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The Involvement of DFF45 and c-fos in Hippocampal Plasticity and Function

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Abstract

Neuroplasticity is an important aspect of neurodevelopment and function. The hippocampus is an area of the brain that undergoes many types of plasticity during development and in adulthood, including neurogenesis, apoptosis and changes in synaptic strength due to changes in gene expression. Hippocampal functions such as learning and memory and regulation of the stress response rely on this plasticity.

To study how changes in plasticity might affect hippocampal function, we generated two knockout mice, the first of which lacks a component of the apoptotic pathway, DFF45. The cells of the mice are resistant to apoptotic stimuli and the mice have an increased number of dentate gyrus granule cells. When hippocampal-dependent learning was tested in these mice we found that they display enhanced spatial and non-spatial learning abilities.

The second model we used is a hippocampal specific knockout of c-fos, an immediate early gene and part of the AP-1 transcription factor complex. The spatial learning abilities are normal in these mutant mice. The mice have more severe kainic-acid induced seizures than wild-type mice and greater CA3 cell death following seizures. While the mice have a normal response to acute restraint stress, they display enhanced habituation to chronic restraint stress. Female mutants also display less anxious behavior in the elevated plus maze than wild-types. Studies to discover the underlying molecular causes for these differences revealed changes in the expression of receptors including GluR6, GR, and ERα. Increased CA3 apical dendritic length was also found in the mutant mice.
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List of Abbreviations

ACTH  adrenocorticotropic
AMPA  $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA analysis of variance
AP-1 activator protein - 1
BDNF brain derived neurotrophic factor
BNST bed nucleus of the stria terminalis
CNS central nervous system
CORT corticosterone
CRE cAMP-response element
CREB cAMP-response element-binding protein
CRH corticotropin-releasing hormone
DFF40 DNA fragmentation factor 40
DFF45 DNA fragmentation factor 45
DG dentate gyrus
EEG electroencephalogram
ER estrogen receptor
ES embryonic stem cell
GluR glutamate receptor
GR glucocorticoid receptor
HPA hypothalamo-pituitary-adrenal
IEG immediate early gene
KA kainic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LTP</td>
<td>long term potentiation</td>
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<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>PTZ</td>
<td>pentetrazole</td>
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<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SAP</td>
<td>stretched attend posture</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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Chapter 1: Introduction

1.1 Neuroplasticity

Neuroplasticity is an important aspect of the development and function of the central nervous system (CNS). During development neurons migrate through the brain both radially and tangentially [1]. This migration is regulated by both intrinsic and extrinsic signals [2,3]. Once a cell has reached its target area, it develops in another way: through development of its dendritic arbors and outgrowth of its axon. This allows the formation of synapses with other neurons. The regions where synaptic connections are made with other cells can be seen as small outgrowths or spines on the dendrites. Throughout development, decreases in the number and density of dendritic spines are seen [4,5]. This form of neuroplasticity can define the final connections and, therefore, functions of the cell.

Neuronal plasticity does not end with maturity, however. The adult brain can undergo many different types of changes to various stimuli. Evidence of neurogenesis in the adult brain has been found [6,7]. Dendritic sprouting and pruning are also forms of plasticity within the adult brain [8-12]. Various studies in the human have shown that the mean dendritic length of cortical neurons is substantially smaller at birth than at adulthood [13,14]. Finally, on the smaller level, plasticity within synapses is also possible. Both changes in synaptic strength as well as changes in synaptic number are found in the adult brain [15-17].

Neuroplasticity underlies neuronal function. This has been shown to be the case in the visual system where visual input leads to the development of the ocular
dominance columns. If one eye is sutured shut, the ocular dominance columns from that eye are smaller than normal [18-22]. It has also been shown in the whisker/barrel system in rodents. The normal rodent has one barrel in the somatosensory cortex for each whisker. During a critical period of development – up to postnatal day 7- the removal or implantation of whiskers results in a lesser or greater number of barrels in the cortex, respectively [23].

1.2 Hippocampus

1.2.1 Hippocampal structure

The hippocampus is made up of Ammon's horn, or the hippocampus proper, which contains the CA1-CA3 regions, the dentate gyrus (DG), and the subiculum. The CA1-CA3 regions are composed of five layers: the stratum oriens, the pyramidal layer, the stratum radiatum, the stratum lacunosum, and the stratum moleculare [24]. The pyramidal layer contains the cell bodies of the pyramidal cells of the hippocampus. These cells exhibit extensive apical and basal dendrites and make the majority of connections between regions in the hippocampus and out of the hippocampus. The neurotransmitter used by these cells is glutamate. The majority of the cells within the dentate gyrus region are granule cells. There are also neuroglia, stellate and fusiform cells present within the hippocampal formation, as well as interneurons [24].
1.2.2 Hippocampal connections

Many of the pathways that connect the hippocampus with other brain regions are known. The majority of the input to the hippocampus is the input to the dentate gyrus granule cells from the entorhinal cortex through the perforant pathway [25-28]. The dentate gyrus then connects to the CA3 pyramidal cells through the mossy fibers. The CA3 then connects to the CA1 region through Schaffer collaterals and also send afferents through the fornix. Finally, the CA1 sends output to the subiculum and the entorhinal cortex [29,30] (Figure 1).

The output from the hippocampus is through two pathways: the fornix, which connects the hippocampus to subcortical structures including septal nuclei such as the bed nucleus of the stria terminalis (BNST), and connections through the entorhinal cortex to the neocortex [31-33]. The hippocampus, specifically the ventral subiculum, also has connections to many other brain regions through the septal-hippocampal afferent system, including various septal nuclei, the bed nucleus of the stria terminalis (BNST) being one of these [31].

1.2.3 Hippocampal Function

Many hippocampal functions are also known. The hippocampus is important in many types of learning. The first of these is spatial learning. This has been shown through lesion studies in both humans and rodents [34-36]. The hippocampus has also been found to be important in recognition memory [37-39]. It seems that the commonality among all hippocampal dependent memory is that it involves contextual-recall [40].
Another important function of the hippocampus is negative feedback onto the hypothalamo-pituitary-adrenal (HPA) axis following the stress response [41-47]. The hippocampus is well equipped to feedback onto the system due to its high levels of both type I (mineralocorticoid or high affinity) and type II (glucocorticoid or low affinity) glucocorticoid receptors (MR and GR) [46-49]. C-fos is expressed in the hippocampus following various types of stress, including restraint stress and swim stress [50-52]. Lesions to the hippocampus lead to elevated levels of corticotropin-releasing hormone (CRH) both basally and following stress [41,42,44,53,54]. Hippocampectomy also leads to increases in corticosterone (CORT) levels following stress [55,56]. On the opposite end of the spectrum, stimulation of the hippocampus lowers both basal and stress induced levels of CORT [57-60].

While the hippocampus does not have direct connections to the hypothalamus, it is possible that the BNST serves as a relay for efferents from the hippocampus to the paraventricular nucleus of the hypothalamus (PVN) [61]. Since hippocampal efferents are mainly glutamatergic, or positive, and BNST efferents are mostly GABAergic, or negative, this allows for a final negative effect of the hippocampus onto the HPA axis [62] (Figure 2).

### 1.2.4 Hippocampal Plasticity

The adult hippocampus is capable of many different types of plasticity. Neurogenesis has been shown to occur within the dentate gyrus region of the hippocampus in the adult brain [6,7,63-65]. Changes in the morphology of the pyramidal cells have also been shown, with dendritic atrophy following chronic
stress in rodent models [12,66]. Changes in synaptic strength occur within the hippocampus, specifically, long-term potentiation (LTP) [15,67,68]. LTP is a long lasting increase in synaptic efficacy following high-frequency stimulation. LTP is thought to underly the functions of learning and memory [69].

1.3. The HPA axis

1.3.1 HPA axis structure
The HPA axis is the critical circuit in the stress response of the animal. It begins in the (PVN) where parvocellular neurons release CRH in response to stress. The CRH goes into the hypophysial portal circulation in the median eminence to the anterior pituitary where it causes the release of adrenocorticotropin (ACTH) from corticotrope cells. ACTH stimulates release of glucocorticoids (GC) such as corticosterone from the adrenal cortex, which enters the bloodstream and acts throughout the body (Figure 2).

1.3.2 HPA axis function
Glucocorticoids activate a number of adaptive responses in the body on many organ systems. The actions are largely catabolic, mobilizing glucose in the liver, increasing cardiovascular tone, altering immune surveillance and inhibiting nonessential endocrine systems [70,71]. CORT also acts as the key to the negative feedback mechanism onto the HPA axis in order to restore homeostasis following a stressor.
Glucocorticoids also affect processes at the cellular level such as neurotransmitter release and cell survival. They increase the release of glutamate and decrease the rate of its reuptake while decreasing GABA inhibition [72,73]. GCs cause a decrease the expression of neurotrophins and increase the vulnerability of neurons to hypoxia and metabolic insults [74-76].

1.3.3 HPA axis plasticity
The HPA axis is activated in response to stress and also in a circadian rhythm in the absence of a stressor. In both cases, there is a feedback system to keep the catabolic actions of the adrenocortical response under control. If the stress response is not controlled properly it can result in many disease states, from colitis, to depression, to post-traumatic stress disorder (PTSD), and even neurodegenerative diseases such as Alzheimer’s disease [77-80]. It is, therefore, very important for the feedback mechanisms of CORT onto the HPA axis to work properly. This is a highly integrated process with many levels of action. CORT can act directly at the hypothalamus and pituitary to slow the stress response by decreasing release of CRH or ACTH [81-84]. Feedback also occurs through actions in other circuits in the brain. One of these, as stated earlier, is believed to be the hippocampus. Others include the brainstem catecholaminergic neurons, the serotonergic dorsal raphe nucleus, the amygdala and the medial prefrontal cortex [85-89] (Figure 3).
1.4 Factors controlling plasticity

1.4.1 Genetic vs. Environmental factors on development

As stated earlier, genetic and environmental inputs are both important in determining the fate of the developing brain [92-93]. It has been shown that intrinsic and extrinsic cues are important in determining both cell migration and cell differentiation. Genetic control can regulate cell growth and differentiation [94]. Specifically, transcription factors such as neurogenin activate neuronal gene expression to lead to neuronal differentiation [95]. Transplant studies have revealed that the environment of the cell can also determine its fate [96-99]. Growth factors can also play a role in determining cell fate [100-102].

Environmental input can be detrimental to normal development as well. For example, exposure to toxins such as alcohol can lead to serious developmental disorders [103-106]. On the other end of the spectrum, vitamin and mineral deficiencies can also lead to defects, such as spina bifida [107,108]. The developing brain is also sensitive to corticosterone. Cognitive deficits have been found following prenatal exposure to corticosterone, specifically those in visual memory, a hippocampal function [109]. In fact, prenatal exposure to dexamethasone has been shown to lead to hippocampal developmental deficits in rhesus monkeys [110].

1.4.2 Apoptosis in plasticity

A form of plasticity that is important in the development of all brain areas, including the hippocampus, is programmed cell death, or apoptosis. During brain development, apoptosis leads to the elimination of neurons that fail to form
synapses with other neurons [111]. This prevents the proliferation of unnecessary or redundant neuronal populations. A number of knockout mouse models lacking molecules important in apoptosis have shown the importance of apoptosis in proper brain development. These knockouts all result in late embryonic to perinatal lethality due to deficits in the CNS. They also display neuronal hyperplasia, structural disorganization, and ectopic cell masses [112-117].

Apoptosis is a method of cell death that eliminates cells in a controlled and protected manner. This is in contrast to necrotic cell death, during which cell membranes rupture allowing leakage of the cellular contents. This can cause tissue inflammation and damage to the surrounding cells. During normal development the majority of cells die through apoptosis in order to remove excessive or damaged cells [111] without causing damage to surrounding cells. There are a number of morphological changes that occur during apoptosis including cell and nuclear shrinkage, chromatin condensation, and the formation of apoptotic bodies, followed by phagocytosis by neighboring cells [118]. Additionally, the chromosomal DNA is broken down. This process is considered the hallmark of apoptotic cell death [119].

Many of the molecular players of the apoptotic process are known. The Bcl-2 family proteins are upstream regulators of apoptosis. Various caspases, including caspase-3, caspase-7, and granzyme B, also play an important role in apoptosis by cleaving downstream molecules [120]. One molecule that these caspases can cleave is DFF45, part of the heteromeric DNA fragmentation factor (DFF) along with
DFF40. The cleavage of DFF45 causes it to be dissociated from DFF40. This allows
the DNase DFF40 to cleave chromosomal DNA [121-126] (Figure 4). It appears that
DFF45 is not only an inhibitor of DFF40’s endonuclease activity, but that it also plays
the role of chaperone. DFF 45 is, in fact, required for synthesis and proper folding of
DFF40 [122,126,127]. Therefore, the loss of DFF45 could lead to a disruption of
DNA fragmentation during apoptosis, and possibly a disruption of apoptosis itself.

Disruption of apoptosis can lead to various disease states, including neuronal
degeneration, autoimmunity disorders and cancer [111,128,129]. While
chromosomal DNA degradation is not required for apoptosis, [130-132] disruption of
proper DNA fragmentation can lead to less efficient apoptosis [132]. This could lead
to functional deficits throughout the brain, and specifically in the hippocampus where
neurogenesis and apoptosis continue into adulthood [6,7,63-65].

1.4.3 Transcriptional regulation in plasticity

Immediate early gene (IEG) expression is increased during neuronal plasticity. For
example, c-fos expression in the hippocampus has been shown following both
spatial learning as well as odor discrimination [133,134]. It is also expressed in the
PVN, the amygdala and the hippocampus following acute stress [52].

Changes in synaptic strength, such a LTP, represent a short-term plastic effect of
stimulation and could play a role in learning. In order for these changes to lead to the
long-term plasticity necessary for learning and memory formation, gene transcription
and protein synthesis are required [135-137]. IEGs, such as c-fos and c-jun, have
the capability of coupling the short-term activation of a cell to long-term changes
This is due to the fact that not only are they expressed so quickly following cell activation, but they are also able to dimerize to form the activator protein-1 (AP-1) transcription factor complex.

The AP-1 transcription factor complex binds to a specific seven-base nucleotide sequence (TGACTCA) present in the promoter region of a large number of genes [139,140], including the gene encoding the glucocorticoid receptor [141]. Based on its makeup of Fos and Jun family members, AP-1 can act as either a positive or negative regulatory element on both basal and induced levels of gene expression [142].

Hormone receptors such as GR can also act as transcription factors. When inactive, they are part of a cytoplasmic multiprotein complex that includes several heat shock proteins (hsp) and an immunophilin [143,144]. Once the hormone binds to the receptor, the receptor is dissociated from the complex and is translocated to the nucleus of the cell. Here they act at their response elements on the promoter regions of genes.

Transcription factors such as GR and AP-1 can also interact with each other to alter resultant gene expression. This interaction has been shown to occur in both directions, with GR repressing AP-1 activity and AP-1 repressing GR activity [145,146].
1.5 Hypothesis and Approach

We believe that alterations in plasticity within the hippocampus will lead to changes in hippocampal function. Our overall approach is to use genetically engineered mouse models that have plastic changes in the hippocampus.

Previous work has focused on the role of apoptosis in development and focused on upstream factors. Few have addressed the question of whether it is also important in adult plasticity and little is known about why DNA degradation always accompanies apoptosis. To begin addressing this issue, we created a mouse that lacked DFF45. The cells of this mouse were resistant to various apoptotic stimuli [132]. The dentate gyrus of this mouse was shown to have an increased number of cells as well as in increased density of cells. Based on this finding, we tested two types of hippocampal-dependent learning. We tested the spatial learning abilities of the mice in the Morris water maze. We then tested the recognition abilities of the mice in the novel object recognition test.

A second form of neuronal plasticity that we altered was the ability of the cells to transcribe genes properly. This was done by creating a regional knockout of the immediate early gene c-fos, which, as stated earlier, can act as a part of the AP-1 transcription factor complex. This approach was used because the complete c-fos knockout results in 60% of the mice dying shortly after birth and the remaining suffering severe deficits [147-149]. These deficits prevent any serious behavioral analysis from being performed. c-fos was the chosen target based on its IEG properties and the fact that it is activated following many stimuli, including
neurotransmitters and neurotrophins, and in many situations, including stress, learning, seizures, and preceding cell death [133,150-158].

The regional knockout was created using the loxp/cre method and is both temporally and spatially regulated, with the loss of c-fos beginning during postnatal week three and complete loss in the hippocampus by week 10. At this time, the knockout is restricted to the hippocampal formation, with a 95-98% loss of induced c-fos expression in the CA1-CA3 regions and a 65% loss in the dentate gyrus. The mouse is normal developmentally.

We tested the seizure response in this mouse using kainic acid (KA) induced seizures. The cell death following these seizures was also tested. The mutant mice were found to have a greater seizure response than wild-type mice and the resultant CA3 cell death was increased in the mutant mice following the seizures.

We also tested the learning response in this mouse. Since various upstream molecules, including CREB, CAMKII and NMDARI, have been shown to be essential for learning, we wanted to explore whether c-fos was another molecular component in this pathway [159-162]. To test the learning abilities of this mouse we tested it in two spatial learning paradigms, the Morris water maze and the Barnes maze.

Finally we tested the ability of this mouse to regulate its stress response. The first approach was to measure the plasma CORT levels of the mouse during a 1-hour restraint stress test. This would test the basal CORT levels of the mouse at both the peak and trough of the circadian cycle. It would also test the stress response and the ability of the mouse to recover from the stressor. We also tested the plasma CORT levels in a chronic restraint stress paradigm, during which the mice are restrained for
one hour for 22 consecutive days. This would test the abilities of the mice to habituate to the stressor.

The anxiety behavior of the mice was tested using the elevated plus maze [163]. This task is often considered a test of amygdala function, but it has been shown that the hippocampus plays an important role in the behavior as well [164-167].

To explore the molecular changes that might result from the loss of c-fos that might impact the hippocampal feedback onto the stress response, we performed Western analysis to measure hippocampal protein levels of target molecules including GR, brain derived neurotrophic factor (BDNF), and estrogen receptor alpha (ERα). Finally, we wanted to see how the changes in the hippocampus might affect the plasticity of the pyramidal neurons that is known to follow chronic stress in other rodent models [12,66]. To do this, we performed 21 days of 6-hour chronic restraint stress and then performed Golgi analysis on the perfused brains, which allowed us to visualize the dendritic arbors of the pyramidal cells.

These tests allow us to evaluate how two different types of manipulations of hippocampal plasticity might affect hippocampal function in different ways. Both the change in the number of hippocampal cells and the change of the makeup of the transcriptional machinery have a great impact on the resulting hippocampal plasticity and function.
**FIGURES**

**Figure 1.** Schematic of the hippocampal structure and its interconnections. Adapted from McEwen Frontiers in Neuroendocrinology 1999 [168].
Figure 2. Schematic of the HPA axis. Adapted from Engler et al. 1999 [169].
Figure 3. Schematic diagram of feedback pathways onto the HPA axis.

Adapted from Herman and Cullinan, 1997 [62].
Figure 4. Mechanism of action of DNA fragmentation factor (DFF) in apoptotic DNA degradation. Adapted from Zhang and Xu, 2002 [170].
REFERENCES


40. Redish AD. The hippocampal debate: are we asking the right questions? Beh Brain Res 2001; 127:81-98.


76. Sapolsky RM. A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults. J Neurosci 1985; 5:1228-1232.


105. Gressens PM, Lammens M, Picard JJ, Evrard P. Ethanol-induced disturbances of gliogenesis and neurogenesis in the developing murine brain: an in vitro and

106. Miller MW, Robertson S. Prenatal exposure to ethanol alters the postnatal development and transformation of radial glia to astrocytes in the cortex. J Comp Neurol 1993; 337:252-266.


152. Dragunow M, Robertson HA. Kindling stimulation induces c-Fos protein(s) in granule cells of the rat dentate gyrus. Nature 1987; 329:441-442.


Chapter 2: DNA Fragmentation Factor 45 (DFF45/ICAD) Deficient Mice Exhibit Enhanced Spatial Learning and Memory Compared to Wild-type Control Mice

2.1 ABSTRACT

Programmed cell death or apoptosis is a highly regulated physiological process critical in development particularly in the central nervous system. The DNA fragmentation factor 45 (DFF45 or ICAD) is a subunit of a heterodimeric DNase complex crucial for DNA fragmentation and normal apoptosis. To examine the neurobiological consequences of lacking DNA fragmentation and timely apoptosis during mouse development in vivo, we compared spatial learning behaviors in DFF45 mutant and wild-type control mice. We found that DFF45 mutant mice exhibit enhanced spatial learning and memory compared to wild-type mice. Moreover, both the granule cell density and total granule cell number in the hippocampal dentate gyrus region are higher in the DFF45 mutant brains than in the wild-type brains. We propose that the increase in granule cell number in the dentate region due to the DFF45 mutation changes the neuronal network underlying spatial learning and memory in DFF45 mutant mice.
2.2 INTRODUCTION

Programmed cell death or apoptosis is a highly regulated physiological process critical in development, particularly in the central nervous system. Roughly half of the neurons in mammals undergo apoptosis during normal development\textsuperscript{1}. Neuronal cell death occurs mostly at a time when neurons innervate their targets. The mechanisms for the death of such a large number of neurons during development remain unclear. One hypothesis is that excess number of neurons compete for limited amounts of neurotrophic factors produced by their targets. Only those neurons that are successful in the competition will make connections with the targets and thus survive to participate in the proper wiring and functioning of the nervous system [1-5]. The molecular components of the apoptotic machinery used in neuronal cell death, including Bcl-2 family of proteins, adapter proteins and caspases, appear to be identical to those used elsewhere [6-17]. Most of these proteins are expressed not only during development but also in adults. However, the functional significance of neuronal cell death during development and continued expression of molecules in the cell death machinery in adults remain poorly understood.

DNA fragmentation and chromatin condensation are hallmarks of apoptosis. These processes have been shown to depend critically on a heterodimeric protein complex composed of DNA fragmentation factors 45 and 40 (DFF45 or ICAD and DFF40 or CAD, respectively) [18-22]. DFF45 is ubiquitously expressed during early embryonic development and in adult tissues, including the brain, suggesting an important role of DFF45 throughout mouse development [23]. To understand the in

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vivo function of DFF45, we previously generated DFF45 mutant mice [24]. We found that DFF45 deficient cells exhibit more resistance to apoptosis than wild-type control cells in response to several apoptotic stimuli [23]. These results suggest that DFF45 is essential for normal apoptosis and mutation of DFF45 could produce functional effects in vivo, particularly when timely apoptosis is necessary.

To test this possibility, we determined whether spatial learning and memory might be altered in DFF45 mutant mice. Spatial learning behavior in mammals is believed to depend critically on the proper development of the hippocampus [25]. Neurons in the hippocampus, including granule cells and CA3 and CA1 pyramidal cells, can undergo multiple forms of enduring plastic changes. For example, the strength of synaptic connections in the hippocampus can be enhanced for prolonged periods of time upon a short burst of high frequency stimulation, a process termed long-term potentiation (LTP) [26-27]. Neurotransmitter receptors, cell surface adhesion molecules, intracellular kinases and transcription factors have been shown to affect spatial learning, and in many cases, LTP in rodents [28-35]. To understand the neurobiological consequences of lacking DNA fragmentation and timely apoptosis during mouse development in vivo, we performed the Morris water maze using both DFF45 mutant and wild-type mice. In addition, we measured and compared granule cell density and total granule cell number in the dentate gyrus region in DFF45 mutant and wild-type brains. We found that DFF45 mutant mice exhibit enhanced spatial learning and memory compared to wild-type mice. Moreover, DFF45 mutant mice exhibit higher granule cell density and total granule neurons in the dentate gyrus region than in wild-type mice.
2.3 METHODS

2.3.1 DFF45 mutant mice.

Mice homozygous for the DFF45 gene mutation were produced by crossing heterozygous mutant mice and were identified by Southern blotting [24]. Twelve DFF45 mutant and 10 wild-type F3 mice eight to fourteen weeks of age were used for behavioral analysis. Seven each age-matched DFF45 mutant and wild-type mice with ages similar to those in the behavioral studies were used for histological studies. The genetic background of the mice was 129/Sv x C57BL/6. Equal numbers of male and female mice were used. All mice were single housed with food and water ad libitum in a 12 hour light cycle room. Behavioral testing was performed during the light phase of the light/dark cycle.

2.3.2 The Morris water maze.

Our water maze set up consists of a round pool 1.2 meters in diameter filled with water that is made opaque by adding white tempura paint. The water temperature is kept at 28°C [33]. A 10 x 10 cm platform with a white cover is placed 1 cm below the water. A video camera connected to a computer is mounted from the ceiling directly above the pool. Blue colored paintings with different geometric shapes hang on the walls of the behavioral room serve as distal cues for the hidden platform tests. A black disk that serves as the proximal cue is placed on top of the platform in the visible-platform test and the distal cues are concealed by a black curtain that surrounds the pool.
2.3.3 The water maze protocol.

The water maze test was divided into four phases. Day 1 was a straight channel test in a 15 cm x 150 cm water-filled corridor the water temperature of which was 28°C. The trial is completed when the mouse touches an escape ladder at the opposite end of the straight channel from which it is placed. Four trials were given with 1 minute maximum per trial and escape latencies were recorded. Intertrial intervals were 1 minute each. All mice used were able to perform this test and thus all were used in the Morris water maze test.

Day 2 to 11: This was the spatial learning phase, consisting of an acquisition phase with a hidden platform placed in the southeast (SE) quadrant and probe trials during which the platform was removed. Initial training of mice to climb the platform was done on day 2 before testing. Following training, four trials were given per day with 1 minute maximum per trial. Distributed trials with 1 hour intertrial intervals were used. Following completion of each trial or if a mouse did not find the platform within 1 minute, it stayed or it was placed on the platform for 30 seconds. Starting positions were varied on each trial with each cardinal position being used once each day and the order of starting positions was varied each day. Path length, cumulative distance from the platform and escape latencies were recorded. Cumulative distance from the platform is the sum of the distances of a mouse from the platform measured every 55 milliseconds during each trial and is based on Gallagher et al’s learning index [40]. Probe trials were given at the beginning of day 6 and at the end of day 11 and were for 1 minute. Number of platform site crossings, time spent in
each of the four quadrants and average distance from the platform site were recorded.

Days 12 to 13: This was the random platform phase. On day 12, two trials were given with the platform at the original location followed by two trials with platform at NE and NW locations. On day 13, one trial was given with the platform at the original location and 3 more trials with the platform at NW, SW and NE locations. Number of platform site crossings, escape latencies, path length and cumulative distance again were recorded.

Day 14 to 19: This was the visible-platform phase. A black curtain was pulled around the maze during testing and all lights except the overhead light were turned off. Tests were performed using the same protocol as in the hidden platform except the platform now had a proximal cue which is a black disk mounted on a rod that protruded 14 cm above the platform. Both start and platform position were varied randomly on each trial. Escape latencies were recorded manually.

2.3.4 Statistical analysis of behavioral data.

Straight channel and Morris water maze data were analyzed by split-plot analyses of variance (group by day by trial or group by trial) with day and trial as repeated measure factors. Non-spherical variance-covariance matrices had F-ratios corrected using the Greenhouse-Geisser method. Significant (p < 0.05) interactions were further analyzed for each level of the interacting variable by t-tests for independent samples (2-tailed) after adjustment, where necessary, for homogeneity of variance. All significant interactions were analyzed in detail, but only the one best
representing the overall trend is presented.

2.3.5 Histology, neuronal cell counting and total volume of the granule cell layer of the dentate gyrus.

For histological studies, two age- and sex-matched DFF45 mutant and wild-type mice were sacrificed and their brains frozen. Coronal sections were cut at 10 µm and stained with cresyl violet. For neuronal cell counting and dentate volume measurement, five age- and sex-matched DFF45 mutant and wild-type mice were perfused with 4% paraformaldehyde and their brains post-fixed, cryoprotected, and cut at 16 µm and 25 µm coronally.

For estimation of cell density, four representative 16 µm hippocampal sections from each brain approximately 550 µm apart were stained with cresyl violet and counted. A 60X oil objective with a numerical aperture of 1.4 was used at 1.5X magnification. A grid was used to count cells within a 1296 µm² area through the 16 µm depth (18 X 72 X 16 sampling volume) of the section for the dentate region. For the CA1 and CA3 regions, a 1620 µm² area (18 X 90 X 16 sampling volume) was counted. Five samplings were taken per section per region and five each DFF45 mutant and wild-type mice were used.

Total volume of the granule cell layer of the dentate gyrus was measured using the Cavalieri method [41-44]. Briefly, 25 µm sections from perfused brains were stained with cresyl violet. Ten successive sections from each brain, each 200 µm apart, were photographed with a 2X objective at 1.25X ocular magnification. A 60 X 60 grid was overlaid on each image using Metamorph and the number of grid points
overlapping the granule cell layer were counted. Total volume was then estimated using the following formula: \( V = t \times a(p) \times P \), where \( V \) = total volume per brain; \( t = \) distance between successive sections (200 \( \mu \)m), \( a(p) = \) area associated with each grid point (4123.7 \( \mu \)m\(^2\) as measured and calculated using a micrometer), and \( P = \) total number of points counted per animal. Two-tailed t-tests were used to analyze neuronal cell density, total volume and total granule cell number results from DFF45 mutant and wild-type control brains.

2.4 RESULTS

2.4.1 DFF45 mutant mice exhibit enhanced spatial learning and memory compared to wild-type mice

The hidden-platform version of the Morris water maze tests for spatial learning as mice learn the relative location of the platform through distal cues in the room surrounding the pool [28]. Both DFF45 mutant and wild-type mice showed improvements in their ability to find the platform since both groups of mice showed decreases in mean path length, mean cumulative distance from the platform and mean escape latency with more training (Figure 1). However, DFF45 mutant mice exhibited significantly shorter mean path length and shorter mean cumulative distance from the platform than wild-type mice on days 2, 3 and 11 (Figure 1A and 1B). Moreover, there was no difference between the two groups of mice in mean path length, mean cumulative distance from the platform and mean escape latency.
for the first two trials on day 2, indicating that DFF45 mutant mice do not have inherent performance advantages compared to wild-type mice. The difference between the two groups of mice was due to a difference in trials 3 (p < 0.05) and 4 (p < 0.01) on day 2. Therefore, even though there was no significant difference in the mean escape latencies between the two groups of mice overall in hidden platform performance (Figure 1C), these results suggest that DFF45 mutant mice exhibit moderately but significantly enhanced spatial learning and memory compared to wild-type mice.

An analysis of the improvement in hidden platform performance across days further shows the enhancement of learning in the DFF45 mutant mice. Performance for both groups of mice improved significantly as a function of day (Figure 1A and 1B). However, analyses of mean path length and mean cumulative distance from the platform for wild-type mice showed that day 2 differed significantly from all subsequent days and day 3 differed significantly from day 2 and all subsequent trials, but that days 4-6 did not differ from one another, nor did days 5-11. By contrast, for DFF45 mutant mice only day 2 differed from all subsequent days, whereas days 3-5 did not differ from one another, nor did days 6-10 or 8-11. Therefore, based on rate of learning, DFF45 mutant mice approached asymptotic performance 50% faster than did wild-type mice.

To test further for enhanced spatial learning and memory in DFF45 mutant mice, we performed probe trials at the beginning of day 6 and at the end of day 11. Probe trials are thought to be better indicators of spatial learning and memory. As shown in Figure 2, DFF45 mutant mice crossed the original SE platform site significantly
more than the other three equivalent platform sites on day 6. In comparison, wild-type mice crossed the original SE platform site significantly more than only the NW equivalent site on day 6. Both groups of mice crossed the original SE platform site significantly more than the other three equivalent platform sites on day 11. Moreover, both DFF45 mutant and wild-type mice showed improved performance in crossing the original SE platform site on day 11 compared to those on day 6. These results indicate that both groups of mice learned the platform site gradually presumably using distal cues with more training. Most importantly, DFF45 mutant mice had a significantly higher number of SE platform site crossings than wild-type mice on both days, suggesting that the mutant mice exhibit better spatial learning and memory than wild-type mice.

To strengthen this argument further, we performed a random platform test. As shown in Figure 3, DFF45 mutant mice also exhibited significantly more crossings of the original platform site than the wild-type mice. The mean path length and mean cumulative distance from the platform were significantly different between DFF45 mutant and wild-type mice when the platform was in the original SE location and they were not significantly different between the two groups of mice when the platform was in random positions (data not shown). As in the hidden platform trials, there was no significant difference in mean escape latencies between DFF45 mutant and wild-type mice when the platform was in either the original location or in random locations. Both groups of mice showed shorter escape latencies when the platform was in the original position than when the platform was in random positions (data not shown). The significant difference between DFF45 mutant and wild-type mice in
platform crossing, mean path length and mean cumulative distance from the platform suggest further that DFF45 mutant mice exhibit enhanced spatial learning and memory compared to wild-type mice.

2.4.2 DFF45 mutant mice do not differ significantly from wild-type mice in swimming speed and visible platform performance

To test for their swimming performance, all mice were tested on day 1 in a straight channel in which latency to escape was used as an index prior to Morris water maze trials. In the absence of directional options, this task requires minimal learning. DFF45 mutant and wild-type mice performed this task similarly and there was no significant difference in escape latencies between the two groups of mice (Figure 4A). To control further for their ability to perform the water maze test, we performed a visible-platform test. DFF45 mutant mice and wild-type mice had similar mean escape latencies in visible platform performance during the last 5 days of test and only on day 1 did the mutant mice have shorter mean escape latencies than wild-type mice (Figure 4B). The shorter mean escape latencies exhibited by the mutant mice in the first day of visible platform test were due to trials 1 (p < 0.01) and 2 (p < 0.05) but not trials 3 and 4.

2.4.3 DFF45 mutant mice exhibit apparently normal central nervous system development

We previously showed that DFF45 mutant mice are more resistant to cell death than wild-type mice upon activation of apoptosis. This resistance may result in altered
central nervous system development including that in the hippocampus. To examine this possibility, we performed a systematic comparison of brains from age-matched DFF45 mutant and wild-type mice. Every tenth coronal brain section was stained with cresyl violet. As shown in Figure 5, there is no obvious difference between the DFF45 mutant and wild-type mice in the brain in general (Figure 5A and 5B) and in the hippocampal regions in particular (Figure 5C-F).

2.3.4 DFF45 mutant mice exhibit higher granule cell density and total granule neurons in the dentate gyrus region than wild-type mice

Even though DFF45 mutant mice exhibit apparently normal central nervous system development, it is possible that because of the resistance to cell death during neuronal development or in adults, there are more neurons in the hippocampus of DFF45 mutant mice than in wild-type mice. To investigate this possibility, we counted neuronal cell density and measured total volume particularly in the dentate gyrus region in DFF45 mutant and wild-type mice. As shown in Figure 6A, DFF45 mutant mice exhibit a significantly higher mean granule cell density in the dentate region of the hippocampus than in wild-type mice. There is no significant difference in mean neuronal cell densities in either the CA3 or CA1 regions between the DFF45 mutant and wild-type mice (data not shown). The mean total volumes of the granule cell layer are very similar between the DFF45 mutant and wild-type brains (Figure 6B). Thus, the mean total number of granule neurons in the DFF45 mutant mice (464,000) are higher than in the wild-type mice (428,000) due to the higher granule cell density (Figure 6C).
2.5 DISCUSSION

To examine the neurobiological consequences of lacking timely DNA fragmentation and apoptosis during mouse development in vivo, we evaluated and compared Morris water maze performance of DFF45 mutant and wild-type mice. In the hidden platform test, we found that the DFF45 mutant mice took shorter paths to reach the platform and had shorter cumulative distances from the platform during the first two days and the last day. Importantly, there was no difference in mean path length, mean cumulative distance from the platform and mean escape latency for the first two trials between the two groups of mice during the first four trials on day 2, indicating equal baselines. The difference between the two groups of mice on the first day was due to a difference in trials 3 and 4. These results suggest that DFF45 mutant mice navigate using presumably distal cues more efficiently than wild-type mice during that period of training. DFF45 mutant mice also displayed a shorter mean escape latency during the first 2 days and the last day compared to wild-type mice although this difference was not significant. Apparently DFF45 mutant mice learn to swim more directly to the platform but not at the identical speed as wild-type mice hence control mice swim farther in the same time.

The probe trial results indicate that, in the absence of the platform, DFF45 mutant mice crossed the exact location where the platform had been significantly more frequently than wild-type mice. This enhanced memory performance was very precise, as it was reflected in site crossings, but not in the more general measure of
time or distance spent in the target quadrant as a whole. This result suggests strongly that DFF45 mutant mice learned and remembered the original platform site better than wild-type mice. In the random probe trials in which the platform is moved unpredictably to a new location, DFF45 mutant mice also crossed the original platform site significantly more often than wild-type mice, suggesting that they were checking and rechecking, with high spatial precision, for its reappearance in the original location. This is consistent with the fact that DFF45 mutant mice exhibit enhanced hidden platform searching ability only selectively for the original platform site. Together, our results suggest that DFF45 mutant mice exhibit enhanced spatial learning and memory compared to wild-type mice.

Despite the greater number of site crossings by the DFF45 mutant mice, these mice did not show a reduction in mean cumulative distance from the platform site on probe trials. This result suggests that even though they knew the exact location of the platform better than wild-type mice, DFF45 mutant mice did not remain close to that site between crossings, but rather swam away and then returned. It is possible that DFF45 mutant mice may follow a check-explore-recheck strategy that is efficient yet ready for changes in the reinforcement contingencies that may arise. DFF45 mutant mice unexpectedly also found the platform faster on the first day of the visible platform trials. This may reflect that DFF45 mutant mice detect and use salient cues better than wild type mice, at least until both groups of mice reach optimal performance, which they do by trial 3 on day 1. Alternatively, this may reflect an order effect since all the mice were previously trained in the hidden platform test and the platform was in the original SE position in the first trial of the
visible platform test. Lastly, in pre-maze trials in a straight swimming channel, DFF45 mutant mice performed comparably to wild-type mice. Thus, the enhanced learning in the DFF45 mutant mice cannot be explained in terms of faster speed of swimming.

To examine the possible reasons for the enhanced spatial learning and memory capacity of the DFF45 mutant mice, we determined granule cell density and total granule cell number in the hippocampus. We found that there is an 8% increase in granule cell number in the DFF45 mutant mice than in wild-type mice. This increase is presumably due to a resistance to cell death in DFF45 mutant mice. The neuronal densities in the CA3 and CA1 regions are not significantly different between DFF45 mutant and wild-type mice, although whether this is also the case for the total volumes of these two hippocampal subregions still needs to be determined.

Increased neuronal numbers can correlate with enhanced learning and memory. Mice transgenic for the bcl-2 gene exhibit enhanced learning ability and have increased cell number in the brain due to a blockade for apoptosis [36]. In addition, mice with more neurons in the dentate gyrus region of the hippocampus due to living in an enriched environment perform better than the control mice in the hidden platform test [37]. Further experiments are needed to determine whether the increase in granule cell number in DFF45 mutant mice is due to resistance to apoptosis during development or in adulthood [37-39].

Although the increase in granule cell number is relatively small, this increase could well influence the neuronal network underlying spatial learning and memory. The increase in granule cells in the DFF45 mutant mice could be accompanied by
an increase in the number of synapses both from entorhinal cortex to dentate and from dentate to the CA3 region in the hippocampus. Alternatively, the increase in granule cells could change how neurons are connected in the hippocampus. The increase in granule cells could also influence how neurons process signals and synaptic strength. All these changes could result in higher spatial learning capacity. Future studies will be directed to addressing these issues. Since neuronal cell death could contribute to the loss of memory during aging, it will be interesting to investigate whether the enhanced learning and memory capacity in the DFF45 mutant mice can be sustained when they age.
**Figure 1.** DFF45 mutant mice exhibit shorter mean path length and shorter mean cumulative distance from the hidden platform than wild-type mice on days 2, 3 and 11. Mean path length ± SEM (A), mean cumulative distance from the platform ± SEM (B), and mean escape latencies ± SEM for the hidden platforms (C) for each day with four trials per day are shown. For path length, a group by day by trial ANOVA with day and trial as repeated measures factors showed effects of group (F1,20 = 6.78, p < 0.02) and group by day (F9,180 = 5.72, p < 0.01). A posteriori t-tests were done on each day. Similar analysis for cumulative distance showed effects of group (F1,20 = 6.32, p < 0.03) and group by day (F9,180 = 7.70, p < 0.01), and for latency showed no significant group-related effects. *p < 0.05, **p < 0.01.
Figure 2. DFF45 mutant mice exhibit more original platform site crossings than wild-type mice in probe trial performance. Mean ± SEM platform site crossings were plotted versus the equivalent locations in the other three quadrants. A group by trial ANOVA with trial a repeated measures factor showed an effect of group (F1,20 = 6.22, p<0.03). Trial was significant (p<0.0001), but the group by trial interaction was not. *p<0.03 compared to wild-type SE crossings averaged across day 6 and day 11. +p < 0.05 compared to the other three platform site crossings within genotype and test day. Analysis of percent time in the target quadrant and average distance from platform showed no effects of group.
**Figure 3.** DFF45 mutant mice exhibit more crossings for the original platform site than wild-type mice in random platform performance. Mean $\pm$ SEM original platform site crossings were plotted. A group by trial ANOVA with trial a repeated measures factor showed an effect of group ($F_{1,20} = 8.82, p < 0.01$) and of trial ($p<0.0001$) but no group by trial interaction. There were no significant differences in mean escape latencies, path length and cumulative distance between the two groups of mice. **p<0.01 compared to wild-type.**
Figure 4. DFF45 mutant mice do not differ from wild-type mice in swimming speed and visible platform performance. Mean ± SEM escape latencies for the straight channel (A) and visible platforms (B) for each day with four trials per day are shown. A group by day by trial ANOVA with day and trial as repeated measures factors showed a group by day effect (F5,100 = 4.39, p < 0.04). The main effects of day and of trial were significant in all analyses (p < 0.01 to p < 0.0001). *p < 0.05.
Figure 5. Lack of obvious developmental deficiencies in the brains of DFF45 mutant mice. Wild-type (A, C,E) and DFF45 mutant (B, D,F) mouse brains (n = 2 each) were processed, sectioned at 10 µm coronally and stained with cresyl violet. Scale bars are 1 mm (A-F).
Figure 6. DFF45 mutant mice exhibit increased granule cell density and number in the dentate gyrus region of the hippocampus than wild-type mice. Mean + SEM granule cell density (A), volume (B) and cell numbers (C) in the dentate regions of hippocampi of DFF45 mutant and wild-type mice (n=5 each) were plotted. There is a significant difference between the granule cell density and total granule cell number (*p < 0.05) but not granule cell layer volume (p=0.693) between the DFF45 mutant and wild-type mice. White and black bars represent results from the wild-type mice and DFF45 mutant mice respectively.
REFERENCES


2. Raff MC, Barnes BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD.

3. Henderson CE. Programmed cell death in the developing nervous system.


7. Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS.
   Development 1994; 120:301-311.


33. Silva AJ, Paylor R, Wehner JM, Tonegawa S. Impaired spatial learning in α-

34. Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER. Impaired long-
term potentiation, spatial learning, and hippocampal development in fyn mutant

long-term memory in mice with a targeted mutation of the cAMP-responsive

36. Coleman GJ, Bernard CC, Bernard O. Bcl-2 transgenic mice with increased
number of neurons have a greater learning capacity. Brain Res 1999; 832:188-
194.

37. Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice


39. Van Praag H, Kempermann G, Gage FH. Running increases cell proliferation
and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 1999; 2:266-
270.

40. Gallagher M, Burwell R, Burchinal M. Severity of spatial learning impairment in
aging: development of a learning index for performance in the Morris water

41. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K,
Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al. Some new,

42. West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Record 1991; 231:482-497.


Chapter 3: DNA fragmentation factor 45 knockout mice exhibit longer memory retention in the novel object recognition task compared to wild-type mice

3.1 ABSTRACT

Apoptosis is an important process in the development and function of the central nervous system (CNS). To study the role of DNA fragmentation factor 45 (DFF45/ICAD) in CNS function, we previously generated DFF45 knockout mice. We found that whereas they exhibit apparently normal CNS development, DFF45 knockout mice exhibit an increased number of granule cells in the dentate gyrus and enhanced spatial learning and memory compared to wild-type mice in a Morris water maze test. In this study, we examined the performance of the DFF45 knockout mice in a novel object recognition task to measure short-term non-spatial memory that is believed to depend on the hippocampal formation. Both wild-type and DFF45 knockout mice exhibited novel object recognition 1 hour post-training. However, whereas wild-type mice no longer did so, DFF45 knockout mice were still able to differentiate the novel versus the familiar object 3 hours post-training. The longer memory retention in DFF45 knockout mice did not last to 24 hours as neither wild-type nor DFF45 knockout mice demonstrated novel object recognition 24 hours post-training. These results suggest that a lack of DFF45 facilitates hippocampus-dependent non-spatial memory as well as hippocampus-dependent spatial memory.
3.2 INTRODUCTION

Apoptosis is a highly regulated physiological process that is important in development and function of the CNS [1-4] and also plays a critical role in neurodegeneration [5]. DFF45 is a subunit of the heterodimeric DNase complex that is necessary for DNA fragmentation and normal apoptotic cell death [6-8]. In the DFF45 knockout mouse, DNA fragmentation is lessened and normal apoptotic cell death is attenuated [9-12]. Although general development, including that in the CNS, appears to be normal, the loss of DFF45 may affect homeostasis in specific brain regions. Indeed, our previous studies showed that the dentate gyrus region of the mutant mouse has a greater number of granule cells compared to the wild-type mouse [13], indicating that DFF45 plays a role in determining neuronal cell number in the dentate gyrus.

The hippocampal formation is a brain area important for learning and memory [14]. In adult mice, neurogenesis and apoptosis occur in the dentate gyrus region. Changes in cell numbers in the dentate gyrus may affect functions of perforant path and/or mossy fiber projections and therefore impact learning and memory. Mice living in an enriched environment possess more dentate gyrus neurons and perform better in Morris water maze tests than control mice [15]. We previously showed that DFF45 knockout mice exhibit enhanced learning and memory as compared to their wild-type littermates when tested in a spatial, hippocampal-dependent, test the Morris water maze [13]. To further understand the role of DFF45 in hippocampal functions, we examined non-spatial hippocampal-dependent short-term memory in DFF45 knockout mice using a
novel object recognition task [16-24]. This task does not depend on the motivation to seek food after food deprivation or to avoid aversive conditions such as to escape from water or bright light. We found that whereas DFF45 knockout mice exhibit normal investigation of unfamiliar objects, these mice exhibit longer memory retention in the novel object recognition test compared to wild-type mice. Longer retention in novel object recognition task occurred in DFF45 knockout mice habituated to the test setting only at 3 hours post-training with similar performance at 1 or 24 hours post-training compared to the wild-type control mice.

3.3 METHODS

3.3.1 DFF45 knockout Mice

An animal use protocol was approved by the Institutional Animal Care and Use Committee, and we followed the guidelines of the NIH for the care and use of laboratory animals. All mice used in the study were bred in-house. Mice were allowed free access to food and water. The animals were housed with a 12:12 hour light: dark cycle with lights turning on at 6:00 am. Heterozygote mating was maintained to generate wild-type and DFF45 knockout mice. The genotypes of the offspring were identified by genomic Southern [9]. The eventual mice used in the experiments were from several independent litters of breeding from the identified wild-type and DFF45 knockout mice. The genetic background of these mice and the wild-type controls is 129Sv x C57BL/6. Separate groups of 14 mice
of each genotype were used for each test interval of the novel object recognition test. In each group 7 mice were males and 7 were females. The mice were approximately 12 weeks old. All testing was carried out during the light phase of the light: dark cycle.

3.3.2 The novel object recognition test

The novel object recognition test was performed using plastic chambers (41 x 41 x 30 cm) in low light conditions during the light phase of the light-dark cycle. All mice were handled every other day from the time of weaning until the time of testing at approximately 9 weeks of age. All mice used had similar general appearances.

The test was divided into three phases. The first phase was habituation during which all mice were placed in a chamber for 10 minutes. The second phase was the acquisition phase and was run on the following day. During this phase, all mice were placed in the chamber for 5 minutes with two identical objects placed 5 cm from the walls in opposite corners. The objects used were two stacked jumbo Legos or two orange fishing bobs glued together. The object not used in the acquisition phase was used as the novel object in the recognition phase. The choice of object for novel and familiar was counterbalanced. All objects were washed with 70% ethanol between trials. The objects were attached to the floor using Velcro. A circle with a 2.5 cm radius was drawn around the object for scoring purposes. The third phase is the retention testing phase during which the mice were divided into three separate groups to test for retention of memory
at one of the three time points, i.e., 1, 3 or 24 hours post-acquisition. Separate
groups of mice were used for each retention interval. The same chambers were
used for each phase of the test. The retention testing phase also lasted for 5
minutes. During both acquisition and retention testing phases, the mice were
videotaped and these tapes were later scored. Time spent exploring each object
was recorded.

Although not blind to the genotype when the age-matched DFF45
knockout and wild-type mice were selected for the experiments, the experimenter
numbered each mouse before the experiments and videotaped each individual
performance. Then the experimenter scored the mouse behaviors by watching
the videotapes according to their numbers after the experiments. A large number
of mice were used in this study. Data analysis and summary were performed
after the experiments. All these factors made the experimenter bias minimal.

3.3.3 Data analysis

Exploration of an object was defined as the mouse having its front paws within
the scoring circle and its body oriented toward the object. Total exploration time
was obtained by adding the time spent exploring the two objects. This measure
was used to allow evaluation of the overall performance of DFF45 knockout and
wild-type mice in the task. The percent time spent exploring the novel object was
calculated by dividing the amount of time spent exploring the novel object by the
total time spent exploring both objects. This index reflects the recognition of the
familiar object. For the acquisition phase, the percent time spent exploring object
A was calculated as the time spent exploring the object in the position where the novel object was placed divided by the time spent exploring both objects. This number was transformed for statistical analysis due to the ratio nature of the data by a square-root transformation for each animal and the resulting number was used for statistical purposes. The experiments were counterbalanced for the position of the novel object, and for the selection of object as the original or the novel object. Statistical significance was determined on transformed data, but untransformed data are presented for illustrative purposes. Data comparing genotypes on acquisition and on retention were analyzed separately using t-tests for independent samples (two-tailed). Data comparing each genotype to chance performance (50%) was analyzed using one-sample t-tests (two-tailed).

### 3.4 RESULTS

During the acquisition phase, all groups of mice, regardless of genotype, spent equal amounts of time exploring either of the two objects (Figure 1A, 1 hour: \( t = 0.41, \text{df} = 26, p = 0.68 \); 3 hour: \( t = 0.72, \text{df} = 26, p = 0.48 \); 24 hour: \( t = 0.37, \text{df} = 26, p = 0.71 \)). In addition, total exploration time was not different between wild-type and DFF45 knockout mice (Figure 1B, all t-tests not significant, \( p < 0.05 \)). These observations indicate that DFF45 knockout mice and wild-type mice have similar sensory motor function, motivation, curiosity and interest in exploring novel objects.
Separate groups of mice were tested for recognition at the 1, 3, and 24 hour post-acquisition intervals. For the short-term 1 hour interval groups, both genotypes showed recognition of the familiar object and spent three times as much time exploring the novel object as the familiar object, indicating that mice with both genotypes possess comparable short-term memory function (Figure 2A, acquisition versus retention ratios for wild-type mice: $t = 4.75$, df = 26, $p < 0.0001$; for DFF45 knockout mice: $t = 6.05$, df = 26, $p < 0.0001$). For the intermediate-term 3 hour interval groups, the wild-type mice spent equal amounts of time exploring each object, showing no recognition of the object that the mice had been exposed to (acquisition versus retention ratios $t = 0.25$, df = 26, $p = 0.80$). In contrast, the DFF45 knockout mice spent significantly more time exploring the novel object than the familiar object (acquisition versus retention ratios $t = 3.53$, df = 26, $p = 0.0016$), showing retention of memory at this time interval (Figure 2A). Importantly, both wild-type and DFF45 knockout mice spent less time exploring the familiar object at 1 hour retention testing compared to that at the acquisition phase (wild-type: 18 seconds in acquisition, 12 seconds in 1 hour retention testing; DFF45 knockout: 20 seconds in acquisition, 13.5 seconds in 1 hour retention testing), whereas only DFF45 knockout mice spent less time exploring the familiar object at 3 hour retention testing compared to that at the acquisition phase (23 seconds in acquisition, 19 seconds in 3 hour retention testing). These results indicate a longer retention memory in DFF45 knockout mice compared to the wild-type mice. For the long-term 24 hour interval groups, both DFF45 knockout mice and wild-type mice spent equal amounts of time
exploring each object, showing no recognition of the familiar object (Figure 2A, acquisition versus retention ratios for wild-type mice: $t = 1.19$, df = 26, $p = 0.24$; for DFF45 knockout mice: $t = 0.32$, df = 26, $p = 0.75$). No differences in total exploration time in the retention phase of the novel object recognition task between DFF45 knockout and wild-type mice at any of the post-acquisition intervals were observed (Figure 2B, $p>0.05$). We did not observe any differences between male and female mice in either genotype at any of the intervals in total exploration time or in novel object recognition in either the acquisition or the retention testing phase of the novel object recognition task (Table 1).

To ensure the validity of the results, we also ran one sample t-tests comparing each result to chance. Using this test both genotypes still showed significant differences in exploration of the novel object at the one hour time point (wild-type mice: $t = 5.18$, df = 14, $p<0.01$; DFF45 knockout mice: $t = 10.2$, df = 14, $p<0.01$). At the 3 hour time point, only the DFF45 knockout mice continued to show significant differences in novel object exploration (wild-type mice: $t = 0.26$, df = 14, $p>0.05$; DFF45 knockout mice: $t = 4.29$, df = 14, $p<0.01$). At the 24 hour time point, neither group showed significant differences in exploration of the novel object (wild-type mice: $t = 1.31$, df = 14, $p>0.05$; DFF45 knockout mice: $t = 0.48$, df = 14, $p>0.05$). These tests as compared to chance supported the previous results.
3.5 DISCUSSION

Eliminating apoptotic nuclease activity by mutating the *DFF45* gene in mice results in animals exhibiting an increased number of granule cells in the dentate gyrus and enhanced spatial learning and memory in the Morris water maze test compared to wild-type mice [13]. To understand further the impact of resistance to DNA fragmentation and apoptosis on CNS function, we used a novel object recognition task to examine the short-term non-spatial memory ability of the *DFF45* knockout mice. Most evidence suggests that novel object recognition is associated with the function of the hippocampal formation and cortical regions in humans, monkeys and rodents [16-28]. For example, a direct lesion of the hippocampus by radio frequency or ibotenic acid reliably induces impairments of novel object recognition in rats [17], suggesting that the hippocampal formation plays a critical role in novel object recognition memory. We found that all mice recognized the familiar object 1-hour post-training and no longer recognized the familiar object 24 hours post-training. However, at the intermediate, 3-hour post-training interval, the differential effect of the *DFF45* gene disruption is evident. The absence of recognition of the novel object by both groups at 24 hours is apparently the result of the mice not exploring the object sufficiently to form a longer lasting memory. Such problems have been circumvented by setting a minimum object exploration period during the familiarization phase of testing [17]. Even when this is done, novel object preference at 24 hours is no better than 58%. The failure of even wild-type mice to prefer the novel object at 24 hours in our study merely shows that this interval was too long to be effective for the
amount of initial object familiarization, but does not detract from the finding that DFF45 knockout mice exhibit longer retention at 3 hours when preference is at an intermediate stage of decay. Importantly, both DFF45 knockout and wild-type mice spent similar amounts of total time exploring the objects. These results, together with our previous findings that DFF45 knockout mice exhibit apparently normal CNS development, argues that the longer retention of memory in the novel object recognition task in DFF45 knockout mice compared to the wild-type mice is not due to non-specific effects such as changes in sensorimotor function or motivation.

We used mice with a mixed genetic background in our study. Whereas future work with congenic strains of mice will further clarify the contributions of genetic background to novel object recognition performance, the clear differences between wild-type and DFF45 knockout mice in novel object recognition test, and our previous finding that DFF45 knockout mice also showed enhanced spatial learning and memory, argue that the better novel object recognition performance in DFF45 knockout mice is due to the DFF45 gene mutation rather than an effect of the genetic background.

Genetic, behavioral and pharmacological studies have demonstrated that perturbing any of many cellular functions can elicit learning and memory deficits [16-24,27,29-31]. In contrast, only a limited number of studies showed enhanced learning and memory by changing neurotransmitter receptor composition and intracellular signal transduction [20,24,32]. Interestingly, mice with an increased cell number in the hippocampal formation have also been previously shown to
exhibit enhanced hippocampus-dependent learning and memory ability in the Morris water and Hebb-Williams mazes [13,15,33]. Our results demonstrate that mice with compromised cell death machinery exhibit longer retention in novel object recognition task memory. Since DFF45 knockout mice appear to develop normally, yet exhibit an increased neuron number in the dentate gyrus as adults, DFF45 may be important for cell death in the adult brain as opposed to the developing brain. Further studies into the mechanisms of the enhanced memory abilities of the DFF45 knockout mice will include histological and electrophysiological studies of the perforant and mossy fiber connections into and out of the dentate gyrus. Moreover, cellular morphological studies of other brain regions, including the rhinal cortices that have also been implicated in novel object recognition memory [34,35], may also reveal whether additional brain regions contribute to the enhanced memory in DFF45 knockout mice. Future studies with hippocampal-specific, inducible and reversible DFF45 knockout mice may further establish if memory storage and consolidation can be affected by proper apoptosis as well as by neurotransmitter receptors and intracellular signal transduction molecules [20,36,37]. Finally, hippocampal-specific and inducible DFF45 knockout mice will help to determine whether the hippocampus is a more general memory structure that is important for both spatial and non-spatial memory or whether it is primarily a spatial processing organ in the brain.
Figure 1. DFF45 knockout mice exhibit A) normal acquisition of object recognition and B) normal total object exploration time during the acquisition phase of the novel object recognition test compared to wild-type mice. Values represent percent exploration time of one of the objects versus the total object exploration time + SEM. N=14 for each group of mice. Open bar, wild-type mice. Closed bar, DFF45 knockout mice. Separate groups of mice were used for the 1, 3, 24 hour retention intervals. p>0.05 in percent time spent exploring object A, total exploration time among groups and between genotypes.
Figure 2. DFF45 knockout mice exhibit A) longer retention of novel object recognition memory and B) normal total object exploration time in the retention phase of the novel object recognition test compared to wild-type mice. Values represent percent exploration time of the novel object + SEM 1, 3 or 24 hours after acquisition. N=14 for each group of mice. Open bar, wild-type mice. Closed bar, DFF45 knockout mice. Separate groups of mice were used for the 1, 3, 24 hour retention intervals. * p<0.05 between percent time spent exploring novel object in retention testing phase and the acquisition phase of the same group of mice. † p<0.05 between wild-type and DFF45 knockout mice in the 3 hour post-training group (t = 3.032, df = 26, p = 0.0054).
Table 1. No difference in novel object recognition task performance between male and female mice in wild-type and DFF45 knockout mice. Values represent percent exploration time of one of the objects versus the total object exploration time ± SEM for acquisition test, and percent exploration time of the novel object ± SEM for retention test. Separate groups of mice were used for the 1, 3, 24 hour retention intervals. Seven each male and female mice were in each group at each test interval.

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REFERENCES


33. Coleman GJ, Bernard CC, Bernard O. Bcl-2 transgenic mice with increased number of neurons have a greater learning capacity. Brain Res 1999; 832:188-194.


Chapter 4: c-fos regulates neuronal excitability and survival

4.1 ABSTRACT

Excitotoxicity is a process in which glutamate or other excitatory amino acids induce neuronal cell death. Emerging evidence suggests that the immediate early gene c-fos plays a key role in regulating neuronal cell survival versus death. To address whether and how c-fos is involved in neuronal excitotoxicity, we generated a mouse in which the c-fos expression is eliminated in the hippocampus. We found that these mutant mice exhibit increased kainic acid-induced seizure severity, neuronal excitability and neuronal cell death compared to control mice. Moreover, c-Fos regulates AP-1 transcription complex formation in the hippocampus both before and after the KA exposure. c-fos also regulates GluR6 and BDNF expression both in vivo and in vitro. Our results suggest that c-fos is a key regulator for cellular mechanisms mediating neuronal excitability and survival.
4.2 INTRODUCTION

Excitotoxicity is a process in which glutamate or other excitatory amino acids induce neuronal cell death in the central nervous system. Accumulating evidence suggests that excitotoxicity may contribute to the pathogenesis of human neuronal cell loss by sustained epilepsy, trauma, ischemia and hypoglycemia. Excitotoxicity may also underlie certain neurodegenerative diseases such as Huntington’s disease and amyotrophic lateral sclerosis [1-5]. Mechanistic studies suggest that excessive stimulation of neuronal ionotropic glutamate receptors (GluRs), including the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainic acid (KA) GluRs, produces neuronal cell death by increasing intracellular Ca$^{2+}$ and Na$^+$, and consequently drastically changing normal cellular physiology [3-5]. Although the activation of these GluRs is a key step leading to neuronal degeneration, poorly defined post-receptor mechanisms are also crucial for excitotoxicity [3-5].

c-fos is an immediate early gene (IEG) that encodes a transcription factor [6-9]. The c-Fos proteins can form heterodimers with Jun family proteins and the resulting AP-1 transcription complexes can bind to the AP-1 site, TGACTCA, a transcriptional regulatory sequence found in a large number of cellular genes [10-12]. c-fos can be rapidly activated by a variety of stimuli, ranging from neurotransmitters to neurotrophins both in vitro and in vivo [13-18]. c-fos is also expressed during central nervous system development [19]. Although the exact role of c-fos in brain function remains unanswered, it is thought that c-fos,
together with c-jun or other IEGs, plays a key role in coupling short-term stimuli received at the cell surface to long-term neuroplastic changes via regulating gene expression [8,18,20,21].

The unique properties of c-Fos, being a transcription factor and being able to be rapidly induced by a variety of stimuli, suggest that it is a key molecule to participate in mechanisms underlying neuronal plasticity in general and excitotoxicity in particular. c-fos is continuously expressed before neuronal cell death [22], suggesting an active role of c-fos in neuronal survival or death. The role of c-fos in this balance of life versus death is highly dependent on environmental stimuli and/or cell type [23,24]. Moreover, the c-Fos and c-Jun family proteins, various combinations of AP-1 heterodimers, and regulation of AP-1 complex activity by Jun kinases (Jnk) can all influence neuronal survival, death and plasticity [25-29]. c-fos is also induced by neuronal activity, such as KA-induced seizures [30,31], and has been widely used as a marker to reflect neuronal activity. However, whether and how c-fos is involved in neuronal excitotoxicity remains unknown.

Genetically engineered mice are particularly good models to study the molecular basis of neuronal excitotoxicity. Standard c-fos mutant mice have been used for evaluating the role of c-fos in neuronal responses in a kindling model [32]. However, since the mutation occurs early and in every cell during development, 60% of c-fos mutant mice die soon after birth with only 40% surviving to adulthood [33,34]. Moreover, those mutant mice who do survive develop osteoporosis and defects in lymphogenesis as well as gametogenesis.
These severe developmental deficiencies and the widespread mutation make the analysis of c-fos function difficult [35]. To overcome this problem and to precisely determine the role of c-fos in neuronal excitotoxicity, we used the loxP/cre system to generate mice with a c-fos mutation in the hippocampus. We found that c-fos expression is eliminated in the hippocampus and is significantly reduced in the dentate gyrus. Though apparently normal developmentally, the mutant mice show an increase in KA-induced seizure severity, neuronal excitability and cell death compared to control mice. Moreover, c-Fos regulates AP-1 transcription complex formation in the hippocampus both before and after the KA exposure. c-fos also regulates GluR6 and BDNF expression both in vivo and in vitro. Our results suggest that c-fos is a key regulator for cellular mechanisms involving neuronal excitability and survival.

4.3 METHODS

4.3.1 Generation of f/fc-fos-cre Mice

A mouse c-fos cDNA was used to clone the genomic DNA sequence from a 129 mouse genomic library. A loxP-c-fos-loxP gene targeting construct with two loxP sites flanking the third and fourth exons of c-fos was made through a series of subcloning steps. The loxP sites consisting of 5'-TATAACTTCGTATAATGTATGCTATACGAAGTTATC were generated by oligonucleotide synthesis. One loxP site was inserted into the BglII site in the
second intron of c-fos. The other loxP site was cloned 3’ of the PGK-neo gene and the neo-loxP sequence was subcloned into the KpnI site 2.5 kilobase pairs (kb) downstream of the fourth exon of c-fos. The two loxP sites flanked a 3.5 kb sequence consisting of the third and fourth exons of c-fos. The targeting vector contained 7 kb of homologous DNA upstream of the first loxP site and 4 kb of homologous DNA downstream of the second loxP site.

To obtain homologous recombinants, we transfected mouse J1 embryonic stem (ES) cells with 70 µg of linearized targeting construct by electroporation using a Bio-Rad Gene Pulser [36]. G418 selection was applied 24 hours after the transfection at 200 µg/ml. G418-resistant colonies were picked and genomic DNA samples were isolated and digested with BglII and hybridized with a 3’-probe. Candidate homologous recombinants were confirmed by digesting their genomic DNA with SpeI/ClaI and hybridizing with a 5’-probe.

ES cells amplified from 4 of the homologous recombinants were injected into blastocysts from C57BL/6 female mice. The injected blastocysts were implanted into the uteri of B6xDBA2 F1 females. The resulting male chimeric mice were bred with C57BL/6 females, and germ-line transmission was identified initially by screening for agouti offspring. Mice heterozygous for the loxP-c-fos-loxP homologous recombination (+/fc-fos mice) were confirmed by genomic Southern analyses of DNA isolated from their tails. Mice homozygous for the loxP-c-fos-loxP homologous recombination (f/fc-fos mice) were produced by crossing heterozygous animals and were identified by Southern blotting.

We crossed f/fc-fos mice with the T50 mice carrying a cre transgene driven by
a CaMKIIα promoter [37]. The T50 transgenic mice were generated in the BCF1 background and were later bred extensively into the C57BL/6 background. We further rederived this mouse line in the C57BL/6 genetic background. The genotypes of the offspring carrying both the cre gene and the loxP-c-fos-loxP homologous recombination were identified by genomic Southern blotting with 5’ and 3’ probes for loxP-c-fos-loxP, and with a 0.4 kb BamHI fragment from the cre gene. Finally, f/fc-fos-cre mice were obtained by intercrossing of +/fc-fos-cre mice. Mutant and their various control litter-mates 10 to 18 weeks of age were used for all subsequent analyses.

4.3.2 Seizure Scoring and Statistical Analysis

Wild-type, f/fc-fos, cre transgenic and f/fc-fos-cre mice 12 to 18 weeks of age were injected with KA i.p. at 20 or 30 mg/kg of body weight. Four to 24 mice in each group were used for each drug dose. KA-induced seizures were scored every 5 minutes for 2 hours according to Yang et al. [25]: 1: arrest of motion; 2: myoclonic jerks of the head and neck with brief twitching movements; 3: unilateral clonic activity; 4: bilateral forelimb tonic and clonic activity; 5: generalized tonic-clonic activity with loss of postural tone including death with continuous convulsions. Pentetrazole (PTZ) was similarly injected at 30 or 50 mg/kg of body weight using 4 to 7 mice in each group. Seizures were assigned a score based on the highest degree of seizure within 15 minutes of the PTZ injection.
For seizure data analysis, we tested the independence of the degree of seizure response to KA administration among the four groups of mice during the first 35 minutes. A 2x4 contingency table was constructed with the degree of response to KA injections grouped as <3 and ≥3 followed by Chi-square analysis. The comparison between the \textit{f/fc-fos-cre} group and the other three control groups were carried out with Freeman-Tukey's multiple comparison after the percentage data were transformed with an Arcsine function. The KA-induced death rate comparison was carried out by Chi-square analysis. PTZ seizure results were analyzed using an unpaired t-test between the \textit{f/fc-fos-cre} and the three control groups of mice.

**4.3.3 \textit{In Situ} Hybridization and Quantification**

Wild-type, \textit{f/fc-fos}, \textit{cre} transgenic and \textit{f/fc-fos-cre} mice 10 weeks of age treated with 20 mg/kg of KA i.p. together with genotype-matched untreated mice were used for all \textit{in situ} hybridization experiments. Seizures were observed for 60 minutes. Brains were then removed, freshly frozen in powdered dry ice and stored at -80°C for 48 hours. Twelve µm cryostat sections were fixed for 5 minutes in paraformaldehyde and delipidated with chloroform (Moratalla et al., 1996). An 800-basepair fragment of the c-terminus of a mouse c-fos cDNA was used to make an $^{35}$S-UTP labeled antisense riboprobe (NEN, specific activity of 1 x $10^8$ cpm/µg). The sense probe was used as a negative control. Hybridization was performed by incubating various brain sections with the riboprobes ($10^6$ cpm/100µl) at 50°C for 16 hours. After being rinsed at room temperature and
washed at 60°C for 1 hour, the slides were dehydrated, dipped in NTB2 photographic emulsion (Kodak) and stored for 2 weeks. After development, brain sections were stained with cresyl violet.

Pictures were taken of each area of each brain section at 50x magnification using NIH Image for quantification. These images were then printed. The silver grains on each cell were counted in defined areas of CA1, CA2, CA3 and dentate gyrus with matching sections for all mice, as well as in cell sized areas of the background. Any cell that contained six times or more the number of grains as the background was counted as a positive cell. This number is derived by comparing values from known positives and negatives and determining a fair value for what is positive.

4.3.4 Immunohistochemistry

For all immunohistological analysis, mice 10 to 18 weeks of age were either treated with 20 mg/kg KA for 1.5 hours or with 30 mg/kg KA for 2 hours, and were perfused with PBS and then with 4% paraformaldehyde along with genotype-matched untreated mice. The brains were removed, postfixed in paraformaldehyde for 2 hours and submerged in 20% sucrose at 4°C for 2 days [36]. Brains were sectioned at 40 µm using a cryostat. An anti-c-Fos antibody (Ab-5, Oncogene Research Products) was used at 1:40,000 dilution; an anti-c-Jun antibody (sc-1694, Santa Cruz Biotechnology) was used at 1:40,000 dilution; an anti-pSer73-c-Jun antibody (New England Biolab) was used at 1:500 dilution; and an anti-GFAP antibody was used at 1:40,000 dilution (Dako). Primary
antibodies were incubated with brain sections at room temperature for 24 hours. Then, the sections were incubated with a biotinylated secondary antibody (1:600, Vector Laboratories) for 2 hours followed by ABC reagent (ABC kit, Vector Laboratories) for 1 hour. The immunoreaction was visualized by treating the sections for 5 minutes at room temperature in 0.05% diaminobenzidine with 0.01% hydrogen peroxide.

4.3.5 Electroencephalogram Analysis
Six each f/fc-fos-cre and wild-type mice 12-18 weeks of age were anesthetized with 5 g/kg urethane. For recording electroencephalogram (EEG), we placed two blunt tip tungsten electrodes on the dura above the sensory motor cortex. A reference electrode was placed on the nasal bone [39]. A high input impedance amplifier with bandwidth of 1-300 Hz was used for recording. The signals were recorded on a digital tape and analyzed off line. Baseline EEGs were recorded in the absence of KA. KA was then injected i.p. at 5 mg/kg, followed by 90 minutes of EEG recording. This was followed by a 10 mg/kg of KA injection and another 90 minutes of EEG recording. Samplings of 2.5-second EEG recordings at every 5-minute interval for each mouse were compared between f/fc-fos-cre and wild-type mice for spike-wave discharges. Student t-tests were performed between groups to determine the significance in genotypes. Records for all mice from each genotype were used.
4.3.6 Evaluating Neuronal Damage

Five days after KA treatment, surviving mice from all four groups at each of the above doses were perfused, their brains removed, post fixed and sectioned coronally at 40 µm as before. Serial brain sections were stained with cresyl violet for neuronal damage evaluation as described [25,40-42]. Neuronal loss was quantified by measuring the areas of severe neuronal damage on every second section using the Metamorph Imaging system, and sections were integrated to calculate volumes of damage of the four genotypes in dorsal hippocampus. An unpaired t-test was performed on the lesion volumes between the f/fc-fos-cre and each of the three control groups of mice. Neuronal damage-induced gliosis was evaluated in all four groups of brain sections by immunostaining for GFAP as described above. The above brain sections from all four groups of mice were also subjected to the TUNEL assay. The In Situ Cell Death Detection Kit in conjunction with the peroxidase-conjugated anti-fluorescein antibody detection system (TUNEL POD) from Boehringer Mannheim was utilized for the assay.

4.3.7 Protein Extract Preparation and Analysis of DNA-Binding Activities

Hippocampi from f/fc-fos-cre and wild-type mice were isolated under a dissecting microscope and nuclear extracts were prepared essentially as described by Mandelzys et al. [43]. All samples were manually pulverized with a Teflon pestle and suspended in 0.5 ml of buffer A in the presence of various protease inhibitors including PMSF, aprotinin, leupeptin and pepstatin A (Sigma). After incubation for 15 minutes on ice, NP40 was added to make a final concentration of 1%
before centrifugation for 1 minute at 4°C. The crude pellet was resuspended in buffer B and incubated for 15 minutes at 4°C. After centrifugation for 15 minutes, the supernatants were aliquoted and stored at -70°C. The protein content was estimated with Bio-Rad Protein assay (Bio-Rad).

DNA-binding activities of the nuclear proteins were analyzed using the electrophoretic mobility shift assay as described by Mandelzys et al. [43]. Twenty µg of nuclear protein were preincubated for 10 minutes at room temperature in a binding buffer with 20 µg/ml poly (dI-dC) and subsequently incubated with 10 pmol (50,000 cpm) of an end-labeled AP-1 oligonucleotide for 15 minutes at room temperature. The AP-1 oligonucleotides were end-labeled with [³²P]-dATP using polynucleotide kinase and purified on NICK columns (Amersham Pharmacia). Control experiments using excess amounts of unlabeled normal or mutated AP-1 sequences were performed to ensure the specificity of AP-1 binding. After incubation, the samples were electrophoresed at 130 V for 3 hours in a nondenaturing 4% polyacrylamide gel. Gels were dried and exposed. Autoradiographs were scanned using a Molecular Dynamics scanner and analyzed by ImageQuant software.

Supershifts were performed according to described [43]. Polyclonal antibodies (Santa Cruz Biotechnology) against c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD were used. One µg of each antibody was added to 15 µl of reaction volume containing 10 µg of nuclear protein extract. The mixture was incubated for 1 hour at 4°C. The labeled AP-1 oligonucleotide was then added to
the reaction mixture and the gel shift protocol was carried out as described above. Individual mouse gives parallel results within each group.

4.3.8 Cell Transfection And Protein Extract Preparation

Neuro2A, which is a mouse neuroblastoma, and U251, which is a human glioblastoma, were cultured in DMEM plus 10%FBS. We previously found that they both express endogenous BDNF. NT2, which is a human teratocarcinoma that exhibits endogenous GluR6 expression [44], was obtained from and was cultured as suggested by ATCC. 80% confluent cells were transfected with 1-4 µg of pCMV-c-fos that contains the rat c-fos with a deletion of the 3’ UTR to stabilize the c-Fos products by lipofection (Fugene6, Roche). 48 hours after the transfection, cells were harvested, washed once with PBS (pH 7.4), and homogenized in an extraction buffer for 1 minute at 4°C. The extracts were spun at 3,000 rpm for 15 minutes at 4°C and the supernatants were used for western blotting. All transfections were performed in duplicates and each transfection was repeated at least three times.

4.3.9 Western Blot Analysis

Wild-type and f/fc-fos-cre mice 12 to 18 weeks of age were injected i.p. with 30 mg/kg of KA or saline. Hippocampi from each mouse were isolated individually from both groups of mice at different time points and homogenized in a RIPA buffer with protease inhibitor cocktails. These extracts, and those from the transfection experiments, were quantified by the Biorad Bradford assay and were
separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with antibodies (Santa Cruz Biotechnology) against c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD, BDNF, TrkB, p75NTR, NMDA R1, GluR2-3, GluR6-7 and actin respectively and secondary antibodies. The results were visualized (Amersham) after exposure of membranes to x-ray films. Western blotting for each sample was performed at least twice.

4.4 RESULTS

4.4.1 Generation of loxP-c-fos-loxP Homozygous Mice

We used a mouse c-fos cDNA to clone the entire c-fos gene and its flanking sequences. A restriction map of c-fos and the targeting construct used are shown in Figure 1A. This targeting construct was designed to, upon proper homologous recombination, insert one loxP site in the second intron of c-fos and a second loxP site together with the Neo selectable marker downstream of c-fos. The third and fourth exons of c-fos encode the DNA binding domain and the leucine zipper domain that are important for heterodimerization with the Jun family of proteins for transcriptional regulation. Thirteen embryonic stem (ES) cell clones harboring the desired homologous recombination were identified by Southern hybridization with both c-fos-specific 5’ and 3’ probes. The ES cells were used to generate male chimeric mice, which were bred with C57BL/6 females to obtain heterozygous mice. Mice homozygous for the loxP-c-fos-loxP
insertion (f/fc-fos) were obtained by intercrossing of heterozygous mice. Genomic Southern blotting with a 3’ probe (Figure 1B) and a 5’ probe (data not shown) identified and confirmed the desired loxP site insertion.

In sharp contrast to the severe developmental deficiencies exhibited by the standard c-fos mutant mice [33,34], f/fc-fos mice appeared healthy and did not show any obvious growth abnormalities compared to their normal and heterozygous litter-mates. The genotype distribution of the first 66 offspring produced by heterozygous breeding was 18 wild-type, 32 heterozygous floxed and 16 f/fc-fos mice, indicating normal Mendelian segregation. Both male and female f/fc-fos mice were fertile when crossed with each other and normal numbers of pups were obtained with no obvious sex bias. These observations suggest that the insertion of the loxP sites did not alter the normal function of c-fos.

To determine precisely the effects of the loxP site insertion on c-fos expression, we quantified both basal and induced c-fos expression in the hippocampus of f/fc-fos and wild-type mice both before and after KA injections. KA is a glutamate analog that elicits seizures by directly stimulating GluRs and indirectly increasing the release of excitatory amino acids from nerve terminals [45,46]. KA can induce abundant c-fos expression, particularly in the CA3, CA2 and CA1 regions of the hippocampus and in the dentate gyrus in rodents [30,31]. We injected f/fc-fos and wild-type mice intraperitoneally (i.p.) with KA and measured c-fos expression in the brains. In situ hybridization results indicate that whereas basal levels of c-fos expression are very low in non-treated wild-
type and f/fc-fos mice, equal and significant levels of c-fos induction are seen in pyramidal neurons in CA3, CA2 and CA1 regions of the hippocampus, and in granule cells in the dentate gyrus region in both genotypes upon KA treatment (Figure 2A-D). Quantification of silver grains of in situ hybridization from the brain sections confirmed that there is no difference between wild-type and f/fc-fos mice in the percent of cells positive for c-fos expression in the hippocampus either before or after the KA injection (Figure 2G). Immunostaining further confirmed that there is no difference in c-Fos protein levels in the hippocampus either before or after KA treatment in these two groups of mice (Figure 2H-M). Thus, insertion of the loxP sites did not change either the basal expression or the inducibility of c-fos in the f/fc-fos mice, and these mice are suitable for crossing with the cre transgenic mice for loxP-mediated c-fos mutation.

4.4.2 Generation of Hippocampal c-fos Mutant Mice

We crossed the f/fc-fos mice with a line of CaMKIIα-cre transgenic mouse, T50, generated and described by Tsien et al. [37]. Previous work demonstrated clearly that this line of cre transgenic mouse produces the Cre recombinase at a high level in the hippocampus, which is sufficient to carry out loxP site-mediated activation of a lacZ reporter gene in virtually all CA1 pyramidal neurons in the hippocampus [37]. Moreover, the loxP site-mediated activation of the lacZ reporter gene was not observed in the cortex, amygdala, striatum, cerebellum, or brain stem [37]. Southern blotting indicates that the genotypes of offspring from heterozygous floxed c-fos /heterozygous cre transgenic mice breeding show
normal Mendelian distribution and mice carrying both the homozygous \textit{f/fc-fos} gene (Figure 1B) and the \textit{cre} transgene, which are designated as \textit{f/fc-fos-cre}, can be identified (Figure 1C).

To verify the mutation of \textit{c-fos} in the hippocampus of \textit{f/fc-fos-cre} mice, we compared both basal and KA-induced \textit{c-fos} expression in the putative \textit{c-fos} mutant mice and control mice. \textit{In situ} hybridization results indicate that \textit{f/fc-fos-cre} mice exhibit virtually no basal \textit{c-fos} expression and a drastically reduced level of \textit{c-fos} induction by KA compared to wild-type and \textit{f/fc-fos} mice (Figure 2A-G) and to \textit{cre} transgenic mice (data not shown) in the CA3, CA2 and CA1 regions of the hippocampus and in the dentate gyrus. Quantitative analysis shows that there is a significant reduction in basal \textit{c-fos} expression in the CA3, CA2, CA1 and the dentate regions in \textit{f/fc-fos-cre} mice 10 weeks of age or older compared to those in the controls (Figure 2G, \textit{p} < 0.05). There is at least a 95% reduction in \textit{c-fos} mRNA induction in the CA3, CA2 and CA1 regions, and a 70% reduction in the dentate region in \textit{f/fc-fos-cre} mice compared to those in the controls after the KA injection (Figure 2G). Significantly, there appears to be equal \textit{c-fos} induction by KA in other brain regions, including the median eminence and entorhinal cortex, a major input and output zone of the hippocampal formation, in \textit{f/fc-fos-cre} and control mice at this particular age (data not shown). \textit{f/fc-fos-cre} mice 8 weeks of age show an 80-90% reduction in \textit{c-fos} expression in the CA3, CA2 and CA1 regions compared to control mice (data not shown). Immunostaining confirmed that c-Fos protein expression is also significantly reduced in the hippocampus in \textit{f/fc-fos-cre} mice 10 weeks of age or older.
compared to that in the controls after the KA induction (Figure 2H-M). Thus, the Cre recombinase is effective in carrying out loxP site-mediated c-fos mutation in the hippocampus, resulting in a significant reduction in both basal expression and induction of c-fos in the f/fc-fos-cre mice.

The f/fc-fos-cre mice look normal and can breed with each other to produce normal number of offspring with no obvious sex bias, suggesting that the Cre recombinase-induced mutation of c-fos in the hippocampus does not interfere with mouse survival and reproduction.

4.4.3 f/fc-fos-cre Mice Exhibit Apparently Normal Central Nervous System Development

The mutation of c-fos via the loxP sites in the hippocampus in f/fc-fos-cre mice presumably starts around postnatal week three, and would have minimum impact in central nervous system development [37,47,48]. To substantiate this assumption, we performed three experiments. First, we did a systematic histological comparison of brains from age-matched f/fc-fos-cre, cre transgenic, f/fc-fos and wild-type mice (n=5 each). Every tenth coronal section was stained with cresyl violet and examined in parallel. There is no obvious difference in general brain structure or in the hippocampus in particular among the four groups of mice (data not shown).

As a second test, we determined whether c-Jun induction is altered in the f/fc-fos-cre mice. The Jnk3-c-Jun signaling pathway is implicated in KA-induced seizure and cell death in the hippocampus [25]. We treated f/fc-fos-cre, cre
transgenic, f/fc-fos and wild-type mice (n=5 each) with KA i.p. at 0 mg/kg, 20 mg/kg and 30 mg/kg doses, and compared c-Jun and phospho-c-Jun expression in the hippocampus in the four groups of mice by immunostaining using coronal brain sections. Counting of immunoreactive signals indicates that there is no detectable difference among the four groups of mice in either the level or pattern of induction of c-Jun and phospho-c-Jun in the CA3 and CA1 regions of the hippocampus, and in the dentate gyrus (data not shown). Thus, regional mutation of c-fos did not significantly alter the Jnk3-c-Jun-mediated signal transduction in the hippocampus in f/fc-fos-cre mice.

As the third test, we investigated whether the c-fos mutation affected hippocampal-dependent spatial learning. Spatial learning behavior in mammals is believed to critically depend on the proper functioning of the intact hippocampus [49]. We first compared performances of f/fc-fos-cre, f/fc-fos, cre transgenic and wild-type mice in the Morris water maze test [50]. We found that f/fc-fos-cre mice performed similarly in both the hidden platform and visible platform version of the water maze when compared to the three control groups of mice [51]. To further test the effect of the c-fos mutation on spatial learning, we performed the Barnes maze test, a slightly less challenging task than the Morris water maze [52]. We found that all four groups of mice performed similarly in both the spatial and cued versions of the Barnes maze [51]. Thus, hippocampal-dependent spatial learning appears to be normal in f/fc-fos-cre mice. Together, these three sets of findings suggest that f/fc-fos-cre mice exhibit apparently normal central nervous system development.
4.4.4 *ffc-fos-cre* Mice Exhibit Increased KA-Induced Seizure Severity Compared To Control Mice

KA can induce seizures in rodents [46]. To investigate the role of c-fos in KA-induced seizures, we treated *ffc-fos-cre* mice together with wild-type, *ffc-fos* and cre transgenic mice with two different doses of KA i.p., and scored the degrees of seizure [25]. KA induced seizures of progressive severity in all groups of mice (Figure 3A and 3B). Whereas there are no appreciable differences among the three control groups, *ffc-fos-cre* mice exhibit a higher degree of seizure at 20 mg/kg of KA than the control groups, including occasional continuous convulsions and death (Figure 3A, \(p < 0.001\)). At 30 mg/kg of KA, all four groups of mice exhibited apparently similar degrees of seizure (Figure 3B). However, the seizure scoring system we used does not distinguish between generalized tonic-clonic activity and death, with death clearly representing a higher degree of seizure. Taking this into consideration, the frequency of death was also compared. *ffc-fos-cre* mice died more frequently following extensive convulsive seizures than the three control groups of mice (Figure 3C, \(p < 0.05\)), suggesting that KA also induced more severe seizures in *ffc-fos-cre* mice than in control mice at the 30 mg/kg dose. The increased seizure induction is specific to KA since *ffc-fos-cre* mice do not differ from the three control groups of mice in seizures induced by PTZ, an epileptogenic agent that blocks GABAergic systems, at both 30 mg/kg (Figure 3D) and 50 mg/kg (Figure 3E) doses (\(p >

112
0.05). These results indicate that hippocampal mutation of c-fos leads to an increase in KA-induced seizure severity in the f/fc-fos-cre mice.

4.4.5 f/fc-fos-cre Mice Exhibit Increased KA-Induced Neuronal Excitability Compared To Wild-type Mice

The increased KA-induced seizure severity exhibited by the f/fc-fos-cre mice may reflect increased neuronal excitability in these mice. To test this hypothesis, we compared the EEG patterns in the cortex of wild-type and f/fc-fos-cre mice [39]. As shown in Figure 4A, there was no significant difference in baseline EEGs between f/fc-fos-cre and wild-type mice in the amplitudes of spike-wave discharges in the absence of KA. This is consistent with the fact that no obvious spontaneous seizures were observed in either f/fc-fos-cre or wild-type mice. When KA was injected i.p. at the 10 mg/kg dose, which is a sub-threshold for seizure induction, both f/fc-fos-cre and wild-type mice showed higher amplitudes of spike-wave discharges than baseline levels (Figure 4A and 4B). However, both the amplitudes of KA-induced spike-wave discharges (Figure 4B), and the frequency of spike-wave discharges whose amplitudes are higher than baseline levels (Figure 4C, $p < 0.05$), are significantly higher in f/fc-fos-cre mice than in wild-type mice. Thus, the c-fos mutation leads to increased KA-induced neuronal excitability that may underlie the increased KA-induced seizure severity in the f/fc-fos-cre mice.
4.4.6 *f/fc-fos-cre* Mice Exhibit Increased KA-Induced Cell Death Compared To Control Mice

Rodent hippocampal CA3 pyramidal neurons are more susceptible to KA-induced death than other neurons in the hippocampus [46,53]. To test the hypothesis that c-fos may play a neuroprotective role against KA-induced excitotoxicity, we assessed the degree of neuronal damage in the hippocampus in the four groups of mice that survived the 30 mg/kg of KA treatment. Since the surviving mice exhibited similar degrees of seizure, a difference in neuronal damage would be directly attributable to the loss of c-fos rather than indirectly to a difference in KA-induced seizure severity.

We perfused *f/fc-fos-cre* mice together with wild-type, *f/fc-fos* and *cre* transgenic mice five days after KA-induced seizures, and analyzed each group of brains. Nissl staining of brain sections from the four groups of mice indicates that *f/fc-fos-cre* mice exhibit a higher level of KA-induced neuronal damage in the CA3 region of the hippocampus (Figure 5B) than wild-type (Figure 5A), *f/fc-fos* and *cre* transgenic mice (data not shown). Higher magnification pictures reveal clearly an increased neuronal cell loss in the CA3 region in *f/fc-fos-cre* mice (Figure 5D) compared to wild-type (Figure 5C), *f/fc-fos* and *cre* transgenic mice (data not shown). Immunostaining for the glial fibrillary acidic protein (GFAP) indicates that there is more gliosis in the CA3 region in the *f/fc-fos-cre* mice (Figure 5F) than in wild-type (Figure 5E), *f/fc-fos* and *cre* transgenic mice (data not shown). The excitotoxic neuronal cell death in the mutant mice is also accompanied by more TUNEL staining in the CA3 region (Figure 5H) than in
wild-type mice (Figure 5G), suggesting apoptosis is potentially involved in the observed neuronal degeneration. Quantification of lesion volumes in the CA3 region in all groups of mice clearly demonstrates that there is a marked increase in KA-induced CA3 cell death in \textit{f/f-}\textit{c-fos-cre} mice compared to the other three control groups (Figure 5I, \(p < 0.05\)). There was less significant neuronal damage and DNA fragmentation in the CA1 region (data not shown), and no obvious damage in the dentate region including the hilus in all four groups of mice (Figure 5A and 5B). Thus, though similar in seizure severity at 30 mg/kg of KA, \textit{f/f-}\textit{c-fos-cre} mice exhibit more cell death in the CA3 region than control mice following KA administration.

4.4.7 \textit{c-fos} Regulates AP-1 Transcription Complex Formation \textit{In Vivo}

c-Fos is thought to function via AP-1 transcription complexes to regulate target gene expression. c-Fos and other Fos family proteins have different affinities for Jun family proteins and can compete for AP-1 complex formation [54]. Moreover, several IEGs, including \textit{fosB}, also have AP-1 binding sites in their promoters and can be regulated by AP-1 complexes [55,56]. These observations suggest that \textit{c-fos} may regulate AP-1 complex formation either by a direct participation or by regulation of other IEG expression. To examine these possibilities, we compared the level and composition of the AP-1 complexes as well as levels of various IEGs in the hippocampus of \textit{f/f-}\textit{c-fos-cre} and wild-type mice before and after KA exposure.
In the absence of KA exposure, basal levels of AP-1 complexes are very low and comparable in wild-type and f/fc-fos-cre mice (Figure 6A). Competition using an increasing amount of unlabeled AP-1 sequences gradually reduced the AP-1 binding whereas AP-1 sequences with a point mutation for the AP-1 binding failed to do so, indicating the specificity of the protein/DNA interaction (data not shown). Supershift experiments using antibodies against various IEG products indicate that, similar to those reported by Mandelzys et al. [43], FosB and JunD, and to a lesser extent, c-Fos and JunB, are the main components of AP-1 complexes in wild-type mice (Figure 6A). In contrast, due to a lack of participation by or competition from c-Fos, only FosB and JunD participate significantly in basal AP-1 complex formation in f/fc-fos-cre mice (Figure 6A). Western blotting indicates that while basal c-fos expression is very low, fosB (Figure 7A), fra-1, fra-2, c-jun, junB and junD (data not shown) expression appear to be comparable in the two groups of mice.

Four hours after KA injections, both f/fc-fos-cre and wild-type mice exhibit 15-30 fold higher levels of AP-1 complexes than basal levels. However, f/fc-fos-cre mice exhibit a 2 fold less AP-1 complexes compared to wild-type mice (Figure 6B). Supershift experiments showed that, similar to those reported by Mandelzys et al. [43], the main components of the AP-1 complexes in the wild-type hippocampus are c-Fos and JunD, and to a lesser extent, FosB and c-Jun (Figure 6B). In contrast, the main components of the AP-1 complexes in the hippocampus of f/fc-fos-cre mice are FosB and JunD (Figure 6B). Moreover, due to a lack of participation by or competition from c-Fos, there are more FosB and
JunD in AP-1 complexes in f/fc-fos-cre mice than in wild-type mice (Figure 6B). Western blotting indicates that in the absence of c-fos induction, absolute FosB levels are reduced (Figure 7A) whereas Fra-1 and Fra-2 levels are increased in f/fc-fos-cre mice compared to wild-type mice. c-Jun, JunB and JunD levels are similar in the two groups of mice (data not shown).

Five days after the KA treatment, the relative AP-1 levels in each group of mice are lower than those after 4 hours of KA exposure but still higher than the basal levels respectively. The main components of the AP-1 complexes in the hippocampus of both f/fc-fos-cre and wild-type mice are FosB and JunD (Figure 6C). This is consistent with those reported by Mandelzys et al. [43]. However, there are less FosB and more JunD participating in AP-1 complexes in f/fc-fos-cre mice than in wild-type mice (Figure 6B). Western blotting results suggest that the expression of all IEGs appears to be similar in f/fc-fos-cre and wild-type mice at this KA treatment time point (Figure 7A and data not shown).

We did not detect significant levels of Fra-1 in either basal or KA-induced AP-1 complexes, whereas Fra-2 levels are similar in AP-1 complexes both before and after the KA induction in the two groups of mice (data not shown). Together, our results suggest that c-fos regulates the overall composition of AP-1 transcription complexes both by a direct participation and by regulation of other IEG expression.

4.4.8 c-fos Regulates GluR6 And BDNF Expression Both In Vivo And In Vitro
Dynamic changes in both the level and composition of AP-1 transcription complexes in the hippocampus occur as a consequence of a transient induction of c-Fos followed by a longer term induction of FosB family proteins in rodents upon KA exposure [43,57]. The changed dynamic regulation of AP-1 complexes due to the c-fos mutation may lead to changes in target gene expression, resulting in a KA-induced increase in seizure severity and neuronal excitability as well as cell death in f/fc-fos-cre mice compared to control mice. To identify such c-fos-regulated target genes, we selected several candidate genes that are involved in either neuronal excitability or survival, and investigated whether c-fos regulates their expression both in vivo and in vitro.

The major class of molecules in mediating the fast excitatory neuronal transmission is the ionotropic GluRs that include the NMDA, AMPA (GluR1-4) and KA receptors (GluR5-7 and KA1-2) [58-61]. In the absence of basal c-Fos, the level and/or composition as well as function of various GluRs or their modulators might be altered, resulting in hyperexcitability. To test this possibility, we selected several GluR genes that have AP-1 binding sites in their promoter regions respectively and compared the expression levels of these GluRs in the hippocampus of f/fc-fos-cre and wild-type mouse individually. As shown in Figure 7B, there is a 2 fold increase in basal GluR6 expression in the hippocampus in naive f/fc-fos-cre mice compared to wild-type mice. Basal expression of the NMDA R1 subunit, GluR2 and 3 are similar in naive f/fc-fos-cre and wild-type mice (data not shown). Thus, basal levels of c-Fos regulate the expression of specific GluR subunits in the hippocampus.
Neurotrophins and their receptors are key regulators of neuronal cell survival [62]. For example, BDNF can attenuate apoptotic cell death of cortical neurons [63], and can play a neuroprotective role against KA-induced seizures in the developing brain [64]. An enriched environment can induce BDNF expression and protect against KA-induced seizures and excitotoxicity [65]. KA can induce BDNF expression in the hippocampus [66,67]. TrkB and p75NTR, the high and low affinity BDNF receptors, are also induced in the hippocampus after seizures [66-70]. c-fos may be involved in regulating the expression of these molecules after seizure induction [5,32]. To test this possibility, we treated f/fc-fos-cre and wild-type mice i.p. with 30 mg/kg of KA and isolated extracts from hippocampi of both KA-treated and untreated mice. We then measured the expression levels of BDNF, TrkB and p75NTR from each individual mouse at different time points. As shown in Figure 7C, BDNF expression is induced compared to basal levels in both f/fc-fos-cre and wild-type mice by KA. However, the time course of BDNF induction differs between the two groups of mice. Four hours after KA exposure, BDNF levels in f/fc-fos-cre mice remain similar to basal levels, whereas they are 2 fold higher than basal levels in wild-type mice (Figure 7C). Basal and KA-induced BDNF expression at the 24 or 48 hour time points are not overtly affected by the c-fos mutation. Both basal and induced levels of TrkB and p75NTR are similar in f/fc-fos-cre and wild-type mice at all time points measured (data not shown). These results indicate that, in the absence of c-fos induction, there is a delayed BDNF induction by KA in f/fc-fos-cre mice compared to wild-type mice.
The GluR6 gene promoter has not been characterized and sequence analysis indicates that there is an AP-1 binding site in the promoter region (unpublished results). The complex BDNF promoters can be regulated by multiple transcription factors such as CREB [71,72]. We noted that the BDNF promoter regions also contain an AP-1 binding site. There is also an AP-1 binding site in the fosB promoter [55]. To further test whether c-fos regulates GluR6, BDNF and fosB expression, we transiently transfected several cell lines with c-fos and measured the expression of the three candidate genes. Similar to those observed in vivo, an increasing amount of c-Fos reduces GluR6 expression (Figure 7D), while increases BDNF or fosB expression in vitro (Figure 7E). The parallel changes observed both in vivo and in vitro suggest that the increased basal GluR6 expression and the delayed BDNF induction by KA in f/fc-fos-cre mice compared to wild-type mice are due to a lack of direct c-Fos regulation rather than to indirect effects of the c-fos mutation.

4.5 DISCUSSION
To investigate whether and how c-fos is involved in neuronal excitotoxicity, we generated a mouse with a hippocampal mutation of c-fos. The c-fos mutation leads to an increased KA-induced seizure severity, neuronal excitability and neuronal cell death in f/fc-fos-cre mice compared to wild-type mice. Moreover, c-Fos regulates AP-1 transcription complex formation both before and after the KA exposure. c-fos also regulates GluR6 and BDNF expression both in vivo and in
Our results suggest that c-fos is a key regulator for cellular mechanisms mediating neuronal excitability and survival.

4.5.1 c-fos Regulates Neuronal Excitability By Regulating GluR6 Expression

Our in situ hybridization results indicate that there is a significant reduction in basal c-fos expression in f/fc-fos-cre mice compared to that in control mice. Moreover, there is at least a 95% reduction in c-fos expression in the CA3, CA2 and CA1 regions of the hippocampus, and a 70% reduction in c-fos expression in the dentate region after KA administration in f/fc-fos-cre mice compared to those in control mice. The c-fos mutation did not appear to occur significantly elsewhere in the brain as KA induced comparable c-fos expression in the entorhinal cortex and median eminence in f/fc-fos-cre and control mice. c-Fos immunostaining results support the in situ hybridization results. Together, these results indicate that the loxP/cre-mediated c-fos mutation was effective in the hippocampus specifically. We observed a broader c-fos mutation pattern in our f/fc-fos-cre mice than that in the T50/lacZ reporter mice in which the reporter gene activation was restricted to the CA1 region of the hippocampus [37]. This may be because we used older mice in our studies, or that the extent of lacZ gene activation underrepresented the extent of loxP/cre-mediated deletion in the original study [37]. Whether the c-fos mutation extends beyond the hippocampus with age is yet to be determined.
We found that f/fc-fos-cre mice exhibit increased KA-induced seizure severity. At 20 mg/kg of KA, f/fc-fos-cre mice exhibit a significantly higher degree of seizure than control mice. At 30 mg/kg of KA, all groups of mice exhibited seizures, but a significantly higher percentage of f/fc-fos-cre mice died compared to control mice. The increase in KA-induced seizure severity in f/fc-fos-cre mice cannot be explained simply by a deficiency in hippocampal structure. Using the T50 CaMKIIα-cre transgenic line, Tsien et al. [37] detected the lacZ reporter gene activation starting around postnatal week 3. The mutation of c-fos via the loxP sites in the hippocampus in the f/fc-fos-cre mice presumably also starts at this time and consequently would have minimum impact on central nervous system development [47]. Indeed, the basic structure and function of the hippocampus appear normal in the f/fc-fos-cre mice as judged by Nissl staining, by the induction of c-Jun and phospho-c-Jun by KA in the hippocampus, as well as by hippocampal-dependent spatial learning. Moreover, PTZ induces comparable levels of seizures in all four groups of mice at two different doses. Finally, both the amplitudes of KA-induced spike-wave discharges, and the frequency of spike-wave discharges whose amplitudes are higher than baseline levels, are significantly higher in f/fc-fos-cre mice than in wild-type mice. These results demonstrate that a c-fos mutation leads specifically to an increased KA-induced seizure severity and neuronal excitability in the f/fc-fos-cre mice compared to those in control mice.

The GluR6 subunit is abundantly expressed in the CA3 neurons in the hippocampus and granule cells of the dentate gyrus, whereas neither GluR5 nor
GluR7 is highly expressed in the pyramidal neurons [73]. KA receptors containing the GluR6 subunit are involved in modulating mossy fiber synaptic plasticity [74,75]. Standard GluR6 mutant mice are less susceptible to KA-induced seizures and excitotoxic neuronal cell death in the hippocampus [42], and GluR6 Q/R site editing-deficient mice are more susceptible to KA-induced seizures than wild-type mice [76]. These studies clearly demonstrate the importance of proper GluR6 expression in neuronal excitability and plasticity. We found that due to a lack of participation by or competition from c-Fos, FosB and JunD constitute most of the basal AP-1 transcription complexes. Moreover, naive f/fc-fos-cre mice exhibit an increased basal GluR6 expression in the hippocampus compared to that in control mice in vivo, and an increasing amount of c-Fos reduces GluR6 expression in vitro. Together, these observations indicate that basal c-Fos regulates the inherent neuronal excitability by regulating proper GluR6 expression. c-Fos may do so by directly participating in AP-1 complexes that negatively regulate GluR6 expression. Alternatively, c-Fos may limit the availability of Fos-less AP-1 complexes that positively regulate GluR6 expression. Either way, our results demonstrate that the increased GluR6 expression causes the f/fc-fos-cre mice to over-respond to KA stimulation and this finding provides a mechanistic explanation for the increased neuronal excitability and seizure severity observed in f/fc-fos-cre mice compared to control mice.

We currently do not know whether the hippocampal c-fos mutation affects seizure induction and/or propagation in f/fc-fos-cre mice. Standard c-fos mutant
mice exhibit attenuated kindling development and mossy fiber sprouting [32]. Our current study shows an essential role for c-fos in neuronal excitability using mice with a much more restricted c-fos mutation and minimal developmental deficiency. Although both studies suggest the importance of c-fos in neuronal plasticity, because the c-fos mutation in the dentate region is not complete in f/fc-fos-cre mice at the age we used, we cannot directly compare the implications of the two studies at this time.

4.5.2 c-fos Protects Neurons From Excitotoxic Death By Regulating BDNF Expression

The hippocampus is the most sensitive brain region to KA-induced neuronal damage [46,53,77]. In our mouse model, the c-fos mutation occurs in the hippocampus and not elsewhere. We thus focused the lesion evaluation on the hippocampus for the current study. We found increased neuronal damage, as marked by increased cell loss, gliosis and DNA fragmentation, in the CA3 region of the hippocampus in f/fc-fos-cre mice compared to control mice following KA injections. The greater extent of neuronal damage observed in f/fc-fos-cre mice can not be simply explained by greater KA-induced seizure severity. The CA3 lesion studies were carried out using f/fc-fos-cre and control mice that survived 30 mg/kg of KA treatment. All surviving mice exhibited similar levels of seizures, yet f/fc-fos-cre mice clearly exhibited more CA3 region neuronal damage. Thus, our results argue strongly that c-fos plays a neuroprotective role against KA-induced cell death. There was less significant neuronal damage and DNA
fragmentation in the CA1 region both in \textit{f/fc-fos-cre} and control mice. In addition to the hippocampus, KA can elicit neuronal damage in other brain regions including the piriform cortex and amygdala [46,77,78]. KA also induces c-fos in these brain regions [22,31]. If the c-fos mutation expands into these regions in \textit{f/fc-fos-cre} mice at older ages, it will be very interesting to investigate whether KA induces more lesions in these brain regions in \textit{f/fc-fos-cre} mice than in control mice.

BDNF has been reported to protect against KA-induced seizure or apoptotic cell death [63-65]. One likely way for c-Fos to play a neuroprotective role is to selectively up-regulate \textit{BDNF} expression following KA-induced seizures, thus limiting the neuronal damage. Indeed, we found that in the absence of c-fos induction, both the level and composition of AP-1 transcription complexes are changed in \textit{f/fc-fos-cre} mice compared to wild-type mice after KA exposure. Moreover, the lack of c-fos expression resulted in a delayed \textit{BDNF} induction in the hippocampus of \textit{f/fc-fos-cre} mice compared to wild-type mice, and an increasing amount of c-Fos induces increasing \textit{BDNF} expression \textit{in vitro}. These results strongly argue that \textit{BDNF} is a direct target for c-fos regulation and that timely \textit{BDNF} induction is crucial for neuronal survival after excessive excitation. We noted that the \textit{BDNF} induction at later time points does not appear to depend on c-Fos. Other factors may be involved.

TrkB and p75NTR, both receptors for BDNF, are also induced in the hippocampus after seizures [66,68-70]. We found that both basal and KA-induced expression of TrkB or p75NTR are similar in the two groups of mice. It is
possible that c-Fos does regulate TrkB and p75NTR expression within the hippocampus. Both TrkB and p75NTR are mostly induced in the dentate gyrus, and less so in the CA3, CA2 and CA1 areas, following seizures [68,70]. Since we used extracts prepared from, in addition to the whole hippocampus, the dentate gyrus in which the c-fos mutation is incomplete, local TrkB or p75NTR expression changes due to the c-fos mutation can be over-shadowed by their overall expression changes following KA administration. Alternatively, mechanisms involving factors other than c-Fos, such as phosphorylation [69], could be responsible for regulating TrkB or p75NTR expression in the hippocampus after KA injections.

FosB has been implicated in a predisposition to developing spontaneous and evoked seizures [43]. We found that c-fos up-regulates fosB expression both in vivo and in vitro. These results suggest that, in addition to a direct participation, c-Fos also regulates AP-1 transcription complex formation by regulating other IEG expression. c-fos and fosB may regulate certain common target genes involving neuronal excitability.

4.5.3 A Molecular Model For The Participation of c-fos In Excitotoxicity

Together, our results suggest a molecular model in which c-fos acts as a key regulator in cellular mechanisms mediating neuronal excitability and survival (Figure 8). In normal neurons, basal c-Fos or c-Fos induced by neuronal activity orchestrates the formation of AP-1 transcription complexes either by a direct participation or by regulation of other IEG expression. Basal AP-1 complexes
regulate the expression of specific GluRs to ensure proper neuronal excitability.
Induced AP-1 complexes regulate specific neurotrophins to ensure cell survival after excessive stimulation (Figure 8). The dual regulation of the two cellular mechanisms by c-Fos ensures appropriate neuronal excitability and protects neurons from potential excitotoxicity.

In the current study, we only selectively tested several candidate genes involved in neuronal excitability and survival. It should be emphasized that a wide range of key molecules are involved in neuronal network assembly and/or plasticity [79,80], and that there are almost certainly more c-Fos-regulated molecules and cellular programs that can influence KA-induced seizures and excitotoxicity. For example, in a preliminary experiment using the cDNA microarray technology, we found differential gene expression changes, among others, in the apoptosis program in the hippocampus of f/f-c-fos-cre and wild-type mice upon KA exposure. This finding correlates with the observation that there are more TUNEL positive cells in the CA3 area in f/f-c-fos-cre mice than in control mice. How c-fos regulates the apoptosis process and how other c-fos-regulated processes contribute to neuronal excitotoxicity are important future questions.

We used mice with a mixed genetic background in our study. Inbred strains of mice differ in sensitivity to KA-induced seizure and excitotoxic cell death. Kosobud and Crabbe reported that C57BL/6 mice are more resistant to KA-induced seizures than other inbred strains of mice, such as the BALB/c mouse strain [81]. Schauwecker and Steward showed that whereas they exhibit similar levels of KA-induced seizures, C57BL/6 and BALB/c mice are more resistant to
KA-induced cell death than other inbred strains of mice [82]. Hu et al. demonstrated that there is a good correlation between KA doses and the extent of neuronal damage as assayed by DNA fragmentation in C57BL/6 mice [83]. These reports demonstrate the importance of studying the function of c-fos in excitotoxicity in defined inbred strains of mice. Whereas future work with congeneric strains of mice will further clarify the contribution of genetic background to seizure and excitotoxicity, the clear differences between $f/fc$-fos-cre mice and control mice in KA-induced seizure severity, neuronal excitability, cell death as well as changed dynamic regulation of gene expression in the hippocampus argue that the deficits in the $f/fc$-fos-cre mice are due to the c-fos mutation rather than an effect of the genetic background.

The continuous use of $f/fc$-fos-cre mice has the potential of allowing us to identify additional molecular targets and neuronal pathways underlying excitability, survival and plasticity. Such studies may provide new information for treating neuronal loss caused by both acute insults and chronic degeneration in the central nervous system.
Figure 1. Generation of f/fc-fos-cre mice. A, The c-fos genomic DNA locus, the targeting vector, the floxed c-fos gene locus, and the 5′ and 3′ hybridization probes. The neo transcription direction is the same as that of the c-fos. Open boxes represent c-fos exons; black arrows indicate the loxP sites. Restriction sites shown: Bg: BglII; Cl: ClaI; Sp: Spel. B, Identification of the f/fc-fos mice. Tail DNA from two litters of heterozygous intercross were digested with BglII, electrophoretically separated, transferred onto membranes and hybridized with a 3′ probe for c-fos. C, Identification of the f/fc-fos-cre mice by Southern hybridization of the same litters of mice as in B using a cre-specific probe.
Figure 2. Basal expression and induction of c-fos is significantly reduced in the hippocampus in f/fc-fos-cre mice. Three to five coronal brain sections from each wild-type (A-B), f/fc-fos (C-D), and f/fc-fos-cre mouse (E-F) were hybridized with a c-fos specific probe. A, C and E are brain sections from two each of untreated mice. B, D and F are brain sections from three to four each of mice treated with 20 mg/kg of KA. G, Quantification of c-fos expression in the hippocampus and dentate of f/fc-fos-cre and two groups of control mice using in situ hybridization. Data represent mean ± SEM. NT: no treatment; DG: dentate gyrus. Three to five coronal brain sections from each wild-type (H-I), f/fc-fos (J-K), and f/fc-fos-cre mouse (L-M) were stained with an anti-c-Fos antibody. H, J and L are brain sections from two each of untreated mice. I, K and M are brain sections from three to six each of mice treated with 20 mg/kg of KA. Mice treated with 30 mg/kg of KA gave parallel results. The scale bars are 1 mm.
Figure 3. *f/fc-fos-cre* mice exhibit increased KA-induced seizure severity compared to control mice. We administered wild-type (+/+), *cre* transgenic (*cre*), *f/fc-fos* (*f/f*) and *f/fc-fos-cre* (-/-) mice with KA i.p. at 20 mg/kg (A, *n*=4-6 mice each) or 30 mg/kg (B, *n*=15-24 mice each), or PTZ at 30 mg/kg (D, *n*=4-5 mice each) or 50 mg/kg (E, *n*=4-7 mice each). Seizures were recorded and plotted. Data represent mean ± SEM (A-B) and mean + SEM (D-E). The percent of mice that died after KA administration at 30 mg/kg (C) is also presented.
Figure 4. *f/fc-fos-cre* mice exhibit increased KA-induced neuronal excitability compared to wild-type mice. We recorded EEG patterns in *f/fc-fos-cre* (−/−) and wild-type (+/+) mice (n=6 each) in the absence (A) or presence (B) of 10 mg/kg of KA. (C), Histogram of the frequency of spike-wave discharges whose amplitudes are higher than baseline levels in the two groups of mice after KA injections. Data represent mean + SEM of 2.5 s of sampling. *p < 0.05.
Figure 5. *f/fc-fos-cre* mice exhibit more KA-induced CA3 cell death than control mice. Five d after seizure induction by 30 mg/kg of KA, mice were perfused and their brains sectioned. We performed Nissl staining (A and C, n=7 wild-type mice and B and D, n=5 *f/fc-fos-cre* mice). We also performed immunostaining for GFAP (E-F) and TUNEL staining (G-H) using the above brain sections for both wild-type (E,G) and *f/fc-fos-cre* (F,H) mice. The scale bars indicate 1 mm for A-B and E-F, and 65 µm for C-D and G-H. Arrows indicate lesioned brain regions. We also performed quantification of lesion volumes in the CA3 region of the hippocampus for all groups of mice at 30 mg/kg of KA (I, n=5-7 mice each). Data represent mean ± SEM. +/-, cre, *ff*, and -/- represent wild-type, cre transgenic, *f/fc-fos* and *f/fc-fos-cre* mice respectively. *p < 0.05. There is no major difference in neuronal damage among the four groups of mice in the amygdala and pyriform cortex after KA exposure.
Figure 6. c-fos regulates AP-1 transcription complex formation in vivo. We isolated nuclear extracts from hippocampi and dentate of wild-type (+/+) and f/fc-fos-cre (-/-) mice either before (n=3 mice each) or after (n=4 mice at each time point) 30 mg/kg of KA injection. We performed gel-shift and supershift analyses using extracts from before (A), after 4 h (B) and 5 d (C) of KA treatment. Competition using an increasing amount of unlabeled AP-1 sequences gradually reduced the AP-1 binding whereas AP-1 sequences with a point mutation for the AP-1 binding failed to do so, indicating the specificity of the protein/DNA interaction. To visualize basal supershift results, we used four times the nuclear extracts as the 4 h or 5 d KA-treated samples and prolonged exposure. Four h after KA injections, f/fc-fos-cre mice exhibit a 1.8 fold less AP-1 complexes compared to wild-type mice. Moreover, there are more FosB and JunD in AP-1 complexes in f/fc-fos-cre mice than in wild-type mice, as judged by the ratio between the supershifts and regular shifts (B). Thus, although the absolute levels of FosB are reduced, there appear to be more FosB participating in AP-1 complexes in f/fc-fos-cre mice than in wild-type mice. Five d after the KA treatment, there are less FosB participating in AP-1 complexes in f/fc-fos-cre mice than in wild-type mice as judged by the ratio between the supershifts and regular shifts (C).
Figure 7. c-fos regulates fosB, GluR6 and BDNF expression in vivo and in vitro.

We isolated nuclear extracts from hippocampi, dentate and other brain regions of individual wild-type (+/+) and f/fc-fos-cre (-/-) mouse (n=4 mice for each time point) either before or after 30 mg/kg of KA injections. We performed western blotting for the indicated IEG products (A) and BDNF (C) before (0 h) and after 4 h or 5 d of KA treatment, and for GluR6 (B) before KA exposure. Equal amounts of protein were loaded in each lane. We included the status of only the full-length FosB since our pan-Fos antibody and FosB antibody do not consistently detect ΔFosB upon KA administration. Expression of the R1 subunit of NMDA, GluR2, GluR3 and GluR7 receptors are not different between the mutant and control brains. Individual mouse gives parallel results within each group. Hip: hippocampus; Ctx: cortex; CPu: caudoputamen; Cb: cerebellum. We transfected increasing amounts of c-fos (1 and 2) into the indicated cells in duplicates with mock-transfected cells (M) as controls. 48 h after the transfection, we isolated cell extracts and used equal amounts of proteins for GluR6 (D), BDNF and FosB (E) as well as c-Fos (D-E) western blotting. We obtained parallel results both within each transfection and among different transfections.
Figure 8. A model for c-fos acting as a key regulator in cellular mechanisms mediating neuronal excitability and survival. In normal neurons, basal c-Fos or c-Fos induced by neuronal activity orchestrates the formation of AP-1 transcription complexes either by a direct participation or by regulation of other IEG expression. Basal AP-1 complexes regulate the expression of specific GluRs to ensure proper neuronal excitability. Induced AP-1 complexes regulate specific neurotrophins to ensure cell survival after excessive stimulation. The dual regulation of the two cellular mechanisms by c-Fos ensures appropriate neuronal excitability and protects neurons from potential excitotoxicity.
SUPPLEMENTAL FIGURES

Graphs depicting percent positive cells across different conditions:

- **ER**, **ME**, and **CTX**
- Legend: +/+ and other conditions

Images showing cellular details for various conditions labeled as a, b, c, d, e, f, g, h, i.
**Figure 1.** Similar induction of c-fos mRNA by KA outside of the hippocampal formation in wild-type (+/+, A-C), f/fc-fos (D-F) and f/fc-fos-cre (G-I) mice. Shown are c-fos expression patterns in the entorhinal cortex (ER: A, D and G), median eminence (ME: B, E, and H) and cortical regions (CTX: C, F and I). Three to five coronal brain sections from each mouse were hybridized with a c-fos specific antisense probe. The sense probe was used as a control. We treated four mice ten wk of age from each genotype with 20 mg/kg of KA. The scale bar represents 50 µm. (J), Quantification of c-fos expression in the above areas of f/fc-fos-cre and two groups of control mice. Data represent mean + SEM.
**Figure 2.** Lack of obvious developmental deficiencies in the brains of $f/fc$-fos-cre mice. Wild-type (A, D and G), $f/fc$-fos (B, E and H) and $f/fc$-fos-cre (C, F and I) mice (n = 5 each) were perfused, their brains removed, sectioned coronally and stained with cresyl violet (A-C). There is no obvious difference in general brain structure or in the hippocampus in particular among the four groups of mice. We also treated the three groups of mice (n=5 each) with 20 mg/kg of KA i.p. for 1.5 h, perfused and sectioned their brains coronally, and performed immunostaining for c-Jun (D-F) or phospho-c-Jun (G-I). Quantification of immunopositive cells indicates that there is no detectable difference among the four groups of mice in either the level or pattern of induction of c-Jun and phospho-c-Jun in the CA3 and CA1 regions of the hippocampus, and in the dentate gyrus by KA. There is no difference for c-Jun immunoreactivity in the non-treated brains of the four groups of mice. Similar age mice treated with 30 mg/kg of KA for 2 h gave parallel results. The scale bars are 1 mm.
REFERENCES


36. Xu M, Moratella R, Gold LH, Hiroi N, Koob GF, Graybiel AM, Tonegawa S. Dopamine D1 receptor mutant mice are deficient in striatal expression of


67. Rudge JS, Mather PE, Pasnikowski EM, Cai N, Corcoran T, Acheson A, Anderson K, Lindsay RM, Wiegand SJ. Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective. Exp Neurol 1998; 149:398-410.


Chapter 5: Hippocampal expression of c-fos is not essential for spatial learning

5.1 ABSTRACT

The formation of long-term memory is thought to involve underlying changes in synaptic strength. Many studies have focused on the mechanisms of spatial learning behavior in mammals that is critically dependent on the proper function of the hippocampus. Because of the enduring nature of long-term memory, it is thought that gene expression is involved in this process. The immediate early gene (IEG) c-fos encodes a transcription factor. The c-Fos proteins form heterodimeric proteins with the c-Jun family proteins and the resulting AP-1 transcription complex plays a key role in coupling short-term events elicited by stimuli received at the cell membrane to long-term neuroplastic changes by regulating gene expression. c-fos is induced in the hippocampus after spatial learning. Despite this knowledge, the precise role of c-fos in memory formation and the underlying mechanisms remain unknown. To start investigating the role of c-fos in learning and memory and underlying mechanisms, we evaluated spatial learning capabilities using mice carrying a hippocampal region-specific mutation of c-fos. We found that the c-fos mutant mice exhibit normal spatial learning behaviors in both the Morris water maze and the Barnes maze tests compared to control mice. Our results suggest that hippocampal c-fos expression is not essential for spatial learning.
5.2 INTRODUCTION

The formation of long-term memory is thought to involve long-term underlying changes in synaptic strength [1]. Many studies have focused on the mechanisms of spatial learning behavior in mammals that critically depends on the proper function of the hippocampus [1]. Neurons in the hippocampal formation, including CA3 and CA1 pyramidal cells and granule cells in the dentate gyrus, can undergo multiple forms of long lasting changes [1]. Because of the enduring nature of long-term memory, gene expression is thought to be involved in this process [2-6]. Protein kinase A and the transcription factor cAMP-responsive element-binding protein (CREB)-dependent transcription have been shown to be essential for certain aspects of spatial learning [6-12]. Nonetheless, the molecular targets of CREB and the sequence of events that constitute the learning process are mostly unknown.

\( \text{c-fos} \) is an immediate early gene (IEG) that encodes a transcription factor [13-16]. The c-Fos proteins can form heterodimers with Jun family proteins and the resulting AP-1 transcription complexes can bind to the AP-1 site, TGACTCA, a transcriptional regulatory sequence found in a large number of cellular genes [17-19]. \( \text{c-fos} \) can be rapidly activated by a variety of stimuli, ranging from neurotransmitters to neurotrophins both in vitro and in vivo [20-25]. \( \text{c-fos} \) is also expressed during central nervous system development [26]. Although the exact role of \( \text{c-fos} \) in brain function remains unanswered, \( \text{c-fos} \) is thought to play a key role in coupling short-term stimuli received at the cell surface to long-term
neuroplastic changes via regulating gene expression together with c-jun or other IEGs [15,25,27,28].

The unique properties of c-Fos, being a transcription factor and being rapidly induced by a variety of stimuli, suggest that it may be a key molecule to participate in mechanisms underlying neuronal plasticity such as changes in synaptic strength. Phosphorylated CREB can bind to the CRE sequence in the promoter region of c-fos and activate its transcription [28-30]. Moreover, c-fos is induced in the hippocampus after spatial learning [31]. Despite such suggestive evidence, the precise role of c-fos in memory formation and the underlying mechanisms remain unknown.

Genetically engineered mice are particularly good models to study the molecular basis of learning and memory [32-34]. Although straightforward c-fos mutant mice have been a useful model system for evaluating how c-fos might be involved in neuronal function, 60% of the homozygous c-fos mutant mice die soon after birth and only 40% survive to adulthood [35,36]. Moreover, those c-fos mutant mice that do survive develop osteoporosis and defects in lymphogenesis as well as gametogenesis. These severe developmental deficiencies make the use of the c-fos mutant mice to evaluate the role of c-fos in learning very difficult [37]. To overcome this problem and to start investigating the role of c-fos in learning and memory, we have used the loxP/cre system [38,39] to generate mice with a c-fos mutation only in the hippocampal formation of the brain [40]. In the current study, we compared the spatial learning capabilities of the c-fos mutant mice with those of the control mice using two
hippocampal-dependent learning paradigms, the Morris water maze and the
Barnes maze test. We found that the c-fos mutant mice exhibit normal spatial
learning behaviors in both tests compared to control mice. Our results suggest
that hippocampal c-fos expression is not essential for spatial learning.

5.3 METHODS

5.3.1 Mice
The hippocampal c-fos mutant mice (f/fc-fos-cre mice) together with their wild-
type, homozygous floxed c-fos (f/fc-fos) and cre transgenic littermates were
generated by intercrossing of heterozygous f/fc-fos-cre parents as described by
Zhang et al. [40]. The genotypes of all mice were identified by genomic Southern
blotting using both a c-fos-specific and a cre-specific probe as described [40].
The genetic background of all the mice used was 129/SvxC57BL/6. All mice
were housed with littermates (no more than 4 per cage) with food and water ad
libitum in a room with a 12 hour light cycle [41]. Behavioral tests were performed
during the light phase of the light/dark cycle.

5.3.2 The Morris water maze set up
The water maze set up is exactly as described by Slane et al. [41]. Briefly, it
consists of a round pool 1.2 meters in diameter filled with opaque water. The
water temperature was kept at 28°C. A 10 x 10 cm platform is placed 1 cm
below the surface of the water. A video camera connected to a computer is mounted from the ceiling directly above the pool and a video tracking system was used to record performance (Polyteack System, San Diego Instruments, San Diego, CA). Distal cues for the hidden platform task, probe trials and the random platform test consisted of various geometric shapes made of blue paper that were hung on the walls. During the visible-platform test, a black cylinder serving as the proximal cue was placed on top of the platform and the rest of the cues were concealed by a black curtain surrounding the pool. 13 wild-type, 12 \textit{f/fc-fos}, 14 \textit{cre} transgenic, and 17 \textit{f/fc-fos-cre} F3 mice were used. There were roughly equal numbers of male and female mice in each genotype. All mice were 10 to 20 weeks of age at the time of experimentation. Previous experiments showed that the \textit{c-fos} mutation is limited to the hippocampal formation within this time window [40].

5.3.3 The Morris water maze procedure

The water maze test was divided into four consecutive phases as described before [41]. Day 1 was the straight channel test in a water-filled corridor with the water temperature of 28°C. The trial was completed when a mouse reached an escape ladder at the opposite end of the straight channel from which it was placed. Four trials were given with 1 minute maximum per trial. Intertrial intervals were 1 minute each. Escape latencies were recorded. All mice were able to complete this task and all were used in the Morris water maze test.
Days 2 to 11 were the spatial learning phase, including an acquisition phase with a hidden platform placed in the southeast (SE) quadrant and probe trials, during which the platform was removed. On day 2, before any testing, we first trained the mice to climb the platform. Following such training, four swimming trials were given per day with 1 minute maximum per trial. We used distributed trials with 1 hour intertrial intervals. After completion of each trial, or if a mouse failed to find the platform within 1 minute, the mouse stayed on or was placed on the platform for 30 seconds. We varied the starting positions on each trial with each cardinal position used once per day and the order of starting positions varied each day. We then recorded the pathlength, cumulative distance from the platform and escape latencies using a computerized system. Cumulative distance from the platform is defined as the sum of the distances of a mouse from the platform measured every 55 milliseconds during each trial and is based on a learning index developed by Gallagher et al. [42]. We performed probe trials at the beginning of day 6 and at the end of day 11 and the trials were for 1 minute. During the probe trials, we recorded the number of platform site crossings, time spent in each of the four quadrants and average distance from the platform site.

Days 12 to 13 were the random platform phase. On day 12, we performed two trials with the platform at the original location followed by two trials with the platform at NE and NW locations. On day 13, we performed one trial with the platform at the original location and 3 more trials with the platform at NW, SW
and NE locations. We recorded number of platform site crossings, escape latencies, pathlength and cumulative distance.

Days 14 to 19 were the visible-platform phase. We pulled a black curtain around the water maze to shield the distal cues from the mice during testing and all lights except the overhead light were turned off. We performed the tests using the same protocol as in the hidden platform except the platform now had only a proximal cue. We varied both start and platform positions randomly on each trial and recorded escape latencies manually.

5.3.4 The Barnes maze set up

The Barnes maze consists of a white disk 1 meter in diameter and 90 cm above the ground, similar to that described by Bach et al. [43]. There are 40 holes each 5 cm in diameter around the perimeter of the white disk. A darkened escape tunnel is located under one of the holes. A bright light, a buzzer and a fan were all mounted above the disk and were used as aversive stimuli during the test. A video camera mounted above the disk was used to record the behavior during the test. Distal cues for the spatial version of the test consist of various objects in the room, including the door, a white curtain, and another testing apparatus set up. These distal cues were hidden during the cued (visible) version of the test, and a soda can wrapped in tape was mounted behind the hole of the escape tunnel as a proximal cue. For the spatial version of the Barnes maze test, exactly same number of wild-type, f/fc-fos, cre transgenic and f/fc-fos-cre F3 mice as in the Morris water maze test were used. For the cued version of the
Barnes maze test, 8 F3 mice from each genotype were used. There were roughly equal numbers of male and female mice in each genotype and all mice were 10-12 weeks of age at the start of the experiment.

5.3.5 The Barnes maze procedure

Day 1 included both training and testing phases of the spatial version of the test [43]. The tunnel was beneath the same hole for every trial. The training session consisted of placing a black cylinder around the hole with the escape tunnel. The mouse was placed in the cylinder and the aversive stimuli were turned on. The mouse was given a maximum of 1 minute to escape into the tunnel. Upon completion of the task, the mouse stayed in the tunnel for 1 minute with the stimuli turned off. If a mouse failed to complete the task, it was placed in the tunnel for 1 minute and the stimuli were turned off. The testing sessions consisted of placing the mouse in the center of the disk in a black cylinder. As soon as the mouse was placed on the disk, the aversive stimuli were turned on. After 10 seconds, the cylinder was removed and the mouse was allowed to explore the maze. Testing was stopped when the mouse escaped into the tunnel or when the process lasted for 4 minutes, whichever was shorter. After completion of each trial, the mouse stayed in the tunnel for 1 minute. If a mouse failed to locate the tunnel, it was again placed in the tunnel for 1 minute. One trial was performed each day. The escape latency, distance between the first hole and the escape tunnel, and number of errors exploring the wrong holes were recorded [43]. Search strategy results were obtained by evaluating each
mouse’s daily search performance in the sequence of holes explored. The various search strategies included random, serial and spatial [43,44]. Searches are defined as nose pokes and head deflections over a hole. An error is defined as searching a hole that does not have the tunnel beneath it.

Days 2 to 45 consisted solely of the spatial version of the test as on day 1. A mouse completed testing when it had 3 or fewer errors on 7 out of 8 consecutive days. To test for memory retention, any mouse that completed the spatial test within the original 45 days was given one trial 42 days following the completion of the test. The trial was run and data collected as in the spatial version of the test.

Days 88-133 were the cued version of the test. All mice were tested once a day. The distal cues were shielded from the mice and a proximal cue was used. The location of the escape tunnel varied randomly for each trial. Data were collected as in the spatial version of the test.

5.3.6 Statistical Analysis
Morris water maze acquisition was analyzed using three-way analysis of variance (ANOVA) with one between (group) and two repeated measure factors (day and trial). Morris water maze probe trial data were analyzed using group by trial ANOVA and Morris random maze data were analyzed using group by platform position ANOVA. Barnes maze data were analyzed using group or group by trial block ANOVA. For both maze tests, significant interactions were further analyzed using simple-effect ANOVAs. Individual group comparisons following a
significant group effect were performed using Duncan pairwise comparisons. Significance was defined as $p < 0.05$.

5.4 RESULTS

5.4.1 $f/fc$-fos-cre mice exhibit similar spatial learning abilities compared to control mice in the Morris water maze test

The hidden platform version of the Morris water maze tests the ability of a mouse to locate the platform by learning the position of the platform relative to distal cues placed around the test room. We compared performance of the $f/fc$-fos-cre mice with those of the wild-type, $f/fc$-fos and cre transgenic control mice. We previously showed that $f/fc$-fos-cre mice exhibit virtually no basal c-fos expression and over 97% reduction in c-fos induction by kainic acid in the CA3, CA2 and CA1 regions of the hippocampus and a 70% reduction in c-fos induction in the dentate gyrus compared to wild-type, $f/fc$-fos and cre transgenic mice [40]. We included the $f/fc$-fos mice in the current study to control for possible effects of inserting the loxP sequences in the c-fos gene, and the cre transgenic mice to control for potential influence of the cre transgene expression in the brain. We previously showed that the three control groups of mice are similar in basal and kainic acid-induced c-fos expression [40].

All four groups of mice learned to perform the hidden platform task, as shown by a gradual decrease in mean escape latency, pathlength and
cumulative distance from the platform over time (Figure 1). Importantly, no significant differences were found in escape latency or cumulative distance from the platform among the four groups of mice on all days (Figure 1, $p > 0.05$).

There was also no difference in pathlength among the four groups of mice on days 1, 5-8 and 10 ($p > 0.05$). On days 2-4, $f/fc$-fos mice exhibited a longer total pathlength than wild-type mice ($p < 0.05$ on days 2 and 3, $p < 0.005$ on day 4). On day 9, $f/fc$-fos-cre mice had a longer pathlength than wild-type mice ($p < 0.05$). These results suggest that $f/fc$-fos-cre mice exhibit similar spatial learning abilities compared to control mice.

To investigate further spatial learning and memory capabilities in $f/fc$-fos-cre mice, we performed probe trials at the beginning of day 6 and at the end of day 11. All four groups of mice spent more time in the platform quadrant than in any other quadrants on both days (Figure 2A, $p < 0.05$). Moreover, all groups of mice crossed the original platform site significantly more than the other three equivalent platform sites on both days (Figure 2B, $p < 0.05$). Furthermore, all groups also showed improvement from Day 6 to Day 11 in both amount of time spent in the platform quadrant and the number of platform site crossings, indicating that all mice continued to learn the position of the platform.

Importantly, as in the hidden platform test, no significant differences were found among the four groups of mice in time spent in the platform quadrant and in platform site crossings on both days ($p > 0.05$), supporting the finding that $f/fc$-fos-cre mice do not exhibit spatial learning deficits compared to control mice.
To gain additional evidence that *f/fc-fos-cre* mice exhibit normal spatial learning, we performed a random platform test. In this test, all groups of mice showed an ability to recall the original platform position (Figure 3). When the platform was placed in the original (SE) position, all mice took far less time to locate it than when it was placed in an equivalent position in any of the other three quadrants (Figure 3, *p* < 0.05). Once again, no significant difference was found in the performance of the four groups of mice (Figure 3, *p* > 0.05).

Together, the results of these three different tests indicate that *f/fc-fos-cre* mice exhibit similar spatial learning compared to the three control groups of mice in the Morris water maze, suggesting that the spatial learning capabilities of the *f/fc-fos-cre* mice are not compromised by the loss of c-fos expression in the hippocampus.

5.4.2 *f/fc-fos-cre* mice exhibit a similar swimming ability and visible platform performance compared to the three control groups of mice

To compare the ability of the mice to swim, we used a straight channel task. There are no directional options in this task, little learning is required to complete the task [41]. All groups of mice were able to complete this task. The swimming speed of all mice was similar, and no significant differences were found in the escape latencies of the four groups of mice (Figure 4A, *p* > 0.05). We then performed the visible platform version of the Morris water maze test. This test controls for the ability of the mice to swim to a platform without having to use spatial cues. This task does not require hippocampal function. There were no
significant differences in escape latencies of the four groups of mice over the entire test period (Fig 4B, p > 0.05). Together, these results suggest that f/fc-fos-cre mice do not differ significantly from the three control groups of mice in swimming speed and in visible platform performance.

5.4.3 f/fc-fos-cre mice exhibit similar spatial learning compared to the three control groups of mice as shown by the Barnes maze test

The Barnes maze is also a task designed to test the spatial learning abilities of rodents [43,44]. In this task, mice learn to escape from aversive stimuli using distal cues. Because the mice are not forced to swim, this test is not as physically demanding as the Morris water maze. Therefore, we used this paradigm as an alternative to analyze the consequence of the hippocampal c-fos mutation by comparing the performance of f/fc-fos-cre mice with those of the three control groups of mice.

In the spatial version of the test, all four groups of mice exhibited spatial learning abilities and 60-80% of the mice were able to complete the task (Figure 5A). A mouse completes testing when it had 3 or fewer errors on 7 out of 8 consecutive days. The mean time to reach such a criterion was 36-38 days (Figure 5B). Importantly, all four groups of mice had a similar task completion percentage and mean time to criterion (Figure 5, p > 0.05). Over the entire spatial version of the test, all groups of mice showed reduced mean number of errors, mean distance from tunnel and mean escape latency, again indicating that all four groups of mice were able to learn to perform this task (Figure 6).
There were no significant differences in any of the measures on any day, suggesting that f/c-fos-cre mice exhibit similar spatial learning compared to the three control groups of mice (Figure 6, $p > 0.05$).

Search strategies in the spatial version of the Barnes maze test are another indicator for the acquisition of spatial learning. With more testing, mice use a fixed sequence of search strategies including random, serial and spatial to find the escape tunnel [43,44]. The random search strategy represents mice exploring many holes in a nonsystematic way whereas the serial search method involves mice going to the perimeter and then exploring consecutive holes in either a clockwise or a counterclockwise manner [43,44]. The spatial search strategy is characterized by mice going directly to the escape tunnel [43,44]. We used this information to further analyze whether the c-fos mutation in the hippocampus might affect the learning abilities of the mice. As shown in Figure 7, all groups of mice exhibited a general decrease in the percent of trials using either the random or the serial search strategy, and an increase in the percent of trials using a spatial search strategy with more training during the spatial version of the test. Importantly, all groups of mice exhibited a similar change in search strategies and there were no significant differences in the search strategies employed by all mice to find the escape tunnel (Figure 7, $p > 0.05$).

Memory consolidation may depend on regulation of gene expression [45-48]. To examine whether c-fos plays a role in this process, we examined the Barnes maze performance of the four groups of mice six weeks after the last day of the spatial version of the Barnes maze test. As shown in Figure 8, all four
groups of mice showed similar mean distance from tunnel, mean number of errors and mean escape latency ($p > 0.05$), again indicating that all mice were able to perform this task similarly. Together, these results suggest that $f/fc$-fos-cre mice exhibit similar spatial learning compared to the three control groups of mice.

5.4.4 $f/fc$-fos-cre mice exhibit similar performance in the cued version of the Barnes maze compared to the three control groups of mice

As in the Morris water maze, a cued version of the Barnes maze controls for the ability of the mice to complete the task without any distal spatial cues [43,44]. This task does not require hippocampal function. A proximal cue was used to mark the escape tunnel and all distal cues were hidden. As in the spatial version of the task, 50-70% of the mice were able to complete the task and all four groups of mice showed an equal ability to perform the cued version of the Barnes maze (Figure 5A). No significant differences were found on any of the measurements taken including mean distance from the tunnel, mean number of errors, and mean escape latency (Figure 9, $p > 0.05$). Thus, $f/fc$-fos-cre mice exhibit similar performance in the cued version of the Barnes maze compared to the three control groups of mice.
5.5 DISCUSSION

Long-term changes in synaptic strength are thought to underlie the formation of long-term memory. Changes in gene expression are an attractive mechanism to accommodate such enduring changes in synaptic strength. c-fos encodes a transcription factor that is turned on rapidly by a variety of stimuli including neuronal activity. c-fos is induced in the hippocampus after spatial learning. To start investigating whether c-fos might contribute to learning, we used mice with a hippocampal-specific c-fos mutation and compared their performance with those of the control mice in two hippocampal-dependent spatial learning paradigms, the Morris water maze and Barnes maze test. The Morris water maze results indicate that the f/fc-fos-cre mice performed similarly compared to wild-type, f/fc-fos and cre transgenic control mice in the hidden platform, probe trials and random platform tests. Results from the Barnes maze test also demonstrate that all mice showed similar learning abilities as judged by the percent of mice completing the task, days to criterion, escape latency, number of errors, distance from the tunnel and search strategies used. All mice also performed similarly in the cued version of both the Morris water maze and the Barnes maze. Taken together, our results suggest that hippocampal c-fos expression is not essential for spatial learning.

We derived the f/fc-fos-cre mice by crossing the f/fc-fos mice with the T50 CaMKIIα-cre transgenic mouse line [40]. Using the T50 CaMKIIα-cre transgenic line, Tonegawa, Tsien and colleagues detected the lacZ reporter gene activation starting around postnatal week 3 [38]. The mutation of c-fos via the loxP sites in
the hippocampus in the f/fc-fos-cre mice presumably also starts at this time and consequently has minimum impact on central nervous system development including the hippocampus. We previously showed that the basic structure of the brain, including the hippocampus, appear normal in the f/fc-fos-cre mice as judged by Nissl staining, and by the number of neurons exhibiting induction of c-Jun and phospho-c-Jun by kainic acid in the hippocampus [40]. Moreover, in the absence of basal c-fos expression, we did not detect obvious changes in basal IEG gene expression, including the Jun family and particularly the Fos family of IEGs in the hippocampus [40]. These results argue that, during the time window we tested these mice, hippocampal c-fos expression is not essential for spatial learning. It should be emphasized that we can not exclude the possibility that additional mechanisms could compensate for the lack of c-fos. For example, gene expression mediated by other IEGs such as Arc [31,49] or by transcription factors such as ATF [28] may partially compensate for the loss of c-Fos during spatial learning.

Different hippocampal-dependent learning mechanisms may use different forms of synaptic plasticity. For example, Kandel and colleagues generated a line of transgenic mouse carrying a Ca$^{2+}$-independent form of CaMKII and showed that this mouse exhibits a selective loss of hippocampal long-term potentiation in the range of the $\theta$ frequency [43]. Interestingly, the transgenic mouse also exhibits an impairment in spatial yet not contextual learning even through both tasks depend on an intact hippocampus [43]. The role of c-fos in contextual learning and other hippocampal-dependent learning tasks, such as
novel object recognition, olfactory discrimination, working and episodic-like memory tasks [50,51], needs to be determined.

Neuronal plasticity in cortical areas is thought to be critical in establishing permanent memory [52,53]. The hippocampus is considered to be critical for converting short-term memories into long-term memories and it is a site for temporarily storing new memories before transferring them to the cortex [54]. Our results show that, six weeks after the last day of the spatial version of the Barnes maze test, f/fc-fos-cre mice and the three control groups mice exhibited similar mean distance from tunnel, mean number of errors and mean escape latency (Figure 8, p > 0.05), suggesting c-fos is also not essential for transfer and storage of spatial memory. Whether c-Fos is critical for the transfer and consolidation of other hippocampal-dependent types of memory and other forms of memory remain to be determined.

We used mice with a mixed genetic background in our study. Future work with congenic strains of mice will help to clarify the contributions of genetic background to spatial learning performance. Nevertheless, the similarity in performance between f/fc-fos-cre and the three control groups of mice in both the Morris water maze and the Barnes maze suggest that hippocampal c-fos expression is not essential for spatial learning and that this finding is unlikely to be due to an effect of the genetic background.
FIGURES

Figure 1. Mice with a hippocampal mutation of c-fos perform similarly to control mice in the hidden platform version of the Morris water maze test. Mean escape latency (A), pathlength (B) and cumulative distance from the platform (C) were compared between mutant (f/fc-fos-cre) and three groups of control mice (wild-type +/+, f/fc-fos and cre transgenic mice). Data represent mean ± SEM. There are no differences in escape latency or cumulative distance from the platform among the four groups of mice. There is also no difference in pathlength among the four groups of mice on days 1, 5-8 and 10. Pathlength of f/fc-fos mice is significantly different from that of the wild-type mice on days 2-4. Pathlength of f/fc-fos-cre mice is significantly different from that of the wild-type mice on day 9. *p < 0.05. **p < 0.005.
Figure 2. Mice with a hippocampal mutation of c-fos exhibit a similar performance compared to control mice in probe trials of the Morris water maze. There are no differences in time spent in the original platform quadrant (A) and number of original platform site crossings (B) among the four groups of mice. Probe trials were performed on day 6 and day 11 respectively. Data represent mean ± SEM.
Figure 3. Hippocampal-specific c-fos mutant mice perform similarly to control mice in random platform test of the Morris water maze. Mean ± SEM escape latency to the platform at the original position as well as at random positions are plotted. All four groups of mice took significantly longer time to escape to the random platform than to the original platform position.
Figure 4. Hippocampal-specific c-fos mutant mice exhibit similar performance compared to control mice in the visible platform test of the Morris water maze and in the straight channel test. Mean ± SEM escape latencies for the straight channel (A) and visible platform (B) for each day with four trials per day are shown.
Figure 5. Mice with a hippocampal mutation of c-fos exhibit a similar percentage in completing the Barnes maze task as control mice. Percent of mice that completed the task are shown for both the spatial and the cued version of the test. The number of days to reach criterion for all groups of mice, defined as having 3 or fewer errors on 7 out of 8 consecutive days, are also presented.
Figure 6. Hippocampal-specific c-fos mutant mice exhibit similar performance compared to control mice in the spatial version of the Barnes maze test. Mean number of errors (A), mean distance from the tunnel (B), and mean escape latency (C) are plotted for the four groups of mice for the first two and last two blocks of the test. There are no differences among the four groups of mice in any of the three parameters presented.
Figure 7. Mice with a hippocampal mutation of c-fos use similar strategies to reach the escape tunnel as control mice. Percent of trials using a random search strategy (A), a serial search strategy (B) and a spatial search strategy (C) are shown for the first and last two blocks of trials. There are no differences among the four groups of mice in any of the three search strategies used.
Figure 8. Hippocampal-specific c-fos mutant mice perform similarly as control mice in the spatial version of the Barnes maze test after 6 weeks of retention. Mean distance from the tunnel (A), mean number of errors (B) and mean escape latency (C) are plotted for the four groups of mice. There are no differences among the four groups of mice in any of the three parameters presented.
Figure 9. Mice with a hippocampal mutation of c-fos perform similarly as control mice in the cued version of the Barnes maze. Mean distance to the tunnel (A), mean number of errors (B) and mean escape latency (C) are shown for the first and last two blocks of trials in the cued version of the Barnes maze. There are no differences among the four groups of mice in any of the three parameters presented.
REFERENCES


22. Dragunow M, Robertson HA. Kindling stimulation induces c-Fos protein(s) in granule cells of the rat dentate gyrus. Nature 1987; 329:441-442.


53. McClelland JL, McNaughton BL, O'Reilly RC. Why there are complementary learning systems in the hippocampus and neocortex: insights from the
successes and failures of connectionist models of learning and memory.


Chapter 6. Hippocampal c-fos expression is essential for regulating stress responses in female mice

6.1 ABSTRACT

The response to stress is an important neurobiological function that, when abnormal, can lead to various disease states. The hippocampus plays an important role in the negative feedback onto the major player in the stress response, the hypothalamo-pituitary-adrenal (HPA) axis. While a few of the molecular players in this feedback mechanism, such as the glucocorticoid receptor (GR), are known, how the expression of these molecules is regulated is unknown. A possible regulator of the molecules in this system is c-fos, an immediate early gene and part of the AP-1 transcription factor complex. An AP-1 binding site is found in the promoter region for GR and c-fos is activated in the hippocampus of mice following a number of stressors, including restraint stress. We used a region specific knockout model to study the effects of the loss of c-fos in the hippocampus on the stress response of the mouse. Mutant mice had normal diurnal corticosterone (CORT) patterns and responded to acute restraint stress. In chronic restraint stress studies, female mutant mice habituated faster than female wild-type mice. In the elevated plus maze, female mutants displayed lower anxiety-like behavior than the female wild-types. Western blot analysis revealed differences in the expression levels of both the estrogen receptor (ERα) and GR in the hippocampi of female mice. Finally, Golgi analysis of the CA3 pyramidal neurons in female mice revealed an overall greater apical dendritic
length in mutants than in wild-types. The findings of these studies suggest that the loss of c-fos in the hippocampus leads to an abnormal stress response specifically in female mice.
6.2 INTRODUCTION

The stress response is an important neurobiological function. Equally important is the termination of the stress response and return to homeostasis. An improper response to stress can lead to various disease states, such as depression [1,2] and post-traumatic stress disorder [3,4]. Therefore, it is important that the stress response and its termination be highly regulated processes.

The major pathway involved in the stress response is the hypothalamo-pituitary-adrenal (HPA) axis. In order to control the stress response, negative feedback onto the HPA axis by its end product, corticosterone (CORT), occurs at many levels. This feedback is both direct – onto the pituitary itself [5-7] – and indirect. One area of the brain that is believed to exert this negative feedback is the hippocampus [8-14]. The hippocampus is thought to be important following “processive” stressors, such as restraint stress or swim stress[15]. The mechanism by which the hippocampus exerts it feedback is through its high levels of both high affinity, type I or mineralocorticoid (MR), and low affinity, type II or glucocorticoid, receptors. The hippocampus is one of the few areas of the brain that contains both types of corticosteroid receptors, has the highest levels of glucocorticoid binding, and has high expression of GR and MR mRNA [14,16].

The regulation of the expression of MR and GR is unknown. One possible candidate for the molecular regulation of these receptors is c-fos. C-fos is an immediate early gene and a component of the AP-1 heterodimeric transcription factor complex [17-19]. C-fos is activated in the rat hippocampus following various types of stressors, including restraint stress [20-23]. Its actions as part of
a transcription factor complex can be exerted on the feedback response to stress in two ways. First, it can alter the expression of GR by acting directly at the AP-1 binding region on its promoter region [24,25]. Secondly, when GR is translocated to the nucleus to act as a transcription factor following activation by its ligand, AP-1 and GR can interact to alter downstream gene transcription [26-31].

To determine the role of c-fos in the molecular regulation of the hippocampal feedback on the stress response, we used a region specific knockout mouse with 95-98% decreased expression of c-fos in the CA1-CA3 regions of the hippocampus and a 70% decrease in the dentate gyrus region. Since the loss of c-fos does not occur until the third postnatal week, the neuronal development of the mutant (mt) mouse is normal [32]. In the present study we examined the endocrine response of the mutant mouse to acute and chronic restraint stress. Anxiety-like behavior of the mouse was examined using the elevated plus maze. To determine the underlying causes of the differences found in these tests, Western analysis was performed to examine protein expression differences. Finally, neuroanatomical changes in response to chronic stress were examined using Golgi analysis.
6.3 METHODS

6.3.1 Mice

The hippocampal c-fos mutant mice (f/fc-fos-cre mice) together with their wild-type (wt), homozygous floxed c-fos (f/fc-fos) and cre transgenic littermates were generated by intercrossing of heterozygous f/fc-fos-cre parents as described by Zhang et al. [32]. The genotypes of all mice were identified by genomic Southern blotting using both a c-fos-specific and a cre-specific probe as described previously [32]. The genetic background of all the mice used was 129/Sv x C57BL/6. All mice were housed with littermates (no more than 4 per cage) with food and water ad libitum in a room maintained on a 12-hour light cycle [33]. All mice were tested between 12 and 20 weeks of age. Previous studies showed that the c-fos mutation is limited to the hippocampal formation during this time [32].

6.3.2 Acute Restraint Stress

Four testing groups were used, male wt, female wt, male mt and female mt, with n = 6 for each group. Mice were placed in ventilated plastic restraint tubes for one hour. Blood samples were taken by tail nick at the beginning (basal sample) and end of the hour (stress sample). Following restraint stress, the mice were returned to their home cage for one hour. After this time, another blood sample was taken (recovery sample). Experiments were performed at the beginning of the light phase and at the beginning of the dark phase to test for normal diurnal
fluctuations in plasma CORT. Blood was centrifuged at 3700 rpm for 10 minutes at 4°C and plasma was collected for plasma CORT determination.

6.3.3 Chronic Restraint Stress

Chronic restraint stress experiments were performed in the same manner as described for the acute restraint stress but repeated for 22 consecutive days. The same testing groups were used with n = 6 in each group. Blood samples (basal, stress and recovery) were collected on days 0, 7, 14 and 21. The test was performed toward the end of the light cycle. Animals were sacrificed by decapitation the day following the last day of restraint and brains frozen on dry ice for Western analysis. The six-hour chronic restraint stress took place for 21 days and blood samples were taken on days 1, 7, 14 and 21. The testing took place during the light cycle.

6.3.4 CORT Radioimmunoassay

Plasma corticosterone was measured by radioimmunoassay using rabbit antiserum raised against corticosterone (B3-163, Endocrine Sciences, Tarzana, CA). Statistical significance was tested with two-tailed t-tests at each time point.

6.3.5 Elevated Plus maze

Four testing groups were used, male wild-type, female wild-type, male mutant and female mutant, with n = 6 in each group. The elevated plus maze was
constructed of black 1/8” plexiglass with four arms (5 x 35.5 cm) adjoined at a center square (5 x 5 cm). Two opposing arms had walls 29 cm high (closed arms) and the remaining two arms had walls 0.3 cm high (open arms). The maze was raised 75 cm off the ground. For the test, the mouse was placed in the center square of the maze facing an open arm. The mouse was allowed to freely explore the maze for 5 minutes. The test was performed during the dark phase and was recorded for behavioral analysis by a video camera using infrared light. Dependent variables included time spent in open arms, number of head dips, and number of stretch attend postures (SAPs). Head dips were defined as when the mouse dipped its head over the side of the maze. SAPs were when the mouse was in the center square of the maze and stretched into an open arm, with its front paws in the open arm and its back paws in the central square, and immediately returned back to the center square. A single observer blind to the genotype of the mice performed behavioral analyses. Statistical significance was tested using two-tailed t-tests for each measure.

6.3.6 Western blotting

Hippocampi were dissected from fresh frozen brains from males and females of each genotype (wt and mt) for both stressed (S) and non-stressed (NS) conditions (n = 4 for each of the 8 groups). Cytosolic and nuclear protein fractions were isolated as described by Mandelzys et al. [34]. All samples were manually pulverized with a pestle and suspended in buffer A with a protease inhibitor cocktail including PMSF, aprotinin, leupeptin and pepstatin A (Sigma).
After incubation for 15 minutes on ice, NP40 was added to make a final concentration of 1% before centrifugation for 1 minute at 4°C. The supernatants were aliquoted and stored at -80°C as the cytosolic fraction. The crude pellet was resuspended in buffer B and incubated for 15 minutes at 4°C. After centrifugation for 15 minutes, the supernatants were aliquoted and stored at -80°C as the nuclear fraction. The protein content was estimated using Bio-Rad Protein assay (Bio-Rad). The proteins were separated by SDS-PAGE and transferred to PVDF membrane. Western blotting was performed using primary antibodies to BDNF, GR, and actin (Santa Cruz Technologies), ER-α and ER-β (provided by Dr. Nira Ben-Jonathan), secondary antibodies and then visualized. Autoradiography was performed and relative band intensity was analyzed using actin as a loading control. Statistical significance was tested with two-tailed t-tests for each protein.

6.3.7 Golgi staining

Female mice (n = 8 each wt-NS, mt-NS, mt-S and n = 9 wt-S) were used. The stressed group was subjected to 6 hours restraint stress for 21 consecutive days. A non stressed control group was also included in this study. Blood samples were taken at 0 hours and 6 hours on day 1 and 0 hours, 1 hour and 6 hours on days 7, 14 and 21. On day 22, mice were perfused with 0.9% saline with heparin followed by 4% paraformaldehyde and brains were removed and placed in 4% paraformaldehyde overnight on ice. Brains were then processed using a modified version of the single section Golgi impregnation technique [35]. The following
day, brains were sectioned at 100 µm on a vibratome under 3% potassium dichromate and placed into 3% potassium dichromate solution for two days. The sections were then removed and mounted onto slides and rinsed briefly with double distilled water. A coverslip with a single drop of superglue at each corner was placed over the sections. The slides were immersed in 1.5% silver nitrate solution for 72 hours in the dark. Sections were then removed from the slides, rinsed in double distilled water, dehydrated in 95% ethanol, 100% ethanol and xylene, mounted onto new slides and coverslipped.

6.3.8 Golgi analysis

Neurons were selected for analysis according to the following criteria: 1) location within the CA3c subregion of the hippocampus, 2) dark and consistent staining throughout the dendrites, 3) distinguishable from neighboring cells, and 4) location of cell bodies within the middle one-third of the brain section. A total of 5-9 neurons were selected from each brain and drawn at 400 x using a camera lucida drawing tube. Drawings were digitized and the lengths of the apical and basal dendrites were measured using Metamorph. Additionally, the number of dendritic branching bifurcation points was determined for the apical and basal dendrites. All analyses was performed blind to the genotype of the mice. Two way ANOVAs were performed with genotype and treatment (non-stressed, stress) as between subjects factors.
6.4 RESULTS

6.4.1 Mutant mice and wild-type mice had similar plasma corticosterone levels during acute 1-hour restraint stress studies.

The acute restraint stress test was used to evaluate the corticosterone levels of the mice under basal and stressed conditions, as well as the recovery ability of the mice. In addition, by testing during both phases of the light/dark cycle, we verified that both groups of mice (wild-type and mutant) had normal diurnal CORT patterns, with higher basal CORT levels during the dark cycle and lower levels during the light cycle (Figure 1A and B). There were no differences between genotypes in either gender. As expected, females had a higher CORT response than males at the 1-hour stress time point, however there were no differences between genotypes for either sex. As was the case with the other time points, the recovery time point showed a normal recovery of all mice with no differences between genotypes.

6.4.2 Female mutant mice habituated more quickly than female wild-type mice to chronic one-hour restraint stress.

The chronic restraint stress test evaluates the ability of mice to habituate to a stressor over a long period of time. Plasma CORT levels for the chronic 1-hour restraint stress are shown in Figure 2. In the chronic 1-hour restraint stress study, a 3-way ANOVA with sex and genotype as factors and day as a repeated measure did not reveal a three way interaction (F(3, 60) = 1.26, p<0.297) (Figure
3B-3C). However, our focus is on the genotype main effect and genotype interactions. The genotype main effect was not significant ($F(1, 20) = 1.735$, $p = 0.203$). The 2-way interaction of genotype and day was significant ($F(3, 60) = 3.48$, $p<0.021$) (Figure 3A), revealing an overall greater change in CORT responses in the mutants compared to the wild-type mice over the course of the test. Additionally, Duncan post-hoc tests revealed a significant difference between day 0 and day 21 in the mutant mice ($p = 0.011$), but not in the wild-type mice ($p = 0.27$). The mutant mice have a greater decline in overall CORT levels over the 22-day testing period, which can be attributed to a greater habituation to the stressor. While it is not possible to determine whether the interaction is influenced primarily by the responses of the female mice, the 2-way interaction mirrors that of the female, causing us to believe that the females are the main influence in this effect.

**6.4.3 Mutant female mice displayed less anxious behavior than wild-type females in the elevated plus maze.**

The elevated plus maze tests anxiety levels of mice [36]. Less anxious behavior in the elevated plus maze is typically characterized by greater time spent in the open arms of the maze, greater number of head dips, and a lower number of SAPs [37]. While anxiety tests are classically used to test amygdala function, the hippocampus is believed to be involved in anxiety behavior as well [39-44]. In this experiment, we found that female mutant mice spent a significantly greater percentage of time exploring the open arms of the elevated plus maze than
female wild-type mice (p<0.0001, t = 7.205, df = 16) (Figure 4A). This response is considered to reflect a less anxious behavior. Additionally, female mutant mice had significantly greater number of head dips than wild-type females (p = 0.0011, t = 3.970, df = 16) (Figure 4B). This also is considered to reflect a less anxious behavior. Finally, the female mutant mice had significantly fewer SAPs than wild-type females (p = 0.0022, t = 3.690, df = 16) (Figure 4C). This is consistent with the other measures in that it reflects a less anxious behavior. All three measures together strongly support a behavioral difference between the female mutant and wild-type mice in the elevated plus maze, with the mutant mice exhibiting a less anxious behavior. To ensure that differences in the hormonal state of the female mice did not influence their behavior in the elevated plus maze, the stage of estrus cycle of the mice was determined. There were no differences in estrus stage of the wild-type versus mutant mice. When the estrus cycle was evaluated, all mice were in diestrus for four consecutive days, which strongly suggests that they were not cycling. No differences were found between male wild-type and mutant mice in any of the measures taken (Figure 4D-F).

6.4.4 Female mutant mice had different expression levels of both ERα and GR as compared to female wild-type mice.

Western blot analysis was performed to determine whether certain target molecules, including ERα, GR, and BDNF, exhibited differential expression in the hippocampus of the wild-type female as compared to the mutant female in the basal as well as the chronically stressed brain. These molecules were chosen
because each gene has a putative AP-1 binding site in its promoter region. The estrogen receptor was chosen due to the response we found in females and not males in both the elevated plus maze behavioral response and in plasma corticosterone levels following chronic restraint stress. Both ERα and ERβ were examined, but clear results were not obtainable from the ERβ blots. The expression of GR could have great consequences on the stress response of an animal, which could contribute to the differences that were found in the behavioral and endocrine responses of the mutant female mice. BDNF has already been found to have differential expression in this mouse following kainic acid injections and is known to be activated during the stress response, therefore it was also chosen as a possible target for differential expression in the mutant mouse. A representation of Western blots with the actin controls is shown in Figure 5A.

When the expression of ERα was analyzed, a 2-way ANOVA revealed a significant genotype by treatment interaction in both the cytosolic and nuclear fractions (cytosolic: F(1,12) = 13.59, p = 0.003; nuclear: F(1,12) = 11.76, p = 0.005). Duncan’s post tests revealed significantly higher levels of expression of ERα in the cytosol of both nonstressed and chronically stressed mutant mice as compared to wild-type mice (nonstressed: p = 0.00017; stressed: p = 0.0093). These tests also revealed significantly higher expression in the nucleus of the mutant unstressed mice as compared to wild-type unstressed mice (p = 0.00017) but no difference in the nuclear fractions of chronically stressed mice (Figure 5B). When GR Western analysis was performed, 2-way ANOVA revealed a significant
genotype and treatment interaction in the nuclear fraction, (p = 0.042, F(1,12) = 5.17) with Duncan’s test post hoc analysis revealing significantly lower expression levels in the mutant untreated mice as compared to the wild-type untreated mice (p = 0.0492) (Figure 5C). There were no differences between genotypes in the chronically stressed group. BDNF Western analysis showed no difference of expression levels between wild-types and mutants in either the nonstressed or chronically stressed mice (Figure 5D).

6.4.5 Six-Hour chronic restraint stress

To prepare the mice for Golgi analysis, the mice were first exposed to 21 consecutive days of 6-hour restraint stress. This protocol has been found to cause dendritic pruning in rats [35]. When six hour chronic restraint stress was performed on female mice, there were no differences found in the area under the curve between wild-type and mutant mice (Figure 6). However, there was a trend towards lower CORT levels by the mutant females on day 7. The wild-type females CORT levels fall to the level of the mutant females by day 14. This difference as compared to the one-hour restraint data could be explained by a faster habituation to the stressor by both genotypes when the restraint occurs for a longer time period each day.
6.4.6 Golgi staining revealed a genotype difference in the apical dendritic length of wild-type and mutant female mice

It has been found that following repeated stress, such as chronic restraint stress, the pyramidal neurons in the CA3 region of the hippocampus of rats exhibit remodeling of the dendritic branches, with the chronically stressed animal exhibiting shorter apical branches and fewer apical branch points [35]. By using the Golgi staining method, the dendritic branches of these neurons can be measured to observe whether this remodeling has occurred. Golgi analysis following chronic restraint stress in mice has not been published previously. Representative drawings of the four groups tested can be seen in Figure 7A. It can be noted that the number of subjects in the mutant stressed group is only 4. This is due to a low number of adequately stained neurons available for analysis in the brain sections.

When the brains of chronically restrained female mice were evaluated, there were no differences found between wild-types and mutants in the total basal dendritic length in either stressed animals or their nonstressed controls (Figure 7B). However, there was an overall genotype effect on total apical dendritic length, with the mutant females having greater apical dendritic length than the wild-type females (p=0.0381, F=4.898) (Figure 7B). Unlike what has been observed in rats, there was no dendritic atrophy in the wild-type mice following chronic stress (Watanabe 1992). There were no significant differences between genotypes on the number of dendritic branching points, whether apical or basal, however there was a trend towards a greater number of branch points
in the apical dendrites of the mutant mice as compared to the wild-type mice (Figure 7C). With a greater number of subjects, it is possible that this trend would reach significance.

6.5 DISCUSSION

The overall goal of this study was to determine how a hippocampal c-fos deficiency affects the stress response. A regional and temporal knockout mouse was used for the study. We found that the mutant mice habituated more quickly to chronic restraint stress than the wild-types, especially the females. Additionally, the female mutant mice exhibited less anxious behavior in the elevated plus maze. To determine the underlying causes of the differences we found in these tests we examined the hippocampal protein expression of target molecules GR, ERα and BDNF and also examined the dendritic structure of the CA3c pyramidal cells both basally and following chronic restraint stress.

In one-hour acute restraint stress no differences were found between genotypes at the basal, stress or recovery time points in either males or females. However, when the restraint stress was repeated for 22 consecutive days, significant differences were found between wild-type and mutant mice, with the mutant mice habituating faster to the stressor. This response appeared to be stronger in the female mice than in the males.

In the elevated plus maze, a gender specific difference was found, with the female mutants displaying a less anxiety than the female wild-types while no
differences were found in the males. Both plasma CORT levels and behavior of female mice can be affected by the estrus cycle phase. While the estrus cycle of the restrained mice was not determined, the estrus cycle of the mice used in the plus maze was tested. All mice tested were in diestrus on all testing days, pointing to the likelihood that none of these mice were cycling. Since the mice used in all tests were housed under the same conditions as these mice, it is unlikely that the mice used for the other tests were cycling. An additional explanation for these results is the extension of the c-fos deletion to the amygdala, an area known to be important in anxiety-like behaviors. This possibility needs to be addressed in future experiments.

Since differences in both the chronic restraint stress and the elevated plus maze were found only in females, we restricted the remainder of our study to females only. We first analyzed the hippocampal protein expression of various molecules that may be playing a role in altering the stress response in female mutant mice. The glucocorticoid receptor (GR) expression was found to be lower in the cytosol of the mutant females under basal conditions. It is possible that this change in expression might contribute to lower anxiety levels displayed in the plus maze. This would be consistent with previous findings that the neural specific GR knockout mouse shows less anxious behavior in the elevated plus maze [45].

The estrogen receptor (ERα) levels were found to be elevated in the cytosol and the nucleus of the mutant female under basal conditions and in the cytosol of the female following chronic restraint stress.
to affect neurite growth as well as hippocampal pyramidal cell dendritic spine density [46-48], which could contribute to increased feedback output from the hippocampus to the HPA axis. This could account for the faster habituation to chronic restraint stress that we found in the female knockout mice. Estrogen and the estrogen receptor also play an important role in the anxiety behavior of mice, therefore the elevation in ER$\alpha$ could play a role in the behavioral differences we found in female mice the elevated plus maze [49-52].

While no differences were found in BDNF expression, it is possible that this is because the brains were not isolated until 24 hours following the final restraint. While it is possible that BDNF expression is not altered under the stress conditions, in order to determine this, brains isolated immediately following the final restraint would need to be used for the study.

Finally, the morphology of the CA3c pyramidal cells was evaluated in both chronically stressed as well as nonstressed female mice using the Golgi staining technique. This experiment revealed that the mutant females had an overall greater total apical dendritic length than the wild-type females. It is possible that the change in the AP-1 transcription factor complex led to changes in neurofilament expression and this change resulted in the change in dendritic length. This increased dendritic length might contribute to an increased hippocampal feedback onto the HPA axis and might be a factor in the changes found in the previous experiments in the study, similar to affects found in previous studies relating hippocampal mossy fibers to anxiety related behaviors [42,43].
While it is true that the only differences we discovered in this mouse model were in the female mice, it is possible that the restraint stress and the elevated plus maze are not sensitive enough for male mice to reveal differences and that other tests, such as a resident intruder test, might be more appropriate. Since we did not use males in the Western analysis or the Golgi analysis, we can not be sure that the protein and dendritic changes that we found in the females are not present in the males. This analysis should be performed in the future. Additionally, BDNF and other growth factor expression levels should be evaluated directly following restraint stress.

Overall, the hippocampal cell that lacks c-fos may be more susceptible to the actions of extrinsic factors such CORT. This increased response could lead to a greater feedback onto the HPA axis and a greater habituation ability. This same increase in sensitivity to CORT could leave the hippocampus more susceptible to injury resulting from seizures. Glucocorticoids increase the vulnerability of neurons to kainic acid induced seizures; the injury resulting from seizures is worsened by glucocorticoids [53,54]. This could be a link between the change in stress response in these mice and the increased susceptibility to KA induced seizures that has been found previously [32].

This sensitive state of c-fos deficient cell might be due to the changes in many of the receptors that we have found. Changes in the expression of GluR6, GR, ERα all could have many implications on the cell and, therefore, hippocampal function.
A final caveat that needs to be addressed is the genetic background of the mice. We used mice with a mixed genetic background in our study. It has been shown that mice of different strains differ in their stress response [55,56]. Whereas future work with congenic strains of mice will further clarify the contribution of genetic background to the stress response, the clear differences between the mutant and wild-type mice in the stress response argues that the changes found are due to the c-fos mutation rather than an effect of the genetic background.
Figure 1. Mutant mice do not differ from wild-type mice in their CORT response at either the AM (A) or PM (B) testing times following one hour restraint stress. n=6 for all groups tested. Data shown is mean plasma CORT ±SEM at basal, stress and recovery time points. Plasma CORT measured by RIA.
Figure 2. CORT levels during 22 day 1-hour chronic restraint stress. A) female mice, B) male mice, n=6 for all groups. Data shown represent mean plasma CORT levels ± SEM at the basal, stress, and recovery time points on days 0, 7, 14, and 21. Plasma CORT levels measured by RIA.
Figure 3. Mutant mice habituate more quickly to chronic restraint stress than wild-type mice. CORT levels shown are area under the curve from Figure 2.  
A) Results from 2-way ANOVA – day by genotype. *p<0.05 comparing Day 0 to Day 21. B) Male data measured by area under the curve. C) Female data measured by area under the curve. n = 6 in each group.
**Figure 4.** Female mutant mice displayed less anxious behavior than wild-type mice in all measures of the elevated plus maze. A) Percent time spent in open arms \(\pm\) SEM, B) number of head dips \(\pm\) SEM and C) stretch attend postures (SAP) \(\pm\) SEM *p<0.05. n=9 for wild-type and mutant female mice. Male mice show no differences by genotype in any measures. D) Percent time spent in open arms \(\pm\) SEM, E) number of head dips \(\pm\) SEM and F) stretch attend postures (SAP) \(\pm\) SEM. n=6 for wild-type and mutant male mice.
**Figure 5.** Western analysis showed differences in the expression of ERα and GR but not BDNF protein levels in mutant and wild-type female mice. Hippocampal extracts were isolated from mutant and wild-type mice with and without 1-hour chronic stress treatment. A) Representative bands from western blots are shown for each protein with the actin control. B) ERα protein levels ± SEM C) GR protein levels ± SEM D) BDNF protein levels ±SEM. *p<0.05 mutant compared to wild-type of same treatment. Data shown represent mean optical density minus background optical density multiplied by the area of the band divided by the actin control. n=4 for each group.
Figure 6. The plasma CORT levels of female wild-type and mutant mice do not differ during 6 hour chronic restraint stress (B) (A) represents nonstressed control females of each genotype. Data shown as mean plasma CORT levels ± SEM at the basal time point, 1 hour and 6-hour stress time points on days 1, 7, 14 and 21. Plasma CORT levels measured by RIA.
Figure 7. A) Camera lucida drawings of a representative Golgi stained CA3c pyramidal neuron from each of the four groups of mice tested. B) Mean total dendritic length ± SEM of basal and apical dendrites. Brains were perfused sectioned and Golgi stained following 6-hour chronic stress. ANOVA revealed an overall genotype effect in apical dendrite length but no interaction between genotype and treatment. C) Mean number of branch points ± SEM of basal and apical dendrites.
REFERENCES


25. Vedeckis WV. The glucocorticoid receptor and c-jun promoters contain AP-1


42. Roullet P, Lasselle JM. Genetic variation, hippocampal mossy fibers distribution, novelty reactions and spatial representation in mice. Beh Brain Res 1990; 41:61-69


Chapter 7: Discussion and Future Directions

7.1 General Conclusions

To understand the molecular mechanisms of neuroplasticity, we used two different mouse models to examine how molecular changes in the mouse might lead to changes in neuronal plasticity and neuronal function. Through the use of these models, we studied how the change in the apoptotic abilities of the mouse affects neuronal number and function in the hippocampus. We also studied how a change in the transcriptional machinery at the cellular level alters the plasticity and function of the hippocampus at the behavioral level.

To determine how DFF45, a player in the apoptosis pathway, affects neuronal plasticity in the hippocampus, we used the DFF45 knockout mouse. We found that the mutant mouse has a greater number of dentate gyrus granule cells than the wild-type mouse and greater cell density within the dentate gyrus region of the hippocampus. The reason for the selectivity of the mutation to affect dentate gyrus cells is unknown. It is possible that this is due to the ongoing neurogenesis and apoptosis in the dentate gyrus that does not occur in other brain regions. Apoptosis during neurodevelopment may not be affected due to the importance of this event and, therefore, may have compensatory mechanisms that do not continue into adulthood. Behaviorally, we found that the mutant mice have enhanced spatial learning abilities as compared to the wild-type mice and also increased retention of recognition in a non-spatial task.
The novel object recognition task revealed a difference in recognition abilities at a three hour timepoint while the Morris water maze revealed a difference in spatial learning abilities over a long term paradigm (5 and 10 days). This difference might be accounted for by the difference in the tasks themselves. The novel object recognition task involves only the recognition of an object while the Morris water maze involves the use of distal cues to acquire the ability to locate the platform.

These results indicate that changing the numbers of hippocampal cells can alter the functions of the hippocampus. It is possible that the increased number of dentate gyrus granule cells leads to increased number of synaptic connections with the CA3 pyramidal cells. The increased connectivity could allow for increased ability for synaptic plasticity changes when learning challenges are presented. This increased synaptic strength could then lead to the enhanced learning abilities that we have found.

Hippocampal function and plasticity can be influenced by many factors. In addition to apoptosis, transcriptional regulation provides a powerful mechanism to modulate hippocampal function. To determine the role of gene regulation by c-fos in hippocampal plasticity and function, we used a hippocampal specific c-fos knockout mouse to study mechanisms of neuronal excitability, excitotoxicity, learning and memory, and regulation of the stress response. We found increased seizure behavior to kainic acid injections, increased neuronal activity during the seizure, increased cell death in the CA3 region following the seizures, and altered gene expression levels in a variety of genes, including GluR6 and BDNF.
Interestingly, in contrast to previous assumptions, we found that the spatial learning abilities of this mouse in both the Morris water maze and the Barnes maze are normal compared to the wild-type mice. It is possible that other molecular pathways are used for this process, or are compensating for the loss of c-fos in the hippocampus. For example, it has been shown that different molecular pathways are used for different types of learning [1]. It is also possible that the water maze task is not a sufficient challenge to reveal c-fos expression differences. A priming of c-fos expression prior to the test might reveal differences that were not found in our paradigm.

The regulation of the HPA axis by the hippocampus is important for controlling the stress response. To determine the role of c-Fos in this process we tested various aspects of the stress response in the knockout mouse. The mutant mice have normal diurnal CORT patterns. The stress response and recovery from acute restraint stress are also normal. However, when the mice undergo 22 consecutive days of 1-hour restraint stress, the mutant mice show a faster habituation to the stress than the wild-type mice. In addition the mutant females also display a less anxious behavior in the elevated plus maze.

To determine what might be underlying these differences we analyzed possible molecular and morphological changes in the female mice. We found that mutant female mice have less GR and more ERα in the nucleus in the basal state. Mutant female mice have higher levels of ERα in the cytosol both basally and following chronic restraint stress. We believe the changes in the levels of these hormone receptors might play an important role in the changes seen in the behavior and
habituation of the mutant mouse. Estrogen and ER have been shown to play a role in the stress response of female mice [2-5]. Additionally, GR has been shown to play an important role in the behavior in the plus maze [6].

Both of these receptors are transcription factors and both interact with the AP-1 transcription factor complex to affect their transcription factor activity [7-10]. GR inhibits the activity of AP-1 while ER activates it. The changes in these transcription factors combined with the change in the makeup of the AP-1 complex could lead to a variety of changes in downstream molecules which could lead to many changes in plasticity and function of the hippocampus. Due to the fact that c-fos and AP-1 are ubiquitous, it is almost impossible to predict how many and which genes will be affected by these changes.

The morphology of the CA3 pyramidal cells of the females was also examined following 21 days of 6-hour restraint stress. The apical dendrites of the pyramidal cells of the mutant females were longer than those of the wild-type females. This change may reflect changes in the previously described transcription factors. It is possible that the expression of cell skeleton molecules, such as neurofilaments, is altered by the changes in all of the transcription factors. It is also possible that the increase in the overall length of the apical dendrites of the female mutant mice might lead to an increased number of synapses and, therefore, stronger connections in the feedback mechanisms onto the HPA axis. These stronger connections involved in stress feedback mechanisms may explain the increased habituation demonstrated in the mutant mice.
It is possible that the molecular and cellular alterations responsible for the altered stress response also can account for the increased seizure vulnerability. The hippocampus of the mutant mouse might be more sensitive to the effects of CORT. Due to the increased sensitivity, the feedback response onto the HPA axis would be greater. Additionally, CORT increases the vulnerability of neurons to kainic acid induced seizures; an increased sensitivity to CORT could cause similar vulnerability [11,12].

The increased sensitivity of the cell may extend beyond CORT and KA. The cells might be more sensitive to many extrinsic factors. This could be due to the changes in expression levels of receptors. Thus far, we have found expression level differences in three receptors, GluR6, ERα, and GR. It is possible that many more receptors are expressed differently as well. By changing receptor levels in the cell, alterations occur in the cell’s reaction to the ligand of the receptor. Whether the receptors are ion channels that can control the degree of excitability of the cell, or hormone receptors that act as transcription factors and control gene expression, alterations of the expression of these molecules may have impact on numerous cellular processes, such as synaptic plasticity.

7.2 The use of knockout mice

Transgenic and knockout mice provide a useful tool for studying the role of specific molecules in an animal. In contrast to pharmacological studies, which can be “dirty” and non-specific, transgenic mice have specific, controlled changes. Knockout mouse models such as the PKCα and *-/- and mGluR1/-/-
have contributed to the knowledge of the molecular pathways in learning and memory [13-16]. Knockout mice have also helped establish an understanding of the molecular mechanisms behind sexual behavior, seizure behavior, and feeding behavior [17-19].

However, there are caveats that need to be addressed when using transgenic or knockout mice. Many questions have been raised as to the use of knockout mice to study the affects of specific molecules. The loss of a molecule can lead to developmental deficits, which make it difficult to perform and interpret behavioral tests on the mice. At times, the knockout even results in lethality of the animal. Additionally, it is possible that the animal has compensatory or redundant mechanisms that would mask any functions of the knocked out molecule [22,23].

The DFF45 knockout mouse is a complete knockout, so the molecule is absent throughout development and throughout the entire mouse. As stated earlier, this can lead to developmental deficits, as well as compensation by related molecules. These factors often make it difficult to interpret behavioral findings using the mice. However, we used proper controls to ensure that all changes we found were due to the loss of DFF45 and not the genetic manipulations. Furthermore, we examined the animal for developmental deficits, which indicated developmentally the mouse was normal, thereby allowing us to rule out these parsimonious explanations for the behavioral changes we found in the mice.

One way to circumvent many of the issues involved with the use of knockout mice is to use the loxp/cre system to develop a temporally and spatially specific knockout mouse. In this system, the expression of cre determines when and where
the knockout will occur. Our system uses a mouse with cre expression driven by the CAMKIIα promoter. This results in cre expression in the hippocampus proper and the dentate gyrus beginning in the third postnatal week. When the mouse expressing cre is crossed with a mouse that has a gene flanked by two loxp sites, or ‘floxed’ the cre expression causes that gene to be deleted when and where the cre is expressed. In this case, the deleted gene is the c-fos gene. Because the c-fos gene is present during development, these mice do not suffer from the severe defects that the complete c-fos knockouts do [22-24]. The conditional knockout, therefore, allows us to perform behavioral tests in which complete knockouts simply cannot be used. There is also less chance that the mice have developed compensatory mechanisms for the loss of c-fos. Additionally, since the loss of c-fos is only in the hippocampus at the time of testing, we can interpret the data as a result of changes in the hippocampus. With such a ubiquitous molecule, it would be almost impossible to interpret any changes found in a complete knockout. Again, the proper controls must be used, with the cre expressing mouse and the mouse with the “floxed” gene also being tested to ensure that neither of these manipulations contributed to our findings.

Finally, these studies have all used mice with mixed backgrounds. Data indicate mouse strain has profound effects on behavior [25-27]. Therefore, interpretation must account for strain differences. Future work with congenic strains of mice will further clarify the contribution of genetic background to the behaviors that we have tested. However, the clear differences we found in all of our studies along with the controls that we ran argue that the changes we found
are likely due to the specific mutations and are not an effect of genetic background.

7.3 Future Directions:

7.3.1 DFF45 knockout mouse

There are many questions to answer regarding molecular mechanisms of neuroplasticity. We found that the loss of DFF45 results in a greater number of DG granule cells and enhanced learning and memory abilities. This immediately brings about additional questions. What molecular mechanisms of a greater number of cells leads to the increased learning abilities?

The synapses on CA3 pyramidal cells need to be studied. The question of whether a greater number of dentate gyrus cells directly leads to a greater number of neurites connecting with mossy fibers of the CA3 region needs to be addressed. If this is the case, there are two possible results from that change in the number of connections: a greater number of synapses or simply a lower density of synapses. If the number of synapses in the mossy fibers is greater, that could begin to explain the enhanced learning abilities found in the mouse. The electrophysiology of the system should also be examined. The neuronal excitability following stimulation of the hippocampus should be measured. These studies would answer questions as to whether additional DG cells results in stronger hippocampal circuitry and whether this contributes to the resulting enhanced learning abilities. Once the molecular and
cellular mechanisms underlying the enhanced learning abilities in the mutant mouse are better understood, it is possible that this knowledge could be used to develop therapies to alleviate memory deficits, especially in aging.

Finally, other hippocampal functions, such as the stress response, should be studied in the DFF45 mutant mouse. The additional granule cells in the dentate gyrus could strengthen the feedback mechanism onto the HPA axis, thus lowering the overall stress response of the mouse. The plasma CORT levels following both acute and chronic restraint stress should be measured. The anxiety behavior of the mice in the elevated plus maze could also be evaluated.

### 7.3.2 Hippocampal c-fos knockout mouse

To continue to address the interesting phenotype that has been revealed in the hippocampal c-fos knockout mouse, there are many studies that can be performed. First, estrogen levels need to be measured in the female mice that are undergoing the chronic restraint stress to discover whether or not estrogen could be directly affecting the stress response or the expression of ERα. The stress tests should also be performed on ovariectomized mice to rule out the chance that the phase of the estrus cycle is causing the effects.

In order to determine whether or not the stress response change is gender specific, behavioral tests that male mice are more sensitive to should be performed, and plasma CORT levels should be measured during these tests. The Western analysis and Golgi analysis should also be performed on male mice, to see if the molecular and morphological changes are specific to female mice. Depending on the
results of both the behavior studies and the molecular studies, various conclusions can be drawn. If the molecular and morphological changes are specific to female mice, it means that the cells of female mice are more sensitive to the loss of c-fos than male mice. If molecular and morphological changes similar to those found in female mice are also found in male mice, but the behavioral changes are not, that would mean that the behavior of female mice are more sensitive to the molecular and morphological changes than male mice. This could be due to the hormonal differences between the genders and the fact that the steroid hormone receptor ERα is one of the molecules whose expression is changed. Finally, if male mice show similar molecular, morphological and behavioral differences to female mice, this would reveal that the differences we have found in the mutant mice are not gender specific, but must be studied differently in the two genders, based on intrinsic behavioral differences between the two.

The mechanisms behind the plastic changes in these mice need to be determined as well. The molecular mechanisms underlying the difference in dendritic branch length in the mutant mice need to be studied. Additional experiments such as Western analysis or proteonomic analysis should be performed to examine protein expression levels of molecules, such as neurofilaments, that might play a role in the neurite length differences. Due to the number of transcription factors that are altered in this model and their wide spread distribution throughout the brain, it would be difficult to predict which downstream genes would be altered. A possible way to address this is to examine gene expression changes on a large scale by performing microarray assays.
The number of mice used for the Golgi analysis needs to be increased also to see if there is a stress effect, as it appears that there might be. If there is a stress affect, the cause behind this change in plasticity needs to be determined. It could be directly related to the change in the ERα as ER has been shown to play a role in neurite outgrowth [30,31].

Learning experiments in c-fos mutants following chronic stress may provide further insight into the stress response of the mutant mice. Stress has been shown to cause learning deficits [32]. It is possible that the habituation abilities and the possible morphological changes in the mutant mice might prevent or attenuate those deficits.

In conclusion, these experiments have facilitated the understanding of neuronal plasticity and how the knockout of specific genes leads to changes in neuroplasticity. They have provided evidence indicating the importance of certain aspects of the mechanisms underlying neuroplasticity and examined how these changes lead to functional and behavioral changes. Most importantly, the results found in the experiments have uncovered a number of directions for future studies to address.
REFERENCES


