Activity- and pharmacology-dependent modulation of adult neurogenesis in relation to Alzheimer's disease

Marlatt, M.W.

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Chapter 9: General Discussion

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Preface

Adult neurogenesis is known to play a critical role in learning and memory while Alzheimer disease is primarily viewed as a disease of cognition and memory dysfunction. In terms of neurobiology, the two topics converge in the hippocampus, a brain structure critically important for learning, memory, pattern separation, and executive functions that manifest as human behavior. This thesis aimed to improve our understanding of neurogenesis and the relevance of this process to Alzheimer disease. To do this, experiments were pursued across different models to evaluate distinct research questions. Ultimately, as basic research is collected on each topic we are approaching a more integrated understanding for the therapeutic potential of adult neurogenesis for AD. A few paragraphs will conclude each topic covered, followed by a discussion of new directions relevant to work compiled here.

1. CHAPTER SUMMARY

In Chapter 3 we studied the consequences of psychosocial stress on neurogenesis in middle-aged common marmosets, a new world primate. Importantly, neuroblasts were found in the basal and lateral nuclei of the amygdala at a dramatically higher density when compared to the hippocampal dentate gyrus. We further showed that these DCX-positive immature neurons were migratory as shown by PSA-NCAM co-expression. Similar cells were also seen in the entorhinal cortex of this species. Our findings, and those of others, show that immature neurons are present in the amygdala and entorhinal cortex respectively, where they may serve an important role in structural plasticity and behavior. We established that hippocampal neurogenesis in marmosets is not sensitive to psychosocial stress exposure when measured after two-week recovery period. Similarly, stress failed to change the density of neuroblasts present in the amygdala.

In Chapter 4, we studied proliferation of neuroinflammatory cells in the aged human hippocampus (>70 years age) in relation to amyloid pathology and AD. Our work followed up a primary publication showing that proliferation was increased in AD but occurred largely in glia-rich regions of the hippocampus. In our study, we co-labeled the proliferation marker PCNA with GFAP expressing

astrocytes, and Iba1 expressing microglia, two glial subtypes found in the brain. While astrocytes failed to co-express proliferation marker PCNA, we demonstrated that Iba1 expressing microglia proliferate in the AD brain. This phenomenon was observed across disease conditions and in the presence of Aβ plaques, indicating that Aβ plaques spur microglial proliferation. This suggests that microglial proliferation occurs during the early stages of disease, making an unknown contribution to neuroinflammation and the subsequent progression of cognitive decline and dementia.

In Chapter 5 we tested the ability of a novel antidepressant drug to stimulate neurogenesis in the dentate gyrus of young female C57Bl6J mice. We compared the ability of duloxetine, a dual-pharmacology SSRI/SNRI, to induce neurogenesis against the SSRI fluoxetine and physical activity i.e. wheel running. Our findings indicate that neither drug was able to improve the survival of new neurons in the dentate, although increased neuronal differentiation was observed for fluoxetine. Behaviorally however, the animals treated with fluoxetine and duloxetine experienced higher levels of anxiety compared to control animals. Compared to the minor effect of fluoxetine, wheel running produced a profound increase in neurogenesis that would be the basis for our follow up experiment in middle-aged mice.

In Chapter 6 we tested the ability of physical activity to stimulate neurogenesis and hippocampal function in middle-aged female C57Bl6J mice. We found that mice allowed to exercise through long-term wheel running performed better in a spatial memory test compared to control animals. Our data shows increases in cell survival that were paralleled by elevated BDNF protein levels in the dentate gyrus. This indicates that prolonged wheel running can increase long-term cell survival and reverse age-dependent deficits in spatial memory. BDNF likely plays an important role in mediating the effect of wheel running, but we did not study how this occurs. These data sets reflect that prolonged exercise in mid-life has beneficial effects on both hippocampal structure and function.

In chapter 8 we report on a similar experimental paradigm as described in chapters 5 and 6 that utilizes the 3xTg Alzheimer mouse model. In this experiment, pharmacology and physical activity are combined to evaluate the ability of synergistic treatment to reverse deficits in neurogenesis. Wheel running significantly increased neurogenesis in 3xTg mice but did not significantly elevate BDNF as

seen in the C57Bl6 study. Surprisingly the 3xTg mice never exhibited deficits in spatial learning and memory, disassociating the relationship between neurogenesis and spatial learning and memory performance. This finding is reviewed carefully, with special attention for the genetic composition of the 3xTg mice, the phenotype of these animals, and the behavioral tests employed.

2. REVISTING NEUROGENESIS IN ADULT MARMOSETS

2.1 Evidence for the temporal stream and amygdala bound neuroblasts

Our findings in the marmoset study follow up on earlier work carried out in rhesus and squirrel monkeys by Rakic and Bernier, among others (1-3). We found extensive populations of DCX expressing neuroblasts in the amygdala and entorhinal cortex of the middle-aged marmoset. Previous work established that adult neurogenesis decreases dramatically with aging paralleled by significantly reduced DCX expression in the rat hippocampus (4-7). This paradigm is conserved in humans, stem cell proliferation and new neurons survival are significantly reduced as age increases (8-10). As such, these large populations of neuroblasts in the marmoset amygdala were unpredicted. Migration from the SVZ to the amygdala was observed via the temporal stream, a unique migratory path from the sub ventricular zone to the amygdala. However we don't know if migration during adulthood is responsible for the entire population of immature cells, or if alternatively, the cell population exists during development and is continuously supplemented by the temporal stream over time.

As DCX was widely expressed in the basal and lateral nuclei of these middle-aged animals, our finding challenges the view of an exclusive transient expression of DCX. Other studies have shown that persistent expression of DCX does occur to a lesser extent in other brain regions(11). Additional evidence strongly suggests that maturation of new neurons is significantly longer. This phenomenon, delayed maturation, has been documented in rodents (6,12) and extended to primates, where the maturation time for new DG neurons in the aged macaque monkey was shown to exceed 6 months (13). While this finding has not been demonstrated for the amygdala, such an extended maturation/differentiation time, and thereby a longer time window to

detect DCX expression, can at least partially explain the presence of the high number of cells. This also explains why DCX cells were much more prevalent than BrdU-labeled cells in the same area.

Despite the high density of DCX+ cells in the amygdala, particularly when compared to the hippocampus, we can only speculate on the exact functional role of these cells in the amygdala. DCX has an established functionality as a microtubule associated protein with additional roles in intracellular vesicle transport, migration(14) , and glia-to-neuron interactions (15). Long-term expression, starting during early life, of DCX and PSA-NCAM has been documented previously, consistent with a functional role for DCX in mature cells of aged animals(16). The nature of these cells can be better defined once their location and connections are more thoroughly mapped e.g. compared with lower species.

Regarding stress and the amygdala, other studies have shown that stress can impact plasticity. Early social-isolation stress affected protein markers of structural plasticity, namely synaptophysin, GAD65, GAD67, and PSA-NCAM, in the basolateral amygdala of postnatal rats (17). Maternal separation during early development alters behavior and neuronal systems in the offspring of rats and mice. Early weaning in mice results in a persistent increase in anxiety-like and aggressive behavior in adulthood, neuroendocrine stress responses, features that have been associated with specifically precocious myelin formation in amygdala(18). Whether the amygdala of marmosets is also affected by early life events remains to be determined. For this, addition experiments need to be undertaken to study the marmoset amygdala during development. Interestingly, in human studies, psychosocial factors from early childhood are correlated with amygdala volumes in adulthood(19), while prenatal stress is known suppress cell proliferation in various developing brain areas including the amygdala(20,21). Ideally, a longitudinal study for the effect of aging could be carried out in marmosets to identify the persistence of cell population in the amygdala.

In adulthood we found that the amygdala and hippocampal bound DCX cells were not reduced by psychosocial stress exposure. This contrasts with the situation in rats, social stress was recently reported to reduce DCX cell numbers for months after the stressful event(22). This suggests that the recovery period after stress in the marmosets may not have influenced our results to a major extent.

However, this would require dedicated experiments to be substantiated (see section 2.3). At this time we cannot exclude species differences in the sensitivity to stress. This is supported by the fact that we also did not observe any effect of sex differences, whereas in the developing rat amygdala clear sex-differences in neurogenesis were reported (23).

2.2 The case for neural stem cells in the entorhinal cortex

We also documented the presence of DCX /PSA-NCAM labeled cells in the entorhinal cortex. When our study was initiated no concrete evidence had been published regarding neural stem cells in this area for primates, although earlier reports had identified their presence in rodents(11). DCX/PSA-NCAM labels migrating cells, but cells in the entorhinal cortex are not derived from the temporal stream. We found no evidence of a migratory path from the SVZ to the entorhinal cortex as BrdU-labeled cells and PSA-NCAM would have identified a migratory path between the two areas. Rather, experimental evidence suggests neural progenitors are indeed present in the entorhinal cortex of marmosets. Work in a transgenic rodent model has established that stem cell progenitors capable of producing DCX-positive cells are present in the entorhinal cortex (24). This is consistent with the BrdU-positive cells we found in this region. These published findings complement our study and strongly suggest that progenitor cells, capable of generating new neurons, do exist in the entorhinal cortex of middle-aged marmosets. This could be further supported through cell fate analysis, co-imaging BrdU-labeled cells with phenotypic protein markers to trace maturation and cell lineages.

2.3 Brain region specific stress effects

So far, relatively few studies have investigated regulation of neurogenesis by stress, running, or antidepressants in primates. Regarding stress, previous work had established that psychosocial stress reduced proliferation of stem cells within the primate SGZ (25) but other regions were not studied. In our paradigm, animals were exposed to 2 weeks of psychosocial stress, defined by an isolation and dominant-intruder paradigm, which was followed by 2 weeks of re-

socialization before sacrifice. This paradigm induced a significant cortisol response. It remains to be seen whether a more severe stressor, or e.g. a shorter recovery period, might have resulted in a different impact of neurogenesis in this brain region. More severe stressors, with appropriate ethical oversight, can be administrated to determine the acute and long-term responses of the DCX+ cell populations. Likewise, more careful biochemical analysis of the amygdala can yield hints about the susceptibility of these cell populations to stress-induced changes.

2.4 Future directions for research on the primate amygdala

Our first report on neuroblasts in the marmoset amygdala is important because the amygdala plays an important role in behavior and emotion, further study – in addition to the issues raised in sections 2.1-2.3- should provide a more detailed understanding of the specific nuclei that contain DCX+ neuroblasts and their functional role in the amygdala. Some important remaining questions are:

- Aging reduces stem cell proliferation in the SGZ and SVZ in marmosets(25) and maturation of new neurons has been shown to exceed 6 months in middle-aged macaque monkey(13). What specific changes occur during aging of the brain that mediate the reduction in progenitor proliferation and the delayed maturation of new neurons in the dentate gyrus of primates?

- Does aging delay the maturation of neuroblasts in the amygdala in a similar manner?

- What role do these proliferating cells play in emotional memory and behavior?

3 MICROGLIAL CHANGES IN THE ADULT BRAIN: RELEVANCE TO ALZHEIMER'S DISEASE AND NEUROGENESIS

3.1 Shifting the neuropathological criteria to include earlier events

Chapter 1 describes the behavioral and clinical changes associated with Alzheimer's disease and the important role that microglia play in amyloid plaque metabolism. One of the main efforts of Alzheimer's disease research is to distinguish normal physiological changes within the aging spectrum from pathological changes leading to dementia and AD. The pathological cascade of events that eventually manifest in Alzheimer's disease has a detectable pre-clinical phase, where subtle changes in the brain may be measured as subtle behavioral deficits. As more biochemical, imaging, and behavioral data has been gathered, the criteria for diagnosing AD has been revised under these principles (26). As described earlier, a normal immune response to Aβ accumulation occurs to remove excess Aβ from the brain.

Chapter 4 explores the role glia cells play during amyloid plaque accumulation and disease progression. Whether microglia can remove, or limit Aβ plaque growth, successfully without spurring a downstream inflammatory cascade remains to be determined. The topic is particularly interesting because removing Aβ plaque remains a primary endpoint for many drugs currently in clinical development. Our study was designed to measure proliferative changes in individuals with AD pathology (aged >70 yrs). Cohorts with matching mild pathology (Braak stage 1-2) but different cognitive status i.e. demented vs non-demented were compared to an AD cohort with heavy pathology (Braak stage 4-5).

3.2 Microglial proliferation in human tissue samples

Our study shows that microglia proliferate in the brain regardless of mental state or severity of AD neuropathology. While we saw proliferating microglia across cohorts, there was a trend for increased proliferation in the dementia cohort. This suggests that microglial proliferation is a distinct feature of disease progression

correlated with a change in cognitive state. Clearly, the study should be expanded dramatically to increase the number of samples included.

There is wide interest in using human brain samples to more clearly identify factors responsible for Alzheimer's disease progression. A similar study evaluated reactive astrocytes in the vicinity (<50 μm) of dense-core Aβ plaques and tangles showed that Aβ load reached a plateau early in the disease, while glial responses increased linearly throughout the disease course(27). Stereology-based quantification was performed on 15 controls and 91 AD patients, identifying a positive correlation between GFAP reactive astrocytes and tangle burden. The authors hypothesized that a certain threshold of Aβ plaque burden is needed to trigger a glial response; this triggering effect leads to a pathogenic cascade increasing independent of Aβ plaques(27). This study highlights that large sample cohorts are often needed for statistically accurate quantification of cellular and morphological changes occurring during Alzheimer disease; AD-type neuropathology is known to have high biological variability(28).

Proliferation is strongly suggested to occur due to increases in Aβ plaque volume(29). Accurately measuring plaque volume in vivo was previously conducted by two-photon excitation (2PE) microscopy. 2PE-scanning microscopy allows for high-resolution and high-sensitivity in intact brain tissue, but it is not used in living human subjects for a number of ethical reasons(4). While the number of Aβ plaques load is known to increase in a hierarchical manner through the brain(30), there has been no simultaneous effort produced to identify plaque volumes with disease stages, i.e. hierarchical Braak stages.

Postmortem quantitation of individual plaques was formerly the only standard method of evaluating plaque density. Currently a number of imaging modalities allow AD to be measured in living patients. Whole brain imaging can identify glucose utilization and Aβ plaques in living subjects (reviewed by Johnson)(31). Longitudinal cognitive decline is associated with Aβ plaque deposition(32), however Aβ deposition occurs prior to clinical decline the presence of Aβ plaque alone is not sufficient produce cognitive deficits(33). It is now possible to detect individual Aβ plaques in vivo at a resolution of 35 μm, and ex vivo at a resolution of 20 μm(34). If we assume that early accumulation of Aβ corresponds with increases in plaque volume, we would therefore conclude the microglial proliferation occurs during the early stages of disease. A dedicated study of plaque volumes and

subtypes, in coordination with microglial engagement would answer these questions.

We know that ultimately, Aβ is not successfully removed from the brain during Alzheimer's disease, suggesting that progressive neuroinflammation accompanies progressive amyloid plaque accumulation. The data presented in this thesis argues that early changes in microglial activation and proliferation linked to Aβ deposition are critical for the disease outcome.

3.3 Microglia and synaptic plasticity

To be clear, we did not measure neurogenesis in the human subjects described in Chapter 4. Previous unpublished observations indicated that markers of neurogenesis would be expressed at low levels precluding a valid comparison between the test cohorts. However, microglia play an important and possibly even critical role in neurogenesis (reviewed by Ekdahl)(35); microglia are known to shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis within 2-4 days of cell birth (36), further highlighting the importance of the neurogenic niche in regulating neurogenesis(37). Interestingly, this work has shown that microglia, identified by Iba1 and low levels of CD11b and CD68, are not activated by LPS injection and maintain their ability to carry out phagocytosis(36). In a similar manner, during ischemic stroke, microglia are known to migrate into the SVZ; these microglia are considered proneurogenic and allow for persistant SVZ neurogenesis (38).

Adult neurogenesis is complemented by synaptic plasticity and an appropriate regulation of synaptic connections. Normal functionality of microglia is seen in association with synaptic pruning (reviewed by Temblay) (39,40). We do not know at this point if aged microglia and/or activated microglia can exert these synaptic plasticity functions during AD. There is reason to believe that impaired synaptic pruning can have dramatic effects. Autism and schizophrenia are developmental problems characterized by aberrant connectivity; improper microglial pruning extranumerary synapses is suspected to contribute to these disorders(41). The etiology of schizophrenia is unclear, however there has been a recent focus on microglial activation and inflammation(42) including models documenting morphological changes in microglia of the dentate gyrus(43). Microglial cells are

likewise implicated in autism disorders. Rett syndrome is an X-linked autism spectrum disorder characterized by a mutation in MECP2(44). The disease can be experimentally prevented by peripheral IV-injection of bone marrow of C57Bl6 mice into transgenic mice with Rett syndrome (MECP -/y). This results in the production of bone-marrow-derived microglial cells; pharmacologically inhibiting phagocytic activity of these microglia significantly diminishes the benefits of transplantation(45).

3.4 Neuroinflammation and adult neurogenesis

Microglial activation and proliferation are known to occur simultaneously, however the temporal expression of activation and proliferation markers has not been systematically documented. Our study identified activated microglia (Iba1+), with a strictly proliferative marker, PCNA, which has no direct role in inflammation. As discussed in Chapter 4, the P2X7 receptor is thought to drive microglial activation and proliferation(46). Blocking the receptor severely decreases proliferation, while overexpression through transfection drives proliferation(47).

Our findings regarding microglial proliferation correspond with results produced in a mouse model of AD; activated microglia, but not the astrocytes, were found to proliferate in the presence of Aβ plaques (48). During aging and increasing Aβ plaque deposition, the authors found a 41-fold induction of microglial proliferation in AD transgenics compared to non-transgenic mice(48). Proliferation corresponded with transcriptional changes in neuroprotective and inflammatory markers. If this accurately reflects the human condition, we would expect a large induction of microglial proliferation by Aβ plaques. Microglia are known to be significantly altered by aging and appear to become more pro-inflammatory under pathological conditions. For instance, ex-vivo microglia cultures isolated from aged mice have shown elevated production of proinflammatory molecules Il-6 and TNF-α(49). The ability of microglia to internalize Aβ may be further compromised as microglia from old animals have a decreased ability to phagocytose Aβ compared to young animals (49).

There is substantial evidence indicating that inflammation reduces neurogenesis. Acute inflammation, induced by LPS, is known to have a deleterious effect on neurogenesis(50). Interleukin 1 (IL-1β)

reduces neurogenesis through activation of the kynurenine pathway(51). This suggests that neuroinflammation can nonetheless reduce the production of new neurons.

Our present data further reflect that microglia proliferate within the brain. Since peripheral microglia cells are capable of invading the brain, where they can contribute to or prevent further neuropathological progression, further study of the Iba1 immunopositive cells can identify resident cells or infiltrating microglia cells.

3.5 Future directions on microglial proliferation in AD

Studying the neuropathology of Aβ deposition, microglia, and inflammation systematically during progressive disease stages would provide definite results about the contribution of microglial proliferation and inflammation in aged individuals. Single time-point measurements cannot address the role of microglia in disease causality. This can be accomplished by monitoring microglial proliferation around Aβ plaques through in-vivo 2PE imaging. This technique can additionally be combined with longitudinal mouse study to establish when microglia become inflammatory. In this regard, stereological methods have been established for counting astrocytes and microglia in the brains of aging mice(52,53).

- Do proliferating microglia excrete the proinflammatory cytokines? Is this response dependent on disease stage or the proximity of astrocytes to an Aβ plaque?

Proliferation of endogenous, resident microglia has been documented as occurring prior to infiltration from bone marrow derived microglia(54,55). To address the relevance of peripheral blood-derived microglia to inflammation, parabiotic mice i.e. mice with conjoined blood supply can be studied. This allows labeled bone-derived microglia to be identified, along with their involvement with A deposition.

- Does Aβ deposition trigger the infiltration of bone-marrow derived cells?

Passive immunotherapy in a mouse model of AD shows that acute treatment has a therapeutic effect on dendritic spines, allowing for a significant increase in dendritic spine formation far away from plaques. This treatment did not have any effect on spine plasticity near plaques(56).

- Will therapy directed at removing Aβ via activation of microglia reduce neuroinflammation and restore the normal function of microglia?

4. DISCUSSION ON ACTIVITY AND PHARMACOLOGY-DEPENDENT NEUROGENESIS

4.1 Defining basic questions

In chapters 5, 6, and 8 we tested methods of inducing adult neurogenesis through pharmacology and wheel running in inbred wildtype mice and in a triple transgenic mouse model of Alzheimer's disease. By inducing neurogenesis we aimed to reverse age and disease-related deficits in neurogenesis and behavior. We addressed the following questions:

1. Can duloxetine, a dual pharmacology, SSRI/NRI stimulate neurogenesis in young C57Bl6 mice? How does this compare to stimulatory effect of running?
2. Can long-term running, administered from middle age onwards, modulate neurogenesis and age-mediated behavioral changes?
3. Can a combination of pharmacology and running show a synergistic effect in the 3xTg model of AD? Will these methods show neuroprotective effects that prevent behavioral deficits or alter the accumulation of AD neuropathology in these animals?

In the following sections I discuss advances made toward answering these questions and explore associated areas that improve our understanding for these topics.

4.2 Sexual dimorphisms in behavior

Female mice were used in all three studies. Sex differences in spatial learning and memory have been document; male and female humans and rodents show remarkable differences in the visual cues

they use to orient themselves. This has been described for the radial arm maze where gonadal hormones induce qualitative differences in visual-spatial navigation in rats. The two sexes perform comparably in the test under standard conditions, however female rats are highly dependent on landmarks within the context while male rats use the geometric shape of the context as an anchor and can navigate better when the landmarks are moved or hidden(57) .

In humans, men and women have distinct advantages for different spatial tasks, as demonstrated in mental rotation tasks(58). We know that the hippocampus is involved in these tasks however the different behavioral performance seems to be linked to subtle differences in pre-frontal and parietal cortex activation during navigation. Unfortunately imaging data at this time is contradictory so we don't know with certainty why these differences in behavior occur.

Overall, data from studies that exclusively use females cannot be generalized indiscriminately to male subjects. Our conclusions in chapters 5, 6 and 8 therefore may not apply to male rodents and male vs. female performance should be evaluated prior to generalizing about behavior in both sexes.

4.3 Refining antidepressant-induced neurogenesis

An escalating dose scheme showed that duloxetine, a dual SSRI/SNRI, did not alter neuronal survival or cell fate, when compared to running and fluoxetine. Fluoxetine had a partial effect in increasing neuronal differentiation. Our results were somewhat surprising as previous work had established that subcutaneous injection of fluoxetine (10 mg/ml for 15 days) increased proliferation of amplifying progenitor cells, as measured by BrdU and cyan protein expression in a transgenic reporter mouse (59) (60). Since we were interested in the behavioral impact of the new neurons we only measured survival of BrdU-positive cells and not proliferation. Our data suggested that both mouse age and strain influence sensitivity to fluoxetine-induced neurogenesis. This was also established by other studies showing that fluoxetine-mediated survival only occurs in mice less than 3 months-old; when older animals are included, aging abolishes the effect on new cell survival in a strain dependent manner (61) (62). Our results also agree with comparisons of several inbred mouse strains in which responses to fluoxetine were found to depend on inherent predisposition for serotonin-induced

neurogenesis (63,64). Separate experiments have now confirmed that chronic, i.e. 4-week administration fails to induce neurogenesis in 9-week old BALB/C mice(65) or in Sprague-Dawley rats, aged 1, 2.5, and 12 months (66).

When I started my experiments, the generally accepted view was that antidepressants promote proliferation and neurogenesis. Meanwhile it has become evident –also from work described in this thesis- that the effect of antidepressants on neurogenesis cannot be generalized and depends, among other things, on age and strain.

4.4 Antidepressants and anxiety related behavioral changes

In regard to behavior, we reported on anxiety phenotypes that were measured in an open field task. Under the hypothesis established earlier, increases in neurogenesis were expected to result in lower levels of anxiety in the animals. However, the drugs fluoxetine and duloxetine reduced the time spent in the center of the maze, unexpectedly indicating higher levels of anxiety. Although running produced a robust effect on the survival of new cells in the dentate, this did not correspond with any measureable change in the open-field assay. Clearly, in this mouse strain, there is no correlation between induction of neurogenesis and decreased anxiety-related behavior when measured in the open-field task. A study utilizing the more anxious Balb/C mouse strain found that fluoxetine did increase center time i.e. reduce anxiety, suggesting that only anxious mouse lines will show a decreased anxiety in the test (67).

The open-field assay was used consistently at the National Institute on Aging to evaluate anxiety related behaviors. However, the open-field test may not be the ideal behavioral assay to measure the anxiolytic effects of pharmacology-induced neurogenesis. In the seminal findings regarding fluoxetine and neurogenesis, the authors tested animals in a Novelty Suppressed feeding (NSF) task (68). Recently an ablation model showed that mice lacking neurogenesis exhibited longer latencies to feed in the same task, i.e. an anxiety phenotype(69). Collectively the experiments confirm that the NSF task is sensitive for measuring anxiety behaviors associated with a gain or loss of neurogenesis. Both articles conclude that increased neurogenesis is needed for antidepressants to induce their behavioral effects when measured in these specific tests.

While the NSF is well accepted by the field, only one study has established that wheel running simultaneously increases neurogenesis and decreases latency to feed in the NSF task(70). In addition to a dependency on the age or strain of the mice, or on the class of drugs tested, behavioral effects of antidepressant drugs are known to have neurogenesis-dependent and neurogenesis-independent effects(63). Measured anxiety is often specific for a mouse-strain and particular experimental conditions. Ideally, a behavioral phenotype is measureable across behavioral tests. So far, there is no standardization of procedures between institutions that ensure that protocols are carried out in a similar fashion. Behavioral experiments are additionally sensitive to factors that are not systematically documented.

4.5 Future directions for studies on pharmacology-dependent neurogenesis and behavior

- What mechanism is responsible for fluoxetine-induced neurogenesis? Perhaps this will lead to more potent compounds?

The study of duloxetine established that that this drug does not induce neurogenesis in this model, however research on fluoxetine and duloxetine is far from complete. Fluoxetine is known to pharmacologically block the serotonin reuptake transporter (SERT) receptor, blocking uptake of serotonin, chemically known as 5-hydroxytryptamine (5-HT), from the synaptic cleft. Historically, this singular pharmacology is the most referenced mechanism of action for fluoxetine. However, additional research indicates that fluoxetine has high affinities for other 5-HT receptor subtypes, acting as an agonist or antagonist depending on the particular receptor(71,72), modifying behavior independent of SERT(73). Genetic analysis has shown that fluoxetine eliminates 5-HT from specific neurons and acts independently of the SERT receptor to regulate 5-HT receptors and downstream targets such as acetylcholine, GABA, and glutamate neurotransmission.(74).

GABA and glutamate play critical roles in the maturation of new neurons in the dentate (reviewed by Mattson) (75,76). GABAergic excitation promotes differentiation and integration during adult neurogenesis(77,78). As such, the downstream effects of fluoxetine

continue to be updated. If fluoxetine does impact the maturation of new neurons in a GABA-dependent fashion, we would anticipate activation of cyclic-AMP response element binding protein (CREB)(79). By manipulating the expression of SERT, 5-HT, and GABA receptors, identifying the major pathways responsible for neuronal maturation and survival could lead to the further identification of new pharmaceuticals with the ability to modulate these pathways.

4.6 Wheel running as a potent inducer of neurogenesis and BDNF

As we documented, wheel running mediated an increase in neurogenesis and prevented an age-mediated loss of spatial memory. Age-related deficits in spatial navigation tasks, such as the Morris water maze (Morris et al., 1982) occur earlier in females than males (80). The sex-dependent effect on MWM performance has not been systematically analyzed. Isolating sex-hormone effects from other genes contained in the X chromosome can be accomplished by using mice transgenic for sex-determining Sry gene, making it possible to create chromosomally female mice that develop male testes and vice versa(81). This provides a model system for evaluating sexually dimorphic neural and behavioral traits(82).

We observed increased BDNF protein in the dentate gyrus in mice with access to running wheels, but stopped short of experimentally proving that BDNF is solely responsible for increased neurogenesis and preventing the age-related behavioral deficit. BDNF KO mice have been created to evaluate the role of BDNF in the brain. BDNF null mice die with the first few weeks of birth, so only heterozygous mice have been tested. These mice show reduced levels of BDNF mRNA(83), learning deficits, and impaired hippocampal LTP(84,85). However, other reports have shown that heterozygous BDNF mice, while having reduced LTP, show no change in spatial memory(86). This underlines, that in the wheel running paradigm, BDNF is not the only neurotrophic factor positively induced. Exercise, such as wheel running, is known to increase levels of BDNF, but simultaneously increases a host of other factors predicted to benefit brain plasticity (reviewed by Cotman)(87).

It had been established earlier that BDNF is reduced during aging (80) and reduced BDNF has also been observed in human AD

brain tissue(75), suggesting it may serve as a therapeutic target for maintaining neurogenesis, preserving neuronal integrity, and protecting against Alzheimer disease. Indeed, the neuroprotective effects of BDNF have been verified in rodent and primate models of AD (88). While many beneficial downstream effects of BDNF have been observed, there are multiple mechanisms activated by BDNF binding. BDNF binds multiple receptors, namely p75NTR and TrkB, which contribute to the downstream effects of BDNF induction(89).

In conclusion: we observed upregulation of neurogenesis and BDNF protein after wheel running that was associated with improved behavioral performance, however we there is no experimentally derived evidence that BDNF was the critical mediator of behavioral effects. This would require dedicated experiments, preferably with transient inactivation of specific pathways downstream of BDNF.

4.7 Future directions for activity-dependent neurogenesis

Wheel running induces a number of changes in the hippocampus, not only promoting the survival of new cells in the dentate. We did not get to explore the fact that wheel running acutely and transiently increases the number of type-2 progenitor cells(90) in addition to the survival effects(91). How these effects are mediated will continue to be explored. We established that BDNF is induced during wheel running, but that BDNF does not exclusively mediate the effects of wheel running.

- What other neurotrophic factors are critical to maintaining the brain? Do these factors have a therapeutic capacity for treating Alzheimer disease?

FGF2 is known to directly stimulate adult neurogenesis(92), and to protect the brain during traumatic brain injury(93). Fibroblast-derived growth factor (FGF2) can restore neurogenesis in the brain of an AD mouse model (94). Whether activity increases FGF2 remains underdetermined but this neurotropic factor may play a critical role in neurogenesis and neuroprotection.

Walking has alone, shown an ability to increase hippocampal CA1 long-term potentiation(95). Repetitive motor movements, such as walking, trigger changes in hippocampal theta rhythms, which may be responsible for these changes in LTP and neurogenesis. Kempermann points out that cholinergic input from the medial septal area is responsible for theta rhythms and adult neurogenesis(96,97). At this time, theta rhythms are mostly seen very briefly during sleep/wake cycles(98). This does not rule out that activity with the ability to induce specific oscillatory patterns could be exploited for therapeutic use.

4.8 Running, motor impairments, and BDNF in 3xTg mice

As shown in chapters 5 and 6, running is a potent activator of neurogenesis in female C57Bl6 mice. A great deal of variability was present in the distance traveled in the running wheel for the 3xTg mice, parallel to a wide range in the values for the BrdU+ surviving cells. This is evident in the acute study of BrdU+ survival in the 3xTg mice as no significant increases in survival were found when evaluated at 4 weeks. We expected to see an acute effect on cell survival, as documented in Chapter 5. At 11 months, running and fluoxetine increased long-term survival however cell survival was not correlation with total distance ($r = 0.16$, $p<0.38$ One-tailed Pearson). For middle-aged C57Bl6 mice, described in chapter 6 shows that BrdU+ cell survival was correlated to total distance ($r = 0.52$, $p<0.01$ One-tailed Pearson). This reflects that wheel running activity is not equivalent between wildtype C57Bl6 and 3xTg mice (see also section 5).

It appears that fluoxetine may have further complicated our ability to interpret any synergistic effect of combined use because the drug influenced motor coordination and appeared to reduce running distance in animals receiving the drug. Indeed, there was a trend for decreased total distance in the fluoxetine treated runners (Student's t-test, $p = 0.09$). There was earlier evidence that fluoxetine could impact activity: several reports in the same dose range showed that fluoxetine increased activity(99,100) while other groups reported that a lower dose of fluoxetine reduced voluntary wheel running(101) and distance traveled in the open field (67). Exercise is known to have antidepressant and anxiolytic effects in rodents and humans(102). Single-housing mice i.e. isolation, has been shown to increase locomotor activity and reduce anxiety in C57Bl6J mice(103).

The 3xTg mice were tested for motor performance at 10 month and 20 months of age in the Rotor-rod assay. Fluoxetine-treated 20 month-old mice had significantly more frequent falls compared to untreated mice (One-way ANOVA $p<0.05$). Notably, motor impairments following fluoxetine treatment have also been seen in other species(104). Reduced activity in the running wheel could reflect a problem with motor coordination that occurs earlier but was only apparent at 20 months-of-age. Among the known effects of SSRIs, fluoxetine has been shown to impact the HPA axis, by increasing hippocampal GR expression(105). In vivo work established that fluoxetine decreased levels of thyroxine (T4) and triiodothyronine (T3)(106), two hormones secreted by follicular cells of the thyroid gland. The two related hormones, T3 and T4, regulate production of thyroid-stimulating hormone (TSH). The effect of fluoxetine on the HPA axis and thyroid function may have thus contributed to this somewhat unexpected phenotype of the fluoxetine-treated 3xTg mice.

Variability in running distance also should be considered when evaluating BDNF expression in the brain of 3xTg mice. Chapter 6 provided solid evidence that running elevated neurogenesis and expression of BDNF in female C67Bl6 mice. In the 3xTg mice we did not see increased expression of BDNF in running or combined running-fluoxetine treatment groups. This is not consistent with the expectation that combined running and fluoxetine treatment would have an additive effects as previously measured by total BDNF mRNA(107).

Motor impairments, anxiety, and motivation could be systematically tested in the future. For instance, an alternative to free access wheel running is a forced running paradigm when mice are placed on a treadmill in constant motion. This needs to be tested carefully because a stress response might interfere or normalize any stimulatory effect of the exercise. If motivation to run is affected by the drug treatment, it could be measured by counting the frequency of stops over a fixed distance.

4.9 Induction of neurogenesis: new drugs and new pathways

Since I began work on activity and pharmachology-dependent mechanisms of neurogenesis, some fundamental principles of adult neurogenesis have been reported with clear implications as pharmacological targets. Earlier, the role of microglia in apoptotic cell

death was mentioned (36). If new neurons enter programed cell death, one strategy to increase neurogenesis would be to block entry into apoptosis. A proneurogenic chemical, potentially working through this mechanism, was described in 2010. The chemical, P7C3, was discovered through an unprecedented in vivo screen of 1000 compounds for biological activity (108). While the pharmacological target of P7C3 has not yet been published, the evidence presented indicates that P7C3 protects new neurons from entering apoptosis. Similarly, the expression of anti-apoptotic BCL-xL, is implicated in steering proliferating stem cells to produce more neurons and respectively fewer glia(109).

Additional work has uncovered essential new findings regarding the glucocorticoid receptor (GR). As discussed previously, elevated cortisol levels are known to decrease hippocampal neurogenesis. This is thought to occur through the GR. Now, additional research has established that the GR may mediate neuronal differentiation. At least two studies have shown that the GR plays an important role in mediating cell differentiation to neuronal phenotypes. The SSRI sertraline, increased neuronal differentiation through a GR-dependent mechanism and proliferation of a stem cell line was dependent on co-treatment with GR agonist dexamethasone (110). There is more to explore regarding GR, because it's also known that antagonism of GR with mifepristone prevents stress-induced apoptosis of new cells in the dentate(111).

5. REVISITING THE BACKCROSSED 3XTG MODEL

5.1 Rationale, behavioral phenotype, and other considerations

At the end of Chapter 8, I discuss the fact that 3xTg mice in this study had been backcrossed to the C57Bl6 line; they were originally and most frequently maintained on a hybrid Sv129 background. Backcrossing was done because the Sv129 strain is generally considered to have poor performance in learning and memory tasks. Based on published literature we can assume that backcrossing resulted in a suppression of the AD behavioral phenotype.

The 3xTg mice were tested in an identical testing paradigm as the C57Bl6 mice described in Chapter 6. While intraneuronal plaques

and tau deposits were present, these neuropathological features had no detectable effect on spatial learning or memory as measured in the MWM. Our data is interesting because we show that a model of Alzheimer's is able to maintain spatial learning and memory behaviors at an old age when plaques and tangles are present.

Whereas the C57Bl6 mice showed age-related deficits in spatial memory at 15 months of age, the 3xTg mice did not show any behavioral deficit at 20 months of age and appeared to be cognitively intact. A few considerations, however, should be taken into account. While not extensively documented, repeated trials in the MWM are known to improve performance in the task (personal communication Charles Vorhees, author of the MWM protocol). The 3xTg mice underwent three MWM trials, i.e. at 11, 15, and 20 months of age; this could have contributed to their maintenance. Sexual dimorphisms have been reported in the 3xTg mice regarding stress response, motor skills, and endocrine function. However, these data sets suggest opposing effects that would not benefit female 3xTg in MWM performance. For instance, young female mice performed *worse* than male mice in stress related tasks; this correlated with elevated cortisol levels during MWM testing(112). Previous work, in a forced running task with young 3xTg mice, showed that exercise had a strong gender component, namely that female mice performed better in motor skill assays. Female 3xTg mice also showed significant gender effects for immunoendocrine status; female Tg mice have increased white adipose tissue and decreased thymus weight compared to their non-Tg littermates, a difference not present in the male cohorts(113).

Backcrossing the mice may have changed their genetic background in such a manner that alterations could have occurred in their brain structures and/or physiological responses to MWM testing. This might involve associated brain regions, such as the amygdala, pre-frontal cortex, and additionally physiological responses e.g. blood pressure, heart rate, and cortisol concentration. Only systematic testing of the mice across a series of generations and a battery of behavioral tests would shed light on the sex and backcrossing components of the behavioral phenotype. This was clearly beyond the scope of this thesis.

5.2 Intraneuronal Aβ in backcrossed and hybrid 3xTg mice

Chapters 1 and 7 provide reviews of Alzheimer pathology and cleavage of APP to Aβ peptide. The 3xTg mouse model is unique because itwas the first model to show a relationship between intraneuronal Aβ and behavioral deficits (114). It has become widely accepted that Aβ burden, i.e. plaque deposition, is uncoupled from behavioral phenotypes in mouse models, while soluble higher-ordered Aβ oligomeric species show a more conserved relationship with behavioral deficits (reviewed by Ashe) (115). These findings suggested that measuring intraneuronal Aβ would be the most sensitive method to correlate pathology to the behavioral changes.

My staining protocol was therefore specially designed to identify intraneuronal Aβ accumulations, using a primary antibody directed against N-terminal Aβ peptides (IBL Japan #18584). This protocol described in chapter 8 was initially published in formalin-fixed paraffin embedded (FFPE) sections cut at 4 μm(116). We reproduced the staining by mounting 40 μm sections on special slides prior to formic acid antigen retrieval. While intraneuronal Aβ was seen in the amygdala and cortex, there was no strong intraneuronal staining in the hippocampus and subiculum of the backcrossed 3xTg mice.

Comparing the amount of pathology we see in Chapter 8 with 3xTg mice on the hybrid background is difficult because, at this time, no studies exist that utilize the same conditions. However, a monoclonal antibody directed at amino acids 1-5 of N-terminal Aβ (3D6, Elan, South San Francisco, CA, USA) has been used to visualize Aβ deposits in the hybrid 3xTg strain at ages of 2, 14, and 20 months(117). The authors found that 3D6 labels intraneuronal perisomatic Aβ puncta that, in 14-month-old animals, is found as robust intraneuronal staining in the CA1. This evidence suggests that that the IBL N-terminal Aβ would be able to detect the same intraneuronal pattern in the CA1 of the backcrossed mice. Without a direct comparison between the hybrid and backcrossed strain using the same antibody, it is very difficult to gauge if this is truly the reason for the loss of behavioral phenotype in the backcrossed strain.

It should be noted that pathology findings in the original hybrid stain have also been revised recently. Investigators currently working with the mice consistently note that the presence of pathology is significantly later than first reported(118,119). Intraneuronal Aβ accumulations in the 3xTg mice have become the subject of intense debate. One research group found that when brain tissue from 3xTg

mice was stained with a combination of antibodies, that APP was preferentially found within neurons not the cleavage product Aβ (120). This finding has been extended by reports showing that intraneuronal Aβ is only a minor component of the intraneuronal pool of Aβ and APP species in these mice (119). Our staining with the N-terminal antibody is sensitive for selective intraneuronal and extracellular Aβ plaques and there is no evidence of cross reactivity with APP or APP C-terminal fragments (CTFs). Our data shows that intraneuronal Aβ is present predominantly in the cortex and amygdala, but not sufficiently present in the CA1 and subiculum to disrupt hippocampal-mediated behavior.

5.3 Future directions for neurogenesis research in 3xTg mice

Successful experiments have recently been conducted with the 3xTg mouse and wheel running intervention at two different ages (105). Investigators maintained the animals on the original hybrid background and compared Tg mice with non-Tg littermates; interventions of 1- and 6-months in duration were started when the animals were 1 or 6 months of age. Running had broad beneficial effects and ameliorated cognitive deterioration as measured by the Morris water maze (121). Wheel running partially protected the mice from changes in synaptic strength and improved antioxidant defense, supporting the notion that exercise protects broadly against neurodegeneration. Interestingly, and similar to our study, no changes in open field behavior or Aβ or tau pathology in the animals were observed. A separate study further found that exposure to wheel running or an enriched environment for 6 months could restore adult neurogenesis to levels seen in non-Tg mice(122).

Based on these observations, experiments to evaluate activity and pharmacology-induced neurogenesis in the 3xTg mouse should be carefully designed. The model should not be backcrossed to the C57Bl6 strain as this altered the phenotype and may have introduced a higher degree of phenotype variability. Backcrossed 3xTg mice have low levels of neurogenesis and show intraneuronal Aβ accumulations in the amygdala and cortex. The deficiencies in adult neurogenesis and low levels of intraneuronal Aβ accumulations were not responsible, nor were they correlated with poor performance in learning and memory tests.

We must therefore question the predictive value for these models, which are pursued in an effort to learn more about human health and disease. Here, we see that manipulating the genetic background of an animal can have unanticipated consequences resulting in an inability to reproduce earlier results. Until more advanced and creative methods can be agreed upon, we must read and discuss these findings in good faith that this work will aid future development.

6. FINAL REMARKS

The discovery and characterization of resident neural stem cells has completely reversed earlier notions of the brain as a static and unchangeable structure. We now have a basic knowledge of how neural stem cells produce new neurons in the adult brain and how these new cells can impact specific aspects of behavior. Our understanding of the significance of neurogenesis and the ability to control this process for therapeutic purposes will likely expand prodigiously in coming years. The findings presented here provide insight into the complex regulation of this process and the difficulties encountered when attempting to stimulate stem cell proliferation in the brain for modifying age- and disease-associated behaviors. The work further highlights the important role for glial cells during health and disease. Although a number of daunting questions wait to be answered, hope for those afflicted by neurodegenerative diseases continues to build as research on neurogenesis advances.

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