«Astrocyte-mediated effect of hyperglycemia on mouse neural stem cells»

Master Thesis

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«Η επίδραση της υπεργλυκαιμίας στα νευρικά βλαστικά κύτταρα μέσω των αστροκυττάρων»

Διπλωματική Εργασία

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Prologue

The present work was carried out in the framework of my Master thesis in Regenerative Neuropharmacology Lab, during the academic year 2020-2021.

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Abstract

Diabetes and hyperglycemia are well-known for inducing deleterious effects on physiological processes and are associated with several pathological conditions such as cardiovascular diseases, peripheral neuropathy, retinopathy, and nephropathy. The impact of metabolic disorders such as diabetes and obesity on the central nervous system (CNS) has been widely studied. In specific, diabetes impairs brain morphology and function and especially normal adult neurogenesis in the hippocampus. Therefore, diabetes is strongly associated with a higher risk to develop Alzheimer's disease (AD) as the hippocampus is among the first brain structures to be affected by AD pathology. However, diabetes does not only have an effect on neurons and neural stem cells but also on glial cells. Astrocyte-neuron interactions are involved in the pathogenesis of several neurologic diseases while molecules secreted by astrocytes can affect adult neural stem/progenitor cells and can increase their differentiation into neurons. High glucose exposure has been also shown to alter astrocytes function and to increase the expression of inflammatory cytokines. Inflammation and apoptosis in the brain have been associated with the p75 neurotrophin receptor (p75NTR). Moreover, the p75 neurotrophin receptor is involved in a variety of other cellular responses, as it is capable of mediating cell survival and neuronal differentiation. p75NTR can also regulate specific aspects of neurogenesis while it is also expressed by glial cells and oligodendrocytes.

In this context, our study focused on the effect of hyperglycemia on neural stem cells (NSCs) and astrocytes properties as well as on the astrocyte-mediated effect of hyperglycemia on NSCs. It was shown that hyperglycemia impairs different aspects of neurogenesis as well as it induces astrocytes reactivity. High glucose exposure not only affects directly the NSCs but it can also affect neurogenesis via astrocytes. P75NTR activation may contribute to hyperglycemia-mediated alterations in stemness, proliferation and apoptosis of NSCs as well in astrocyte response to high glucose exposure.

Περίληψη

Ο διαβήτης και η υπεργλυκαιμία είναι γνωστοί για την πρόκληση επιβλαβών επιδράσεων σε φυσιολογικές διεργασίες και συνδέονται με πολλές παθήσεις όπως καρδιαγγειακές παθήσεις, περιφερική νευροπάθεια, αμφιβληστροειδοπάθεια και τη νεφροπάθεια. Η επίδραση των μεταβολικών διαταραχών όπως ο διαβήτης και η παχυσαρκία στο κεντρικό νευρικό σύστημα (ΚΝΣ) έχει μελετηθεί ευρέως. Συγκεκριμένα, ο διαβήτης βλάπτει τη μορφολογία και τη λειτουργία του εγκεφάλου και ιδιαίτερα τη φυσιολογική νευρογένεση των ενηλίκων στον ιππόκαμπο. Ως εκ τούτου, ο διαβήτης συνδέεται στενά με υψηλότερο κίνδυνο εμφάνισης της νόσου του Alzheimer (AD), καθώς ο ιππόκαμπος συγκαταλέγεται μεταξύ των πρώτων εγκεφαλικών δομών που επηρεάζονται από την παθολογία AD. Ωστόσο, ο διαβήτης δεν έχει μόνο επίδραση στους νευρώνες και τα νευρικά βλαστικά κύτταρα αλλά και στα γλοιακά κύτταρα. Οι αλληλεπιδράσεις αστροκυττάρων-νευρώνων εμπλέκονται στην παθογένεση αρκετών νευρολογικών ασθενειών, ενώ μόρια που εκκρίνονται από τα αστροκύτταρα μπορούν να επηρεάσουν τα ενήλικα νευρικά βλαστικά / προγονικά κύτταρα και μπορούν να αυξήσουν τη διαφοροποίησή τους σε νευρώνες. Η υψηλή έκθεση στη γλυκόζη έχει επίσης αποδειχθεί ότι μεταβάλλει τη λειτουργία των αστροκυττάρων και αυξάνει την έκφραση των φλεγμονωδών κυτοκινών. Η φλεγμονή και η απόπτωση στον εγκέφαλο έχουν συσχετιστεί με τον υποδοχέα νευροτροφίνης p75 (p75NTR). Επιπλέον, ο υποδοχέας νευροτροφίνης p75 εμπλέκεται σε μια ποικιλία άλλων κυτταρικών αποκρίσεων, καθώς είναι ικανός να μεσολαβεί στην επιβίωση των κυττάρων και στη νευρωνική διαφοροποίηση. Ο p75NTR μπορεί επίσης να ρυθμίσει συγκεκριμένες πτυχές της νευρογένεσης, ενώ εκφράζεται επίσης από γλοιακά κύτταρα και ολιγοδενδροκύτταρα.

Σε αυτό το πλαίσιο, η μελέτη μας επικεντρώθηκε στην επίδραση της υπεργλυκαιμίας στα νευρικά βλαστικά κύτταρα (NSCs) και στις ιδιότητες των αστροκυττάρων, καθώς και στην επίδραση της υπεργλυκαιμίας στα NSCs μέσω των αστροκυττάρων. Αποδείχθηκε ότι η υπεργλυκαιμία βλάπτει διάφορες πτυχές της νευρογένεσης καθώς επίσης προκαλεί την αντιδραστικότητα των αστροκυττάρων. Η υψηλή έκθεση σε γλυκόζη δεν επηρεάζει μόνο άμεσα τα NSCs, αλλά μπορεί επίσης να επηρεάσει τη νευρογένεση και έμμεσα μέσω των

αστροκυττάρων. Η ενεργοποίηση του P75NTR μπορεί να παίζει κάποιο ρόλο στις μεταβολές που παρατηρούνται στη διαφοροποίηση, τον πολλαπλασιασμό και την απόπτωση των NSCs εξαιτίας της υψηλής συγκέντρωσης γλυκόζης, καθώς και στην απόκριση των αστροκυττάρων στην υψηλή έκθεση στη γλυκόζη.

1. Introduction

1.1 Diabetes mellitus

Diabetes mellitus (DM) is one of the most common metabolic diseases worldwide and according to the International Federation[1] of the disease, it has reached epidemic levels in recent years [2]. Over 500 million people worldwide are affected by DM [3] and the number of patients is estimated to be 693 million until 2045 [4]. DM often leads to the development of secondary complications in various organs, such as eyes, kidneys, heart, brain, and skeletal muscle[5]. The main cause of mortality in individuals with DM is the complications in both the macrovascular and microvascular system such as cardiovascular disease and diabetic neuropathy and retinopathy [6].

However, diabetes cannot be defined as a single disease, but rather as a group of conditions sharing a mutual diagnostic criterion — hyperglycemia, a common feature of several metabolic disorders[6]. Hyperglycemia refers to high blood glucose levels (>1.26 g/L in fasting conditions)[7] and occurs due to a dysfunction either in insulin secretion which characterizes Type 1 Diabetes Mellitus (T1DM) or in insulin action leading to the development of Type 2 Diabetes Mellitus (T2DM) or a dysfunction in both [8].

DM is also associated with increased production of reactive oxygen species (ROS) and diminished lipid oxidation[9],[10]. It often leads to reduced mitochondrial activity and consequently to an impairment in regulation of metabolic pathways and the maintenance of the appropriate energy balance in tissues[11]. Similar to mitochondria, the functions and the abilities of stem cells are impaired under diabetic conditions in several tissues including the neural tissue[11].

The central nervous system (CNS) has been reported to be critically affected by DM [11]. As early as 1922, impairments in cognitive function were recognized as a result of diabetes [12]. Moreover, DM is strongly associated with vascular dementia, depression and Alzheimer's disease (AD)[11]. More specifically, T2DM is associated with a 1.5–2.5-fold increased risk of dementia onset including Alzheimer's disease[13],[14]. These disorders may be caused by

morphological changes, such as white matter leukoaraiosis and hippocampal, cortical, and amygdala atrophies as it was shown by the brains of DM patients[15]. According to studies in rat models of diabetes, plasma and brain glucose levels increase on average by approximately 3-fold, with mean values in brain rising from 2.2 mmol/g in controls to 6.9 mmol/g in diabetic animals[16]. To conclude, DM is strongly associated with symptoms leading to accelerated brain aging[17].

1.2 Diabetes-associated complications in the Brain

Alterations in glucose metabolism affect brain function and its ability to deal with insults indicating a correlation between DM complications and the brain's functional capacity [18]. Alzheimer's disease (AD) is the most common neurodegenerative disease and the leading cause of dementia worldwide [2], [18]. The main pathological hallmarks of the disease are the presence of senile plaques and neurofibrillary tangles (NFTs)[19]. Other common features in AD are reactive gliosis, oxidative stress, reduced neurogenesis and reduced mitochondrial activity[20]. AD and T2D share some common properties including dysfunction in glucose metabolism and impairment of insulin signaling. Metabolic alterations have been found to contribute to acceleration of AD processes and specifically in the impairment of normal adult NSCs proliferation and neurogenesis[21],[22]. Therefore, insulin resistance and hyperglycemia are considered risk factors for AD[23].

Diabetes is notably linked to changes in brain morphology and functions. These changes are reflected as decreased neurogenesis including changes in cell proliferation, differentiation and survival, reduced brain volume, alterations in blood-brain barrier physiology of cerebral microvessels and increased vulnerability to cognitive deficits and behavioral changes[7],[24]. Chronic hyperglycemia, as reflected by higher levels of glycated hemoglobin (A1C), is linked to poor cognition in patients with diabetes. This is probably a result of microvascular changes, alterations in synaptic plasticity, oxidative stress and the accumulation of advanced glycation end products[25],[26].

1.2.1 Effect of diabetes on normal adult neurogenesis

Until a few years ago, neurogenesis was believed to take place only in the developing mammalian brain[27]. Nevertheless, since advances in cell labeling techniques occurred, it was confirmed that neurogenesis is sustained and persists throughout adulthood in specific brain regions, such as the dentate gyrus (DG) and the subventricular zone (SVZ) of the hippocampus[24], [28]. Neurogenesis, the genesis of newborn neurons, is thought to include 5 stages: proliferation, differentiation, migration, targeting, and integration phases, respectively[7],[29]. This process represents the plasticity of the adult brain in response to environmental stimuli while neurogenesis deficits are correlated with several neurodegenerative disorders such as Alzheimer's disease[30].

The DG and the SVZ give birth to 3 different types of cells: the neural stem cells (NSCs), the transit amplifying cells (TACs) and newborn cells of the 3 neural lineages namely the neuronal, the astroglial and the oligodendroglial[30]. NSCs of DG are called type 1 cells while TACs are called type 2. Postmitotic immature neurons are called type 3 cells[30]. Typical markers of type 1 cells which are characterized by self-renewal and multipotency, are the glial fibrillary acidic protein (GFAP) and brain lipid binding protein (BLBP), as well as Sox2 and Nestin[30],[31]. Type 2 cells also express GFAP, Sox2 and nestin and have different morphology from type 1 cells[30],[32]. Finally, type 3 cells are postmitotic immature neurons that express markers of the neuronal lineage (PSA-NCAM, DCX, NeuroD, Prox1) but lack glial markers[33]. *In vitro*, NSCs are able to form free-floating aggregates, known as neurospheres[34]. Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) are two essential growth factors used in neural precursor cultures to maintain cells in their mitotic and undifferentiated state[35].

It has been shown that the neurogenic process continues in healthy people until the end of their life, however it is significantly reduced in aged people with AD. Moreover, postmortem studies in AD patients have reported that the expression of common neurogenesis markers such as nestin and doublecortin (DCX) is decreased in the DG and the SVZ[28], [36].

However, impaired neurogenesis is not only a feature of neurodegenerative diseases. Alterations of the hippocampus, one of the two main neurogenic niches of the adult brain, are

present in both diabetic cognitive impairment and Alzheimer's disease[30],[37]. Accordingly, adult neurogenesis is impaired in the diabetic brain[38]. Reduced hippocampal size has been reported in older diabetic patients following MRI studies. Cortical and hippocampal atrophies are also observed in older diabetic (db/db) mice[39]. These changes are probably a result of decreased neurogenesis and increased neuronal death[40]. Evidence indicates that the older diabetic patients are more susceptible to aging-associated cognitive decline than older people without diabetes[41].

The most usual method to analyze changes in proliferation, differentiation, and survival of neural progenitors in diabetic neurogenic niches is labeling of DNA-synthesizing cells with the thymidine analog 5-bromodeoxyuridine (5-BrdU)[30],[42]. Immunohistochemistry using typical proliferative markers such as PCNA (Proliferative Cell Nuclear Antigen) and Ki-67 are also common methods to detect changes in brain cell proliferation[7]. Several *in vivo* studies in T1DM and T2DM animal models have shown that brain cell proliferation was significantly decreased in the DG compared to the control groups[7], [43]. However, some results contradict this pattern. For example, a study reports that the amount of BrdU+ cells was higher in the SVZ and the subgranular zone (SGZ) of the DG in Goto-Kakizaki rat (model of T2DM) while the cell death was increased in these regions[44], [45]. However, the survival of newly generated neurons was decreased in DG[46]. Another study also showed increased percentage of BrdU+ or DCX+ cells in the SVZ, SGZ and cortex of db/db mice[39]. Moreover, results vary between males and females as recently it was found that proliferation rate was lower in the SGZ of the hippocampus of male rats submitted in a high-fat diet (HFD) compared to females[47]. Interestingly, neurogenesis seems to be differentially affected during development. HFD had a negative impact on neurogenesis in young rats while it did not affect neurogenesis in adults, as it was indicated by DCX immunostaining[48].

Furthermore, diabetes leads to a dysfunction in brain cell differentiation in neurogenic niches. Studies have reported that in several DM animals there was a significant decrease in newly generated neurons[49], [50]. DM also results in a reduced survival of newborn cells as indicated by the number of TUNEL-positive neurons and the higher expression of pro-apoptotic proteins such as caspase-3 in the hippocampus of STZ mice[51].

In summary, diabetes seems to impair hippocampal cell proliferation and survival as well as neuronal differentiation (Figure 1). However, results vary between different animal models, indicating that further investigation is needed.

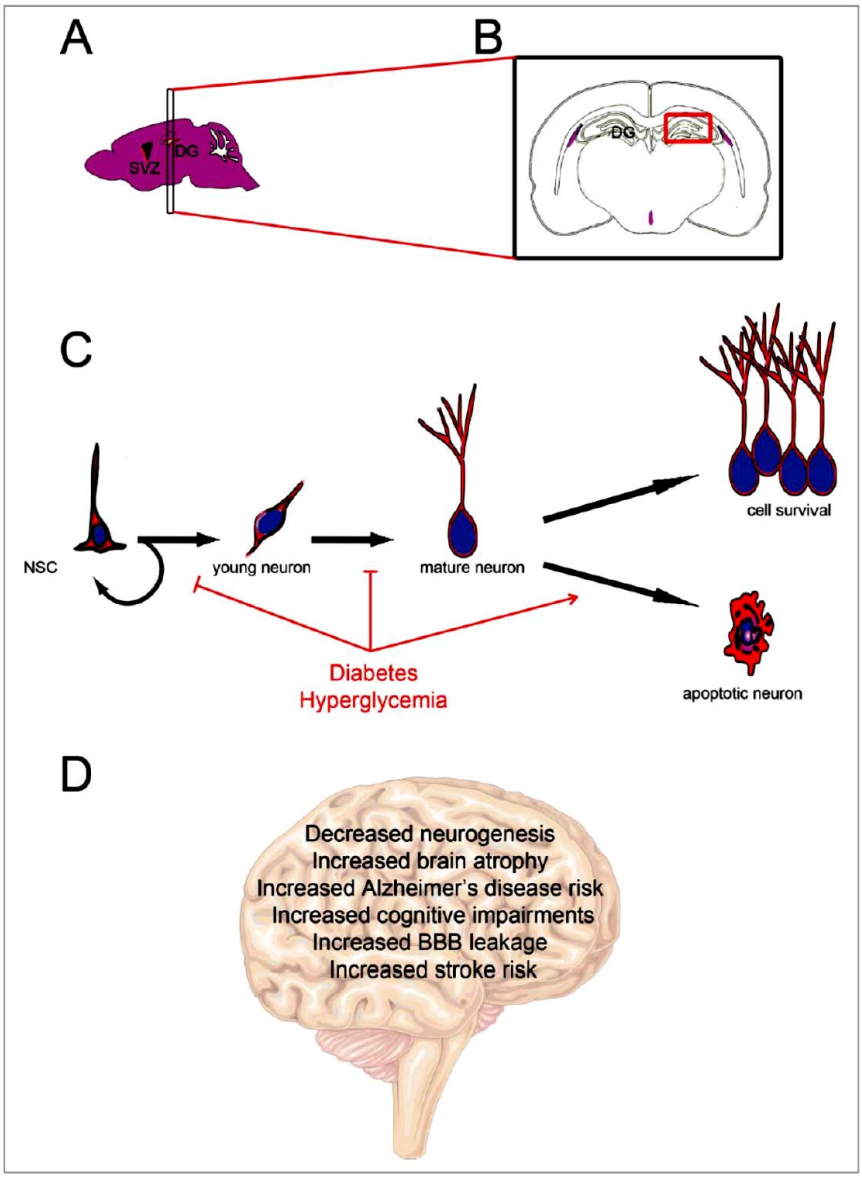


Figure 1. Diabetes-associated impairments in adult neurogenesis and brain functions. Diabetes complications are found mainly in the main neurogenic regions of the brain: the subventricular zone of the lateral ventricle (SVZ) and the subgranular region of the dentate gyrus of the hippocampus (DG). Neurogenic processes involve neural stem cell proliferation (including self-renewing) and the generation of neuronal precursors that differentiate into mature neurons. Diabetes/hyperglycemia has been shown

to inhibit neural stem cell proliferation and neuronal differentiation and to promote cell death. Diabetes is also linked to increased cognitive impairment and AD risk[7].

1.2.2 Effect of diabetes on astrocytes

The mammalian brain depends upon glucose as its main source of energy[52]. Neurons specifically need high levels of glucose to function properly and disruption of normal glucose metabolism leads to several brain disorders[53], [54]. Nonetheless, hyperglycemia in diabetes could radically increase neuronal glucose levels resulting in neuronal damage, a phenomenon known as glucose neurotoxicity[55], [56]. Glucose neurotoxicity is probably driven by several mechanisms such as oxidative stress and protein glycation. Moreover, indirect effects through glial cells might be involved in the glucose neurotoxicity[55]. Astrocytes are the most abundant glial cells in the CNS and are essential for maintaining normal brain functions, such as physical support, neurotransmitter uptake, energy storage and supply as well as synaptic transmission regulation[56]. Additionally, they contribute to the formation and maintenance of the blood–brain barrier and provide neurons with glucose, lactate, and trophic factors[53]. Glial fibrillary acidic protein (GFAP) has been the most widely used marker for astrocytes, however it should be noted that it only labels 30% of all astrocytes, with a very low expression in grey matter[57].

Astrocytes react to all forms of CNS dysfunction by a process commonly referred as reactive astrogliosis[58]. Astrocytes grown in high-glucose media are exposed to several well-established consequences of severe, chronic hyperglycemia[16]. High glucose exposure has been reported to alter astrocyte energy metabolism and function phenotype in primary astrocyte culture[56],[59]. It also leads to an increase in reactive oxygen species (ROS) production, inflammatory cytokines expression, and cell apoptosis in primary astrocytes[60]. Recently, it has been reported that high glucose (25 mM) had a dramatic effect on astrocyte phenotype *in vitro*. Specifically, it caused an irreversible inhibition of astrocyte proliferation possibly caused by cell cycle arrest without having an effect on apoptosis[56]. Diabetes has been shown to inhibit astrocyte activation after ischemic stroke indicating that inhibition of

astrogliosis in diabetes might be attributed to the inhibition of astrocyte proliferation due to high glucose[56], [61].

1.3 Astrocytes and inflammation

1.3.1 Astrocyte reactivity

Astrocytes are activated in the context of trauma, infection, and neurodegenerative diseases in order to contribute to tissue repair and promote CNS pathology[62]. Increased expression of glial fibrillary acidic protein (GFAP) has been found as a result of astroglial activation and gliosis during neurodegeneration, therefore it is commonly used as a marker for astrocyte reactivity[63],[64]. However, GFAP expression in reactive astrocytes varies significantly depending on their location in the CNS, their proximity to the injury site, and the type of injury[64]. Early formation of reactive astrocytes after CNS injury is considered to be neuroprotective and helps limit damage and inflammation[65]. There are two categories of reactive astrocytes based on the expression of a specific set of genes: 'A1 and A2', resembling the 'M1 and M2' macrophage polarization states[66]. A1 astrocytes display neurotoxic activity *in vitro* and were suggested to contribute to the pathogenesis of multiple neurologic diseases such as Alzheimer's disease (AD), Parkinson's disease, and multiple sclerosis (MS). On the other hand, A2 astrocytes, identified during the analysis of ischemia samples, are considered to have neuroprotective activity[66].

Astrocytes play multiple roles in CNS inflammation. They can limit the influx of peripheral immune cells into the CNS as well as they produce neurotrophic factors to promote tissue repair. However, they can also promote neurodegeneration and inflammation through the recruitment of peripheral inflammatory cells, the activation of surrounding microglia, and their own intrinsic neurotoxic activities[67].

Astrocyte reactivity is modulated by multiple signaling pathways, including the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway[68], [69] the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway[70], the

calcineurin (CN) pathway[71], and the mitogen-activated protein kinase (MAPK) pathway [62],[72]. Dysfunctional neurons, activated microglia as well as astrocytes themselves release a wide range of molecules, which recognize and bind specific receptors at the astrocyte plasma membrane and activate the above-mentioned intracellular pathways[73]. For example, the JAK/STAT3 pathway is activated by interleukins such as IL-6 or CNTF[73]. IL-6 is considered to regulate the expression of GFAP, vimentin, STAT3 and other genes with STAT responsive elements[62]. A few studies have examined the direct role of astrocyte-derived IL-6 on neuroinflammation and specifically on EAE development[74]. Moreover, a recent study that investigated the role of environmental factors to the pathogenic activities of astrocytes during neuroinflammation further supports the observation that astrocytes promote microglia proinflammatory functions through the secretion of IL-6 and other signaling factors[67],[75]. Finally, it was shown that high glucose (30.5 mM) increased mRNA expression of interleukin (IL)-6 and secretion of both IL-6 and IL-8 in human astrocytes indicating that hyperglycemia in T2DM may contribute to the observed increased risk of AD by aggravating astrocyte-mediated neuroinflammation[76].

1.3.2 Astrocytes interaction with neurons during neuroinflammation

Astrocytes were first considered to provide a structural scaffold necessary for neuronal function, however it is now clear that astrocyte-neuron interactions go further than just structural aid[77], [78]. Astrocyte-neuron interactions are involved in the pathogenesis of several neurologic diseases while the neurotoxic capabilities of reactive astrocytes have been widely studied[67], [78]. For example, multiple studies have reported that the activation of NF-kB signaling in astrocytes in the context of CNS inflammation, triggers the production of nitric oxide which is harmful for neurons when in excess[75], [79]. In addition to neurotoxicity caused by excessive NO, reactive astrocytes can promote neuronal death when there is a dysregulation of neurotransmitter uptake and release. This is partially a result of microglial CXCR4-dependent

release of high amounts of glutamate during neuroinflammation, leading to excitotoxicity and neuronal loss[80].

Physiologically, astrocytes provide lactate to neurons in order to support their metabolic needs[81]. Nevertheless, the reduced metabolic support of neurons by astrocytes promotes neurodegeneration[67]. Similar observations of dysfunctional metabolic coupling between astrocytes and neurons have been made regarding AD[82], Parkinson's disease[83] and ALS[84]. These findings suggest that astrocytes can promote neuronal death by multiple mechanisms, such as excitotoxicity and dysfunctional metabolism[67]. However, these results come from *in vitro* work, therefore further investigation is needed to validate our present understanding of astrocyte-induced neurotoxicity *in vivo*[67].

1.3.3 Astrocytes interaction with neural stem cells (NSCs)

Astrocytes are the dominant cell type of the neurogenic niche[85]. Radial glia-like cells, a subpopulation of precursors generated before embryonic neurogenesis, that give rise to neurons are able to interact with numerous cells of the dentate gyrus through their multiple extensions[86]. A study showed that molecules produced by astrocytes have an effect on adult neural stem/progenitor cells and can increase their differentiation into neurons[87]. Astrocytes can also negatively affect neurogenesis by astrocyte–stem cell contact mediated by Notch signaling through the intermediate filament proteins GFAP and vimentin[88]. They are also known to secrete molecules such as gliotransmitters, neuromodulators, trophic factors, and hormones in order to communicate with neurons and other glial cells[89]. These neuroactive molecules can act positively or negatively in different steps of normal adult neurogenesis[85].

In specific, ATP and FGF2 released from astrocytes increase the proliferation of adult NSC (aNSC) *in vitro* via PI3K signaling[90],[91]. Moreover, astrocytes secrete N-methyl-D-aspartate receptor (NMDAR) co-agonist D-serine that is known to enable the proliferation of neural progenitor cells *in vitro*[92]. Astrocyte-secreted molecules may also modulate other stages of adult neurogenesis, such as migration and differentiation of progenitor cells into neurons, or the maturation, synaptic integration, and survival of newborn neurons[85]. Song et al. showed

that in vitro, aNSC differentiation into neurons is increased by cell culture medium conditioned by astrocytes[87]. The astrocyte-derived soluble factor thrombospondin-1 (TSP1), has been found to increase aNSC proliferation and neuronal differentiation *in vitro* while *TSP1* deficient mice show reduced proliferation of aNSC[93], [94]. Furthermore, neurogenesin-1, IL-1b, IL-6, and WNT3 promote neuronal differentiation while insulin-like growth factor binding protein 6 (IGFBP6), enkephalin and decorin, also secreted by astrocytes, decrease neuronal differentiation of aNSC/ progenitor cells *in vitro*[95], [96],[97].

To sum up, it is obvious that astrocytes are in close contact with aNSC and they control their proliferation or differentiation based on their state and the secretion of specific molecules[85].

1.4 The role of p75NTR

The neurotrophin family of neurotrophic factors includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophins NT3 and NT4 and their respective precursors (proNGF, proBDNF, proNT-3)[98],[99]. These factors are responsible for the regulation of neuronal survival and function by interacting with distinct receptor complexes. Their role is to promote survival, axonal growth, and synaptic activity by signaling via Trk receptors as well as to induce apoptosis by signaling via the p75 neurotrophin receptor (p75NTR)[100].

The neurotrophin receptor p75NTR is able to bind all aforementioned neurotrophins (NGF, BDNF, NT3, NT4) and is the preferred receptor for pro-neurotrophins[98],[101]. It is involved in a variety of cellular responses, including apoptosis, neurite outgrowth and myelination[102]. p75NTR can also regulate specific aspects of neurogenesis. p75NTR is expressed by neurogenic stem cells in the postnatal and adult rodent ventricular/subventricular zone and its activation leads to the differentiation of these cells into neurons[103]. It has been also shown that p75NTR mediates the survival and death of newly born neurons, as the conditional *p75NTR* gene deletion from neural precursors led to increased apoptosis of these cells and a decrease in brain volume and abnormalities in telencephalic structures formation in adult knock out

mice[104]. In addition, adult hippocampal neurogenesis is affected by p75NTR deficiency, however it is still unknown whether this effect is direct or indirect[103]. Furthermore, the proliferation and differentiation of neural and non-neural cells has been found to be regulated by p75NTR mediated signals *in vitro*[105]. Finally, the p75NTR receptor mediates the death of specific populations of neurons during the development of the nervous system or after cellular injury indicating its significance in the apoptosis of brain cells[106].

The p75NTR receptor is widely expressed during the development of the nervous system, but also selectively during adulthood. It is expressed by many neuronal cell types, as well as by neural stem cells, astrocytes, oligodendrocyte precursors, Schwann cells and glia. However, some regions maintain p75NTR expression at lower levels including the primary cholinergic neurons of the forebrain, aesthetic neurons and motor nerves of the spinal cord[107].

1.4.1 p75NTR pathway

One of the most studied function of p75NTR is its ability to promote cell death[108]. Back in 1993, a research group showed that the ectopic expression of p75NTR in a number of nerve cells in serum-free medium, increased apoptosis [109]. Apoptosis induced by p75NTR contributes to the formation of correct target innervation during development and the elimination of cells during periods of naturally occurring cell death[110]. Apoptosis induced by p75 is also manifested after seizure or inflammation[111],[112].

On the one hand, activation of NF-kB, mediated via the interaction of RIP2 and p75NTR, can lead to neuronal survival or inflammation[113]. This interaction can also lead to the release of RhoGDI from the receptor and consequently to the activation of RhoA[114]. Binding of Rho-GDI to p75NTR can be accomplished by a complex consisting of myelin-associated glycoprotein (MAG) and the simultaneous dimerization of NgR / Lingo1R receptors, which serve as p75NTR co-receptors[115] (Figure 1).

On the other hand, apoptotic function of p75NTR is related to an increase of Rac and JNK activities, which are necessary for NGF-dependent death[116]. Specifically, p75NTR-induced phosphorylation of cJun / JNK induces cell death. Activation of JNK is achieved through

interactions of the family of TNF receptor-related factors (TRAFs) with the ICD of the p75NTR[117] (Figure 2).

P75NTR-induced cell death is also related to other co-receptors such as sortilin, SorCS2 and SorL1, which are selectively activated through binding to pro-neurotrophins that can initiate p75NTR signaling[101],[118].

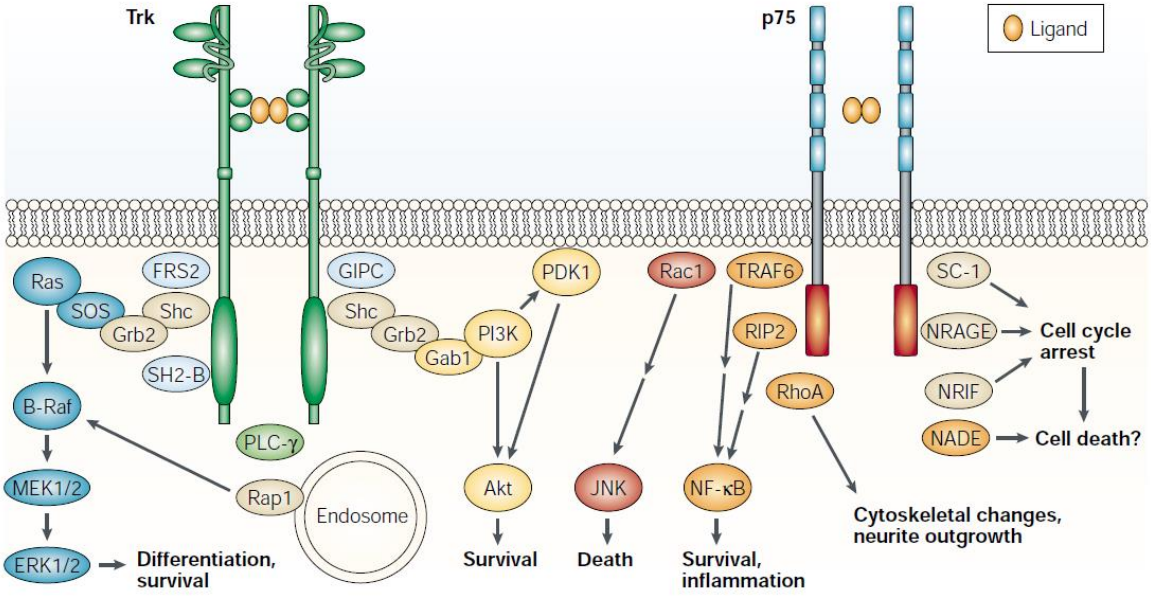


Figure 2. Neurotrophin receptor signalling. Trk receptors act as mediators of differentiation and survival signalling through extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways. The p75 receptor can activate NF-κB and Jun N-terminal kinase (JNK), and modulates RhoA activity. These responses occur through adaptor proteins that bind to the cytoplasmic domain of p75, including neurotrophin-receptor interacting factor (NRIF) and receptor-interacting protein 2 (RIP2), which can lead to apoptosis, survival and cell cycle arrest[119].

1.4.2 The role of p75NTR in Alzheimer's disease

P75NTR is also involved in the pathogenesis of Alzheimer's disease as it can bind to amyloid β and amyloid precursor protein. Studies have shown a biphasic role of this interaction as it has

been associated with neuronal damage whereas the extracellular domain of this receptor seems to play a neuroprotective role by inhibiting Aβ deposition[120], [121].

P75NTR mediates programmed cell death[121],[122], and the expression of p75NTR makes cells sensitive to β-amyloid (Aβ) toxicity[123]. In addition, p75NTR has been shown to induce apoptosis in response to pro–nerve growth factor (pro-NGF), which is increased in AD brain[124]. This observation, together with studies that showed increased or stable p75NTR levels coupled with decreased Trk levels in AD, and others showing that high p75NTR/Trk ratios can lead to degeneration, led to the suggestion of targeting p75NTR as a possible treatment for AD[125]. Therefore, small-molecule neurotrophin mimetics that bind p75NTR have been suggested as possible therapeutics in Alzheimer's disease[126], [127].

1.4.3 The role of p75NTR in Astrocytes

Astrocytes produce and secrete neurotrophins and proNGF[128],[129] especially after CNS injury and they also act as a target for these mediators[130]. P75NTR expression in astrocytes is limited in the normal adult rodent brain. *In vitro*, there is low expression of p75 in primary cultures of rat type I astrocytes, but treatment with NGF significantly up-regulates mRNA expression of p75NTR[131]. Treatment of rat hippocampal astrocytes with NGF in primary cultures results in a significant reduction in the cell number, however this effect is not mediated through apoptosis via p75NTR[132]. Astrocytes proliferation is increased after different types of injury, resulting in the formation of a glial scar while it has been reported that there is an increased activation of p75NTR on astrocytes under these conditions. This interaction may possibly restrict glial scar formation after injury *in vivo*[132]. Therefore, it is believed that p75NTR may regulate the proliferation of activated astrocytes to limit the glial scarring that occurs after injury[130]. It has been also shown that global transient cerebral ischemia in rats, led to an up-regulation of both p75NTR and TrkA in the majority of astrocytes in the cornu ammonis 1 area of the hippocampus[133]. Nonetheless, the consequence of this up-regulation of p75NTR in astrocytes remains unclear and further investigation is needed.

1.5 Aim of the study

Hyperglycemia, one of the main features of diabetes, has been shown to impair normal adult neurogenesis. This impairment is reflected in changes in proliferation, differentiation and survival of neural progenitor cells. However, *in vivo* results regarding the effect of hyperglycemia on proliferation of NSCs are contradictory indicating that further investigation is needed.

Astrocytes are the most abundant glial cells in the CNS and are essential for maintaining normal brain functions while supporting the metabolic needs of neurons. High glucose exposure has been reported to alter astrocyte energy metabolism and function in primary astrocyte cultures and to lead to an increase in the secretion of pro-inflammatory markers. During CNS inflammation, astrocytes can either act as neuroprotective or they can promote neuronal death by multiple mechanisms.

The neurotrophin receptor p75NTR is expressed by many neuronal cell types including NSCs and astrocytes. It is involved in a variety of cellular responses such as neurogenesis and cell death and its biphasic role has been studied a lot especially during inflammation.

In this context, we wanted to study the hyperglycemia-mediated brain deficits. More specifically, we were interested in investigating the effect of hyperglycemia on neural stem cells P7 (post-natal day 7) and astrocytes as well as the astrocyte-mediated effect of hyperglycemia on neural stem cells. We focused on the effects of high glucose exposure on NSCs proliferation, survival, stemness and the involvement of p75NTR signaling in NSCs dysfunction. We also examined the effect of hyperglycemia on astrocytes proliferation, cell death and the role of p75NTR and astrocytes- induced inflammation. Last but not least, we studied the effect of hyperglycemia on NSCs via astrocytes, by treating P7 with astrocyte conditioned medium. More specifically, we analyzed changes in stemness as well as the involvement of p75NTR in NSCs-astrocytes interaction.

2. Materials and Methods

2.1. Primary Astrocytes culture

Astrocytes were isolated from the cortex of P2 (post-natal day 2) mice. Briefly, the cortex of P2 mice was split in the two hemispheres and the meninges were removed. The dissected brains were put in HBSS 1X and then the suitable amount of trypsin 2.5% was added. After 10 minutes at 37 ^{0}C, trypsin solution was removed and the pellet was resuspended in 10 ml of HBSS 1X with 2 ml FBS. After a number of centrifugations and trituration, the cells were homogenized in 16 mL of cortex medium (DMEM High Glucose, 10% Fetal Bovine Serum (FBS), 2% Penicillin-Streptomycin) and 4 mL were added in 75 cm^2 flasks in a final volume of 10 mL per flask and were put in the incubator at 37 ^{0}C, 5% CO_2. After 13 days (DIV 14), astrocytes and microglia were separated from oligodendrocyte precursor cells (OPCs) as the astrocytes remain firmly attached to the flask surface. As microglia sometimes grow on the surface of the astrocyte monolayer, the addition of cytosine β-D-arabinofuranoside (araC) (Sigma-Aldrich #1768) in the medium (dilution 1:2500), helps to clear it.

Astrocytes were grown in astrocyte low-glucose medium containing DMEM low-glucose (5.5 mM glucose) (Sigma Life Sciences #D5546), 10% FBS, 1% Pen-Strep and 1% L-glutamine. The medium was changed after 3 days and astrocytes were cultured in flasks.

When the cells were confluent, astrocytes were passaged into plates. Firstly, the medium was removed and 4–5 ml of trypsin 0.25-0.3% were added (dilution in PBS 1X from 2.5% stock) and cells were incubated at 37 ^{0}C, 5% CO_2 until cells began to lift off (around 5 minutes). Equal amount of astrocyte low-glucose medium was added to stop the trypsinization and cells were released by repetitive pipetting. The cell suspension was transferred to a falcon and centrifuged at 300g for 5 min. The pellet was resuspended in astrocyte low glucose medium (1-3 mL) and passaged into plates. Cell density was determined by adding 10 µl of the cell suspension to a hemacytometer. The number of cells seeded in plates usually was:

- In 6-well plates: around 300.000 cells/well
- In 12-well plates: around 120.000 cells/well

- In 24-well plates: around 30.000 cells/well
- In 48-well plates: around 15.000 cells/well

2.2. Primary hippocampal NSCs (P7) culture

NSCs were isolated from the hippocampus of P7 mice. Cells were cultured in 25 cm^2 flasks in a final volume of 5 mL adult NSC medium that contains: DMEM/F12 (Gibco #11330-032), B27 supplement without vitamin A 1:50, D-glucose ~1:75 (Sigma #68769-45% stock), Primocin 1:1000 (InvivoGen 50 mg/mL stock), L-glutamine 1:100 (200mM stock), FGF 1:1000 (20 µg/mL stock), EGF 1:1000 (20 µg/mL stock) and Heparin 1:1000 (50mg/mL stock). After the first passage, cells are cultured in NSC low-glucose medium (17 mM glucose) that contains the above reagents except for D-glucose and L-glutamine.

In flasks, NSCs are able to form free-floating aggregates, known as neurospheres. When neurospheres were enough in size and quantity, NSCs were passaged into plates. Cells were transferred to a falcon and centrifuged at 300g for 5 minutes. Next, the medium was removed and the pellet cells were resuspended in around 200 µl of accutase and incubated for around 7 minutes at 37^0C, 5% CO2 and cells were released by pipetting every 2-3 minutes. A volume of DMEM/F12 around 10-fold higher than accutase volume was added for the inactivation of accutase. The mixture was centrifuged at 300g for 5 minutes and the pellet was resuspended in 1 mL of NSC low-glucose medium. Cell density was determined by adding 10 µl of the cell suspension to a hemacytometer. The number of cells seeded in plates usually was:

- In 12-well plates: around 100.000 cells/well
- In 24-well plates: around 41.000 cells/well
- In 48-well plates: around 22.000 cells/well
- In 96-well plates: around 9.000 cells/well

Before plating the cells, Matrigel coating is needed for cells to adhere to the surface of the wells. Matrigel needs to thaw in ice and is mixed with appropriate amount of cold DMEM/F12

(usually to a final concentration of 1:18). Then, Matrigel/media mixture was transferred to plates at the following volumes:

- In 12-well plates: around 320µl/well
- In 24-well plates: around 160µl/well
- In 48-well plates: around 90µl/well
- In 96-well plates: around 50 µl/well

Plates were put in the incubator (37^0C) for at least 30 minutes or were wrapped in parafilm and stored in 4°C fridge for up to 1 week. Matrigel/media mixture was removed before plating the cells.

2.3 Collection of astrocyte-conditioned medium

Astrocytes were seeded in 6-well plates. Treatment with D-glucose (30mM) was performed on Days 0 and 2. D-glucose concentration of 5.5mM was considered the control condition. Astrocyte supernatant was collected on Day 4 of the experiment and was concentrated using MilliporeSigma™ Amicon™ Ultra Centrifugal Filter Units (MWCO 3000 Da). More specifically, after rinsing the filter units with water for 15 minutes at 5000g, 4^0C, supernatants were collected and submitted into subsequent centrifugations (as many as needed at 5000g, 4^0C). Finally, concentrated supernatants were aliquoted and stored at -80 ^{0}C until usage.

The control and high glucose supernatants were added in the wells on Day 0 and Day 2 of the experiments (experiment ends on Day 4) in a dilution of 1:50.

2.4 Protein extraction (cell lysis)

1) Astrocytes:

Astrocytes were seeded on 6-well plates as described above. Control (5.5mM) and high glucose (30 mM) conditions were used and glucose was added on Day 0 and Day 2 of the

experiment. After removing astrocytes supernatant on Day 4, lysis buffer was added in each well. Lysis buffer has to remain on ice and consists of:

a) Pierce™ Lysis Buffer (Thermo, cat. #87787)
b) Protease inhibitors 1X (Merck, cat. #53913) (stock concentration 100X)
c) Phosphatase inhibitors 1X (Merck, cat. #524629) (stock concentration 50X)

Around 100 µl of Lysis Buffer was added to each well considering the confluency of the cells. After cells were lysed, the mixtures of cells and Lysis Buffer is transferred to tubes and are incubated on ice for 10 minutes. Then, tubes are centrifuged at full speed (14.800 rpm) for 7 minutes at 4^0C. Supernatants are transferred to new tubes and stored at -80^0C and the pellets are discarded.

2) P7 NSCs:

NSCs were seeded on 12-well plates and the conditions that were used were control (17 mM glucose) and two high glucose conditions (40 mM and 100 mM). Treatment with D-glucose (40mM/100mM) was performed on Days 0 and 2. After removing the medium on Day 4, lysis buffer was added in each well, similarly to the process that was followed for astrocytes.

2.5 Western Blot

Protein concentration was measured by Pierce™ BCA Protein Assay Kit (#23225). A total of 25 µg of protein for each sample were separated on a 10, 12 or 15% polyacrylamide gel, depending on the molecular weight of the proteins to be studied. The divided proteins were subsequently transferred to a nitrocellulose membrane. The membranes were incubated in blocking buffer (5% BSA in TBS-Tween 0.1%) for an hour at room temperature (RT), and then with primary antibodies overnight at 4^0C. After washing the membranes in TBS-T (3x10 minutes) membranes were incubated for an hour at room temperature in corresponding secondary horseradish peroxidase-conjugated antibodies (HRP) in 2% BSA in TBS-T 0.1% solution. Then, membranes were washed in TBS-T (3x10 minutes) and developed by

chemiluminescence (ChemiDoc Imaging System-BioRad), according to the manufacturer's instructions. The primary antibodies used can be found in the table below (Table 1):

Primary Abs	Species	Company	concentration
GAPDH	mouse	Sigma (G8795)	1:1000
p75	rabbit	promega	1:1000
SOX2	rabbit	Abcam (ab15830)	1:1000
SOX1	rabbit	Cell signaling (#4194)	1:500
Ki67	rabbit	Abcam (ab15580)	1:500
GFAP	mouse	Millipore (MAB360)	1:1000
RIP2	rabbit	Enzo Life Sciences	1:1000
Total-JNK	rabbit	Cell signaling (9252S)	1:1000
Phospho-JNK	rabbit	Cell signaling (#9251)	1:1000

Table 1. Primary antibodies used in Western Blot analysis.

The secondary antibodies used in Western blot analysis were HRP anti-mouse (Thermo Fisher Scientific, A10521) and anti-rabbit (Invitrogen, #656120) antibodies, both in dilution 1:6000.

TBS-T weas prepared as described below:

1) **TBS (10X) 1L**

- 24.2 g Tris-Base
- 80 g NaCl

Up to 1 L in dH_2O (pH=7.6)

2) **TBS-0.1% Tween20 (TBST)**

- 100 mL TBS 10X
- 1 mL Tween-20

Up to 1 L in dH2O

2.6 Growth curve

On Day 0 of the experiment, a specific number of cells was seeded in each well in full NSC medium. Glucose and NGF (nerve growth factor Millipore 50 μg, dilution 1:1000) were added in

the medium on Day 0 and Day 2 of the experiment and cells were counted on Day 2 and on final Day 4 of the experiment.

2.7 BrdU/Ki67 immunostaining

Four hours or 48 hours prior to the completion of the experiment, BrdU (bromodeoxyuridine) analog (1μM) was added to NSC or astrocyte cultures respectively (BrdU stock 1mM) and the cultures were incubated at 37^0C, 5% CO_2. Cells were then washed with PBS 1X at RT for 1 minute and fixed with 4% PFA for 10 minutes. After 2 washes with PBS 1X for 5 minutes, HCl 2N was added and cells were incubated for 30 minutes at 37 ^{0}C. Then, a 10-minute wash in 0.1M sodium tetraborate pH 8.5 and three 5-minutes washes with PBS 1X followed. Cells are washed with 0.1% PBS-Triton at RT for 5 minutes and incubated for 1 hour in blocking solution (10% Normal Horse Serum, 0.1% BSA in 0.3% PBS-Triton) and then overnight at 4 ^{0}C with primary BrdU antibody (Invitrogen, #B3512, 1:200) and Ki67 antibody (Abcam, ab15580, dilution 1:1000) diluted in 1% Normal Horse Serum in 0.1% PBS-Triton. The next day, cells are washed 3 times in PBS 1X for 5 minutes and incubated for 1 hour at RT with secondary antibodies (anti-mouse Alexa 488, 1:1000 and anti-rabbit Alexa 546, 1:1000). After a 5-minute wash with PBS 1X, cells are incubated in Hoechst 33342 (dilution 1:10000 in PBS 1X, Invitrogen) for 10 minutes and washed 3 times with PBS 1X. Fluorescent images of BrdU/Ki67 labeling were captured using Leica DM500 Fluorescence Microscope.

P75 inhibitor was added in astrocytes medium on Day 0 and Day 2 in a final dilution of 1:1000000 (abcam #6172).

2.8 MTT assay

Four hours prior to the completion of the experiment, the MTT solution is added to NSC cultures (MTT final concentration 0,5 mg/ml-stock 5mg/ml) and they were incubated at 37^0C, 5% CO_2. Then, the supernatant was removed and cells were incubated in DMSO/isopropanol solution (1:1 ratio) for 15 minutes at RT and another 15 minutes at 4^0C. Absorbance is measured at 545 and 630 nm using SPECTROstar® Omega Absorbance Plate Reader.

2.9 Statistical analysis

The results were analyzed using GraphPad Prism, version 8 (GraphPad Software Inc.). All results are reported as mean +SEM. The comparison between two samples was performed using the unpaired t-test. A *P* value of <0.05 was considered statistically significant. The results represent the average of at least three independent biological replicates.

3. Results

3.1 Effect of hyperglycemia on NSCs

Firstly, we studied the effect of hyperglycemia on NSCs E13.5. However, these cells were resistant to high glucose treatment, so the investigation was then focused on NSCs P7 (post-natal day 7). Hyperglycemia was represented by 40 mM and 100 mM glucose concentrations and 17 mM glucose concentration was used as the control condition. 40 mM glucose concentration is considered the most relevant to *in vivo* levels of glucose under "diabetes mellitus" condition.

1) Expression of apoptosis, stemness and proliferation markers

In order to compare the expression of apoptosis-, stemness- and proliferation-related proteins between control and high glucose conditions, Western Blot analysis was used. More specifically, protein levels of p75NTR, SOX2, SOX1 and ki67 were assessed in NSCs grown in control and high glucose medium (40 and 100 mM).

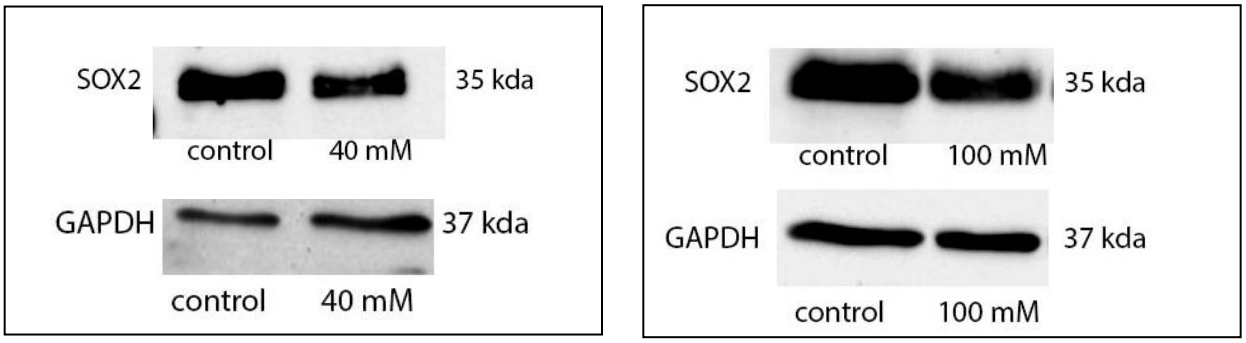
SOX2 is a well-established marker of neural stem and progenitor cells and SOX1 is expressed in only a subset of stem/early progenitor cells. SOX2 and SOX1 protein levels were examined as it was supposed that high glucose would result in the reduction of stemness (self-renewal and differentiation capacity) of NSCs. Even though SOX2 levels were significantly reduced in both hyperglycemic conditions compared to the control (Figure 3) indicating that indeed high glucose led to a reduction in stemness of NSCs, SOX1 did not show a consistent pattern of upregulation or downregulation among the 3 conditions.

Then, changes in the proliferation and differentiation of NSCs were assessed by comparing changes in the p75NTR expression. p75NTR has been found to regulate proliferation and differentiation of NSCS *in vitro* and it can be used as a cell marker for neural progenitor cells with a primary neurogenic potential[98]. P75NTR levels, were remarkably reduced in NSCs

grown in high glucose conditions (40 and 100 mM) compared to the control condition which is consistent with the observed reduction in growth capacity of these cells (Figure 5).

Lastly, we assessed changes in the expression of Ki67, a pan-proliferative marker. Ki67 protein levels were expected to be reduced in high glucose conditions, however there was not a consistent pattern of upregulation or downregulation between the 3 conditions. A slight reduction in ki67 levels in 40 mM and 100 mM was seen only once (Figure 6).

A)



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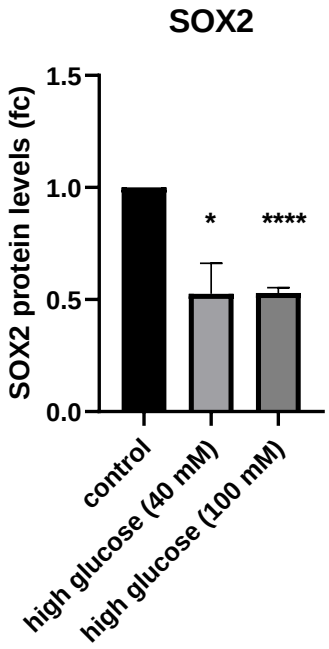
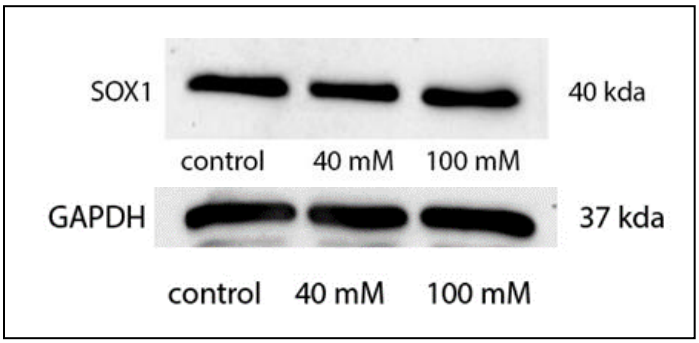


Figure 3. Representative Western blots (A) and quantitative analysis of SOX2 protein levels (B) among control and high glucose conditions (40 mM and 100 mM). The values are mean + SEM (*P < 0.05, ****P < 0.0001).

A)



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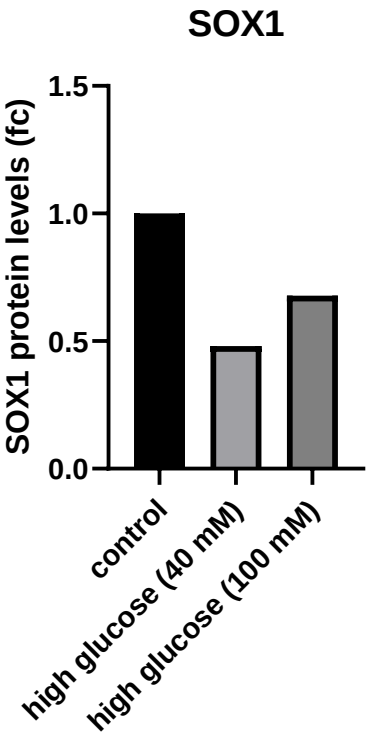
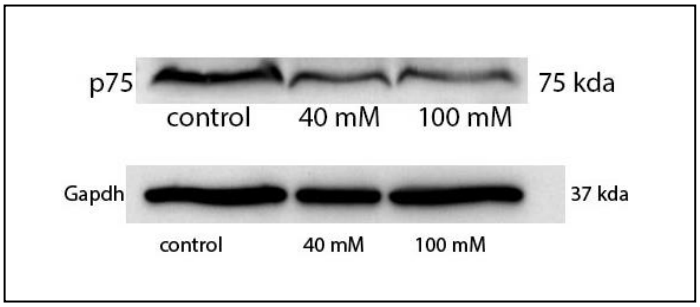


Figure 4. Representative Western blots (A) and quantitative analysis of SOX1 protein levels (B) among control and high glucose conditions (40 mM and 100 mM).

A)



B)

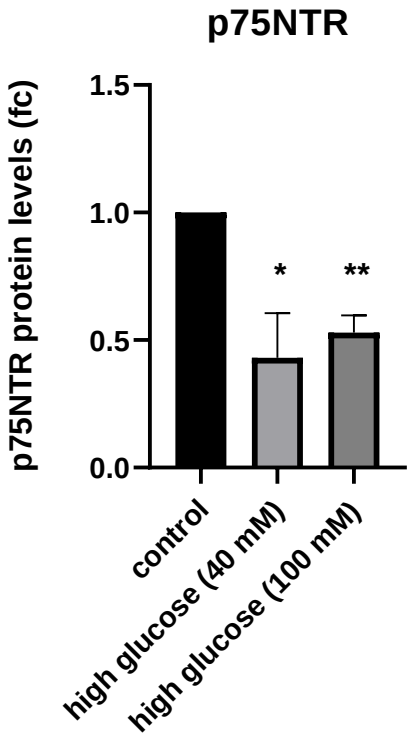
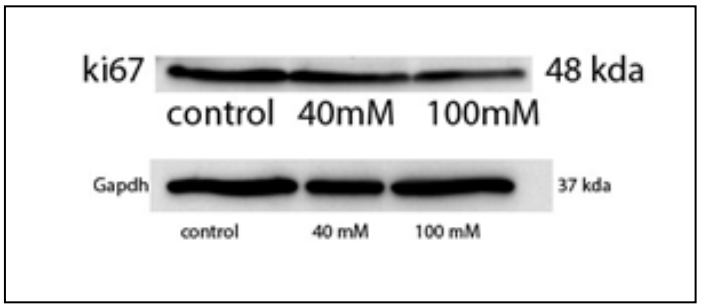


Figure 5. Representative Western blots (A) and quantitative analysis of p75NTR protein levels (B) among control and high glucose conditions (40 mM and 100 mM). The values are mean + SEM (*P < 0.05, **P < 0.01).

A)



B)

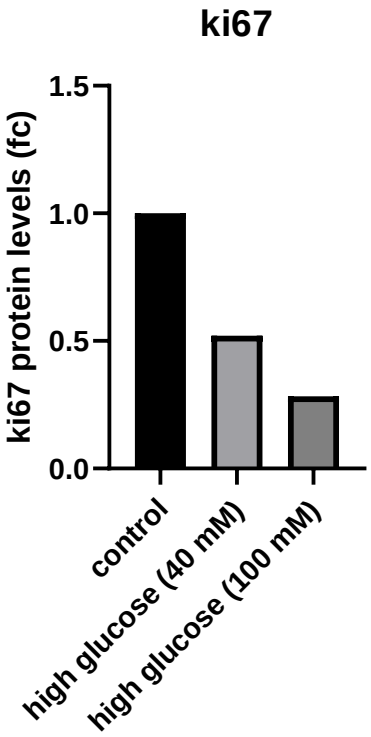


Figure 6. Representative Western blots (A) and quantitative analysis of ki67 protein levels (B) among control and high glucose conditions (40 mM and 100 mM).

2) NSC growth

In order to study how high glucose treatment affects NSC growth, a growth curve analysis was performed using Day 2 and Day 4 after glucose treatment as the time points. It was shown that the number of cells was significantly lower in 40 mM and 100 mM glucose conditions both on Days 2 and 4 (Figure 7).

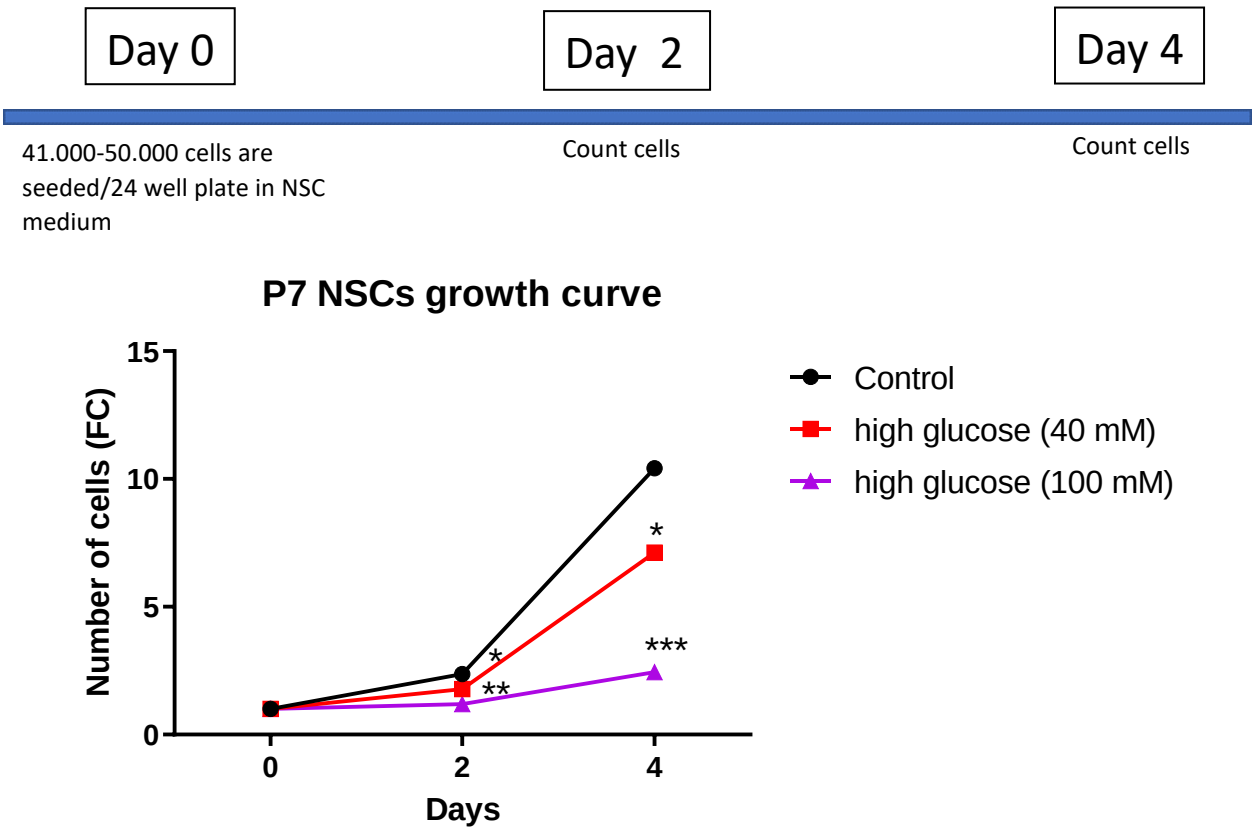


Figure 7. Number of cells per well on Day 2 and Day 4 after glucose treatment. The values are mean +SEM. (*P<0.05, **P<0.01, ***P<0.001)

As a reduction in p75NTR protein expression was observed in high glucose treated cells, NGF, which signals through binding to p75NTR and plays a key role in neuronal development, function, survival, and growth, was added in NSC medium in order to investigate whether the

reduction in the number of the cells grown in 40 mM and 100 mM glucose is associated to p75NTR and could be reversed. P7 NSCs do not express TrkA which is the one of the two NGF receptors other than p75NTR, so it could only act through p75NTR. There is a tendency of increase in NGF-treated cells on Day 2, especially in the high glucose-treated cells, nevertheless the difference is non-significant. On the other hand, NGF did not seem to affect the growth of NSCs on Day 4 after glucose treatment (Figure 8). This could be attributed to the lack of pre-starvation. To more accurately determine the effect of NGF in the growth of NSCs, an additional growth curve with pre-starvation of growth factors (EGF and FGF) for 24 h needs to be done. 100 mM glucose condition was not included in this experiment.

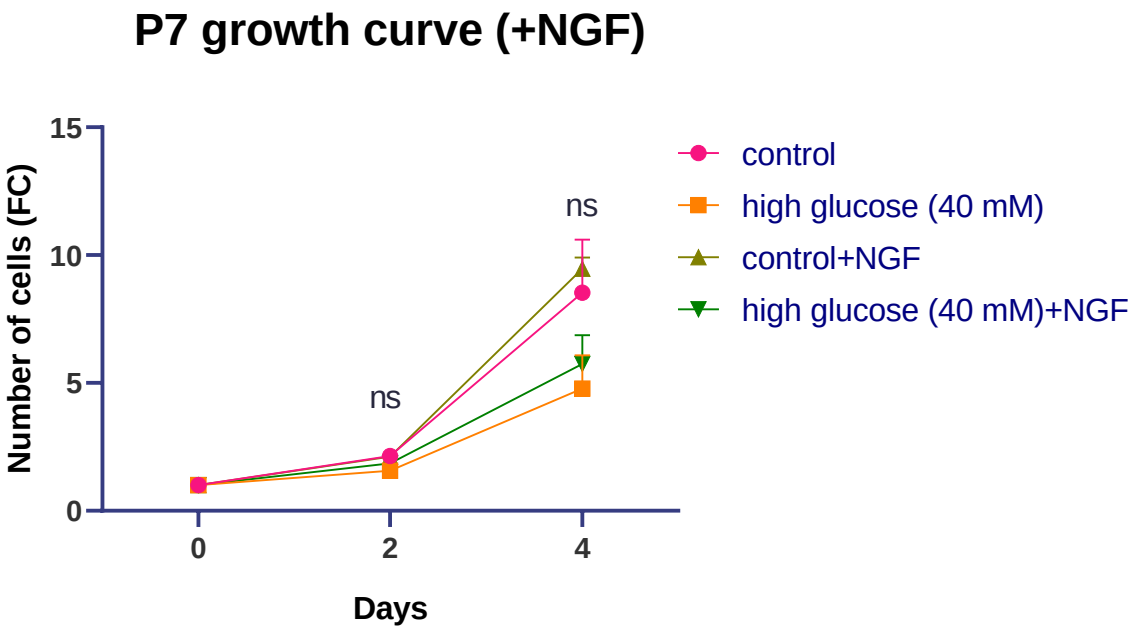


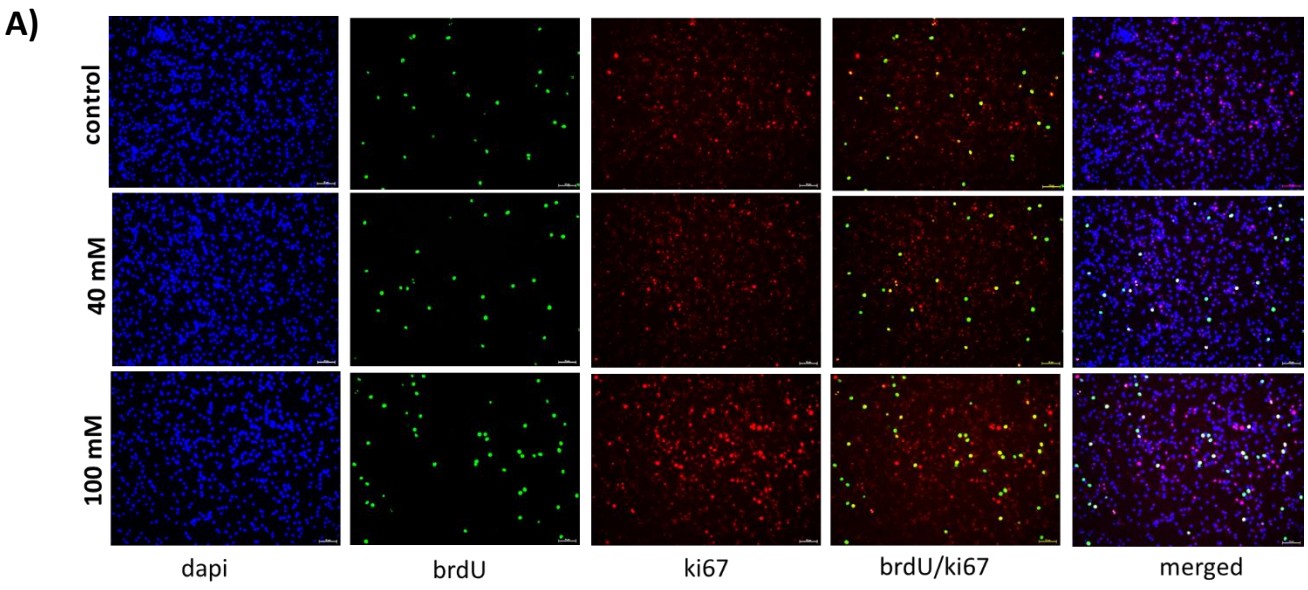
Figure 8. Number of cells per well on Day 2 and Day 4 after glucose and NFG treatment. The values are mean +SEM. (ns: P>0.05)

3) Proliferation

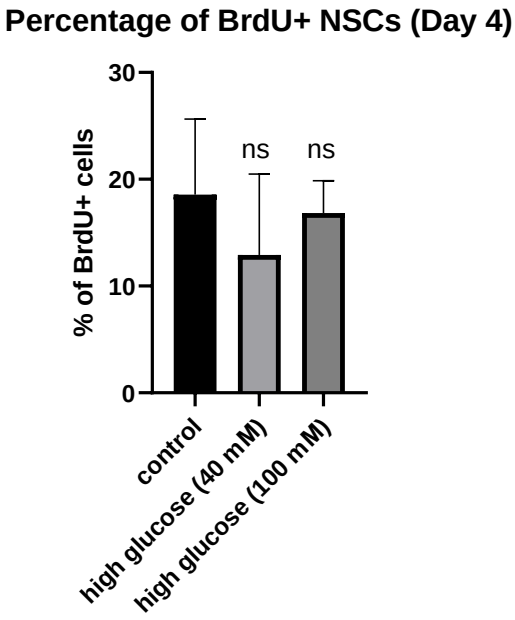
To assess whether the reduction in the number of the cells after glucose treatment was a result of decreased proliferation, BrdU labeling combined with Ki67 (pan-proliferation marker) staining was performed. BrdU and ki67 labeling were performed either 2 or 4 days after glucose treatment.

A. BrdU analysis on Day 4 (BrdU pulse 4 h)

Treatment with D-glucose (40mM/100mM) was performed 24h (Day 0) and 72h (Day 2) after seeding the cells in plates. Four hours prior to the completion of the experiment on Day 4 after glucose treatment, BrdU analog was added to NSC medium and BrdU/ki67 immunostaining was performed. A slight decrease in the proliferation of NSCs grown in high glucose conditions compared to control was observed as expected, but this difference was not statistically significant (Figure 9a, 9b). Interestingly, although the number of the cells in 100 mM glucose was lower than in 40 mM glucose (Figure 7), the percentage of BrdU+ cells was higher in 100 mM compared to 40 mM. Similarly, there were minor but no significant differences in the percentage of ki67+ cells between the 3 groups (Figure 9a, 9c).



B)



C)

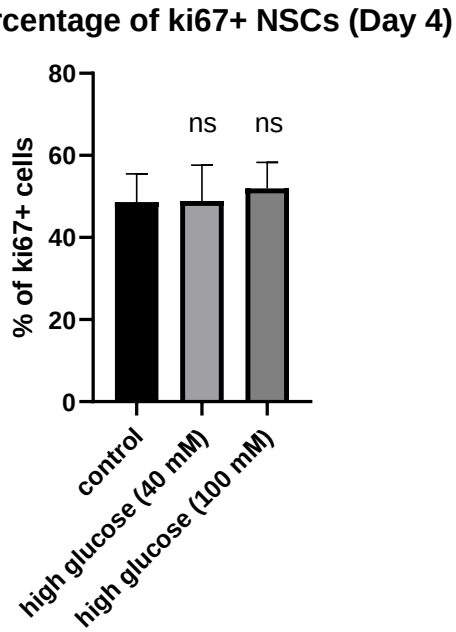
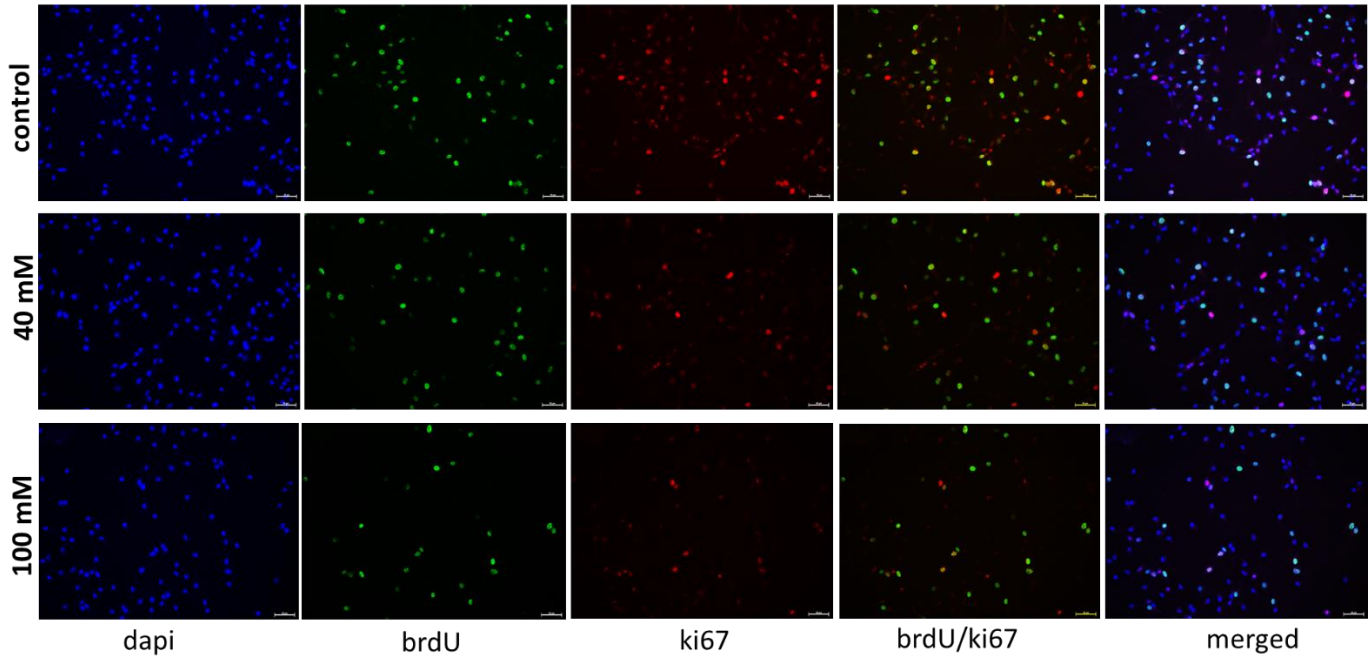


Figure 9. Representative images from BrdU/Ki67 staining (A) and quantitative analysis of the percentage of BrdU+ (B) and ki67+ cells (C) among control and high glucose conditions (40 mM and 100 mM) on Day 4 after glucose treatment. The values are mean + SEM. (ns=non-significant difference, P> 0.05)

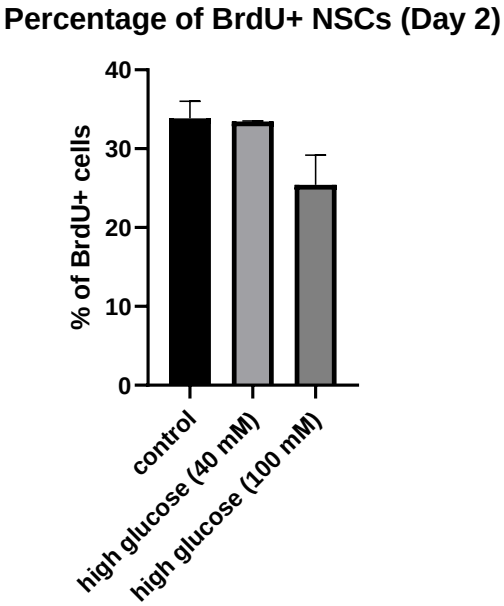
B. BrdU analysis on Day 2 (BrdU pulse 4h)

As no significant differences were observed on Day 4, the investigation was focused on the effect of hyperglycemia on NSCs on Day 2 after glucose treatment. Treatment with D-glucose (40mM/100mM) was performed 24h (Day 0) after seeding the cells in plates. Four hours prior to the completion of the experiment on Day 2 after glucose treatment, BrdU analog was added to NSC medium and BrdU/ki67 immunostaining was performed. However, no differences were observed between control and 40 mM glucose condition, but there was a slight decrease of BrdU+ cells in 100 mM glucose condition (n=2) (Figure 10a, b). Moreover, there were no differences in the percentage of ki67+ cells among the 3 groups (n=2) (Figure 10a, c).

A)



B)



C)

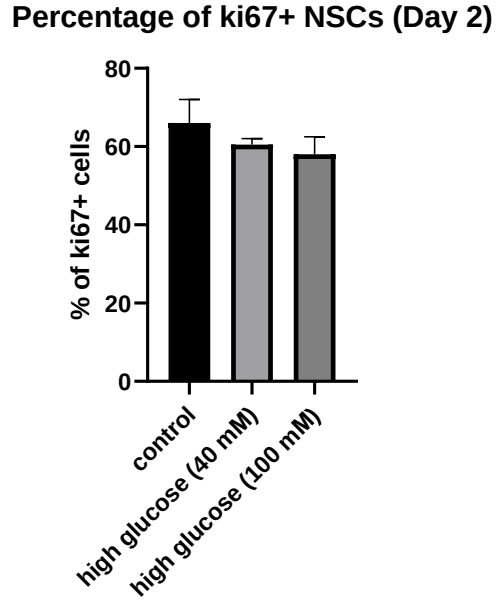


Figure 10. Representative images from BrdU/Ki67 staining (A) and quantitative analysis of the percentage of BrdU+ (B) and ki67+ cells (C) among control and high glucose conditions (40 mM and 100 mM) on Day 2 after glucose treatment.

To sum up, no significant differences in the proliferation rate of control and high glucose-treated NSCs were found on Day 2 and 4 after glucose treatment. Even if there was a small reduction in the number of proliferative cells in 100 mM glucose on Day 2 compared to control, this difference disappeared on Day 4. The opposite pattern was observed for the 40 mM glucose condition, as the decrease in the proliferation of these cells compared to control was bigger on Day 4 than on Day 2 after glucose treatment.

4) Cell death

As no significant differences in the number of proliferative cells were observed, in order to assess whether the reduction in the number of the cells after glucose treatment was a result of an increase in cell death, Cell-Tox assay was performed. This assay measures changes in membrane integrity that occur as a result of cell death. Results showed that cell death was significantly increased in NSCs grown in 40 mM glucose on Day 4 after glucose treatment, while no differences were observed in 100 mM glucose (data from the lab). Conclusively, apoptosis is increased in hyperglycemia conditions, however even in high glucose concentrations, some cells can still survive.

5) Mitochondrial activity

The metabolic activity of NSCs P7 was assessed using MTT assay in control and 100 mM glucose conditions on Day 4 after glucose treatment. The MTT assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. A significant decrease was observed in the high glucose condition compared to control as the cellular metabolism and the rate of cell proliferation were probably reduced due to high glucose related-stress (Figure 11). This data agrees with the reduced number of NSCs grown in 100 mM glucose compared to the control

condition as it was indicated by the growth curve (Figure 7). NSCs grown in 40 mM glucose have to be included in future experiments.

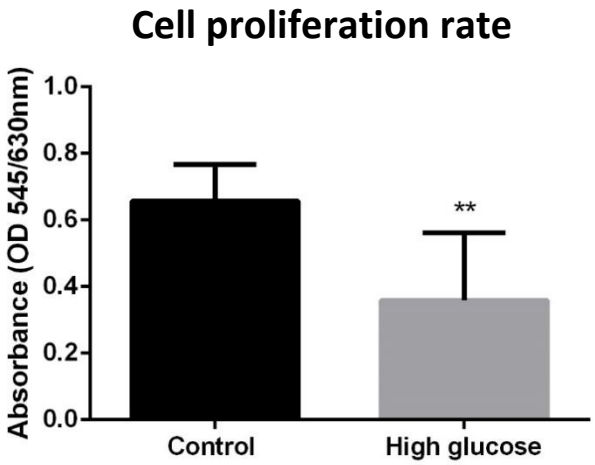


Figure 11. Cell proliferation rate in 17 mM and 100 mM glucose medium. The values are mean + SEM (**P<0.01).

3.2 Effect of hyperglycemia on astrocytes

In order to assess the effect of hyperglycemia on astrocytes, astrocytes grown in 5.5 mM glucose concentration were used as the control condition and astrocytes grown in 30 mM glucose concentration were used as the high glucose condition. The above glucose concentrations were determined based on studies in the literature [56].

1) Expression of reactivity markers and p75NTR downstream mediators

Alterations in the expression of reactivity markers and p75NTR levels and its downstream mediators protein levels in astrocytes were detected by Western Blot analysis. More specifically, protein levels of GFAP, p75NTR, RIP2, total JNK and phospho-JNK were assessed in astrocytes grown in control and high glucose medium.

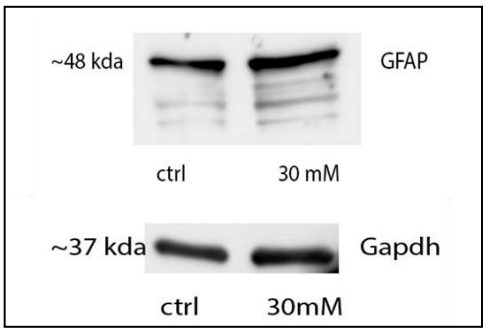
Firstly, astrocytes reactivity was evaluated using the GFAP marker. It has been shown that increased GFAP levels, indicate astroglial activation in terms of inflammation or neurodegeneration. Here, GFAP protein levels were found to be increased under hyperglycemic conditions, as expected, indicating a reactive phenotype of these cells (Figure 12).

The p75NTR can recruit various adaptor proteins to activate multiple pathways including those involved in inflammation, degeneration and cell death[134], [135]. Moreover, Dr. Chanoumidou saw increased expression of pro-inflammatory markers in hyperglycemic astrocytes. Thus, p75NTR protein levels were assessed to examine whether hyperglycemia leads to p75NTR-induced cell death or inflammation. P75NTR levels were significantly higher in high glucose-treated astrocytes, indicating possible downstream activation of p75NTR signaling in response to high glucose exposure that could be related to increased astrocyte reactivity (Figure 13).

RIP2 mediates the activation of p75NTR pathway that leads to neuronal survival. Changes in RIP2 protein expression between the control and high glucose condition were also studied. RIP2 levels were expected to be reduced in the high glucose condition however, there was not a consistent pattern of upregulation or downregulation between the 2 conditions, as it was once slightly downregulated in the high glucose condition (Figure 14) and twice there was no difference between them. So additional replicates are needed to determine whether RIP2 expression is altered under hyperglycemic conditions in astrocytes.

p75NTR mediated activation of JNK signaling pathway is related to induction of apoptosis. Therefore, based on the increased expression of p75NTR we were interested in investigating whether this pathway was activated in response to high glucose. Expression of total JNK and its activated phosphorylated form, phospho-JNK, was analyzed. No significant differences between the control and high glucose conditions were observed, as indicated by the phospho-JNK/total JNK ratio (Figure 15). Thus, apoptosis in astrocytes is not mediated through the interaction of p75NTR with JNK. P75NTR mediated NFk-B pathway that results in increased inflammation has to be examined in the future.

A)



B)

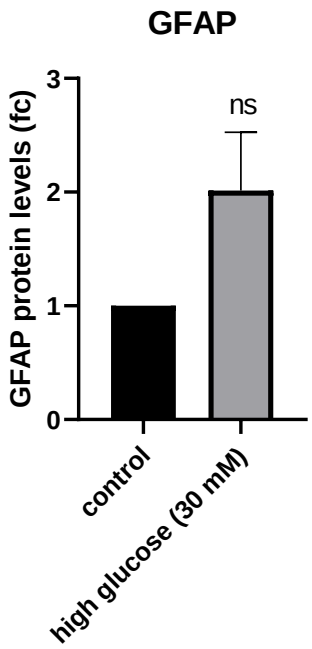
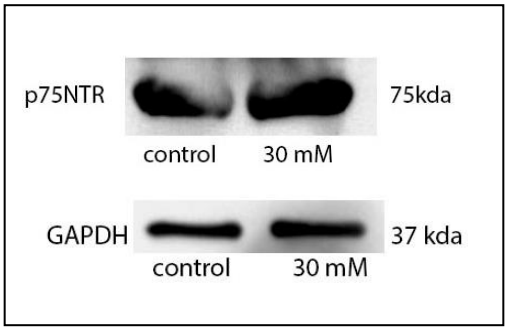


Figure 12. Representative Western blots (A) and quantitative analysis of GFAP protein levels (B) among control and high glucose conditions. (ns=non-significant difference, P>0.05)

A)



B)

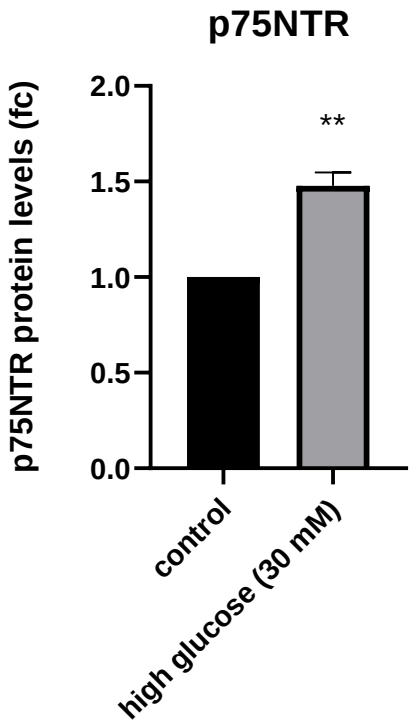
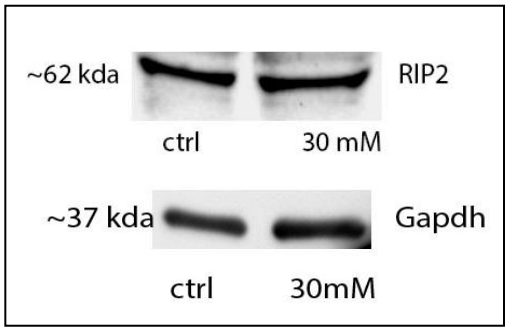


Figure 13. Representative Western blots (A) and quantitative analysis of p75NTR protein levels (B) among control and high glucose condition. The values are mean + SEM (**P < 0.01).

A)



B)

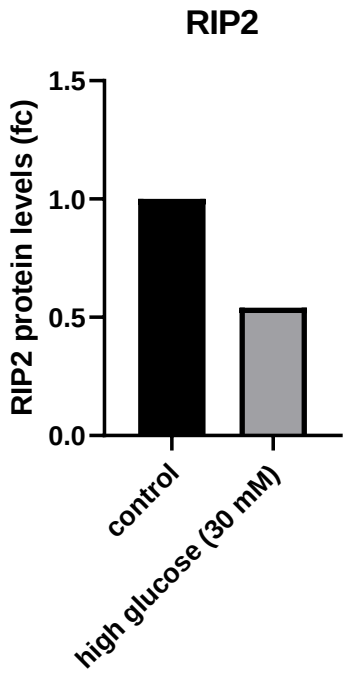
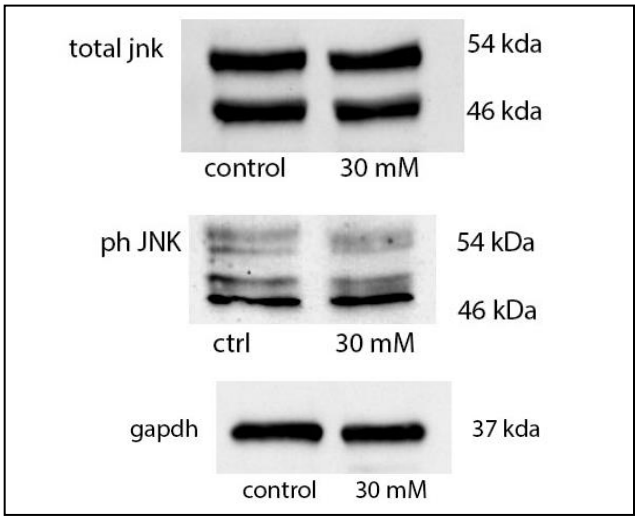


Figure 14. Representative Western blots (A) and quantitative analysis of RIP2 protein levels (B) among control and high glucose condition.

A)



B)

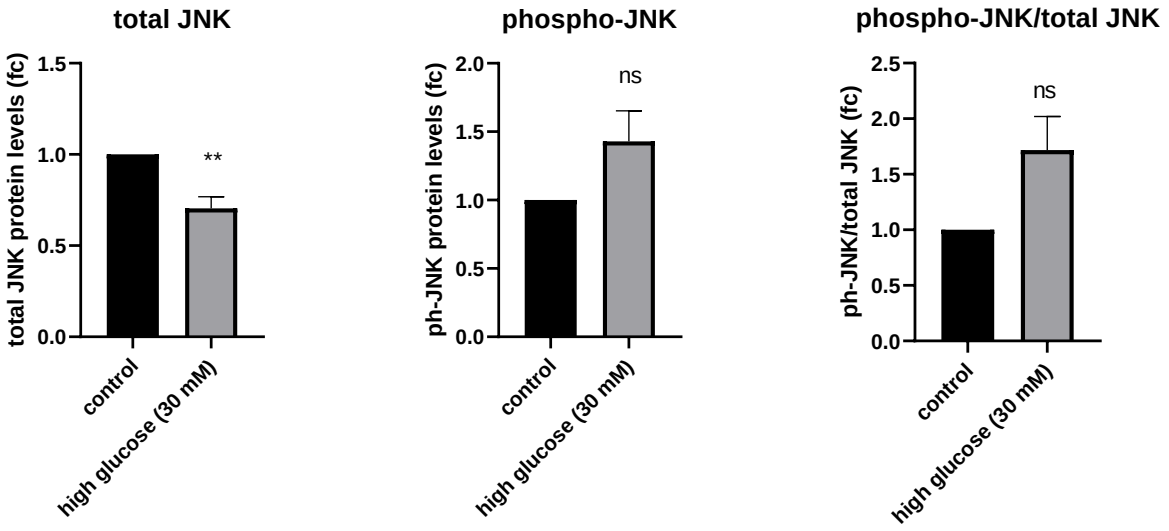
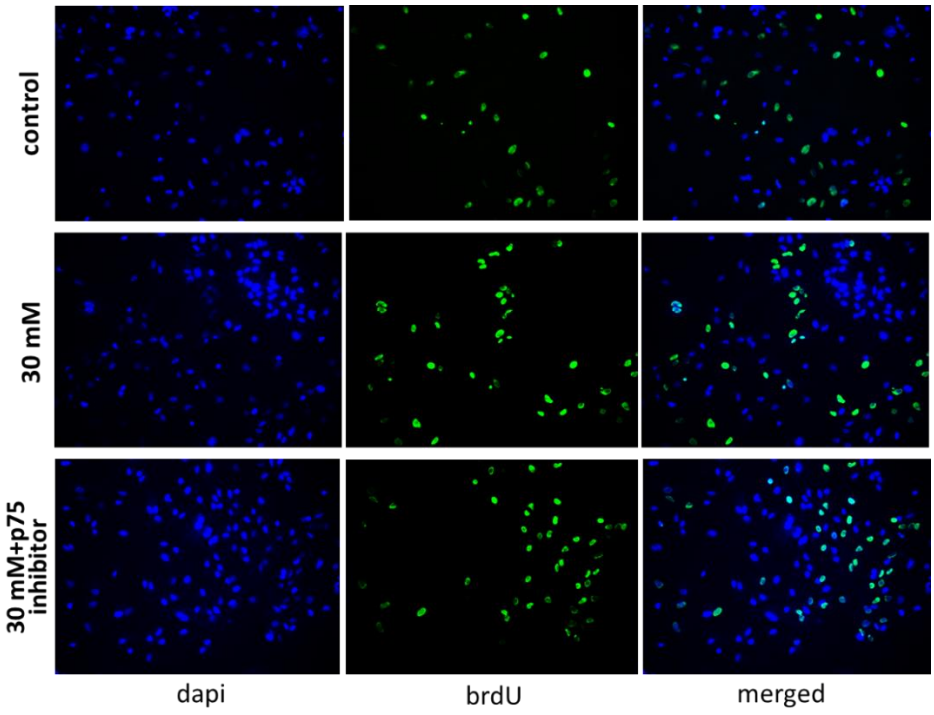


Figure 15. Representative Western blots (A) and quantitative analysis of total JNK, phospho-JNK protein levels (B) and phospho- JNK/total JNK ratio (C) among control and high glucose condition. The values are mean + SEM (**P < 0.01, ns: P>0.05).

2) Proliferation

Based on preliminary results by Dr. Chanoumidou in the lab that showed that high glucose reduces the proliferation of astrocytes *in vitro*, BrdU assay was performed on Day 4 after glucose treatment, to assess the effect of p75NTR inhibitor on astrocytes proliferation. The 3 conditions that were compared were: control (5.5 mM glucose), 30 mM glucose and 30 mM glucose with the addition of p75NTR inhibitor. P75NTR inhibitor was added to examine whether astrocytes response to high glucose was mediated by p75NTR signals. Treatment with D-glucose and p75NTR inhibitor was performed 24h (Day 0) and 72h (Day 2) after seeding the cells in plates. 48 hours prior to the completion of the experiment, BrdU analog was added to astrocyte cultures and BrdU analysis was performed on Day 4 after glucose and p75NTR inhibitor treatment. Nonetheless, this time no differences were found in the number of BrdU+ cells between the 3 conditions (Figure 16). Neither P75NTR inhibitor nor high glucose seemed to affect astrocytes proliferation in this experiment.

A)


control
30 mM
30 mM+p75 inhibitor
dapi
brdU
merged

B)

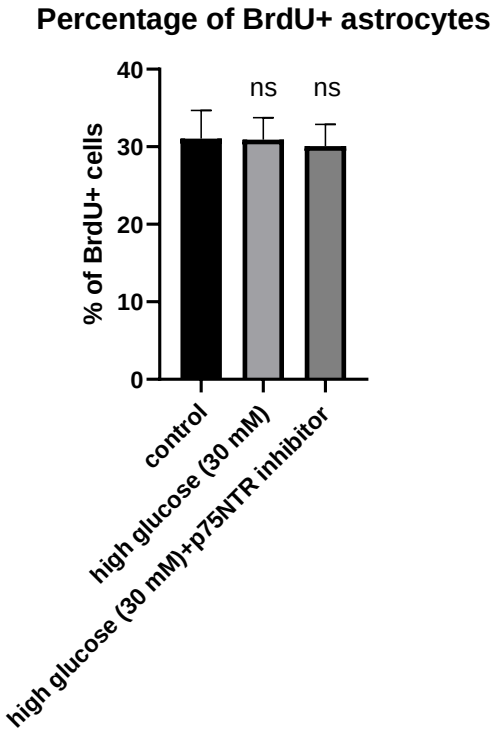

Percentage of BrdU+ astrocytes
% of BrdU+ cells
40
30
20
10
0
ns
ns
control
high glucose (30 mM)
high glucose (30 mM)+p75NTR inhibitor

Figure 16. Representative images from BrdU staining (A) and quantitative analysis of the percentage of BrdU+ (B) among control, high glucose condition and high glucose plus p75NTR inhibitor condition on Day 4 after glucose treatment. The values are mean + SEM. (ns: P>0.05)

3.3 Effect of hyperglycemia on NSCs via astrocytes

The effect of hyperglycemia on NSCs via astrocytes was assessed by adding the concentrated control and high glucose astrocytes supernatants in plates where NSCs P7 were seeded. Treatment was performed 24h (Day 0) and 72h (Day 2) after seeding the cells in plates and the analysis was performed on Day 4 after treatment.

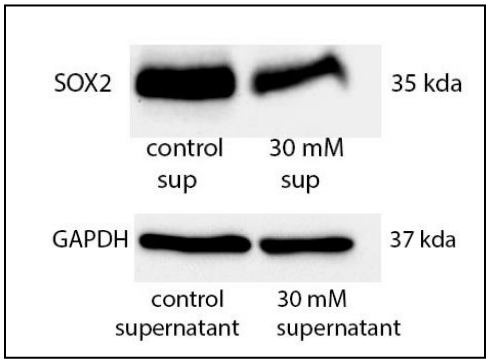
1) Expression of apoptosis and stemness markers

The effect of hyperglycemia on NSCs via astrocytes was evaluated by detecting alterations in the expression of apoptosis- and stemness-related markers by Western Blot analysis. More specifically, protein levels of p75NTR and SOX2 were assessed in NSCs treated with astrocytes control (5.5 mM glucose) or high glucose medium (30 mM).

SOX2 was used to determine the effect of high glucose astrocytes supernatant on NSCs stemness. Interestingly, SOX2 expression was significantly reduced in NSCs treated with high glucose astrocyte supernatant indicating a reduction in the stemness of these cells (Figure 17).

P75NTR protein levels were assessed to examine whether the response of NSCs to astrocytes conditioned medium was mediated by p75NTR signaling. P75NTR expression varied between control and high glucose treated NSCs as it was found once slightly upregulated in high glucose condition and twice slightly downregulated (Figure 18). The downregulation of p75NTR is in accordance with the reduction of SOX2, however this experiment has to be repeated once more to reach a conclusion.

A)



B)

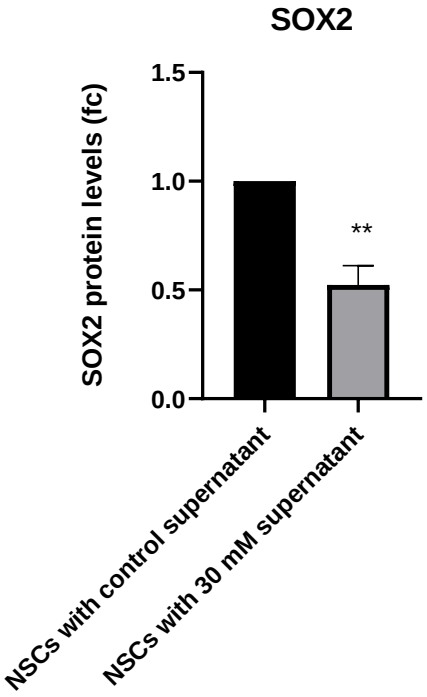
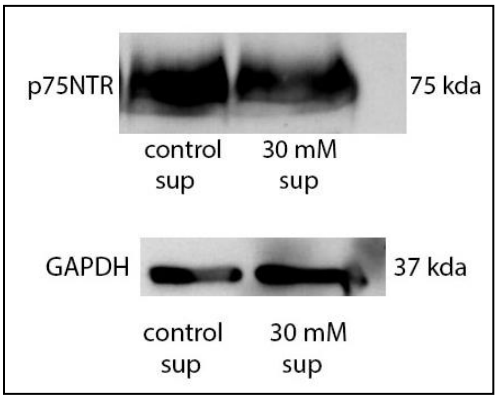


Figure 17. Representative Western blots (A) and quantitative analysis of SOX2 protein levels (B) among NSCs treated with control and high glucose astrocytes supernatant. The values are mean + SEM (**P < 0.01).

A)



B)

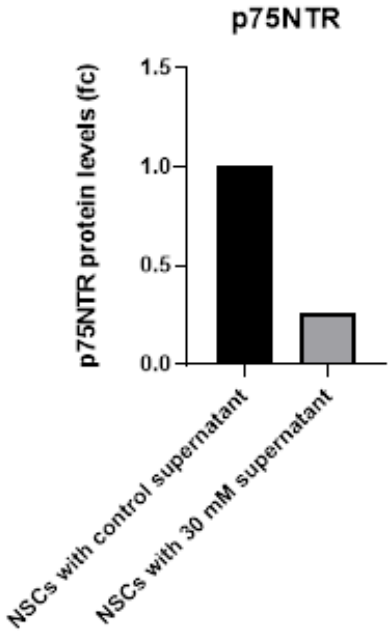


Figure 18. Representative Western blots (A) and quantitative analysis of p75NTR protein levels (B) among NSCs treated with control and high glucose astrocytes supernatant.

4. Discussion

A growing number of *in vivo* studies have revealed a correlation between hyperglycemia and alterations in brain function[11]. These changes refer mainly to decreased neurogenesis including changes in cell proliferation, differentiation and survival[24]. Diabetes has been shown to affect adult neurogenesis by inhibiting neural stem cell proliferation and differentiation and promoting cell death[7]. However, *in vivo* results vary significantly between different animal models, indicating that there are many unknown variables regarding the mechanisms underlying the effect of hyperglycemia on neurogenesis. Moreover, hyperglycemia does not only affect neurons but also the glial cells which are known to support critical neuronal functions. In specific, proliferation of astrocytes has been found to be impaired due to their exposure to high glucose[56]. It is also known that astrocyte-secreted molecules may also modulate specific stages of adult neurogenesis[85], indicating that an impairment in astrocytes function may indirectly affect NSCs properties.

In the present study, it was shown that hyperglycemia leads to reduced growth of NSCs *in vitro*. Therefore, due to the research focus of our lab on neurotrophin receptors and the important role of p75NTR in neural progenitor growth, we examined changes in expression of p75NTR in NSCs under hyperglycemic conditions. P75NTR levels were found to be significantly decreased in high glucose-treated NSCs. This reduction is in accordance with the reduction in SOX2 levels, indicating that high glucose exposure impairs the maintenance of NSCs stemness. The addition of NGF which signals through p75NTR in NSCs P7 did not seem to reverse this effect in 40 mM glucose. However, our approach had some limitations, as an additional growth curve with pre-starvation of growth factors (EGF and FGF) has to be done to determine the effect of NGF in the growth of NSCs and to unravel the role of p75NTR to this process. Although there was a slight reduction in the proliferation rate of NSCs grown in high glucose medium (40 and 100 mM), the differences with the control condition were not statistically significant as it was shown by BrdU labelling and Western blot analysis. Only MTT assay showed a significant decrease in the proliferative rate of NSCs grown in 100 mM glucose compared to control, which reflects the reduced cell number that

was reported in growth curve in this condition. NSCs grown in 40 mM glucose which is a more relevant glucose concentration than 100 mM *in vivo* were not involved in this experiment, therefore they have to be included in future experiments. Hyperglycemia is reported to have a diverse effect on neurogenesis and our data are not consistent with the general observation that high glucose reduces the proliferation of NSCs [7]. Additional replicates are needed to validate the effect of hyperglycemia on NSCs proliferation *in vitro*. Importantly, high glucose led to reduced NSCs stemness, which refers to the molecular processes underlying the fundamental stem cell properties of self-renewal and generation of differentiated daughter cells. This was shown by decreased SOX2 protein levels in both 40 mM and 100 mM glucose conditions.

Astrocytes are activated in the context of trauma, infection, and neurodegenerative diseases[62]. Exposure of astrocytes to high glucose results in increased reactivity of these cells as indicated by increased GFAP protein levels in astrocytes grown in 30 mM glucose, as it has been previously observed[56]. In addition, p75NTR protein levels were significantly higher in astrocytes under hyperglycemic condition compared to control. Studies have shown that increased expression of p75NTR in astrocytes is associated with astroglial activation in response to injury in order to restrict the glial scarring that occurs[133]. According to this observation, hyperglycemia leads to astrocytes activation as indicated by increased GFAP levels and preliminary results by Dr. Chanoumidou in the lab and increased expression of p75NTR may be implicated in this phenotype. Moreover, it is known that p75NTR-induced phosphorylation of cJun / JNK induces cell death while p75NTR interaction with RIP2 mediates neuronal survival[113], [116]. In our study, there was an increase in phospho-JNK levels in high glucose-treated astrocytes, suggesting that p75NTR activation may be mediated through JNK pathway, although the difference was not statistically significant. Therefore, additional replicates are needed to determine whether JNK pathway is activated in response to p75NTR activation under high glucose conditions. On the other hand, RIP2 did not show a consistent pattern of upregulation or downregulation as a result of high glucose exposure, therefore further investigation is required to define its

involvement in the observed phenotype. More important is the future investigation of the involvement of NFk-B pathway in astrocyte activation in a p75 dependent manner.

Astrocytes are in close contact with neurons during inflammatory conditions and they can also control the proliferation and differentiation of NSCs through the secretion of specific molecules[85]. The addition of high glucose-treated astrocyte medium on NSCs showed a significant reduction in NSCs stemness as indicated by the decreased SOX2 levels. This data is in agreement with published data that show that astrocytes secrete molecules that modulate several stages of adult neurogenesis[85]. The involvement of P75NTR in astrocytes-NSCs interaction cannot be determined as its expression was twice downregulated in NSCs treated with high glucose astrocyte medium and one more replicate is needed to confirm it. To further support our hypothesis that astrocytes under hyperglycemic conditions can affect aspects of neurogenesis, the effect of high glucose astrocyte medium on NSCs cell death and proliferation needs to be investigated.

5. Conclusion

Hyperglycemia had a detrimental effect on the growth of neural stem cells (NSCs). This impairment is represented by a reduction in the proliferation rate and stemness of NSCs as well as an increase in cell death. Hyperglycemia led to the activation of astrocytes in the context of inflammation and increased expression of p75NTR indicates that p75NTR may be involved in this response. Hyperglycemic astrocytes can interact with NSCs and reduce their self-renewal and differentiation capacity. P75NTR activation may be the mechanism underlying hyperglycemia-mediated alterations in NSCs as well as it may play a significant role in astrocytes response to high glucose exposure.

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