The Yin and Yang of L-type Calcium Channels: Age-related Roles in Learning and Memory

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ABSTRACT

L-Type calcium channels (LTCCs) are intricately involved in the process of learning and memory. In the young population, the LTCCs are beneficial to the learning process and help facilitate the learning process. As we age, our ability to recall and form new memories deteriorates. We hypothesize that there is a paradoxical shift in the role of LTCCs in learning and memory during ageing. Specifically, there is an increase in the number of or hyperfunction of the LTCCs present in the cell as we age, increasing calcium entering the cell. This causes divergence from calcium homeostasis, thus being a mechanism contributing to a decline in one's cognitive ability. In this work, we have set out to systematically investigate the potential effects of blocking LTCCs using a calcium channel antagonist, nimodipine, at various ages. Using an odour associative learning task and a spontaneous location recall test, we find that LTCC blockade impairs performance of young rats while rescuing performance of aged rats, demonstrating the shift in their role during ageing. Further, nimodipine infusions into the CA1 region of the hippocampus and the piriform cortex block learning as well as prevent c-Fos activation in the piriform cortex, showing the critical role of LTCCs in synaptic plasticity in young rats. These findings further elucidate our current understanding of the roles that LTCCs play when it comes to memory formation, function, and duration.

GENERAL SUMMARY

L-type calcium channels (LTCCs) are critical for calcium entry into the brain cells, leading to a cascade of events, forming memories. However, a paradoxical role of the LTCC associated with memory decline has been observed during ageing. One theory suggests that it results from dysregulation of calcium within the brain nerve cells. In this work, we have systematically tested the roles of LTCCs in adult and aged animals in odour and spatial learning paradigms, using an LTCC blocker nimodipine. We have found that in both learning models, LTCCs in adult animals promoted and facilitated the learning process as its blockade prevented learning. However, in aged animals, LTCCs were detrimental to the same memory formation. Blocking LTCCs rescued the learning deficiency in aged animals. Our data are consistent with current literature that overexpression of LTCCs may underlie calcium dysregulation and altered neural plasticity in aged population.

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CO-AUTHROSHIP STATEMENT

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

- Afterhyperpolarization phase – AHP
- Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors – AMPARs
- Analysis of variance – ANOVAs
- Calcium/calmodulin-dependent protein kinase II – CaMKII
- cAMP Response Element-Binding Protein - CREB
- Cellular compartment analysis of temporal activity by fluorescence in situ hybridization – catFISH
- Cerebellar purkinje cells – P/Q-type
- Conditioned stimulus – CS
- Cyclic adenosine monophosphate-dependent protein kinase – PKA
- Dihydropyridines - DHPs
- Dorsal root ganglion – DRG
- Early phase long term potentiation – E-LTP
- Ethanol – EtOH
- High-voltage activated calcium channels - HVAs
- Immediate early genes – IEGs
- Immunohistochemistry – IHC
- Late phase long term potentiation – L-LTP
- Long term memory - LTM
- Long-lasting type calcium channels – LTCCs
- Long-term depression – LTD
- Long-term potentiation – LTP

- Low-voltage activated calcium channels – LVAs
- Magnesium - Mg^{2+}
- Main olfactory bulb - MOB
- Neither L nor T type calcium channels – N-type
- N-methyl-D-aspartate receptors - NMDARs
- Odour detection and discrimination test - ODADT
- Open-field test - OFT
- Paraformaldehyde – PFA
- Piriform cortex – PC
- Polyvinylpyrrolidone – PVP
- Posterior piriform cortex – pPC
- Postnatal day – P(number)
- Postsynaptic density – PSD
- Resistant type calcium channel – R-type
- Short term memory – STM
- Simple odour discrimination – SOD
- Spontaneous location recognition – SLR
- Transient type calcium channels – T-type
- Unconditioned Stimulus – US

APPENDICIES

- Ethics approval

Introduction

1.0. *Overview*

1.1. Calcium Channels and Their Function

There is a large diversity of the calcium channels that are found throughout the mammalian body, giving rise to a variety of functions, ranging from hormone control to muscle contraction (Terrar, 1993). Calcium channels fall into one of two types, high voltage-activated calcium channels (HVAs) or low voltage-activated calcium channels (LVAs) (Armstrong, and Matteson, 1985). However, despite the differences in their voltage activations, the general structure of the calcium channels is persevered throughout both main classifications.

Calcium channels consist of four subunits: α1, α2δ, β, and γ. The α1 subunit contains the calcium channel pores as well as the binding site for calcium channel blockers (Dolphin, 2006). The α1 subunit consists of six transmembrane segments (S1-S6). The S5 and S6 regions form the pore loop, allowing the calcium channel to open and close through a series of conformational changes (i.e., temporary changes in structure). Glutamate residues within the pore loop to allow for calcium selectivity, a unique property of calcium channels. Furthermore, the positively charged S4 region contains the voltage-sensing region of the calcium channel. The S4 region achieves this through the detection of changes occurring within the membrane potential. This region brings forth another feature unique to calcium channels; when a depolarizing event occurs a change in voltage is detected (due to an event that causes the membrane potential to shift from resting membrane potential), the S4 region moves outward and rotates, causing a conformational change. This conformational change of the calcium channel leads to the opening of the pore, allowing calcium to enter the cell. These six transmembrane segments make up the four domains

(I-IV) of an α1 subunit, which, when joined together with the remaining subunits, leads to the formation of a functional calcium channel (Takahashi, et al. 1987; Zamponi, 2005).

The β subunit is made up of non-transmembrane α helices, while the glycoprotein γ subunit is made of four transmembrane segments (Ruth et al. 1989 and Jay et al. 1990). The α2δ subunit is constructed by two units, α2 and δ, joined together by a disulphide linkage. Several hydrophobic sequences on the outside of the membrane make up the α2 unit, while the δ unit is attached to the membrane by a glycophosphatidylinositol membrane anchor. Interestingly, a single gene encodes for both the α2 and δ subunits (Ellis et al. 1988; Davies et al. 2010). Furthermore, the α2δ subunit can be split apart in regards to its functions within the calcium channel. The α2 subunit has been shown to have a role in enhancing the calcium currents, while the δ subunit has been found to play a role in modifying the voltage-dependent properties of the calcium channels (Felix, Gurnett, De Waard, and Campbell, 1997; Gurnett, De Waard, and Campbell, 1996).

Over the years, it has been discovered through various techniques that the categories of the HVA calcium channels (not LVA), can be further classed into various subtypes based on their structure (Catterall, Perez-Reyes, Snutch, and Striessnig, 2005; Curtis, and Catterall, 1984). A study conducted by Nowycky, Fox, and Tsien, (1985) identified three different types of calcium channel currents within the dorsal root ganglion (DRG) based on recorded calcium currents and different pharmacological properties of each channel tested. Their results led to discovering the T, N, and L-type calcium channels (LTCCs). The L-type channels were found to have a large, long-lasting single channel conductance, and also found to be highly sensitive to antihypertensive drugs (e.g., Nifedipine). Similarly, the T-type channels are also sensitive to such drugs, while the Dihydropyridines (DHPs) are specific for the L-type, they do block T-type

currents at higher concentrations. However, they were activated at a lower voltage and were termed transient (T-type) due to their tiny amount of conductance during the recording. The third type of current that was recorded was activated at high voltage but were insensitive to antihypertensive drugs and were termed N-type (Neither T nor L). Further studies also discovered the existence of P/Q-type, found to be within the Purkinje neurons and the cerebellar granule cells. In addition, a the remaining current was left after blocking the above-mentioned types of calcium channels, it was found to be a current-resistant type of channel, termed R-type calcium channels, these were also discovered in the cerebellar granule cells. This type of calcium channel was found to be resistant to the drugs that inhibited the function of the previously mentioned channels (i.e., L, P, Q, N, T- calcium channels) (Llinás, and Yarom, 1981; Randall, and Tsien, 1995). In light of the information above, it is evident that several different forms of calcium channels can be found throughout the mammalian body. This study will focus on expanding the understanding of the role of LTCCs in age-related changes to learning and memory. Past work has shown that the LTCCs are involved in such processes, which will be discussed in a future chapter.

Overall, 10 different forms of the HVA family have been identified based on the channel type and variation of the α1 subunit, including 4 LTCCs ($Cav_{1.1\text{-}1.4}$), one N-type ($Cav_{2.1}$), one P/Q-type ($Cav_{2.2}$), one R-type ($Cav_{2.3}$), and three T-type ($Cav_{3.1\text{-}3.3}$). Despite their differences in location and function, the general structure of the four domains (α1, α2δ, β, and γ) is preserved (Catterall. 2010; Catterall. 2011). The overall function of any calcium channel is to permit calcium ions across the cell membrane and into the cell. However, given the abundance of calcium channels found throughout the body, the result of activating calcium channels is vast, as calcium is widely used as a principal intracellular messenger, acting in a variety of different

pathways (Terrar, 1993). Of these, LTCCs have been shown to play a significant role in learning and memory, in a subtype specific manner. In addition, LTCC expression and distribution has also been extensively examined. Aged animals have an increased expression of $Cav_{1.2}$ within the somatic regions of the CA1 and CA3 of the hippocampus when compared to young animals (Núñez-Santana et al., 2014), while $Cav_{1.3}$ was only found to increase in the CA3 region of the hippocampus. Moreover, our lab has recently reported that an age-related increase of $Cav_{1.2}$ expression within the piriform cortex along with an increase in LTCC-mediated long-term depression (Rajani, Maziar, Man, Hell, and Yuan, 2021).

1.1.1. Neurophysiological roles of L-Type Calcium channels

Some of the defining features of the various forms of calcium channels are the differences in the α1 subunit amino acid sequence identity. For the LTCCs, there is approximately 75% shared homology (Catterall. 2011). Given the nature of the high voltage dependency and the prolonged calcium influx of LTCCs, it has been shown that they are critical players in the activation of pathways involved in synaptic plasticity (Ghosh et al., 2017; Mermelstein et al., 2000; Schafe. 2008; Tsien, Deisseroth, and Heist, 1998). However, subtypes $Cav_{1.2}$ and $Cav_{1.3}$ have varied involvement in different types of valence learning. The LTCCs have also been found to play a regulatory role in the electrophysiological properties within the basolateral amygdala, upon activating the LTCCs with an LTCC agonist (S)-BayK 8644, resulted in increased excitability proprieties at both postnatal day 7, and 21 (Zhang et al., 2020). These results suggest that at different neurodevelopment stages, LTCCs have distinct contributions to normal neuronal development. This notion of the LTCCs contributing to normal neuronal development is further supported by the findings of Pinggera and colleagues (2015) through mutating the $Cav_{1.3}$ gene in

human cell cultures to express one of two mutations identified in patients with autism. These mutations induce a gain-of-function (i.e., genetically altering organisms resulting in enhancing biological functions). The researchers found that it resulted in a significant shift in the steady-state activation and inactivation to more negative voltages of the $Cav_{1.3}$'s, or significantly slowing the current inactivation during depolarizing events. Meanwhile, the $Cav_{1.2}$ gene CACNA1C, has also been found to be a possible candidate risk gene that when mutated leads to the development of other mental illnesses such schizophrenia and major depressive disorder (He et al., 2014). Further suggesting that LTCCs play critical roles in normal neurological development.

1.1.2. Subtypes of LTCCs

As noted previously, there are four subtypes of LTCCs, $Cav_{1.1, 1.2, 1.3, \text{and } 1.4}$, which are found throughout the body. $Cav_{1.1}$ is found predominately within skeletal muscle, $Cav_{1.2}$ in heart/smooth muscles, neurons and endocrine cells, $Cav_{1.3}$ in the heart, neurons, endocrine and sensory cells, and $Cav_{1.4}$ is found within the retina and immune cells (Striessnig, Pinggera, Kaur, Bock, and Tuluc, 2014). Given their varying locations, their functions are different and mainly depend on the location in which they are expressed (Zamponi, Striessnig, Koschak, and Dolphin, 2015). Of interest to this work are $Cav_{1.2}$ and $Cav_{1.3}$. These channels have been found to be most commonly expressed in the nervous system and play specific roles in spatial and fear learning and memory, respectively. Animals with blocked or deleted $Cav_{1.3}$ channels are unable to learn the fear paradigm; while animals lacking $Cav_{1.2}$ function, are unable to learn spatial learning tasks (McKinney, and Murphy, 2006; White et al. 2008).

N-methyl-D-aspartate receptors (NMDARs; discussed in detail below) play a critical role in learning and memory formation via inducing calcium influx and triggering long-term potentiation (LTP). However, Moosmang and colleagues (2005) further investigated the role of the LTCC Cav1.2 in NMDAR-independent LTP within the hippocampus in the formation of spatial memories. To investigate this, the authors produced a knockout mouse line that did not express $Cav_{1.2}$ mainly within the hippocampus and neocortex ($Cav_{1.2}^{HCKO}$) by inactivating the CACNA1C gene (the gene encoding α1 of $Cav_{1.2}$) with the use of a hippocampus specific Cre recombinase. In the presence of the NMDAR antagonist (D-APV) or the potassium channel blocker TEA; (to examine NMDAR-independent synaptic plasticity), the mutant mouse line, had a significantly decreased LTP compared to controls suggesting that $Cav_{1.2}$ mediates LTP induction in an NMDAR-independent manner. Another striking finding is that the $Cav_{1.2}^{HCKO}$ mice behave normally (compared to control) when it comes to motor function, exploratory behaviour, anxiety and visual acuity. However, despite these normal behavioural functions, when introduced to tasks that depend on hippocampus spatial learning, the $Cav_{1.2}^{HCKO}$ performed significantly worse in both tests (Morris water maze and a spatial learning labyrinth). $Cav_{1.3}$ channel knock out mice, on the other hand, exhibited no impairments to learning, or hippocampal LTP, indicating that the $Cav_{1.3}$ channel is not involved in spatial memory formation (Clark et al. 2003). Taking this into consideration, it is evident that the $Cav_{1.2}$ but not $Cav_{1.3}$ channel is involved in the plasticity of memory.

As noted above, $Cav_{1.2}$ is involved in spatial memory formation. $Cav_{1.3}$, on the other hand, is involved in the consolidation of fear memories, such as conditioned fear learning via foot shock. In 2006, McKinney and Murphy set out to elucidate the role of LTCC $Cav_{1.3}$ channels by establishing a $Cav_{1.3}$ knockout mouse, which behaves normally compared to wild

type in regards to general behaviour (Clark et al. 2003). The researchers found that the mutant animals had an impaired ability to consolidate a fear memory in the exposure to a noxious stimulus, when compared to control animals. These findings suggest that the $Cav_{1.3}$ subunit is required for the process of fear learning. Additionally, unlike what is seen in $Cav_{1.2}$ mutant animals, $Cav_{1.3}$ knockout mice showed no impairment in spatial learning tasks (McKinney, and Murphy, 2006). Fittingly, $Cav_{1.3}$ is also found to play an important role in the amygdala, a region involved in fear-related memories, as animals that lack the $Cav_{1.3}$ subunit showed significant impairment in their ability to consolidate a contextual fear memory. In addition, it was illustrated that $Cav_{1.3}$-deficient animals have a reduced electrophysiological response to LTP-provoking stimuli within the amygdala, despite basal synaptic transmission being unaffected (McKinney, Sze, Lee, and Murphy, 2009). These results may explain why the knockout animals were unable to consolidate the contextual fear memories as it depends on an unimpaired plasticity within the amygdala.

1.1.3. LTCC Pharmacology

Over several decades ago, Albrecht Fleckenstein, a German physiologist, found that organic substances such as verapamil mimicked the effects that were seen in the cardiovascular system when treated with β-adrenergic receptor antagonists (cardio depressants) (Striessnig, Ortner, and Pinggera, 2015). However, despite this action, the effects seen were reversed by administering adrenergic agonists, and they also did not alter sodium-dependent action potentials. Therefore, he suggested that they did not bind to the same site of ß-adrenergic receptor antagonists. Additionally, he observed that the effects of this substance could not be induced by the removal of calcium ions from the medium. He concluded that such drugs

specifically targeted the inhibition of cellular entry of calcium into the cell, and as a result of this, he termed such drugs as calcium antagonists.

The LTCC family of channels, despite their location of expression, are all affected by calcium channel blockers such as: Dihydropyridines (Nimodipine, Nifedipine), phenylalkylamines (Verapamil), and benzothiazepines ((+)-cis-diltiazem) (Catterall et al. 2015; Striessnig, Pinggera, Kaur, Bock, and Tuluc, 2014). Despite the drugs being different in their chemical make-up, the drugs all bind near the α1 pore forming subunit of the LTCCs, however, they bind at their respective binding sites of the α1 subunit (Tikhonov, and Zhorov, 2009; Striessnig, Hoda, Wappl, and Koschak, 2005). In addition to the many drugs available to inhibit LTCC function, there are others that act as agonists (promote their function), such as (-)-BayK8644 (BayK) and (+)-SDZ202-791 (Hamilton, Yatani, Brush, Schwartz, and Brown, 1987), that increase calcium influx.

1.2. The Basis of Learning and Memory

Memory formation is a critical component that finalizes the learning process into stored and accessible information. Without memory formation, individuals would not be able to obtain vital information that is essential for survival and interacting with our environment. In order for memory formation to occur and be stored as long-term memories, it is protein synthesis that conducts this crucial process, which transforms learned information or experienced stimuli into long-lasting consolidated memories on a cellular level. Therefore, understanding the mechanisms that underlie memory consolidation is essential as it allows us to investigate the components individually and clarify their roles and functions. The widely accepted mechanism that is thought

to underlie this process is LTP, which will be discussed in the following sections. However, other possible mechanisms may also play a part or underlie cognition. For example, synaptic scaling is a well-studied form of non-Hebbian synaptic plasticity, which keeps the firing rate of cells within a range by synaptic modification that leads to an increase of decrease in alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Turrigiano et al., 1998).

1.2.1. NMDARs and AMPARs

As one of the most abundant neurotransmitters within the nervous system, glutamate acts as the principal excitatory neurotransmitter within the brain, by binding to ionotropic glutamate receptors and initiating excitatory responses via the entry of positive ions such as sodium and calcium into the cell or resulting in a cellular cascade triggered by the activation of metabotropic glutamate receptors.

Two of the most widely studied glutamatergic receptors are NMDARs and AMPARs (Isaac, Ashby, and McBain, 2007; Wright, and Vissel, 2012). NMDAR structure is encoded by a total of seven genes, one GRIN1, four GRIN2, and two GRIN3, which encode the subunits GluN1, GluN2A-D, and GluN3A-C respectively (Traynelis et al., 2010). NMDARs have a heterotetrametric structure in which two GluN1 subunits (glycine-binding) must join together with either two GluN2 subunits (glutamate-binding), a mix of GluN2 and GluN3 subunits (glycine-binding), or a combination of GluN1 and GluN3 without GluN2, forming the central ion channel pore.

Similarly, the AMPAR structure is also formed as a tetramer comprising of the receptor subunits GluA1-GluA4. The overall structure of the AMPAR is divided into domain layers, an N-terminal, ligand binding domain and a transmembrane domain, consisting of four folds. The transmembrane domain is where a single change in the amino acid for the GluA2 can be modified (glutamine to arginine) to enhance calcium permeability. In addition, each of the subunits differ from one another, thus allowing for different channel kinetics, ion selectivity as well as receptor trafficking (Seeburg and Hartner, 2003; Herguedas et al., 2016). AMPARs play a role in synaptic plasticity as they mediate the initial depolarization of the cell, and from their quick activation they will then lead to the activation of NMDARs which are also paramount to synaptic plasticity and learning.

Both AMPARs and NMDARs require glutamate-binding for their activation to occur. However, NMDARs also require the co-agonist glycine to bind and are voltage-dependent due to the magnesium (Mg^{2+}) block (Rao, and Finkbeiner, 2007). Depolarization causes removal of the Mg^{2+} block, and an increased calcium influx through both NMDARs and AMPARs, initiating downstream calcium-dependent pathways and resulting in an upregulation of both AMPAR and NMDAR signaling. The resulting changes in receptor trafficking and signaling lead to changes in synaptic strength, classified LTP. In order to ensure these changes are long-lasting, gene transcription is required to occur for this to happen (Derkach, Oh, Guire, and Soderling, 2007). These changes within the synapse are typically achieved through the process of NMDAR activation via second messenger cascades as a result of different firing patterns of presynaptic signals which will designate either LTP or long-term depression (LTD) to occur thus either facilitating or hindering the process of learning. It is important to note that LTP and LTD are distinctive processes from one another. Generally, LTD is induced by a repetitive low frequency

stimulation, while LTP is induced via high frequency stimulation (Dudek and Bear, 1992; Malenka, and Bear 2004).

1.2.2. Long-Term Potentiation

Long-term potentiation is the strengthening of an existing connection, or the formation of new synapses, from experiences that cause activity-dependent long-lasting changes (Citri, and Malenka, 2007). Bliss and Lømo (1973) were the first to explore the idea put forward by Donald Hebb (1949) that memories are formed by synaptic modification. They found that in the hippocampus, repeated activation of synapses by high frequency stimulation caused a potentiation lasting hours after stimulation. As a result of these findings, LTP has been thoroughly studied over the years, and findings have supported the idea that LTP is one of the key features to the formation of memories (Martin, Grimwood, and Morris, 2000; Whitlock, 2006).

1.2.2.1. The Mechanism Behind the Process of LTP

Two of the most critical units for LTP induction and expression are the glutamate receptors NMDARs and AMPARs, respectively (Citri, and Malenka, 2007). Of the two receptors, the AMPARs are permeable to sodium and potassium ions upon glutamate binding and are responsible for the majority of the excitatory inward current. Consequently, upon sufficient depolarization via activation of the AMPARs, the Mg^{2+} block is displaced from within NMDARs, thus allowing sodium (Na^{+}), potassium (K^{+}) and, more importantly, calcium ions into the postsynaptic cell (Nowak, Bregestovski, Ascher, Herbet, and Prochiantz, 1984). Calcium

entry via NMDAR activation is critical for triggering the downstream cascades necessary for LTP in CA1 neurons of the hippocampus, a well-studied region involved in memory formation (Malenka, and Nicoll, 1993). Generally, LTP can be induced by applying high-frequency tetanic stimulation to the synapses or by using what is known as a 'pairing protocol' where the direct postsynaptic cell depolarization is paired with a low-frequency synaptic activation throughout. Due to the Mg^{2+} block, the NMDAR is a coincident detector, and requires depolarization before allowing increased ion flux. (Citri and Malenka, 2007). At basal synaptic activity, glutamate is released, which binds to both AMPARs and NMDARs, however, the glutamate binding alone is not sufficient enough to displace the Mg^{2+} block, thus the NMDARs provide little to the postsynaptic response during basal synaptic activity. In order for the NMDARs to be fully opened and allow for an increased influx of ions, they must undergo; co-agonist binding, glutamate binding and sufficient depolarization (i.e., remove the Mg^{2+} block) simultaneously. Upon the expulsion of the Mg^{2+} block, and glutamate binding result in calcium influx, acts as a trigger for synaptic plasticity (Tabone and Ramaswami, 2012). This unique property of NMDARs also helps explain the basic properties of LTP (i.e., cooperativity, associativity and input sensitivity) (Nicoll, Kauer, and Malenka, 1988). Cooperativity in relation to LTP illustrates that it is possible to induce LTP through the activation of coincident activation (i.e., occurring together) of a critical number of synapses. Associativity is the ability to take a weak input (i.e., an activation of a small number of synapses) and strengthen its signal input when it is activated along with a strong input (i.e., many synapses activated). Finally, input selectivity of LTP is the property that states that LTP only occurs within the activated synapses and not those that surround them, or those that are inactive on the same postsynaptic cell.

As previously mentioned, the calcium signal is a crucial component in the induction and maintenance of LTP via the activation of signal transduction molecules (Malenka, and Bear, 2004; Sanes, and Lichtman, 1999). In order to investigate whether or not a protein/molecule is important in LTP, pharmacology is often used to explore if blocking the activation of the molecule of interest or intentionally activating it during LTP has any effect. (Citri, and Malenka, 2007). If, using pharmacological compounds prevents the molecule from being activated and LTP induction is prevented or hindered, then it can be concluded that this molecule is essential. Conversely, if activating the molecule leads to a potentiated response and prevents further LTP, the same can be concluded. One key molecule that has been identified is calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) (Barria, Muller, Derkach, Griffith, and Soderling, 1997; Fukunaga, Muller, and Miyamoto, 1995). CaMKII undergoes autophosphorylation after LTP is triggered, and Silva and colleagues (1992) found that mutant animals, which lack the α-CaMKII (a synaptic protein), prevented the induction of LTP. Additionally, over-expression of CaMKII present in the postsynaptic side leads to an increase in synaptic strength, and the prevention of further LTP (Pettit, Perlman, and Malinow, 1994). Other kinases have been suggested to play a role in LTP induction such as cyclic adenosine monophosphate-dependent protein kinase (PKA) and src-family tyrosine kinases also underlie different forms of LTP (Blitzer et al. 1998; Rajani, Sengar, and Salter, 2021).

After LTP has been induced, it must be able to express the changes in synaptic transmission as a result. The main feature is an increase in the number of AMPARs on the postsynaptic membrane (Bredt, and Nicoll, 2003; Song and Huganir, 2002), and this is mediated by activity-dependent changes in the AMPAR trafficking. A critical mechanism that is important for this to occur is the phosphorylation of AMPARs at different sites. Support for this stems from Derkach

and colleagues (1999), in which CaMKII phosphorylation of Ser831 leads to an increase in single-channel conductance of homomeric GluR1 receptors. During this phosphorylation, the AMPARs are also undergoing translocation to the postsynaptic density (PSD) via exocytosis as well as other mechanisms. These newly incorporated AMPARs, a result of protein synthesis, are believed to be important in the maintenance of LTP due to the structural changes that take place in synapse and dendritic spines (Sutton and Schuman, 2006; Zhou et al., 2006).

As discussed above, it is evident that for LTP in the CA1 area of the hippocampus, NMDARs, as well as AMPARs, play critical and different roles. NMDARs are critical for the induction of LTP, while AMPARs are key to the expression and maintenance of LTP. The process involves first NMDARs, leading to an influx of calcium ions into the cell, which allows for the necessary signal transduction proteins to be activated. Of particular importance is CaMKII, which leads to the phosphorylation of AMPARs, which causes the insertion of new AMPARs or the emergence of previously internalized AMPARs into the PSD as a result of protein synthesis that is activated. These changes result in the structural modifications of the synapse and at dendritic spines (Luscher, and Malenka, 2012).

In addition to NMDAR-dependent LTP, it has been found from past research that there are two distinct forms of LTP. The first being the NMDAR-dependent form and the other an L-type voltage dependent calcium channel-dependent form, which can be induced by multiple tetanus or high frequency stimulation (e.g., 200Hz) (Cavus, and Teyler, 1996). Furthermore, it has also been established that there are two stages of LTP, early and late phase (E- and L-LTP). L-LTP is characterized by its dependence on protein synthesis, which leads to lasting changes in synaptic plasticity. (Vickers, Dickson, and Wyllie, 2005). Interestingly, L-LTP, induced by high-frequency stimulation, has been found to steadily reduced in ageing animals as a result of the

change in intracellular calcium levels through LTCCs (discussed in section 1.3), and is suggested as an indicator of age-related memory loss (Huang, and Kandel, 2006). Specifically, it is suggested that the $Cav_{1.2}$ L-type calcium channel may be critical player in L-LTP, as suggested by Moosmang and colleagues (2005).

1.2.3. Different Types of Memory: Long-Term and Short-Term

When it comes to the general sense of what we perceive as memory, we usually think of it in two broad categories. Long-term and short-term memories are memories that last a varying length of time (LTM and STM, respectively). Long-term memories are on the order of hours to days, while short-term is on the order of seconds to minutes. These two broad categories can be further split into subdivisions. Long-term memory, for example, can be split into declarative memories (such as recalling facts and events) and non-declarative memories (procedural tasks, e.g., tying one's shoes). Short term memory, on the other hand is sometimes referred to as working memory as it is only able to hold a few items at a time, generally in the range of 7 ± 2 (Murman 2015) However, despite the limited capacity of short-term memory, it is crucial to our ability to learn. Without it, we would not be able to form the new information we obtain into long-term memories. These types of memories are what we depend on in order to interact and adapt to our ever-changing environment.

However, given the two types of memory, being able to define how they differ is paramount in studying memory formation in animals. Thankfully we are able to achieve this through testing the animal's "memory" during various time points after a learning task. The defining characteristic between short-term and long-term memory in animals is that long-term memories

cannot be formed without de novo (extrasomal) protein synthesis. This was illustrated by Ghirardi, Montarolo, and Kandel in 1995, in which the authors showed in aplysia (sea slugs) that long-term, not short-term memory is dependent on protein synthesis. This process has since been demonstrated in rat pups (Grimes et al., 2011). To explore this hypothesis animals were injected with either anisomycin or actinomycin (a translation inhibitor or a transcription inhibitor, respectively) in an odour preference paradigm. In an odour preference model, the animal first learn to associate an odour with a positive stimulus. In this experiment Grimes and colleagues (2011) established this by placing rat pups on warmed peppermint scented bedding for 10 minutes. During this period, they administered either drug after the exposure to a peppermint scented bedding. To assess the pup's preference to the odour, they measured the percentage of the time the pup spent with the peppermint bedding. It was shown that either drug administered during exposure prevented the rat pup from forming any LTM (24) hours after, as indicated by a lack of preference towards the bedding. However, the animal's short-term association with the odour was intact (STM) 1-3 hours after the odour exposure as indicated by the amount of time they spent over the peppermint scented bedding.

1.2.4. Discrimination Learning and Pattern Separation

One of the vital processes that we depend on in our daily lives is the ability for us to discriminate between similar experiences. This ability relies on recall of specific memories, specifically episodic memories. Episodic memories, are memories that we can recall on in relation to personal experiences and recall events when, where and what happened (Gillund, 2012). Typically, this type of learning is referred to as discrimination or pattern separation and is defined as: "A mode of processing in neural networks in which complex inputs are represented

by a limited number of active units. This protects potentially overlapping inputs from interference effects. It is facilitated by sparse processing." ("Pattern Separation," 2012). Interestingly, it has been established that in both animals and humans, that there is an age-induced decline in one's ability to detect small changes in object's positions or features between trials. (Reagh et al., 2013; Holden, Toner, Pirogovsky, Kirwan, and Gilbert, 2013). Aged animals (mice; 18-months old) performed significantly worse in their ability to discriminate between two similar objects (object pattern separation) when compared to young (4-months old) (Cès et al., 2018), but not in their ability to discriminate between two highly dissimilar objects. Moreover, mice lacking the specific CACNA1C gene (encodes for $Cav_{1.2}$) are unable to successfully learn tasks that require the discrimination of environmental cues or in which the cues are limited (Temme, Bell, Fisher, and Murphy, 2016). These findings support the role of LTCCs in the age-induced decline in similar object discrimination.

1.2.4.1. Spatial Learning

One subtype of discrimination learning that is a major focus of this paper is spatial learning, involving animal's ability to distinguish the same object between familiar and novel locations. Rodents have a tendency to explore the same object displaced within a familiar context (Ennaceur, Neave, and Aggleton, 1997). Moreover, young animals preferred a displaced object (by 20cm) compared to aged animals, indicating a decline in the animal's spatial learning performance with age (Cès et al. 2018). At various age points (3, 7, 11, 16 and 21 months of age), there was a steady decline in the animal's discernment of a displaced object as a novel object. This study illustrated that there are clear impairments in the aged animals in spatial learning when compared to the young animals, and this was able to be rescued by D-serine an

endogenous co-agonist of NMDAR, suggesting that in aged animals, NMDARs can be activated to restore the animal's ability to learn the task.

1.2.4.2. Odour Discrimination Learning

Being able to distinguish between two odours is another form of discrimination learning that we will investigate. In early odour preference learning rat pups learn and utilize odour cues to locate their mother (Leon and Moltz, 1971), in which the rat pups show a preference towards the lactating mother, compared to the non-lactating mother. This behaviour is an example of how odour discrimination is used in early development to satisfy an innate need. Investigators are able to explore this behaviour in adult rodents by classical conditioning, by pairing a novel odour with a number of stimuli that are either positively associated with a reward or negatively associated with a punishment (Sullivan and Leon, 1987; Sullivan, and Wilson, 2003). Apart from appetitive forms of odour preference learning, another type, aversive odour conditioning has also been studied in adult rodents. Aversive odour conditioning is when an animal is exposed to an odour paired with an aversive stimulus (i.e., foot shock), a paradigm involving the amygdala, an area of the brain important for emotions and fear related memories. (Rasia-Filho, Londero, and Achaval, 2000). As mentioned previously, the pairing of a neutral odour (Conditioned Stimulus; CS) with a foot shock (Unconditioned Stimulus; US) is a form of an aversive learning, which is sufficient in producing a significant amount of freezing to the odour when presented alone following the fear conditioning, compared to controls (Shionoya et al., 2013; Carew et al., 2018).

Past research suggests that the piriform cortex is critical for odour encoding and discrimination (Shakhawat, Harley, and Yuan, 2014). The authors used cellular compartment

analysis of temporal activity by fluorescence in situ hybridization (catFISH) to measure Arc, an immediate early gene, to index the neuronal ensembles activated in an olfactory discrimination paradigm in the piriform cortex. In this behavioural model, animals learned to discriminate between odours, one associated with a reward, and the other with no reward, therefore the animal is presented with two stimuli to encode (i.e., activating two neuron ensembles). This study showed that with completion of the odour discrimination training, encoding for an individual odour stimulus is strengthened, while the overlap between the two neuronal ensembles that encoded for the odours decreases. This suggests that odour discrimination training leads to better pattern separation. Furthermore, the same group demonstrated that the adrenoreceptor's activity within the anterior piriform cortex, but not the olfactory bulbs, was essential for the similar odour discrimination learning to occur (Shakhawat, Harley, and Yuan, 2014; Shakhawat et al., 2015). Interestingly, there is an innate preference or aversion to certain odourants (Devore, Lee, and Linster, 2013). The roles of the L-type calcium channels in regards to olfactory-based learning will be discussed in chapter 1.3.

1.3. The Roles of LTCCs in Learning and Memory Formation

Given the evidence that show the importance of the LTCCs in spatial and aversive learning paradigms, as well as the role that they play in LTP, it is clear to see that they may be a significant player in our everyday learning and memory formations. One of their main roles, aside from allowing calcium entry into the cell, is to facilitate coupling to other cellular functions. The main types of coupling include excitation-contraction (a series of events in the muscle cells leading to a contraction), excitation-secretion (the transduction of an electrical stimulus into the synaptic vesicle fusion), and excitation-transcription (via the influx of calcium

leads to diverse cellular responses such as gene expression) (Berger, and Bartsch, 2014; Hofmann, Flockerzi, Kahl, and Wegener, 2014; Jerome, Hou, and Yuan, 2012). Excitation-transcription is critical in neuronal function and memory formation of various types (e.g., fear memory, olfactory memory). As a result, LTCCs are involved in a variety of learning types. LTCCs have been found to be involved in hippocampus-dependent spatial memory (Ingram et al., 1994; Quevedo et al., 1998) as well as amygdala-dependent fear memory (Bauer, Schafe, and LeDoux, 2002; Cain, Blouin, and Barad, 2002). The roles that they play in different forms of learning and LTP suggests that there is a large diversity in their function. This raises the question: do the LTCCs play differential roles during development? There is some evidence supporting this claim, as some types of memories are not present in young ages (e.g., the fear memory is not present in neonate rats due to the fact that the amygdala is not fully developed). Additionally, as we age there are changes in LTCCs that also support this, which will be discussed in section 1.3.3.

1.3.1. LTCCs Role in Neonate Olfactory Learning

LTCCs are involved in early odour preference learning in neonatal mice (Mukherjee, and Yuan, 2016). Blocking NMDARs prevented the formation of short-term (3hr) and long-term (24hr) memory, while blocking LTCCs with Nifedipine did not prevent short-term memory but did prevent long-term memory. However, when BayK-8644 (a LTCC agonist) was used to activate LTCCs in the presence of NMDAR blockade, the activation of the LTCCs allowed for the formation of short-term memories as well as long-term memories. Another critical finding from this study was the role of the LTCCs in odour specificity memory. They found that NMDARs, but not LTCCs, are required for the stimulus-specificity of odour associative

learning. Blocking NMDARs, but not LTCCs, prevents the pups from forming a preference for a rewarded odour as opposed to a similar odour. This illustrates that LTCCs are critical in some aspects of odour learning and memory. However, they are not required for others, such as odour specificity memory, which persists the absence of NMDAR activation.

1.3.2. LTCCs in Adult Learning

A study done by Ghosh et al., (2017) examined the developmental changes in the anterior-PC pyramidal neurons through whole-cell calcium recordings. Interestingly the LTCC mediated calcium current decreased beyond the critical period of rat pups, up to the weaning age. However, the LTCC mediated calcium currents increase with age in adult life. In the prefrontal cortex from postnatal day 25 (P25) through P80, there is an increased function of LTCCs, which is believed to be involved with improved working memory and decision making in early adulthood (Heng, Markham, Hu, and Tseng, 2011).

1.3.3. LTCCs in the Age-Related Decline of Learning and Memory

Landfield and Pitler (1984) were among the first that suggested that there is an increased amount of calcium as we age, which leads to the age-related cognitive decline. This was termed, ‘the calcium hypothesis of brain ageing’. In aged hippocampal slices, there is a marked increase in the afterhyperpolarization phase (AHP) of an action potential. When comparing the aged animal’s AHPs to that of young healthy animals, they tested to see if calcium was responsible for this significant difference they observed. When placing young hippocampal slices into solutions varying in calcium concentration, they found that in the high concentration of calcium, young

slices exhibited AHPs similar to aged slices, while the other levels did not. This suggested that an increased amount of calcium is responsible for this increase in the AHP (Landfield 1987; Landfield and Pitler 1984).

Moreover, from the idea that increased amount of calcium entry into the cell leads to the decline in learning and memory in the aged animals, experiments were conducted to investigate source of this increased calcium. The study conducted by Moyer and Disterhoft (1994) set out to explore the roles of LTCCs to answer this question. It was found that that when examining calcium-dependent action potentials, which characterized by their initial fast phase and slow plateau phase. The Fast phase consists of the initial spike of the action potential, while the plateau phase is followed by this spike, and consists of the cell returning to rest. Aged hippocampal slices had a significantly larger amplitude and increased duration of the slow phase, while the fast phase was unaffected, indicating that the slow phase may be the underlying cause of increased calcium entry. However, when the aged slices were treated with as little as 100nM of nimodipine (LTCC antagonist), it resulted in a calcium-dependent action potential that mimicked what was seen in young slices, that is, a reduction in the slow phase. This indicates that there is an increased amount of calcium entering the cell via LTCCs.

In addition to electrophysiological studies, the LTCCs have been found to be harmful to the learning and memory process of aged animals. In an eye-blink condition task, in which animals learn to pair an unconditioned stimulus (air puff to the eye) with a conditioned stimulus (a tone), there is a clear deficit observed in aged animals when compared to young animals. Aged animals require a significant higher number of trials to meet the learning criteria of the learning paradigm. However, when the aged animals were treated with nimodipine and the LTCCs were blocked, the number of trials the aged animals required to undergo to learn was significantly

reduced to a level similar to that of young animals (Deyo, Straube, and Disterhoft, 1989). Furthermore, it has also been shown that in the same behavioural task (eye-blink conditioning), aged humans, who were also given nimodipine treatment, had significant improvements in learning (Carrillo et al., 1994, as cited in Disterhoft, Thompson, Moyer, and Mogul, 1996).

1.4. C-Fos: The Immediate Early Gene as a Marker of Neuronal Activity

The formation of new memories and the ability to store information requires changes in synaptic structure via protein synthesis mediated synaptic plasticity. However, identifying what creates these proteins is critical to our understanding the mechanisms of how they work and how they are altered. Proteins are encoded by a number of genes but are also encoded by immediate early genes (IEGs). One of the most well studied genes of the IEGs following learning is the Fos family of transcription factors. C-Fos, a specific form of the Fos family which are distinguished most prominently distinguished via structural properties (Funk, Poensgen, Graulich, Jerome, and Müller, 1997), has been found to be involved in the process of LTP and a marker of neuronal activity (Kaczmarek, 1993; Nikolaev, Tischmeyer, Krug, Matthies, and Kaczmarek, 1991).

The structure of c-Fos is formed by 380 amino acids and acts as a pair of dimers, which allow binding to either promoter or enhancer regions in numerous mammalian genes (Szalóki, Krieger, Komáromi, Tóth, and Vámosi, 2015). C-Fos has not only been found to be a marker of neuronal activity (Bullitt, 1990), it has also been found to be involved in various forms of learning. For example, in a conditioned taste aversion paradigm conducted by Navarro, Spray, Cubero, Thiele, and Bernstein (2000). They found that there was an increase of Fos-like immunoreactivity was observed, not only in the central nucleus of the amygdala but also in the

intermediate nucleus tract of the solitary tract and in the parabrachial nucleus, which are all regions considered to be involved in this form of learning. Similar findings were also found by the study conducted by Bullitt (1990) using various forms of noxious stimuli and by measuring c-Fos in multiple regions of interest.

C-fos has also been found to be involved in olfaction-based learning. In the past, researchers have shown that in odour discrimination learning task, many regions within the brain are involved in this process (Tronel, and Sara, 2002). Furthermore, some observers found that in the piriform cortex, the trained animals had higher levels of c-Fos expression compared to controls, as well as different levels of expression based on the length of training (Datiche, Roullet, Cattarelli, 2001). Researchers also saw markedly increased levels of c-fos within the hippocampus (CA1 and CA3) and various regions within the amygdala (Hess, Gall, Granger, and Lynch, 1997; Hess, Lynch, and Gall, 1995). Significant to our current study, c-Fos measurements indicate that enhanced neuronal excitability within the hippocampus occurs prior to rule learning in olfactory tasks, and leads to increased learning abilities. Which suggests that when the hippocampus is in this enhanced state, prior to rule learning, it allows for a more rapid and efficient acquisition in a variety of behavioural tasks such as spatial and olfactory based paradigms (Zelcer et al. 2006).

1.5. Hypothesis: What is Missing From the Current Understanding?

In the past, it has been shown that young hippocampus slices exposed to high concentrations of calcium mimics what is seen in aged hippocampal slices, led to the calcium hypothesis of brain ageing. Research expanding on this idea, showed that when aged rabbits were treated with nimodipine (i.e., blocking the LTCCs), lead to a rescue in their ability to learn the eye-blink

conditioning task. Additionally, LTCCs have been shown to critically involved in the formation of LTM olfactory based memories in rat pups. Given the above information and the evidence that the LTCCs are involved in the processes of learning and memory formation in different behavioural models, there still remains unanswered questions: Do LTCCs differentially regulate olfactory memory formation in healthy adult and ageing rats? Do the LTCCs roles shift in hippocampus-dependent spatial memory in adult and aged rats? We hypothesize that LTCC activity in young animals promotes associative learning as well as prolongs the memory. However, increases in LTCC activity leads to impairment of the same processes in ageing animals.

2.0. Methodology

2.1. Ethics

All procedures, experiments and surgeries were conducted following approval and in guidance with the Animal Care Committee at Memorial University of Newfoundland and the Canadian Council on Animal Care (as seen in section 7.0. appendix).

2.2. Animals

The animals used for this set of experiments were Sprague Dawley of both sexes, ordered from Charles River, Ontario. The age range of the animals were 6-9 months old for the young cohorts, and >18 months for the old cohort. All animals were housed in a reverse dark-light cycle, and all experimental procedures were conducted during the animals light phase. Animals were given *adlib* access to food and water unless otherwise undergoing the food-restricted paradigm. During the time of food restriction, animals were fed daily 20g of standard rat chow.

2.3. Surgical Procedures: Cannula Implantation Surgeries

To allow for direct infusion into the piriform cortex and CA1 the animals underwent a cannula implantation surgery. The animals were anesthetized using isoflurane via an induction chamber, and then mounted in a stereotaxic apparatus and placed in the skull flat position. The holes for the piriform cortex cannulas were drilled at -2.3 AP, and ± 5.5 ML and for the hippocampus CA1 coordinate were drilled at -3.6 AP, and ± 2.4ML. After the holes for the two cannulas were drilled, the cannulas were lowered into place (DV: -8.0 PC; DV: -2.2 CA1) from

brain surface, and then affixed to the skull via dental cement. Following this, the incision was sutured and was then covered with dental cement in order to ensure the cannulas would not move.

2.4. Behavioural Studies

Behavioural studies were carried out in a dimly lit behavioural room to observe the effects of systemic i.p. injections, as well as the targeted infusion of nimodipine versus vehicle in various behavioural tasks to elucidate the differential roles of LTCCs in ageing animals.

All drugs or vehicle were given either by i.p. (nimodipine, 5.5 mg/kg; to ensure the full effect of LTCC blockade), based on previous research using nimodipine at similar dosages, and show the nimodipine crosses the blood brain barrier within 30 minutes (Martin et al., 2004; Woodruff-Pak et al, 1997). Alternatively, infused nimodipine into the brain (1 µM or 100 µM; 0.5 µl per site; dissolved in 25% DMSO and 75% PBS) (Quevedo et al., 1998), 30 min before training via a 10 µl Hamilton syringe and infusion pump. The infusion was done over 3 min followed by a 1 min wait before withdrawing the syringe.

2.1.1. Odour Associative Learning Paradigm

To test the differential role of the LTCCs during different age periods (6-9-month-old vs. >20- month-old), we used simple odour discrimination (SOD) learning, in a food-deprived rat model. This model uses food pellets as a reward within an open chamber (60cm x 60cm) in a dimly lit room. The animal must learn to discriminate between two dissimilar odours; one with

the food reward (60µL of almond extract) and one with no food reward, (60µL of coconut extract). This procedure consisted of a training phase, followed by a test phase. The training phase consisted of exposing the animal to an unscented sponge baited with food. This procedure was repeated for 4-10 days or until the animals reached a threshold value indicating they had learned the task of retrieving food from the center of the sponge.

Afterward, the animals moved to the test phase. They were split into two groups, one given the LTCC blocker nimodipine (i.p. or locally infused to the piriform cortex), and the other vehicle (5.5mg/kg i.p..; 0.5uL/side of 100uM of Nimodipine.), 30 minutes prior to the start of the testing session, consisting of six trials each day. The animal was then placed in a designated home corner (location fixed) and was presented with two scented sponges (locations vary each trial randomly). They were given a maximum of 300 seconds to retrieve the food pellets from the positive odour. Reese's puff cereal was used as the food reward. To measure whether the animals had learned the rule or not, we recorded the animal's first nose poke (either correctly sniffing the scented sponge associated with a reward or incorrectly sniffing the scented sponge not associated with a reward). The number of correct nose pokes was divided by the total number of correct and incorrect nose pokes during each trial to obtain a % correct nose poke.

2.4.1. Memory Duration Test

We tested the odour memory at various time points (3, 6, or 12 weeks) following the odour associative learning paradigm training. In order to do this, we exposed the animal to the same apparatus for the appropriate paradigm and then measured the appropriate response of the animal. For the food retrieval task, the animals were once again exposed to two scented sponges

(one with the previously rewarded odour and the non-rewarded odour) for a single 300s trial. In which we recorded the time spent in the positive or negative quadrant (facing/exploring the sponge, at least within 2cm), as well as the number of the correct and first nose pokes, thus allowing us to observe if the animals had a preference to the previous appetitive odour.

2.4.2. Odour Detection and Discrimination Test

The animals were subjected to an odour detection and discrimination test (ODADT) to examine whether or not the infusion or injections of nimodipine affected the animal's ability to discriminate between odours. This consisted of serial exposures to odours. The animals were placed in a clean cage, and odours were presented through the drinking hole via a small vial with several holes which contained a filter paper with the odour dissolved in mineral oil. Thirty minutes prior to starting the test, the animals were given either a vehicle or nimodipine injection or infusion, following the exact dosages as mentioned previously. The presentation of odours went in this order: 3 presentations of mineral oil, 3 presentations of odour one (0.001% heptanol) dissolved in mineral oil, and finally, 1 presentation of a second similar odour (a 1 to 1 ration of 0.001% heptanol and octanol) dissolved in mineral oil. Each presentation lasted for 50 seconds, with an inter-trial interval of 5 minutes between each presentation. The amount of time the animals spent sniffing the tube (i.e., within 1cm of the presented vial) was recorded for each trial. Once completed, the amount of time spent sniffing was examined, and a discrimination index was calculated.

2.4.3. C-Fos Expression

To measure the extent of neuronal activity to the conditioned odour following the odour associative learning paradigm, we measured the level of c-Fos expression. The animals first had to complete the odour associative learning paradigm. Once they completed this task, the animals were placed in their home cages overnight. After 24 hours, animals were individually exposed to a freshly scented sponge (as used in the odour associative learning task) containing one of the two odours (the reward odour or the non-rewarded odour) for 10 minutes within their own cage with the lid completely shut. The odour was removed after the 10-minute period. Ninety minutes after the end of the odour exposure, the animals were perfused transcardially, and their brains were stored in a vial of 4% paraformaldehyde (4% PFA). Brains were sectioned coronally using a Compresstome (Compresstome® VF 310-0Z Vibrating Microtome, Precisionary Instruments LLC) with phosphate buffer saline, and sections from -1.4 to -3.6 bregma were stored in polyvinylpyrrolidone (PVP) until used for immunohistochemistry (IHC). Sections were selected based on quality of tissue and put through a series of washes, in the following order: Tris buffer (10 Mins), Tris A (10 Mins), Tris B (10 Mins), a 10% normal goat serum solution (1 Hr), Tris A (10 Mins) and finally Tris B (10 Mins) again, before being incubated overnight (24 Hrs) in a c-Fos primary antibody solution (#2250, Cell Signalling, Host: Rabbit; at a concentration of 1:2000 in Tris B). The following day, sections were again washed in Tris A (10 Mins) and Tris B (10 Mins), followed by a 45 minute incubation period with secondary antibody AlexaFluor647 (A-21244, Invitrogen, Host: Goat, anti-rabbit (target); at a concentration of 1:1000 in Tris B), and a wash in Tris buffer (10 mins) before being mounted on microscope slides. Slides were cover slipped using Fluoroshield mounting medium with DAPI. The DAPI stain allows us to visualize the neurons, which we compare to the c-Fos positive cells which were counted using a

fluorescent microscope (EVOS M5000, Thermofisher). For c-Fos count, cage animals (directly taken out from the home cage and perfused) were used to determine background staining. Thresholding and segmentation were performed by "TWS" function in ImageJ plug in (Arganda-Carreras et al., 2017). Analysis was conducted by a blind experimenter.

2.4.4. Spontaneous Location Recognition Task

Using a previously defined protocol, the spontaneous location recognition task (SLR) (Bekinschtein, 2013), animals were handled and habituated daily, for 3-6 days to the open field chamber (60cmx60cm) and room location. After the habituation phase, the animals underwent the training phase. They were given an injection/infusion of nimodipine or vehicle 30 minutes prior to their exposure to three identical objects. Object 1 (A1) was placed in the center of one wall (30cm from either side) by itself. On the opposite side, objects 2 and 3 (A2 and A3) were placed side by side, equidistant from the wall, leaving the center between the two open. The animals were then given 10 minutes (600s) to explore the objects freely, and the time spent exploring each object was recorded. The following day (24 hours later), the animals underwent the test phase to examine if the injection/infusion prevented learning of the previous object's location. This consisted of exposing the animal to two new objects identical to the previous three (A4 and A5). A4 acted as the familiar object as it shared the same position as A1, and A5 served as the novel object, as its location was directly between A2's and A3's position. The animal was then given 600s to explore the two objects freely, and the time spent exploring was recorded.

2.4.5. Open-Field Test

From the open-field test (OFT) test, were able to observe if and how blocking the LTCCs affected the animal's locomotive behaviour, as compared to the vehicle treated animals. The animals underwent 3-6 days of habituation to an open-field chamber (60cm x 60cm x 60cm) in a different context than that of the previous tests. Following this, animals underwent a single trial inside the box. 30 minutes prior to the trial, the animals were given an injection of nimodipine (5.5mg/kg) or vehicle to observe the effects on the animal's behaviour in this task. The OFT is designed to allow us to measure the animal's general behaviour to examine the effects of nimodipine. The total distance travelled was measured in order to assess the animal's locomotion activity as a measure of their exploratory behaviour and physical response to nimodipine.

2.4.6. Cannula Placement Targeting

Following the completion of all behavioural tasks, animals were transcardially perfused to confirm the placement of the cannulas. For this, brains were placed in a 20% sucrose solution for 2 days for cryoprotection. Following this, the brains were flash frozen by submerging them into -80°C 2-Methylbutane. Brains were then sectioned coronally using a Cryostat (Microm HM 550, Thermofisher Scientific). Sections were mounted onto gelatin coated microscope slides and left dry overnight before performing a Nissl stain. The Nissl procedure consisted of placing each slide into the following solutions: 75% ethanol (EtOH) for 2 minutes, 95% EtOH for 2 minutes, 100% EtOH for 2 minutes, dH_2O rinse, cresyl violet up for to 15 minutes for stain to penetrate, dH_2O rinse, and then the slides were put through the first three steps again for 1 minute each,

before being placed into xylene until cover slipped with permount. The sections were viewed under a compound light microscope and cannula tracts were labeled onto a brain atlas.

2.5. Statistical Analyses

All statistical analysis procedures were carried out using IBM Statistical Package for the Social Sciences (SPSS). Data are presented as mean ± S.E.M. Odour associative learning in figure 1 was measured by either a one-way (memory duration) or a two-way (learning curves by groups) repeated analysis of variance (ANOVAs), followed by Tukey tests. In the ODADT in figure 2 two-way (treatment x age) ANOVAs were used, followed by post-hoc Tukey tests. C-Fos expression data in Figure 3 was analyzed by independent samples t-tests as well as a one-way ANOVA. SLR data in figure 4 was measured by two-way (treatment x age) ANOVAs were used, followed by post-hoc Tukey tests. Open field data in figure 5 was analyzed by independent sample t-tests.

3.0. Results

The goal of this present study was to elucidate the roles of LTCCs in various types of learning as animal's age. Specifically, we assessed LTCCs in olfaction-based and spatial-based learning paradigms. We started with systemic injections of nimodipine and then moved onto targeted infusions of the areas responsible for specific types of learning. In order to explore LTCCs in olfactory based learning, an odour discrimination paradigm was used. To evaluate spatial learning, a spontaneous location recognition task was implemented. Overall, from these experiments we found that in young animals, LTCC blockade impaired spatial and olfactory performance by either delivery system. In contrast, the performance of aged animals was rescued by blocking LTCCs.

3.1. Associative Odour Learning Task

In order to explore the aged-related role that the LTCCs play in olfactory based learning, we used nimodipine in an odour associative learning task in adult and aged animals where they learned to discriminate between two odours (Figure 1A). Two-way repeated measures ANOVA revealed a significant effect of day of testing and group ($F(5,75) = 15.418$, $p < 0.001$). Following post-hoc testing was conducted using Tukey tests. Young-adult vehicle animals that received i.p. injections ($N = 11$) or direct infusions of vehicle into the posterior-PC (pPC) or CA1 ($F(10, 115) = 4.836$, $p > 0.001$; $N = 11$ for both groups) performed significantly better compared to young-adult animals that received nimodipine (Both i.p. 5.5mg/kg, $N = 6$; and direct infusion of nimodipine 100µM into the pPC or CA1, $N = 10$ and $N = 5$ respectively) (figure 1B and 1C). Furthermore, when looking at the aged animals we see a reversed trend. Ageing animals that received i.p. injections of nimodipine ($F(5,45) = 3.679$, $p = 0.007$; $N = 6$) or infusions of nimodipine into the pPC or CA1 ($F(5,60) = 2.401$, $p = 0.018$; both $N = 6$), performed drastically better from ageing animals that received vehicle treatment (i.p. $N = 5$; cannula both pPC and CA1 $N = 6$) (Figure 1 D and E). Further post-hoc testing was conducted to see what groups differed across ageing time-points. It was found that young-adult vehicle animals in either delivery method differed significantly from ageing vehicle treated animals ($p < 0.001$). Furthermore, ageing animals treated with nimodipine in either treatment method, also performed significantly better ($p < 0.001$) from young animals that received either i.p. injections or direct infusions of nimodipine. Interestingly, when comparing young-adult vehicle-treated animals (i.p. vs direct infusions), they did not differ from one another ($p = 1.000$), nor did they differ from ageing animals that received nimodipine ($p = 1.000$). In addition, young-adult animals that received nimodipine showed no significant difference ($p = 1.000$) when comparing delivery

methods. These findings suggest that in young-adult animals, LTCCs in both the hippocampus and PC are required for odour associative learning, while in aged animals, the LTCCs in these regions prevent the animal from learning the rule of this task. Additionally, in aged animals, PC infused animals appeared to learn at a slower rate when compared to CA1 infused animals. PC infused animals performed better than the vehicle group on day 4 ($p < 0.01$) while CA1 animals performed better on day 3 compared to vehicle infused animals ($p < 0.01$). Moreover, we assessed whether LTCC blockade affected the duration of memory. The odour memory duration in young-adult rats with vehicle injection was compared to that in aged rats whose learning was rescued by nimodipine injection (as these groups learned the rule). In both young-adult ($F(3,40) = 16.683$, $p < 0.001$; Fig. 1F) and aged rats ($F(3,13) = 11.124$, $p < 0.001$, Fig. 1G), there is a decline of the associative memory with time. Memories in both the young-adult ($p < 0.01$) and aged rats ($p < 0.05$) lasted for 3 weeks compared to the first day of the training. By 6 weeks, the memory of the odour associative rule they learned was lost in both groups ($p > 0.05$ compared to day 1 of the training).

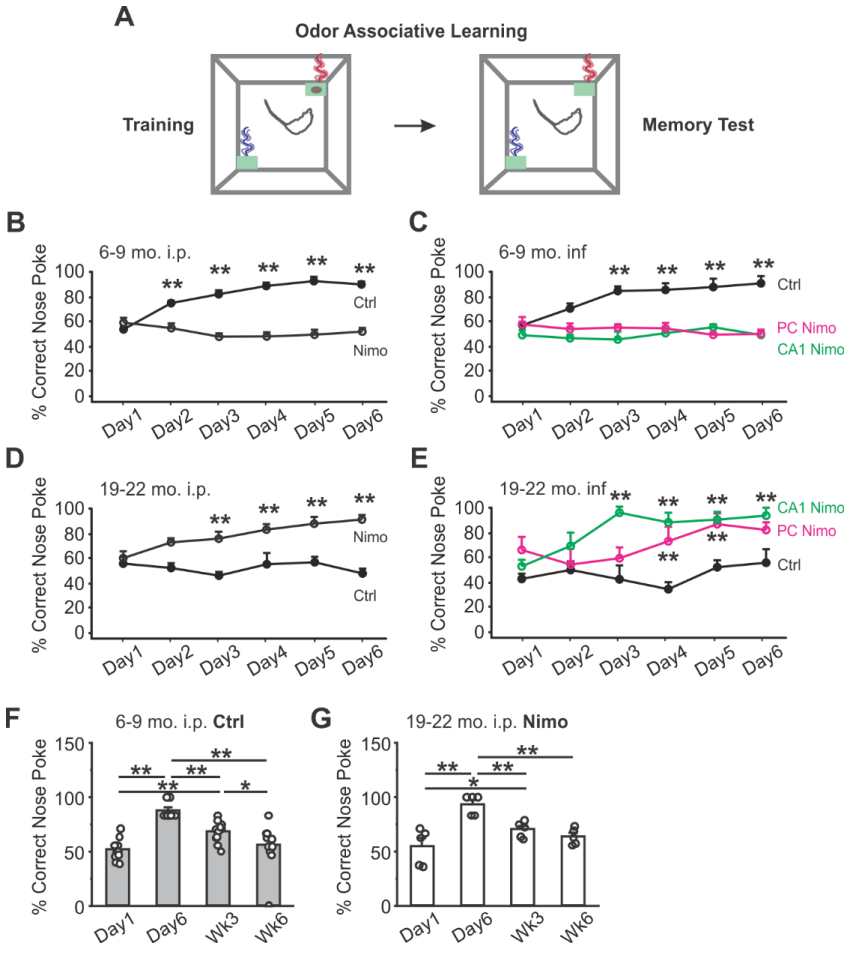

Figure 1 - ODOUR ASSOCATIVE LEARNING TASK. Panel A. Schematics of odour associative learning and memory test. Panel B. Nimodipine i.p. administration prevent odour associative learning in young-adult animals (*N* = 11 vehicle; *N* = 6 nimodipine). Panel C. PC or CA1 nimodipine infusions (*N* = 10; *N* = 5, respectively) prevented odour associative learning in young-adult rats (*N* = 11, vehicle). Panel D. Nimodipine i.p. injection allowed odour associative learning in aged rats (*N* = 6 vehicle; *N* = 6, Nimodipine). Panel E. Both PC (*N* = 6) and CA1 (*N* = 6) infusion of nimodipine allowed for odour associative learning in aged rats (*N* = 6, vehicle). Panel F. Memory duration in young-adult rats treated with vehicle injection. Panel G. Memory duration in aged rats with nimodipine injections. **p*<0.05, ***p*>0.01.

3.2. Odour Detection and Discrimination Test

To assess whether or not the blockade of LTCCs impairs the animal's ability to discriminate similar odours, animals were put through serial presentation of odours Figure 2A. Through the use of a two-way ANOVA followed by Tukey test, we compared discrimination indices. When animals were treated with nimodipine in either delivery method, i.p. injection (5.5mg/kg) or direct infusion into the PC (100μM), had no effect on the animals ability to discriminate between the odours in either young-adult or aged animals (age: $F(1,38) = 0.246$, $p = 0.622$; treatment: $F(2,38) = 1.144$, $p = 0.329$; age x treatment interaction: $F(2,38) = 2.522$, $p = 0.094$; Figure 2B). Since the animal's ability to discriminate between similar odours was not affected by nimodipine, this suggests that LTCCs do not play a significant role in similar odour discrimination.

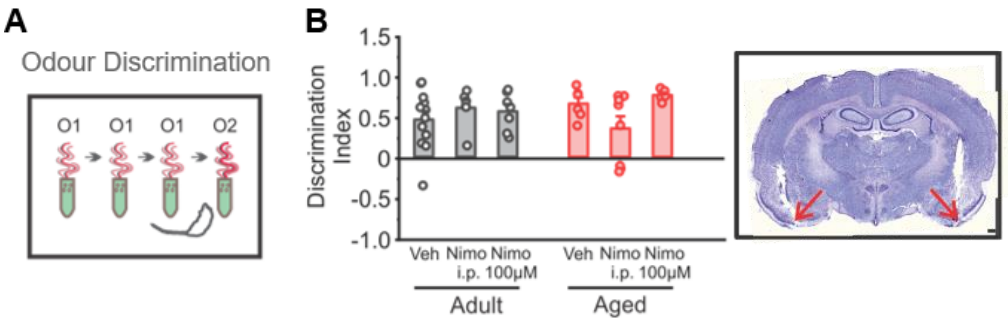

Figure 2 - ODOUR DECTION AND DISCRIMINATION TEST. Panel A. The experimental schematic behind the odour detection and discrimination test. Panel B, young-adult animals, nimodipine administration i.p (*N* = 6) or PC infusion (*N* = 4), had no effect on the animal's performance (grey bars), vehicle treated animals were combined, young-adult vehicle (*N* = 12*)*. In aged animals nimodipine administration i.p. (*N* = 8*)* or PC infusion (*N* = 5) had no effect on the animal's performance, aged vehicle (*N* = 6). Additionally, a sample target image of the piriform cortex cannula tract stained with nissl, scale bar 500µM.

3.3. c-Fos Expression

To further evaluate the effects nimodipine on the learning process, we examined the amount of c-Fos expressed in response to a presentation of one of the two trained odours in the associative odour task. To do this, we used pseudotrained animals, which are animals that were trained in the odour associative task, however the food reward was not fixed to a specific odourant A set of conditioned animals, that underwent normal training consisting of one reward odour and one negative odour (no reward) with no drug administration, and finally, a set of animals that did receive nimodipine during training. When comparing pseudotrained animals (N = 7) to conditioned animals (N = 7) we found that there was a significant difference in the animal's performance ($t(12) = -8.2272$, $p < 0.001$) (Figure 3A) and followed the same experimental design as depicted in Figure 1 A. When examining the level of c-Fos in the CA1 region of the hippocampus of these animals, we observed no significant difference in the number of c-Fos activated cells (Figure 3 B1-B3). However, this finding was not observed within the piriform cortex, when examining c-Fos levels in the PC of the pseudotrained group (N = 8) compared to the conditioned group (N = 8); there was a significant difference observed in the amount of positively expressed c-Fos levels in the conditioned animals ($t(14) = -3.7084$, $p < 0.01$) (Figure 3 C1-C3). Interestingly, when comparing the conditioned animals (no drugs), to the animals that were given infusions into the piriform cortex (N = 3) or hippocampus CA1 (N = 5), there was a significant difference. The infused animals had significantly reduced levels of c-fos activation within in the PC ($F(2) = 6.17572$, $p < 0.05$.) (Figure 3 D1-D4). Further post-hoc testing (Fisher Test) revealed that when comparing both the piriform and the CA1 nimodipine infused groups to the conditioned group, that they had significantly decreased levels of c-Fos expression ($t = -2.92477$, $p < 0.05$; $t = -2.68377$, $p < 0.05$., respectively). These results suggest

that when nimodipine is infused in young-adult animals, in either region, it leads to a reduction of c-Fos activity within the piriform cortex. When the LTCCs were inhibited in the hippocampus, it prevented c-Fos activation in the PC. Suggesting that there is a connection between the hippocampus and the piriform cortex which is in line with previous research as it has been demonstrated that the PC sends input directly to the hippocampus via the lateral entorhinal cortex, and that the CA1 axons also directly project into the PC (Cenquizca, and Swanson, 2007; Kerr, Agster, Furtak, and Burwell, 2007).

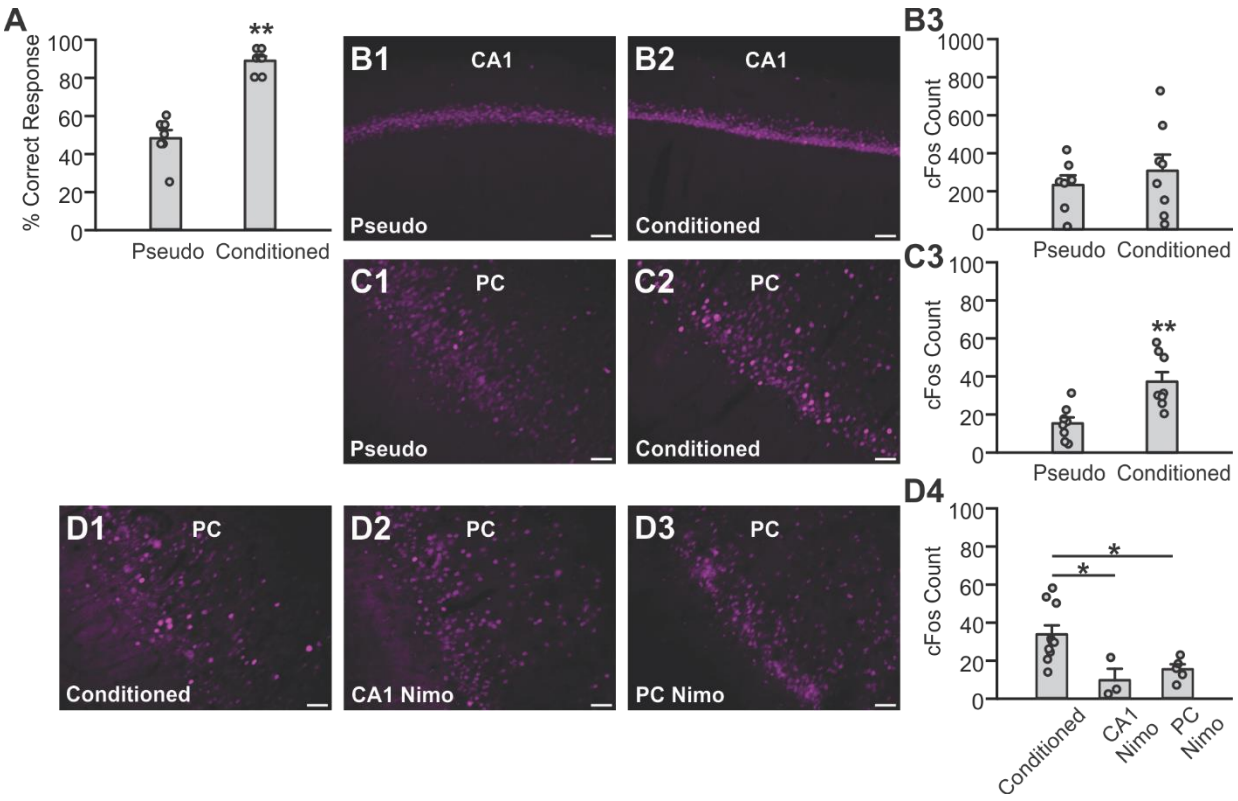

Figure 3 - C-FOS EXPRESSION DATA. Panel A shows the performance of pseudo-conditioned (*N* = 8) and regularly conditioned animals (*N* = 8) in the odour associative task. Panel B1-B2. Shows the level of c-Fos expression (before thresholding) of the pseudotrained animals (*N* = 7) and the conditioned animals (*N* = 8) within the CA1 region. Panel B3. Shows that the level of c-Fos expression in the CA1 region do not differ as a result of the different training methods. Panel C1-C2 shows the c-Fos expression (before thresholding) within the piriform cortex, comparing the pseudotrained (*N* = 8) and conditioned animals (*N* = 8). Panel C3. Shows that regularly trained animals in the odour associative paradigm have higher levels of c-Fos. Panel D1-D3, show the amount of c-Fos expression (before thresholding) within the piriform cortex of the conditioned animals (*N = 8*, control) and animals that received infusions of nimodipine in the CA1 (*N* = 5) and piriform cortex (*N* = 3). Panel D4. Shows that nimodipine in either region results in a reduction of c-Fos levels within the piriform cortex. Scale bars: 50µM *p<0.05

3.4. Spontaneous Location Recognition Task.

To assess whether the blockade of the LTCCs would affect the animal's ability to discriminate between novel and familiar objects, we administered nimodipine via i.p. injection or direct infusion into the hippocampus dorsal CA1 region during a spontaneous location recognition task (Figure 4A,). The dorsal CA1 was chosen as the target for this study as past research has shown that the dorsal aspect encodes for spatial and cognitive information, while the ventral aspect encodes emotion-related information (Kjelstrup et al., 2002; Moser et al., 1995). There were significant effects of age ($F(1,21) = 6.247$, $p = 0.021$) and an age x treatment interaction ($F(1,21) = 70.741$, $p < 0.001$), and a significant effect of nimodipine on both age groups ($F(2,51) = 59.089$, $p < 0.001$). When comparing young-adult nimodipine-treated (i.p.) animals ($N = 6$) to young-adult vehicle-treated (i.p.) animals ($N = 6$), the vehicle treated animals performed significantly better ($p < 0.001$) (Figure 4B, Left). When comparing young-adult nimodipine-treated (cannula) animals (1µM - 100µM, $N = 7$) to young-adult vehicle-treated (cannula) animals ($N = 7$), the same significant result was found ($p < 0.001$), in which the vehicle treated animals performed better compared to the nimodipine animals (Figure 4C, Left). Strikingly once again, when comparing the aged nimodipine i.p. animals ($N = 8$) to aged vehicle i.p. animals ($N = 5$), it was found that the significant difference was that the nimodipine animals performed better ($p < 0.001$) (Figure 4B, Right). Aged animals that received CA1 ($N = 5$) 100µM nimodipine infusions also performed significantly better than vehicle infused aged ($N = 6$) animals as well as performing better than the 1µM infused aged animals ($N = 5$) ($p < 0.001$) (Figure 4C, Right). Further analysis showed that when comparing aged vehicle treated animals, to either young-adult nimodipine treated animals (i.p. or CA1 infusion), their performance did not differ ($p > 0.05$). However, aged vehicle treated animals differed significantly from young-

adult animals in the vehicle treatment conditions ($p < 0.001$). When comparing aged nimodipine i.p. or 100μM infused animals to young nimodipine treated animals (i.p. or direct infusions), it was found the aged animals, performed significantly better ($p < 0.001$). These results suggest that LTCCs are beneficial in spatial learnings tasks in young rats, while impairing learning in aged rats.

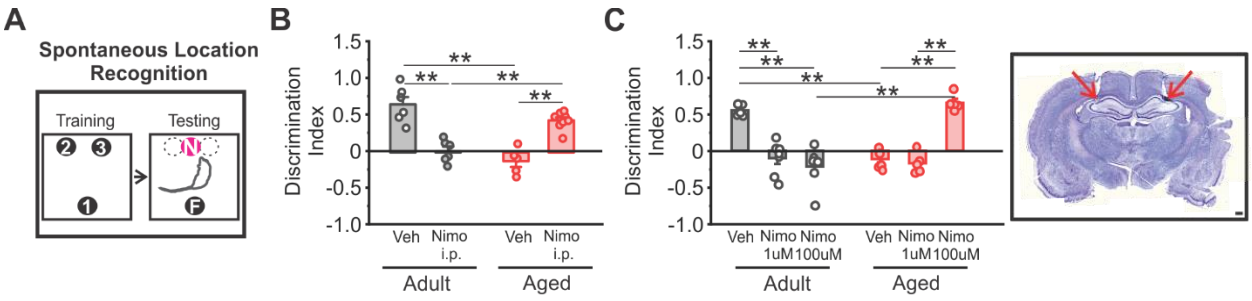

Figure 4 - SPONTANEOUS LOCATION RECOGNITION TASK. Panel A. Schematic representation of the setup for the spontaneous location recognition task. Panel B. Nimodipine i.p. administration in young-adult animals prevent the spatial learning process (*N* = 6 vehicle; *N* = 6 nimodipine). In aged animals nimodipine i.p. (*N* = 8) allowed for the spatial learning process (*N* = 5 aged vehicle i.p.). Panel C. In young-adult animals both the 1µM and 100µM infusion of nimodipine (Both *N* = 6 for each concentration) prevented the spatial learning process. In aged animals 100µM infusion (*N* = 5) permitted the spatial learning process (*N* = 6 vehicle infusion; *N* = 5 1µM infusion). In addition, panel C shows a sample target of the CA1 cannula tract stained with nissl. ***p*>0.01, scale bar 500µM.

3.5. General Behaviour

To examine if the blocking of the LTCCs affected the animal's general locomotive behaviour and exploratory, the animals were placed in an open field chamber to freely explore. It was found that in young-adult animals, there were no significant differences in total distance travelled when the vehicle animals ($N = 6$) were compared to the nimodipine treated animals ($N = 6$) ($t(5) = 1.4038, > p = 0.281$) (Figure 5). When examining aged animals, we found a similar result, that the total distance travelled between each group, showed no significant differences between vehicle treated ($N = 4$) when compared to nimodipine treated animals ($N = 4$) ($t(3) = -0.7617, p = 0.501$) (Figure 5). This suggests that nimodipine does not affect the general behaviour of young adult and aged animals. We conclude that the differences found throughout learning paradigms used in this present study, are in response to the LTCCs involvement specific to the processes of learning and memory.

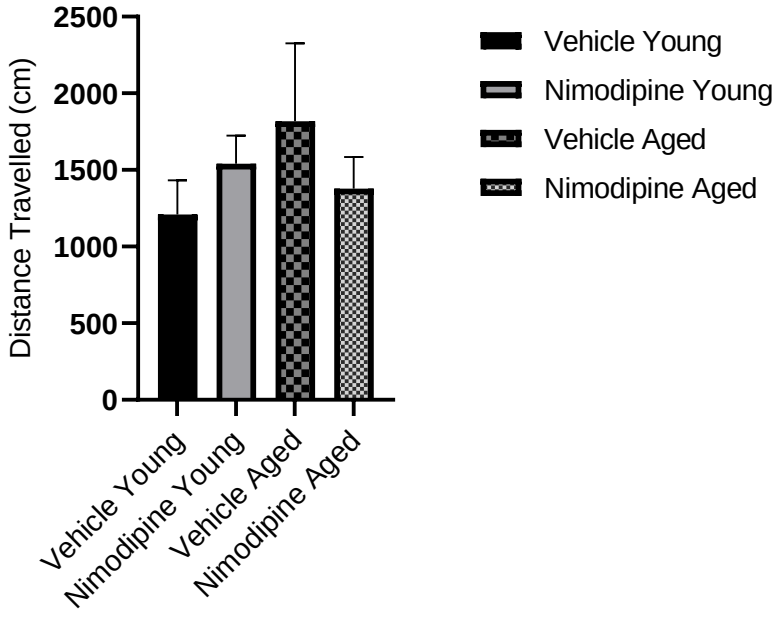

Figure 5 - OPEN FIELD TEST. Shows the total distance travelled by both groups of adult animals given i.p. injections vehicle (*N* = 6) or nimodipine (*N* = 6) (5.5mg/kg), resulted in no significant differences in the total distance travelled between the two groups. We see the same outcome in that the aged animals that received either vehicle treatment (*N* = 4) or nimodipine (*N* = 4) resulted in no significant differences between the two groups.

3.6. Cannula Placement Targeting

The confirmation of the cannula locations was assessed under a compound light microscope, which were then mapped onto a rat brain atlas by comparing the section of tissue under a compound light microscope to the images in the rat brain atlas (Paxinos, and Watson, 1998) for confirmation of a successful surgery. Our results showed localized clusters of cannula locations around the areas of interest (Figure 6). This further validates the location specific actions of nimodipine in our behavioural experiments.

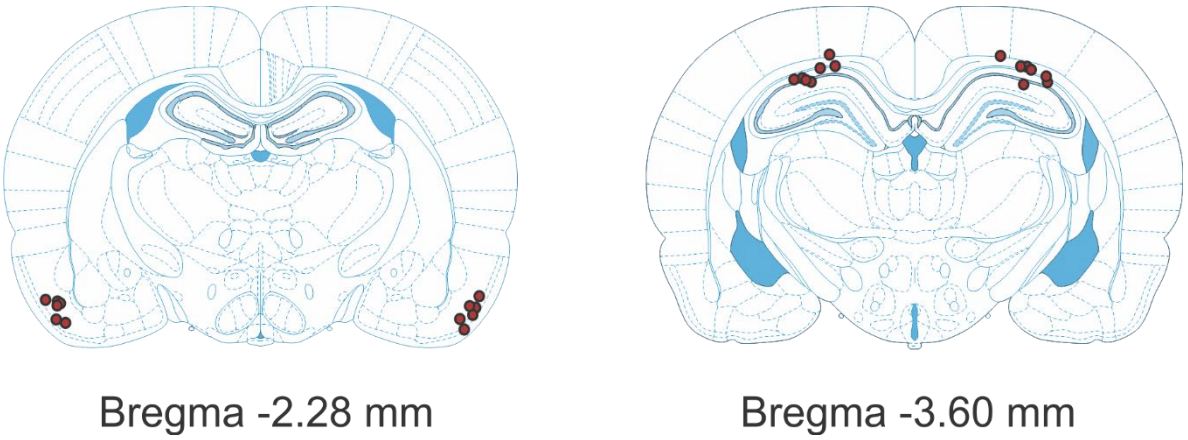

Figure 6 - CANNULA TARGETTING. Representative targeting data of cannula placement for both the Piriform cortex (Left) and the CA1 (Right). Cannula tracts were confirmed through Nissl staining viewed under a light microscope. Red dots represent the end of the cannula tract.

4.0. Discussion

4.1. Brief Overview

Our results of blocking L-type calcium channels with nimodipine in young-adult animals (6-9 months) and aged animals (>18 months) suggest a paradoxical shift of the role of LTCCs in the process of learning and memory. In young-adult animals, performance in both an odour associative and spatial learning task was impaired with nimodipine, while in aged animals, their performance after nimodipine treatment showed a significant improvement. Meanwhile, blocking the nimodipine channels in regards to the animal's general behaviour showed that nimodipine did not have any significant impact.

4.2. LTCCs Promote Odour Associative Learning in Young-adult but Impair the Learning Process in Aged Animals

In the odour associative task, it was found that young-adult vehicle (i.p. or cannula infused) animals were clearly able to demonstrate that they had learned the rule that one of the scented sponges contained the food reward while the other one did not. Meanwhile, in young-adult animals that received either i.p. injections or infusions of nimodipine into either the PC or the CA1, it was found that the blockade of LTCCs led to a significant impairment in their ability to learn the task when compared to the vehicle animals.

However, what was seen in the young-adult animals in the odour associative task, was not seen in aged animals. Instead, aged vehicle (i.p. or cannula) animals showed a significant impairment in their ability to learn this task. Interestingly, when compared to young-adult

animals treated with nimodipine, they showed similar levels of performance. Strikingly, when the aged animals were given i.p. injections of nimodipine, the age-related impairment was rescued; they demonstrated a steady increase in their learning of the rule when compared to aged vehicle animals and showed a significant difference in their performance. When it came to the aged nimodipine treated animals with infusions into the PC and the CA1, we again observed this marked difference in the animal's ability to perform this task when compared to the aged vehicle animals. Nimodipine infusion into the CA1 and the PC both resulted in a rescue of the learning behaviour of the ageing rats. However, infusion into the PC resulted in a slower rate of learning when compared to the rate of the CA1 infused animals. In figure 1E, it is clear that by the third day, the CA1 infused animals demonstrated that they had learned the rule and continued to stay at this level throughout the remaining testing days. Meanwhile, the PC infused animals showed a steady rate of learning, but it was not until the fifth day that they demonstrated that they had learned this rule.

As discussed previously, blocking the LTCCs in neonatal animals in the anterior-PC with a calcium channel blocker led to a significant impairment in their long-term memory in regards to associating an odour with either an aversive stimulus or a positive one (Mukherjee and Yuan, 2016; Zhang et al. 2010). In our odour associative task (Figure 1), we see similar results that in our young-adult animals, LTCC blockade in either region leads to impairment, while in our aged animals, it leads the rescue of the age-related impairment. In line with previous research, our results suggest that communication between the piriform cortex and the hippocampus is essential for contextual spatial and olfactory learning (Zelcer et al., 2006). As a result, our odour associative task will allow us to further assess the hippocampus' role in integrating the contextual odour information in the formation of new memories. This makes it unique in

providing evidence of the roles of LTCCs in the crosstalk of different brain regions involved in learning and memory. Infusion of nimodipine into either region (CA1 or PC) produced an observable effect, suggesting extensive connections between the two areas.

Our c-Fos expression data (Figure 3) further supports this notion, as CA1 or PC infusions of nimodipine in conditioned adults led to a significant decrease in the level of c-Fos in the piriform cortex, implying a dependency on regional connections in odour associative learning. However, we did not observe a significantly higher level of C-fos in the CA1 of conditioned animals. It has previously been shown that the CA1 region receives a high level of input from the piriform cortex via the lateral entorhinal cortex (Kerr, Agster, Furtak, and Burwell, 2007) and that CA1 axons themselves directly project into the PC (Cenquizca, and Swanson, 2007). Additionally, a similar odour associative learning model in which animals had to choose between two odours to receive a reward demonstrated that prior to rule learning, there is an increase in the overall excitability of CA1 neurons and later potentiation in the neurons of the PC after animals reached successful learning (Zelcer et al., 2005). Taking these reports in combination with the finding from our present study, we propose it is first the hippocampus that begins the learning process of the odour discrimination task. The CA1 neurons transiently acquire enhanced excitability at the beginning of the training, leading to increased synaptic plasticity or LTP in the PC in the later phase of the training. As a result, the c-fos expression observed in the PC is likely due to a late potentiation of PC excitability following the transient activation of CA1 neurons. The resulting synaptic potentiation in the PC is correlated with successful learning in young-adult animals, which is prevented by blocking normal LTCC function.

Evaluation of the effect of nimodipine on the animals' ability to discriminate between similar odours was another important finding. We determined that neither i.p. injection nor infusions of

nimodipine into the PC resulted in any significant differences. For young-adult animals, neither nimodipine-treated groups showed differences when compared to vehicle animals. The same result was also observed in aged animals, in that there were no significant differences in response to receiving nimodipine compared to aged vehicle animals. Together, these results suggest that the injection nor the infusions of nimodipine affected the animals' ability to discriminate between odours during the associative odour-learning paradigm. Which is in line with previous findings. For example, in a neonatal mouse model, blocking LTCCs did not impair the animal's ability to discriminate between two dissimilar odours (Mukherjee and Yuan, 2016). Here, when it came to the animal's ability to discriminate between two similar odour mixtures, it was again found that blocking LTCCs also did not impair the animal's ability to differentiate between the odours. Additionally, when blocking the activation of NMDARs while still activating the LTCCs, the animals then showed the inability to discriminate between the odours. These findings suggest that the NMDARs are required for odour specificity and not LTCCs. This is further shown by impaired pattern separation due to hypo-functioning NMDARs in the dentate gyrus of the hippocampal formation (Eadie et al., 2012).

4.3. LTCCs Promote the Process of Spatial Discrimination Learning in Young-adult Animals but Impair Spatial Learning in Aged Animals

The final set of experiments used in this present study, the SLR, was conducted to clarify further the involvement of hippocampal L-type calcium channels in regards to their spatial abilities. For young-adult animals, vehicle-treated animals (i.p.) were able to successfully discriminate between the novel and familiar objects (Figure 4B). From the nimodipine-treated animals' discrimination index, we observed a significant impairment of the animal's ability to

recognize the novel position compared to the vehicle animals. Furthermore, this is also illustrated in animals infused by nimodipine via cannulas into the CA1. Once again, while the vehicle animals were quickly able to recognize the novel position, this ability was impaired in animals that received either 1uM or 100µM of nimodipine. In addition to this, the shift in the effects of nimodipine we observed in aged animals in the odour associative task presented itself again in the SLR task. Aged i.p. vehicle-treated animals showed an evident impairment in their ability to recognize the novel position, while the aged animals that received nimodipine demonstrated that they were able to identify the novel position successfully.

Moreover, for aged animals with cannula infusion (Figure 4C), the vehicle-treated animals again showed a significant impairment in their ability to recognize the novel position. Interestingly, when the aged animals received 1uM of nimodipine into the CA1, their ability to recognize the object was not rescued, as they still showed significant impairment (in contrast to the effect of 1uM had in young-adult animals). However, aged animals that received 100uM of nimodipine showed a drastic increase in their performance with this task. This dose-dependent effect of nimodipine in spatial learning could be due to the differential roles that the LTCCs play in learning as the animal ages. It has been found that 1µM of nimodipine inhibits a more significant portion of the current from the $Cav_{1.2}$ calcium channel (~90%) and only partially inhibits the current from the $Cav_{1.3}$ channel (~50%) (Xu and Lipscombe, 2001). It is possible that $Cav_{1.2}$ may be critical in the process of synaptic plasticity in young-adult rats, while in aged rats, both $Cav_{1.2}$ and $Cav_{1.3}$ may be important in contributing to the learning process. In Xu and Lipscombe (2001), they reported that the use of 10µM of nimodipine blocked both channels. Here, we used 100µM for brain infusions, even though there is some dilution with distance since we used a ten-fold increase in concentration. We speculate that we are safe to say it mostly

blocks both channels at the dosage of 100μM; both the $Cav_{1.2}$ and $Cav_{1.3}$ channels could be fully blocked, alleviating the age-related impairment caused by increased calcium entry through the LTCCs. Alternatively, the inability to learn this task seen in aged vehicle rats could be attributed to an increased amount of calcium entry into the cell, which could allow for an easier induction of LTD as opposed to LTP, leading to a deficiency in the learning process (Neveu, and Zucker, 1996). A higher concentration of nimodipine is thus required to sufficiently reduce intracellular calcium levels to successfully induce LTP.

LTCCs have roles in many different forms of learning and memory. As a result, the role of the LTCC within the hippocampus is one of the most well-studied areas, from electrophysiology recordings to behavioural studies, demonstrating their importance. Landfield and Pitler (1984) showed that aged hippocampal slices exhibited increases in the afterhyperpolarization and calcium conductance in the hippocampal neurons, caused by higher amounts of calcium entering into the cell. From this research stemmed the 'calcium hypothesis of brain ageing' in that an increased elevation of intracellular calcium is what underlies the age-related decline in cognition. Several cognitive studies have supported the hypothesis. Blocking LTCCs in aged animals is beneficial to their ability to learn in an eyeblink conditioning task. Animals given dietary nimodipine exhibited a reduction in the number of trials required for the aged animals to learn (Deyo, Straube, and Disterhoft 1989), indicating that the LTCCs may indeed be related to or cause the decline in their cognitive ability we see in the ageing population. In contrast, evidence has also shown that the LTCCs are important in young-adult animals. It was found that animals lacking $Cav_{1.2}$ showed significant impairment in the Morris water maze, a spatial learning task (Moosmang, 2005). Animals with a deletion of $Cav_{1.2}$ showed a substantial impairment in this task, compared to controls, illustrating that the LTCCs are essential for facilitating learning in

young animals (White et al., 2008). From our present study, we have shown further evidence that LTCCs shift throughout the ageing process. In our young-adult animals, inhibition of LTCC function led to the impairment of the animal's spatial ability. Additionally, in a consistent fashion, LTCC blockade in aged animals led to a beneficial outcome in the same behavioural paradigm. This supports that an increased amount of intracellular calcium via LTCCs is believed to be the underlying cause in age-related learning deficits as a result of overexpression or the prolonged activation of LTCCs present in the cell. L-type calcium channels can directly mediate the calcium signalling required for synaptic plasticity, such as the induction of LTP, as well as translate the calcium signal to nuclear gene expression in order to achieve long-term memory formation (Rajadhyaksha Barczak, Macias, Leveque, Lewis, Konradi, 1999). In young rats, this effect dominates; therefore, when blocking LTCCs, it prevents their learning. Moreover, LTCCs can also affect AHPs and associated neuronal excitability (Landfield and Pitler, 1984), and in aged rats, this effect dominates due to the abnormal levels of calcium. The increased function of LTCCs may lead to a shift away from normal calcium homeostasis, resulting in the increase of the AHP and reduced neuronal excitability, thereby increasing the threshold required for the induction of LTP. Nimodipine treatment in aged animals may lead to a reversal of these processes and therefore result in a rescue of the animals learning.

Apart from spatial learning and memory, L-type calcium channels have also been highlighted to have roles in olfactory-based learning. In an aversive learning paradigm, blocking LTCCs in neonatal animals' main olfactory bulb (MOB) showed a significant impairment in the animal's ability to associate the odour with an aversive stimulus (Zhang et al. 2010). Further evidence stems from our lab's past research, which shows that blocking L-type calcium channels in neonatal rats in an odour preference paradigm prevented the development of long-term based

memory (Mukherjee, and Yuan, 2016). The memory, in this case, was a preference towards an odour associated with a positive stimulus. This indicates that the LTCCs are important in both valances of olfactory learning. Most likely, the effects seen in the present study are occurring during L-LTP, since $Cav_{1.2}$ LTCCs have been previously shown to be a key component of this form of LTP (Moosmang et al., 2005).

In summary of the findings above, Figure 7 suggests a hypothesized mechanism underlying the age-related changes in the vehicle animal's performance. The figure illustrates that in young-adult animals, a larger contribution of calcium entry comes from the NMDAR channel activity than LTCCs and normal functioning potassium channels (Figure 7 A). In aged animals, we see an increase in the amount of LTCCs present in the cell, and as a result, significantly increased amounts of calcium influx, causing changes to calcium homeostasis, cell toxicity and prevention of normal learning from occurring (Figure 7 B). In aged animals, the contribution of the NMDARs has decreased, with increases in potassium channel activity in line with that of LTCCs. Furthermore, once the calcium has entered the cell, a series of downstream cascades will be activated via the calcium entry. For example in this proposed system is CaMKII, which when activated will activate cAMP Response Element-Binding Protein (CREB). CREB once activated leads to the creation or regulation of expressed genes as well it has been found it play a role long-term memory and synaptic plasticity (Alberini, 2009; Dyson and Wright, 2016).

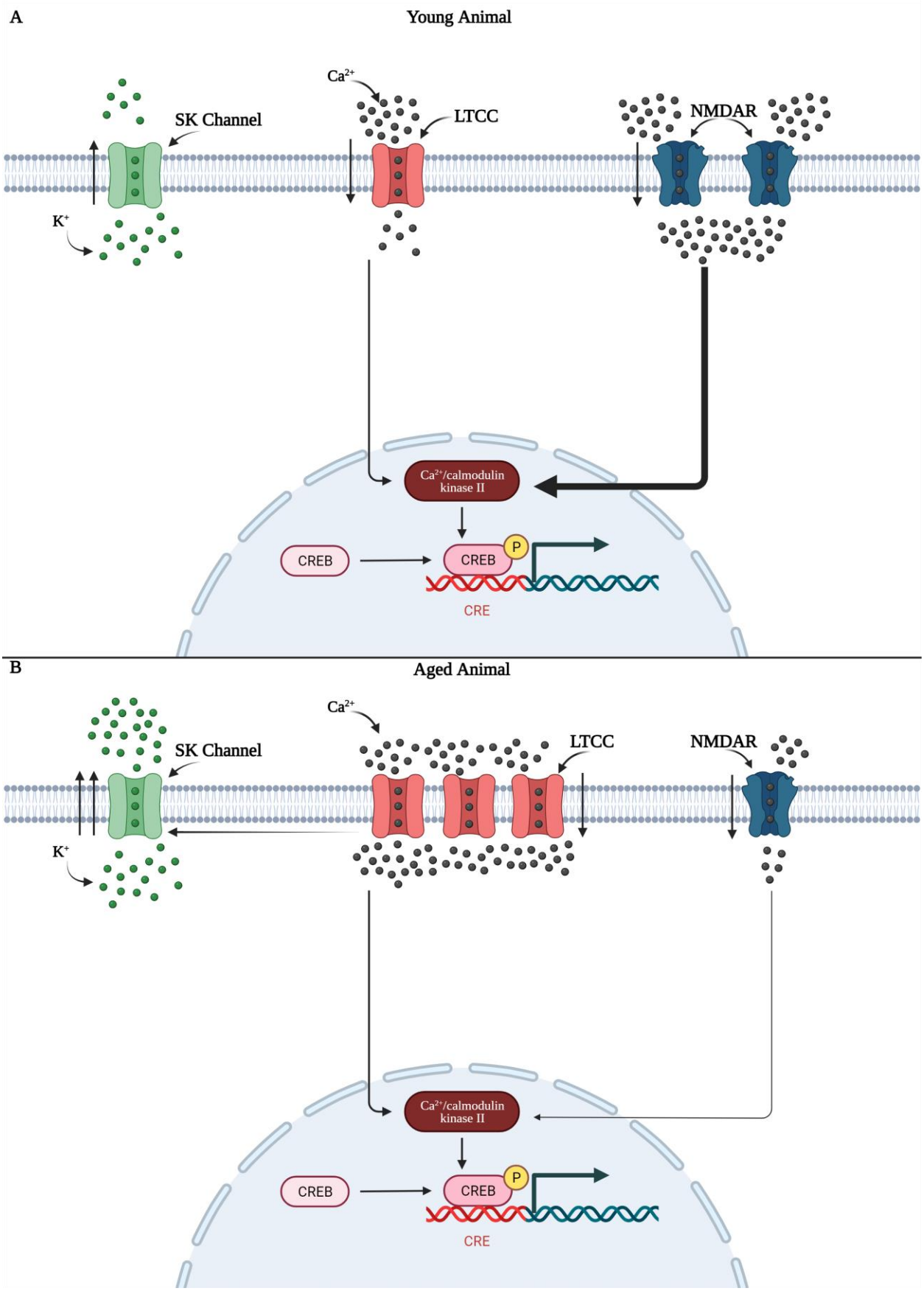
A
Young Animal
Ca2+
SK Channel
LTCC
NMDAR
K+
Ca2+/calmodulin kinase II
CREB
CREB
P
CRE
B
Aged Animal
Ca2+
SK Channel
LTCC
NMDAR
K+
Ca2+/calmodulin kinase II
CREB
CREB
P
CRE

Figure 7. *HYPOTHESIZED AGE-DEPENDENT MECHANISM OF ACTION OF LTCCS.* Panel A. Shows a hypothesized mechanism underlying the formation of new proteins following LTP in young animals. A larger contribution comes from NMDARs while a lesser amount from the LTCCs. Panel B. Shows the same mechanism, and the hypothesized shift in the amount of LTCCs present/hyperfunction, meanwhile, also leading to increase potassium channel activity. Meanwhile, the amount of calcium entering the NMDARs has decreased significantly. Adapted from "CREB Signalling Pathway", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

4.4. Clinical Significance of Findings and Future Directions

While a number of studies test the roles of LTCCs in odour or spatial learning independently, there are no consistently tested models of behaviour that explore both odour and spatial learning. Our study is the first to assess the importance of LTCCs in contextual odour learning directly and provides insight into their age-related roles in communication between the hippocampus and PC that is required for this type of learning.

According to the World Health Organization (2021), the ageing population (60 years or older) is increasing at an exceptional pace; in 2019, it was over one billion, and it is projected to be over 2 billion by 2050. In addition to this, they report that approximately 50 million people worldwide are affected by some form of mild cognitive decline or dementia, and by 2050, this number is predicted to triple in size (‘Risk reduction of cognitive decline and dementia’ 2019). As a result, our findings are crucial in providing evidence on ameliorating the cognitive decline that we see in the aged population. We have shown that the roles of LTCCs shift as we age, from beneficial in young animals to detrimental in aged animals. Upon blocking the LTCCs in aged animals, we see an amelioration of this age-related cognitive decline. Our data suggest that blockade of the L-type calcium channels may prove as a useful target or as an area of interest when it comes to treating and possibly preventing cognitive decline in our populations.

Clinically, the effects of LTCC inhibitors have also shown some beneficial effects. Patients that are at risk for developing schizophrenia, as indicated by the CACNA1C gene (a validated risk gene for schizophrenia), have significantly increased activity in the prefrontal cortex during a working memory task (Bigos et al., 2010). The researchers then investigated the potential effects of blocking the LTCCs with nimodipine in these patients and found that it led to a significant decrease in the neural activity of the parietal and frontal cortices during the same

working memory task and improved cognitive assessment scores. This suggests that the blockade of the LTCCs led to improved cortical efficiency and rescued them from the learning impairment (Zink et al. 2020). In patients with mild cognitive impairment, nimodipine treatment resulted in significant improvements in memory tests and in a mini-mental state examination (Wang et al., 2006). Previous research has also suggested that LTCCs are also involved in forms of dementia, such as Alzheimer's disease (Anekonda, and Quinn, 2011; Arispe, Pollard, and Rojas, 1994; Ueda, Shinohara, Yagami, Asakura, and Kawasaki, 1997). Amyloid-beta facilitates calcium influx through LTCCs, leading to cellular disruption of homeostasis and neuronal death. This is termed as the ß-amyloid Ca^{2+}-channel hypothesis for neuronal death in Alzheimer's disease. This is similar to the calcium hypothesis of brain ageing and provides further support that LTCCs are involved in not only age-related cognitive decline but the development of dementia as well, indicating that they are channels worth exploring when it comes to the treatment and prevention of such diseases. Clinically, nimodipine was tested for its potential therapeutic effects in treating old age dementias. Ban et al. (1990) found that patients who received an oral dose of 90mgs of nimodipine for 12-weeks, performed or scored better in a variety of measures such as the global deterioration scale (a scale for determining the stage of dementia), and many others.

Furthermore, a study conducted by Lovell and colleagues (2015) tested several calcium channel blockers (including nimodipine, among others) and their potential benefits regarding stopping or slowing down the progression of Alzheimer's disease via levels of ß amyloid proteins. They reported that the use of nimodipine (1µM) in H4 neuroglioma cells transfected to overexpress amyloid precursor protein, as well as other calcium channel antagonists, significantly reduced the rate of progression of dementia in the patients and reduced the amount of ß amyloid proteins. In addition to this, the authors reported that patients who used calcium

channel blockers and no other forms of antihypertensive medications showed a significant decrease in the rate at which the patient progressed to dementia. This further suggests that the LTCCs are involved not only in the calcium hypothesis of brain ageing but also involved in overexpression or hyperfunction in age-related disease pathophysiology.

Although the present study provides further clarification on the roles of the LTCCs throughout the span of life of young-adult animals to aged animals, there still remains a few unanswered questions. One of which is: what are the possible long-term effects of blocking the LTCCs over a long period of time? More specifically, as there is no clearly defined age in which cognitive decline begins, would taking nimodipine chronically throughout mid-late adulthood into an aged time point have any adverse or beneficial effects*?* Pinpointing the exact age at which one should start 'treatment' of nimodipine would prove quite tricky, as not everyone will experience the same time course when it comes to this impairment. It would be interesting to explore whether starting 'treatment' during adulthood would lead to the same beneficial results that we see in our acute treatment in this study. In addition, what would be, if any, negative effects that would be present? Is it possible that the chronic blocking of the LTCCs leads to an overall reduction in the amount of LTCCs expressed, thus making blocking no longer beneficial to the same extent? Furthermore, something that was not considered in this study was the effects of nimodipine on animals with induced cognitive impairments. Ghosh and colleagues (2019) have established a pre-tangle tau rat model in which the animals at 7-8 months post-infusion of a virus that contains the genetic construct of human pseudo-hyperphosphorylated tau showed apparent deficits in an odour discrimination task. If we adopted such a model, it would be interesting to see whether during this time period of 7-8 months post infusion, animals would show the same response to nimodipine as in our present study (i.e., impairment in the olfactory

learning task) or if the treatment of nimodipine would ameliorate the deficits seen in this model. It would also be interesting to explore aged animals in the same model to explore once again if nimodipine leads to the same result or another.

Like all studies, this study is not without its own limitations. In this present study, only one dosage of nimodipine was used for both the i.p. injections and direct infusions in olfactory learning tasks. Previous studies have used a variety of dosages ranging from 1mg/kg to 40mg/kg for i.p. injections (Cain, 2005; Levere, and Walker, 1992). This brings forth the question of whether or not the dosage used was an appropriate amount. A way to address this possible concern would be to use a range of dosages to explore the therapeutic effects of them from a lower dosage, a mid-range dosage, to a high dosage, to examine if the same results are found and the potential differences that arise. Another possible limitation of this study would be the number of animals used. Ageing studies are difficult to conduct as the animals often develop health issues as a result of their age and often had be euthanized before or during behavioural experiments. A simple solution to this would be to increase the number of animals used to yield more data from different animals. Due to the widespread expression of LTCCs, Nimodipine may have also affected the cardiovascular and neurovascular system (Terrar, 1993). Vascular effects of systemic and local nimodipine administration may have had physiological effects leading to changes in animal behaviour. However, Nimodipine injections of a similar dosages has been previously used in rabbits and rats, and it was found to have little effect on the cardiovascular system in response to the administration of the drug (Woodruff-Pak et al., 1997; Chakrabarti et al., 1998; Michaluk et al., 1998). In addition, in Sprague-Dawley rats, nimodipine injection (5mg/kg i.p.) had no effect on the blood flow of the animal (Chakrabarti et al., 1998). However, it has been found that nimodipine does have effects on cerebral blood flow (Scriabine and van

den Kerckhoff, 1988), which could possibly lead to enhanced blood flow and improved cognitive abilities. Despite this, it does not account for the opposite effects of nimodipine we observed in young-adult animals vs. aged animals.

5.0. Conclusion

This study is the first to explore the roles of L-type calcium channels in adult and aged animals in the same behavioural models. It has also established behavioural models to explore the LTCC associated brain plasticity and has helped to explain the LTCC-associated calcium ageing hypothesis further. Here we show that in adult animals (6-9 months old), when treated with nimodipine via i.p. Injection or direct infusions to the piriform cortex or the CA1 region of the hippocampus, leads to the significant impairment in their ability to learn an odour associative task, as well as a spatial discrimination task. In the same behavioural tests, aged animals (>18 months old) given identical treatments of nimodipine show a complete reversal in their performance. Nimodipine treated animals show a significant improvement in their abilities to perform these tasks while their vehicle counterparts showed an apparent deficit. Our results suggest that the LTCCs roles do indeed switch from being beneficial and critical to everyday learning and memory formation in the young population to impairing the same processes in aged populations. Given that there is an ever-increasing rise in our ageing population and the development of age-related dementias, further understanding of all contributing players to the progression of such aliments is paramount in providing not only effective treatment but prevention as well.

6.0. References

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7.0. Appendices

Dear: Dr. Qi Yuan, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20192537
Animal Care File: 18-02-QY
Entitled: (18-02-QY) The roles of L-type calcium channels in memory formation
Related Awards:

Awards File No	Title	Status	
20160955	Memory: Modifiable odor representations, adaptive behavior and Alzheimer's disease	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20181548	L-type calcium channels in memory	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Approval Date: February 05, 2019
Next Annual Report Due: February 05, 2022
Ethics Clearance Expires: February 05, 2022

Your Annual Report was reviewed by the ACC and approved.

Animal use records will be compiled and reported to the Canadian Council on Animal Care.

NOTE: You can access a copy of this email at any time under the "Shared Communications" section of the Logs tab of your file in the Memorial Researcher Portal.

Sincerely,

ANULIKA MBAKWE | ACC COORDINATOR
Department of Animal Care Services
Memorial University of Newfoundland
Health Sciences Centre | Room H1848
P: 709-777-6621
E-Mail: ambakwe@mun.ca