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Glucose, Glucose Transporters and Neurogenesis

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# GLUCOSE, GLUCOSE TRANSPORTERS and NEUROGENESIS

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the MSc degree in Psychology

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*Your file Votre référence*
*ISBN: 978-0-494-50926-5*
*Our file Notre référence*
*ISBN: 978-0-494-50926-5*

**Acknowledgements**

I would like to acknowledge and thank my thesis supervisor, Dr. Claude Messier, for his never-ending support, encouragement and training that allowed me to continuously develop my research skills. In addition, I would also like to thank Dr. Bill Staines (Cellular and Molecular Medicine) for all of his help and expertise, especially with the thesis document. I also like to thank my family for their continued support during my years in Ottawa. Finally, I would like to acknowledge and thank my friends and fellow graduate students whom, in a variety of ways, provided to be role models in my pursuit of higher education.

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**Abstract**

Since the pioneering work of Altman in the late 60's, much has been learned about the generation of neurons in the adult brains of several species, including mice, rats, and humans. An underlying assumption is that these newborn neurons acquire their energy, in the form of glucose, in a similar manner to mature neurons: via glucose transporters. Using BRDU and double immunohistochemistry, we investigated the relationship between hippocampal neurogenesis and glucose transporters, as well as monocarboxylate transporters. Unexpectedly, the results suggest that newborn neurons *do not* acquire their energy via the major glucose transporters (1, 3, 4, and 8), nor via either monocarboxylate transporter tested (1 and 2). Future studies will have to resolve whether lesser known glucose transporters carry this function or if other mechanisms are used to provide metabolic energy to newborn neurons.

## Glucose, Glucose Transporters and Neurogenesis

### *Background*

At the turn of the century, both Santiago Ramon y Cajal and Camillo Golgi, two of the world's foremost neuroscientists of their times, shared the conviction that the regeneration and growth of neurons and dendrites ended with development (cited in Colucci-D'Amato, Bonavita, & di Porzio, 2006). For more than half a century, this conviction dominated the scientific community. However, in the 1960's, work on adult neurogenesis appeared, suggesting that Cajal and Golgi may have been too quick to come to a conclusion (Lie, Song, Colamarino, Ming, & Gage, 2004). Although this early research was given little attention in its time, it provided the impetus for further inquiry. Only recently has a preponderance of evidence confirmed that neurogenesis does take place, albeit in discrete areas, of the adult brain (Alvarez-Buylla & Garcia-Verdugo, 2002; Gage, 2002; Kempermann & Gage, 2000).

Using a technique developed by (Sidman, Miale, & Feder, 1959) to label newly dividing cells with [$H^3$]-thymidine, Smart (1961) was among the first to observe the development of new neurons in young mice. Later, the research of Altman (Altman, 1969; Altman & Das, 1966) provided further evidence for neurogenesis, in various regions of the rat brain. Despite this evidence, and because the research was not taken seriously enough, the field stagnated, although interest was rekindled in the early 1980's with the discovery of neurogenesis in the vocal control centre of adult female canaries (Goldman & Nottebohm, 1983). With the introduction of bromodeoxyuridine (BrdU) in

the early 80s as a new marker of cell birthdates, however, the study of neurogenesis was facilitated (Ming & Song, 2005). In the early 1990s, two important research results were published. First, Reynolds and Weiss (1992) were able to isolate progenitor cells from the adult mouse striatal area (Reynolds & Weiss, 1992). Subsequently, Gage and colleagues (1995) isolated progenitor cells from the adult rat hippocampus (Gage et al., 1995). As a result of this seminal work, the field exploded and neurogenesis has since been detected in many species of adult mammals, including humans (Eriksson et al., 1998).

## Neurogenesis: From stem cell to mature neuron

Neurogenesis is the generation of neurons from progenitor cells. Although widely generalized in development, this phenomenon is localized to two distinct regions of the adult brain: the sub-ventricular zone (SVZ) of the lateral ventricle, and the sub-granular zone (SGZ) of the dentate gyrus (Couillard-Despres et al., 2005). Minor neurogenesis has also been reported in the cortex (Au & Fishell, 2006; Gould, Reeves, Graziano, & Gross, 1999), amygdala (Bernier, Bedard, Vinet, Levesque, & Parent, 2002), and substantia nigra (Yoshimi et al., 2005), although these findings are not universally accepted.

First isolated in the central nervous system of an adult rat in the early nineties, neural stem cells are the source of the progenitor cells (Ming & Song, 2005). Due to the vast amount of neurogenesis research in recent years, the difference between *stem cell* and *progenitor* has become somewhat blurred (Gage, 1998). Most researchers agree that neural *stem cells* are mitotic cells, albeit slowly dividing ones, that have the ability to proliferate, self-renew and are multipotent (Abrous, Koehl, & Le Moal, 2005). While

also mitotic, *progenitor* cells divide at a greater rate than stem cells. Moreover, unlike the stem cells from which they derived, the progenitor cells are much more committed to a particular cell lineage (Abrous, Koehl, & Le Moal, 2005). Stem cells do exist in other areas of the brain, but most remain dormant. Only stem cells in the two major neurogenic regions are active. Granted that there is still much to learn about the process of neurogenesis, but what is known (discussed below) is that neuronal progenitors in the SGZ eventually give rise to new hippocampal granule neurons, integrating within the molecular layer of the CA3 region of the hippocampus (Lie, Song, Colamarino, Ming, & Gage, 2004). Neuronal progenitors in the SVZ give rise to new neurons in the olfactory bulb that migrate to this site via the rostral migratory pathway (RMS) (Lie, Song, Colamarino, Ming, & Gage, 2004).

*Methods for Investigating Adult Neurogenesis*

There are two strategies to study neurogenesis (Christie & Cameron, 2006). In the first, experimental animals are injected with a particular compound that labels cells undergoing cell division at the time of injection, followed by detection of that label, usually by immunohistochemical means. The second strategy involves labelling and detecting proteins that characterize dividing cells. Each of these strategies has its advantages and disadvantages, and both will be examined in turn below.

*I. Strategy one: Injection, labelling and detection*

In the 1960's, peripheral injections of [$H^3$]-thymidine were used to detect dividing cells in the brain (Christie & Cameron, 2006). When injected, usually intraperitoneally (i.p), the [$H^3$]-thymidine incorporated itself into the DNA. This can only occur when the DNA is being synthesized (during the S-phase of the mitotic cycle), thus, the [$H^3$]-

thymidine is a marker of mitotically active cells (Abrous, Koehl, & Le Moal, 2005). [$H^3$]-thymidine can be visualized by autoradiography but it is a time-consuming and expensive procedure (Abrous, Koehl, & Le Moal, 2005). Moreover, it does not enable the double-labelled or triple-labelled immunofluorescent microscopy required for current criteria for documentation of neurogenesis (Christie & Cameron, 2006).

The introduction of the use of the synthetic thymidine analog bromodeoxyuridine (BrdU) in the early eighties has had a great impact on the study postnatal neurogenesis (Gratzner, 1982). BrdU is incorporated into cells during DNA synthesis (Abrous, Koehl, & Le Moal, 2005). BrdU incorporation is evaluated by immunohistochemical staining, and may be co-labelled with other cellular markers

The use of BrdU has a number of limitations that merit discussion (Christie & Cameron, 2006). One commonly raised issue relating to the use of BrdU is that, in addition to its labelling of mitotically active cells, it will also label cells undergoing DNA repair, where some of the DNA is also being synthesized (Rakic, 2002b). Although this argument appears reasonable, the number of nucleotides that are synthesized during DNA repair is much smaller than during the S-phase of mitosis. Thus, it is unlikely that the BrdU can actually label the repairing DNA to a large extent (Christie & Cameron, 2006). This conclusion is supported by the observation that when irradiation is used to cause DNA damage and thus, an increase in DNA repair, BrdU staining only reveals dividing cells (Parent, 2002).

Secondly, the dosage and, perhaps more importantly, the timing of the injection(s) will determine to certain extent the number of cells clearly labelled. The most common dosage (50 mg/kg) injected once a day for several consecutive days labels an acceptable

number of cells (Abrous, Koehl, & Le Moal, 2005). However, some recent work by Cameron and McKay (2001) suggests that this dose is, in fact, not high enough and only labels a portion of dividing cells, which may result in false negatives. To remedy this, they suggest using a dose of 200-300 mg/kg that will label a larger number of cells. Alternatively, it has been suggested that multiple doses of the 50 mg/kg dose can be used to successfully label more dividing cells (Abrous, Koehl, & Le Moal, 2005). Although there is empirical evidence to suggest that embryonic or postnatal injections (60 mg/kg) of BrdU result in various physical and behavioural abnormalities (Kolb, Pedersen, Ballermann, Gibb, & Whishaw, 1999), doses as high as 200-300 mg/kg will not cause cell death in adults, thus making them safe for use (Christie & Cameron, 2006).

In addition to deciding *how much* BrdU is injected, *when* it is injected is another important consideration. This decision depends heavily on the neurogenic phenomenon being examined, either proliferation or survival. When studying proliferation of new cells, it is best to give the BrdU injection(s) within a few hours of treatment because the BrdU, upon injection, labels nearly all cells within the first half hour (Cameron & McKay, 2001). For studying cell survival, it is best to inject BrdU several days after treatment in order to avoid any false negative that would arise from the dilution of BrdU that occurs even during the first division after injection (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003).

Overall, the popularity of BrdU has exploded insofar as it is probably the most prevalent method to measure neurogenesis. Having said that, however, researchers rely on another strategy to complement BrdU labelling and in the next section, this strategy will be discussed in depth.

Finally, before proceeding to the next section, it is worth mentioning that the use of retroviruses expressing green fluorescent protein (GFP) has been used to study neurogenesis (eg. ((Zhao, Teng, Summers, Ming, & Gage, 2006)). This technique, where a retrovirus is used to infect target cells, has two distinct advantages over BrdU. First, and perhaps most importantly, the retrovirus *only* labels dividing cells during the mitotic stage of the cell cycle, and not during a state of DNA repair, as BRDU might (Roe, Reynolds, Yu, & Brown, 1993). Secondly, the associated GFP allows for the entire cell (including arborization in neurons) to be visualized at once, making it much simpler to identify the cells in question (Okada, Lansford, Weimann, Fraser, & McConnell, 1999). Despite the higher specificity, the retrovirus method is not as efficient at labelling cells as is BrdU, mainly because of the need for rigorous specificity and direct injection into a population of dividing cells (Ackman, Siddiqi, Walikonis, & LoTurco, 2006). Another problem that can occur with the use of retroviruses is the potential for false positives that may arise when infected microglia fuse to a neuron, whereupon it fills the neuron with GFP. Thus, researchers will have to be much more judicious when using the retrovirus method in situations that lead to increased microglia activation.

*II. Strategy two: Labelling of endogenous proteins*

There are endogenous markers of the cell cycle, such as Ki-67, associated with cell proliferation (Taupin, 2006). Ki-67 labelling is similar to BrdU, although Ki-67 labelling is expressed throughout the majority of the cell cycle (Eadie, Redila, & Christie, 2005). In addition to Ki-67, PCNA (proliferating nuclear antigen) and RNR (ribonucleotide reductase) are measures of adult neurogenesis (Taupin, 2006). There is, however, one major difference between these markers and BrdU that limits the use of the

cell-cycle markers. Unlike BrdU, which gets permanently incorporated into the DNA, the cell-cycle markers are only expressed during the cell cycle and not at any point during the maturation process (Taupin, 2006). Therefore, these markers can be used to indicate which cells are in the midst of the cell cycle and, presumably, DNA synthesis but are limited to this use.

In addition, cell cycle markers do not provide phenotype information. Rather, these markers are used to identify maturing neurons and glia, some have been identified as markers of stemlike cells (Abrous, Koehl, & Le Moal, 2005). The most widely used amongst this small group is the intermediate filament protein nestin, and several studies have described nestin expression in both of the previously described neurogenic regions in the adult brain (e.g. (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997; Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003). Another putative marker of stemlike cells is the traditional glial marker, glial filament acidic protein (GFAP) (Alvarez-Buylla, Seri, & Doetsch, 2002; A. D. Garcia, Doan, Imura, Bush, & Sofroniew, 2004). In the SVZ and SGZ of rodents (see below), GFAP-positive astrocytes can act as neural progenitors and give rise, eventually, to migrating neuroblasts and immature neurons, respectively (Ming & Song, 2005).

For immature neurons, one of the more viable and prominent markers is the microtubule-associated protein doublecortin (DCX), which is expressed in migrating neuronal precursors in both neurogenic regions (Couillard-Despres et al., 2005). Furthermore, empirical evidence has shown that DCX can label these immature neurons up to 2 weeks after they become post-mitotic (Rao & Shetty, 2004). However, DCX has also been localized to other non-neurogenic regions of the brain, such as the corpus

collosum and striatum (Nacher, Crespo, & McEwen, 2001), which may make interpretation of the results more difficult. Other markers of immature neurons include class III β-tubulin (or Tuj1), the neuron-specific protein that is expressed in each post-mitotic neuron, and polysialylated-neuronal cell adhesion molecule (PSA-NCAM) (Abrous, Koehl, & Le Moal, 2005). Although the cytoplasmic protein TOAD-64 (or TUC-4) has also been used as a marker, quality antibodies are difficult to find (Christie & Cameron, 2006).

Finally, fully mature neurons are identified with antibodies against NeuN (Neuron Specific Nuclear Protein) or NSE (Neuron Specific Enolase) (Abrous, Koehl, & Le Moal, 2005). However, there has been some ambiguity surrounding the specificity of these markers, especially NeuN, as they reportedly label other non-neuronal cells (Rakic, 2002a). To get around that problem, markers for astrocytes, such as S100β or GFAP, and oligodendrocytes (e.g. CNP) are used (Abrous, Koehl, & Le Moal, 2005).

This, in conjunction with the appropriate controls, enables researchers to properly interpret results and his generated much of what is known about adult neurogenesis in the brain. In the next section, the specifics of this phenomenon will be examined.

*Adult Neoneurogenesis: The process*

Before proceeding, it is important to note that there are two periods of neurogenesis: one in the developing brain (gestational (or embryonic) neurogenesis) and another in the adult brain. The development of a single neuron is comparable during both periods of neurogenesis as newborn neurons in both times of neurogenesis express similar markers, namely nestin and doublecortin. There are also a couple of other interesting points of comparison. For instance, migration plays a key role in both stages

of neurogenesis. During embryonic development, developing neurons in the cortex and cerebellum use different types of glial cells to migrate to their final destination. Similarly, in the adult brain, newborn neurons need to migrate from their birth zone (SGZ or SVZ) to their final destination in the hippocampus or olfactory bulb. In addition to these similarities, there are also some differences between the two processes. One example is that, although adult neoneurogenesis is a limited process in to two distinct regions, in the developing brain neurogenesis occurs in more areas and produces an entire brain's worth of neurons. Also, the function of developmental neurogenesis is clear: the development of the brain and its circuitry. This is completely opposite from the function of adult neoneurogenesis which is still debated and remains unknown (see below).

*I. The Sub-ventricular zone (SVZ)*

In general, there has been a large body of research dedicated to studying the SVZ of many species and, as one might expect, there is a lack of consensus among researchers regarding the particulars of the SVZ. The goal of this review is to provide a basic summary of neurogenesis in the SVZ, and not anything beyond that. For a more in depth review of adult neurogenesis in the SVZ (and in general) see Abrous et al. (2005). Before proceeding, however, it is important to briefly define some of the key terms in the area of neurogenesis. Although the terms get interchanged on occasion, a *neural stem cell* and a *progenitor cell* do not have the same properties. A *neural stem cell* is a cell that is multipotent (generate many types of neural cells) and has the capacity for unlimited self-renewal (Seaberg & van der Kooy, 2003). Very often, the term *neural precursor* is used interchangeably with neural stem cell and both refer to the same

properties. On the other hand, a *progenitor cell* is unipotent (committed to a particular lineage) with limited self-renewal.

One of two neurogenic regions in the adult brain, the SVZ is spread throughout the wall of the lateral ventricle and contains the largest collection of proliferating cells in the brains of several species, including humans (Bernier, Vinet, Cossette, & Parent, 2000; Taupin & Gage, 2002). Several different cell types have been identified using various markers associated with the SVZ: Type A cells (migrating neuroblasts, PSA-NCAM$^+$, DCX$^+$), Type B cells (SVZ astrocytes, GFAP$^+$, Nestin$^+$), Type C cells (transit amplifying progenitors, Nestin$^+$, DCX$^+$) and Type E cells (ependymal cells)(Abrous, Koehl, & Le Moal, 2005; Ming & Song, 2005).

Which (if any) of these cell types is *the* neural stem cell is still a matter of a debate (Ming & Song, 2005). Based on some empirical evidence, which showed their ability to divide and differentiate into neurons, it was proposed that type E cells, which are a layer of endothelial cells that lie between the SVZ and the ventricle, were the long sought after neural stem cell (Johansson et al., 1999). However, subsequent research has been unable to reproduce these findings, and in fact, has shown that ependymal cells do not have stem cell characteristics (Chiasson, Tropepe, Morshead, & van der Kooy, 1999). Along with type E cells, SVZ astrocytes (type A cells) have also been identified as potentially being the neural stem cell (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999). Unlike the ependymal hypothesis, the SVZ astrocyte hypothesis has still to be refuted. In this study, type A and C cells were eliminated following a week-long infusion of the antimitotic drug cytosine-β-D-arabinofuranoside (AraC) onto the surface of the brain, leaving B cells to continue dividing (Doetsch et al., 1999). Previous

research by the same group had confirmed that this dose of AraC effectively eliminated Type A and C cells but did not affect B cells, even after two weeks of infusion (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1999). A few days after the treatment ended, type C cells reappeared, followed quickly by type A cells a few days after that. The conclusion was that the SVZ astrocytes are the precursor cells, and give rise to type C cells, which will generate the migrating neuroblasts (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999). In fact, their conclusion, known as the astrocyte hypothesis, remains prevalent among current research (Ming & Song, 2005).

Once the type A migrating neuroblasts (upwards of 30000 in adult rodent brain each day) are generated, they begin their migration from the SVZ to the olfactory bulb (Lois & Alvarez-Buylla, 1994). To reach the olfactory bulb, the newborn neurons migrate tangentially along the rostral migratory stream (RMS), from which the new cells exit upon their arrival in the olfactory bulb (Doetsch & Alvarez-Buylla, 1996). As well as being a restricted pathway for this type of neural migration, travel along the RMS facilitates a unique form of migration called chain migration. The neuroblasts join in clusters and move through special tubular structures formed by glial cells (Lois, Garcia-Verdugo, & Alvarez-Buylla, 1996). Several mechanisms are involved in directing the movement of the chain, most notably PSA-NCAM (Doetsch & Alvarez-Buylla, 1996), it has recently been proposed that an unknown chemical attractant is secreted into the layers of the olfactory bulb and plays a critical role in guiding the neurons to the olfactory bulb (Liu & Rao, 2003).

At their destination, the neuroblasts detach from the chain and migrate, this time radially, to the various layers of the olfactory bulb, where the neuroblasts will develop

into mature neurons (Lledo, Alonso, & Grubb, 2006). More specifically, they will develop into the two main interneurons of the olfactory bulb – granule and periglomer cells – and become fully functional, forming connections with the olfactory bulb's projection neurons (Lledo, Alonso, & Grubb, 2006). Thus, what begins has an astrocyte in the SVZ, eventually becomes a functioning neuronal element of the olfactory system.

*II. The Sub-granular zone (SGZ)*

Located between the granule cell layer (GCL) of the hippocampus and the hilus, the SGZ of the adult dentate gyrus produces a number of neural progenitors daily, much like the SVZ. However, approximately 9000 cells are generated per day in the SGZ, substantially fewer than the number produced in the SVZ (Cameron & McKay, 2001). In addition to rodents, the proliferating activity of the SGZ has been demonstrated in both monkeys and humans (Eriksson et al., 1998; Kornack & Rakic, 1999). The research of Eriksson et al (1998) used the brains of deceased cancer patients who had been injected with BRDU for diagnostic purposes to first show neurogenesis in the SGZ of the adult human brain.

Despite the consensus on the occurrence of neurogenesis in the adult SGZ, there is still some debate, although a little one-sided, as to the type of proliferating cells involved (Abrous, Koehl, & Le Moal, 2005). The preponderance of evidence favors a lineage of two cells that give rise to the granule neurons of the dentate gyrus: type B cells ($GFAP^+$, $Nestin^+$) and type D cells ($DCX^+$, $PSA\text{-}NCAM^+$)(Seri, Garcia-Verdugo, Collado-Morente, McEwen, & Alvarez-Buylla, 2004). Like the type B cells in the SVZ, the type B cells of the SGZ are astrocytes, and are considered to be the initial precursor to the granule neurons of the hippocampus whereas type D cells, which are generated by

type B cells, act as the transient cell between type B cells and immature granule neurons (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). The best evidence for this theory comes from the use of the antimitotic agent, AraC, which eliminates most D cells and astrocytes in the SGZ. Soon after cessation of treatment with AraC, the remaining B cells start terminally differentiating and 4 days later, D cells reappear leading to the conclusion that B cells act as the dentate stem cells and give rise to D cells, which eventually differentiate and produce mature granule neurons (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Recent work challenging this conclusion is based on in vitro evidence that, while the adult hippocampus does contain a population of progenitor cells, it does not contain true stem cells capable of self-renewal (Bull & Bartlett, 2005; Seaberg & van der Kooy, 2002).

Although the particulars of their origins are under some scrutiny, the fate of newly generated neurons is much better known. Unlike newborn cells of the SVZ, the newly born neurons generated in the SGZ migrate only a short distance to the granule cell layer where they mature into granule neurons (Ming & Song, 2005). In addition to NeuN (a standard marker of mature neurons), this maturation can be marked by the expression of two distinct calcium-binding proteins. Early in the postmitotic stage, the immature granule neurons express calretinin and, as the cell matures, calretinin gets switched for calbindin (Brandt et al., 2003; Kempermann, Jessberger, Steiner, & Kronenberg, 2004).

Within 10 days of their birth, the new neurons have integrated into the granule cell layer of the hippocampus and extended axons toward the CA3 region of the hippocampus (Hastings & Gould, 1999; Zhao, Teng, Summers, Ming, & Gage, 2006). Also during this period, the cells develop dendrites, which extend into the molecular

layer, within which they will branch in increasing complexity over a four month period (van Praag et al., 2002). The molecular mechanisms that underlie the maturation process await future research.

*III. Controversial Cortical Neurogenesis*

Aside from the focus on the SGZ and SVZ as the most prominent areas of adult neurogenesis, there has been much progress made on neurogenesis in other areas of the brain, such as the amygdala and substantia nigra (see above for references). Of these other areas, cortical neurogenesis has received the greatest attention and controversy (Abrous, Koehl, & Le Moal, 2005). Although evidence for cortical neurogenesis was reported in the early years of neurogenesis research by Gould and colleagues (1999) has provided some of the current evidence used to support the theory. In their work, BrdU injected into adult macaque monkeys labelled cells in prefrontal, inferior temporal and posterior parietal cortices that were positive for neuronal markers. They proposed that these new cortical neurons originated from the SVZ and migrated through white matter to their respective neocortical destinations.

Other papers have refuted the finding of neurogenesis in the cortex of mice (Magavi, Leavitt, & Macklis, 2000), humans (Au & Fishell, 2006) and monkeys (Koketsu, Mikami, Miyamoto, & Hisatsune, 2003). In a replication of the experiment Kornack and Rakic (2001) found BrdU labelling in macaque cortex but determined that none of these cells were neuronal.

*Neurogenesis: Factors and Functions*

Now that a basic overview of neurogenesis has been provided, I will present some of the factors that can affect neurogenesis and discuss the possible functions of neurogenesis.

*I. Factors Influencing Neurogenesis*

Of all the factors that affect adult neurogenesis, the most studied are environmental stimuli. An oft-used paradigm, especially for rodents, is placing the animal in an enriched environment, which usually involves putting them in a bigger cage (than normal) with several other mice and a large array of toys and running wheels (Kempermann, Kuhn, & Gage, 1997). In general, exposing rodents to this type of environment increases the survival of newborn granule neurons in the SGZ, but does not affect SVZ neurogenesis (Nilsson, Perfilieva, Johansson, Orwar, & Eriksson, 1999).

In addition to an enriched environment (EE) situation, physical exercise, such as voluntary wheel running, is another commonly used paradigm when studying neurogenesis. When voluntary wheel running is used, a running wheel is placed in the animal's cage, and the animal is allowed to run in the cage when it wants to. As with EE, physical exercise increases both the proliferation and survival of granule neurons in the SGZ (Ra et al., 2002; van Praag, Kempermann, & Gage, 1999). Furthermore, not only does voluntary running increase proliferation and survival, but also dendritic complexity, spine density and dendritic length (Eadie, Redila, & Christie, 2005; Redila & Christie, 2006). Finally, a recent study explored the possibility of an imaging correlate for neurogenesis. After analyzing the relationships between angiogenesis and neurogenesis, as well as angiogenesis and cerebral blood volume (CBV), the researchers found that, in exercising mice, increases in cerebral blood volume (CBV) in the dentate gyrus

positively correlated with levels of BrdU in the same area (Pereira et al., 2007). Thus, the researchers surmised that CBV could be used as an imaging correlate for neurogenesis. Using MRI technology, this imaging correlate was applied to exercising humans and exercise also increased CBV in the dentate gyrus of these subjects. The similarity between the mice and human data suggests that the exercise-induced change in the CBV of the dentate gyrus of humans is likely mediated by the same process, neurogenesis, as it is in the mouse hippocampus (Pereira et al., 2007).

Even though physical activity increases neurogenesis in the adult hippocampus, physical stress, and stress in general, have a negative impact on cell proliferation in the SGZ of rats and monkeys (Fuchs & Gould, 2000). This effect is likely due to the stress activation of the hypothalamic-pituitary-adrenal (HPA) axis which has been shown to block neurogenesis in the hippocampus (Cameron & Gould, 1994). In addition to stress, cell proliferation and neurogenesis (mostly in the SGZ) are decreased by opiate abuse, alcohol (Crews et al., 2003) and age (Cuppini et al., 2006).

Both injury and various pathologies can modulate neurogenesis. Ischemic insults, focal and global, lead to an increased proliferation in the SVZ and SGZ of rats and mice (Zhang, Zhang, & Chopp, 2005). Brain tissue from human stroke victims shows new cells expressing various markers of new neurons (DCX, βIII-tubulin) in areas surrounding the infarct (Jin et al., 2006). Two questions arise, i) whether ischemia triggers innate neurogenesis or just a random mitogenesis through local disruption of the blood brain barrier that exposes the brain to bloodborne growth factors and ii) whether any of these newborn neurons actually get integrated and become functional. The answers to these questions are unknown, although some studies suggest that the local

environment surrounding the injury cannot adequately support the long-term survival of new neurons (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002).

Similar to brain injury, several chronic neurodegenerative diseases can result in the modification of adult neurogenesis. Like all neurodegenerative diseases, Parkinson's disease (PD) has been well-studied with respect to neurogenesis. Based on the results of animal and human research, it is generally agreed that neurogenesis is adversely affected in both neurogenic regions (Phillips, Michell, & Barker, 2006).

Neurodegenerative influence on neurogenesis is often manifested as an *increase*, rather than a decrease and can even show opposite effects on the two main neurogenic regions. For instance, the quinolinic acid lesion model of Huntington's disease (HD), increases neurogenesis in the SVZ of rats, as evidence by increased BrdU and DCX labelling (Tattersfield et al., 2004) mimicking the SVZ neurogenesis in the brains of HD patients (Curtis et al., 2005). However, using a transgenic mouse model of HD, no change in proliferation in the SVZ and an actual decrease in hippocampal neurogenesis was observed (Gil et al., 2005; Lazic et al., 2006).

In Alzheimer's disease (AD) there seems to be discrepancies between findings in animal and human research. The SGZ of patients with AD shows a significant increase in the neurogenic markers DCX and PSA-NCAM (Jin et al., 2004). In contrast, several different mouse models, including the PDAPP model, have demonstrated a significant decrease in hippocampal neurogenesis (Donovan et al., 2006).

Recent research has provided a greater understanding of the relationship between neurogenesis and various factors. The potential for repair of the nervous system, long thought impossible, is an enticing thought and will continue to drive research.

*II. Function of Neurogenesis*

As long as research has been conducted in the field of postnatal neurogenesis, there has been much speculation as to its function and there is no definitive evidence for one particular function over another. There are, however, an abundance of hypotheses. In one hypothesis, new neurons have special properties that allow them to play a key role in synaptic plasticity. Other proposed functions of neurogenesis include a maintenance system (Manev & Manev, 2005) and an explicit role in the formation of temporal associations in memory (Aimone, Wiles, & Gage, 2006).

Because the hippocampus is a critical brain substrate for learning and memory, it is not surprising that shortly after the discovery of neurogenesis in the 60's, it was suggested that these new neurons play some role in learning and memory. Credence was given to the suggestion when, in the late 1970s and early 1980s, study of the song system in the brains of adult canaries revealed that new neurons were being added to the Higher Vocal Centre (HVC), a component of the song system (Paton & Nottebohm, 1984). Soon thereafter, it was widely speculated that these additional new neurons resulted from the learning of new songs by adult canaries. However, it remains speculative as there has yet to be any definitive evidence indicating that the learning of new songs directly led to neurogenesis in the adult brain of canaries (Nottebohm, 2002).

Despite the lack of evidence for a direct relationship in canaries, research has provided evidence for some sort of relationship between neurogenesis and behaviour (Bruel-Jungerman, Laroche, & Rampon, 2005). For instance, when physical exercise is

used to increase hippocampal neurogenesis, there is also an enhancement of learning during hippocampal-dependent behavioural tasks and, vice-versa, when hippocampal neurogenesis is reduced, there is a corresponding reduction in behavioural task performance (Gould, Tanapat, Hastings, & Shors, 1999). However, these relationships are correlational in nature. To better isolate the contribution of neurogenesis to the effect of enrichment/physical activity on memory performance, a recent study used an anti-mitotic agent to dramatically reduce the enrichment-generated neurogenesis (Bruel-Jungerman, Laroche, & Rampon, 2005). While the control rats had the expected increase in SGZ neurogenesis (from the enrichment) and better memory performance, the rats injected with the anti-mitotic drug did not. Thus, this observation provided strong support for the memory-enhancing effect of the newborn hippocampal cells (Bruel-Jungerman, Laroche, & Rampon, 2005). Further investigation into this potential causal link has been done using X-ray irradiation to block hippocampal neurogenesis. In this case, however, mice that were exposed to the irradiation showed similar improvements in spatial memory to mice that were not exposed to the irradiation. Contrary to the results of Bruel-Jungerman et al., this evidence suggested that hippocampal neurogenesis was *not* required for the memory-enhancing effect (Meshi et al., 2006). Clearly, more research is needed before any conclusion can be reached.

Still, for the time being, the search for the function of adult neurogenesis continues. It is remarkable that after more than a decade of research, there is still so much left to understand and until it becomes, if ever, fully elucidated, there may always be an element of mystery about it.

## Glucose: Transport and the brain

As key as glucose is for energy production in the body, it is even more important in the brain, where it acts as the primary source of energy (Pardridge, 1983). As chief energy source for the brain, one would expect that glucose would always be in abundance, and that it could move freely from the blood to the brain and vice versa. This, however, is not the case.

The brain is invested by a protective barrier, referred to as the blood-brain barrier (BBB), which restricts the flow of most substances (including glucose) in and out of the brain (Reese & Karnovsky, 1967). This barrier is essential in protecting the brain from any molecule or chemical in the blood, while still allowing essential metabolic functions. Having said that, certain molecules do cross the BBB through specialized transport channels that are specific for one type of molecule. Glucose, as an essential component of brain function, is amongst this group of molecules.

In order for glucose to perform its role as primary energy source for the brain, it relies on a family of glucose transporters (GLUTs) to gain entry, first into the brain itself, and second, into the various cells within the brain (Maher, Vannucci, & Simpson, 1994). Several transporters have been identified, both in the brain and the periphery, although only a few of these are expressed in the brain (McEwen & Reagan, 2004). Located primarily within the endothelial cells that line the blood vessels of the BBB, GLUT1 is the transporter by which glucose crosses from the bloodstream into the cerebrospinal fluid of the brain (Messier, 2004). Once inside the brain, glucose must gain access to the

interior of the various cell types that make up the brain. GLUT3, often referred to as the neuron-specific glucose transporter, is located on neurons and transports glucose into the cell (Mantych, James, Chung, & Devaskar, 1992). Together, GLUT1 and GLUT3 are thought to be the most prominent of the glucose transporters to be found in the brain. Despite being fewer in number, other glucose transporters including GLUT4 and GLUT8, also have an important purpose. These purposes, as well as details about the roles of the various transporters will be examined in the next section. Moreover, the developmental expression of some of the key transporters will also be examined and is relevant for the later discussion on adult neurogenesis.

*The Major Glucose Transporters*

*Glut 1*

Found all over both the periphery and brain, GLUT1 is one of the major glucose transporters and also, one of the first to be identified in the brain (Dick, Harik, Klip, & Walker, 1984). Responsible for glucose transport across the blood-brain barrier and, perhaps, into astrocytes, research has since discovered two distinct isoforms of the GLUT1 transporter, 55 kDa and 45 kDa, each of which has a unique role in the transport of glucose and differ only in the extent of their glycosylation (D. S. Dwyer, Vannucci, & Simpson, 2002). Originally identified using an antibody raised against the human red blood cell glucose transporter, the 55 kDa isoform of GLUT1 was localized to the endothelial cells of the blood-brain barrier in the rat brain and further immunohistochemical studies using rodent and human brain section concurred with this result (Dick, Harik, Klip, & Walker, 1984; Maher, Vannucci, & Simpson, 1994). In addition, there is some evidence to suggest that this vascular isoform of GLUT1 is also

found in the choroid plexus, but it is a matter of debate (Dobrogowska & Vorbrodt, 1999). Despite originating from the same gene, the 45 kDa isoform of GLUT1 is not localized to endothelial cells, but rather, to the astrocytes surrounding the vasculature, as well as other astrocytic cell bodies and processes (Leino, Gerhart, van Bueren, McCall, & Drewes, 1997).

The distribution of GLUT1 reflects its importance in providing the brain with a steady supply of glucose. Both western blot and immunohistochemical analyses demonstrated the presence of GLUT1 over the majority of the brain, however, it is more abundant in certain cortical areas, as well as the hippocampus and cerebellum (Brant, Jess, Milligan, Brown, & Gould, 1993; Choeiri, Staines, & Messier, 2002). Furthermore, western blot analysis has also revealed that the ratio of the 45/55 kDa isoforms is skewed heavily in favour of the 45 kDa isoform, at least when considering the brain as a whole (S. J. Vannucci, Maher, & Simpson, 1997). Interestingly, even within the endothelial cells there is an uneven distribution of GLUT1, although it is all the 55 kDa isoform. Relative to the luminal (blood vessel) surface, there is nearly four times as much GLUT1 on the abluminal (brain) surface which eases the quick transport of glucose into the brain and, ostensibly, into the surrounding astrocytes via the 45 kDa isoform of GLUT1 (Dobrogowska & Vorbrodt, 1999).

Now that a basic overview of the function of GLUT1 has been presented, the focus now turns to the developmental expression of GLUT1. Although not the only source of energy (see discussion on monocarboxylate transporters), glucose is an important source of energy for the fetus and newborn, and eventually becomes the primary source of energy in the adult (S. J. Vannucci & Simpson, 2003). Several studies

in various animals, including humans, indicate that the brain's glucose utilization increases as it matures and that this utilization is not distributed evenly across the brain, but rather, are associated with increased activity and demands (R. C. Vannucci & Vannucci, 2000). However, what of GLUT1? Is there a corresponding change in GLUT1 levels during this time period?

Indeed, the results from several different studies suggest that there is a change in GLUT1. An early studying examined mRNA expression of various glucose transporters of embryonic and post-natal rat brains and found abundant amounts of GLUT1 mRNA in several structures of the embryonic brain, including the vasculature, choroid plexus and most other non-neuronal components (Bondy, Lee, & Zhou, 1992). Moreover, immunohistochemical analysis of embryonic rat brain revealed significant levels of GLUT1 in endothelial cells, but little in the brain parenchyma (Devaskar, Zahm, Holtzclaw, Chundu, & Wadzinski, 1991).

Despite the significant GLUT1 (protein and mRNA) in the embryonic brain, the expression of GLUT1 in the post-natal brain is low. Studies in rats (S. J. Vannucci, 1994), rabbits (K. J. Dwyer & Pardridge, 1993) and mice (Khan, Rajakumar, McKnight, Devaskar, & Devaskar, 1999) established that, in general, there is an initial decrease in GLUT1 protein in the first ~14 days followed by a large up-regulation that does not stabilize until the adult level is reached. Interestingly, during the early postnatal period when GLUT1 protein levels have decreased, the corresponding mRNA levels remain fairly stable indicating that there may be a translational or other posttranscriptional process that regulates GLUT1, although that has yet to be elucidated (K. J. Dwyer & Pardridge, 1993; Khan, Rajakumar, McKnight, Devaskar, & Devaskar, 1999).

Thus, the empirical evidence available suggests that the developmental expression of GLUT1 parallels the increased glucose utilization and energy demands of the brain. Meeting these demands is of an utmost importance to the proper development of the newborn. In rare cases, a defect in the GLUT1 transporter results in a dramatic decrease in glucose levels of the cerebrospinal fluid (CSF), which leads to a metabolic deficit in the brain, termed to as GLUT1 deficiency syndrome (Klepper, 2004). The results of this deficiency can include seizures, motor disorders and developmental delays (Klepper, 2004). Although this is an extremely rare condition, it exemplifies the necessary role that both glucose and GLUT1 play in proper development.

*Glut 3*

Along with GLUT1, glucose transporter three (GLUT3), a 45 kDa protein, is thought to be the most prominent neuronal glucose transporter in the brain. Despite similar affinities for glucose, GLUT3 has a much higher rate of transport than GLUT1, which makes it able to keep pace with the high demand of glucose by neurons. GLUT3 is commonly referred to as the neuron specific glucose transporter, although it also has a limited expression in other human tissues including the testis and spermatozoa. By comparison, GLUT3 has only been observed in the brains of rodents, and not in the rest of the body (Maher, Vannucci, Takeda, & Simpson, 1992). GLUT3 has been localized to virtually all areas of the rodent brain, including the striatum, cerebellum, hippocampus, cortex and thalamus (Brant, Jess, Milligan, Brown, & Gould, 1993) and, conversely, is virtually absent from the vascular compartment.

Unlike GLUT1, which has an easily identifiable staining pattern due to its vascular location, GLUT3 staining is much less distinct. This is due to the fact that

GLUT3 is localized to the neuropil (referring to the network of glial, axonal and dendritic fibres) leaving the cell bodies unstained, a result that has been confirmed in numerous immunohistochemical studies as well as a study using electron microscopy (McEwen & Reagan, 2004). Furthermore, in situ hybridization histochemistry and northern blots revealed that GLUT3 mRNA is co-localized with its protein in many locations across the brain, including the cortex, hippocampus cerebellum and, to a lesser extent, the thalamus, pituitary and septum (Apelt, Mehlhorn, & Schliebs, 1999).

Glucose is an important source of energy for the developing brain and, as such, the expression of GLUT3 (protein and mRNA) in the embryonic and early postnatal brain has been studied. An early study by Bondy et al (1992) used in situ hybridization to examine the ontogeny of GLUT3 in embryonic and postnatal rat brains. Beginning a few days before birth (~E14), low levels of GLUT3 mRNA can be detected in differentiated neurons and, as development continues, GLUT3 mRNA gradually increases until approximately 3 weeks after birth when adult levels are reached. Two areas involved in adult neurogenesis, the olfactory bulb and hippocampus, had some of the highest levels of GLUT3 mRNA reported, although the distribution of GLUT3 mRNA in the developing rat brain was relatively diffuse. Further studies using rat (S. J. Vannucci, 1994) and mouse (Khan, Rajakumar, McKnight, Devaskar, & Devaskar, 1999) tissue confirmed the findings of Bondy et al (1992) and added to them by using western blotting to identify GLUT3 protein levels in the developing brain. Following the pattern of the mRNA, GLUT3 protein can be found in the brain at relatively low levels ~8 days before birth followed by a large increase at postnatal day 14 leading to adult levels by the third week after birth. Similar results were also found using human brain tissue with the adult

brain expressing significantly more GLUT3 than the neonatal brain (Mantych, James, Chung, & Devaskar, 1992). Interestingly, Vannucci (1994) noted a regional difference in the developmental expression of GLUT3 whereby GLUT3 levels in the cerebellum took longer to attain adult levels than in the brainstem. The major difference between these two areas is that, while the brainstem is mature at birth, the neurons that make up the cerebellum are still migrating and differentiating. This provides further credence to the notion that GLUT3 is expressed only in differentiated neurons, and GLUT3 levels will correspondingly increase as the brain (and its neurons) matures and becomes solely (nearly) dependent on glucose for its energy needs.

*Glut 4*

Commonly referred to as the insulin-sensitive glucose transporter, GLUT4 has been extensively studied in the peripheral system where it is localized to several different tissues including heart, skeletal muscle and adipose. The common thread between these tissues is that each responds to insulin or contraction by moving GLUT4 to the cell membrane in order to increase glucose uptake into the cell (Saltiel & Pessin, 2002).

Although not nearly as prominent as GLUT1 or GLUT3, there is a relatively wide distribution of GLUT4 across the brain. However, in most areas of the brain where GLUT4 can be found, it is found in relatively low amounts. Initially localized to neurons in the hypothalamus and cerebellum through western blot analysis of rat brain tissue (Brant, Jess, Milligan, Brown, & Gould, 1993), further study extended these results to include neural cell bodies and dendrites in other areas of the rat and mouse brain, including the hippocampus, cortex and olfactory bulb (Choeiri, Staines, & Messier, 2002; El Messari et al., 1998; S. J. Vannucci et al., 1998). Moreover, studies have also

demonstrated that GLUT4 mRNA generally co-localizes with the protein, although there is some discrepancy in the hippocampus and substantia nigra (El Messari, Ait-Ikhlef, Ambroise, Penicaud, & Arluison, 2002).

The distribution of GLUT4 and the insulin receptor overlap in the brain as in the periphery leading to the suggestion that GLUT4 has a similar role in both the periphery and brain (McEwen & Reagan, 2004). In fact, research has shown that in the genetically diabetic (*db/db*), hyperinsulinemic mouse there is an increase in GLUT4 expression in the cerebellum relative to control mice; moreover, in *hypo*insulinemic mice, the opposite has been observed (S. J. Vannucci et al., 1998). When these results are combined with the observation of a pool of seemingly available GLUT4 in the cytoplasm, as well as the membrane, it provides evidence that GLUT4 protein in the brain may have a similar function to peripheral GLUT4.

There has not been much research on the ontogeny of GLUT4. Early research concluded that GLUT4 mRNA could not be detected at *any* stage of development (Bondy, Lee, & Zhou, 1992) and, perhaps, in some way this slowed research into the developmental aspect of GLUT4. In the brains of embryonic mice, GLUT4 mRNA can be detected in the last few days preceding birth. It is localized within the neuroepithelium, cerebellar plate and choroid plexus (S. J. Vannucci, Rutherford, Wilkie, Simpson, & Lauder, 2000). Further to this, Vannucci and colleagues (2000) also detected GLUT4 protein as early as E9 in the neural folds and its expression increased throughout the gestation in areas such as the cerebellum. GLUT4 levels in the cerebellum continued to increase postnatally until they peaked at approximately P21. In adults, however, the levels of GLUT4 were reduced. As this pattern of expression of GLUT4 closely follows

the neurogenesis of cerebellar granule cells, the authors suggested that perhaps it has a key role during brain development, and that the role might be different than that in the adult brain. Further research found that GLUT4 expression peaked at P21 and localized GLUT4 at P15 to the dentate, hypothalamus and piriform cortex (Sankar, Thamotharan, Shin, Moley, & Devaskar, 2002). Despite these results, there is still an incomplete picture of GLUT4 in the developing brain, especially in species other than the mouse, and more research will be needed to further examine some of the mechanisms that direct the ontogeny of GLUT4.

*Glut 8*

Once referred to as GLUTx1, the glucose transporter isoform GLUT8 was only recently identified by comparing its genetic sequence to other known GLUT's and discovering that, indeed, it represented a novel transporter of the GLUT family (Ibberson, Uldry, & Thorens, 2000). Primarily expressed in the testis, the results of several studies have revealed a widespread, albeit low level distribution of GLUT8 mRNA and protein in several brain areas including, but not limited to, the hypothalamus, cerebellum and hippocampus (Ibberson, Uldry, & Thorens, 2000). Within these areas, GLUT8 is localized to neural cell bodies (McEwen & Reagan, 2004). In addition, GLUT8 protein does not co-localize with glial markers, suggesting that it is expressed only in neurons which means that, along with GLUT3, it is a neural-based glucose transporter, although the two distributions do not necessarily overlap (Reagan et al., 2002).

Functionally, there is a certain level of ambiguity surrounding GLUT8. One interesting facet about GLUT8 is that it is not found on the plasma membrane, but rather in an intracellular compartment, leading to uncertainty regarding its physiological role

(Membrez et al., 2006). One hypothesis has GLUT8, in response to changes in insulin levels, playing a key role in maintaining glucose homeostasis within the neural cell body by transporting glucose out of the endoplasmic reticulum (ER) and into the cytoplasm (McEwen & Reagan, 2004). Evidence supporting this hypothesis comes from the observation that, in diabetic rats, increased insulin levels promote an increased GLUT8 expression in the ER. Furthermore, in the embryo GLUT8 responds to increased insulin levels in embryo by translocating to the plasma membrane in blastocysts (Membrez et al., 2006). Although these findings support the hypothesis that GLUT8 maintains glucose homeostasis, ultimately, the function of GLUT8 remains to be determined. Recently, Membrez et al (2006) generated mice with an inactive *glut8* gene, thus suppressing GLUT8 expression. Aside from a few minor physiological alterations, the mice developed normally and had normal glucose homeostasis but also had increased proliferation in the hippocampus, which suggests an effect on neurogenesis. Although no hypotheses were put forward to explain these findings, these results suggest that the function of GLUT8 may not be as critical as first thought, but only further research can shed some light on the situation.

As with GLUT4, there has been little research on the ontogeny of GLUT8. There is evidence that GLUT8 is found in mouse blastocysts and more recent research has also identified GLUT8 in rabbit blastocysts (Navarrete Santos, Tonack, Kirstein, Kietz, & Fischer, 2004). In one of the few papers to have examined the developmental expression of GLUT8, Sankar and colleagues (2002) used western blots and immunohistochemistry to probe the brains of early postnatal mice for GLUT8. In general, the total amount of GLUT8 peaked at P15 and by P60, western blots revealed an extremely faint band of

GLUT8, indicating a relatively low amount of it in the brain. The immunohistochemistry revealed a similar distribution pattern throughout the brain except for some very intense labelling in the substantia nigra, a novel finding that, at this point, has not been explained. Regardless, the results from this study produce a relatively clear image of postnatal development of GLUT8. Unfortunately, there is little on the prenatal development of GLUT8, aside from the research on blastocysts. But, from the early embryonic stage to birth, the ontogeny of GLUT8 remains unknown and should remain a goal for future research.

*Other Glucose Transporters (Glut2, Glut5 and others)*

In general, these particular glucose transporters have a sparse distribution in the brain, although some, such as GLUT2 and GLUT5, may be fairly prevalent in the peripheral organs and tissues. However, relatively little is known about the function and distribution of the other transporters, GLUT6 and GLUT10. Although mRNA for both transporters is expressed in the brain, little is known beyond that. The following section will give the reader a brief overview of the "other" GLUTs, 2 and 5.

*Glut 2*

Primarily found in peripheral tissues, including the liver, pancreas and small intestine, GLUT2 acts as a glucose-sensor detecting plasma glucose levels. Although the majority of published research involving GLUT2 focuses on its peripheral function, there has been a small body of work dedicated to clarifying its central action. In the brain, the GLUT2 mRNA is expressed in various nuclei of the hypothalamus, neurons of which have been tentatively identified as being glucose-sensing, acting in a similar manner to pancreatic β cells (Levin, Dunn-Meynell, & Routh, 2001). GLUT2 mRNA has also been

found in specialized hypothalamic glial cells, called tanycytes, which may play a role in the glucose-sensing capability of the hypothalamus, although this remains speculative (M. A. Garcia et al., 2003).

At the protein level, a set of studies by Arluison and colleagues used immunohistochemistry and electron microscopy to examine the distribution of GLUT2 in the rat brain (Arluison et al., 2004; Arluison, Quignon, Thorens, Leloup, & Penicaud, 2004). The results of these studies indicate that there are low levels, at least relative to other more prominent glucose transporters, of GLUT2 protein expression in a number of areas in the brain. Prominent labelling was observed in nuclei of the hypothalamus (arcuate and paraventricular), the amygdala, perirhinal cortex and in the nucleus of the solitary tract. Additional labelling was also observed in an extremely small number of oligodendrocytes and astrocytes. Generally, the labelling was punctate and resembled small dots surrounding larger neural cell bodies, although some cell bodies in the hypothalamus also appeared to be labelled. Using electron microscopy to further analyze the staining, the researchers found that the bulk of this circular punctuate staining was actually labelled synaptic terminals forming synapses with neuron cell bodies. Moreover, they also observed that some neurons were labelled intracellularly. Thus, there are two potential explanations for the distinctive GLUT2 labelling and it is not clear from the results presented here which role GLUT2 has in the brain.

Nevertheless, to correctly determine which explanation fits, the function of GLUT2 in the brain must be fully elucidated. Although the exact mechanism of neural glucose sensing is not known, what is known is that glucose sensing the brain is accomplished by two sets of neurons. As glucose levels rise, glucose-excited neurons

increase their activity and use glucokinase to phosphorylate the glucose. As a result, ATP sensitive potassium channels close (Leloup et al., 2006). On the other hand, glucose-inhibited neurons increase their activity at low glucose levels (Levin, Routh, Kang, Sanders, & Dunn-Meynell, 2004). Although the research conducted by the Arluison group is the most complete examination of GLUT2 distribution in the (rat) brain, it did not necessarily shed new light on the role of GLUT2 in the brain. Because GLUT2 was in areas where glucose-sensitive neurons are known to be found *and* in other areas not known to contain any glucose-sensitive neurons, it makes the conclusions ambiguous. Perhaps GLUT2 does have a role in glucose sensing, but it is also entirely possible that it may not be its only role, or even its role at all. Clearly, future research is needed to shed more light on the situation.

*Glut 5*

Much like GLUT2, the glucose transporter isoform five (GLUT5) is found in a number of peripheral tissues, including the testis, intestine, skeletal muscle and spermatozoa (Kayano et al., 1990). GLUT5 has also been detected in human macrophages (Maher, Vannucci, & Simpson, 1994). In the brain, GLUT5 protein has been localized to the microglia of rat and human brain tissue, and more recently, research found that the mRNA for the gene responsible for GLUT5 (*glut5*) was found in Purkinje cells of the cerebellum, as well as the corresponding protein (Funari, Herrera, Freeman, & Tolan, 2005).

Functionally, little is known about the physiological role of GLUT5. Generally, GLUT5 does not effectively transport glucose, and acts primarily as a fructose transporter. Thus, its localization in the periphery is not surprising as one might guess

that fructose in the plasma does not pass the blood-brain barrier. However, this leads to question of why the *brain* would need such a transporter, when it has such a heavy reliance on glucose. Moreover, why is GLUT5 localized to microglia only? Perhaps microglia have a unique fructose-sensing property and might be able to sense fructose release from ruptured neurons. However, until more is known about the role of GLUT5 in the brain, these questions will remain unanswered.

**Monocarboxylate Transporters: Doors for an Alternate Source of Energy?**

Although it is accurate to say that glucose is the pre-eminent source of energy for the brain, it would be incorrect to suggest that it is the *only* source of energy. In particular, the developing brain is just as dependent on other, alternative sources of fuel as it is on glucose until a certain point in development, when the brain switches to a near complete reliance on glucose (S. J. Vannucci & Simpson, 2003).

As an alternative fuel source, monocarboxylates such as lactate and ketone bodies play a key role in providing energy to the growing brain and this has been shown in both human and rodent brains. For instance, the high-fat content of maternal milk in both rodents and humans generates ketone bodies at a high enough concentration to provide substantial energy to the developing brain (S. J. Vannucci & Simpson, 2003). To further the point, research has also shown that cerebral uptake and utilization of ketone bodies in newborn rats peaks during the first few weeks after birth before declining to adult levels, whereas this pattern is reversed for glucose uptake and utilization (S. J. Vannucci & Simpson, 2003).

Similar to glucose, monocarboxylates cannot cross the blood-brain barrier or cell membrane by simple diffusion. Thus, to facilitate transport, there is a family of

moncarboxylate transporters (MCT) that includes fourteen different isoforms, with MCT1, 2 and 4 being the main cerebral transporters (Pierre & Pellerin, 2005). Over the last decade or so, substantial research has examined their distribution and a short overview of these three important cerebral MCTs will be presented. For a more in depth review, see Pierre and Pellerin (2005).

Generally, MCT1 is distributed throughout the rodent brain, primarily within neuropil, the BBB and astrocytes (Pierre & Pellerin, 2005). Although this is the case in both the young and adult rodent brain, MCT1 is much more prominent at the BBB during embryonic and early postnatal development, but decreases as the brain matures and becomes more glucose-dependent (Baud et al., 2003; S. J. Vannucci & Simpson, 2003). On the other hand, non-vascular MCT1 remains elevated in the adult brain (at least relative to BBB MCT1), although it does decrease slightly from its peak at three weeks (S. J. Vannucci & Simpson, 2003)

Another monocarboxylate transporter, MCT2, has a distribution and neuropil localization similar to MCT1, but is predominantly localized to neurons, rather than astrocytes or endothelial cells (Pierre & Pellerin, 2005). In particular, strong labelling occurs in the hippocampus, Purkinje cells of the cerebellum and in the cerebral cortex. In terms of developmental changes, MCT2 follows a pattern similar to non-vascular MCT1 in that levels increase and peak by P15, and decrease only slightly thereafter (S. J. Vannucci & Simpson, 2003).

Predominantly localized to skeletal muscle, MCT4 has not been the focus of much neuroscience research. However, a few studies have examined the distribution of MCT4 in the rat and mouse brain where it is localized to astrocytic processes. Although

the labelling is astrocytic, it has a relative widespread distribution in the brain, including the cerebellum, hippocampus and corpus callosum (Pellerin, Bergersen, Halestrap, & Pierre, 2005). Unfortunately, little is known about the developmental profile of MCT4.

Overall, the role of monocarboxylates as a source of cerebral energy is still not fully elucidated. It is interesting that both MCT1 and MCT2 remain elevated during adulthood, indicating that monocarboxylates play a greater neuroenergetic role than previously thought. An important question raised from the developmental findings is: How do MCTs potentially relate to neurogenesis? From what has been presented here, it is clear that, during early neurodevelopment, there is a greater dependence on monocarboxylates as an alternative energy source. Thus, it remains possible that in lieu of glucose transporters, developing neurons could express MCT transporter(s) and acquire their energy via monocarboxylates.

In fact, a neuroenergetics theory, the lactate-shuttle hypothesis, has recently been proposed to explain the role of monocarboxylates, and lactate in particular, in the brain. According to this hypothesis, lactate is first produced in astrocytes (as a by-product of glycolysis) before being shuttled into the parenchyma via MCT1/MCT4 (Pellerin & Magistretti, 2004). As this pool of lactate builds up in the brain parenchyma, it is taken up by neurons, via MCT2, where the lactate is converted into pyruvate and can take its appropriate place in the citric acid cycle/oxidative phosphorylation to produce energy in the form of ATP (Pellerin & Magistretti, 2004). In the years since its conception, the aptly named astroctye-neuron lactate shuttle hypothesis (ANLS) has generated a substantial amount of supporting data, although a recent *in vivo* study found that the changes in lactate over time are incompatible with the shuttle hypothesis (Fillenz, 2005).

Nevertheless, the ANLS remains the most well-supported theory regarding the role of lactate in the brain and represents an intriguing alternate energy source for newly differentiated neurons.

The present study aims to examine the distribution of glucose and monocarboxylate transporters in newly born neurons of the adult mouse dentate gyrus. Based on what is known about the relevance of glucose and monocarboxylates to neuroenergetics, we predict that one (or more) of the major glucose transporters will co-localize with either BRDU, DCX or both. Thus, the results will explicitly demonstrate which transporters the newly differentiated cells use to acquire energy.

## Experimental Procedures

*Animals*

Eight CD1 mice (Charles River, Canada), aged 15-20 weeks, were used for the BRDU portion of the study. The eight mice were maintained in individual cages on a 12-hour light/dark cycle, and given free access to food and water. In order to increase neurogenesis, mice had unlimited access to a running wheel (Ra et al., 2002; van Praag, Kempermann, & Gage, 1999). All experiments conformed to the guidelines of the Canadian Council on Animal Care on the ethical use of animals and were approved by the Animal Care Committee of the University of Ottawa.

*5-Bromo-2'-Deoxyuridine Administration and Tissue Preparation*

All animals received a series of five BRDU injections (50 μg/g, 10mg/ml in PBS) over three days, with two injections 4-5 hours apart on days one and two, followed by a final injection 24 hours later. At 7 or 14 days following the final injection, the mice were sacrificed. Briefly, mice were deeply anesthetized and perfused via the left ventricle with 20 ml of saline followed by 20 ml of fixative (4% paraformaldehyde, 0.2% picric acid in 0.16 M sodium phosphate buffer, pH 7.1). The brain was removed and following a four hour post-fixation at 4°C, the brain was transferred to a 10% sucrose solution (in 0.1 M sodium phosphate buffer, pH 7.1) for cryoprotection. Later, 14 μm sagittal frozen sections were cut on a cryostat and collected on gelatin-coated slides before being stored at -80°C.

*Immunohistochemistry*

Sections were briefly rinsed in 10 mM phosphate-buffered saline (PBS) before an overnight incubation at 4°C with the primary antibody. Antibodies were diluted with 0.3% Triton X-100 in PBS containing 3% bovine serum albumin (BSA). Following the overnight incubation, tissue sections were washed (3 x 5min) in PBS and incubated, for an hour at room temperature, with an appropriate secondary antibody (see Table 1 for the list of antibodies). After a second series (3 x 5min) of washes, the sections were coverslipped using a non-commercial anti-fade mounting medium.

For immunofluorescence of BRDU, a few additional steps to the above protocol were required to expose the BRDU epitope within the tissue before proceeding to the primary antibody incubation. First, the sections were incubated in 2N HCl for 1 hr at 37°C to denature the DNA, followed by several rinses (3 x 5min) in 0.1M boric acid (pH 8.5) and further rinses in PBS. After the rinses, the immunohistochemistry proceeded as outlined above.

Finally, to double-label the tissue with a combination of BRDU and other antibodies, the labelling protocol had two successive steps. This was to ensure that the harsh acid treatment needed for proper BRDU staining would not affect the antibody reaction of the other marker (GLUT etc). Therefore, the immunohistochemical reaction for GLUT (or DCX etc.) was performed first and the results documented using digital images. This was followed by immersion of the slides in HCl (as above) and BRDU staining. Recording of stage coordinates ensured that the documented image locations could be reimaged following BRDU staining.

**TABLE 1.** List of antibodies used in this study

| Primary Antibodies | Dilution | Source |
|---|---|---|
| Guinea Pig Anti-Doublecortin | 1 : 2000 | Chemicon (Temecula, CA, USA) |
| Mouse Anti-BRDU | 1 : 50 | Roche Diagnostics (Laval, QC, Canada) |
| Rabbit Anti-Glucose Transporter 1 | 1 : 1500 | Alpha Diagnostic (San Antonio, TX, USA) |
| Rabbit Anti-Glucose Transporter 3 | 1 : 2400 | Chemicon (Temecula, CA, USA) |
| Rabbit Anti-Glucose Transporter 4 (intracellular) | 1 : 800 | Chemicon (Temecula, CA, USA) |
| Rabbit Anti-Glucose Transporter 8 | 1 : 2000 | Gift (Dr. Maureen Charron, Albert Einstein College of Medicine) |
| Rabbit Anti-MCT1 | 1:500 | Gift (Dr. Luc Pellerin, Universite de Lausanne) |
| Rabbit Anti-MCT2 | 1:500 | Gift (Dr. Luc Pellerin, Universite de Lausanne) |
| **Secondary Antibodies** | **Dilution** | **Source** |
| Goat Anti-Rabbit $Alexa_{488}$ | 1 : 200 | Molecular Probes c/o Invitrogen (Burlington, ON, Canada) |
| Goat Anti-Guinea Pig $Alexa_{488}$ | 1 : 400 | Molecular Probes c/o Invitrogen (Burlington, ON, Canada) |
| Donkey Anti-Rabbit $Alexa_{594}$ | 1 : 300 | Molecular Probes c/o Invitrogen (Burlington, ON, Canada) |
| Donkey Anti-Mouse $Alexa_{594}$ | 1 : 400 | Molecular Probes c/o Invitrogen (Burlington, ON, Canada) |
| Donkey Anti-Mouse $Alexa_{488}$ | 1 : 400 | Molecular Probes c/o Invitrogen (Burlington, ON, Canada) |

## Results

*Specificity of Antibodies and other controls*

The double immunohistochemical results can be seen in Figures 1-12. To begin with, the specificity of each GLUT antibody used in the present thesis (GLUT 1, 3, 4 & 8) has been previously demonstrated (Choeiri, Staines, & Messier, 2002; Reagan et al., 2001). Furthermore, the absence of non-specific binding of the secondary antibody was shown by omitting the primary antibody, which eliminated all staining (Fig. 13). In many cases, however, faint staining of certain cells remains due to the presence of autofluorescent lipofuscin. This pigment accumulates in the cytoplasm of cells with age, and is particularly a problem in mice (Moore & Ivy, 1995).

In addition to the specificity control, other measures were taken to ensure that the results were accurate. To verify that the BRDU was truly labelling newly differentiated neurons, it was double-labelled with doublecortin (DCX), a marker of migrating neuronal precursors. Figure 1 shows double-stained neurons, showing that BRDU labels recently differentiated neurons. Moreover, to confirm that BRDU actually labelled cells (in general), a BRDU stained section was counterstained with Hoechst dye, a stain that labels cell nuclei. The results of this staining (Fig. 10) showed that in all cases BRDU labelling was restricted to nuclei.

*Immunohistochemistry: BRDU and DCX double-labelling*

The distribution of positive immunoreactivity for each individual glucose transporter was consistent with that previously described (Choeiri, Staines, & Messier, 2002). The GLUT1 signal is present throughout the dentate gyrus and is identifiable from its strong staining of blood vessels (see Figs. 2 & 3), while the GLUT8 immunoreactivity is characterized by its prominent immunoreactivity in neuron cell bodies in the hippocampus (see Figs. 8 & 9). Although GLUT3 is also quite prominent throughout the hippocampus, it is not nearly as easy to identify, which is attributed to its localization to neuropil (rather than cell bodies) staining in the dentate gyrus (see Figs. 4 & 5). Finally, unlike the other GLUT antibodies examined here, GLUT4 immunoreactivity is much less prominent and the dentate gyrus contains very few labelled cells (see Figs 6 & 7).

In conjunction with the GLUT staining, the tissue sections were also labelled with BRDU (Figs 2, 4, 6, 8) or doublecortin (DCX, Figs 3, 5, 7, 9), a marker of neurogenesis (see earlier description). In general, the staining for these two antibodies was consistent and worked as expected. BRDU labelled a small number of cells in the dentate gyrus of each section. In contrast, each section had a significantly greater amount of DCX labelled cells. The reason for this is that BRDU labels a cell undergoing division during a specific time period (DNA synthesis) whereas DCX labels cells over a greater time period as the cell differentiates into an immature neuron and begins migrating. Thus, the combination of these two labelling strategies allowed me to capture a greater number of labelled cells and improve the strength of the conclusion. However, when comparing the staining of GLUTs to that of BRDU or DCX, no overlap was found in either the subgranular zone, indicating that there was no double-labelling present. Of particular

note, there was no co-labelling of the doublecortin-positive fibres in migrating newborn neurons with GLUT3, the neuronal glucose transporter that has a neuropil distribution. Another surprising result is that none of the doublecortin- or BRDU-positive cells were stained for GLUT8, a transporter found in most neurons (Messier, unpublished observations).

Lastly, tissue sections labelled with DCX were also labelled with MCT1 or MCT2 antibodies to ascertain the presence of monocarboxylate transporters in newly differentiated neurons. Similar to the results for the GLUTs, no co-labelling was observed.

## Discussion

The aim of the present study was to look for energy (GLUT and MCT) transporters during early neurogenesis and the results showed that, of the major glucose transporters (1, 3, 4, 8), none co-localized with either a marker of neurogenesis (DCX) or a marker of newborn cells (BRDU). These results provide an interesting dilemma because they are, at the same time, disappointing and interesting. As mentioned earlier, the individual staining patterns for the antibodies are consistent with our previous observations, but we found no overlap between the GLUTs and either neurogenesis marker, which was unexpected.

Originally, the hypothesis was that one or more of the major glucose transporters (1, 3, 4 or 8) would co-localize with either or both of BRDU/DCX, thus providing explicit evidence of the transporters that newly differentiated neurons were dependent on to acquire energy. More specifically, we had predicted that newborn neurons would express GLUT3 and GLUT8 because they are both neuronal glucose transporters and

were more likely to co-localize with developing neurons. Although GLUT1 and GLUT4 are major glucose transporters, GLUT1 is primarily a vascular transporter – facilitating glucose transport across the blood brain barrier, and there is little GLUT4 in the dentate gyrus, making it less likely that these transporters would co-localize with developing neurons.

However, these results raise a number of interesting questions. First, although prior research has established how mature neurons acquire their supply of glucose, the results presented here suggest that developing neurons do not acquire glucose via the same mechanism(s). This leads to a second question: If these developing neurons do not acquire their energy via glucose uptake, how do they acquire their energy?

Of the potential candidates, the monocarboxylates appeared the most likely. Monocarboxylates, including lactate and ketone bodies, act as alternative sources of fuel for the brain during times when there is a lack of glucose, such as during early development or during exercise. Moreover, monocarboxylate transporters (MCT) have been localized to neurons (MCT2), astrocytes (MCT4) and endothelial cells (MCT1) in rodent brains, although there is some debate as to whether MCT1 also labels astrocytes (Bergersen, 2007). With this information, we tested the hypothesis that neurogenesis in the dentate gyrus would indicate any co-localization between MCT1 or MCT2 and DCX (see Figures 10, 11). This brief, preliminary work did not reveal co-localization between these antibodies, suggesting that newborn neurons in the dentate gyrus (at least at the DCX stage) do not express MCT transporters, and therefore do not take up monocarboxylates.

The results of this study, however, do not necessarily preclude glucose as the source of energy for cells in early post-neurogenesis. One possibility is that the glucose may be transported into the neurons via other glucose transporters (GLUT6, 10 and 12) present in the brain; however, these were not tested because of the lack of suitable antibodies for immunohischemistry. A more likely explanation is that other, less prevalent glucose transporters provide the necessary energy. GLUT2 and GLUT5 are found in low levels in the brain and while their presence on newly formed neurons is possible, they seem to be unlikely candidates given their specific localization and the greater affinity for fructose by GLUT5. The more recently discovered GLUT6 and GLUT10 have been detected in the brain using western blot and the mRNA distribution from the mouse brain (from the Allen Brain Atlas data) suggests a neuronal distribution but their specific localization remains to be examined.

The results of this study present more questions than answers. However, the results do lead to an important conclusion about the delivery of energy to developing neurons and it is that it seems to differ from mature neurons. Although previous research has ascertained how mature neurons acquire glucose, the present study is the first to examine glucose transporters in developing neurons *in vivo*. Thus, the results dispute assumptions concerning the energy acquisition in developing neurons. At this point, it is worth mentioning that a recent *in vitro* study found that adult neural stem cells from the SVZ express *both* GLUT1 and GLUT3 (Maurer, Geomor, Burgers, Schelshorn, & Kuschinsky, 2006). These results directly contradict the *in vivo* results presented here; however, there is a major issue that cannot be ignored when examining the results from Maurer et al (2006). As the authors readily admit, the *in vitro* environment that the

cultured cells were raised in contained a significant amount more glucose (nearly 5X in this case) than the *in vivo* environment. Because of this, one cannot discount the possibility that the GLUT1 and GLUT3 present on the neural stem cells was a cellular response to the increased level of glucose and, as such, would not occur *in vivo*. Moreover, it seems peculiar that glucose transporters would be expressed in neural stem cells and mature neurons, but not at all during the developmental phase.

*Future Directions and Limitations*

These two topics are related as only by addressing some of the current limitations can future research in the area be greatly improved. Any research involving BrdU has potential limitations. Foremost is that BrdU may also label cells undergoing DNA repair, leading to false positives. However, the likelihood of this occurring is low, and regardless the outcome or results of the research would remain the same because the labelling of DNA under repair is *in addition* to the labelling of dividing cells. In addition, BRDU will label astrocytes, microglia and oligodendrocytes, further complicating matters. Either way, there was no double labeling in this study making it a moot point.

A second, more general, limitation of using BrdU is that the protocol required to stain the BrdU-labelled DNA is harsh, making double staining difficult. In the case of the GLUT antibodies, a more involved double-staining protocol (see Experimental Procedures) had to be implemented and, because of this, there is a higher risk of errors being made, especially when capturing image even though this did not limit the study or the results, per se.

By and large, however, the main limitations of the study are derived from one of the most important components of any immunohistochemical study: the antibodies.

Certainly, this is the same for most studies of this nature and, for the most part, the antibodies used in the study (see Table 1) and presented in the results worked as previously reported in the literature. Initially, however, the scope of the project was larger and meant to encompass antibodies against a wider variety of neurogenesis markers and glucose transporters. Yet, as the project moved forward the scope became narrower by necessity. For the "lesser" glucose transporters (2, 5, 6, 10 etc), the number of quality, commercially available antibodies that are compatible with mouse brain tissue were limited. Until these higher quality antibodies are produced, studies of this nature will be limited.

Another part of the study that was cut from the end result was *in vitro* analyses of developing neurons and their GLUT expression. As conceived, it was meant to mirror the *in vivo* results, thereby strengthening the overall conclusion. However, our initial attempts at labelling the cultures with the GLUTs were unsuccessful (data not shown). Unfortunately, it is possible that the in vitro environment that these neurons are maturing in affects normal GLUT expression. Although future research could determine the nature of the effect, suffice it to say that any effect imposes severe limitations on the usefulness of cell cultures to study GLUT expression in developing neurons.

In summary, there is a need to address these limitations before further work can be done. When that occurs, it would be interesting to see if one of the other GLUT transporters is responsible for glucose transport during adult neurogenesis. In addition, considering what is known about the developmental expression of monocarboxylates (see above), it would be disadvantageous to not consider examining these although, again, it

would require valid, specific and clean antibodies to clearly identify such a small population of cells.

## Conclusion

From the available data presented in this thesis, it is clear that during adult neurogenesis, newborn neurons do not express any of the major glucose transporters in abundance. The results presented here are the very antithesis of the expectations arising from what is known about the neuroenergetic process, in particular, the acquisition of energy in mature neurons. Therefore, rather than confirming a priori assumptions, this study opens up the possibility that the process of supplying newborn neurons (in adulthood) with energy is substantially different than the process for mature, integrated neurons. Although not the expected result, it remains intriguing and future research will be needed to determine the nature of energy acquisition in adult neurogenesis.

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APPENDIX

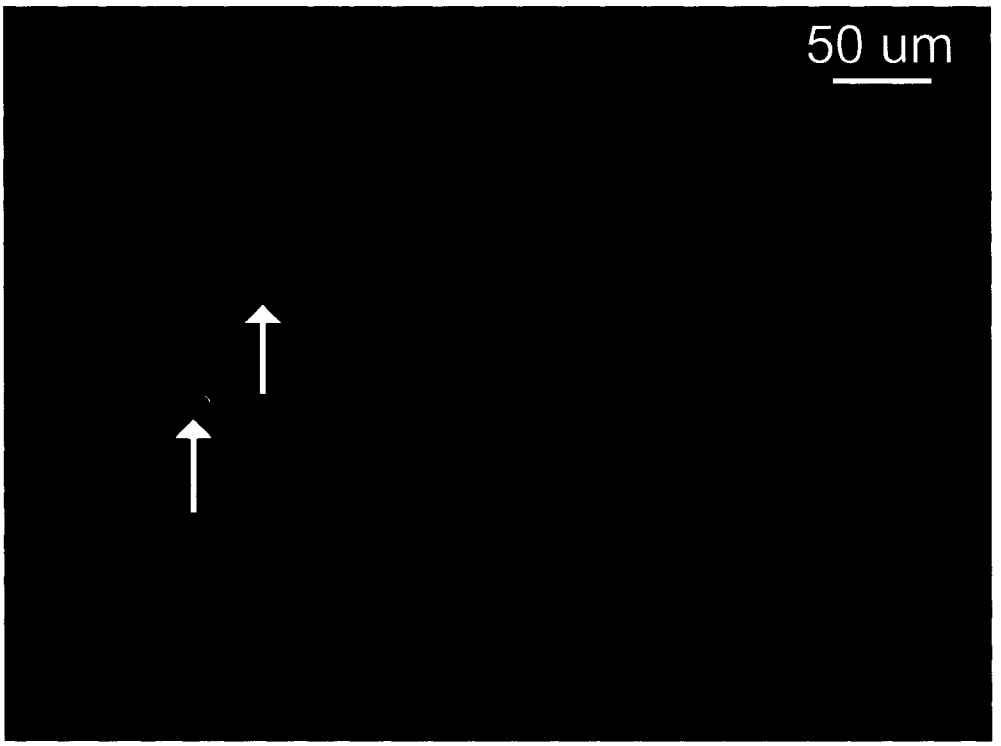


Figure 1. Double immunofluorescent labelling of DCX and BRDU. Arrows indicate two cells double-labelled for the two antibodies.


Figure 2. Double immunofluorescent staining for GLUT1 and BRDU. Note the prominent vascular location of GLUT1.


Figure 3. Double immunofluorescent labelling GLUT1 and doublecortin.

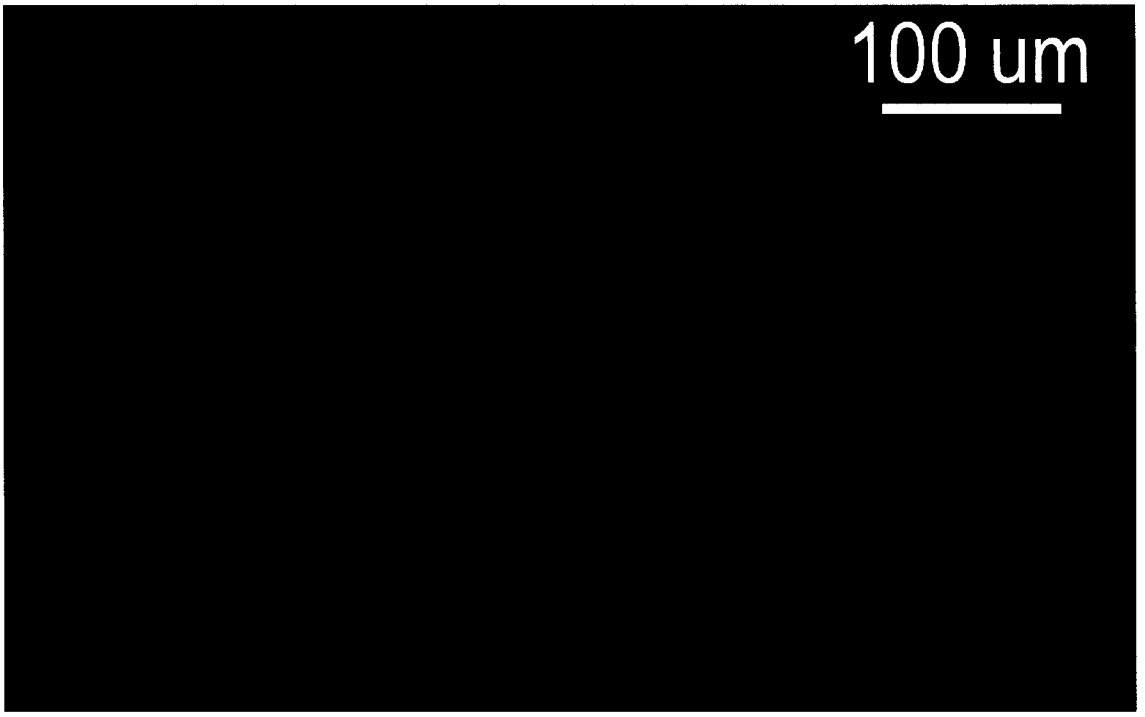


Figure 4. Double immunohistochemical labelling of GLUT3 and BrdU in the dentate gyrus. Note the punctate staining of GLUT3 and the inset shows a closeup of the BrdU-labelled cell.

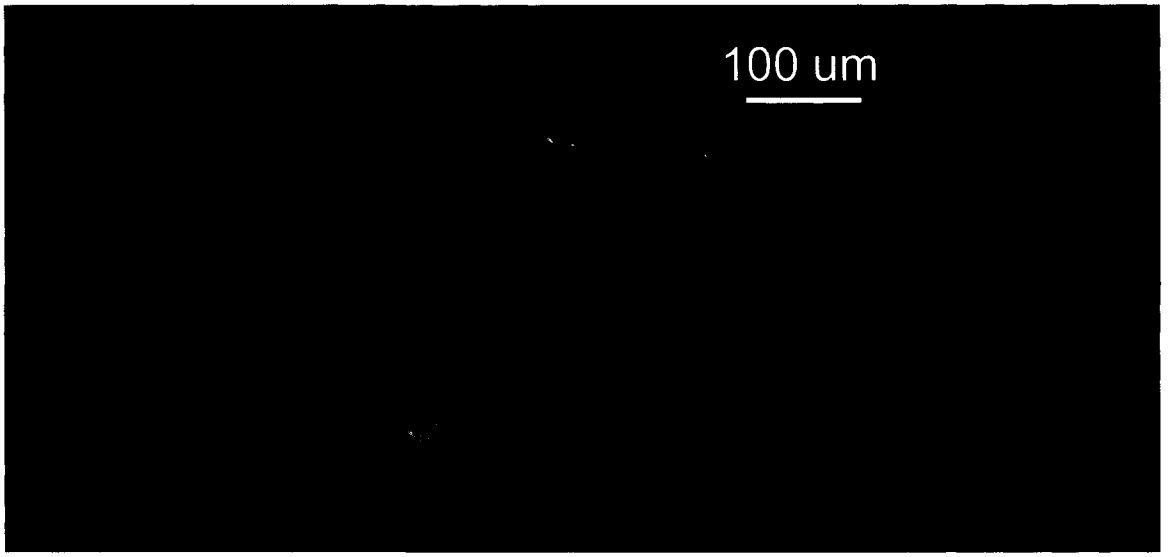


Figure 5. Double immunohistochemical labelling of GLUT3 and doublecortin. Note the punctuate neuropil labelling of GLUT3, and the inset better shows the relationship between GLUT3 and DCX.

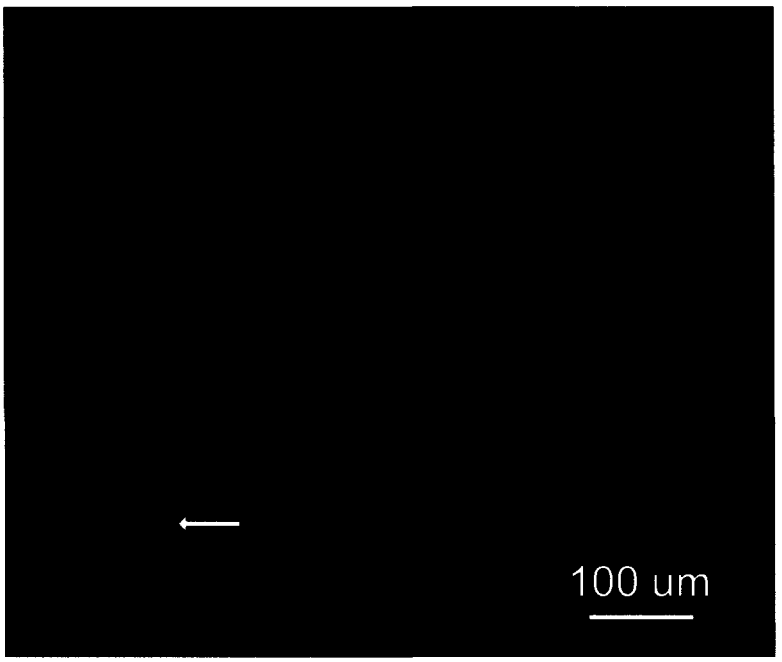


Figure 6. Double immunofluorescent labelling of GLUT4 and BrdU. Note the low levels of GLUT4 in dentate gyrus and the lack of co-labelling.

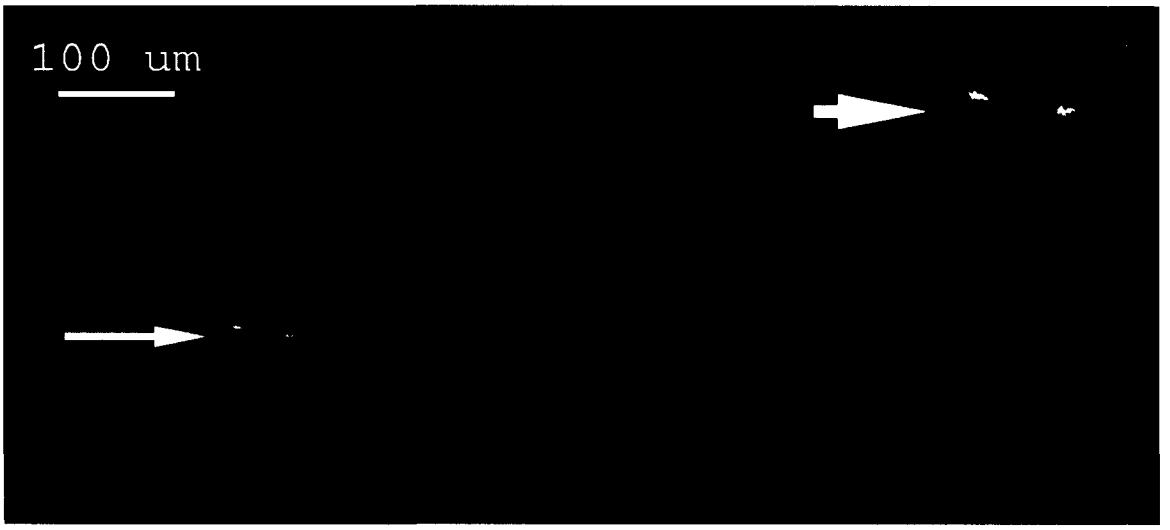


Figure 7. Double immunofluorescent labelling of GLUT4 and DCX. Note the low levels of GLUT4 in dentate gyrus and the arrow identifies one of the few GLUT4 positive cells. The inset picture provides a close-up for a better view.

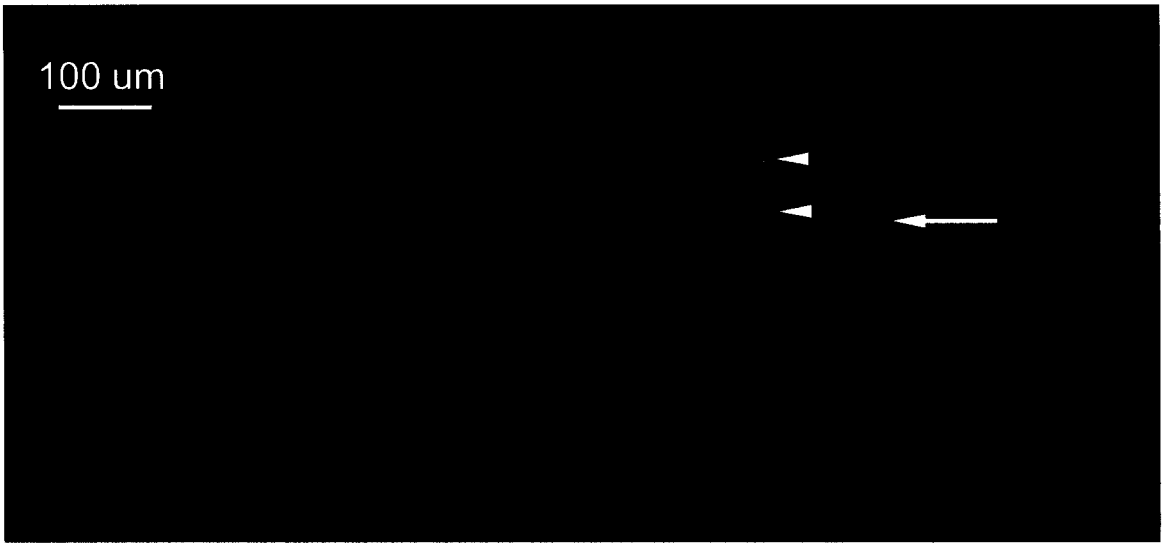


Figure 8. Double immunofluorescent labelling of GLUT8 and BRDU. Note the prominence of GLUT8 in cells of dentate gyrus (the arrow is an example), and the arrowheads identify BRDU-positive cells.


Figure 9. Double immunofluorescent labelling of GLUT8 and DCX.

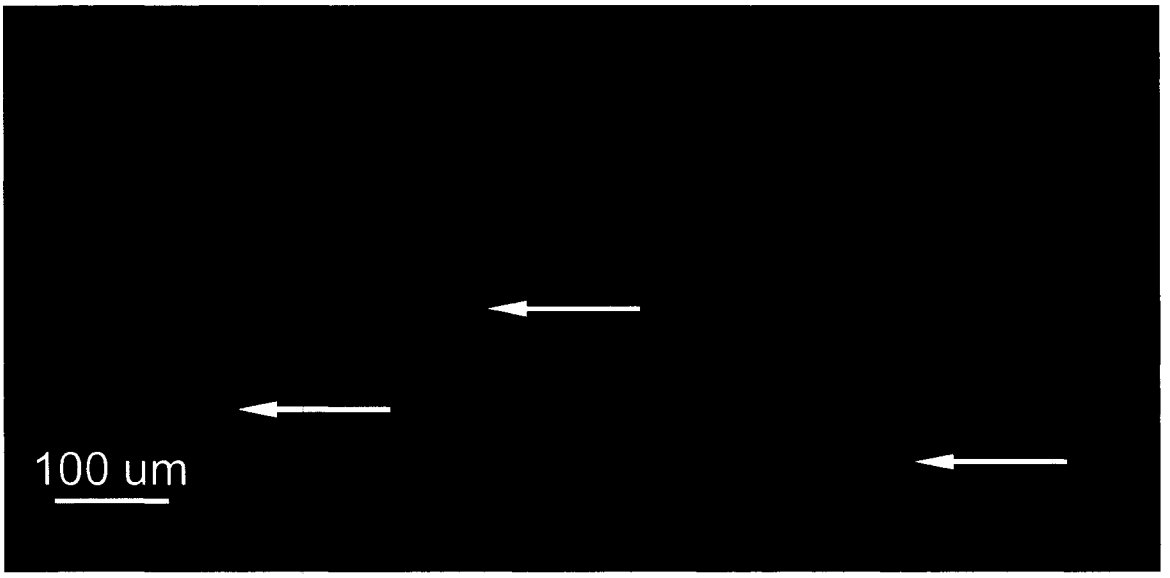


Figure 10. Double immunofluorescent labelling of BRDU and Hoechst dye. The arrows identify three purple coloured cells that are double labelled.

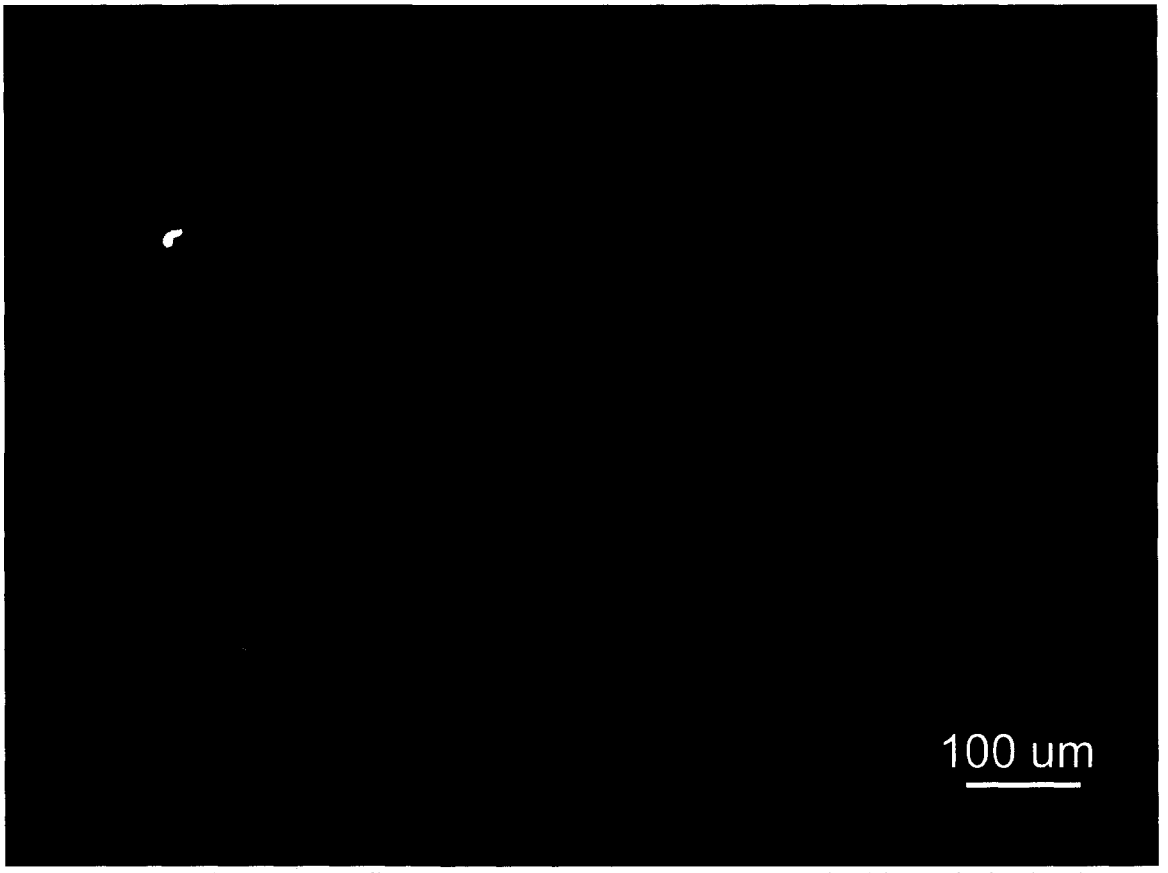


Figure 11. Double immunofluorescent labelling of MCT1 and doublecortin in the dentate gyrus. Note the vascular labelling of MCT1, mirroring GLUT1.

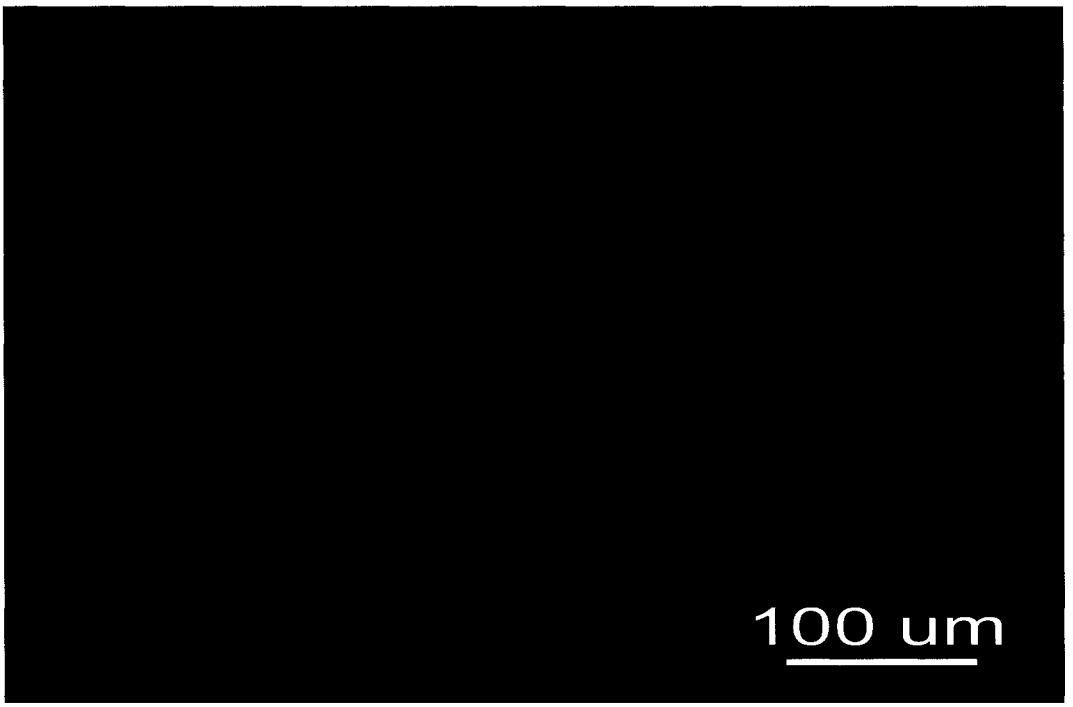


Figure 12. Double immunofluorescent labelling of MCT2 and doublecortin in the dentate gyrus. Note the neuropil labelling of MCT2.

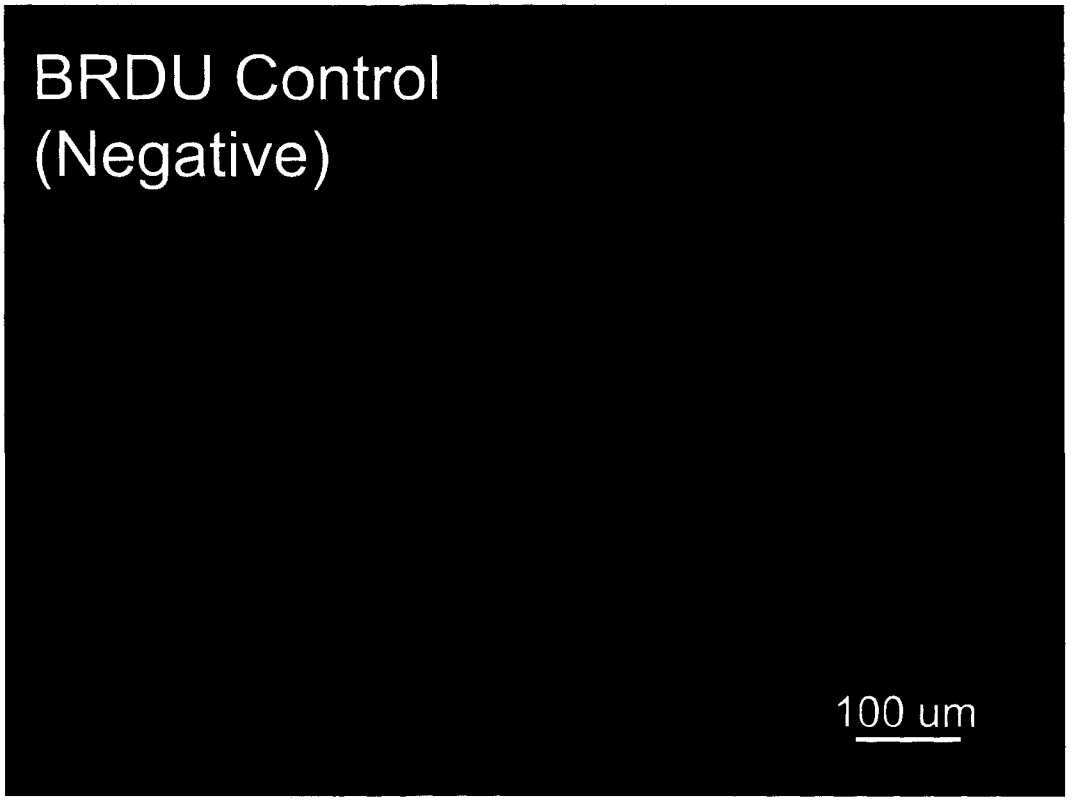


Figure 13. BRDU Negative Control, note the lack of BRDU labelling.