brain injury, such as ischemia (Simon et al., 1984). A similarly important role for NMDA has also been reported in the excitotoxic component of AD. Although the NMDA-induced excitotoxicity might not be the initiating factor of the disease, it can interact with essential intracellular processes to further develop neurotoxicity and disease progression (Dodd et al., 1994). The most significant event in the NMDA-induced neuronal degeneration is the excessive Ca\(^{2+}\) influx, which results in a cascade of various events. An increase in the intracellular Ca\(^{2+}\) concentration can activate protein kinases that disrupt the cytoskeleton, or cause mitochondrial dysfunction. Likewise, cell death could occur through the disintegration of the mechanism that maintains the excitatory amino acid concentration at physiological level (Dodd et al., 1994; Lee et al., 1999). Excessive stimulation by NMDA may also trigger the production of free radicals such as nitric oxide or superoxide anions. The accumulation of these radicals can result in a necrotic and/or apoptotic neuronal death, depending on the dosage of NMDA used (Bonfoco et al., 1995). Further evidence suggested that the NMDA receptor mediated glutamate excitotoxicity is a major step in the neurodegenerative process triggered by different Aβ peptides (Mattson et al., 1992). Thus, the injection of NMDA at various toxic concentrations into the SI-NBM is followed by cholinergic cell and thus fiber loss that originate from the damaged SI-NBM. This NMDA-induced cholinergic lesion is a well-established experimental model in rodents (Luiten et al., 1995; Oosterink et al., 1998; Abraham et al., 2000; Horvath et al., 2000; Harkany et al., 2001a, 2001b; Dolga et al., 2009).

Ethylcholine aziridinium ion (AF64A) treatment of cholinergic targets has also been used in various studies. AF64A induces a persistent central cholinergic hypofunction of presynaptic origin, providing a selective cholinotoxic method (Fisher and Hanin, 1986). Intracerebral or systemical injections of monoclonal antibodies against AChE (Rakonczay et al., 1993) or the ribosome-inactivating protein, saporin, coupled to a monoclonal antibody against the low-
affinity nerve growth factor (p75NTR) receptor (McGaughy and Sarter, 1999; Lehmann et al., 2000; Galani et al., 2002) have also been widely used by different laboratories for cholinergic lesions. The p75NTR receptor, expressed exclusively by cholinergic neurons (Sobreviela et al., 1994), is a transmembrane protein that binds all neurotrophins; therefore providing a selective target approach of the cholinergic system.
1.2 Actions of estrogen on BFC neurons

The gonadal steroid, estrogen, is known to be one of the most important female steroid hormones. It exists as three different forms in humans and rodents; 17β-estradiol (E2), which is the most dominant form, followed by estrone, and estriol. Estrogen(s) is a group name commonly used to refer to all three estrogen forms. For this thesis, “E2” represents 17β-estradiol and used as a single form in treatments, whereas “estrogen” is used in the introduction and discussion sections to discuss general estrogenic actions.

Apart from the essential importance in reproductive functions, estrogen has effects on various tissues throughout the body, including the central nervous system in both sexes. It has been demonstrated that estrogen is a growth and trophic factor for the brain in all ages, influencing neurogenesis, differentiation, and neuronal survival (Behl, 2002a; Toran-Allerand, 2004). Among various factors, controlling the vulnerability of BFC neurons, the gonadal steroid estrogen is one of the most important contributors. The effects of estrogen have been studied on several different BFC models. Ovariectomy (OVX) has been shown to decrease the length of cholinergic dendrites (Saenz et al., 2006), whereas estrogen treatment enlarges the soma of cholinergic neurons in the BF in vivo (Ping et al., 2008) as well as increases the total neurite length and branching in vitro (Dominguez et al., 2004). In addition to the morphological alterations, estrogen exposure also increases ACh synthesis in BFC neurons and their projection areas, underlining the functional role of estrogen in neurochemical regulation of these neurons. Estrogen exposure induces ACh synthesis via an increase in ChAT activity (McMillan et al., 1996; Gibbs, 1997), while OVX brings a reduction in the ChAT mRNA level and protein expression in the MS and SI-NBM (Gibbs, 1998). These findings were supported by studies in which estrogen treatment increased the ACh concentration in BFC cortical projection areas (Ping et al., 2008). Lesion studies, mimicking neurodegenerative pathological conditions, demonstrated that estrogen treatment could enhance cognitive
functions and restore synaptic connectivity following NMDA or 192 IgG-saporin-induced cholinergic cell death in the SI-NBM (Horvath et al., 2002; Saenz et al., 2006). The clear gender differences in human neurodegenerative diseases, such as AD or Parkinson’s disease, further highlight the importance of this steroid hormone in pathological brain functions and neuroprotection (Leranth et al., 2000; Vina and Lloret, 2010).

1.2.1 Mechanism of estrogenic actions

It is commonly accepted today that estrogen can modify cellular behavior in two different ways: classical and non-classical. Estrogen exerts its “classical” effects through direct DNA binding of liganded estrogen receptor (ER) and consequent gene transcription. Besides this classical action, estrogen also influences intracellular second messenger pathways such as the protein kinase A (PKA), mitogen-activated protein kinase (MAPK) and cAMP response element-binding protein (CREB) in a rapid manner (Carlstrom et al., 2001; Kim et al., 2002; Abraham et al., 2003; Guerra et al., 2004; Abraham and Herbison, 2005; Vasudevan et al., 2005; Zhao et al., 2005). This latter mechanism is defined “non-classical”, as it indirectly acts on gene transcription via signaling systems (Figure 3). Some articles use the genomic and non-genomic terms, indicating direct and indirect gene transcription, respectively. However, these expressions can be misleading because the non-classical estrogen pathway eventually leads to gene transcription as well. It is also common, although similarly misleading, to use the word “rapid” to refer to the non-classical actions, as the fast activation of signaling events is a key feature of this pathway. Estrogen has been shown to activate both classical and non-classical pathways in BFC neurons (Marin et al., 2003a; Szego et al., 2006).

Following the pioneer discovery almost 50 years ago, describing estrogen’s effects in the uterus, subsequent findings revealed that estrogen has two classical receptors; the ERα (Green et al., 1986; Greene et al., 1986) and ERβ (Kuiper et al., 1996). Both types of receptors share common features such as structure, ligand binding domain and nuclear localization; however,
their distribution in the brain and physiological relevance differ (Behl, 2002a). The highest neuronal and glial ER expression is in the forebrain, preoptic area and hypothalamus. In some areas the two types of ERs are co-expressed in the same cell (Behl, 2002a). BFC neurons have been shown to express predominantly ERα, providing an effective platform for estrogenic actions (Shughrue et al., 2000; Kalesnykas et al., 2004). In estrogen’s classical pathway, the binding of estrogen to the ERs (α or β) activate conformational changes that eventually lead to gene transcription. Both ERα and ERβ form homo or heterodimers and bind to the estrogen response element (ERE) in the DNA to initiate a direct gene transcription (Behl, 2002a). The main difference between the classical and non-classical pathways is that estrogen through the non-classical pathway does not initiate an ERE dependent gene transcription. The change in gene transcription is rather thought to be dependent on other transcription factors, such as CREB, which are activated indirectly through intracellular signaling events (Figure 3).

**Figure 3: Classical versus non-classical estrogen pathway**

Diagrams showing the activation of the classical (A) and non-classical (B) estrogen pathways. The classical mode involves binding of estrogen to its receptors (α and β), which then translocates into the nucleus to initiate ERE dependent gene transcription. The non-classical pathway consists of the activation of intracellular signaling events followed by an interaction with several ERE independent transcription factors.
Classical ERs (ERα and ERβ) can participate in both the classical and non-classical pathways. Moreover, it has been demonstrated that the non-classical estrogen actions can involve membrane associated ERs (Watson et al., 1999; Guerra et al., 2004; Vasudevan and Pfaff, 2007). The initiation point for the non-classical actions of estrogen at the membrane can be linked to various receptors with different molecular structures. Studies reported the importance of the classical ERs (ERα and ERβ) (Watson et al., 1999), a newly discovered membrane associated ER (ER-X) that shares some homology with ERα (Toran-Allerand, 2004), G protein coupled receptors such as the GPR30 (Qiu et al., 2003), and growth factor-like members (Anuradha et al., 1994). Another important initiation point for non-classical estrogenic actions is the specialized invagination of the cellular membrane, called caveola (Boulware et al., 2007). There are three caveolin isoforms (1-3), which are crucial for the structural integrity of the caveola. It has been shown that these structures are able to compartmentalize specific elements of signal transduction pathways and locally bring them together (e.g. ERs and members of the MAPK pathway) (Boulware et al., 2007). Caveolin isoforms, such as caveolin-1 can aid in the anchoring mechanism of signaling molecules. Thus, the caveola might help to initiate membrane-related estrogenic actions (Okamoto et al., 1998; Razandi et al., 2002; Boulware et al., 2007) (Figure 4).

Of the intracellular signaling pathways, the MAPK, PKA and CREB activations by estrogen are reported to be among the most essential (Lee et al., 2004; Boulware et al., 2005; Zhao et al., 2005; Szego et al., 2006). MAPKs are major components of intracellular pathways, influencing cell differentiation, proliferation and cell death (Pearson et al., 2001). One of the key molecules in this complex pathway is the p44/42 MAPK, also known as extracellular signal-regulated kinase (ERK) 1/2, which translocates into the nucleus in its activated form and phosphorylate CREB (Barabas et al., 2006). ERK 1/2 is activated by a pair of closely related mitogen activated protein kinase kinase (MEK)s, MEK1 and MEK2 (Crews et al., 1992). Selective blockade of MEKs has been reported to successfully inhibit the activation of
ERK 1/2 (Han and Holtzman, 2000; Szego et al., 2006). CREB is a member of a large family of transcription factors, which binds to the cAMP response element (CRE) on target genes (Shaywitz and Greenberg, 1999). Activation of CREB can be completed through the cAMP/PKA pathway as well. When intracellular cAMP levels are elevated, the regulatory subunit of PKA gets released to translocate into the nucleus where it phosphorylates CREB (Shaywitz and Greenberg, 1999). The selective blockade of the PKA pathway by specific inhibitors, such as H-89, has also been reported in earlier studies (Chijiwa et al., 1990; Kawasaki et al., 1998).

Research has shown that the MAPK pathway or its specific elements (ERK 1/2 and MEK 1/2) are involved in the estrogen-induced neuroprotection of neurons (Singer et al., 1999; Guerra et al., 2004). It has also been suggested that the activation of CREB via various kinases such as the MAPK or PKA is important in neuronal survival (Walton and Dragunow, 2000). Furthermore, estrogen-induced CREB phosphorylation occurs in BFC neurons, highlighting its essential role in these types of neurons (Szego et al., 2006). Figure 4 demonstrates the MAPK and PKA pathway activation by membrane-linked ERs.
Figure 4: Membrane-linked estrogenic actions

Diagram demonstrating different types of ERs located at the cell membrane. Binding of estrogen to these various receptors activates the non-classical estrogen pathway, resulting in a cascade of intracellular signaling mechanisms, which then lead to altered cellular functions and/or initiate gene transcription.

1.2.2 Estrogen-induced amelioration of BFC neurons

Several studies have indicated the importance of the estrogen-induced ameliorative actions on BFC neurons during neurodegenerative processes, such as AD (Mesulam, 1996; von Linstow Roloff and Platt, 1999; Marin et al., 2003a). The major mechanisms involve: 1) the alteration of Aβ or amyloid precursor protein processing (APP), 2) neurotrophin signaling, and 3) classical or non-classical actions on intracellular second messenger systems.

Effects of estrogen on Aβ and APP processing

Aβ peptides and their plaque formations, one of the major hallmarks of AD, are believed to contribute to the degeneration of BFC neurons. *In vivo* animal models, human observation,
and in vitro experiments demonstrated that several forms of the Aβ peptides, such as Aβ1-40 and Aβ1-42 cause cytotoxic death of cholinergic cells (Harkany et al., 2000c; Auld et al., 2002; Marin et al., 2003a). Estrogen exerts its ameliorative effects on BFC neurons via attenuation of Aβ related neurodegenerative processes involving a variety of mechanisms, such as reduction of Aβ accumulation and enhancement of microglial Aβ uptake. In a large number of in vivo AD animal models transgenic mice are used and they show high levels of Aβ cause amyloid deposits in the brain and display several other features seen in human AD (Games et al., 1995; Hsiao et al., 1996). In an effective cross of two transgenic mouse lines, amyloid aggregates can be visualized. In these mice, Aβ levels were higher after OVX compared to intact animals and this effect could be reversed by the administration of estrogen (Zheng et al., 2002). The APP, an integral membrane protein, also plays a critical role in the pathogenesis of AD. The abnormal processing of APP triggers the aggregation, deposition, and toxicity of its Aβ derivative. APP can be processed through amyloidogenic and non-amyloidogenic routes (Selkoe, 1999). In the α-secretase pathway, APP is cleaved within the amyloidogenic Aβ domain producing a large non-amyloidogenic soluble APP. In contrast, the β- and γ-secretase pathways lead to amyloidogenic Aβ that can form neurotoxic aggregates (Selkoe, 1999). It has been shown that estrogen can encourage the non-amyloidogenic breakdown of the APP, thereby acting against the Aβ plaque formation (Xu et al., 1998). This activity of estrogen is thought to be mediated by the MAPK pathway and is independent of ERs (Manthey et al., 2001).

**Effects of estrogen on neurotrophin signaling**

Several experiments suggest that estrogen may help to prevent the loss of cholinergic functions associated with aging or disease by increasing the responsiveness of BFC neurons to endogenous neurotrophins. Nerve growth factor (NGF) is thought to be an essential neurotrophin for BFC neurons. Its effects are mediated through two receptors, the low-affinity
(p75NTR) and high-affinity nerve growth factor receptors (TrkA) (Chao and Hempstead, 1995; Huh et al., 2008). Sobreviela and colleagues (1994) have shown that cholinergic neurons extensively express both receptors in the BF. Decreased neurotrophic support for cholinergic neurons has been associated with AD (Phelps et al., 1989). Binding to TrkA and activation of the receptor tyrosine kinase are essential for mediating trophic effects of NGF. Although the role of p75NTR is less clear, it may have anti-trophic action on BFC neurons. Indeed, p75NTR deficient mice have larger cholinergic neurons which suggest that neurotrophins acting through p75NTR receptors negatively regulate neuronal size (Yeo et al., 1997). Data indicates that an imbalanced NGF signaling can lead to altered neurodegeneration through neurotrophin receptors. TrkA neutralization can lead to potential Aβ production, whereas removing p75NTR signaling can be protective against induced amyloidogenesis (Capsoni et al., 2010). Studies have demonstrated that estrogen upregulates TrkA expression and downregulates p75NTR in the BF (Gibbs, 1998; Ping et al., 2002). It appears that estrogen may have an impact on the delicate TrkA and p75NTR balance.

The brain derived neurotrophic factor (BDNF) (another member of the neurotrophin family) and its receptor, the tropomyosin-related kinase B (TrkB), have also been shown to be important in maintaining the functionality of BFC neurons (Nonomura and Hatanaka, 1992). Moreover, there is clear evidence that estrogen upregulates TrkB (Jezierski and Sohrabji, 2001), indicating that estrogen influences BDNF-induced neuronal survival (Sohrabji and Lewis, 2006). We have been limited by no information about estrogen sensitivity of neurotrophins in the SI-NBM; however, some experiments have demonstrated that concentrations of BDNF, but not NGF, in BFC projection areas such as the hippocampus show a positive correlation with circulating estrogen levels (Bora et al., 2005; Franklin and Perrot-Sinal, 2006).
Chapter 1 - Introduction

**Classical and non-classical signaling and the role of ER**

Long-term estrogen administration alters gene expression via the classical pathway where liganded ERs interact with the ERE in the nucleus, inducing an ameliorative effect on cholinergic neurons (Marin et al., 2003a). Twenty-four hours treatment with estrogen effectively reduced Aβ neurotoxicity in the cholinergic SN56 cell line (Marin et al., 2003a), suggesting a classical way of estrogen action. This finding was supported by the fact that estrogen induced a translocation of ER to the nucleus and enhanced the activity of ERE-driven transcriptional machinery in this experiment (Marin et al., 2003a). Moreover, the estrogen-induced luciferase activity was blocked by the ER antagonist ICI 182,780, indicating ER dependent transcriptional activity in the SN56 cell line (Marin et al., 2003a).

The possible role of ERs in ameliorative actions is also suggested by human postmortem brain samples from patients with AD. Comprehensive immunohistochemical studies demonstrated that AD patients have a significantly higher number of ERα and ERβ in the SI-NBM (Ishunina and Swaab, 2001). In contrast, the metabolic activity of the neurons in AD usually shows a characteristic decrease in brain areas such as hippocampus, hypothalamus or even the SI-NBM (Salehi and Swaab, 1999). Besides the classical action, the non-classical estrogen effect on signaling molecules also appears to play a significant role in estrogen-induced ameliorative mechanisms. In this regard, the membrane bound ER plays a pivotal role. Using a membrane impermeable estrogen-horseradish peroxidase, Marin and colleagues (2003b) and Guerra and colleagues (2004) effectively ameliorated SN56 cholinergic cells against Aβ toxicity. *In vitro* SN56 cell line experiments explored the estrogen sensitive signaling pathways involved in the ameliorative actions. Using a specific MAPK pathway inhibitor or an upstream element inhibitor against Raf-1, the ameliorative effect of estrogen can be successfully blocked, indicating that the MAPK pathway is a critical signaling system against Aβ toxicity (Guerra et al., 2004). Estrogen-induced effects on signaling systems can also alter neurite outgrowth and branching in cholinergic cell cultures providing an effective...
regenerative potential under pathological conditions. Detailed morphological analysis revealed that BF primary cultures from rat respond to estrogen treatment with an increase in total neurite length and in total branch segment number (Dominguez et al., 2004). In these experiments, an upstream element (ERK1/2) inhibitor blocked the ability of estrogen to enhance neurite outgrowth. HACU or the newly synthesized ACh reflects the viability of BFC neurons. Recent in vitro findings reported that estrogen at physiological concentration increases cholinergic neuronal viability by modifying HACU and ACh synthesis (Pongrac et al., 2004; Bennett et al., 2009). Selective blockade of the estrogen-induced ERK1/2 phosphorylation resulted in the attenuation of estrogen-mediated changes in HACU and ACh synthesis (Pongrac et al., 2004). Another study demonstrated that estrogen induces ER-mediated CREB phosphorylation via the MAPK and PKA pathways in cholinergic neurons in the SI-NBM, in vivo (Szego et al., 2006). Although the role of ER-mediated CREB activation is uncertain in estrogen-induced ameliorative mechanisms, CREB has been linked as a key transcription factor in many ameliorative effects (Finkbeiner et al., 1997; Walton and Dragunow, 2000). A number of proteins with anti-apoptotic effects, such as the B-cell lymphoma 2 or BDNF contain CRE in their promoters (Tao et al., 1998; Pugazhenthi et al., 2000; Saini et al., 2004), suggesting an important role for CREB regulated transcriptional activity in neuronal survival. However, it remains to be determined whether estrogen sensitive CRE-mediated transcription has a role in neuronal survival in the BFC system.

Taken together, a great body of evidence supports the idea that estrogen plays a critical role in the amelioration of BFC neurons in animal models (both in vivo and in vitro) as well as in human neurodegenerative diseases. Figure 5 demonstrates the estrogen-induced ameliorative effects on cholinergic neurons.
Chapter 1 - Introduction

Figure 5: Estrogen-induced ameliorative actions on cholinergic neurons

Estrogen induces the activation of classical and non-classical pathways in cholinergic neurons to initiate amelioration. Hollow arrows indicate the alteration of receptors (neurotrophin and estrogen) and signaling molecules. These changed cellular functions then lead to altered gene transcription, which is important for potential amelioration. Figure adapted from Abraham et al., 2009.

Alternatives to estrogen therapy

During the past century, the average life span of women has increased dramatically, so women may now spend more than one-third of their lives in a chronic hypoestrogenic postmenopausal state, which raises critical medical concerns. It is known that women are at greater risk against age-related cognitive and neurodegenerative diseases such as AD, as the level of estrogen decreases (Musicco, 2009). Estrogen replacement therapy (ERT) has been shown to ameliorate cholinergic neuronal dysfunctions and cognitive impairments (Gibbs, 2000; Kompoliti et al., 2004; Tinkler et al., 2004). However, it has also been reported that ERT has several side effects, increasing risk of dementia or stroke in postmenopausal women (Shumaker et al., 2003; Maalouf et al., 2010). Clinical trials and observations have shown that these side effects depend on age and the initiation of the therapy. These factors may influence the benefit-risk ratio associated with therapy (Manson et al., 2006). Although ERT has
positive effects, especially starting it immediately after the cessation of the menstrual cycle, the treatment potential of estrogen is arguable. Our recent review highlighted the importance of alternative estrogen treatment therapies (Abraham et al., 2009). Several studies indicated the significance of the non-classical estrogen pathway, in which MAPK, PKA, and CREB signaling events are involved (Kim et al., 2002; Guerra et al., 2004; Zhao et al., 2005). These findings suggest that specific activation of the non-classical pathway may potentially be beneficial in future medical treatments, without side effects. In line with this hypothesis, estren (4-estr-3α, 17β-diol) has been investigated in several studies as a non-classical pathway activator (Kousteni et al., 2002, 2007; Moverare et al., 2003; Cordey et al., 2005; Otto et al., 2006). Additional selective activators (compounds A and C) have also been reported in another study (Otto et al., 2008) (Figure 6).

![Figure 6: Non-classical estrogen pathway activators](image)

Figure 6: Non-classical estrogen pathway activators

Figure showing the structures of different non-classical estrogen pathway activators. Adapted from (Otto et al., 2006).

Estren was the first reported molecule having non-classical effects and preventing bone loss in mice, without any activity on reproductive organs (Kousteni et al., 2002). Another study indicated that estren treatment induced non-classical pathway activation to protect cultured neurons against Aβ toxicity (Cordey et al., 2005). In this study, the neuroprotection, induced by estren, was insensitive to both estrogen and androgen receptor antagonists. The observed effects depended on the PKC pathway; however, the MAPK and PKA pathways were not
involved. Compounds A and C have also been demonstrated to be able to bind to ERs and activate signaling molecules influencing non-classical signaling events; however, their ability to activate the classical estrogen pathway is greatly reduced (Wessler et al., 2006). These newly discovered compounds with low binding affinity to ERα and ERβ may be still potent by activating membrane associated ERs (Toran-Allerand et al., 2002). Another alternative mechanism might occur through an inadequate binding to classical ERα or ERβ, which is not sufficient to activate the classical transcription but sufficient to initiate the non-classical estrogen pathway (Kousteni et al., 2001). A few years ago Manolagas and colleagues suggested the concept of ANGELS (activators of non-genotropic estrogen-like signaling) (Manolagas et al., 2002). This concept hypothesized that the non-classical estrogen signaling contributes to the preservation of bone mass, whereas the classical signaling is important for uterotrophic effects. Estren, compound A, and compound C have been associated with this concept as potent non-classical activators (Otto et al., 2008). Among these three molecules, estren has been used in the most number of studies (Kousteni et al., 2001, 2002; Islander et al., 2005; Krishnan et al., 2005; Neill, 2006; Otto et al., 2006; Wehling et al., 2006; Wessler et al., 2006). However, the exact mechanism by which these new molecules act on signaling events initiating protection is yet to be understood. Nevertheless, they may help in the identification of alternative ligands for hormone therapy in neurodegenerative processes, including the impairments of the BFC system.

**Indirect estrogenic effects**

Although estrogen has a potent effect on cholinergic cells through the classical and non-classical pathways, it is worth to note that estrogen also has antioxidant properties. Similarly to α-tocopherol, estrogen is a monophenolic compound. These phenolic compounds are reported to be inhibitors of lipid peroxidation. Estrogen is effective in preventing oxidative cell death induced by Aβ, glutamate or hydrogen peroxide and this activity is fully
independent of the activation of ERs (Behl et al., 1997; Behl, 2002a). However, as the required concentration for antioxidant effects is much higher than the physiological estrogen level, the physiological relevance is questionable. Estrogen has also been reported to have vascular effects as well as target glia cells and mitochondria (McNeill et al., 2002; Yang et al., 2005; Yager and Chen, 2007; Arevalo et al., 2010). However, we have been limited by no information about these indirect estrogenic effects on BFC neurons.
1.3 Rationale

Previous *in vitro* experiments have demonstrated that estrogen has ameliorative effects on cholinergic neurons (Marin et al., 2003a, 2003b; Guerra et al., 2004). These investigations also suggested the possible role of the non-classical estrogen pathway. However, the mechanism of the estrogen-induced ameliorative effects remains to be studied.

In order to examine the effects of estrogen on cholinergic neurons, we aimed to establish an *in vivo* neurodegenerative animal model. In our model, the glutamate receptor agonist NMDA is used to induce lesion of BFC neurons. NMDA injection in mM concentration causes significant cell loss in the injected area. This NMDA lesion experiment is a well-established model in studying neurodegeneration in rats (Luiten et al., 1995; Abraham et al., 2000; Harkany et al., 2001a). Our aim was to establish a mouse model, which provides an added advantage to incorporate the use of transgenic animals. NMDA-induced cholinergic neuronal death offers a chance to examine the ameliorative effects of the estrogen treatment. Our approach was to focus only on post-injury treatments, representing an animal model suitable for future medical applications. Most of the estrogen treatment models use OVX female animals for research, which does not fully represent potential human conditions. Therefore, we also aimed to investigate estrogenic effects in a wider physiological range, including intact female, male, and aged groups. To investigate the mechanism behind the estrogen-induced amelioration, we intended to further dissect the non-classical estrogen pathway. With the use of neuron-specific ERα knockout (KO) animals we aimed to investigate the involvement of the ERα. Inhibition of key elements of intracellular signaling pathways (MAPK and PKA) allows for the further dissection of these non-classical actions. Finally, the use of a synthetic non-classical pathway activator (estren) makes it easier to investigate and focus on these non-classical effects specifically.
1.4 Aims

- To establish a neurodegenerative mouse model by injecting NMDA into the SI-NBM complex of the BF to induce cholinergic cell and thus fiber loss (for results, see Chapter 3).
- To determine the ameliorative effects of estrogen on cholinergic neurons, using an acute E2 treatment model in OVX mice (for results, see Chapter 4).
- To determine the ameliorative effects of estrogen on cholinergic neurons under different physiological conditions (intact females, males, and aged animals) (for results, see Chapter 5).
- To characterize the mechanism of the ameliorative effects of estrogen on cholinergic neurons, using ERα KO animals, signaling pathway inhibitors (MAPK and PKA) and a non-classical pathway activator (estren) (for results, see Chapter 6).
Chapter 2

METHODS

For a list of laboratory materials and suppliers, see Appendix (Page 133).

2.1 Animals

The experiments were performed on male and female adult (6-8 week old) wild-type (C57BL/6; 20-30g) and neuron-specific ERα KO mice. The total number of mice used in each experimental group was n=4-8 (unless otherwise specified). In some of the experiments, aged wild-type male and female mice (1.5-2 years of age) were used. The animals were housed 1-6 per cage at constant humidity and temperature with a 12 hour light cycle. Food and water were available ad libitum. For some experiments, neuron-specific ERα KO mice were used, provided by the Herbison laboratory (University of Otago). In these experiments, mice were generated by crossing a floxed exon 3 ERα mouse line with Ca^{2+}/calmodulin-dependent protein kinase II α (CamKIIα)-Cre mice, controlled by PCR genotyping (by Mr Rob Porteous, University of Otago, based on Wintermantel et al., 2006). CamKIIα is expressed from birth in forebrain neurons but not in glial cells, providing neuron-specific targets for crossing (Ouimet et al., 1984; Burgin et al., 1990). The Cre recombinase enzyme catalyzes recombination between two loxP sites. This CamKIIα-Cre transgenic mouse line is crossed with another mouse line, in which the target gene (ERα) is flanked by loxP sites (Casanova et al., 2001) (Figure 7). The CamKIIα-Cre mouse line has been shown to effectively delete loxP target sequences (Marsicano et al., 2003). All experimental procedures were approved by the Animal Ethics Committee (University of Otago, New Zealand; under AEC protocol: 96/07).
Diagram showing the generation of neuron-specific ERα KO mouse. A Cre transgenic mouse line (A) is crossed with one carrying ERα flanked by two loxP sites (B). In the next generation, the original gene function will be disrupted (C).

2.2 Gonadectomy

In order to eliminate the effects of endogenous estrogen or testosterone secretion, animal groups were gonadectomized two weeks prior to experiments. Mice were anesthetized with a single dose intraperitoneal injection of 0.1ml/10g body weight avertin (containing: 1.97g 2-2-2-tribromoethanol, 1.18ml amyl hydrate, 7.9ml ethanol, and 90.9ml saline). For OVX, animals were placed on their abdomen and the skin at the back was shaved and disinfected. A small incision was made in the skin and then in the muscle layer on both sides. The ovaries were visualized in the abdominal cavity and retracted from the body using forceps. The junction between the fallopian tube and the uterine was tied using sterile suture to avoid bleeding. The ovaries were carefully removed and the wound was closed by suture. Animals were placed on a heating pad for post-surgical recovery. For orchidectomy (ORX), animals were placed on their back and the skin was disinfected at the surgical area. A small incision
was made in the scrotum and the testes were retracted with the epididymis, which then was
tied and the testes were removed. Likewise, the wound was closed by suture and the animals
were placed on a heating pad for post-surgical recovery. Prior to surgeries, pain control was
used by a single subcutaneous injection of lidocaine-hydrochloride (5mg/kg body weight) for
local anesthesia and carprofen (5mg/kg body weight) for general pain control (provided by the
Animal Welfare Office, University of Otago); both dissolved in sterile saline.

2.3 Intracerebral injections and treatments

To investigate the effects of estrogen on cholinergic cell bodies and fibers, we used a
neurodegenerative and acute treatment mouse model by injecting NMDA into the BF SI-
NBM complex followed by a steroid treatment (E2 or estren). For NMDA injections, animals
were anaesthetized with halothane (1.4% v/v; 1L/min flow) and placed on a stereotaxic device
using a mouse adaptor. The head of the animals was secured using plastic ear bars.

Temperature was controlled throughout the surgeries using a heating lamp. After disinfecting
the skin, a small incision was cut to reveal the surface of the skull. Hydrogen peroxide
solution (33%) was used to clean and disinfect the skull and visualize the sutures. After the
Bregma was set as zero the following coordinates were measured: AP=-0.58; ML=1.75 on the
right hemisphere (Paxinos and Franklin, 2000). At these coordinates, a small hole was made
on the skull, using an electric driller. The dura was gently removed using a needle to reveal
the brain surface. A Hamilton syringe (25S) - attached to the stereotaxic device - was lowered
to DV=4.5 and DV=4 coordinates (from dura). Next, NMDA (freshly dissolved in tris-
buffered saline - TBS) was slowly (approximately 0.1μl/min) injected into the SI-NBM
complex on the right hemisphere at various concentrations (1mM, 10mM, and 20mM) by
hand. Half μl was injected at DV=4.5 coordinate and another 0.5μl at DV=4. The Hamilton
syringe was kept in the brain for another 5 minutes before retracting it slowly. The skin was
closed using wound clips and animals were placed on a heating pad for post-surgical
recovery. Given that we performed unilateral lesions, the contralateral brain side remained intact and served as a control throughout all the experiments. Figure 8 indicates the position of the NMDA injection.

**Injection and treatment protocols:**

1. In our first experiment series, the degree of cholinergic neuronal death was studied with six different survival times (3, 6, 9, 12, 15, and 28 days) and three different NMDA doses (1, 10, and 20mM) in adult wild-type mice. Based on the results of the first experiment series, 10mM NMDA dose and 12 days survival time were selected for the following experiments. For results, see Chapter 3.

2. In the following experiment, wild-type OVX mice were administered a single subcutaneous injection of 3.3 or 33ng/g E2 in 0.1ml ethyl-oleate or vehicle (0.1ml ethyl-oleate/30g body weight), 1 hour or 24 hours following NMDA infusion. After 12 days survival time, animals were deeply anaesthetized with avertin and perfused transcardially (see 2.4). Based on the results of this experiment, 33ng/g E2 dose and 1 hour treatment timing were selected for the following experiments. This dose of E2 is supraphysiological as it causes a significant increase compared to the E2 level in the blood in cycling animals (Szego et al., 2006). Figure 9 demonstrates the time-line of the investigations. For results, see Chapter 4.

3. In the subsequent experiments, intact (non-OVX) female, ORX male and aged male and female mice were used. Animals were injected with NMDA and treated with 33ng/g E2 (in 0.1ml ethyl-oleate) or vehicle (0.1ml ethyl-oleate/30g body weight) one hour following lesion. After 12 days survival time, animals were deeply anaesthetized and perfused for brain tissue collection and further analyses. For results, see Chapter 5.

4. In the last experiment series, various animal groups and injection protocols were utilized.
First, neuron-specific ERα KO OVX mice (and heterozygous wild-type siblings) were used. Animals were injected with NMDA and treated with 33ng/g E2 (in 0.1ml ethyl-oleate) or vehicle (0.1ml ethyl-oleate/30g body weight) one hour following lesion.

Next, we used intracellular signaling pathway inhibitors along with the NMDA infusion and E2 treatment in wild-type OVX mice. Signaling pathway blockers were injected intracerebroventricularly on the right hemisphere (AP=-0.58, ML=1, DV=2) 30 minutes after the NMDA lesion. In order to inhibit the MAPK pathway, we injected 1μl MAP kinase kinase 1/2 inhibitor (MEK1/2; U0126; 0.1μg/μl; dissolved in 2.5% DMSO/artificial cerebrospinal fluid - aCSF). To block the PKA pathway, 1μl PKA inhibitor (H-89; 0.67μg/μl; 1μl dissolved in 2.5% DMSO/aCSF) was used. The dose and timing of the inhibitors were established previously (Cervo et al., 1997; Han and Holtzman, 2000; Rahmouni et al., 2004; Szego et al., 2006). Figure 8 indicates the position of the inhibitor injection.

In the final experiment, we studied the effects of estren treatment in wild-type OVX mice. Animals were treated with 3.3, 33 or 330ng/g estren (in 0.1ml ethyl-oleate) or vehicle (0.1ml ethyl-oleate/30g body weight) one hour following the NMDA infusion. Figure 9 demonstrates the time-line of the investigations. For results, see Chapter 6.
Chapter 2 - Methods

Figure 8: Intracerebral injections

Figure showing the injection sites of NMDA and signaling pathway inhibitors. Modified from “The Mouse Brain in stereotaxic coordinates” (Paxinos and Franklin, 2000).

Figure 9: Time-line of the investigation of estrogenic actions

Animals were injected with NMDA unilaterally to induce cholinergic cell loss in the SI-NBM and thus fiber loss in the ipsilateral cortex. In some of the experiments, animals received signaling pathway inhibitors intracerebroventricularly 30 minutes following NMDA infusion. 1 or 24 hours later, animals received a single E2 or estren injection as a treatment. After 12 days, animals were perfused transcardially and brains were removed for further analyses.

2.4 Brain tissue collection

Unless otherwise specified, at the end of all experimental procedure, animals were deeply anaesthetized with an overdose of avertin (0.2ml/10g body weight) and perfused transcardially. Heart was exposed and a cannula was inserted into the left ventricle; the right atrium was cut. Thirty ml 4% paraformaldehyde (dissolved in 0.1M phosphate buffer, pH 7.6)
was pumped through the heart using a Masterflex pump (3ml/min flow rate). Brains were removed from the skull and post-fixed at room temperature for 4 hours on an orbital shaker. Thereafter they were transferred to 30% sucrose solution (dissolved in TBS) for cryoprotection for 24 hours at 4°C. Four series of coronal sections (30μm thick) were cut on a freezing microtome. Sections were collected in TBS and stored at 4°C for further immunohistochemical and histochemical analyses.

2.5 Methods to study the uterotrophic effects of estren

To study the uterotrophic effects of estren, OVX mice received two subcutaneous hormone injections (33ng/g estren or E2 as control in ethyl-oleate or vehicle: 0.1ml ethyl-oleate/30g body weight) with 24 and 3 hours survival times. Animals were decapitated and uteri were removed immediately and weighed on a laboratory scale. The tissues were then transferred into 4% paraformaldehyde solution for overnight fixation. Next, 30% sucrose solution was used as cryoprotectant overnight at 4°C. Thirty μm sections were cut on a freezing microtome and mounted on gelatin coated slides. Haematoxylin-eosin staining was used to visualize the epithelium of the uteri. Sections were analyzed using Olympus microscope (BX51) under 40x magnification. Uterus weight was expressed in mg ± standard error of mean (SEM) while the epithelium thickness in μm ± SEM.

2.6 Methods to study the ERα distribution in cholinergic neurons

To investigate the intracellular distribution of the ERα in cholinergic neurons, brain sections were obtained from other experiments that involved NMDA-induced lesion of the SI-NBM. For detailed protocol see 2.3. In this experiment, only vehicle treated groups were used to rule out the influence of the hormone treatment on the receptor expression. After 12 days, animals were deeply anaesthetized and perfused. Brains were removed and sectioned. Double-labeled
ERα and ChAT fluorescence immunohistochemistry was performed to reveal the intracellular receptor distribution in ChAT-positive cells.

2.7 Histochemistry and immunohistochemistry

2.7.1 AChE histochemistry

To visualize cholinergic fibers in the cortex, AChE histochemistry was used with silver nitrate intensification (Hedreen et al., 1985). Adjacent series of sections were chosen from plate 28-40 (Paxinos and Franklin, 2000) to ascertain that we covered most of the cholinergic projections to the cortex. All procedures were performed at room temperature on an orbital shaker. Sections were washed in sodium acetate for 3x2 minutes (0.1M, pH = 6) then incubated in sodium acetate buffered incubation solution for 2x45 minutes. Fifty ml incubation solution contained 32.5ml sodium acetate (0.1M), 2ml sodium citrate (0.1M), 5ml copper sulphate (0.03M), 9.5ml ddH2O, 1ml potassium ferricyanide (5mM), and 25mg acetylthiocoline-iodide. Sections were then rinsed in sodium acetate buffer for 3x2 minutes following 1 minute incubation in freshly made ammonium sulphide solution (1%). After washing in sodium nitrate (0.1M) for 3x2 minutes, sections were intensified in silver nitrate solution (1%) for 1 minute to reveal dark brown staining for AChE rich neurons and fibers. Sections were then rinsed in sodium nitrate, sodium acetate and TBS buffers for 3x2 minutes each. All reactions were performed in foil covered incubation trays to protect sections from light. Sections were then mounted on gelatin coated slides, dehydrated in ascending alcohol solutions and xylene (ddH2O, 50%, 70%, 90%, 100% ethanol and xylenes – each for 2 minutes) and coverslipped with DPX mounting medium.

2.7.2 ChAT, VACHT, and ERα peroxidase immunohistochemistry

All procedures were performed at room temperature, except incubation in primary antibodies (which was at 4°C) using an orbital shaker. The exclusion of primary antibodies in these experiments resulted in a complete absence of immunoreactivity. Before the
immunohistochemical procedures, sections were thoroughly washed in TBS solution (5x10 minutes) in a washing tray.

We performed peroxidase immunohistochemistry on free floating sections to visualize cholinergic cell bodies (ChAT), cholinergic cell bodies and fibers (VACHT) and to verify the absence of ERα in ERα KO animals. In the first step, we blocked the endogenous peroxidase activity using hydrogen peroxide solution (containing 0.1% hydrogen-peroxide and 40% methanol in TBS) for 15 minutes, followed by a wash in TBS solution 3x10 minutes. Non-specific binding sites were blocked using TBS/bovine serum albumin (BSA) solution (containing 3μl/ml Triton X-100 and 25mg/ml BSA). Next, goat anti-ChAT primary antibody (1:2000) or rabbit anti-VACHT primary antibody (1:10000) or rabbit anti-ERα primary antibody (MC-20, 1:250) was used for 48 hours at 4°C. Then sections were washed in TBS (3x10 minutes) before adding biotinylated anti-goat or anti-rabbit antibody (1:200) for 1 hour. After three more washes in TBS, sections were incubated in avidin (A) biotin (B) peroxidase complex (Vectastain Elite ABC kit, 1:500) for 2 hours (A and B components were mixed 30 mins in advance). Sections were then washed again in TBS (3x10 minutes). Peroxidase labeling was visualized with nickel-diaminobenzidine tetrahydrochloride (DAB) using glucose oxidase. Nickel-DAB solution for the reaction was prepared and filtered in advance (1.25g nickel sulphate, 25ml 0.2M sodium acetate buffer, 200mg glucose, 40mg ammonium chloride, 12.5mg DAB and 25ml ddH₂O). Glucose oxidase was added right before sections were transferred to the nickel-DAB solution. The reaction was stopped once a dark black immunoreactive product was detected by visualization under a light microscope. Sections were then mounted on gelatin coated slides, dehydrated in ascending alcohol solutions and xylene (ddH₂O, 50%, 70%, 90%, 100% ethanol and xylenes – each for 2 minutes) and coverslipped with DPX mounting medium. The specificities of the ChAT, ERα and VACHT antibodies have been reported previously in the mouse (Yeo et al., 1997; Omoto et al., 2005; Darrow et al., 2006).
2.7.3 ERα and ChAT double-labeling fluorescence immunohistochemistry

As part of the study, we investigated the ERα expression and distribution in cholinergic neurons in the SI-NBM. In order to visualize the receptor distribution in ChAT-positive neurons, we used fluorescent double-labeling immunohistochemistry. Sections were incubated in TBS/BSA solution (containing 3μl/ml Triton X-100 and 25mg/ml BSA) to block non-specific binding sites. After three washes in TBS, sections were further processed in rabbit polyclonal anti-ERα antibody (MC-20, 1:250) for 48 hours at 4°C. Next, sections were carefully washed in TBS (3x10 minutes) then incubated in goat anti-ChAT primary antibody (1:2000) for 48 hours at 4°C. After 3x10 minutes rinse in TBS, ERα was visualized using Cy5 conjugated F(ab’)2 fragment anti-rabbit secondary antibody (1:400) for 1 hour. Then all sections were washed in TBS (3x10 minutes). Next, donkey biotinylated anti-goat antibody (1:200) was used for one hour, followed by wash in TBS buffer. ChAT-positive neurons were visualized using streptavidin Alexa Fluor 488 (1:400) for 1 hour. After a few final washes (3x10 minutes) in TBS, sections were mounted on gelatin coated slides and coverslipped with Vectashield mounting medium for fluorescence with 4’,6-diamidino-2-phenylindole (DAPI) for further analysis. DAPI was used to label the nuclei of neurons in order to determine the location of the ERα in ChAT-positive neurons.

2.7.4 Haematoxylin-eosin staining

Perfusion fixed uterus samples were cut on a freezing microtome and immediately mounted on gelatin coated slides. In order to visualize the uteri, haematoxylin-eosin staining was used. First, sections were rehydrated in ddH2O, then Gill II. haematoxylin stain was used for 3 minutes followed by wash in running tap water for 2 minutes. Sections were then further processed in 1% acid alcohol (70% ethanol, 1% HCl) followed by another wash in tap water for 2 minutes. After that, eosin stain was used for 30 seconds followed by brief wash in tap water. Sections were dehydrated in ascending alcohol solutions and xylene (ddH2O, 50%, 70%, 90%, 100% ethanol and xylenes – each for 2 minutes) and coverslipped with DPX. The
uterus epithelium layer thickness was determined using light microscope under 40x magnification (Olympus BX51).

2.8 Analysis and statistics

All measurements were performed without prior knowledge of the case condition.

2.8.1 Cholinergic cell counting

The effects of NMDA lesion and hormone treatment on ChAT-positive cell bodies were determined by counting the number of cells throughout the whole SI-NBM complex in both lesioned and non-lesioned sides of the brain using an Olympus BX51 microscope under 10x and 20x magnification. Since the animals received unilateral NMDA injections, the contralateral (non-lesioned) brain side served as a control. As a result, animals were self-controlled; therefore, cholinergic cell loss was always expressed as a comparison between the lesioned and non-lesioned brain sides without comparing total cell numbers between animals. Sections were chosen from plate 33-39 (Paxinos and Franklin, 2000) using at least six sections per animal with intervals of 120μm to avoid double counting of a single cell. Data are presented as percentage of cell loss between lesioned and non-lesioned hemispheres.

\[
\text{Cell loss \%} = 100 - \frac{\text{cell number on the lesioned side}}{\text{cell number on the non-lesioned side}} \times 100
\]

2.8.2 Cholinergic fiber densitometry

Cholinergic neuronal damage and the effects of different treatments were also assessed by measuring the decrease in cholinergic cortical projections that originate from the SI-NBM complex with quantitative computerized image analysis system (Olympus BX-51 microscope with F-View II camera and Cell-P Image Analysis software) (Harkany et al., 1998). Quantification of AChE-positive fiber density was performed in layers IV and V of the somatosensory cortex in a defined region of interest (ROI) that contained the highest density
of cholinergic innervations. The ROI was set based on our initial experiments with non-lesioned and NMDA lesioned animals. The position of the ROI was determined by locating the most affected region of the somatosensory cortex following NMDA infusion. The size of the ROI was set to cover both layers IV and V in the affected cortical area. Two measurement areas were used per hemisphere (size of ROI for one measurement area: 0.75mm$^2$). The size and position of the ROI was maintained throughout all experiments. After background subtraction and gray scale threshold determination (which results in a binarized image), the surface area of AChE-positive fibers was computed (the area covered by AChE-positive fibers). Density was measured in twelve cortical sections in the primary and secondary somatosensory cortex (S1 and S2) with intervals of 120µm (plate 28-40) and the values were averaged. Due to the unilateral projections of the SI-NBM cholinergic neurons (Pearson et al., 1983; Walker et al., 1985; Kitt et al., 1987; Luiten et al., 1995), the contralateral (non-lesioned) brain side served as a control within each individual. The relative value of fiber reduction was calculated as a percentage difference between the surface area density of the lesioned and non-lesioned sides of the brain. Both the threshold and size of the ROI were constant across all sections in various experiments.

$$Fiber\ loss\ % = 100 - \frac{fiber\ density\ on\ the\ lesioned\ side}{fiber\ density\ on\ the\ non-lesioned\ side} \times 100$$

Figure 10 provides a representative illustration of each step of the fiber surface area calculation.
Figure 10: AChE-positive fiber densitometry

Figure A showing the ROIs (blue and red rectangles) for the AChE-positive fiber surface area density calculations. Red vertical line indicates the position of the NMDA injection. AChE-positive fiber density was measured in layers IV and V (B, white bars indicate the borders). After gray-scale threshold determination, the ROI was set (C, red rectangle shows an example of the ROI). Finally, the background was removed and the images were binarized (D). The density was calculated based on the surface covered by AChE-positive fibers inside the ROI (D). Scale bar: 100μm.
Animals from one experimental group (both hormone and vehicle treated) were incubated in the same incubation solution providing a standardized fiber density for further comparisons.

In some of the experiments, we labeled cholinergic fibers using VACHT peroxidase immunohistochemistry and performed fiber density analyses. The same procedure was used for VACHT-positive fiber densitometry as with AChE (see above).

### 2.8.3 Analysis of ERα distribution in cholinergic neurons

ERα expression in cholinergic cells was detected with the use of a Zeiss LSM 510 confocal laser scanning microscope by means of a 40x objective (oil immersion) using argon (488nm) and helium neon (633nm) lasers with emission filtered at 504nm for Alexa 488 and 655nm for Cy5. For DAPI 388nm excitation wavelength and 655nm filter were applied.

Three different distributions were distinguished based on the location of the ERα in ChAT-positive neurons: 1) nuclear, 2) cytoplasmic, and 3) both (nuclear and cytoplasmic). Cells were counted in the field of view of the 40x objective in the SI-NBM. Both NMDA lesioned and non-lesioned hemispheres were examined. The three different types of ERα distributions were expressed as a percentage of total number of cholinergic neurons expressing ERα:

\[
e.g. \text{nuclear expression} \% = \frac{\text{number of ChAT cells expressing nuclear ERα}}{\text{total number of ChAT cells expressing ERα}} \times 100
\]
2.8.4 Statistical analysis

Statistical analysis of data from two groups was performed by F-test followed by unpaired, two-tailed Student’s t-test. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was used to compare three or more groups with one independent variable. For data with two independent variables, two-way ANOVA followed by Bonferroni’s post hoc test was computed (see Results section for more detail) (GraphPad Prism version 5.04 for Windows). Data was presented as mean ± (SEM). Statistical significance was accepted at P < 0.05.
Chapter 3

THE NEURODEGENERATIVE MOUSE MODEL

Introduction

Cholinergic neurons in the BF have been in the focus of different researches and studied for decades, especially after the establishment of the cholinergic hypothesis in age-related changes and AD (Bartus et al., 1982). The cholinergic anatomy of the BF is well-described in rats, as these animals have been subjects to the majority of the experiments. Immunohistochemical and histochemical protocols based on cholinergic enzymes (ChAT, VACHT, and AChE) are well-known in this species, giving standardized results throughout various studies (Luiten et al., 1995; Abraham et al., 2000; Aggarwal and Gibbs, 2000; Horvath et al., 2000, 2004; Harkany et al., 2001a; Conner et al., 2003, 2005; Gibbs, 2003; Yamamoto et al., 2007; Henny and Jones, 2008; Hammond et al., 2011). The effects of the NMDA-induced cholinergic lesion are also well documented in rats (Luiten et al., 1995; Abraham et al., 1997; Oosterink et al., 1998; Horvath et al., 2000). The non-selective NMDA infusion at the mM range induces an excitatory cell death, in which neurons slowly start to degenerate. Harkany and colleagues have shown that the NMDA-induced neurotoxicity was confined to the lesion core at 4 hours post-lesion and significantly reduced the number of cholinergic neurons throughout the SI-NBM at 24 hours. Changes in p75NTR immunoreactivity and AChE-positive fiber density in the ipsilateral somatosensory cortex were also visible. The loss of AChE-positive fibers was significantly greater at 48 hours post-lesion (Harkany et al., 2001a). Although there are cholinergic studies in mice (Gordon and Finch, 1984; Hohmann and Ebner, 1985; Hohmann et al., 1985; Kitt et al., 1994; Berger-Sweeney et al., 2001; Dumont et al., 2006; Perez et al., 2007; Moreau et al., 2008; Dolga et al., 2009; Nag et al., 2009), the quantification methods for histochemical and
immunohistochemical analyses following NMDA infusion are not well-established in this species. Therefore, in the first part of this study our aim was to establish a neurodegenerative model in mice, in which NMDA was injected into the BF SI-NBM to elicit cholinergic cell and thus fiber loss.
Results

To eliminate the effects of internal estrogen or testosterone secretion, wild-type animals were gonadectomized prior to the experiments.

3.1 Effects of NMDA on cholinergic fibers

We measured the cholinergic fiber loss in the somatosensory cortex as an indication of the extent of the neurodegeneration. All animals that received unilateral NMDA infusion in the SI-NBM complex showed a profound decrease in AChE stained fibers in a large part of the cortex on the lesioned hemisphere. The decrease in the cholinergic fiber density was in agreement with the unilaterally organized anatomical pattern of the SI-NBM cholinergic projections in rodents (Luiten et al., 1985). In our mouse model, the most affected cortical areas after NMDA lesion were the S1 and S2. Our results indicate that NMDA injection into the SI-NBM changes the pattern of the cholinergic fibers mainly in layers IV and V of the somatosensory cortex. We did not find a difference in the amount of damaged fibers between these two layers; both structures were equally disrupted by the NMDA injection. Consequently, for further experiments, we chose these two layers (IV and V) as markers of the cholinergic fiber loss in the cortex. Figure 11 indicates the position of the NMDA injection and the affected cortical areas of a female mouse brain.
Figure 11: NMDA lesioned cholinergic fibers in the cortex

Figures illustrating the effects of NMDA lesion on cholinergic fibers. AChE histochemistry was used to visualize cholinergic fibers in the cortex. Black arrow indicates the position of the Hamilton needle (A). NMDA infusion decreases the fiber density in the ipsilateral S1 and S2 regions (red arrows) while the contralateral side remains intact. The motor (M1 and M2) and piriform cortex (Pir) also remain intact, showing a precise lesion of the SI-NBM projections (A). Photomicrograph B demonstrates the structure of the non-lesioned somatosensory cortex, while C shows the lesioned one (white bars indicate the borders of layers IV and V, scale bar: 100µm). Inserts show high magnification images (scale bar: 50µm).
3.1.1 Different survival times following NMDA lesion

To investigate the effect of different survival time on the NMDA-induced cholinergic lesion in mice, OVX animals were perfused 3, 6, 9, 12, 15 and 28 days after the NMDA-induced lesion (using 1µl, 10mM NMDA). Regression analysis showed that administration of 10mM NMDA into the SI-NBM results in a non-linear relationship between the survival time and percentage of fiber reduction with an apparent maximal loss (40-50%, estimated plateau from fitting curve: 42.4%) starting at 12 days following NMDA infusion. We observed a significant fiber loss (32.4% ± 11.3) 6 days following NMDA infusion. This loss reached 38% ± 8 at day 9, 40 ± 8.9 by day 12 and remained elevated at day 15 (40.1% ± 7.5). The amount of damaged fibers was still similar after 28 days (39.5% ± 9.1). For that reason, we used 12 days survival time throughout the rest of the study (Figure 12).

![Figure 12: Effects of different survival times on NMDA-induced cholinergic fiber loss](image)

The cholinergic fiber degeneration following NMDA infusion reaches its maximum around 12 days and remains elevated. The equation of the non-linear fitting curve is: \( Y = Y_0 + (\text{Plateau}-Y_0) \times (1 - \exp(-K \times x)) \) (n=4-6 for each time point).
3.1.2 Different NMDA concentrations

To identify the most effective dose of the NMDA, we investigated the effects of 1, 10 and 20mM of NMDA exposure using 12 days survival time in OVX mice. Administration of NMDA at these different concentrations evoked a clear dose dependent loss in the cholinergic fiber density in the somatosensory cortex. While 1mM NMDA induced limited fiber loss (15.3% ± 6.4), the highest concentration (20mM) caused profound damage, eliminating almost all of the cholinergic fibers from the somatosensory cortex (fiber loss: 89.8% ± 5.4). The 10mM NMDA elicited AChE-positive fiber loss was 41.3% ± 10.8, thereby providing an effective concentration window to influence the lesion in both negative and positive ways. Accordingly, we used 10mM NMDA concentration in further experiments. As the NMDA was dissolved in TBS, we used 1µl TBS as a control injection. Our results indicate that the control solution does not change the fiber density in the cortex (fiber loss: 3.3% ± 3) (Figure 13).

Figure 13: Effects of different NMDA concentrations on cholinergic fibers

NMDA infusions at different doses showed a typical concentration dependent cholinergic fiber loss in the cortex while TBS did not change the AChE-positive fiber density (** P < 0.01, *** P < 0.001, one-way ANOVA, n=4-6 in each group).
3.1.3 Fiber loss detection using different histological approaches

In this methodological experiment, we compared two cholinergic fiber staining techniques for cholinergic fiber loss, AChE histochemistry and VAChT peroxidase immunohistochemistry. Although the analysis of AChE- and VAChT-positive fibers showed similar results, AChE histochemistry was shown to have a better signal-noise ratio. AChE staining provides a detailed fiber structure visualization and optimal platform for further fiber density analysis. VAChT immunohistochemistry showed too much variability making it difficult to keep the same parameters for the fiber detection (threshold) analysis. During our staining procedures we did not observe differences between the two different techniques using male and female or intact and gonadectomized animals. Figure 14 shows cholinergic fibers visualized by AChE histochemistry and VAChT peroxidase immunohistochemistry.

![Figure 14: AChE- and VAChT-positive fibers in the cortex](image)

Photomicrographs demonstrating two different fiber visualization methods; AChE histochemistry (A) and VAChT peroxidase immunohistochemistry (B). Black bars indicate the borders of layers IV and V, scale bar: 100µm.

Although AChE histochemistry provided adequate contrast for visualization of cholinergic fibers and to analyze fiber loss following NMDA infusion, as a comparison we also measured fiber loss using VAChT immunohistochemistry. We used brain sections from the same female animals for both staining procedures. Our results indicate that there is no difference in the
fiber loss between the two staining methods. We obtained 47.5% ± 8.1 fiber loss for AChE and 44.3% ± 11.3 for VACHT (Figure 15).

![Figure 15: AChE and VACHT fiber loss comparison](image)

Histogram showing cholinergic fiber loss using two different visualization techniques (AChE histochemistry and VACHT peroxidase immunohistochemistry). Both staining procedures showed approximately 40-50% decrease in the overall fiber density in the cortex following NMDA lesion (Student’s t-test, n=5 in each group).

### 3.2 Effects of NMDA on cholinergic cell bodies

The cholinotoxic potential of NMDA on cell bodies can be visualized with different immunohistochemical and histochemical techniques. We compared ChAT and VACHT peroxidase immunohistochemistry and AChE histochemistry in the detection of cholinergic cells. Although all three protocols are widely used in several laboratories, our results indicate that different methods give slightly different neuronal pattern and cell morphology in the observed SI-NBM. Results show that ChAT immunohistochemistry provides the most accurate and clear labeling of BFC cell bodies. Using this technique, ChAT-positive cells and some neuronal processes were always visible and the identification of labeled cells was suitable for further analysis. With the other two staining techniques (AChE and VACHT) the background was higher, thus making the cell differentiation difficult. However, in case of an NMDA infusion, the cholinergic cell loss was clearly visible. Cell counting and
differentiation between lesioned and non-lesioned hemispheres could be performed using all three methods. We did not find any difference between the qualities of these various methods using male and female or intact and gonadectomized animals. Based on this experiment, further cholinergic cell body visualizations were carried out using ChAT immunohistochemistry. **Figure 16** demonstrates representative photomicrographs from each staining procedure.

![Photomicrographs showing cholinergic cell bodies in the SI-NBM complex using different staining methods. A illustrates AChE-positive cells; B represents VACHT-positive neurons while C reveals ChAT-positive ones (scale bar: 50µm).](image)

**Figure 16: Cholinergic cell bodies in the SI-NBM**

Photomicrographs showing cholinergic cell bodies in the SI-NBM complex using different staining methods. A illustrates AChE-positive cells; B represents VACHT-positive neurons while C reveals ChAT-positive ones (scale bar: 50µm).

ChAT-positive cell body counting revealed that unilateral NMDA infusion of OVX animals resulted in approximately 80% cell loss in the SI-NBM complex, whereas the control TBS
injection did not change the number of neurons significantly (cell loss: 82.5% ± 12.4 and 2.4% ± 2.5, respectively) (Figure 17).

Figure 17: Effects of NMDA lesion on cholinergic cell bodies in the SI-NBM

Photomicrograph A shows the contralateral (non-lesioned) side with intact ChAT-positive cells. B demonstrates the NMDA lesioned side (scale bar: 100 µm). Inserts show high magnification images (scale bar: 25 µm). Bar graph demonstrates that NMDA lesion resulted in a profound cholinergic cell body loss while TBS injection did not change the number of ChAT-positive cells (** P < 0.01, Student’s t-test, n=4 for TBS and 6 for NMDA).
Discussion

The main aim of this study was to analyze the ameliorative effects of estrogen in a model of an excitotoxic neurodegenerative condition. In this first part, we established a neurodegenerative mouse model that mimics certain neurological disorders such as AD or stroke.

3.3 Visualization of cholinergic fibers and cell bodies

The BFC system, with its widespread unilateral projections, provides an adequate experimental design for our research. We focused on the cholinergic neurons in the SI-NBM as these neurons provide projections to cortical areas. The analysis of the cholinergic projection fibers was a crucial point of this project as most of the results in further experiments were calculated based on the altered fiber densities in the cortex. Therefore, the labeling of cholinergic projections was a crucial technique. We used a silver-nitrate intensification method for AChE detection to reveal cholinergic fibers, providing a valuable technique to analyze the difference between lesioned and non-lesioned hemispheres. Previous morphological and functional studies have shown that the AChE histochemistry is an appropriate method to label cholinergic projection fibers in various cortical areas (Karnovsky and Roots, 1964; Johnston et al., 1979; Wenk et al., 1980; Hedreen et al., 1985; Luiten et al., 1995). To confirm the AChE results, we used a different, immunohistochemical technique (VACHT). This method has also been used in various laboratories to label cholinergic fibers in the cortex (Henny and Jones, 2008). In the present study we used a quantitative computerized surface area densitometry, which requires good contrast. Our results indicate that the AChE histochemistry provides better contrast and the labeling is more consistent (less individual difference) compared to VACHT. With the help of AChE staining, we were able to maintain the same threshold for fiber detection in all sections throughout various experiments and brain
samples. Although AChE histochemistry provided a sufficient method for visualization of non-lesioned and lesioned cholinergic fibers, we could not exclude the possibility that the NMDA lesion itself caused a slight change in the AChE enzyme level, influencing our densitometry results. Therefore, we compared between the AChE fiber loss and VACht fiber loss. Both the histochemical (AChE) and immunohistochemical (VACht) techniques proved to be valid methods to analyze fiber loss in the cortex in our study. A similar decrease in the AChE- and VACht-positive fiber densities were detected following lesioning, suggesting that the observed fiber loss is not due to a change in certain enzyme levels. Although these results do not provide direct evidence, it is unlikely that both AChE and VACht enzyme levels change following NMDA lesion. Our AChE results are in good agreement with previous studies (Luiten et al., 1995; Abraham et al., 2000; Horvath et al., 2000; Conner et al., 2003, 2005), where this technique was used to analyze neurotoxic effects on BFC fibers. Based on our AChE and VACht results, along with previous data found in the literature, we strongly hypothesize that cholinergic axons physically disappear from the somatosensory cortex due to the degeneration of cell bodies in the SI-NBM complex following NMDA infusion. Thus, our method provides a solid and useful technique to detect fiber degeneration in the cortex following NMDA-induced lesion.

Proper visualization of cholinergic cell bodies is also an essential aspect in studying the neurotoxic effects of the NMDA lesion. During our initial experiments, we investigated three different staining techniques to label cholinergic cell bodies in the SI-NBM complex. Among others, three important enzymes can be found in cholinergic neurons based on their ability to synthesize, transport, and break down ACh: ChAT, VACht and AChE, respectively. ChAT immunohistochemistry is used for detection of cholinergic neurons in many studies (Aggarwal and Gibbs, 2000; Horvath et al., 2000, 2004; Gibbs, 2003; Kalesnykas et al., 2005; Yamamoto et al., 2007; Sotthibundhu et al., 2008; Dolga et al., 2009; Hammond et al., 2011). AChE histochemistry is similarly a well-known method to reveal cholinergic cell bodies.
(Mesulam et al., 1983b; Luiten et al., 1987). VACHT immunohistochemistry has also been used to a lesser extent in a number of studies (Gilmor et al., 1999; Bennett et al., 2009). Our results indicate that the ChAT immunohistochemistry provides the most adequate labeling. Using this technique we were able to visualize ChAT-positive neurons and differentiate BFC groups (e.g. SI-NBM, HDB, VDB) with great success, even under low magnification (4x, 10x).

### 3.4 NMDA-induced neurodegeneration

The BFC system is vulnerable against many neurodegenerative processes such as AD. Various laboratories established animal models to mimic the pathological conditions of these degenerative disorders. One of the widely used methods has been to lesion BFC neurons in the SI-NBM either physically (e.g. electrolytic) or chemically by the injection of substances that damage neurons. Chemical lesion models have used different strategies, targeting cholinergic cells specifically or using general neurodegenerative drugs. A widely used technique to selectively lesion cholinergic neurons is through the use of 192 IgG-saporin (Heckers et al., 1994; Leanza et al., 1995; Steckler et al., 1995). The ribosome inactivating protein, saporin, is coupled to a monoclonal antibody (192 IgG) that is specific to the low-affinity p75NTR NGF receptor. As cholinergic neurons express these receptors extensively (Sobreviela et al., 1994), it is practicable to selectively target cholinergic populations in the brain without influencing other neuronal connections. However, the mechanism of cholinergic cell death underlying neurodegenerative disorders is different and more likely to be connected to excitatory neuronal death. The ability of glutamate exposure (or its analogs) to trigger neuronal death has been documented for more than 50 years (Dunnett et al., 1991). Activation of NMDA receptors induces an excessive Ca$^{2+}$ influx leading to potentially excitotoxic events, such as observed in stroke, ischemia and traumatic brain injury (Lee et al., 1999). Therefore, other studies utilized excitatory drug injections to mimic such pathological injuries. The most
frequently used drugs for lesion include NMDA, kainic-, ibotenic- and quisqualic-acid (Dunnett et al., 1991). In other studies the injection of different Aβ fragments has been used to better understand the mechanism by which cholinergic neurons undergo neuronal death in AD (O’Mahony et al., 1998; Abraham et al., 2000; Harkany et al., 2000c). Moreover, evidence has shown that NMDA receptor mediated glutamate excitotoxicity is a major step in the neurodegenerative process triggered by Aβ peptides (Mattson et al., 1992). It is becoming more apparent that Aβ interacts with the NMDA receptor channel resulting in a sustained Ca$^{2+}$ overload that can eventually lead to cell death (Harkany et al., 2000a). Furthermore, Aβ toxicity has been successfully blocked in various studies in the presence of NMDA receptor antagonists (Maurice et al., 1996; O’Mahony et al., 1998). In our study, we used NMDA-induced lesions in the SI-NBM in order to initiate cholinergic cell death. Although NMDA is not a specific cholinotoxin, the mechanism by which it induces degeneration is related closely to that observed in neurodegenerative disorders. Therefore, we hypothesize that with the use of NMDA injection, we have a better understanding of certain neurodegenerative diseases.

Although the scientific literature provides detailed information about cholinergic lesions in the BF in various rat models, insufficient evidence has been accumulated in mice. More recently, the NMDA-induced cholinergic lesion in the SI-NBM of male mice was studied (Dolga et al., 2009). Although the latter study was not available at the beginning of our investigations, we obtained similar results. In the first series of experiments, we characterized the properties of NMDA lesions in mice. As the SI and NBM cholinergic groups are not distinct in the mouse, we decided to lesion both structures together (SI-NBM complex), using them as one continuous cholinergic population. To study the effects of different NMDA concentrations on the BFC system, we analyzed the AChE-positive fiber density in the somatosensory cortex. In further experiments, we investigated the effects of E2 treatment on cholinergic fibers. Therefore, we needed to achieve approximately 50% cholinergic fiber loss in the cortex to provide a concentration window for the treatment to influence the lesion in
both positive and negative ways. Our results indicate that 10mM NMDA concentration induced the most suitable fiber loss in the cortex. Analysis of the injection site revealed that the motor and piriform cortex remained intact indicating a controllable unilateral cortical damage in the cholinergic system. As control injections (TBS) did not modify the overall AChE-positive fiber density in the cortex, we did not use TBS injection on the contralateral hemisphere.

Depending on the time and concentration of the NMDA exposure, a necrotic or apoptotic process starts which eventually leads to cell death (Bonfoco et al., 1995). Previous studies have indicated a maximum cholinergic fiber loss 10 days after NMDA lesion in rats (Harkany et al., 1995; Abraham et al., 2000). We analyzed various survival times (3, 6, 9, 12, 15, and 28 days) in our initial experiment series. The results obtained were consistent with previous rat studies. The maximum cholinergic fiber loss caused by NMDA lesion (approximately 40-50%) was at 12 days and it remained elevated at 15 and 28 days following lesion.

*In vivo* experiments demonstrated that the BFC system has a natural compensatory mechanism against neurodegeneration through neurotrophin receptors, such as TrkA and p75NTR, expressed by cholinergic neurons (Sobreviela et al., 1994). An AD study has shown that the level of the primary ligand of TrkA (NGF) changes, depending on the progress of the pathophysiology (Hellweg et al., 1998). Further research indicated a substantial increase in the NGF level 3 months following quisqualic acid lesion of the BF (Gericke et al., 2003). Modulatory effects of NGF can influence cholinergic neuronal morphology, hippocampal plasticity and behavior (Conner et al., 2009). Although the level of the NGF is unknown in our experiments, the stagnant fiber loss 28 days after NMDA infusion suggests that even an NGF-induced compensatory mechanism is unable to restore the cholinergic fiber density in the somatosensory cortex following NMDA lesion. It is very likely, that cholinergic fibers
will be permanently erased from the cortex following the toxic NMDA lesion, without a possible mechanism of natural restoration.

Anatomical observations of our model reveal that 10mM NMDA causes a high percentage of cholinergic cell loss (approximately 80%) in the SI-NBM complex. In contrast, previous studies on rats showed that NMDA infusion caused around 50% decrease in the number of ChAT-positive cells (Harkany et al., 2001b; Horvath et al., 2002). However, our experiments were performed on mice and that could perhaps explain the differences in the actions of NMDA. Moreover, as opposed to previous rat studies, we lesioned the SI along with the NBM.

Analysis of the somatosensory cortex reveals that NMDA infusion induces a reproducible neurodegeneration in mice. The fiber density analysis was a critical point in this study; therefore, it was necessary to develop a method to accurately calculate fiber density. The ROI selection was crucial in our experiments. Previous studies and our observations show that NMDA lesion induces a unilateral cholinergic fiber loss in the S1 and S2 areas of the somatosensory cortex, without influencing the overall AChE-positive fiber density in other cortical areas. To gain the most accurate result for the calculations, we covered a large portion of the somatosensory cortex. As opposed to another study (Szego et al., 2011), we acquired our photomicrographs under low magnification (10x and 20x) and set the ROI to cover a substantial amount of cholinergic fibers. In this way, we could study 70-80% of the layers IV and V in the S1 and S2 areas.

3.5 Summary

Taken together, in this first part of the study, we established an NMDA-induced neurodegenerative model and successfully applied it to mice. The experimental design, with the involvement of the excitatory cell death of cholinergic neurons mimics important aspects
of the pathophysiology of AD or stroke. Due to the strictly unilateral projections of cholinergic neurons in the SI-NBM complex, it was possible to use well defined lesions. The main advantage of this model is a reproducible cholinergic cell loss in the SI-NBM and thus fiber loss in the cortex.
Chapter 4

RESTORATIVE EFFECT OF E2 IN OVX MICE

Introduction

In the previous experiments, we have established a neurodegenerative model in mice, in order to investigate the effects of estrogen on cholinergic neurons. It has been described before that estrogen has ameliorative actions on BFC neurons (Abraham et al., 2009). A single E2 injection at 33ng/g concentration can cause significant changes in the brain (Abraham et al., 2003, 2004; Barabas et al., 2006), including the BFC neurons (Szego et al., 2006, 2011). Therefore, our aim was to explore the ameliorative changes that a single E2 injection induces following excitotoxic injury. A similar paradigm was tested in rats; however, E2 treatment was applied before, during and after the NMDA lesion (Horvath et al., 2002). Therefore, our approach is the first, providing information about a single post-lesion E2 treatment on BFC neurons. OVX animals are considered the most appropriate experimental subjects in many estrogen treatment studies, although the level of estrogen in the brain during estrous-cycle or after OVX is currently not clear. Bartus and colleagues have highlighted decades ago that BFC neurons and their widespread projections are involved in age-related and pathological memory disturbances (Bartus et al., 1982). Despite the importance of the cholinergic system, there is no effective treatment against diseases or brain trauma with cholinergic deficits. There have been studies targeting the cholinergic system with specific AChE inhibitors in AD patients; however, with limited success (Martorana et al., 2010). Therefore, possible restoration of the BFC system by estrogen following injury could potentially lead to medical applications and may change the future for those suffering from BFC neuronal loss and thus cognitive impairments.
Results

In this series of experiments, we characterized the ameliorative effects of estrogen on cholinergic neurons following NMDA-induced cell and fiber loss. We applied our NMDA lesion model (described above in Chapter 3) to elicit cholinergic cell and thus fiber loss. OVX mice were used to avoid the interaction with endogenous estrogen levels. Animals received a single subcutaneous injection of E2 or vehicle following NMDA lesion. To detect the effects of the E2 treatment, two parameters were measured: cholinergic fiber density in the somatosensory cortex and the number of cholinergic cells in the SI-NBM complex.

4.1  Effects of E2 on cholinergic fibers following NMDA lesion

We tested whether E2 treatment had an effect on the cholinergic fiber loss after NMDA lesion. It has been shown previously that a single subcutaneous injection of 33ng/g E2 causes rapid CREB phosphorylation in cholinergic neurons (Szego et al., 2006). Accordingly, we performed initial experiments by testing the 33ng/g E2 concentration. OVX mice were treated with 33ng/g E2 or vehicle 1 hour after NMDA lesion.

Our results showed that the administration of 33ng/g E2 to OVX mice significantly attenuated the NMDA-induced AChE-positive fiber loss in the somatosensory cortex (vehicle: 57.7% ± 8.1, E2: 20.6% ± 4.1) (Figure 18).
Figure 18: Effects of E2 treatment on cholinergic fibers following NMDA lesion in OVX mice

Photomicrograph A and C represent the contralateral (non-lesioned) brain sides showing intact cholinergic fibers. B and D demonstrate the NMDA lesioned hemispheres (white bars indicate the borders of layers IV and V, scale bar: 100µm). Inserts show high magnification images (scale bar: 50 µm). E2 restores the cholinergic fiber density following NMDA lesion (D and bar graph) (*** P < 0.001, Student’s t-test, n=7 in each group).
4.1.1 Time dependence

The timing of the treatment drug administration is critical in neurodegenerative models. The previous experiment has shown that E2 injection one hour after NMDA lesion is effective in restoring the cholinergic fiber density in the cortex. We also treated OVX animals at a different time point, 24 hours after the NMDA infusion to better understand the importance of timing. We found that E2 treatment 24 hours after NMDA infusion was not effective (fiber loss in vehicle: 45.3% ± 6.9, E2: 40.1% ± 7.5) (Figure 19). Our results indicate that in this model, there is only a limited effective time-frame available for treatment.

![Figure 19: Effects of different E2 treatment timings on cholinergic fibers following NMDA lesion in OVX mice](image)

E2 treatment restores the cholinergic fiber density using it one hour following NMDA infusion, but it does not have an effect using it 24 hours following NMDA infusion (*** P < 0.001, two-way ANOVA, n=5-7 in each group).

4.1.2 Dose dependence

Although the 33ng/g E2 concentration has been shown to be effective in restoring the fiber density in the cortex, we also investigated the effects of a lower dose (3.3ng/g). With the 3.3ng/g dose, we aimed to determine whether the 33ng/g concentration was the optimum dose to see ameliorative effects. OVX animals were treated with 3.3ng/g E2 or vehicle one hour
after NMDA infusion. Our results show that the low E2 dose (3.3ng/g) was not sufficient enough to restore the cholinergic fiber density in the cortex (fiber loss in vehicle: 57.7% ± 8.1, E2: 45.4% ± 5.4) (Figure 20).

![Figure 20: Effects of different E2 doses on cholinergic fibers following NMDA lesion in OVX mice](image)

Administration of 3.3ng/g and 33ng/g doses of E2 evoked a clear dose dependent cholinergic fiber density restoration in the cortex following NMDA lesion (*** P < 0.001, one-way ANOVA, n=5-7 in each group).

4.1.3 Effects of E2 on cholinergic fibers in non-lesioned cortical areas

In order to investigate the effects of E2 treatment on intact cholinergic fibers (without NMDA lesion), we measured the AChE-positive fiber density on the contralateral (non-lesioned) side of the somatosensory cortex. Brain sections from different animals went through the same staining procedure, incubating them together, providing accurate and comparable results. As there was no comparison between lesioned and non-lesioned hemispheres, data is expressed as raw fiber density. OVX animals were used from previous experimental groups that were treated with 33ng/g E2 or vehicle one hour following NMDA lesion.

Our results showed that E2 treatment did not change the raw fiber density in the contralateral (non-lesioned) somatosensory cortex (raw data in vehicle: 4983 ± 477 and E2: 4998 ± 569)
Chapter 4 - Restorative effect of E2 in OVX mice

(Figure 21). This indicates that without NMDA-induced neurodegeneration, E2 treatment does not influence the fiber density.

![Graph showing Cholinergic fiber density ± SEM](image)

**Figure 21: Effects of E2 treatment on non-lesioned cholinergic fibers in OVX mice**

Raw density of AChE-positive fibers revealed that E2 treatment did not have an effect on cholinergic fibers in the non-lesioned cortex (Student’s t-test, n=7 in each group).

### 4.2 Effects of E2 on cholinergic cell bodies following NMDA lesion

In this part of the study, we investigated the effects of E2 treatment on cholinergic cell bodies in the SI-NBM following NMDA lesion. OVX animals were treated with 33ng/g E2 or vehicle, one hour following NMDA infusion. Cell loss was assessed using the contralateral SI-NBM complex and results were expressed as percentage of cell loss.

NMDA infusion induced approximately 80% ChAT-positive cell body loss in the SI-NBM complex in the vehicle treated group (80.5% ± 13.7). E2 treatment did not have an effect on the NMDA-induced cell loss (75.2% ± 11.8) (Figure 22).
Figure 22: Effects of E2 treatment on cholinergic cell bodies following NMDA lesion in OVX mice

Photomicrographs showing the effects of vehicle (A) and E2 (B) treatments (scale bar: 100μm). The reduction in the ChAT-positive cell number illustrates that E2 treatment failed to rescue ChAT immunoreactive cells following NMDA lesion (bar graph) (Student’s t-test, n=7 in each group).
Discussion

In this section of the study, we investigated the effects of supraphysiological E2 treatment on cholinergic fibers and cell bodies following NMDA lesion in OVX mice. Our results indicate that acute E2 treatment restores the cholinergic fiber density in the somatosensory cortex after excitotoxic lesion of the SI-NBM complex. Conversely, E2 treatment failed to rescue cholinergic cells in the NMDA injected area.

4.3 Estrogen treatment model

There is an increasing body of evidence suggesting that estrogen has an effect on cholinergic neurons in various diseases and neurodegenerative models (Abraham et al., 2009). Also, a number of studies have reported that both treatments with physiological and supraphysiological concentration of estrogen influence brain functions in normal and pathophysiological conditions (Strom et al., 2009). It has been reported that estrogen application has ameliorative effects by using it as both prevention (pre-injury) and treatment (post-injury) on BFC neurons, \textit{in vivo} (Saenz et al., 2006; Szego et al., 2011). In our experimental design, animals received an acute E2 injection after the NMDA lesion. Similar to our experimental approach, a recent treatment study by Lebesgue and colleagues (2010) has demonstrated that a single injection of a supraphysiological dose of E2 is able to rescue CA1 pyramidal cells against a global model of ischemia. Another study provided information about the amelioration of cholinergic neurodegeneration by E2 treatment (post-injury) in IgG-saporin lesioned female rats (Saenz et al., 2006). E2 treatment administered post-ovariectomy has also been described to reduce the number of apoptotic cells in the rat hippocampus (Sales et al., 2010). Another approach is to apply estrogen before the injury, as a preventive measure against neurodegenerative processes (Strom et al., 2009). Pre-treatment of OVX mice with E2 decreases cholinergic neuron and fiber loss following Aβ-induced toxicity (Szego et al.,
2011); further highlighting the importance of the ameliorative effects of estrogen on the BFC system. A previous quantitative histochemical study has shown that chronic supraphysiological E2 pre-treatment increased the cholinergic fiber density on both ipsi- and contralateral sides of the somatosensory cortex following NMDA lesion of the NBM in rats (Horvath et al., 2002). These findings were partially corroborated with our data as the acute E2 treatment in our model did not affect the AChE-positive fiber density in the contralateral somatosensory cortex. A possible explanation for the discrepancy is that we are utilizing a mouse model, in which an acute E2 treatment (short exposure) is applied after the NMDA lesion. The dissimilarity in the experimental designs might explain the differences in estrogenic actions. Although the prevention of neurodegenerative processes by estrogen application pre-injury raises important aspects, it does not model treatment methods following acute injury. Our approach was therefore to mimic an acute pathological condition and then attempt to ameliorate the degenerative processes by a single estrogen treatment.

The appropriate concentration of the treatment drug is critical and may determine the outcome of any amelioration. Studies in monkeys have shown a decrease in ChAT activity and cholinergic fiber density following OVX and this decrease could be reversed by physiological estrogen replacement (Kompoliti et al., 2004; Tinkler et al., 2004). Therefore, there is an indication that physiological ERT is able to compensate for the loss of steroid hormones by OVX. Conversely, in animal models of neurodegenerative diseases (AD or ischemia) typically supraphysiological estrogen treatments are applied. BFC lesion experiments provide models of an already well-developed pathological condition with substantial amount of cholinergic cell loss and thus fiber degeneration. The progression of parallel human diseases, such as AD is normally slower, in which the physiological estrogen level can act as a preventive hormone, especially at early stages. However, there is no evidence regarding the ameliorative effects of physiological estrogen levels on cholinergic neurons in acute neurodegenerative animal models such as NMDA or IgG-saporin lesion. In our experiments,
we tried two different supraphysiological E2 doses. The results indicated that the lower E2 concentration was not able to initiate fiber restoration in the cortex, although it was still higher than the physiological E2 level. Our finding is supported by a study where estrogen replacement at physiological levels did not protect cholinergic cells against ibotenic acid lesion in the NBM (Aggarwal and Gibbs, 2000); hence indicating the importance of the supraphysiological estrogen treatment. Bora and colleagues (2005) also highlighted that estrogen, given in physiological doses that exert extensive effect on neuronal phenotype (e.g. ChAT expression), do not contribute to neuronal survival.

### 4.4 Cholinergic fiber restoration

Our results show that E2 treatment restores the AChE-positive fiber density in the cortex. We further examined this restorative action and proposed three different explanations. It has been shown that estrogen is involved in neuroprotective signaling (Behl, 2002b). Therefore, our first possible explanation was that E2 had direct neuroprotective effects on cholinergic fibers, rescuing them following NMDA lesion. Results indicated that the timing of the E2 treatment was crucial in our experiments. The administration of E2 one hour following NMDA infusion resulted in a reduced fiber loss; however, the fiber restoration was diminished using E2 treatment 24 hours after the induced neurodegeneration. Based on the NMDA timing experiment (see 3.1.1), cholinergic fibers slowly start to degenerate following NMDA infusion. According to our first explanation, a possible theory behind the restorative action would be that E2 treatment (using it one hour after NMDA infusion) minimizes the degenerative processes, acting from the time of the treatment; therefore, the full effects of the NMDA lesion cannot develop. However, E2 injection 24 hours following NMDA infusion would meet with an already increased cholinergic fiber degeneration that reduces the possibility for a successful treatment. In contrast, we did not observe any neuroprotective effect in the SI-NBM complex, analyzing cholinergic cell bodies. E2 treatment was not
effective in rescuing cell bodies against NMDA lesion. It is unlikely that cholinergic cell bodies go through neurodegeneration and disappear from the SI-NBM complex but their projections remain in the somatosensory cortex as a result of the E2 treatment. Consequently, we rejected the explanation that E2 treatment directly protects cholinergic fibers but not cell bodies against NMDA-induced neurodegeneration.

Our second explanation was that cholinergic projections reappear in the somatosensory cortex, projecting from other brain areas, providing increased fiber density following NMDA lesion. However, the anatomical properties of the cholinergic projections are unilateral (Pearson et al., 1983; Walker et al., 1985; Kitt et al., 1987) and the contralateral brain side does not influence the fiber density on the ipsilateral (NMDA lesioned) side. On the other hand, the arborization or sprouting of cholinergic fibers on the ipsilateral (NMDA lesioned) side might provide a possible answer. The latter led to the next explanation.

In the third explanation, we proposed a compensatory mechanism for the fiber density restoration. The strictly unilateral structure of the BFC projections from the SI-NBM suggests that the remaining cholinergic cells sprout axon collaterals in the NMDA lesioned SI-NBM. Our results showed an approximately 80% cholinergic cell loss in the SI-NBM complex following NMDA lesion, which resulted in about 50% fiber loss in the cortex. It is well known that the adult brain remains highly plastic and can be regulated by hormones. Previous studies have shown that estrogen has sprouting effects in the entorhinal cortex following lesion and this effect is dramatically reduced in OVX animals (Stone et al., 1998; Kadish and Van Groen, 2002). This regenerative sprouting effect caused by estrogen has also been shown in the lesioned hippocampus in rats (Morse et al., 1992). Therefore, we hypothesize that the cholinergic fiber restoration involves the effective sprouting of the surviving axons in the somatosensory cortex on the lesioned hemisphere, as a compensatory mechanism following injury. The “restorative action” of estrogen in this experimental model does not involve a
rescuing mechanism for already lesioned (dead) fibers but rather the re-establishment of the 
original (or closer to the original) fiber density in the cortex by the recruitment of surviving 
fibers (Figure 23).

![Figure 23: Model of the E2-induced fiber density restoration](image)

Figures demonstrating the hypothetical mechanism by which E2 treatment initiates 
cholinergic fiber density restoration. Figure A shows the effects of the NMDA lesion, reduced 
number of cholinergic cells in the SI-NBM and decreased fiber density in the cortex. E2 
treatment increases the sprouting capabilities of the remaining cholinergic fibers to 
compensate the loss (B).

In the present experiments, it seems unlikely that the effect of estrogen on cholinergic fibers is 
due to the up-regulation of AChE expression in the ipsilateral hemisphere because the single 
dose of E2 injection does not have an effect on the AChE fiber density in the contralateral 
hemisphere. This suggests that E2 treatment in our model does not initiate the arborization of 
cholinergic axons without a neurotoxic insult. Although the enhancing effects of chronic 
administration of estrogen on ChAT expression (Gibbs et al., 1994; McMillan et al., 1996) as 
well as on enzyme activity (Luine, 1985; Gibbs, 2000) is well known, there is no indication in 
the literature that cortical ChAT or AChE expressions change 12 days following a single dose 
of E2.
Another controversial point could arise about the effect of estrogen on non-cholinergic neurons in the BF. The BF consists of heterogeneous neuronal populations and it is well known that non-cholinergic neurons widely project to the cortex along with their cholinergic counterparts (Gritti et al., 2006; Lau and Salzman, 2008). One could argue that the BF provides AChE-positive non-cholinergic projections to the cortex, which would potentially jeopardize our results. NMDA infusion provides a non-selective lesion in the BF, therefore, it is unlikely that NMDA destroyed cholinergic cells but spared AChE-positive non-cholinergic projecting neurons. Although, it is possible that E2 treatment, following NMDA infusion, had an effect on AChE-positive non-cholinergic neurons, the number of these neurons in our experiments is unknown. Previous studies in both rats and mice showed that the selective IgG-saporin injection into the SI-NBM complex provides a complete deletion of AChE-positive fibers in the cortex (Harati et al., 2008; Kaur et al., 2008; Moreau et al., 2008). These results are in good agreement with our NMDA dose experiment, in which the non-selective 20mM NMDA provided the same complete loss of AChE-positive fibers in the cortex (see Chapter 3). Although this is not direct evidence about the absence of AChE-positive non-cholinergic neurons in the mouse BF, it might be a good indication that the observed AChE-positive fiber density in the cortex was indeed originated from BFC neurons.

4.5 Summary

In conclusion, our data demonstrates that an acute supraphysiological E2 treatment restores the AChE-positive fiber density in the cortex following NMDA-induced neurotoxicity. The effective time and concentration window also indicates a therapeutic potential of the treatment after brain trauma. We hypothesize that this amelioration of the neurotoxic insult by E2 treatment occurs through a compensatory mechanism in which the remaining cholinergic fibers will be recruited thereby increasing their sprouting capabilities. As we did not observe ameliorative effects on cholinergic cell bodies in the SI-NBM complex, we focused on the
cholinergic fibers in the cortex in further experiments. In order to investigate the physiological relevance of this treatment model, we intended to use animal groups under different physiological conditions (intact female, male and aged) in the following experiments.
Chapter 5

RESTORATIVE EFFECT OF E2 UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS

Introduction

In previous experiments, we used OVX mice to eliminate the effects of internal estrogen secretion. Although the use of OVX animals in estrogen treatment experiments is well-accepted, these animals do not fully represent all physiological human conditions. OVX in rodents is a good model for mimicking human ovarian hormone loss (Baeza et al., 2010); however, a recent study has shown that it fails to adequately represent the menopause transition (Van Kempen et al., 2011). Moreover, various studies have indicated that levels of physiological estrogen have an influence on the cholinergic system (Kompoliti et al., 2004; Tinkler et al., 2004), raising the question whether the physiological estrogen is sufficient to ameliorate the NMDA lesion. The sex difference in pathological conditions such as AD is also well-known (Musicco, 2009). Therefore, one could hypothesize that this cholinergic lesion study might as well show sex differences in the ameliorative actions of estrogen. Finally, as this study utilizes a cholinergic deficit, such as observed in AD (an aging disease), it is essential to analyze the restorative effects of estrogen in aging animals. We have shown previously that E2 treatment has restorative potential on cholinergic fibers in OVX animals. Therefore, in our next experiments, we aimed to investigate the restorative effects in various animal groups. With the use of female animals with intact gonads, we could potentially reveal the influence of the endogenous estrogen in this neurodegenerative model, while the use of male mice could indicate potential sex differences. Furthermore, aging subjects possibly reveal critical information about cholinergic deficits and estrogen treatments in age-related diseases.
Results

Here, we investigate the effects of E2 treatments under various physiological conditions, such as intact females, males and aged animals.

5.1 Effects of E2 on cholinergic fibers in intact female mice

The concentration of E2 treatment administered in previous experiments was of supraphysiological levels. In this experiment, we investigated the effects of physiological estrogen concentration on the NMDA-induced fiber loss and E2 treatment. Given that estrogen levels fluctuate across the estrous-cycle, we tested animals according to both low and high endogenous estrogen concentrations. Mice in the diestrous stage represented low physiological estrogen level while animals in proestrous represented a high estrogen level (Bronson, 1981). The estrous-cycle was assessed at the time of the NMDA infusion by vaginal smear. Animals received 33ng/g E2 or vehicle one hour following NMDA lesion. The influence of physiological hormone levels on the NMDA lesion was revealed in the vehicle treated group, whereas the E2 treated group shed light on the interaction between natural hormone levels and the E2 treatment.

NMDA infusion elicited around 40% fiber loss in the cortex in both proestrous and diestrous animals in the vehicle treated groups (42.9% ± 4.8 and 40.5% ± 3.5, respectively). The NMDA-induced cholinergic fiber loss was similar to animals that were OVX (see Chapter 4). Our experiments show that endogenous estrogen concentrations are not sufficient to protect cholinergic fibers against NMDA lesion. Our results from the E2 treated groups demonstrated that the treatment significantly reduced the NMDA-induced fiber loss, irrespective of the stage of the estrous-cycle. The fiber loss was around 20% in both proestrous and diestrous animals (20.5% ± 4.5 and 23.4% ± 3, respectively) (Figure 24).
Figure 24: Effects of E2 treatment on cholinergic fibers following NMDA lesion in intact female mice

Proestrous and diestrous stages represent the highest and lowest endogenous estrogen level, respectively. E2 treatment restores the cholinergic fiber density in animals whose endogenous estrogen production was intact. Conversely, endogenous estrogen levels are not sufficient to provide amelioration of cholinergic fibers following NMDA lesion (* P < 0.05, two-way ANOVA, n=4-5 in each group).

5.1.1 Effects of E2 on cholinergic fibers in non-lesioned cortical areas

In this part of the study, we measured the cholinergic fiber density only on the contralateral (non-lesioned) somatosensory cortex of intact female animals. Mice were analyzed from previous experimental groups that were injected with NMDA and treated with 33ng/g E2 or vehicle. Raw fiber density was measured by detecting AChE-positive fibers. We found that E2 treatment did not change the fiber density on the contralateral side (in proestrous vehicle: 4578 ± 449, E2: 4778 ± 301 and in diestrous vehicle: 4974 ± 237, E2: 4778 ± 147). Therefore, E2 treatment did not influence the overall fiber density without NMDA-induced lesion in intact female animals (Figure 25).
5.2 Effects of E2 treatments in male animals

We have shown that E2 treatment has restorative potential on the cholinergic fiber density in female animals, regardless of the endogenous estrogen level. Next, we investigated the restorative effect of E2 in male mice. ORX was performed two weeks prior to NMDA infusions. Animals were treated with the same E2 dose that was shown to be effective in females (33ng/g). Cholinergic fiber loss and cell loss were assessed.

5.2.1 Effects of E2 on cholinergic fibers following NMDA lesion

AChE staining of brain sections revealed that male mice have a similar cholinergic fiber pattern in the somatosensory cortex as their female counterparts. Moreover, the same cortical cholinergic layers are damaged (layers IV and V) following NMDA-induced lesion of the BF SI-NBM complex. We did not find differences in either the anatomy or the sensitivity against NMDA toxicity between male and female mice. NMDA infusion into the SI-NBM complex caused substantial fiber loss in the S1 and S2 areas of the somatosensory cortex in the vehicle

Figure 25: Effects of E2 treatment on non-lesioned cholinergic fibers in intact female mice

Raw density of AChE-positive fibers revealed that E2 treatment did not have an effect on the overall cholinergic fiber density on the non-lesioned hemisphere in any of the intact animal groups (two-way ANOVA, n=4-5 in each group).
treated group (fiber loss: 55.2% ± 6.8). This damage is approximately the same as in OVX animals (57.7% ± 8.1, see Chapter 4). Further results revealed that E2 treatment was able to successfully restore the cholinergic fiber density in the cortex following NMDA lesion (fiber loss: 31.6 ± 4.3) (Figure 26).

Figure 26: Effects of E2 treatment on cholinergic fibers following NMDA lesion in ORX mice

Photomicrographs showing AChE-positive fibers following NMDA lesion and vehicle (A) or E2 (B) treatments (black bars indicate the borders of layers IV and V, scale bar: 100µm). Bar graph demonstrates that E2 treatment restores the cholinergic fiber density in the cortex (** P < 0.01, Student’s t-test, n=5 in each group).
5.2.2 Effects of E2 on cholinergic cells bodies following NMDA lesion

In the following experiment, we analyzed the effect of E2 treatment on cholinergic cell bodies in the SI-NBM complex following NMDA lesion in ORX mice. The number of ChAT-positive cell bodies was assessed and presented as cell loss.

NMDA infusion into the SI-NBM complex resulted in a substantial cholinergic cell loss. Nearly 80% decrease was detected in the number of ChAT-positive cells in the vehicle treated group (76.3% ± 3.6). E2 treatment did not have an effect on the ChAT-positive cell loss (78.1% ± 2.9). These results indicate that, similar to female counterparts, E2 is unable to rescue cholinergic cell bodies in ORX mice in our neurodegenerative NMDA model (Figure 27).
Chapter 5 - Restorative effect of E2 under different physiological conditions

Figure 27: Effects of E2 treatment on cholinergic cell bodies following NMDA lesion in ORX mice

Photomicrographs showing NMDA lesioned and vehicle (A) or E2 (B) treated ChAT-positive neurons in the SI-NBM complex (scale bar: 100µm). Bar graph demonstrates that E2 treatment did not rescue ChAT-positive cells in the SI-NBM complex following NMDA-induced neurodegeneration (Student’s t-test, n=5 in each group).

5.3 Effects of E2 on cholinergic fibers in aged animals

In previous experiments, we used 6-8 weeks old animals. In this part of the study we investigated the effects of E2 treatment and its consequence on the cholinergic fiber density in aged animals (1.5-2 years of age). Male and female animals were used for this experiment with intact gonads. Female animals’ estrous-cycle was not tested as it has been shown that C57BL/6 mice stop cycling before the age of 16 months and maintain persistent diestrous
(Felicio et al., 1986; Nelson et al., 1989). Animals were treated and analyzed in the same manner as detailed above.

NMDA infusion resulted in a significant fiber loss in both vehicle treated male and female animals (41.3% ± 4.9 and 39.4% ± 3.4, respectively). This result demonstrates that there is no difference in the NMDA-induced cholinergic fiber loss between aged and young animals (see 5.1 and 5.2). In contrast, E2 treatment did not restore the cholinergic fiber density following NMDA-induced lesion in aged males and females (fiber loss: 34% ± 3.9 and 34.9% ± 2.3, respectively) (Figure 28).
Figure 28: Effects of E2 treatment on cholinergic fibers following NMDA lesion in aged male and female mice

Photomicrographs showing cholinergic fibers from the non-lesioned brain sides (A and C) and NMDA lesioned and E2 treated ones (B and D) (white bars indicate the borders of layers IV and V, scale bar: 100µm). Bar graph demonstrates that E2 treatment failed to restore the cholinergic fiber density in the cortex after NMDA lesion (two-way ANOVA, n=4 in each group).
Discussion

Using different animal groups in this neurodegenerative and acute treatment model, we investigated the influence of endogenous estrogen levels and sex difference, and the effects of aging. Our results indicate that 1) E2 treatment restores the cholinergic fiber density in the cortex following NMDA lesion in intact female mice, 2) there is no sex difference in the NMDA-induced cholinergic fiber loss and E2-induced fiber density restoration and 3) the E2-induced restorative effect diminishes during the process of aging.

5.4 Influence of endogenous estrogen levels

The effects of physiological estrogen and other female steroid hormones have been studied for decades. Research on human patients indicated the preventive actions of physiological levels of female gonadal steroid hormones against neurodegenerative diseases (Paganini-Hill and Henderson, 1994; Tang et al., 1996). Based on studies from the literature, we expected a slight amelioration of the NMDA lesion by endogenous estrogen in the intact females, as these animals had an intact estrogen production from birth. Interestingly, our results demonstrate that the endogenous estrogen does not influence the outcome of the NMDA-induced neurodegeneration. Data from the vehicle treated animal group show that the cholinergic fiber damage in the cortex is similar in intact and OVX animals following NMDA lesion. Endogenous estrogen level at diestrous stage is low, while proestrous animals provide a higher natural estrogen concentration. Moreover, intact animals likely had normal estrous-cycle prior to the NMDA lesion; hence we saw the ineffectiveness of a physiological estrogen “pre-treatment” in this neurodegenerative model. We also found that the supraphysiological E2 treatment has restorative effects in intact animals after lesion, similarly to OVX counterparts (see Chapter 4). We have shown before that the low E2 treatment dose (3.3ng/g) is not able to initiate restoration. Although the conditions were different (physiological
estrogen levels pre-injury in cycling animals versus supraphysiological treatment post-injury in OVX ones) there is indication that low estrogen levels (physiological and 3.3ng/g treatment dose) do not provide sufficient level for the fiber restorative action. Our results are in agreement with previous findings whereby a supraphysiological E2 treatment dose was needed to ameliorate an induced neurodegeneration (Lebesgue et al., 2010; Szego et al., 2011).

The comparison of contralateral (non-lesioned) brain sides between vehicle treated OVX and intact female animals showed that removal of the ovaries did not influence the overall cholinergic fiber density in the somatosensory cortex. Interestingly, previous studies do not support this finding. Long term OVX has been shown to influence the number of cholinergic cells or fibers (Tinkler et al., 2004; Yamamoto et al., 2007). However, the experimental subjects were different in these studies (monkeys and rats versus mice). In addition, we used only two weeks of post-surgical recovery following OVX, which might not have been enough to initiate changes. Another possible explanation would be that the difference between fiber densities in intact and OVX animals is subtle, compared to an extensive damage by NMDA lesion. Nonetheless, we demonstrated that the physiological circulating estrogen (at any estrous stage) does not influence the NMDA-induced neurodegeneration. Analyses of contralateral (non-lesioned) brain sides reveal that the supraphysiological E2 treatment does not influence the fiber density in the intact animal group without NMDA lesion. This suggests that neither the endogenous estrogen levels at different estrous stages nor the supraphysiological E2 treatment has any measurable effect on the non-lesioned AChE-positive fiber density in the cortex. In contrast, a study has shown that supraphysiological estrogen concentration increases the number of cholinergic fibers in the cortex (Horvath et al., 2002). The difference might be due to different animal models (rats versus mice) and E2 treatment procedures (long-term E2 treatment before NMDA infusion versus acute E2 injection after NMDA infusion).
5.5 Sex differences

There is evidence in the literature that the cholinergic system is sexually dimorphic after neurogenesis (Schaevitz and Berger-Sweeney, 2005). Consequently, the manipulation of the BFC system at various stages of development is likely to have different effects in males and females. Moreover, data suggests that BFC projections to the cortex also mature differently in males and females. Afferent axons advance earlier in females reaching their cortical targets earlier (Berger-Sweeney, 2003). This sexual dimorphism during development may influence the aging process of the cholinergic system and possibly contribute to different behavioral and cognitive decline in neurodegenerative diseases as well. Although cholinergic differentiation and timing can vary between males and females, there is no clear evidence that the BFC system has considerable functional difference among sexes. Galani and colleagues (2002) have shown that male and female rats have similar AChE-positive fiber pattern in the cortex. This data is in agreement with our results as we did not find any variation in the cholinergic fiber densities between age-matched male and female mice.

There is also clear evidence in the literature about the sexual dimorphism in neurodegenerative diseases, such as in AD (Musicco, 2009; Vina and Lloret, 2010). In contrast to these data, we did not find any difference between E2 treated male and female groups. Using the same experimental parameters for male mice, the cholinergic cell loss and fiber damage following NMDA lesion was very much comparable to that of the female groups. Also, E2 treatment had the same restorative action, regardless of the sex. Male animals were ORX prior to the experiments; however, in the previous part of the study with female animals, we found that the endogenous estrogen level does not interact with this treatment model. The lack of sex difference in our study might be due to the limitation of the experimental model, which emphasizes certain pathological conditions. NMDA infusion in mM concentration initiates extensive neuronal death, which makes it difficult to analyze
subtle variations between males and females. Treatment following injury at supraphysiological concentration also contributes to these difficulties. The slow progression of human neurodegenerative diseases, such as AD, is more likely to provide a possibility to detect sex differences.

5.6 Effects of aging

It has been demonstrated that aging has a substantial effect on BFC structures including anatomical changes of cholinergic fibers in the cortex (Nyakas et al., 2010). Data indicates that aging processes may substantially modulate estrogenic actions as well. Savonenko and Markowska (2003) have demonstrated that the protective effects of estrogen on cognition are dramatically reduced in aged animals. Using a T-maze active-avoidance task, estrogen treatment showed a protective effect against scopolamine in middle-aged females but not in older counterparts. Our data supports these findings because E2 treatment did not restore the cholinergic fiber density in aged mice. Although the cholinergic system has been well studied over the last few decades, the normal physiological and pathological consequences of aging on cholinergic neurons are not fully understood. One hypothesis is that aging results in a reduction in the functioning of the NGF signaling system. BFC neurons respond to the administration of NGF and increase their ChAT activity. A study has shown a reduction in the levels of TrkA mRNA in the MS and NBM in aged animals (Gibbs, 2003). Moreover, data suggested that long-term loss of ovarian hormones had an additional negative effect on BFC neurons, beyond the effects of normal aging (Gibbs, 2003). The mechanism by which TrkA mRNA decreases as a result of aging is not clear. The down-regulation of TrkA receptors might result in a decreased trophic support for cholinergic cells. It has also been shown that endogenous NGF in the cortex has a role in maintaining cholinergic boutons (Debeir et al., 1999). In response to unilateral electrical stimulation of NBM neurons in adult rats, NGF levels on the ipsilateral hemisphere significantly increased; however, this upsurge was absent
in aged animals (Hotta et al., 2009). Another study has also indicated that the compensatory elevation of NGF levels after quisqualic acid lesion is impaired in aging (Gericke et al., 2003). Further studies are needed to elucidate whether the lack of estrogenic action in our study was a consequence of the weakened NGF function due to old age.

Another possible explanation for the lack of fiber density restoration in aged animals is the alteration of ER expression in cholinergic neurons. There is evidence in the literature that aged mice show a similar number of ERα-positive neurons in the BF as younger counterparts and that these receptors are still responsive to changes in estrogen levels (Kalesnykas et al., 2004). On the other hand, the intracellular localization of the ERs changes as animals age. The number of ChAT-positive neurons expressing nuclear ERα decreases, leaving an increased number of detectable cytoplasmic ERα. Furthermore, the distribution is unaffected by the loss of estrogen, suggesting that age is a more dominant factor than endogenous estrogen levels (Kalesnykas et al., 2005). A human study has also reported age related differential ERα and ERβ expression in the supraoptic nucleus (Ishunina et al., 2000). Translocation of ERα from the nucleus to the cytoplasm can alter the balance between classical and non-classical estrogen signaling, highlighting the importance of the ERs in our study.

In addition, there is clear evidence that the aged brain shows reduced plasticity. Data demonstrates that aging is associated with increased mortality following traumatic brain injury in rats (Hamm et al., 1991). Furthermore, it has been shown in the hippocampus that lesion-induced neuronal sprouting diminishes with age (Schauwecker et al., 1995). The BFC system has also been a targeted area for age-related plasticity research and the aberrant sprouting response of these neurons is known (Sarter and Bruno, 1998; Crutcher, 2002). SN-BM lesions produce a more profound dendritic atrophy in aging rats than in the young (Works et al., 2004). Other studies, investigating the aging of the cholinergic system
corroborate the hypothesis that plasticity and the sprouting capability in the cortex are altered in aging animals (Wellman and Sengelaub, 1995; Kim et al., 2005). In the previous chapter, we hypothesized that the E2-induced cholinergic fiber restoration was a consequence of an increased sprouting effect of the remaining fibers in the NMDA lesioned somatosensory cortex. The present investigation in aged mice, in which E2 treatment was ineffective, further emphasizes the importance of this sprouting theory.

5.7 Summary

Taken together, we investigated the restorative effects of E2 treatment under different physiological conditions, to better understand the mechanism behind the fiber restoration and obtain a more realistic model of human pathological conditions. Our results indicate that E2 treatment is effective in restoring the cholinergic fiber density in the cortex of young animals, in a gender and endogenous hormone independent manner. However, the mechanism by which E2 was able to initiate enhanced cholinergic fiber density restoration remains unclear. With the use of different physiological conditions we could progress one step forward in understanding this ameliorative action. Nevertheless, the exact cellular mechanisms needed to be analyzed further. Exploring the different theories about the ineffectiveness of E2 treatment in the aged group reveals that the ERα might play an important role in this model. Moreover, there is an increasing body of evidence in the literature indicating that the BFC system is influenced by non-classical estrogen signaling and this signaling is involved in neurodegenerative processes as well. Therefore, in further experiments, we aimed to characterize the intracellular mechanism of the E2-induced restorative effect in association with the ERα and the non-classical estrogen pathway.
Chapter 6

MECHANISM OF THE E2-INDUCED CHOLINERGIC FIBER RESTORATION

Introduction

We have shown in previous experiments that E2 treatment is able to restore cholinergic fibers in the somatosensory cortex following NMDA lesion under various physiological conditions. However, the mechanism behind this restorative effect is still unknown. Based on our previous results and the data in the literature, we hypothesized that the non-classical estrogen pathway was involved in this amelioration. Estrogen is able to initiate rapid changes in BFC neurons, which is a good indication towards the non-classical effects (Szego et al., 2006). The possible involvement of the ER is a crucial point in all estrogenic actions, including those that occur through the non-classical estrogen pathway. Therefore, firstly, we aimed to investigate the link between the ERα and the cholinergic fiber restorative effects with ERα KO mice. Studies have shown that ERα KO animals have reduced responsiveness to estrogen and the ChAT expression upon estrogen treatment is greatly reduced (Dubal et al., 2001; Bora et al., 2005). However, there is no clear indication that ERα KO animals would show AD symptoms in the brain. ERα polymorphisms are associated with age-related memory deficits and an increased incidence of AD among women (Olsen et al., 2006; Corbo et al., 2007). Although there is no direct evidence that ERα KO mice show these symptoms, it is possible that these animals are predisposed to neurodegenerative cholinergic diseases as the ERα is important in all AD-related processes and the ChAT down-regulation is an early symptom of AD.

Participation of intracellular signaling events in the non-classical estrogen pathway is also important. Inhibition of some of the major signaling pathways (MAPK or PKA) can completely block the rapid non-classical effects of estrogen in cholinergic neurons (Szego et
al., 2006). In the light of this, we aimed to utilize signaling pathway inhibitors *in vivo*, to better understand the involvement of the non-classical actions.

The availability of non-classical estrogen pathway activators, such as estren, makes it achievable to selectively initiate non-classical signaling events. In this study, we aimed to use estren as a treatment following NMDA lesion, although the use of estren on BFC neurons is completely unknown. Therefore, our study is the first, analyzing the ameliorative effects of estren on cholinergic neurons following neurodegeneration, *in vivo*. 
Chapter 6 - Mechanism of the E2-induced cholinergic fiber restoration

Results

In these experiments, we used neuron-specific ERα KO animals, intracellular signaling pathway inhibitors and a synthetic non-classical pathway activator (estren), in order to investigate the mechanism behind the cholinergic fiber restoration.

6.1 Effects of E2 on cholinergic fibers in ERα KO mice

To examine whether the estrogen-induced restorative effect on cholinergic fiber density requires classical ERα, we used neuron-specific ERα KO mice. Female animals were OVX two weeks prior to NMDA infusions along with heterozygous siblings used as control (wild-type littermates). All parameters (NMDA injection, E2 treatment) were designed based on experiments with wild-type OVX mice used before (see Chapter 4). In order to validate the KO animals, fluorescence ERα immunohistochemistry was performed.

Analysis of the immunohistochemistry data revealed a high number of ERα (93.2% ± 1.4, n=5) in the heterozygous control animals as well as showing no receptors in the KO ones (n=5) (Figure 29).
Figure 29: ERα in a cholinergic neuron in wild-type and ERα KO mice

Photomicrographs depict ERα immunoreactivity (red) in ChAT-positive neurons (green) in wild-type (A) and ERα KO (B) mice (scale bar: 10µm).

Evaluation of AChE-positive fiber densities following NMDA infusion and E2 treatment demonstrated that E2 had no effect on the fiber density in ERα KO mice (fiber loss in vehicle: 51.3% ± 9.1 and E2: 44.7% ± 11.8). In contrast, the treatment of heterozygous littermates significantly reduced the NMDA-induced AChE-positive fiber loss in the cortex (vehicle: 39.8 ± 3.8 and E2: 17.1 ± 2.3). Data we obtained from heterozygous control littermates corresponded with the results from normal wild-type OVX animals (see Chapter 4). These results suggest that the ERα plays an important role in the ameliorative action of E2 on cholinergic neurons (Figure 30).
Figure 30: Effects of E2 treatment on cholinergic fibers following NMDA lesion in ERα KO mice

Photomicrographs showing the effects of E2 treatment on cholinergic fibers in heterozygous wild-type (A) and ERα KO (B) mice (black bars indicate the borders of layers IV and V, scale bar: 100µm). The fiber restorative action of E2 disappears in ERα KO animals (bar graph) (** P < 0.01, two-way ANOVA, n=5-6 in each group).

6.2 Subcellular distribution of ERα in cholinergic neurons

Previous experiment with ERα KO animals has shown that the ERα has an important role in the estrogen-induced restorative action. Consequently, we further investigated the involvement of the ERα in this model. One possibility is that the subcellular ERα distribution in cholinergic neurons correlates with the NMDA lesion. A specific subcellular location (e.g. nuclear or cytoplasmic) might provide an effective platform for subsequent estrogenic actions. Accordingly, we characterized the intracellular expression of ERα in cholinergic neurons in...
the SI-NBM complex of wild-type OVX mice following NMDA lesion. Animals received NMDA infusion and vehicle treatment. Three categories were differentiated based on the ERα’s location in ChAT-positive neurons: 1. nuclear, 2. cytoplasmic or 3. nuclear + cytoplasmic (both). Cholinergic neurons, ERα, and cell nuclei were visualized using fluorescence immunohistochemistry. DAPI staining for nucleus was used to determine the subcellular distribution of ERα. Both lesioned and non-lesioned hemispheres were analyzed. Using confocal laser scanning microscopy, our results showed that 80-90% of cholinergic neurons express ERα in the SI-NBM, both on the non-lesioned and lesioned sides (87.5% ± 2.4 and 81.9% ± 3.8, respectively). Neurons on both hemispheres showed mainly nuclear ERα expression (non-lesioned: 88.6% ± 1.6 and lesioned: 98.8% ± 1.1). Although the NMDA injected hemisphere has much fewer ChAT-positive cells (an approximately 80% reduction, see Chapter 4), the subcellular distribution of the ERα did not change after the NMDA-induced neurodegeneration. The remaining cholinergic neurons following lesion showed the same (nuclear) receptor localization. Figure 31 demonstrates confocal microscope images, showing ERα expression in cholinergic neurons. Figure 32 shows the quantified receptor expression in cholinergic neurons on the NMDA lesioned and non-lesioned brain sides.
Figure 31: ERα distribution in cholinergic neurons

Photomicrographs showing ChAT-positive neurons (green) (A) with ERα (red) (B), and DAPI labeled nuclei (blue) (C). Merged image (D) demonstrates nuclear ERα distribution in ChAT-positive neurons (scale bar: 20μm).
Figure 32: ERα distribution in cholinergic neurons following NMDA lesion

Cholinergic neurons, regardless of the NMDA lesion, express mainly nuclear ERα (* P < 0.05, *** P < 0.001, one-way ANOVA, n=5).

6.3 Effects of signaling inhibitors on the E2-induced restoration

Studies on cholinergic neurons have shown that the intracellular signaling pathways may play an important role in the E2-induced ameliorative effects (Marin et al., 2003b; Dominguez et al., 2004; Guerra et al., 2004; Szego et al., 2006). In this part of the study, we examined the role of two major pathways in the E2-induced cholinergic fiber restoration. OVX animals were NMDA injected and treated in the same manner as detailed earlier (see Chapter 4). We investigated the MAPK and PKA pathways by means of specific inhibitors (MEK inhibitor - U0126 and PKA inhibitor - H-89, respectively). We applied these signaling inhibitors individually and in a combination as well, 30 minutes following NMDA lesion. As the inhibitors were dissolved in DMSO, 2.5% DMSO was used as a control and its effects tested on the E2-induced fiber restoration in the first experiment.

E2 administration significantly attenuated the AChE-positive fiber loss in the control group, in which animals received intracerebroventricular DMSO injection (vehicle: 45.8% ± 4.4 and E2: 23.4% ± 3.8). In contrast, intracerebroventricular administration of U0126 or H-89 or the combination of U0126 and H-89 blocked the effects of E2 on the NMDA-induced fiber loss.
(for U0126 vehicle: 47.1% ± 8.2, E2: 35.8 ± 7.5; for H-89 vehicle: 44.4% ± 7.3, E2: 32.7% ± 6.2; for the combination: vehicle: 37.6% ± 4.5, E2: 34.6% ± 4.3). The NMDA-induced fiber loss was not altered by the inhibitors in the vehicle treated animal groups. These results suggest that the MAPK and PKA signaling pathways play a critical role in the E2-induced restoration of the cortical cholinergic fiber density following NMDA lesion (Figure 33).

**Figure 33: Effects of E2 treatment on cholinergic fibers following NMDA lesion in the presence of signaling inhibitors in OVX mice**

Photomicrographs showing the effects of E2 treatment in the presence of DMSO control (A) and the combination of U0126 and H-89 signaling pathway inhibitors (B) (black bars indicate the borders of layers IV and V, scale bar: 100µm). Bar graph demonstrates that both pathway blockers (U0126 and H-89) were able to attenuate the fiber restorative effects of E2 (** P < 0.01, two-way ANOVA, n=6 in each group).
6.4 Effects of estren

Using a non-classical pathway activator, estren, we investigated whether the non-classical actions of estrogen are involved in its ameliorative effects. Although several studies use estren as a non-classical estrogen pathway activator, the scientific literature is still controversial about its classical or non-classical action. Therefore, first we examined whether estren induces classical activation in our model. The effects of estrogen on the uterus are considered as classical - genomic mechanisms, because estrogen mediates proliferative effects through a classical ERα dependent, ERE mediated transcription. Accordingly, we tested the uterotrophic effects of estren.

6.4.1 Effects of estren on the uterus

In this experiment, we treated OVX animals with estren, E2 or vehicle (33ng/g), 24 hours and 3 hours (two separate doses of hormone treatment) before an overdose of avertin injection. Uterus weight and overall thickness of the uterus epithelial layer were measured.

Our results show that E2 treatment increases the weight (vehicle: 20.8mg ± 1.2 and E2: 46.8mg ± 4.1) and the epithelium layer thickness (vehicle: 18.1µm ± 1.5 and E2: 25.8µm ± 1.5). However, estren failed to show these classical actions (weight: 18.1mg ± 2 and thickness: 16.3µm ± 1.3). This experiment indicates that estren, at this concentration, does not influence the classical pathway of estrogen. Figure 34 illustrates representative images of uteri following different steroid treatments. Figure 35 summarizes the quantified results of the experiments.
Figure 34: Estren and E2 treated uteri
Photomicrographs showing the effects of E2, estren and vehicle treatments on the uterus in OVX mice. E2 injection caused a profound increase in the size of the uterus (B) compared to the vehicle treated one (A). Estren injection, however, did not cause a significant change (C) (scale bar: 200µm).
Figure 35: Effects of estren and E2 on the uterus

Histogram A showing the effects of E2 and estren injections on the uterus weight in OVX mice while graph B demonstrates their effects on the uterus epithelium layer thickness. E2 increased the size of the uterus; however, estren treatment failed to have an effect (** P < 0.01, *** P < 0.001, one-way ANOVA, n=6 in each group).

6.4.2 Effects of estren on cholinergic fibers following NMDA lesion

As estren did not have classical effects in our study, we further investigated its actions on the cholinergic fibers loss. We performed our experiments on OVX mice and measured fiber density after NMDA injection and estren treatment. We applied three different estren treatment concentrations: 3.3ng/g, 33ng/g and 330ng/g. The middle range was the same as before; the effective dose for E2 (33ng/g).

Results showed that both the low (3.3ng/g) and high (330ng/g) doses of estren were not effective in restoring the cholinergic fiber density following NMDA lesion (fiber loss in vehicle group: 45.3% ± 4.5, 3.3ng/g estren: 39.9% ± 6, 330ng/g estren: 47.1% ± 3.2). However, the same dose as in the E2 experiment series (33ng/g) was able to initiate fiber restorative effects (23.3% ± 3.2) (Figure 36).
Figure 36: Effects of different estren doses on cholinergic fibers following NMDA lesion in OVX mice

Photomicrograph A illustrates the effect of vehicle treatment, B shows 3.3ng/g estren, C 330ng/g estren, and D 33ng/g estren treatments (black bars indicate the borders of layers IV and V; scale bar: 100µm). Thirty three ng/g estren restores the cholinergic fiber density in the cortex (bar graph) (** P < 0.01, one-way ANOVA, n=6 in each group).
Discussion

In this final part of the study, we characterized the mechanism of the E2-induced cholinergic fiber density restoration following NMDA lesion. The ameliorative effect was completely abolished in the ERα KO animals, indicating the critical role of the ERα. Our experiments also showed the importance of the intracellular signaling pathways in the E2-induced effects on cholinergic fibers. Furthermore, the synthetic non-classical activator, estren was shown to have E2-like restorative effect.

6.5 Involvement of the ERα

Previously, it has been shown that cholinergic neurons in the BF express mainly ERα (Shughrue et al., 2000). There are several studies investigating ERα in cholinergic neurons, since the ER is known to be a critical element guiding estrogenic actions (Behl, 2002a). Miettinen and colleagues (2002) showed a significantly lower ERα co-localization in the rat SI-NBM than our study. The difference could be explained by the obvious dissimilarity between rat and mouse species. ERα KO animals have been shown to have a disrupted hypothalamic-pituitary axis, resulting in various altered functions from cellular to behavioral (Kim and Casadesus, 2010). Moreover, it is known that the ERα plays a critical role in the estrogen-mediated actions in cholinergic neurons as well (Szego et al., 2006). Indeed, our findings demonstrated that an acute E2-induced restorative action on cholinergic fibers was completely blocked in neuron-specific ERα KO mice. The scientific literature lacks studies investigating estrogen-induced amelioration on cholinergic neurons following neurotoxic insult in ERα KO animals. However, there is indication that this subtype of ER provides a critical link in estrogen-mediated amelioration against neurodegeneration. Dubal and colleagues (2001) have shown in an in vivo study that deletion of ERα completely abolishes the protective effects of estrogen against stroke injury in every brain region. Estrogen
treatment was also shown to have protective effects against Aβ toxicity in SN56 cholinergic cell line in an ERα dependent manner (Marin et al., 2003b). In the latter study, with the use of an ER antagonist (ICI 182,780), an extranuclear (membrane-linked) ER was proposed to be involved. Extranuclear (cytoplasmic) ERα has been reported in cholinergic neurons in another study as well (Kalesnykas et al., 2005). In contrast, results from our experiment, aiming to determine the subcellular distribution of the ERα showed that cholinergic neurons in the SI-NBM express mainly nuclear receptors in female mice. This distribution did not change after NMDA lesion. Therefore, we hypothesize that extranuclear ERα is not involved in the estrogen-induced fiber restoration at the level of the cholinergic cell bodies in the SI-NBM. Conversely, it is possible that estrogen can act directly at the level of the cholinergic fibers in the cortex; although, there has been no clear evidence about the presence of ERα in cholinergic fibers. Nevertheless, substantial evidence has accumulated about ER localization in axons and dendrites and at synapses (Hart et al., 2007; Woolley, 2007). Extranuclear ERs in cholinergic fibers might provide an initiation point for further signaling mechanisms that are required for the fiber density restoration. Moreover, classical ERs located in close proximity to the plasma membrane play a pivotal role in estrogen-induced non-classical actions (Kousteni et al., 2001). Szego and colleagues (2006) have demonstrated that estrogen induces CREB phosphorylation in BFC neurons and that this effect is completely absent in ERα KO mice; proposing a strong participation of the non-classical signaling events through the ERα. Although another membrane-linked ER (GPR30) has also been reported in cholinergic neurons in a recent study (Hammond et al., 2011), its role in the estrogen-induced non-classical signaling in these neurons remained unknown.

6.6 Participation of intracellular signaling pathways

Several in vitro studies have demonstrated the importance of the activation of intracellular signaling pathways by estrogen in cholinergic neurons. The estrogen-induced MAPK pathway
activation elicits cholinergic neurite outgrowth (Dominguez et al., 2004), activates HACU and ACh synthesis (Pongrac et al., 2004; Bennett et al., 2009). Previously it has also been shown that estrogen can directly activate the MAPK and PKA pathways, leading to the phosphorylation of CREB in cholinergic neurons in the SI-NBM, in vivo (Szego et al., 2006). In vitro findings also suggest that the estrogen-induced neuroprotective effects may depend upon the activation of intracellular signal transduction pathways, including MAPK and CREB (Dominguez et al., 2004; Lee et al., 2004; Zhao et al., 2005). Moreover, CREB has been linked as a key transcription factor in many restorative effects (Walton and Dragunow, 2000).

Our findings demonstrated that the E2-induced restorative effects on cholinergic fibers were blocked by the inhibition of the MAPK or the PKA pathway. These results suggest that estrogen can induce fiber restoration via a coupled MAPK-PKA pathway activation in cholinergic neurons of the SI-NBM complex. Although there are not too many in vivo studies about the estrogen-induced activation of signaling pathways in cholinergic neurons, these results are in agreement with a previous in vitro study, whereby the activation of MAPK pathway played a critical role against Aβ toxicity in cholinergic SN56 cells (Guerra et al., 2004). The activation of intracellular signaling events by estrogen is thought to be a unique feature of the non-classical pathway. Therefore, our neurodegenerative and acute treatment model with the use of specific signaling pathway inhibitors suggest, that the restorative actions occurred through the non-classical estrogen pathway. The disadvantage of this in vivo experimental model is that we cannot evaluate whether the actions of the signaling pathway blockers had a direct effect on cholinergic neurons. MAPK and PKA pathway blockers were injected into the ventricle which inhibited these signaling systems in the whole brain. It is possible that the inhibition of MAPK and PKA pathways in interneurons had an effect on the outcome of the E2 treatments. However, we did not observe any change in the inhibitor injected and vehicle treated animals. Also, there were no changes on the contralateral (non-lesioned) brain sides of inhibitor injected mice. Therefore, we hypothesize that
intracerebroventricular injection of signaling blockers had a direct effect on cholinergic projecting neurons.

### 6.7 Estren as a non-classical pathway activator

By this point, we had accumulated enough evidence to hypothesize that the cholinergic fiber density restoration occurred through an ERα dependent non-classical estrogen pathway. With the use of a non-classical pathway activator, estren, we attempted to further support our “non-classical” hypothesis. However, the scientific literature is controversial about the use of estren as a non-classical estrogen pathway activator. A study suggested that estren behaves as a weak estrogen rather than a non-classical selective activator in the mouse uterus (Hewitt et al., 2006). Hewitt and colleagues used a much higher estren dose (300µg/animal for 3 days) in this study. We found that estren injection at 33ng/g concentration did not have an effect on the mouse uterus. Thus, our experiment indicates that estren, at the concentration that was required for the cholinergic fiber restorative action, does not have classical estrogen-like effects on the uterus. Therefore, we used estren as a non-classical activator in our further experiments. We tried three different concentrations, using 33ng/g as a middle range as it has been shown to be effective for E2. Our results demonstrate that estren has E2-like restorative actions on cholinergic fibers in the cortex following NMDA-induced loss of subcortical cholinergic input. Previous *in vitro* results from the literature are in good agreement with ours, as it was shown that estren protects rat primary cortical neurons against Aβ toxicity (Cordey et al., 2005), while having low binding activity to ERs and poor ability to induce classical transcriptional activity (Kousteni et al., 2002; Moverare et al., 2003). Interestingly, estren treatments in our experimental model did not show linear dose-response effects. The results with the lowest and middle doses (3.3ng/g and 33ng/g) were similar to that of the E2 treated animals; showing restorative action at 33ng/g. However, the highest concentration (330ng/g) failed to restore the cholinergic fiber density following lesion, raising interesting questions
about the mechanism behind these actions. Kousteni and colleagues (2001) have proposed a hypothesis whereby perfect or imperfect binding of hormone molecules to the ER could induce classical or non-classical effects, respectively. In this model, the imperfect binding can rapidly induce the activation of signaling systems, while the classical pathway would require perfect, long-term binding between the hormone and receptor. It is possible that the highest estren concentration (330ng/g), providing sufficient amount of molecules, initiates a perfect, estrogen-like binding, therefore activating classical actions that do not lead to cholinergic fiber restoration. On the other hand, the middle dose range of estren (33ng/g) delivers only an imperfect binding leading to the activation of non-classical signaling events and eventually fiber restoration in the cortex. We did not observe any amelioration using the lowest estren dose (3.3ng/g) which could mean insufficient estren levels even for imperfect binding. Knowing that estren has low affinity to bind to the ER (Wessler et al., 2006; Otto et al., 2008), it is possible that this lowest concentration did not initiate any signaling action and therefore the treatment failed to be successful. Figure 37 demonstrates the hypothesis of these actions at different estren doses.
Diagram demonstrating the hypothetical action of estren influencing cellular behavior. Low estren concentration provides an imperfect hormone-receptor binding that leads to the activation of non-classical signaling pathways. High estren concentration provides enough molecules for a perfect binding that initiates classical genomics response. $E_a$: activation energy, $k_a, k_d$: association and dissociation rate, respectively. Figure adapted from Kousteni et al., 2001.

Another controversial point in the use of estren is the possible androgenic effect. Islander and colleagues (2005) have reported that estren has similar effects to the androgen, dihydrotestosterone, in thymus and bone marrow. An additional study has showed that estren can bind to and cause translocation of androgen receptors, regulating the transcription of two known target genes in an androgen responsive cell line (Krishnan et al., 2005). Although these results depict critical information about the mechanism by which estren might influence
cellular behavior, there is no clear evidence *in vivo* in the brain. Moreover, the distribution of androgen receptors in BFC neurons remains unknown.

### 6.8 Summary

Taken together, the results from these final experiments suggest that the non-classical estrogen signaling, involving MAPK and PKA pathways, through ERα might contribute to the E2-induced restorative action on cholinergic fibers. The disadvantage of the *in vivo* model is that we were not able to show direct estrogenic effects on cholinergic neurons. However, a previous study has shown that estrogen does induce non-classical actions (CREB phosphorylation) on selectively isolated cholinergic neurons using tetrodotoxin (Szego et al., 2006). In addition, we did not see changes on the contralateral brain side. Therefore, we hypothesize that the intracellular signaling inhibitors and E2 treatment acted directly on cholinergic neurons. This treatment model also does not make it possible to differentiate between nuclear and extranuclear ERα on which estrogen could act to initiate cholinergic fiber restoration. In a separate experiment, we could show that almost all cholinergic neurons express nuclear receptors in the SI-NBM; however, we cannot exclude the extranuclear receptors, localized in cholinergic fibers in the cortex. With the use of 33ng/g estren dose, we could successfully restore the cholinergic fiber density following NMDA lesion but not with the lower or higher doses. The interesting mechanism that stands behind the altered dose-response action remains unclear. Nevertheless, based on our last experiment series, we strongly hypothesize that the non-classical estrogen pathway was responsible for the cholinergic fiber density restoration in the cortex.
Conclusion

From our results, we have shown that a single dose of E2 treatment induces restoration of the cholinergic fiber density in the cortex after NMDA-induced loss of subcortical cholinergic input. Our data provides the first in vivo evidence of involvement of ERα and the MAPK-PKA signaling systems in ameliorative actions of estrogen on BFC neurons.

Here, I would like to highlight concerns about the experimental design, potential disadvantages and provide a possible future application of this treatment model.

One of the concerns was that in our study, we used a non-selective toxin (NMDA) to lesion BFC neurons. One could argue that this method gives too much unknown variability, considering that other neuronal phenotypes are also very much affected by the lesion. Moreover, it is known that selective and non-selective BF lesions result in different cognitive deficits, highlighting the importance of other neuronal phenotypes in the BF (Kaur et al., 2008). The use of a selective cholinotoxin, 192 IgG-saporin, was well-established by Leanza and colleagues in rats (Leanza et al., 1995). Since then, numerous studies have reported the success of this highly selective cholinotoxin, based on the fact that cholinergic neurons extensively express p75NTR receptors. However, this immunotoxin does not recognize mouse neurons. The first version of a mouse-specific toxin (mu p75-saporin) was tested by Berger-Sweeney and colleagues (Berger-Sweeney et al., 2001). It was reported in their study that there appear to be some important differences between the rat and mouse toxins. First, the mouse toxin does not have the same potency as the rat one. While the rat toxin generally induces around 90% cholinergic depletion, the mouse toxin causes much less (19%-58%), depending on the brain region (Berger-Sweeney et al., 2001). To achieve a higher rate of cholinergic cell death a much higher dose is needed, which increases the already high mortality rate in mice. Higher doses of toxin also induce non-selective damage, which questions the overall value of this drug. In 2006, an improved version of mu 75-saporin was
released, promising reduced mortality rate and higher specificity. Indeed, bilateral intracerebroventricular injections resulted in a greater ChAT-positive neuronal damage in the striatum and MS as well as AChE-positive fiber loss in the cortex (Moreau et al., 2008; Ho et al., 2009). However, ChAT-positive neurons in the SI-NBM were weakly affected. Considering that the SI-NBM complex is the main target area in our study, even the improved version of this selective drug would not be able to achieve similar cholinergic cell loss as NMDA. The number of studies using mu 75-saporin is very limited, showing that the use of this selective cholinotoxin in the mouse is controversial and far from well-established. It is also important to note that the p75NTR receptor distribution is not fully mapped in the mouse BF, raising another crucial question about saporin injections in mice. It has been reported in mice that not all ChAT-positive neurons co-localize with p75NTR in the SI-NBM (Berger-Sweeney et al., 2001; Nag et al., 2009). A rat study also indicated that some of the SI-NBM cholinergic neurons do not express this receptor (Heckers et al., 1994), although other studies state significant ChAT and p75NTR co-localization (Harkany et al., 2001a; Horvath et al., 2002). In addition, as it was mentioned in Chapter 3, we believe that the NMDA-induced excitatory cell death is closer to certain pathological conditions (e.g. stroke) than the immunotoxin saporin. Taken together, we believe that the NMDA-induced cholinergic cell death in mice was more appropriate for this study over the selective but controversial mu p75-saporin.

Another important argument about this study would be the possible involvement of AChE-positive non-cholinergic neuronal phenotypes. Our experiments involved the analyses of AChE-positive neuronal fibers in the cortex. ChAT immunohistochemistry possibly would have provided higher specificity as the ChAT enzyme can be found solely in cholinergic neurons. However, the computerized fiber density analysis requires good background / signal ratio, which could be achieved only with AChE histochemistry. Numerous studies have used AChE histochemistry to detect cholinergic fiber alterations in the cortex with great success.
(Luiten et al., 1995; Abraham et al., 2000; Horvath et al., 2000; Harkany et al., 2000b, 2001a; Conner et al., 2003, 2005). Moreover, Kitt and colleagues have shown that ChAT and AChE-positive fibers in the mouse cortex are virtually identical (Kitt et al., 1994). Therefore, our results strongly indicate that the measured fiber density in the cortex belonged to cholinergic neurons.

Although the unilateral cortical cholinergic projections from the SI-NBM are well-known in rats, there is no clear evidence in the literature that C57BL/6 mice have the same projection pattern. However, a comparative review highlighted that the mouse and rat cholinergic anatomy show significant similarities (Van der Zee and Keijser, 2011). Our results from all experimental groups clearly showed that NMDA infusion induces a unilateral AChE-positive fiber loss in the cortex. Furthermore, NMDA at the 20mM range induced a complete loss of AChE-positive fibers in the ipsilateral cortex but no loss on the contralateral side (see Chapter 3). Therefore, we conclude that the other hemisphere did not influence the experimental results in our mouse model.

Although the NMDA lesion induced a profound cell loss in the SI-NBM and a significant fiber loss in the cortex, our study did not involve the investigation of functional deficits following lesion. Generally, saporin-induced lesion studies in rats examine functional deficits with an almost complete absence of cholinergic cells and fibers, as the 192-IgG saporin is much more potent than the mouse counterpart (Berdiev et al., 2007; Harati et al., 2008; Kaur et al., 2008; Ramanathan et al., 2009). Although the number of functional studies in mice investigating behavioral deficits following SI-NBM lesions is limited, there is indication that a 35-55% cell loss is enough to induce significant changes. Moreau and colleagues have reported that mu p75-saporin lesioned mice (50% cell loss in the SI-NBM) showed moderate deficits in water-maze and Barnes-maze tests and were hyperactive during locomotor activity test following surgery (Moreau et al., 2008). Anxiety-related behavior was also affected.
following saporin-induced cholinergic lesions in mice (Nag et al., 2009). Dolga and colleagues have shown in mice that NMDA lesion followed by 35% cholinergic fiber loss induces impaired behavioral performance. SI-NBM damage specifically affects neocortical denervation and its memory functions while leaving hippocampal innervations and its learning unaffected (Dolga et al., 2009). Our study reported an approximately 80% cholinergic cell loss and a 40-50% fiber loss, which is not as profound as other rat studies using saporin; however, it is significantly higher than previous data found in mice. Although further behavioral studies are needed, it is likely that NMDA lesion induced significant and long-term functional impairments.

Although the NMDA-induced neuronal death closely resembles to that of observed in pathological conditions such as stroke or AD, our model is not the most appropriate representation of these neurodegenerative conditions. The single injection of NMDA into the brain, inducing acute excitatory neuronal death, shows similarities to stroke symptoms; however, stroke generally occurs in cortical areas rather than in the BF. The cortical plasticity and sprouting mechanisms are known in stroke (Carmichael, 2006; Li et al., 2010); however, we did not investigate other neuronal phenotypes in the cortex. Therefore, based on our study, we cannot conclude that E2 treatment would enhance a restorative mechanism in other cortical neurons following brain injury, although estrogenic effects have been reported in stroke (Leon et al., 2011; Strom et al., 2011). Damage of cholinergic neurons in the BF is usually observed in AD (cholinergic hypothesis), which is a slowly developing neurodegenerative disease (Craig et al., 2011). Therefore, the rapid excitatory death of BFC neurons does not fully represent the disease progress. Our results also indicated that E2 treatment was not effective in aged mice. Considering that AD is an aging disease, the potential medical implications of our results in this age group are questionable. Moreover, we administered a single E2 injection as treatment, which probably would not be sufficient as an alternative therapy in AD.
Our main finding from this study was that E2 treatment restored the cholinergic fiber density in the cortex following NMDA-induced lesion. Maybe the most controversial point of this study is the mechanism behind the increased fiber density following injury and treatment. The neuroprotective effects of estrogen are well-known - numerous studies have investigated it under different pathological conditions (Behl, 2002a; Sherwin, 2009; Suzuki et al., 2009; Amtul et al., 2010; Lebesgue et al., 2010; Yang et al., 2010; Dang et al., 2011). Based on the simple model of this study (induced neurodegeneration and acute treatment) and previous findings in the literature, one could conclude that the observed estrogenic effects were protective rather than restorative. The effective time-window of the treatment further emphasizes the protective effects of estrogen. Treatment one hour following a lesion might indicate a protective mechanism because neuronal damage usually requires a longer time to develop. It has been shown that NMDA-infusion induces ChAT-positive cell loss 4 hours following injury with a maximum damage within 24 hours (Harkany et al., 2001a). The brief time window suggests that cholinergic neurons were already dying at the time of E2 administration. Therefore, E2 treatment 24 hours following injury was not able to rescue cholinergic neurons. Looking at only the cholinergic fibers in the somatosensory cortex, it is convincing that E2 protected cholinergic neurons against neurodegeneration. However, E2 treatment was not protective on cholinergic cell bodies in the SI-NBM. Protection of the cell bodies is essential in order to observe lessened fiber damage in the cortex. Because of the strict unilateral projection of the SI-NMB cholinergic neurons, these findings raise the possibility that the remaining cholinergic neurons sprout into the cortex from the lesioned SI-NBM to restore the original condition. However, the experiments in this study do not provide direct evidence for this E2-induced sprouting action. This experimental design was not able to clarify how the sprouting mechanism might occur. It is possible that there is an immediate natural regenerative mechanism following lesion (e.g. through the neurotrophin signaling), which is further enhanced by a single E2 treatment. However, vehicle treated animal groups
Conclusion

revealed no natural regeneration; therefore, it is more likely that the single E2 injection is an initiating factor rather than enhancing. As the treatment occurred not long after the induced lesion, it is probable that the single E2 treatment induced long-lasting sprouting tendency from the time of injection. As this restorative or sprouting mechanism remained unclear, further experiments are needed for clarification.

The most pressing question regarding the effects of estrogen on the cholinergic fiber restoration is in its physiological and therapeutic relevance. Data from the literature suggest that estrogen holds promise in the amelioration of BFC neurotoxic and neurodegenerative damage. Our experiments on intact female animals revealed that endogenous estrogen levels do not affect the NMDA-induced cholinergic loss, indicating that the physiological estrogen is not sufficient to initiate cholinergic fiber restoration in the cortex. Indeed, restoration was evident only at a supraphysiological concentration of E2 treatment. In addition, the restorative effect of E2 treatment was limited to a time-frame after the NMDA lesion. The effective concentration and time window of this action indicates possible therapeutic potential after brain injury. Other important results from this study are the lack of sex difference and the importance of aging, which reveals critical information for future medical applications. However, it is also known that estrogen therapy, beside its ameliorative action in neurodegenerative processes, has several side effects such as the risk of thrombosis, breast cancer or stroke (Shumaker et al., 2003; Maalouf et al., 2010). Therefore, it is critical to explore more specific estrogen treatment options in order to increase the ameliorative potential of estrogenic compounds and decrease their side effects. Our results reveal that the observed cholinergic fiber restoration is closely linked to the non-classical estrogenic action, providing valuable treatment options to the standard estrogen therapy. Non-classical estrogen pathway activators, such as estren, are promising candidates since they exhibit estrogen-like ameliorative actions without estrogen-like side effects. Such compounds could potentially
Conclusion

offer better safety profiles than the non-selective estrogen and may change the future for those suffering from loss of BFC neurons and related cognitive impairments.

**Future experiment**

In order to better address the physiological relevance of this model, further behavioral studies are needed. Lesion of BFC neurons is known to induce motor learning and memory deficit in rodents (Conner et al., 2003). We intend to determine the effects of estrogenic compounds on lesion-induced deficits in motor learning tasks. We believe that these behavioral experiments would reveal important information about the functional outcome of cholinergic deficits following injury and treatment. In addition, there is no evidence in the literature about the functional effects of selective non-classical estrogen pathway activators, which therefore need to be further elucidated with specific memory and performance tasks.

--
References


Gritti I, Henny P, Galloni F, Mainville L, Mariotti M, Jones BE (2006) Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated...
glutaminase and colocalizing vesicular glutamate transporters. Neuroscience 143:1051-1064.


## Appendix

### List of products and suppliers

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSM 510 microscope, ZEN software 2009</td>
<td>Carl Zeiss</td>
<td>Thornwood, NY, USA</td>
</tr>
<tr>
<td>MEK 1/2 inhibitor (U0126)</td>
<td>Cell Signaling Technology</td>
<td>Danvers, MA, USA</td>
</tr>
<tr>
<td>ChAT primary antibody</td>
<td>Chemicon (Millipore)</td>
<td>Billerica, MA, USA</td>
</tr>
<tr>
<td>Masterflex pump</td>
<td>Cole-Parmer</td>
<td>Vernon Hills, IL, USA</td>
</tr>
<tr>
<td>Stereotaxic device and mouse adaptor</td>
<td>David Kopf Instruments, Stoelting Co.</td>
<td>Tujunga, CA, USA</td>
</tr>
<tr>
<td>Electric driller</td>
<td>Foredom Electric Co.</td>
<td>Bethel, CT, USA</td>
</tr>
<tr>
<td>GraphPad Prism 5.04</td>
<td>GraphPad Software</td>
<td>La Jolla, CA, USA</td>
</tr>
<tr>
<td>Hamilton microsyringe 25S</td>
<td>Hamilton</td>
<td>Reno, NV, USA</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Invitrogen</td>
<td>Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Biotinylated anti-goat serum and Cy5 secondary antibody</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>West Grove, PA, USA</td>
</tr>
<tr>
<td>BX51 microscope and CellP Image Analysis software</td>
<td>Olympus</td>
<td>Tokyo, Japan</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>ProSciTech</td>
<td>Kirwan, QLD, Australia</td>
</tr>
<tr>
<td>ERα antibody (MC-20)</td>
<td>Santa Cruz Biotechnology</td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>DPX mounting medium</td>
<td>Scharlau</td>
<td>Gillman, SA, Australia</td>
</tr>
<tr>
<td>NMDA, PKA inhibitor (H-89), VAcT antibody and E2</td>
<td>Sigma-Aldrich</td>
<td>Poole, UK</td>
</tr>
<tr>
<td>Estren</td>
<td>Steraloids</td>
<td>Newport, RI, USA</td>
</tr>
<tr>
<td>Gill II. Haematoxylin and Eosin</td>
<td>Surgipath</td>
<td>Richmond, IL, USA</td>
</tr>
<tr>
<td>Vectastain Elite ABC kit and Vectashield mounting medium</td>
<td>Vector Laboratories</td>
<td>Burlingame, CA, USA</td>
</tr>
<tr>
<td>Freezing microtome</td>
<td>Wetzlar</td>
<td>Germany</td>
</tr>
</tbody>
</table>

All other unlisted laboratory products were ordered from **Sigma-Aldrich**.