Animal Models of Alzheimer’s Disease, Stroke, Type II Diabetes Co-morbidity: The Role of Hippocampal Adult Neurogenesis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by a progressive decline in cognition and memory in the aging population. This progression is advanced by risk factors for vascular cognitive impairment, such as stroke and diabetes. The present study examines hippocampal adult neurogenesis in the animal models for combined stroke and AD, and combined type II diabetes and AD treatments. Stroke or AD treatment alone significantly impaired dendritic arborization of young neurons 3 weeks after the surgery. The combined treatment further reduced neuronal survival by over 90%. No significant changes in cell proliferation and neuronal survival were observed after combined type II diabetes and AD treatment. Our evidence suggests that combined stroke & AD can impact the survival of young neurons leading to more dramatic consequences in terms of possible cognitive decline. These effects on neurogenesis could contribute to the early onset and progression of Alzheimer’s dementia.
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List of Abbreviations

2VO: two-vessel occlusion
4VO: four-vessel occlusion
Aβ: β-amyloid
AD: Alzheimer’s disease
APP: β-amyloid precursor protein
BB: bio breeding
BrdU: bromodeoxyuridine
CaBP: calbindin D-28k
CD11b: cluster of differentiation molecule 11b
CldU: chlorodeoxyuridine
DCX: doublecortin
EC: entorhinal cortex
EPO: erythropoietin
Et1: endothelin-1
GABA: gamma-aminobutyric acid
GCL: granule cell layer
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein
FGF-2: fibroblast growth factor 2
KCC2: K-Cl co-transporter 2
IdU: iododeoxyuridine
IGF-1: insulin-like growth factor-1
LPS: lipopolysaccharide
RFP: red fluorescent protein
MCA: middle cerebral artery
ML: molecular layer
mPP: medial perforant path
MRI: magnetic resonance imaging
NeuN: neuronal nuclei
NKCC1: Na-K-Cl cotransporter 1
NMDA: N-methyl-D-aspartic acid
NOD: non-obese diabetic
OB: olfactory bulb
PS: presenilin
RMS: rostral migratory stream
SCF: stem cell factor
SGZ: subgranular zone
SVZ: subventricular zone
T1D: type 1 diabetes
T2D: type 2 diabetes
VEGF: vascular endothelial growth factor
ZDF: Zucker diabetic fatty rats
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Part I: The Functional Role of Hippocampal Adult Neurogenesis

Part II: Preliminary Development of Methodology for Using CldU and IdU to Label Two Populations of Adult Born Neurons in the Same Animal
1 Hippocampus

1.1 Structure and circuitry

Hippocampus is a seahorse-shaped brain structure that together with other brain areas: amygdala, anterior thalamic nuclei, septum, limbic cortex and fornix, form the limbic system, which forms the inner border of the cortex. Stellate pyramidal cells in layer II of the entorhinal cortex (EC) located in the parahippocampal gyrus, the cortical region adjacent to the hippocampus, which connected with many parts of the cerebral cortex, projects densely to the granule cells in the dentate gyrus, and less extensively to the apical dendrites of CA3 and CA1 regions of the hippocampus (main output of EC, perforant pathway). The axons of granule cells (mossy fibers) project to the apical dendrites of CA3 pyramidal cells, whose axons then project to the apical dendrites of the CA1 pyramidal cells, which then project back to the EC. Some output also goes to other brain areas, such prefrontal cortex.

1.2 The functional role of hippocampus in learning and memory

It is universally accepted that hippocampus plays important roles in spatial navigation, formation of episodic memories and consolidation of short-term memory to long-term memory (reviewed by Bird and Burgess, 2008). An early famous report by Scoville and Brenda Milner described that the outcome of surgical destruction of hippocampus in an attempt to relieve epileptic seizures in a patient was severe anterograde and retrograde amnesia. The patient was unable to form new episodic memories and to recall previous recent memories, but was able to retain memories from many years ago. It has led to the Declarative Theory, which states that
consolidation of memory takes time and involves transferring of memory from hippocampus to other brain regions. There is also an alternative explanation proposed by the Multiple-Trace Theory, which states that hippocampus stores memory traces of an event whenever it is retrieved from the neocortex, such that remote memories are represented by more traces than the new ones. Thus, the information that represents the remote episodic memories, which is stored in the neocortex, is more integrated with pre-existing knowledge, thus less susceptible to hippocampal damage. Nevertheless, both theories acknowledge that remote episodic memories are more resistant to hippocampal disruption.

O'Keefe and Dostrovsky first discovered that the firing activity of rat hippocampal neurons was associated with the animal’s location in its environment. Subsequent extensive literature has shown that place cell responses are exhibited by pyramidal cells, and to a less extent, inhibitory interneurons. This has led to the idea that hippocampus can create a cognitive map that represents the layout of the environment. In Marr’s model, modification of recurrent connections in CA3 region encodes a representation of an event, which can be retrieved by an incomplete cue through pattern completion. This allows animals to stay oriented in various, but similar environments. But if spatial cues are significantly different, then the place cell representation will remap the new environment.
1.3 The functional role of adult neurogenesis

The functional role of adult neurogenesis in hippocampus-dependent learning and memory processes has been addressed by several computational models and behavioural studies. It has been proposed that young neurons contribute to the formation of contextualized memory. For example, consider an individual who parks his car in one location (event) in the morning (context). If he parks his car again in the same location (second event) in the morning but one week later (second context), he will form a separate memory. Although the two events are highly similar – parking his car in the same location, he does not confuse these two events because each event is associated with a distinct context. On the other hand, if these two events occur under the same context, for instance, parking his car several times in the same location in the same morning, he will likely confuse these events, a condition named memory interference.

Young neurons are proposed to have two functions: pattern integration and pattern separation (Becker, 2005; Becker & Wojtowicz, 2007), both of which prevent the occurrence of memory interference. Pattern integration refers to the function of young neurons that can “tag” an event with its associated context. Because young neurons are hyper-excitible before they become fully mature, they can be activated by both the event and the context at a given time. This links the event with the context, contributing to the formation of a contextualized memory. Pattern separation refers to the function of young neurons that can form different contextualized memories that are separated by longer time scale. Because young neurons are constitutively generated, different populations generated at different times can form separate contextualized memories, which will prevent an individual from confusing similar events that have occurred at different times.

Several previous experimental studies have examined “pattern separation” and “pattern
integration” function of hippocampal adult neurogenesis. Clelland et al., (2009) showed that adult mice with ablated hippocampal neurogenesis 2 months after focal low-dose x-irradiation presented with impairment in spatial discrimination in two behavioural tasks: a spatial navigation radial arm maze and a spatial, but non-navigable task in mouse touch screen. Sahay et al., (2011) showed that increased adult neurogenesis via conditionally knocking out Bax in transgenic mice was associated with better performance in a contextual fear discrimination task. These evidences point to the functional role of new neurons in discriminating similar events that are temporarily close. Yet, there is evidence that suggests the functional role of new neurons in integrating temporarily close events (pattern integration). Recently, Marin-Burgin et al., 2012 showed “pattern integration” role of young neurons by combining calcium imaging and electrophysiology in acute hippocampal slices. Young neurons labeled by red fluorescent protein (RFP, 4 week old) were more likely to be activated in response to medial perforant path (mPP) compared to RFP negative cells, which were presumably mature granule cells. In response to 10 Hz trains delivered to the mPP, young neurons fired repetitively whereas mature neurons fire at most once since they were under more GABAergic inhibition. More importantly, in response to 2 separate stimulation of mPP, young neurons were more likely to be activated by both stimuli, whereas mature granule cells were more likely to be activated by a single stimulus, which suggests that young neurons more likely to act as “pattern integrators,” whereas mature granule cells more likely to act as “pattern separators.” Thus, as new neurons become more mature, their functional roles change, from being “integrators” to “separators.” This is consistent with the model proposed by Becker & Wojtowicz, 2007 that adult neurogenesis is crucial for resolving interference in memories that are separated by longer temporal scale, which allows turnover of different populations of young neurons. In our recent study, we have further provided behavioural evidence on “pattern separation” role of young neurons (Luu et al., 2012; in press).
Briefly, animals were trained to perform two sequential similar odor tasks (odor task 1 and 1’) under same contexts (contexts: A and A) or different contexts (contexts: A and B). Training of odor task 1 under a context (context A), which took a few days, was followed by testing the animals’ ability to learn odor task 1’ under same (context A) or different context (context B). Presumably, learning of odor task 1’ under same context (context A) would be more difficult since odor task 1 and 1’ were similar, and learning of task 1’ under a different context (context B) would be easier since the animals could discriminate task 1 and 1’ based on their contexts: odor task 1 - context A, and odor task 1’-context B. Our results showed that control animals could discriminate odor task 1 and 1' better than irradiated animals (without hippocampal adult neurogenesis. Furthermore, learning of odor task 1’ under different context allowed animals to perform better. Thus, hippocampal adult neurogenesis is involved in resolving interference in memories that are separated by longer temporal scale. This is further supported by our finding that control and irradiated animals did not differ in their ability to discriminate two similar tasks on a shorter time scale.
2 Adult Neurogenesis

2.1 Neurovascular niche

In the adult brain, differentiation of neural progenitors into neurons is primarily restricted to two regions: the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. In the SVZ, neuroblasts migrate toward olfactory bulb (OB) through a tube formed by astrocytes in the rostral migratory stream (RMS). At OB, they migrate radially toward glomeruli and differentiate into various subtypes of olfactory interneurons, such as GABAergic granule cells and GABAergic periglomerular neurons. In the SGZ, neuroblasts migrate a short distance into the adjacent inner granule cell layer (GCL) where they differentiate into granule cells.

These neurogenic regions retain their specialized microenvironment or niche, which contain various cell types and soluble factors and membrane bound molecules necessary to maintain and regulate the stem cell self-renewal and progenitor differentiation, from the embryonic development to the life of the animal (reviewed by Alvarez-Buylla and Lim, 2004). The neurogenic niche is rich in various cell types including endothelial cells, astrocytes, ependymal cells, microglia, mature neurons, and adult neural precursors (reviewed by Ming and Song et al., 2011). Neural precursors are in close proximity to blood vessels and are influenced by blood-derived cues. Neurogenesis is also closely associated with angiogenesis. Astrocytes are also closely associated with blood vessels, and further modulate the influences of various growth factors and cytokines on neurogenesis. Microglia is involved in clearing apoptotic neurons from the niche under basal condition. Under inflammatory condition, microglia can release various factors and cytokines that have both beneficial and deleterious effects on neurogenesis depending on the balance between pro- and anti-inflammatory actions. In the SVZ, ependymal cells are...
closely associated with neural precursors and regulate the neuronal fate specification.

2.2 Proliferation of neural progenitors

In the SVZ, GFAP positive type B cells are neurogenic astrocytes, which are derived from radial glia, are the primary self-renewing precursors that give rise to rapidly dividing transit amplifying cells (type C cells), which then differentiate into committed migratory neuroblasts (type A cells). In the SGZ, GFAP positive radial glia-like cells and/or Sox2 positive nonradial cells give rise to proliferating intermediate progenitor cells, which then give rise to proliferating neuroblasts (reviewed by Ming and Song et al., 2011). Most of the cells are proliferative within their first week of neuronal development.

2.3 Neuronal survival and maturation

Maturing neurons initially receive tonic GABA activation from neuroblasts (SVZ) and local interneurons (SGZ), followed by synaptic GABAergic inputs, and followed by synaptic glutamategic inputs. In this process, they undergo two critical survival periods: the first one occurs during the neuroblasts stage, and the second one occurs during the immature neuron integration stage.

In the dentate gyrus, the survival of new neurons is influenced by neural activity. Kempermann et al., (1997) first showed that enriched environment increases the survival of new neurons. Tashiro et al., (2007) further showed that experience-dependent increase in neuronal survival occurs during the first three weeks of neuronal development with the peak during the second week. It was later shown that certain types of hippocampus-dependent learning tasks, such as trace eyeblink conditioning, can prevent new neurons from dying during their second week of neuronal development (Gould et al., 1999; Waddell and Shors, 2008).
The first critical survival stage occurs during the second week of neuronal development, which is independent of NMDA receptors (reviewed by Aasebo et al., 2011). Since GABAergic inputs regulate early dendritic and synaptic development of new neurons, they are likely involved in activity-dependent regulation of neuronal survival. The second survival period occurs during the third week of neuronal development, which is NMDA receptor dependent, likely involving competition of new neurons for synaptic integration. By the end of fourth week, the young neurons that have survived the previous survival periods are fully integrated into the network and start to enter the synaptic maturation phase, which takes an additional four weeks. During this period, young neurons undergo NR2B-dependent enhanced synaptic plasticity. A study in 4-8 week-old cells labeled by retroviral vector expressing green fluorescent protein (GFP) in live mouse hippocampal slices showed that adult-born neurons functionally integrate into the neural network, receive perforant synaptic input, and exhibit electrophysiological properties that closely resemble mature granule cells (Van Praag et al., 2002).
3 Regulation of Adult Neurogenesis

The proliferation and/or survival of adult-born neurons are influenced by various physiological factors, such as physical exercise and enriched environment. Physical exercise increases proliferation presumably due to increased trophic factors. Praag et al., (1999) first showed that voluntary wheel running doubled the number of surviving adult born neurons by increasing cell proliferation in mice. Jin et al., (2002) showed histological evidence that young neurons express vascular endothelial growth factor receptor 2 (VEGFR2), and i.c.v. administration of VEGF increased cell proliferation in the SVZ and SGZ. Aberg et al., (2000) showed that peripheral infusion of insulin-like growth factor-1 (IGF-1) increased cell proliferation in the SGZ. On the other hand, enriched environment mostly affects survival presumably due to brain activity. Kempermann et al., (1997) first showed that young mice housed in enriched environment had higher cell survival (4 weeks after BrdU injection) compared to mice housed in standard condition. Yet, proliferation of progenitor cells (1 day after BrdU injection) was not influenced by environmental enrichment. The effect of environmental enrichment on cell survival was also observed in the senescent dentate gyrus in mice (Kempermann et al., 1998).

The production and/or survival of adult-born neurons are also negatively influenced by various physiological factors, such as stress and inflammation. Ekdahl et al., (2003) showed that microglia activation associated with inflammation induced by direct infusion of bacterial lipopolysaccharide (LPS) into the brain impairs the survival of young neurons, which can be rescued by administration of microglia activation inhibitor, tetracycline derivative minocycline. Furthermore, brain insults, such as status epilepticus, induce microglia activation, which is shown to suppress the survival of young neurons. In addition, stress elevates the level of glucocorticoid that reduces neuronal proliferation (Gould et al., 1992; Cameron and Gould, 1994) and neuronal survival (Wong and Herbert, 2004).
The main excitatory and inhibitory neurotransmitters: GABA and glutamate, respectively regulate the early and late stages of neuronal development of adult-born neurons (reviewed by Ge et al., 2008). Young neurons receive GABAergic inputs from local interneurons in the dentate gyrus, and glutamatergic inputs from perforant path. Neural progenitors and immature neurons express functional ionotropic GABA receptors (GABA\(_A\)Rs), which are chloride (Cl\(^-\)) permeable channels. They are initially activated by tonic GABAergic inputs followed by phasic GABAergic inputs. Since these cells in the early development stages have higher intracellular Cl\(^-\) concentration due to the expression of NKCC1, a Cl\(^-\) importer, binding of GABA to its GABA\(_A\)Rs leads to Cl\(^-\) efflux, which results in depolarization. As immature neurons become more mature, they gradually decrease the expression of NKCC1, and gradually increase the expression of KCC2, a Cl\(^-\) exporter. Consequently, binding of GABA to its GABA\(_A\)ARs leads to Cl\(^-\) influx, which results in hyperpolarization. However, Chiang et al., (2012) indicated that the action of GABA on mature granule cells could be inhibitory or excitatory, depending on its precise timing and location to other excitatory inputs. Yet, most of the literature suggests that GABA is overall inhibitory in mature neurons. The conversion of GABAergic action from activation to inhibition occurs during the third week of neuronal development when the maturing neurons begin to receive excitatory synaptic glutamatergic inputs, which coincides with the later stage of synaptic integration and subsequent synaptic maturation in the following 6 weeks.
4 Animal Models of Alzheimer’s Disease and Its Risk Factors

4.1 Animal models of Alzheimer’s disease

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by progressive neurodegeneration and cognitive deterioration. It was first characterized by a German physician, Alois Alzheimer, who found a multitude of plaque and tangles in the AD brain. Subsequent studies identified and studied β-amyloid (Aβ), the main component of the plaque, to determine if it was the cause or a symptom of AD. Hardy and Allsop (1991) was the first to propose the most influential theory, Amyloid Cascade Hypothesis, which suggested that excess accumulation and aggregation of Aβ due to mismetabolism of β-amyloid precursor protein (APP) is the triggering event in AD pathogenesis that leads to tau phosphorylation and neurofibrillary tangle formation followed by disruption of synaptic connections and neuronal death. This hypothesis was supported by several notable early studies that generated various transgenic mice as models of the hereditary and dominant form of AD, which is associated with 5 mutations in or near the Aβ domain of APP, as well as presenilin 1 (PS1) and presenilin 2 (PS2) mutations.

Notably, Games et al., (1995) generated an AD transgenic mouse model by over-expressing human APP containing a single mutation with Val
$_{717}$ > Phe. These animals progressively develop many hallmarks of AD, including β-amyloid deposits and plaque, synaptic loss, astrocytosis, and microgliosis beginning at 6 - 9 months of age in various vulnerable brain regions: frontal, temporal, and entorhinal cortex, subiculum, and hippocampus including the outer molecular layer of the dentate gyrus. Hsiao et al., (1996) generated a AD transgenic mouse model by over-expressing a human APP$_{695}$ isoform, which is normally predominantly expressed in neurons, containing double-mutations with Lys
$_{670}$ > Asn and Met
$_{671}$ > Leu found in a large Swedish family. These animals showed impairment in learning and memory starting at 9 months of age, which
was correlated with increased deposition of amyloid plaque and Aβ deposits. Duff et al., (1996) generated an AD transgenic mouse model by over-expressing mutant PS1, which selectively increased Aβ42 deposition. Furthermore, through pronuclear microinjection of two independent transgenes encoding human APP<sub>Swe</sub> and tau<sub>P301L</sub> into embryos harvested from mutant PS1<sub>M146V</sub> knockin mice, Oddo et al. (2003) generated a triple-transgenic mouse AD model (3Tg-AD), which progressively developed both Aβ plaque and neurofibrillary tangles that more closely mimicking that observed in AD.

Although all of these transgenic animal models presented with major phenotypes of AD, they did not fully recapitulate the pathogenic process of all cases of AD. Importantly, the more common late-onset, sporadic form of AD has a more heterogeneous nature; its onset is closely related to many risk factors, such as stroke and diabetes. Specifically, patients with cerebral infarcts and moderate AD-type lesions, adult-onset diabetes, atherosclerotic disease and atrial fibrillation, and hypertension significantly increase the risk of AD later in life (reviewed by Cechetto et al., 2008). Yet, how the risk factors contribute to AD remains unknown. Thus, experimental animal models should be created to address this complex question.

### 4.2 Animal models of cerebral ischemia

Cerebral ischemia is a condition in which there is insufficient blood flow to various parts of the brain that fail to meet metabolic demand of those regions. This leads to rapid drop in ATP, disruption of ionic gradient across the cell membrane, and Na<sup>+</sup>-dependent astrocytic glutamate uptake, which results in excess accumulation of extracellular glutamate. Excess glutamate over-excite NMDA receptors, leading to intracellular Ca<sup>2+</sup> overload that results in damages to protein synthesis, mitochondrial function, cytoskeleton, and eventually cell death (Lipton, 1999). Since glutamate and NMDA receptor are thought to be the key players in mediating excitotoxicity and
neuronal death, NMDA and AMPA receptor blockers have been used in clinical trials (anti-
excitotoxic therapy) to treat ischemia brain damage. Yet, this approach failed to benefit stroke
patients (Davis et al., 2000). Consequently, scientists have started to look for alternative, non-
glutamate related targets that result in ionic imbalance and cell death. These targets include acid-
sensing ion channels, TRP channels, hemichannels, volume-regulated anion channels, sodium-
calcium exchangers, and non-selective cation channels (Tymianski, 2011).

Animal models of ischemia are mostly produced by vessel occlusion. There are two broad
categories of ischemia: global and focal ischemia. Complete global ischemia is produced by
cardiac arrest, neck-cuff, and ligation of all arteries stemming from the heart, which nearly
completely cut off blood flow to the brain. Incomplete global ischemia is produced by vessel
occlusion, which dramatically lowers blood flow to major forebrain regions, such as
hippocampus, striatum, and neocortex, whereas leaving residual blood flow to various
subcortical regions. A rat model of transient bilateral forebrain ischemia was initially produced
by permanently occluding vertebral arteries and temporarily occluding common carotid arteries
(four-vessel occlusion, 4VO model) for 10 to 30min, which resulted in neuronal damages in
several vulnerable areas, such as dorsolateral striatum, hippocampal CA1 regions, and neocortex
(Pulsinelli et al., 1982). The damage to small to medium-sized striatal neurons and hippocampal
hilar neurons was observed early, whereas the damage to hippocampal CA1 pyramidal neurons
was initially delayed for 3 to 6 hours, and progressively became worse in the following 3 days.
However, due to low survival rate and inconsistent cerebral blood flow in this model, Smith et
al., (1984) generated another rat model of forebrain ischemia by temporarily occluding common
carotid arteries for 10min combined with a lowering of mean arterial blood pressure to 50mmHg
(two-vessel occlusion, 2VO model). This model more consistently lowered blood flow rate in
major forebrain areas during ischemia although flow rates in various subcortical structures were variable. The delay between ischemia and cell death in the vulnerable regions depends on the length of ischemia insult: the longer the insult, the shorter the delay.

Focal ischemia is produced by occlusion of one middle cerebral artery (MCA) at different sites. A rat model of focal ischemia was initially produced by permanent or temporary occlusion of the proximal part of the MCA, which resulted in bilateral damages mostly in the frontal, sensorimotor, and auditory cortex, and lateral portion of the striatum (Tamura et al., 1981; Longa et al., 1989). Occlusion of the MCA beginning proximal to the olfactory tract, which isolates lenticulostriate end-arteries from the proximal and distal blood supply, produced consistent infarctions of uniform locations (Bederson et al., 1986). However, occlusion of MCA at its origin and at the olfactory tract produced inconsistent outcomes due to collateral blood supply. Alternatively, a more reliable and convenient rat model of focal cerebral ischemia can be produced by stereotaxic injection of endothelin-1 (Et1), a potent vasoconstrictor that reduces local blood flow, directly into the cortex and striatum (Windle et al., 2006). One of the key differences between global and focal ischemia is that global ischemia results in delayed and selective neuronal death in vulnerable brain areas, whereas the damaged brain areas induced by the latter is characterized by core ischemic regions where blood flow is reduced to <15%, penumbral regions where blood flow is reduced to <40%, and extra-penumbral regions where blood flow is > 40% (reviewed by Lipton, 1999). Core and penumbral regions undergo different mechanisms of cell death in which the former is more rapid and drastic than the latter. Within the first few minutes of ischemia, the core is characterized by severe ATP depletion, rapid depolarization independent of glutamate receptors, rise in extracellular $K^+$, and decrease in extracellular $Ca^{2+}$, resulting in neuronal damage. On the other hand, penumbra does not undergo permanent anoxic depolarization, but sporadic transient depolarization mediated by glutamate
release from the core. It slowly leads to infarct that reaches its full size in a few days or weeks depending on the duration of temporary ischemia.

4.3 Animal models of diabetes

Diabetes mellitus is a metabolic disorder characterized by high blood glucose (hyperglycemia) because the body cannot produce enough insulin and/or the body does not respond to the insulin (insulin resistance). Diabetes is a risk factor for dementia. Hyperglycemia, immune dysfunction, damaged nerves and blood vessels, and changes in insulin and amyloid metabolism can negatively influence the brain in complex ways.

Type 1 diabetes (T1D) is caused by lack of insulin production usually because of beta cell dysfunction mediated by autoimmune attack by T-cells. Rodents are the most commonly used to generate models of diabetes. T1D model is commonly generated by injection of streptozotocin, which is particularly toxic to beta cells. Spontaneous T1D model usually involves non-obese diabetic (NOD) mouse and bio breeding (BB) rat, which have been inbred for many generations by selecting for hyperglycemia. Type 2 diabetes (T2D), the most common type of diabetes, is caused by insulin resistance combined with reduced insulin secretion. It has a more heterogeneous nature, and is known to associate with many factors, such as obesity. Cytokines released by fat cells, such as TNFα and IL-6, contribute to insulin resistance (reviewed by Powell, 2007). T2D model commonly involves obese animals with single gene mutations such as ob/ob (leptin deficient), db/db (leptin resistant), and fa/fa (leptin resistant). In addition, transgenic animals over- or under-expressing various genes in specific tissues, such as insulin receptor and glucose transporters, are used to study the pathogenesis of T1D and T2D.
5 Adult Neurogenesis in Pathological Conditions

5.1 Adult neurogenesis and Alzheimer’s disease

Previous studies have reported inconsistent results on the effects of amyloid beta on cell proliferation in the dentate gyrus (Jin et al., 2004a, Jin et al., 2004b; Haughey, et al., 2002; Wen et al., 2002; Verret et al., 2007; He and Shen, 2009; Hamilton et al., 2010). This discrepancy seems to stem from examination of different AD transgenic mouse models and utilization of different fragments of β-amyloid. A recent study has revealed that cell proliferation is increased at later stage of AD when β-amyloid plaque is present, likely as a compensatory response to neurodegeneration (Yu et al., 2009). On the other hand, long-term survival of adult-born neurons is affected before the formation of plaque (Verret et al., 2007). Soluble Aβ has been shown to cause impairment in the induction of long-term potentiation in the CA1 region (Walsh et al., 2002; Trinchese et al., 2004; Chang et al., 2006), as well as in the medial perforant pathway in the dentate gyrus (Wang et al., 2004; Tomiyama et al., 2010) in various animal models. It also correlates with the onset of reduced expression of a presynaptic marker, synaptophysin, in the molecular layer (Mucke et al., 2000). There is also a strong correlation between soluble Aβ and cognitive decline (Lue et al., 1999).

In conclusion, previous studies have consistently shown that adult neurogenesis is influenced by AD-like pathologies, although the exact mechanism is still unclear. In order to dissect the mechanism underlying AD-like pathologies, consistent amyloid beta fragment and animal model should be used. Aβ protein is a 39 - to 43 - amino acid polypeptide. A polypeptide corresponding to the first 40 amino acids of amyloid β protein (Aβ₁₋₄₀) was initially found to dose-dependently enhance in vitro survival of immature hippocampal neurons (0-2 days) at lower concentration (≤ 0.1nm) (Yankner et al., 1990). However, at higher concentration (≥ 40nm), it caused dendritic
and axonal retraction, and neuronal death of more mature neurons (3-5 days). A portion of Aβ protein corresponding to the 25-35 amino acids (Aβ25-35) was found to mediate both the trophic and toxic effects. Thus, injection of Aβ25-35 into animals can be used to study its effect in vivo.

5.2 Adult neurogenesis and cerebral ischemia

Liu et al., (1998) first showed that proliferation of neural progenitors in the dentate gyrus of adult gerbils was increased after 10 min of transient global ischemia (bilateral common carotid artery occlusion). The rate of proliferation was initially increased by ten-fold between 6 to 10 days after ischemia, and then decreased back to the basal level in the following 10 days. Similarly, Takagi et al., (1999) showed a two-fold increase in proliferation of neural progenitors in the dentate gyrus of male C57B/6 mice 4 to 9 days after 15 min transient global ischemia. Jin et al., (2001) showed that after 90 min of unilateral occlusion of the middle cerebral artery, cell proliferation was increased bilaterally in the SGZ (peak at 1 week after ischemia) and SVZ (peak at 2 weeks after ischemia). Arvidsson et al., (2002) showed histological evidence that 2 hours of middle cerebral artery occlusion (MCAO) increased production of neuroblasts in the SVZ, which migrated into the damaged area of striatum, where they differentiated into striatal medium-sized spiny neurons within 4 weeks after ischemia. Although neuronal proliferation is increased, most of the newly generated neurons cannot survive (Takasawa et al., 2002). The increase in cell proliferation after MCAO was correlated with elevated levels of growth and trophic factors, such as FGF-2 (Yoshimura et al., 2001), and stem cell factor (SCF) (Jin et al., 2002), and cytokine, erythropoietin (EPO) (Shingo et al., 2001), which are induced by ischemia to serve neuroprotective roles. In addition, increased level of glutamate after ischemia is also correlated with increased neurogenesis. Bernabeu and Sharp, (2000) showed that direct injection of MK-801, an NMDA receptor antagonist, and NBQX, an AMPA receptor antagonist, into the hippocampal CA1 region before ischemia prevented ischemia-induced neurogenesis and delayed
neuronal death in the CA1 region starting ~2-3 days after transient global ischemia. The authors postulated that due to close proximity between CA1 region and DG, glutamate could diffuse to the DG and lead to activity-mediated increase in adult neurogenesis. Further, some early studies have also suggested that cell death in the hippocampus after ischemic or epileptic insults can directly stimulate neurogenesis. Yet, neurogenesis is not necessarily triggered by neuronal death, since mild brain insults, which do not cause detectable neuronal death, also induce neurogenesis. Indeed, Ekdahl et al., (2001) showed that i.c.v infusions of caspase inhibitors decreased the number of apoptotic cells in the rat dentate gyrus following status epilepticus, which was correlated with an increase, not decrease in neurogenesis. In conclusion, previous studies have shown that ischemia increases hippocampal adult neurogenesis, which is thought to be mediated by various factors. Yet, the net effect of these factors on neuronal production is weak.

5.3 Adult neurogenesis and diabetes
T1D and T2D induced synaptic changes and transmission in the hippocampus in various regions, which were closely associated with impairment in hippocampus-dependent learning and memory (Popovic et al., 2001; Winocur et al., 2005; Stranahan et al., 2008a). Hippocampal adult neurogenesis is reduced in various animal models of diabetes. Beauquis et al., (2008) showed that both proliferation and survival of adult-born neurons in the hippocampus were reduced in non-obese diabetic mouse, a T1D model. Consistently, Zhang et al., (2008) showed that proliferation and survival of adult-born neurons were reduced in streptozotocin-treated rats. Stranahan et al., (2008b) showed that elevated level of glucocorticoid in both streptozocin-treated rats (T1D model) and db/db mice (T2D) impaired perforant path synaptic plasticity, adult neurogenesis, and hippocampus-dependent memory, which were reversed when normal level of corticosterone was maintained. In conclusion, previous studies have consistently shown that the production and survival of adult-born neurons are reduced in diabetic animals.
Chapter 2  
General Objectives, Hypotheses, and Timeline

6 General Objectives

The onset of sporadic AD is closely associated with various risk factors for vascular cognitive impairment, such as stroke and diabetes. We aim to collaboratively study the interaction between AD and its risk factors by establishing animal models containing both AD & stroke-like neuropathology, and AD & diabetes-like neuropathology. In the previous studies, combined AD & stroke model presented with greater inflammation and infarct size around the ischemic region compared to AD and stroke model alone. The objective of this study is to further collaboratively examine pathological changes in various brain areas and processes, including hippocampal adult neurogenesis and associated behavioural impairment in learning and memory in combined AD & stroke model, as well as in combined AD & T2D model.

7 General Hypotheses

We hypothesize that combined models (AD & stroke model, AD & T2D model) will have greater neurodegeneration, inflammation, Aβ deposition in the vulnerable brain areas, such as hippocampus and cortex, resulting in more severe impairment in learning and memory compared to AD, stroke, and T2D models alone.
8 General Timeline

The general experimental timeline of combined AD and stroke study and combined AD and T2D study is shown above. Surgical injections were performed on animals of 11 weeks of age to create stroke, AD, and combined AD and stroke models, and on established animal model of T2D (Zucker diabetic fatty rats) to create combined AD and T2D model. BrdU was i.p. injected into each animals 1 week after the surgery to label a population of proliferating progenitor cells in the hippocampus. To study the effects of treatments on cell proliferation, some animals were perfused 24 hours after the BrdU injection (1 week survival timeline). To study the effects of treatments on neuronal survival of adult born neurons, some animals were perfused 2 weeks after the BrdU injection (3 weeks survival timeline).
Chapter 3
Changes in Adult Neurogenesis in a Rat Model of Stroke and Alzheimer’s Disease

9 Specific Objectives and Hypothesis

9.1 Specific objective

The objective of this study is to examine changes in hippocampal adult neurogenesis, which can provide an explanation for the pathological interaction between stroke and AD-like pathology.

9.2 Specific hypothesis

We hypothesize that combined AD & stroke model will reduce hippocampal adult neurogenesis compared to AD and stroke models alone.
10 Methods

10.1 Animals
All experimental procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of the University of Western Ontario. Twenty-eight (28) ten-week old male Wistar rats were randomly assigned to four treatment groups: sham (n=6), Aβ (AD model) (n=8), Et1 (stroke model) (n=8), and Et1+Aβ (combined AD and stroke model) (n=6).

10.2 Stereotaxic surgery
Each animal was anesthetized by O₂ delivered isoflurane. Lubricant was applied to the eyes to prevent drying. An electrical surgical shaver was used to remove the hair around the incision line. The animal was placed on a David Kopf stereotaxic apparatus, and its head was stabilized by the ear bars and incisor bar. The skin was cleaned and disinfected before making an incision to expose the skull. After bregma and injection sites were clearly marked on the skull, a surgical drill was used to generate holes at the injection sites to allow insertion of injection cannula (30 gauge). Cerebral focal ischemia model (Et1 group) was produced by a single injection (6pmol/3ul saline) of endothelin-1 (Et1, Sigma-Aldrich, Oakville, ON), a potent vasoconstrictor, at a rate of 1ul/30s into the right striatum (anteroposterior +0.5mm and mediolateral -3.0mm relative to the bregma, and dorsal/ventral -5.0mm below dura). The AD model (Aβ group) was created by bilateral injections (50nmol/10ul saline) of Aβ25-35 (Bachem, Rorrance, Calif) at a rate of 1ul/30s into the lateral ventricles (anteroposterior -0.8mm and mediolateral ±1.4mm relative to the bregma, and dorsal/ventral - 4.0mm below dura). The combined stroke and AD model (Et1+Aβ group) was created by bilateral injections of Aβ25-35 into the lateral ventricles followed by unilateral injection of Et1 into the right striatum. The sham controls (Sham group) received saline injections. After each injection, the cannula was left in situ for 3 minutes to allow
dispersion of the solution. After wound suture, 30ug/kg buprenorphine and 0.02ml of antibiotics enrofloxacin (Baytril, Bayer Inc., Toronto, ON, Canada, 50mg/ml) were correspondingly injected subcutaneously and intramuscularly to each animal.

10.3 BrdU administration

5’-Bromo-2-deoxyuridine (BrdU, Sigma) was dissolved in warm saline with 1ul NaOH (10N) to produce a stock concentration of 20mg/ml. Each animal received a single intraperitoneal injection of BrdU (200mg/kg) 1 week after the surgery to catch the highest rate of cell proliferation (Jin et al., 2001)

10.4 Perfusion and fixation

Fourteen animals, which included sham (n=3), Et1 (n=4), Aβ (n=4), and Et1+Aβ (n=3) were sacrificed one week after the surgery (1-week survival time-point) to examine changes in cell proliferation (24 hours after BrdU injection) after each treatment. The remaining fourteen animals, which included sham (n=3), Et1 (n=4), Aβ (n=4), and Et1+Aβ (n=3), were sacrificed three weeks after the surgery (3-week survival time-point) to examine changes in neuronal survival (2 weeks after BrdU injection) after each treatment. Each animal was deeply anesthetized with isofluorane and intracardially perfused with 300ml 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 200ml ice-cold 4% paraformaldehyde (PFA, pH 7.4) in PBS. The brain was carefully removed from the skull and post-fixed in 4% PFA at 4 °C for 24 hours. Then, the brain was stored in 0.1% sodium azide in PBS until sectioning.

10.5 Sectioning and sampling

The right hemisphere from each animal was coronally sectioned at 40µm thickness using a vibratome (Leica VT1000 S, Leica Microsystems). The sections for each animal were stored in a 48-well plate (4 sections/well). Six sections were sampled evenly across the whole length of the
hippocampus (Rod and Auer, 1992). Six sections were sampled evenly across the lateral ventricles (anteroposterior 1.2mm - -0.4mm) to examine adult neurogenesis in the subventricular zone. Three sections were sampled evenly from each injection site (striatum: anteroposterior 0.5±0.16mm; lateral ventricle: anteroposterior -0.8±0.16mm). The sampled sections were immunostained for one or two of the following markers: BrdU (exogenous proliferation marker), doublecortin (DCX, endogenous immature neuronal marker), NeuN (endogenous mature neuronal marker), Ki67 (endogenous proliferation marker), CD11b (endogenous marker for monocyte/macrophage and microglia) or ED1 (activated microglia/macrophage).

10.6 Introduction to histological markers

- **5-Bromo-2’-deoxyuridine (BrdU)** is a synthetic analogue of thymidine, which can be administered to label dividing cells (S phase of the cell cycle).
- **Ki67** is a nuclear protein associated with cellular proliferation. It is present during all active phases of the cell cycle (G1, S, G2, and M phases).
- **Doublecortin (DCX)** is a microtubule associated protein, which is expressed by dividing neuronal precursor cells and their neuronal daughter cells for 2-3 weeks before they become mature neurons in the embryonic and adult brain.
- **NeuN (Neuronal Nuclei)** is a soluble nuclear protein expressed by most post-mitotic neuronal cell types, except cerebellar Purkinje cells, olfactory mitral cells, and retinal photoreceptor cells.
- **Calbindin D-28k (CaBP)** is a calcium-binding protein that buffers cytosolic calcium is highly expressed in mature granule cells.
- **Cluster of differentiation molecule 11b (CD11b)** is a protein subunit that forms the heterodimeric integrin alpha-M beta-2 molecule, also known as macrophage-1 antigen or complement receptor 3. It mediates inflammation, and is expressed on the surface of most
macrophages, dendritic cells, granulocytes and cells with the morphology of microglia in brain.

- CD68 is a single chain glycoprotein, which is expressed on the lysosomal membrane of myeloid cells. It is expressed by majority of tissue macrophages, and can be detected by anti-CD68 antibody (ED1), which recognizes the rat homologue of human CD68.

10.7 Immunohistochemistry
For BrdU staining, the brain sections were incubated with 1N hydrochloric acid for 30 minutes at 45°C followed by three 5-minute washes. The sections were incubated with anti-BrdU monoclonal primary antibody (rat, 1:200 in 0.3% Triton-X PBS, Serotec) for 24 hours at 4°C followed by three 5-minute washes. Then, the sections were incubated with the secondary antibody (chicken anti-rat IgG Alexa Fluor 594, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.

For DCX staining, the sections were incubated with anti-DCX polyclonal primary antibody (goat, 1:200 in 0.3% Triton-X PBS, Santa Cruz Biotechnology) for 24 hours at 4°C followed by three 5-min washes. Then, the sections were incubated with the secondary antibody (donkey anti-goat IgG Alexa Fluor 488, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.

For NeuN staining, the sections were incubated with anti-NeuN monoclonal primary antibody (mouse, 1:200 in 0.3% Triton-X PBS, Chemicon) for 24 hours at 4°C followed by three 5-min washes. Then, the sections were incubated with the secondary antibody (goat anti-mouse IgG Alexa Fluor 488, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.
For Ki67 staining, the brain sections were incubated with anti-Ki67 polyclonal primary antibody (rabbit, 1:200 in 0.3% Triton-X PBS, Vector Laboratories) for 18 hours at room temperature followed by three 5-minute washes. Then, the sections were incubated with secondary antibody (donkey anti-rabbit IgG Alexa Fluor 568, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.

For CD11b staining, the brain sections were incubated with anti-CD11b (OX42) monoclonal primary antibody (mouse anti-rat CD11b, 1:50 in 0.3% Triton-X PBS, Chemicon) for 48 hours at 4°C followed by three 5-min washes. Then, the sections were incubated with the secondary antibody (goat anti-mouse IgG Alexa Fluor 488, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.

For ED1 staining, the brain sections were incubated with anti-ED1 (CD68) monoclonal primary antibody (mouse anti-rat ED1, 1:200 in 0.3% Triton-X PBS, Chemicon) for 24 hours at 4°C followed by three 5-min washes. Then, the sections were incubated with the secondary antibody (goat anti-mouse IgG Alexa Fluor 488, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes.

All the sections were mounted on slides with mounting medium (PermaFluor, Thermo Scientific).

10.8 Quantification of dendritic arborization and cell numbers
The typical, physiological sequential development of DCX positive neurons allows their dendritic development to be specifically measured at four ascending levels in each blade of the dentate gyrus (Rosenzweig and Wojtowicz., 2011). The absolute numbers of primary, secondary,
tertiary, and higher-order dendrites were quantified using a 20X objective lens under a fluorescent microscope (Nikon Optiphot-2), based on their intersections with the corresponding lines drawn in the middle of the GCL, at the boundary between GCL and molecular layer (ML), at the inner one-third of ML, and at the outer one-third of ML across the entire section. Dendritic development index, a measurement of the dendritic arborization at each level compared to its previous level, was expressed as the number of dendrites at each level per millimeter per total number of dendrites at the previous level.

In the hippocampus, cells positive for BrdU, DCX, and Ki-67 in the subgranular zone (SGZ) and granule cell layer (GCL) were quantified under a fluorescent microscope (Nikon Optiphot-2) using an 40X objective lens. Co-labeled BrdU/DCX neurons and co-labeled BrdU/NeuN neurons were quantified under a Leica TCS-SL confocal microscope (Leica Microsystems) using a 40X oil immersion objective lens. The length (mm) of SGZ in the images captured by a SensiCam camera (The Cooke Corp., Michigan, USA) was measured using ImageJ software (NIH). The number of stained cells in each treatment group was either expressed as number of cells per SGZ (mm) or as total number of cells per dentate gyrus. Neuronal maturation was measured as the percentage of co-labeled BrdU/DCX cells and BrdU/NeuN cells of total number of BrdU cells. In the lateral ventricles, due to the large number of Ki67 positive cells in the subventricular zone, neurogenesis was quantified by measuring Ki67 positive area using ImageJ software (NIH).

10.9 Statistical analysis

Statistical analysis was done using SPSS software. One-way analysis of variance (ANOVA) and post hoc comparisons using Gabriel test were used.
11 Results

11.1 Inflammation at injection sites

Figure 1 illustrates the injection sites (top panel) in each animal model in which ED1 positive microglia/macrophage (bottom panel) can be observed 3 weeks after surgery. In the stroke model, ED1 positive microglia were found in the striatum. In the AD model, ED1 positive microglia were found in the lateral ventricle. In the combined model, ED1 positive microglia were found in both the striatum and lateral ventricles. No ED1 positive cells were found in the sham group.
Figure 1. ED1 staining of injection sites in each animal model. Top panel: injection sites for Et1 and Aβ. Bottom panel: representative ED1 staining at the injection sites (red boxes in the top panel). Sham group had no ED1 positive cells at the injection sites. Et1 group had ED1 positive cells in the striatum. Aβ group had ED1 positive cells along the lining of the lateral ventricles. Et1+Aβ group had ED1 positive cells in the striatum and the lateral ventricles.
11.2 Et1, Aβ, and Et1+Aβ treatments did not affect cell proliferation and BrdU uptake 1 week after the surgery

Figure 2 shows that Et1, Aβ, and Et1+Aβ treatments did not significantly affect the number of proliferating Ki67 positive cells and BrdU positive cells in the dentate gyrus 1 week after the surgery. The number of BrdU positive cells 24 hours after BrdU injection was in the same range as the number of Ki67 positive cells in each group, suggesting that BrdU uptake was not influenced by the treatments. Yet, since the sample size was low, the statistical power was low, and this negative result should be interpreted with caution. For instance, the effect size (Cohen’s d) and power between sham and Et1+Aβ groups for Ki67 are correspondingly 0.835 and 0.124 (2-tailed). There is a trend that Et1+Aβ group had more proliferation compared to the sham group, but this would require further confirmation with sufficient sample size. The minimum total sample size (N) required to give us a desired statistical power (0.8) of detecting a large effect size (0.8) is 52 (2-tailed hypothesis).
Figure 2. Cell proliferation and BrdU uptake were not changed by any treatment 1 week after the surgery. BrdU was injected 1 week after the surgery, and the animals were perfused 24 hours later. Top panel: representative images of Ki67 and BrdU staining. The arrows point at Ki67 positive and BrdU positive cells. Bottom panel: quantification of Ki67 positive cells in each animal model 1 week after the surgery (left), and quantification of BrdU positive cells in each animal model 24 hours after BrdU injection. No significant differences were found. Error bars represent the standard error of the mean (SEM).
11.3 Et1, Aβ, and Et1+Aβ treatments reduced terminal dendritic arborization of immature neurons

Figure 3 shows that Et1, Aβ, and Et1+Aβ treatments significantly reduced DCX positive cells’ terminal dendritic arborization in the outer one-third of the molecular layer of the dentate gyrus 3 weeks after the surgery (p < 0.05). The DCX positive cells had shorter dendrites with fewer branches compared to the sham treatment. We chose to examine dendritic morphology 3 weeks after surgery because our collaborator’s previous study (Whitehead et al., 2007) has found progressively, significantly higher neuroinflammation and neurodegeneration after Et1+Aβ treatment compared to Et1 and Aβ treatment alone at later timepoint (4 weeks) compared to early timepoint (1 week).
Figure 3. Et1, Aβ, and Et1+Aβ treatments reduced terminal dendritic arborization of maturing adult-born neurons 3 weeks after the surgery. Top panel: representative images of DCX staining in each treatment group. Bottom panel: quantification of dendritic arborization in each treatment group. Et1, Aβ, and Et1+Aβ significantly reduced terminal dendritic arborization in the afferent input termination zone compared to the sham group. * p<0.05 Error bars represent SEM.
11.4 Et1+Aβ treatment, but not Et1 and Aβ treatment alone, decreased neuronal survival of immature neurons

Figure 4 shows that Et1+Aβ treatment reduced the total numbers of BrdU positive cells and BrdU/NeuN co-labeled cells compared to the sham treatment 3 weeks after the surgery (p < 0.05). The statistical power was 0.84 (2-tailed) comparing Et1+Aβ group and sham. The effect of Et1 and Aβ treatment alone on the numbers of BrdU positive cells, BrdU/DCX co-labeled cells, and BrdU/NeuN co-labeled cells were not significant.
Figure 4. Et1+Aβ treatment reduced neuronal survival of adult-born neurons 3 weeks after the surgery. BrdU was injected 1 week after the surgery, and the animals were perfused 2 weeks later. Left panel: representative images of BrdU (red) and NeuN (green) co-staining in each treatment group. White arrows point at BrdU positive cells (appear red) and BrdU/NeuN co-labeled cells (appear yellow). Right panel: quantification of BrdU, BrdU/DCX, and BrdU/NeuN in each treatment group. Et1+Aβ significantly reduced neuronal survival compared to the sham group. * p<0.05 Error bars represent SEM.
11.5 Et1, Aβ, and Et1+Aβ treatments did not affect the total number of immature neurons

Figure 5 shows that the total number of DCX positive cells was not significantly affected by any treatment 3 weeks after the surgery. This result is expected because DCX positive cells are a heterogeneous population consisting of cells of various ages. DCX is expressed in maturing adult-born neurons that are less than 3 weeks old. Considering that more than 50% of adult-born neurons die off during their second week of neuronal development, DCX positive population consists of mostly very young, immature neurons (approximately 1 week old). Since our experimental treatments decreased neuronal survival of older cells (Figure 4) without influencing cell proliferation (Figure 2), significant changes in the total number of DCX positive population were not expected.
Figure 5. Total number of DCX positive cells was not significantly influenced by any treatment 3 weeks after the surgery. Top panel: quantification of DCX positive cells in each treatment group. No significant differences were found. Error bars represent SEM. Bottom panel: a partial correlation between DCX cells and Ki67 cells controlled for experimental treatment. A significant correlation was found (p<0.05).
11.6 Et1, Aβ, and Et1+Aβ treatments did not affect dentate gyrus volume

Figure 6 shows that the volume of the granular cell layer in the dentate gyrus was not affected by Et1, Aβ, and Et1+Aβ treatments 3 weeks after the surgery. This result was expected because the survival time was short in this experiment. If we consider that 9,000 new cells are generated in a young rat dentate gyrus every day, and the basal survival rate after the critical survival periods is approximately 50%, then within a month, around 270,000 new cells are generated, but only around 135,000 cells can survive, which represents 5.6% of the total granule cell population of 2.4 million (Cameron and Mckay, 2001). In this experiment, the animals were approximately 3 months old, which had much lower number of generated cells compared to 1-month-old animals (by over 50%). Thus, approximately 4,500 new cells are generated every day, and after a month, 135,000 cells would be generated, but only 67,500 cells would survive. Combined treatment further decreased neuronal survival by more than 90%, thus within a month, 6,750 cells would survive, and the remaining 60,750 cells, which failed to survive would represent 2.5% reduction of the total granule cell population. Since our survival timeline was only 3 weeks, we would not observe any significant changes in dentate gyrus volume after the surgery.
Figure 6. Dentate gyrus volume was not changed 3 weeks after the surgery. The volume of DG was measured by measuring the area of NeuN positive cells per section (mm^2) multiplied by the total number of hippocampal sections and multiplied by the thickness of each section (40um). Error bars represent SEM.
11.7 Et1, Aβ, and Et1+Aβ treatments did not affect neuronal maturation of adult-born neurons

Figure 7 shows that each treatment did not significantly influence neuronal maturation compared to the sham group 2 weeks after the BrdU injection (3 weeks after the surgery). In the sham group, 60% of BrdU positive cells were co-labeled with DCX, and 77% of BrdU positive cells were co-labeled with NeuN (77%). The sum of percentages is more than 100% most likely because some BrdU positive cells co-express DCX and NeuN (Snyder et al., 2009). Et1, Aβ, and Et1+Aβ treatments had similar percentages of co-labeled BrdU/DCX+/+ neurons (p = 0.898), but apparently reduced percentages of co-labeled BrdU/NeuN+/+ neurons by 43% on average, suggesting a possible underlying deficit in the maturation of immature neurons to become mature neurons. Yet, this effect was expected to be non-significant (p = 0.530) since the animals were sacrificed early. However, since the sample size was small, the statistical power was low (0.2). More experiments are needed to increase the sample size (N=52, 2-tailed, Cohen’s d=0.8, power=0.8).
Figure 7. Neuronal maturation was not significantly affected 3 weeks after the surgery. The numbers of BrdU/DCX⁺/⁺ neurons and BrdU/NeuN⁺/⁺ were expressed in terms of % of the total number of BrdU⁺ cells. There was no significant difference in % DCX and % NeuN expression between sham and treated groups. Error bars represent SEM.
11.8 Et1+Aβ treatment resulted in greater inflammation in the CA1 region compared to Et1 and Aβ treatment alone

Figure 8 shows representative images of CD11b staining of microglia/macrophage in the hippocampal subregions: DG, CA3 and CA1 regions 1 week after the surgery in all the animals. Et1 and Aβ treatment alone did not result in any apparent changes in CD11b staining intensity in the hippocampus compared to the sham group. However, Et1+Aβ treatment led to greater CD11b staining in the CA3 and CA1 subregions compared to the sham group.
Figure 8. Et1+Aβ treatment resulted in greater inflammation in the hippocampus 1 week after the surgery. Et1 and Aβ treatment alone did not result in any apparent changes in CD11b staining, whereas Et1+Aβ treatment resulted in greater CD11b staining in the CA3 and CA1 subregions (at the edge).
12 Discussion

12.1 Summary of our results
We showed that Et1 and Aβ alone reduced dendritic complexity of hippocampal young neurons without altering neuronal survival. Yet, changes in dendritic complexity may be an early stage of neuronal death. Indeed, neuronal survival was significantly reduced when these treatments were combined. The reduction in survival was not caused by changes in cell proliferation at an early time point. Thus, our results suggest that Et1 and Aβ interact, most likely through multiple mechanisms, exacerbating dendritic abnormalities and reducing the survival of adult born neurons in the hippocampus.

12.2 Summary of our collaborators’ results
Other, possibly related mechanisms underlying the interactions between Et1 and Aβ were investigated by our collaborators (paper submitted for publication). Both magnetic resonance imaging (MRI) and histological assessment showed that combined treatment resulted in significantly more pronounced enlargement of forebrain lateral ventricles and tissue loss in the hippocampus and striatum three weeks after the surgery compared to Et1 and Aβ treatment alone (results not shown, Dr. Bartha’s and Dr. Cechetto’s labs, University of Western Ontario). This was also associated with elevated deposition of endogenous beta amyloid, inflammation, and cellular degeneration in the striatum, somatosensory cortex, and hippocampus (results not shown, Dr. Cechetto’s lab), and behavioural deficits in a hippocampus-dependent discriminative fear conditioning to context task (results not shown, Dr. R.J.McDonald’s lab, University of Lethbridge). Furthermore, MRI showed a time-dependent progression of neurodegeneration, which was not obvious at gross level one week after the surgery. Overall, our results and our collaborators’ results suggest that Et1 and Aβ could synergistically interact through multiple mechanisms, which progressively led to observed neuropathology and behavioural deficits.
12.3 Possible Mechanisms Underlying Changes in Hippocampal Adult Neurogenesis and Future Studies

The decrease in dendritic arborization and neuronal survival of hippocampal adult-born neurons was associated with elevated deposition of Aβ, higher expression of class II MHC protein (OX6 staining), and increased number of degenerating neurons (Fluoro-Jade staining) in the dentate gyrus, especially in the hilus region, 3 weeks after the combined treatment (results from Dr. Cechetto’s lab). Higher staining intensity of CD11 was also observed in the CA1 region. The effect on adult neurogenesis could be caused by several possible mechanisms.

First, Aβ could result in a net over-excitation in the hippocampus. Palop et al., (2007) showed that transgenic mice over-expressing APP had spontaneous non-convulsive seizure activity in the hippocampus and associated synaptic plasticity deficits in the dentate gyrus. Aβ could disrupt the strength of GABAergic inhibition by impairing excitatory synapses onto inhibitory hilar interneurons and/or by directly inducing apoptosis of hilar interneurons. This is consistent with our collaborator’s observation that combined treated group presented with more degenerating neurons in the hilus region (Dr. Cechetto’s lab). The lack of GABAergic signaling can have several consequences on adult neurogenesis. First, hyper-excitation in the dentate gyrus can directly lead to excitotoxic dendritic injuries of young neurons (Greenwood & Connolly, 2007). Second, lack of GABAergic inputs onto young neurons can impair early neuronal development (Ge et al., 2006). Consequently, neuronal survival is reduced. To address these possibilities, future studies can address the effects of GABA receptor agonists on neurogenesis after the combined treatment.

Second, Aβ could trigger inflammation in the hippocampus, reducing the survival of young neurons. Although the staining intensity of CD11 positive microglia/macrophage was higher in the CA1 region than in the dentate gyrus, it was likely that diffusible inflammatory mediators...
influenced the survival of young neurons due to the close proximity of the two hippocampal subregions. However, since OX6 positive microglia/macrophage were found in the dentate gyrus, it was also possible that these microglia directly influenced young neurons. Since microglia can exist in different states that determine their beneficial or detrimental roles in neurogenesis, it was likely that in our study, the overall net effect of microglia was detrimental to the young neurons. To address this possibility, future studies can address the effects of anti-inflammatory drugs on neurogenesis after the combined treatment.

Third, since neurogenesis is negatively influenced by glucocorticoid, it was possible that combined treatment resulted in higher levels of stress in the animals. To address this possibility in future studies, we can monitor the level of glucocorticoid and/or surgically remove adrenal glands from animals, and supplement the animals with physiological levels of glucocorticoid, then study changes in adult neurogenesis after combined treatment. Furthermore, future experiments should address the full effects of each treatment by examining at later time points, for example, one or two months after surgery.

In our study, we did not observe any changes in cell proliferation in the dentate gyrus after any treatment. It was most likely due to counteracting effects of various factors. Cell proliferation was not increased in our stroke model most likely because the ischemia lesion was smaller than the lesion induced by other global and focal animal models. Previous studies have suggested that diffusible growth factors released from damaged areas after ischemia increase neurogenesis (Yoshimura et al., 2001; Yan et al., 2006), so it is possible that our stroke model did not significantly increase the level of growth factors. Furthermore, cell proliferation was not altered in our AD model mostly likely because of various factors. Previous studies have shown that Aβ directly stimulates neuronal proliferation (Lopez-Toledano and Shelanski, 2007; Gan et al.,
2008), whereas inflammation reduces neuronal proliferation (Monje et al., 2003), so it is possible that these two factors cancelled each other with net effect of no change in cell proliferation in our AD model. Considering the complex effects of various factors on neurogenesis, such as stress, growth factors, inflammation, and various types of microglia that could increase or decrease neurogenesis, it is likely that the net effect of these factors did not alter cell proliferation after the combined treatment.

Although our comorbid AD & stroke model resembles an extreme case in clinical setting, it does provide a good starting point for basic researchers to directly study the underlying mechanism. Future studies can develop alternative models, such as induction of stroke in young adult rats followed by treatment of Aβ later in life, which would be more applicable to clinical cases. In addition, although injection of Aβ_{25-35} generates a useful AD model and many previous studies have shown some similar results obtained by treatment of Aβ_{25-35} and full length Aβ_{1-42} and Aβ_{1-40} (reviewed by Mattson, 1997), Aβ_{25-35} has not been found in AD patients and treatment with different Aβ isoforms has produced mixed results (reviewed by Mattson, 1997). Thus, future studies should adjust the dosage and further utilize other Aβ isoforms and transgenic models to dissect the underlying mechanism in the comorbid models.
13 Specific Objectives and Hypothesis

13.1 Specific objective

The objective of this study is to examine changes in hippocampal adult neurogenesis, which can provide an explanation for the pathological interaction between obesity-linked T2D and AD-like pathology.

13.2 Specific hypothesis

We hypothesize that combined AD & obesity-linked T2D model will reduce hippocampal adult neurogenesis compared to AD and T2D models alone.
14 Methods

Experimental procedure was carried out in the same way as the combined AD and stroke study (Chapter 3) with the following changes.

14.1 Animals

All experimental procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of the University of Western Ontario. Twelve (12) ten-week old Zucker rats were randomly assigned to four treatment groups: sham (ZDF lean, n=3), T2D (ZDF obese, n=3), AD (ZDF lean+Aβ) (n=3), and AD+T2D (ZDF obese+Aβ, n=3).

14.2 Stereotaxic surgery

The AD model (ZDF lean+Aβ group) was created by bilateral injections (50nmol/10ul saline) of Aβ25-35 (Bachem, Rorrance, Calif) at a rate of 1ul/30s into the lateral ventricles (anteroposterior -0.8mm and mediolateral ±1.4mm relative to the bregma, and dorsal/ventral -4.0mm below dura) of ZDF lean rats. The combined T2D and AD model (ZDF obese+Aβ group) was created by bilateral injections of Aβ25-35 into the lateral ventricles of ZDF obese rats. The sham group (ZDF lean) and diabetic group (ZDF obese) received saline injections.

14.3 BrdU administration

5’-Bromo-2-deoxyuridine (BrdU, Sigma) was dissolved in warm saline with 1ul NaOH (10N) to produce a stock concentration of 20mg/ml. Each animal received a single intraperitoneal injection of BrdU (200mg/kg) 1 week after the surgery to catch the highest rate of cell proliferation.
14.4 Perfusion and fixation

All the animals were sacrificed 3 weeks after the treatment. Sectioning, sampling, and staining procedure was carried out in the same way as the combined AD and stroke study (Chapter 3).
15 Results

15.1 Inflammation at injection sites

Figure 9 shows CD11b staining of microglia/macrophage in the lateral ventricles 3 weeks after the surgery. Inflammation was not detected in the sham and ZDF obese groups, but was present in the Aβ/ZDF lean group and Aβ/ZDF obese groups.
**Figure 9. Inflammation at injection sites 3 weeks after the surgery.** Sham and ZDF obese animals did not have any significant CD11b staining in the lateral ventricles. Aβ/ZDF lean and Aβ/ZDF obese had CD11b staining in the lateral ventricles (white arrows).
15.2 Neuronal survival of adult-born neurons in the hippocampus was not significantly changed

Figure 10 shows neuronal survival reflected by the number of BrdU positive cells in each group 3 weeks after the surgery (2 weeks after the BrdU injection). There was no significant effect of any treatment on the number of BrdU cells. Yet, since the statistical power was 0.33 (2-tailed) comparing Aβ/ZDF obese and sham, more experiments should be done to include sufficient sample size.
Figure 10. Neuronal survival of hippocampal adult-born neurons was not influenced by any treatment. BrdU was injected 1 week after the surgery, and the animals were sacrificed 2 weeks later. Top panel: representative image of BrdU staining in the DG. White arrows point at BrdU positive cells. Bottom panel: quantification of BrdU positive cells in each group. No significant differences were found. Error bars represent SEM.
15.3 Cell proliferation in the DG was not significantly changed

Figure 11 shows cell proliferation reflected by the number of Ki67 positive cells in each group 3 weeks after the surgery. There was no significant effect of any treatment on the number of Ki67 positive cells.
Figure 11. Cell proliferation in the DG was not influenced by any treatment 3 weeks after the surgery. Cell proliferation was measured by the number of Ki67 positive cells. No significant differences were found. Error bars represent SEM.
15.4 The number of DCX positive cells was not significantly changed

Figure 12 shows that the number of DCX positive cells was not significantly influenced by any treatment 3 weeks after the surgery. This result was expected because there were no significant changes in cell proliferation (Figure 11) or cell survival (Figure 10).
Figure 12. The number of DCX positive cells in the DG was not influenced by any treatment 3 weeks after the surgery. Top panel: representative DCX staining in the DG. White arrows point at DCX positive cells. Bottom panel: quantification of DCX positive cells in each group. No significant differences were found. Error bars represent SEM.
15.5 Inflammation was not observed in the hippocampus

Figure 13 shows that inflammation was not detected in any hippocampal subregions: DG, CA3 and CA1, 3 weeks after the surgery. CD11b staining was very similar among all treatment groups.
Figure 13. Inflammation was not detected in the hippocampus 3 weeks after the surgery. Cd11b staining of microglia/macrophage in the DG, CA3, and CA1 regions was similar among all treatment groups.
16 Discussion

We showed that in 14 weeks old ZDF obese rats, obesity-induced hyperglycemia and Aβ treatment alone, as well as combined treatment, did not significantly influence cell proliferation or survival of young neurons in the hippocampus. We did not observe any changes in CD11b staining of microglia/macrophage in the hippocampus after any treatment. ZDF obese rats were significantly heavier than ZDF lean rats at the time of perfusion (14 weeks old, approximately 390g for ZDF obese and 310g for ZDF lean, Dr. Cechetto’s lab, results not shown). ZDF obese rats also had higher glucose and slower clearance during glucose tolerance test compared to ZDF lean rats at the time of perfusion (baseline: approximately 10mM for ZDF obese and 5mM for ZDF lean; peak: 20mM for ZDF obese and 13mM for ZDF lean; after 90min: 15mM for ZDF obese and 5mM for ZDF lean; results not shown, Dr. Cechetto’s lab). We did not observe any significantly changes in adult neurogenesis most likely because we examined ZDF rats too young an age.

The interaction between T2D and Aβ in our animal model can occur at the level of inflammation, and is likely to be exacerbated by aging. T2D is known to associate with obesity as cytokines released by fat cells, such as TNFa and IL-6, contribute to insulin resistance (reviewed by Powell, 2007). Systemic inflammation can exacerbate inflammation in the brain by further converting microglia to the pro-inflammatory state (reviewed by Perry et al., 2007). Since aging is also associated with elevated systemic inflammation, which has shown to reduce neurogenesis (Villeda et al., 2011), aged obese, diabetic rats can have greater inflammatory response after brain insults, such as Aβ treatment, thus experiencing greater decline in neurogenesis. In addition, it is also possible that T2D and Aβ produce more stress in the animals, which lowers adult neurogenesis. Since the extent of hyperglycemia in our animals was not as severe as in other studies (results not shown, Dr. Cechetto’s lab), it is possible that the combined treated
animals did not have significant changes in glucocorticoid levels. Thus, adult neurogenesis was not significantly decreased.

Since brain pathologies induced by hyperglycemia and Aβ are exacerbated by aging, changes in neurogenesis are more likely to be detected at older age. For instance, Hwang et al. shows that ZDF rats have greater reduction in neurogenesis at an older age (30 weeks old) compared to at a younger age (16 weeks old) (2010a, 2010b). Changes in neurogenesis at young age are less likely to be detected unless the animals are challenged. For instance, Yi et al., (2009) shows that neurogenesis in young ZDF rats is less responsive to running, a positive stimulant, despite it appears normal under basal condition. Therefore, future studies can examine changes in adult neurogenesis in aged ZDF obese rats treated with Aβ, and test the effects of anti-inflammatory drugs and restoring glucocorticoid levels on adult neurogenesis.
17 The Overall Picture

In conclusion, T2D, Et1, and Aβ can interact via various mechanisms in our animal models, such as beta-amyloid generation, brain inflammation, and potentially stress, which closely associated with changes in hippocampal adult neurogenesis (Figure 14). These mechanisms do not exist independently, but likely interact with each other in an age-dependent manner. Future studies should further dissect how each treatment, as well as combined treatments, influence adult neurogenesis.
Figure 14. Overall picture of Et1, Aβ, T2D comorbidity. Stroke, AD, and T2D alone is age-dependently associated with inflammation, Aβ deposition, stress, which negatively influence the generation and survival of adult-born neurons in the hippocampus. Growth factors can also be present in the brain as a compensatory mechanism to brain insults. The net effect on adult neurogenesis is influenced by various factors, depending the balance between detrimental and beneficial cues.
References


Appendices

Part 1: The Functional Role of Hippocampal Adult Neurogenesis

Part 2: Preliminary Development of Methodology for Using CldU and IdU to Label Two Populations of Adult Born Neurons in the Same Animal

Section I: Rationale

Previous studies on adult neurogenesis have extensively utilized the birthdate marker BrdU to label one population of adult-born neurons in experimental animals. In order to study adult-born neurons with different ages, BrdU has to be injected into different batches of animals, which are sacrificed at different time-points after the injection. The main issue involved is that the direct relationship between different populations of young neurons at different ages cannot be studied, since in this case, several batches of animals have to be used. Thus, in order to address this issue, we have to label several populations of young neurons at different time-points in the same animals. This preliminary study has directly addressed this approach by utilizing two birthdate markers, CldU and IdU, which are injected into the same animals at different time-points. Here, we provide evidences that CldU and IdU can label 2 separate populations of young neurons in the same animals.
Section II: Experimental Design

Experiment 1: Injection of CldU to Label Adult-born Neurons in the Hippocampus
Equimolar amount of CldU (comparable to 200mg BrdU/kg body weight) was dissolved in 0.9% saline solution, and i.p. injected into a 2-month-old Sprague-Dowley rat. The stock solution could be prepared at 20mg/ml. The animal was sacrificed 24hr after the injection.

Experiment 2: Injection of IdU to Label Adult-Born Neurons in the Hippocampus
Equimolar amount of IdU (comparable to 200mg BrdU/kg body weight) was dissolved in 0.9% saline with a few drops of NaOH, and i.p. injected into a 2-month-old Sprague-Dowley rat. The stock solution could be prepared at 20mg/ml. The animal was sacrificed 24hr after the injection.

Experiment 3: Injection of CldU and IdU into the Same Animals to Label 2 Separate Populations of Adult-born Neurons in the Hippocampus
CldU was first injected into a 2-month-old Sprague-Dowley rat. Three weeks later, the same animal received IdU injection, and perfused 24hr after the IdU injection. In this case, at the time of perfusion, CldU positive cells were 3-week-old, whereas IdU positive cells were 24hr. In another experiment, the animals were perfused 1 week after the IdU injection. In this case, at the time of perfusion, CldU positive cells were 3-week-old, whereas IdU positive cells were 1-week-old.
Section III: Result

3.1 Staining of CldU

The protocol for CldU and IdU staining is the same as BrdU staining, involving 30min incubation in 1N HCl at 45°C for 30min followed by 3 brief rinses and 3×5min washes in PBS. We did 3 rinses to ensure that we extensively washed out the acid, because any remaining of the acid could denature the primary antibodies and influence the staining of CldU and IdU.

In Experiment 1, CldU positive cells could be labeled by rat anti-BrdU antibody (1:1500, Serotec, OBT0030) followed by Alexa Fluor goat anti-rat IgG secondary antibody (1:1500) (Figure 1, left column, top). At this concentration, CldU antibody did not label any IdU cells in Experiment 2, meaning that rat anti-BrdU could specifically label CldU at this concentration (data not shown). Most of the CldU positive cells were also positive for Ki67 (Figure 1, left column, bottom), suggesting that they were proliferating. The staining protocol for CldU/Ki67 involved sequential addition and incubation of CldU primary antibody (1:1500, 24hr at 4°C), followed by Ki67 primary antibody (rabbit anti-Ki67, 1:200, 18hr at room temperature), and followed by co-incubation of secondary antibodies (2hr, room temperature).

3.2 Staining of IdU

In Experiment 2, IdU positive cells could be labeled by mouse anti-BrdU antibody (1:700, Lot 70048) followed by Alexa Fluor goat anti-mouse IgG secondary antibody (1:700) (Figure 1, right column, top). At this concentration, IdU antibody did not label any CldU cells in Experiment 1 (data not shown), meaning that mouse anti-BrdU could specifically label IdU at this concentration. Most of the IdU positive cells were also positive for Ki67, suggesting that they were proliferating (Figure 1, right column, bottom). The staining protocol for IdU/Ki67 involved sequential addition and incubation of IdU primary antibody (1:700, 24hr at 4°C), followed by Ki67 primary antibody (rabbit anti-Ki67, 1:200, 18hr at room temperature), and followed by co-incubation of secondary antibodies (2hr, room temperature).
Figure 1. CldU and IdU positive cells could be specifically labeled. Top: timeline of experiments 1 and 2. Young rats were injected with either CldU or IdU, and sacrificed 24 hours later. Bottom: CldU and IdU cells were co-labeled with Ki67.
Double Staining of CldU/IdU with Neuronal Markers

Considering the concentrations of CldU and IdU antibodies were low, double-staining of CldU/IdU with various neuronal markers should be done by co-incubating primary antibodies followed by co-incubating corresponding secondary antibodies. The reason behind co-incubation of primary antibodies was that sequential staining of various markers would dilute CldU and IdU staining. For example, if CldU or IdU antibody was added first and incubated for 24hr, and then it was washed out upon addition of primary antibodies of neuronal markers followed by incubation period of 1-3 days, the resulting staining would lack CldU or IdU (results not shown). In addition, we have shown that the primary antibodies of CldU and IdU did not cross-react (Experiment 1 & 2), so secondary antibodies should be carefully chosen/tested to ensure absence of cross-reactivity.

3.3.1 CldU/IdU Co-staining

The primary antibodies of CldU and IdU did not cross react (Experiment 1 & 2). However, when the secondary antibodies were added, most labeled cells were co-positive for CldU and IdU, despite that they were 2 different populations (results not shown). This indicated that the secondary antibodies (anti-rat & anti-mouse) cross-reacted. This was further confirmed by the following test: in experiment 1, application of CldU primary antibody (rat anti-BrdU) and IdU secondary antibody (anti-mouse) resulted in false positive staining, indicating that IdU secondary antibody could bind to CldU primary antibody. This false positive staining could not be reduced by dilution, since IdU secondary antibody at a concentration as low as 1:4000 still yielded false positive staining. Thus, without specific secondary antibodies for rat and mouse, CldU/IdU co-labeling is not feasible.

However, it was possible to separately label CldU or IdU with various neuronal markers in different hippocampal sections from the same animals. Since CldU labeled 3-week-old neurons, we could co-labeled CldU with CaBP, a mature neuronal marker. Since IdU labeled 24hr-old or 1-week-old neurons, we could co-labeled IdU with DCX, an immature neuronal marker. In order to show that CldU and IdU could label 2 separate populations, CldU positive cells should also be positive for CaBP, but not DCX, whereas IdU positive cells should also be positive for DCX, but not CaBP.
3.3.2 CldU/CaBP Staining

Co-incubation of CldU primary antibody (rat anti-BrdU, 1:1500) and CaBP primary antibody (rabbit anti-CaBP, 1:200) for 3 days followed by co-incubation of secondary antibodies (Alexa Fluor, goat anti-rat IgG 488, donkey anti-rabbit IgG 568) successfully co-labelled CldU/CaBP (Figure 2, left column). Co-incubation of CldU primary antibody and DCX primary antibody (goat anti-DCX, 1:200) for 1 day followed by co-incubation of secondary antibodies (Alexa Fluor, goat anti-rat 488, donkey anti-goat 594) did not result in co-labeled CldU/DCX positive cells (Figure 2, right column).
Figure 2. CldU labeled a more mature population in experiment 3. Top: Timeline of experiment 3. CldU injection was done 3 weeks before the IdU injection into the same animals. Animals were perfused 24hr or 1 week after IdU injection. Bottom: Representative confocal images of CldU staining. Left column: CldU positive cells (pointed by white arrow) were co-positive for CaBP (pointed by yellow arrow), a mature neuronal marker. Right column: CldU positive cells (pointed by white arrows) were not co-positive for DCX, an immature neuronal marker.
3.3.3 IdU/DCX Staining

Co-incubation of IdU primary antibody (mouse anti-BrdU, 1:700) and DCX primary antibody (goat anti-DCX, 1:200) for 1 day followed by co-incubation of secondary antibodies (Alexa Fluor, goat anti-mouse IgG 488, donkey anti-goat 594) successfully co-labeled IdU/DCX (Figure 3, left column). Co-incubation of IdU primary antibody (mouse anti-BrdU, 1:700) and CaBP primary antibody (rabbit anti-CaBP, 1:200) for 3 days followed by co-incubation of secondary antibodies (Alexa Fluor, goat anti-mouse IgG 488, donkey anti-rabbit IgG 568) did not result in any co-labeled IdU/CaBP positive cells (Figure 3, right column).
**Figure 3. IdU labeled a younger population in experiment 3.** Top: Timeline of experiment 3. CldU injection was done 3 weeks before the IdU injection into the same animals. Animals were perfused 24hr or 1 week after IdU injection. Bottom: Representative confocal images of IdU staining. Left column: IdU positive cells (pointed by white arrow) were co-positive for DCX (pointed by yellow arrow), an immature neuronal marker. Right column: IdU positive cells (pointed by white arrows) were not co-positive for CaBP, a mature neuronal marker.
Section IV: Discussion

This preliminary study has shown that CldU and IdU can be used to label 2 populations of adult-born neurons in the hippocampus in the same animals. Although co-labeling CldU and IdU was not feasible due to cross-reactivity of the secondary antibodies, CldU and IdU co-labeling with various neuronal markers could be done on separate hippocampal sections from the same animals. We showed that 3-week-old CldU positive cells were co-positive for CaBP, a mature neuronal marker, but not DCX, an immature neuronal marker, and 24hr or 1-week-old IdU positive cells were co-positive for DCX, but not CaBP. Our results indicated that CldU and IdU could label 2 populations of young neurons with different ages.

Given the preliminary success of CldU and IdU co-labeling with neuronal markers, a further complete study should be done to address the following: 1. determine the dosages of CldU and IdU required to label the maximal numbers of dividing cells without causing cell death during the timeframe of cell cycle. 2. Since CldU and IdU have different solubility and potentially bioavailability, they most likely label different numbers of dividing cells. Thus, the maximal numbers of dividing cells labeled by CldU and IdU, and their subsequent survival should be studied. This will allow direct comparison between CldU and IdU cell numbers in the same animal.

By using CldU and IdU, future studies can examine various experimental treatments on 2 separate populations of young neurons at different ages of development in the same animals. This will provide more understanding about the fundamental processes of adult neurogenesis and its function in learning and memory.