Age-related LTCC and NMDAR expression in the hippocampus and olfactory cortex in rats

by

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Abstract

Neuronal calcium is a critical mediator for learning and memory. However, with age, calcium dysregulation leads to cognitive decline. L-type calcium channels (LTCCs) and N-methyl-D-aspartate receptors (NMDARs) mediate Ca^{2+} influx in neurons. We hypothesized that there are age-related changes of calcium channel expression in the piriform cortex (PC) and hippocampus, two areas that are crucial for olfactory and spatial learning ability respectively. We measured synaptic and extrasynaptic levels of LTCCs (Cav1.2) and NMDAR subunits (GluN1, GluN2A, and GluN2B) in neonatal, adult, and aging rats using Western blot. PSD-95 colocalizing and non-colocalizing Cav1.2 expression were compared between adult and aging brains using immunohistochemistry and confocal microscopy, and we further investigated somatic and dendritic expression of both Cav1.2 and Cav1.3 subunits. The expression of hippocampal synaptic, but not extrasynaptic, NMDARs was higher in adult and aging groups compared to neonates. However, GluN2A/2B ratios and synaptic:extrasynaptic ratios of NMDAR subunits were similar across age groups. In contrast, in the PC, GluN2A/2B and synaptic:extrasynaptic ratios were higher in adult PC compared to neonates. In hippocampal CA1 and PC, the soma:dendritic ratio of Cav1.2 expression increased with aging, but the soma:dendritic ratio of Cav1.3 expression decreased. Extrasynaptic Cav1.2 non- PSD95 colocalizing expression was also found to have higher expression in the aging PC compared to adult. Our data suggest that PC and hippocampus are different in age-related channel expression. PC maturation is accompanied by a switch from GluN2B to GluN2A subunits. Higher somatic Cav1.2, but not Cav1.3 expression in

CA1 and higher extrasynaptic Cav1.2 in the PC may correlate with aging-associated disruption of calcium homeostasis and cognitive decline.

General Summary

Memory plays an important role in our lives. Our existence would be meaningless without memory. With age, people experience learning and memory impairments, which complicate their lives tremendously. Understanding the mechanism behind this impairment is critical for enhancing the quality of life. The first step toward reaching this goal is to understand the molecular basis of age-related memory loss.

Calcium plays a critical role in memory and learning, but too much calcium may lead to calcium toxicity and can disrupt memory. In this study, we investigated whether the expression or location of calcium channel subunits changes with age. We found that overexpression of calcium channels subunits were more prominent in hippocampal CA1 and posterior piriform cortex, suggesting that these areas are most susceptible to calcium homeostasis disruption caused by aging. This dyshomeostasis may result in impaired memory and learning abilities.

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AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
APC	anterior piriform cortex
BDNF	brain-derived neurotrophic factor
CaMK	calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CREB	cyclic-AMP response element binding
DG	dentate gyrus
EC	entorhinal cortex
EPSC	excitatory post-synaptic current
ERK1/2	extracellular signal-regulated kinases 1/2
Jacob	juxtasynaptic attractor of caldendrin on dendritic boutons
LEC	lateral entorhinal cortex
LOT	lateral olfactory tract
LTCC	L-type calcium channel
LTD	long term depression
LTP	long term potentiation
M	molar
MAPK	mitogen-activated protein kinase
MEC	medial entorhinal cortex

NMDAR	N-methyl-D-aspartate receptor
OB	olfactory bulb
OE	olfactory epithelium
ONL	olfactory nerve layer
OR	odorant receptor
OSN	olfactory sensory neuron
PC	piriform cortex
PFA	paraformaldehyde
PKA	protein kinase a
PKC	protein kinase c
PP	perforant path
PPC	posterior piriform cortex
PSD	postsynaptic density
qPCR	quantitative polymerase chain reaction
s	second
SD	standard deviation
SD[1]	sprague dawley
SEM	standard error of the mean
TA	temporoammonic path
TORC	transducer of regulated CREB
VSCC	voltage-sensitive Ca^{2+} channels

[1] SD rat

1. Introduction

1.1. Role of calcium in learning and memory

Memory makes us who we are and shapes our personalities. Without memories, we would not be able to learn from our mistakes and adapt to an ever-changing environment. Despite learning and memory being one of the most studied areas of neuroscience, there is still so much unknown about this field.

Memory refers to the process of retaining information from previously experienced situations, whereas learning refers to the acquisition of memories (Okano et al., 2000). A memory can be achieved through a series of procedures which require alterations in the brain. Neuroscientists believe memory results from changes in the efficacy of synaptic transmission, a process known as synaptic plasticity. Synaptic plasticity includes activity-dependent changes at pre-existing synapses as well as structural changes in synaptic connections (Korte & Schmitz, 2016).

Long-term potentiation (LTP) and long-term depression (LTD) are two forms of synaptic plasticity that are proposed as cellular mechanism underlying learning and memory (T. V. Bliss & Collingridge, 2013; T. V. P. Bliss & Lømo, 1973). LTP involves synaptic strengthening that lasts at least one hour. (T. V. P. Bliss & Lømo, 1973). LTP is classified into two types: early LTP, which is independent of transcription and translation and which last 1-3 hours, and late LTP, which is dependent on altered gene expression and lasts more than 3 hours (T. V. P. Bliss & Collingridge, 1993). LTD refers to any form of depression in

the synaptic transmission. Following a low-frequency repetitive stimulation, transmission efficiency at synapses decreases, leading to LTD (Ito, 1989). LTP and LTD are synaptic plasticity forms that have been proposed as cellular or biological learning and memory substrates.

The conversion of electrical signals into biochemical activity is mediated by Ca^{2+} levels in the post-synaptic cell (Korte & Schmitz, 2016). The induction of both LTP and LTD is known to be dependent on intracellular Ca^{2+} (Zucker, 1999). For instance, injection of Ca^{2+} chelators has been shown to prevent LTP and LTD induction (Cavazzini et al., 2005; Mulkey & Malenka, 1992).

Elevation of Ca^{2+} levels within the postsynaptic neuron triggers a series of events that ultimately lead to the amplification of synaptic transmission. These events include the activation of kinases (Sacktor, 2011) such as PKA, Ca^{2+}/calmodulin-dependent protein kinases (CaMK), Fyn, Scr, and PKC, which function in various signal transduction cascades to sustain an increase in synaptic activity (Kandel, 2012). Levels of intracellular Ca^{2+} levels can rise via three mechanisms: the activation of NMDA receptors and voltage-sensitive Ca^{2+} channels (VSCC), and internal store release (Segal & Korkotian, 2014).

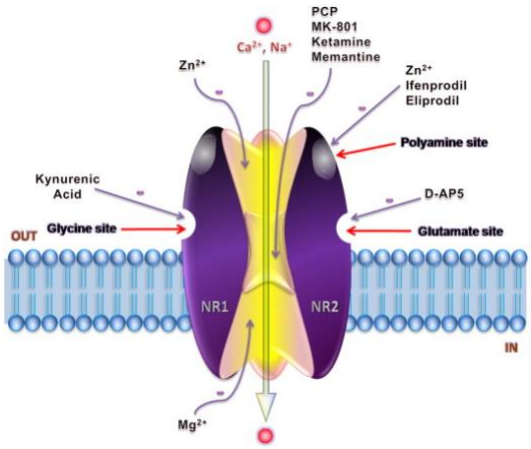
1.2. NMDAR structure and function

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that are involved in many important brain functions such as synaptic plasticity, learning and memory, neuronal survival and death, synaptogenesis and synaptic development (McBain & Mayer, 1994; Nakanishi & Masu, 1994). The elimination of in vivo NMDAR activity causes widespread apoptosis in developing neurons (Gould et al., 1994; Ikonomidou et al., 1999). They are also necessary for normal motor coordination, as the absence of these receptors in rat cerebellum leaves the animals uncoordinated during locomotion (Kadotani et al., 1996).

NMDARs are permeable to sodium (Na^+), potassium (K^+) and Ca^{2+} (Dingledine, 1983; MacDermott et al., 1986; McBain & Mayer, 1994). They are inactive at resting membrane potential due to the voltage-sensitive blocking action of extracellular magnesium (Mg^{2+}) ions (Fig. 2.1). In order for NMDARs to be activated, AMPA receptors must open and allow Na^+ ions into the post-synaptic neurons, resulting in fast excitatory post-synaptic currents (EPSCs) and depolarization of the postsynaptic membrane. Upon membrane depolarization, Mg^{2+} blockade is lifted, leading to NMDARs opening and Ca^{2+} influx to the post-synaptic compartment (Nicoll & Roche, 2013). NMDAR activation results in insertion of more AMPARs in the postsynaptic membrane, causing LTP (Malinow et al., 2000). By contrast, a lower NMDAR activation leads to AMPAR removal from the postsynaptic membrane , causing LTD (Holtmaat & Svoboda, 2009; Malenka & Bear, 2004). The induction of both LTP and LTD is dependent on the amount and kinetics of the calcium influx

into the postsynaptic neuron (Ismailov et al., 2004; McBain & Mayer, 1994) and Ca^{2+} influx is strongly controlled by NMDAR subunit composition (Kehoe et al., 2013).

NMDARs consist of seven subunits, which are classified into three subfamilies: GluN1, GluN2 and GluN3 subtypes. In the central nervous system (CNS), NMDA receptors containing GluN1 and GluN2 form the functional NMDARs (Kehoe et al., 2013). Functional NMDARs are heteromeric structures composed of two obligatory GluN1 subunits and two GluN2 subunits which have four isoforms (GluN2A, GluN2B, GluN2C, and GluN2D). For activation two molecules of glycine and two molecules of glutamate simultaneously bind to unique external binding sites on GluN1 and GluN2 subunits respectively (Fig. 1.1) (Benveniste & Mayer, 1991; Clements & Westbrook, 1991).



[2] Fig. 1.1. NMDARs once activated are permeable to Ca2+

The GluN1 subunit is functionally equivalent to the NMDA receptor, while various GluN2 subunits determine properties of the receptor, including glutamate affinity, open

[2] (Ghasemi & Schachter, 2011) Reproducing this figure with the permission of the copyright holder.

probability, deactivation kinetics, channel conductance and subcellular localization (Erreger et al., 2007; Furukawa et al., 2005; Papadia et al., 2008; Sanz-Clemente et al., 2013; Vieira et al., 2020)

The glutamate affinity of GluN2D-containing receptors is the highest (EC_{50} ~ 0.4 μM) While GluN2A-containing receptors have the lowest sensitivity to glutamate (EC_{50} ~ 4 μM). Both GluN2B (EC_{50} ~ 2 μM) and GluN2C-containing receptors (EC_{50} ~ 1 μM) possess a moderate affinity to glutamate (Brimecombe et al., 1997; Sanz-Clemente et al., 2013; Vieira et al., 2020).

Additionally, the channel open probability of the GluN2A receptors is highest when agonists are fully bound to the receptors (P_o ~ 0.5), then comes the GluN2B receptors (P_o ~ 0.1) and the GluN2C and GluN2D-containing receptors have the lowest open probability (P_o ~ 0.05) (Vieira et al., 2020).

Deactivation kinetics are measured by the excitatory post-synaptic current decay. GluN2A-containing receptors have the fastest deactivation rate (Paoletti, 2011) (τ_{decay} ~ 50 ms), more than 20 times faster than GluN2D-containing receptors (τ_{decay} > 1 s), while Glu2B (τ_{decay} ~ 400 ms) and GluN2C (τ_{decay} ~ 290 ms) display intermediate decay rates (Vieira et al., 2020). Overall, the channel conductance of GluN2A and GluN2B receptors is higher (γ ~ 40–50 pS) than that of GluN2C and GluN2D (γ ~ 18–35 pS) (Vieira et al., 2020). The composition of subunits is highly influenced by brain area and developmental stage.

1.2.1. Age-dependent changes of NMDAR composition and subcellular distribution

GluN1 is the essential unit of NMDARs, while distinct pharmacological and kinetic properties of NMDARs are owed to different GluN2 subunits (Akazawa et al., 1994; Dingledine et al., 1999; Ishii et al., 1993; Meguro et al., 1992; Moriyoshi et al., 1991). Thus the functional properties of GluN2 subunits are responsible for the heterogeneity of NMDA receptors in the brain. In different brain regions, the expression pattern of GluN1 and GluN2 subunits changes with age.

Monyer has shown that GluN1 mRNA increases throughout the developmental stages into adulthood (Monyer et al., 1994). GluN2A and GluN2B are the dominant GluN2 subunits in the hippocampal and cortical neurons (Qiu et al., 2011). In the first 3 weeks of development, GluN2B-containing NMDARs are primarily responsible for NMDAR-dependent neurotransmission, whereas after this period GluN2A-containing receptors predominate (Wenzel et al., 1997). The mRNA levels of GluN2A subunit in the hippocampal neurons rise from birth through 3 weeks postnatally (Guilarte & McGlothan, 1998; Monyer et al., 1994; Ritter et al., 2002; Riva et al., 1994; Zhong et al., 1995) in parallel with GluN2A protein expression level (Luo et al., 1996; Wenzel et al., 1997). As such almost after two postnatal weeks, NMDA EPSCs are unaffected by an GluN2B antagonist, CP101,606 (Rumbaugh & Vicini, 1999), whereas GluN2A antagonists inhibit LTP induction in hippocampal slices of three to four weeks old rats (X.-B. Liu, 2004) suggesting that NMDARs with NR2A subunits mediate LTP signaling in mature tissues. In this

developmental period, the GluN1/GluN2A complex, which has fast offset kinetics, dominates at the synapse, while the GluN1/GluN2B complex, which has slow kinetics, can be often found in extrasynaptic membrane (Rumbaugh & Vicini, 1999) suggesting that subcellular distribution of the NMDAR varies significantly depending on the subunit composition.

According to anatomical, physiological, and behavioral evidence, different cortical sensory modalities such as gustatory, olfactory, somatosensory, auditory, and visual develop at different rates (Fox, 1995). In the critical period of plasticity, GluN2-containing NMDARs are involved. It is during this time that synaptic maturity takes place, which requires a switch from GluN2B to GluN2A in NMDAR subunit composition (Barria & Malinow, 2002). Evolutionarily, this developmental switch occurs in other brain areas as well, including the cortex, hippocampus, amygdala, and cerebellum (Sheng et al., 1994).

From post-natal days 3-6, primary somatosensory cortex responds less sensitively to ifenprodil (an GluN2B antagonist) and LTP-inducing stimulation (Barth & Malenka, 2001; Crair & Malenka, 1995) suggesting that the onset of somatosensory maturation occurs around the first week of development. Replacement of synaptic GluN2B-NMDARs by GluN2A-NMDARs and an increase in GluN2A:GluN2B occurs by the end of second postnatal week in the visual (Quinlan, Olstein, et al., 1999) and auditory cortex (Hsieh et al., 2002). Hippocampus and cerebellum mature more than a week after the visual cortex. At about 2 weeks of age, both GluN2A and GluN2B are highly expressed in hippocampal synapses and LTP and LTD are at their peak (Dudek & Bear, 1995; Harris & Teyler, 1984; Mulkey & Malenka, 1992).–The postnatal third week is the time when the GluN2A: GluN2B ratio

reaches its maximum. During this time the number of GluN2A-NMDARs increases in the hippocampus and replaces GluN2B-NMDARs (Wenzel et al., 1997). So as the animal develops, NMDARs containing the GluN2A, which has fast offset kinetics, dominates at the synapses, while those containing the GluN2B subunit, which have slow kinetics are displaced to extrasynaptic sites (Rumbaugh & Vicini, 1999; van Zundert et al., 2004), such that the composition of NMDAR subunits has a significant impact on their subcellular localization.

Up to three-quarters of all GluN2B containing NMDARs in immature hippocampal neurons are extrasynaptic (Tovar & Westbrook, 2002). After the critical developmental period, when GluN2B-NMDARs are pharmacologically blocked, neither learning (X.-B. Liu, 2004) nor performance in the Morris water maze are changed (Guscott et al., 2003), emphasizing that GluN2B-NMDARs modulate synaptic plasticity as opposed to mediating it (Dumas, 2005). However, GluN2A antagonists inhibit LTP induction in hippocampal slices of three to four weeks old rats (X.-B. Liu, 2004) suggesting that NMDARs with NR2A subunits mediate LTP signaling in mature tissues. Thus, during postnatal development of the brain, NMDARs switch from predominantly GluN2B- to GluN2A-containing receptors that results in changes in their biophysical properties and downstream signaling cascades (Paoletti et al., 2013).

In the postnatal third week through adolescence, the hippocampal DG GluN2A: GluN2B ratio markedly increases, and it continues to increase until adulthood. Age-related upregulation of GluN2A and downregulation of GluN2B is responsible for this increase (Ge et al., 2019).

In adult brains, there is a regional distribution of GluN2B subunit expression. A higher number of GluN2B-containing NMDARs are found at hippocampal synapses on the left side of adult mice compared to the right hemisphere (Kohl et al., 2011). In adulthood through aging, however, there is a continuation of the developmental decrease of GluN2B as mRNA levels decrease (Ontl et al., 2004). As the mRNA levels of the GluN2B subunit decline throughout development and aging, the density of glutamate binding to NMDA receptors decreases significantly as well (Ontl et al., 2004), suggesting that Glu2B subunit changes are likely responsible for some of the changes in NMDA receptor binding (Magnusson, 2012). In the prefrontal/frontal cortex of mouse brain, aligned with mRNA downregulation, GluN2B and GluN1 protein expression decreases with age, and these changes are linked to age-related deficits in spatial memory performance (Magnusson et al., 2007). Within the hippocampus, apart from age-related declines in GluN2B expression (Sonntag et al., 2000; Zhao et al., 2009), dramatic declines in GluN1 subunit protein expression in CA2/3 of the hippocampus have also been observed (P. Liu et al., 2008). This decline in the hippocampus negatively influences the performance of aged mice in the Morris water maze while good spatial learning performance of adult mice is associated with a high level of GluN2B and GluN1 expression in the synaptic membrane of the hippocampus (Zhao et al., 2009). Transgenic mice overexpressing the GluN2B subunit despite declining protein levels over time retain their higher levels of GluN2B and have better novel object recognition, contextual and cued fear conditioning, spatial reference memory, and spatial working memory than wild-type mice (Cao et al., 2007). Hence, maintaining GluN2B levels higher than those seen in normal aging rodents could be beneficial for memory. Some dietary interventions in old rodents such as a long-term intermittent fasting diet, increase mRNA expression for the GluN2B subunit in the hippocampus, but not for GluN2A or GluN1

subunits, which would result in improved learning and consolidation of various tasks and synaptic efficiency (Fontan-Lozano et al., 2007)

Fractionation of hippocampus into whole-tissue homogenate, light membranes and synaptic membranes shows that GluN2B subunit proteins display an age-related decline in all the fractions (Zhao et al., 2009), suggesting that aging does not affect subunit localization, and the decline is more widespread, indicating a problem of production. Nevertheless, other studies show that the decrease occurs together with a change in the localization of the receptors with age, GluN2B containing NMDARs migrate from synaptic to extrasynaptic sites by lateral diffusion (Avila et al., 2017; Potier et al., 2010). The imbalance between synaptic and extrasynaptic NMDARs may contribute to the cognitive decline in the elderly. In other words, aging may change NMDA receptor location, which may alter the signaling cascade for protein synthesis.

1.2.2. Synaptic and extrasynaptic NMDAR

The lateral diffusion between synaptic and extrasynaptic compartments determines the characteristics of the NMDAR induced responses (McQuate & Barria, 2020). The activity of synaptic NMDARs contribute to cell survival, whereas stimulation of extrasynaptic NMDARs causes neuronal death (Hardingham et al., 2002). Hence, the fate of neurons depends not just on the degree of NMDAR activity but also on synaptic and extrasynaptic components.

A more accurate description is the x-shaped model of NMDAR-dependent excitotoxicity which shows the opposing effects of increasing synaptic and extrasynaptic NMDAR activity on promoting neuronal death or survival (Hardingham & Bading, 2010). NMDAR hypoactivation damages neurons while enhancement of NMDAR activity at the synapses helps neurons survive by triggering multiple neuroprotective pathways. Conversely, activation of extrasynaptic NMDARs at low levels does not affect neuronal survival, while activating extrasynaptic NMDARs promotes cell death (Hardingham & Bading, 2010).

If synaptic and extrasynaptic NMDARs play fundamentally different roles on neuronal fate, these two populations of receptors must have different downstream cascades. There are still differences between the two cascades even when the overall Ca^{2+} amounts are similar (Hardingham & Bading, 2010). The Ca^{2+}, which enters the soma through synaptic NMDARs, invades the nucleus where it targets Ca^{2+}/calmodulin-dependent protein kinase IV(CaMKIV). Synaptic NMDAR stimulation also results in an increase in extracellular signal-regulated kinases 1/2 (ERK1/2) activity (Hardingham & Bading, 2010). Both the fast-acting nuclear CaMKIV pathway and the slower acting, longer lasting ERK1/2 pathway mediate the phosphorylation of cyclic-AMP response element binding protein (CREB) (Hardingham et al., 2001a, 2001b; Wu et al., 2001). CREB is activated by phosphorylating Ser133, which recruits CREB binding protein (CBP) and initiates transcription (Chawla et al., 1998; Chrivia et al., 1993; Ghosh & Greenberg, 1995).

CREB is a signal-regulated transcription factor that plays a critical role in neuronal survival, neurogenesis, and learning and memory (Lonze et al., 2002; Mayr & Montminy,

2001; Shaywitz & Greenberg, 1999). Another essential step in the CREB activation process is the import of the transducer of regulated CREB activity (TORC) into the nucleus through calcineurin-dependent dephosphorylation (Conkright et al., 2003). In rat hippocampal neurons activation of CREB induces the expression of brain-derived neurotrophic factor (BDNF) (Jiang et al., 2005; Papadia et al., 2008). The activation of BDNF in the hippocampus protects neurons against glutamate excitotoxicity (Jiang et al., 2005). BDNF enhances antioxidant defense and provides neuroprotection (Parsons & Raymond, 2014).

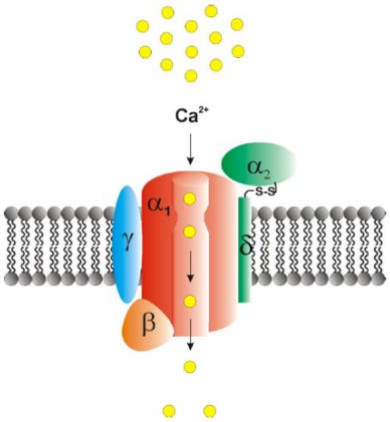
In contrast, activation of extrasynaptic NMDARs triggers signalling pathways that lead to the inactivation of the ERK1/2 (Ivanov et al., 2006) and import of juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob) into the nucleus (Dieterich et al., 2008) that promotes CREB dephosphorylation and activates the CREB shut off pathway (Hardingham et al., 2002; Hardingham & Bading, 2002). Nuclear knockdown of Jacob inhibits CREB shut-off following extrasynaptic NMDAR activation, while its nuclear overexpression induces CREB shut-off without NMDAR stimulation (Dieterich et al., 2008).

Studies indicate that the main factor influencing this dichotomy is the location of the NMDAR, which is referred to as the 'localization hypothesis' (Hardingham et al., 2002) and their location can be identified immunohistochemically (for example, distal from the postsynaptic density (PSD)).

1.3. LTCC structure and function

The voltage-gated calcium channels (VGCCs) are key molecules that allow calcium ions into electrically excitable cells after membrane depolarization. A variety of neuronal functions are mediated by them, including activating neurotransmitters, generating dendritic Ca^{2+} spikes, and regulating gene expression in response to activity. Neurons express different kinds of VGCCs, including L-, P/Q, N, and R-type Ca^{2+} channels (Catterall, 2000).

L-type calcium channels (LTCCs) are voltage gated calcium channels, characterized by their long-lasting activation during depolarization. LTCCs are located post-synaptically at both synaptic and extrasynaptic sites (Hell et al., 1996, p. 1; Leitch et al., 2009; Obermair et al., 2004) and their activation is subsequent to NMDARs activation (Mukherjee & Yuan, 2016). LTCCs are comprised of the Cav1 family, including Cav1.1, Cav1.2, Cav1.3, and Cav1.4 isoforms (Catterall et al., 2005). Cav1.2 and Cav1.3 are the predominant brain LTCCs, while Cav1.2 is the most abundant one (Obermair et al., 2004). As a result of radioreceptor assays with isradipine and quantitative polymerase chain reaction (qPCR) experiments, Cav1.2 accounts for 89% of all brain LTCCs, while Cav1.3 accounts for 11% (Sinnegger-Brauns et al., 2009). They are composed of a pore-forming α1 subunit that contains the voltage sensor and the binding sites for most regulatory modulators and drugs, and the accessory α2/δ and β subunits that are involved in anchorage, trafficking, and regulatory functions (Fig. 1.2). CACNA1C and CACNA1D encode the α1 subunits for Cav1.2 and Cav1.3 respectively (McCarthy et al., 2016).



[3]*Fig. 1.2. L-type calcium channels are composed of a pore-forming α1 subunit that contains the voltage sensor and the binding sites for most regulatory modulators and drugs*

Many important functions are performed by LTCCs in the brain, including contributing to dendritic Ca^{2+} spikes (Sjöström & Nelson, 2002), postsynaptic LTP (Grover & Teyler, 1990), as well as transcriptional regulation of genes important for synaptic plasticity and neuronal survival (West et al., 2002). LTCCs play a central role in neuronal function by converting electrical activity into biochemical signals (Berridge, 2014). It is necessary for signalling to reach the nucleus in order to carry out these functions. Communication with the nucleus occurs via activation of either calmodulin-activated kinase (CaMK) (Bito et al., 1996) or the mitogen-activated protein kinase (MAPK/Erk) pathway (Xing et al., 1996) that phosphorylates CREB in the nucleus (Ma et al., 2012).

The CaMK pathway enables accurate transmission of synaptic stimulations due to its greater speed and sensitivity (Finkbeiner et al., 1997) while the MAPK/Erk pathway

[3] (Wormuth et al., 2016) Image reproduced under Creative Commons Attribution-Non-Commercial 4.0 International Public License (CC BY-NC 4.0).

promotes a slow and late phase of CREB phosphorylation (Dolmetsch et al., 2001; Wu et al., 2001). Both rapid CaMK and slow MAPK signaling differ by only 1 min, yet their differential impacts on CREB phosphorylation last much longer, beginning at 20 min but lasting beyond 60 min which would promote transcription of multiple genes (Wu et al., 2001). Depending on the signaling pathway, changes in gene expression can result in altered protein expression.

Following membrane depolarization, LTCCs are the main responsible calcium gate for activating CREB-dependent transcription in the nucleus (Finkbeiner et al., 1997). The LTCC blocker nimodipine eliminates phosphorylated CREB, but NMDA blockers (APV) appears to be slightly less effective on activation of CREB (Dolmetsch et al., 2001) suggesting that LTCC is the source of Ca^{2+} that is necessary to sustain CREB Ser133 phosphorylation.

1.3.1. LTCC subcellular localization

Cav1.2 and Cav1.3 channels differ in biophysical properties, in subcellular localization (Lacinová, 2005), and in their coupling to the gene transcription machinery (Zhang et al., 2006).

In rat brain, immunocytochemical analysis shows that neurons of the hippocampus express both Cav1.2 and Cav1.3 channels abundantly. However, there is a differential distribution of the two subtypes within individual neurons. Cav1.2 channels are primarily

found in cell bodies and proximal dendrites of the hippocampus, with decreasing density in more distal dendritic areas, whereas Cav1.3 channels are most prominent in cell bodies (Hell et al., 1993). However, Obermair shows that Cav1.2 is expressed fairly uniformly throughout the entire dendritic tree, over the soma, and in the initial segment of the axon of the hippocampal area (Obermair et al., 2004). Furthermore, Cav1.2 and Cav1.3 are localized in both synaptic and extrasynaptic areas in pyramidal cells and interneurons of the hippocampus (Obermair et al., 2004).

In spite of the 75% sequence homology between the CACNA1C and CACNA1D genes, the channels differ significantly in their electrophysiological properties (Catterall et al., 2005). Cav1.3 channels are activated more rapidly and at more hyperpolarised membrane potentials than Cav1.2 channels (Hofmann et al., 2014). Cav1.3 channels also inactivate more slowly than Cav1.2 channels (Koschak et al., 2001).

Cav1.2 and Cav1.3 are expressed by most brain neurons including hippocampal pyramidal neurons, dentate granule neurons, cortical neurons, cerebellar Purkinje cells, many interneurons, and many other classes of central neurons (Chin et al., 1992; Hell et al., 1993). However, both subtypes are distributed differently within individual neurons.

1.3.2. Role of LTCC in hippocampus-dependent learning

Mice with a global homozygous knockout of Cav1.2 (CACNA1C) die before day 14.5 postcoitum (Seisenberger et al., 2000), whereas mice with a global knockout of Cav1.3 (CACNA1D) survive with normal neurological function (Clark et al., 2003), but they develop deafness and sinoatrial node dysfunction (Platzer et al., 2000). On the other hand, when Cav1.2 is deleted in the hippocampus and cortex after birth, significantly impaired spatial memory is observed 30 days after Morris water maze training (Morris et al., 1986), providing evidence that Cav1.2 plays a crucial role in consolidating remote spatial memories (White et al., 2008). The CACNA1C -deficient mice are not just severely impaired in hippocampus-dependent spatial memory, but also show a selective loss of protein synthesis sensitive NMDAR-independent late phase LTP (Moosmang et al., 2005).

1.3.3 Age-dependent changes of LTCC

A newborn rodent is blind, deaf, and has limited motor skills. Their survival depends on odor learning during the first two weeks after birth when they are under the care of the nest (Kojima & Alberts, 2009; Moriceau & Sullivan, 2004). Odor cues play a critical role in nipple attachments as well as orientation to the mother and litter (Sullivan et al., 2000; Wilson & Sullivan, 1994). In the first 10 days following birth, pups associate the mother's odor with maternal care, and this is what defines early odor preference (Moriceau & Sullivan, 2005; Sullivan et al., 2000). Upon exposure to an odor, LTCCs are activated, mediating intracellular

processes that lead to plasticity in olfactory bulb neurons (Jerome et al., 2012). The LTCC expression is higher in pyramidal neurons of the piriform cortex of mice during the highly plastic period including P7–10 compared with mice in the P14–20 period (Ghosh et al., 2017) mediating more LTCC-dependent Ca^{2+} influx critical for LTP (Grover & Teyler, 1990; Kapur et al., 1998; Moosmang et al., 2005).

LTCC currents in hippocampal neurons are altered during aging in rats (Campbell et al., 1996; Landfield, 1994; Shankar et al., 1998; Thibault et al., 2001; Thibault & Landfield, 1996) and rabbits (Moyer et al., 1992; Moyer & Disterhoft, 1994). The age-related current elevation is prominent in CA1 of the hippocampus (Campbell et al., 1996; Moyer & Disterhoft, 1994). In addition to LTCC current changes, there are studies reporting conflicting evidence about changes in LTCC numbers.

The amount of mRNA that encodes the Cav1.3 subtype increases with age in the CA1 of hippocampus of aged Fisher 344, 25-month old rats, compared to adult rats 4 months old (Herman et al., 1998), align with Cav1.3 protein expression (Veng & Browning, 2002), which correlates with LTCC current in single hippocampal neurons in CA1 (Chen et al., 2000). In area CA3, expression of the Cav1.3 subtype has been shown to be decreased with age (Veng & Browning, 2002), although some reports indicate that age-related increases in binding of [3H]nimodipine have been observed in the area CA3 of Fisher 344 24-month old rats (Araki et al., 1997).

According to western blotting and immunohistochemical analysis, one study showed that both young (4-month-old) and older (24-month-old) Fischer 344 rats have unchanged expression levels of Cav1.2 protein in their hippocampal CA1, CA3, and DG regions (Veng

& Browning, 2002). While the results of other studies indicate that in hybrid Fischer 344 X Brown Norway aged rats, there is a reduction in Cav1.2 and Cav1.3 total protein levels in all 3 major areas of dorsal hippocampus: CA1, CA3 and DG (Núñez-Santana et al., 2014) suggesting that age-related calcium toxicity cannot be due to a global change in the LTCC expression. Furthermore, the authors found that there is an age-related increase in Cav1.2 surface expression in CA1 and CA3 of aged hippocampus, as well as Cav1.3 in CA1 (Núñez-Santana et al., 2014) suggesting that changes in channel function rather than number is responsible for the increased Ca^{2+} influx in aged animals. Cav1.2 levels increase in the somatic regions of aged CA1 and CA3 pyramidal neurons, but not in DG granule cells (Núñez-Santana et al., 2014). It is possible that these contradictory reports can be attributed to several factors, including different strains of model organisms, age categories, or differences in analysis techniques in each study.

Some age-related neurodegenerative diseases are associated with an increase in Cav1.3 and decrease in Cav1.2 (Hurley et al., 2013). This suggests that disturbed Ca^{2+} homeostasis might be responsible for certain neurodegenerations correlated with aging.

1.4. Ca^{2+} hypothesis of brain aging

Calcium is a pivotal intracellular signalling molecule, which is important for a wide range of physiological functions including neuronal functions. In both excitatory and inhibitory synapses, postsynaptic Ca^{2+} is crucial for synaptic plasticity. Because of its central role, Ca^{2+} homeostasis must be tightly regulated. In a landmark study, Landfield and Pilter demonstrated for the first time that aged brains contain more Ca^{2+} than young brains suggesting that brain aging might be associated with altered Ca^{2+} homeostasis (Landfield & Pitler, 1984). Later, in mid-1980, the "Ca^{2+} hypothesis of brain aging" was proposed by Landfield and Khachaturian which states that aging alters brain Ca^{2+} regulation resulting in impaired neuronal function and eventually neurodegeneration (Khachaturian, 1987; Landfield, 1987). In other words what they propose is that a key initiating factor in the elderly memory impairment is an age-related increase in neuronal Ca^{2+}. This alteration has been attributed to a variety of factors, but it has been suggested that changes in the number or function of Ca^{2+} permeable channels play a major role (Khachaturian, 1994, p. 1). The molecular mechanisms responsible for this elevation are still not fully understood.

1.5. Olfactory piriform cortex

The odor's molecule is detected by odorant receptors (ORs) in the olfactory epithelium (OE) (Buck & Axel, 1991). The detected odor information is transmitted topographically by olfactory sensory neurons (OSNs) to the olfactory bulb (OB) as a pattern of activated glomeruli (Mori & Sakano, 2011). In turn, projection neurons, mitral/tufted (M/T) cells, transmit information about an odor map in the OB to various olfactory cortex regions (Igarashi et al., 2012).

Olfaction plays an important role in rodent survival. The piriform cortex (PC) is the largest area of the rodent olfactory cortex. It is also called piriform because the PC resembles a pear (Latin: *pirum*, pear). PC is located on the ventrolateral side of the brain next to the lateral olfactory tract (LOT). It receives direct projections from olfactory bulb via LOT (Löscher & Ebert, 1996). Despite the similar cell layers and morphology of the whole PC, it is sometimes divided into anterior (APC) and posterior parts (PPC). A possible boundary between the anterior and posterior PC can be determined by the disappearance of the LOT and thickened layer III in the PPC (Löscher & Ebert, 1996). In olfactory processing, the APC and PPC play different roles (Litaudon et al., 2003). APC encodes synthetic odorant identity and structure while PPC is more broadly tuned to familiar odorants and encodes odor quality (Gottfried et al., 2006; Kadohisa & Wilson, 2006).

PC consists of three neuronal layers, while other sensory cortices are six-layered (Löscher & Ebert, 1996). The outermost layer is layer I, a superficial plexiform layer that

contains dendritic trees from semilunar and pyramidal neurons, and axonal fibers of mitral and tufted cells (Haberly et al., 1987). Layer I is further divided into two parts; layer Ia being the superficial portion containing afferent fibers from the olfactory bulb, and layer Ib being the deeper part, containing afferent fibers from the prepiriform cortex (Price, 1973; Shabangu et al., 2021). The layer II of the PC is the densest, as it contains a large number of cell bodies. It is further subdivided into two layers of IIa and IIb. Layer IIa is dominated by semilunar cells, whereas Layer IIb is primarily composed of pyramidal neurons. Unlike layer II, layer III is relatively lower in density and composed of pyramidal and multipolar neurons (Haberly, 1983; Haberly et al., 1987; Haberly & Bower, 1984; Martínez et al., 1987; N. Suzuki & Bekkers, 2006). In the PC, excitatory glutamatergic neurons are mostly found in layers II/III, whereas GABAergic interneurons, which inhibit excitatory neurons and define stimulus receptive fields, are more evenly distributed across the three layers (Haberly, 1983; Luna & Schoppa, 2008; N. Suzuki & Bekkers, 2011, 2012).

1.6. The hippocampus

The hippocampus is an elongated structure buried deep within the medial temporal lobe. It is called the hippocampus as it resembles a seahorse (Latin: *Hippocampus* genus). In rodents, the hippocampus is a large, cashew-shaped structure just beneath the neocortex (Knierim, 2015). The hippocampus is composed of dentate gyrus and the cornu ammonis (Latin: *Amun's horns*, abbreviated as CA). The CA is divided into three fields: CA1, CA2, and CA3.

The principal neuronal calls in the DG are the granule cells, and in the CA are the pyramidal neurons (Lorente De Nó, 1934). The granule cells have small spherical bodies and their dendrites extend perpendicularly to the granule cell layer. Granule cells are monopolar neurons since their dendrites emerge exclusively from the apical portion of the cell body. Axons of granule cells extend from the basal portion of the cell body, and are referred to as mossy fibers due to their unique appearance (Johnston & Amaral, 2004). On the other hand, pyramidal neurons found in CA1, CA2 and CA3 form layered structures that are three to six cells deep. The dendritic trees of these cells extend perpendicularly from the cell layer in both directions, making them multipolar neurons. Apical dendrites are longer than basal dendrites and extend from the apex of the pyramidal cell body toward the center of the hippocampus.

DG mossy fibers terminate on the proximal dendrites of the CA3 pyramidal cells and interneurons. Pyramidal cells in CA3 project heavily to other levels of CA3 and to CA1.

Projections to CA1 are commonly referred to as schaffer collateral projections. CA1 pyramidal cells form connections with both the subiculum and deep layers of the EC, the subiculum in turn extends into the deep layers of EC. The direct CA1-EC projection supports memory formation, while the CA1-subicular-EC projection is involved in memory retrieval (Ledergerber & Moser, 2017; Roy et al., 2017). EC then projects to many of the same areas of the cortex that originally projected to it, thus, information entering the EC from one cortical area traverses the entire hippocampal circuit using the excitatory pathways and eventually returns to the same cortical area it originated from. The transformations that take place through this traversal are presumably essential for enabling the information to be stored as long-term memories (Johnston & Amaral, 2004).

Hippocampus plays a central role in memory formation and spatial learning. The main cortical input from many cortices arrives at the hippocampus primarily via the entorhinal cortex (EC) (Buzsáki & Moser, 2013). In rats, the entorhinal cortex consists of two distinct regions: the medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC). The MEC is involved in spatial processing, while the LEC is associated with object-recognition (Knierim, 2015). Olfactory information is received by LEC as inputs from both the olfactory bulb and the piriform cortex project directly into the LEC (Burwell & Amaral, 1998; Cleland & Linster, 2003; Kerr et al., 2007; Sosulski et al., 2011).

EC sends information to the hippocampus via two synaptic pathways: indirect perforant path (PP), which connects EC to hippocampal DG and CA3, and direct temporoammonic path (TA), which links EC to hippocampal CA1 (Andersen et al., 2007; Brun et al., 2008; Li et al., 2017). The strongest projections are along the perforant path. EC

projects into the DG (Synapse 1). Through the mossy fiber pathway, the DG sends projections into the CA3 region (Synapse 2). CA3 links to CA1 region through the schaffer collateral pathway (Synapse 3). In the end, CA1 connects back to the entorhinal cortex, completing a loop called the "trisynaptic circuit" (Andersen et al., 2007; Knierim, 2015). During exploration of a novel environment, synaptic plasticity in CA3 is essential to forming localized firing fields in CA1 (Nakazawa et al., 2003) and DG is involved in pattern separation (Jonas & Lisman, 2014). In the indirect path, spatial navigation and declarative memory formation are mediated (Yassa & Stark, 2011), while olfactory cue-place learning is mediated by the direct EC to CA1 circuit (Igarashi et al., 2012).

1.7. Rationales of the current study and hypothesis

The normal ability to learn and remember is influenced by increased intracellular Ca^{2+}, while at the same time, the age-related loss of this ability has been related to elevated Ca^{2+}. Both NMDARs and LTCCs allow Ca^{2+} to enter the cell. The expression of these channels or their intracellular location may change with age, which may explain this dichotomy. Synaptic Ca^{2+} permeable channels promote neuronal survival, however extrasynaptic ones activate phosphatases (e.g. calcineurin) leading to memory erasure.

With NMDARs most research has been done in hippocampus and not much is known about other brain structures. In LTCCs, conflicting past research has shown alteration of LTCCs expression which can result in memory impairment. Whether there is any change in the synaptic or extrasynaptic LTCCs is still unknown. A hypothesis we have proposed is that aged animals express an elevated level of extrasynaptic calcium channels, that leads into disrupting Ca^{2+} homeostasis and impairing synaptic plasticity.

In our lab we focus on olfactory learning due to its being the only sensory modality having circuitry that avoids the thalamus. Additionally, olfactory dysfunction is one of the earliest signs in memory disorders. The aim of this study is to investigate the intracellular distribution of LTCCs and NMDARs in neonate, adult and aging SD rats specifically in the piriform cortex and hippocampus using molecular techniques.

It is evident that the balance of channels and receptors is important for learning and memory to occur normally, and any deviation from this ratio can lead to impairment in one's cognitive abilities. Therefore, elucidating how elevated calcium levels in different locations of the neuron can either promote or impair learning is critical for our understanding of how the normal aging process unfolds with respect to memory impairments..

2. Materials and Methods

2.1. Animal models

Postnatal 10-14 days neonate, 3-6 months adult and 20-24 months aging Sprague Dawley rats of both sexes were used in this study. Animals were bred on site and were housed in individual cages on a standard light-dark cycle, receiving dry food and water ad libitum. All procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and followed the guidelines set by the Canadian Council on Animal Care.

2.2. Tissue collection for Western blot

Rats were anesthetized using light isoflurane and immediately decapitated. Brains were removed. PC and hippocampus were collected and flash frozen on dry ice. Samples were stored at -80ºC until further processing.

2.2.1. Synaptic and Extrasynaptic extraction

Tissue samples were placed on ice and homogenized in sucrose buffer (300 µl) containing: 320 mM sucrose, 10 mM Tris buffer (pH7.4), 1 mM EDTA, 1 mM EGTA, 1X

complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). The homogenized tissue was centrifuged at 2,000 rpm at 4ºC, for 15 min. The supernatant containing cytosolic, synaptic and extrasynaptic fractions was taken and centrifuged at 10,000 rpm at 4 °C, for 30 min to obtain a pellet.

Two hundred µl Triton X-100 buffer containing: 10 mM Triton-100, 10 mM Tris buffer (pH7.4), 1 mM EDTA, 1 mM EGTA, 1X complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche) was added to the pellet for detergent extraction (final 0.5% v/v). This suspension was vortexed and incubated at 4 °C for 30 min with gentle rotation and then centrifuged at 14,000 rpm at 4 °C, for 2 hours.

The obtained pellet contains postsynaptic densities and synaptic junctions that are insoluble in Triton X-100 (Cotman et al., 1971). Fifty µl of STE buffer containing: 100 mM Tris buffer (pH7.4), 10 mM EDTA, 10% SDS, 1X complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche) was added to the pellet. The mixture was labelled as the synaptic fraction, sonicated and stored at -80ºC until further use.

Six volumes of acetone were added to the obtained supernatant containing the extrasynaptic fraction. The mixture was stored at 4°C overnight. The following day the suspension was centrifuged at 5,000 rpm at 4°C, for 15 min. Fifty µl of STE buffer was added to the pellet. The mixture was labelled as the extrasynaptic fraction, sonicated and stored at -80ºC until further use.

2.2.2. Western Blotting

Total protein concentration was quantified by standard BCA assay (Pierce). The volume of lysate required to make 40 μg of protein for extrasynaptic and 10 μg of protein for synaptic fractions of each sample was calculated. Lysate solution, sample buffer (0.3 M Tris-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol), and dH_2O were prepared and boiled for 2 min at 100°C.

Samples and a prestained protein ladder (PM008-0500, Froggabio) were loaded into a 7% SDS-PAGE gel. Samples were separated and then transferred to nitrocellulose membranes (0.45 μm; Thermo Fisher Scientific). The membranes were stained with reversible Ponceau S staining. They were then inserted in between transparency sheets and scanned at 600 dpi to a TIFF file using a standard scanner. After that, the membranes were rinsed in 1X TBST for 10–15 min until the staining was completely eliminated. They were cut horizontally at the 72 kDa level. The upper portions of the membranes were blocked for 2 hr with 5% non-fat dry milk at room temperature, then incubated at 4°C overnight with the following antibodies: rabbit Anti-NR2A (1:5,000; cat. no. 07-632; Millipore Sigma), rabbit Anti-NR2B (1:5,000; cat. no. 06-600; Millipore Sigma), rabbit Anti-NMDA Receptor 1 (GluN1) (1:5,000; cat. no. D65B7; Cell Signalling) and rabbit Anti-Cav1.2 (1:2,000; provided by J.W. Hell; was raised against a peptide covering residues 818 –835 within the cytoplasmic loop between domains II and III of the Cav1.2 protein (Hall et al., 2013; Hell et al., 1993)). Next day membranes were washed 3x5 min with 1X TBST. Anti-rabbit HRP-

bounded second antibody was applied (1:10,000; cat. no. 31460; Thermo Scientific) at room temperature for 1.5 hour within 1X TBST and then washed 3x10 min with 1X TBST.
The protein bands were visualized using chemiluminescent substrate (cat. no. 34577; Thermo Fisher Scientific) and the optical density (OD) of each band were measured using ImageJ software.

2.3. Tissue collection for immunohistochemistry

Both 3-6 months and 20-24 months rats were anesthetized with pentobarbital i.p. (150 mg/kg, Rafter 8 Products) and perfused transcardially with ice-cold saline (0.9%), followed by ice-cold 4% paraformaldehyde (PFA) fixative in a 0.1M phosphate buffer. Brains were collected and stored in 4% PFA for 24 hours at 4°C and then transferred to a 0.1 M phosphate buffered saline (PBS) for an additional 24 hours before slicing.
Brains were embedded in 4% agarose to be ready for slicing. Fifty μm coronal sections were cut using a vibratome (Leica VT 1000S) and transferred to 24 well plates containing PVP using a sterile brush. Free-floating sections were stored at 4°C until further processing.

2.3.1. Double staining Fluorescence Immunohistochemistry

Sections were washed 3x5 min in Tris buffer (0.1M, pH 7.6) to remove the remaining PVP solution from them. Each section was then blocked in 1ml 5% bovine serum albumin (BSA) in Tris buffer, for 1 hour at room temperature on the shaker, to prevent non-specific binding. Sections were then incubated with the FP1 rabbit Anti-Cav1.2 antibody (1:500; provided by J.W. Hell) in 5% BSA in Tris buffer on the shaker at 4°C overnight.

The following day, the sections were washed 2x10 min in Tris buffer. All subsequent processes took place in the dark. Sections were probed with Anti-rabbit secondary antibody Alexa 647 (1:500; cat. no.A-21244; Thermo Fisher Scientific) in 5% BSA in Tris buffer, for 1 hour, at room temperature on a low speed shaker. Afterwards sections were washed 3x5 min in Tris buffer followed by 1x10 min wash in Tris A (0.1% Triton-X-100 in Tris buffer) and 1x10 min wash in Tris B (0.1% Triton-X-100 and 0.005% BSA in Tris buffer). Thereafter the sections were incubated with the second primary antibody; mouse Anti-PSD-95 antibody (1:2000; cat. no. MA1-046; Invitrogen) in 5% BSA in Tris B on a shaker at 4°C overnight.

On the last day, sections were washed 1x10 min in Tris A followed by 1x10 min in Tris B. They were probed with Anti-mouse secondary antibody Alexa 555 (1:500 ; cat. no. A-21424; Thermo Fisher Scientific) in 5% BSA in Tris B, for 1 hour, at room temperature on a low-speed shaker. Sections were then washed 3x5 min in Tris buffer and mounted on chrome-gelatin coated slides. Nuclei were counterstained with 4′-6-diamidino-2-

phenylindole (DAPI; cat. no. ab104139; abcam) and slides were cover slipped. They were kept at 4°C before confocal microscopy scanning.

2.3.1.1 Confocal Scanning

Using an FV10i confocal microscope (Olympus), z-stacks (~0.4 mm^2) were taken from the dendritic layers of dorsal hippocampus (CA1, CA3 and DG) and PC (L1a and L1b) at a magnification of 60x with a 10x zoom (Fig. 3.5A). Z-stacks were acquired from three to four sections from rostral to caudal areas. Photomultiplier tube assignments, confocal aperture size, and contrast remained constant for all sections.

2.3.1.2. Image Analysis

The Otsu method in image thresholding was used to perform automatic image segmentation into foreground and background. Using Grana's (BBDT) algorithm, we identified connected components. In order to remove image noise, two filters of size 11x11 and 5x5 were applied resulting in creating two masks. Using the logical 'AND' operator between these two masks identified regions of overlap between two channels. Next, by using Grana's (BBDT) again, the overlapped regions of two images were counted.

2.3.2. Single staining Fluorescence Immunohistochemistry

All sections from mid-age and aged animals were simultaneously processed in order to minimize the effects of potential inter-batch variability. Sections were washed 3x5 min in Tris buffer (0.1M, pH 7.6) to remove the remaining PVP solution from them. Sections were washed 1x10 min in Tris A followed by 1x10 min in Tris B Each section was then blocked in 1ml 5% BSA in Tris B, for 1 hour at room temperature on a shaker, to prevent non-specific binding. Sections were then incubated at 4°C overnight with the following antibodies: FP1 rabbit Anti-Cav1.2 antibody (1:500; provided by J.W. Hell) and Ab144 rabbit Anti-Cav1.3 (1:500; provided by A. Lee).

The next day, after 1x10 min wash in Tris A and 1x10 min in Tris B, sections were probed with Anti-rabbit secondary antibody Alexa 647 (1:500) in 5% BSA in Tris B, for 1 hour, at room temperature on a low speed shaker. Sections were then washed 3x5 min in Tris buffer and mounted on chrome-gelatin coated slides. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI, cat. no. ab104139; abcam) and slides were cover slipped. They were kept at 4°C before microscopy scanning.

2.3.2.1. Microscopy scanning

Using an EVOS™ M5000 microscope (Invitrogen), images were obtained from four to five sections from the rostral to caudal area. Images were taken from the dendritic and

somatic layers of dorsal hippocampus (CA1, CA3 and DG) and PC at a magnification of 20x (Fig. 3.6, 3.7). The intensity of light and exposure parameters were standardized across all captured images.

2.3.2.2. Image Analysis

Image analysis was performed using ImageJ software. In hippocampal CA1, CA3 and DG, and PC, 2 rectangular regions of interest (ROI) with equal dimensions were drawn in both dendritic and somatic layers. For each ROI, the integrated fluorescent intensity was calculated. In order to plot and analyze each animal's integrated fluorescent intensity, all slices of each animal were averaged.

2.2.3. Statistics

OriginPro 9.0 software was used to analyze all data sets. Data were reported as the mean ± SEM. For group comparisons, one-way ANOVA and post-hoc Tukey tests were used. Differences between groups were considered significant when p values were <0.05.

3. Results

We have established a protocol to separate synaptic and extrasynaptic compartments (McGaraughty et al., 2009; Mukherjee & Yuan, 2016) using Triton X-100, since postsynaptic densities and synaptic junctions are not soluble in Triton X-100 (Matus et al., 1978). The accuracy of this method was validated by demonstrating PSD-95 to be more abundant in the synaptic fraction than the extrasynaptic fraction (Fig. 3.1).

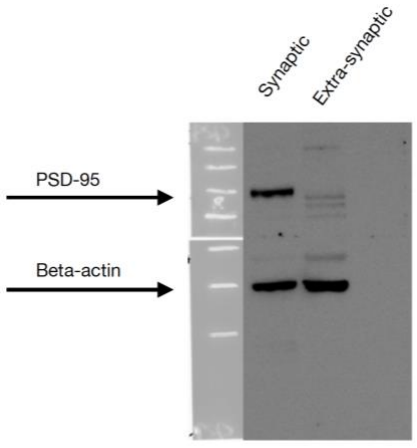


Fig 3.1. Validation of synaptic membrane extraction

With western blotting the expression level of GluN1, GluN2A and GluN2B were examined in the synaptic and extrasynaptic fractions of hippocampus (Fig. 3.2) and PC (Fig. 3.3) of neonate, adult and aging rats. Quantitative analysis through one-way ANOVA and Fisher test revealed that in hippocampus, GluN1 ($F(2, 26) = 7.01$, $p = 0.003$) was statistically lower in the synaptic fraction of neonates comparing to adult ($p = 0.001$) and aging brains ($p = 0.02$). In contrast, GluN1 extrasynaptic ($F(2, 26) = 5.95$, $p = 0.007$) expression was higher in hippocampal neonates comparing to adult ($p = 0.003$) and aging ($p = 0.01$) , although no difference was observed between adult and aging rats (Fig. 3.2A).

GluN2A ($F(2, 27) = 12.6$, $p = 0.01$), and GluN2B ($F(2, 22) = 3.77$, $p = 0.03$) were lower in the synaptic fraction of neonates compared to adult ($p = 0.001$ & $p = 0.01$) and aging brains ($p = 0.001$ & $p = 0.02$) (Fig. 3.2B, 3.2C).

Within the hippocampus, both synaptic ($F(2, 22) = 2.76$, $p = 0.08$) and extrasynaptic ($F(2, 22) = 1.51$, $p = 0.24$) GluN2A/2B ratios were similar among different age groups (Fig. 3.2D)

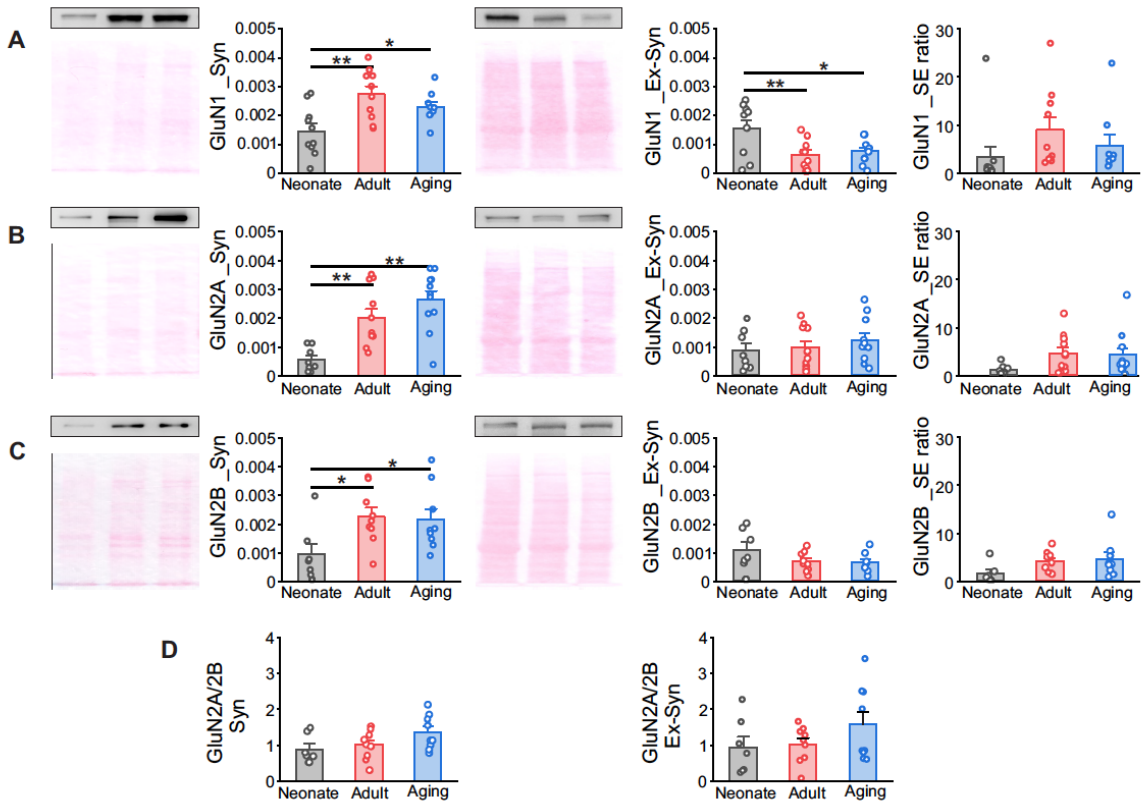


*Fig. 3.2. Hippocampal GluN1, GluN2A and GluN2B expressions at different ages. (A) Comparing expression of synaptic and extrasynaptic GluN1 proteins in hippocampus of neonate, adult and aged rats (n=8). (B, C) Quantitation of GluN2A and GluN2B protein expression normalized to their corresponding ponceau revealed higher expression in the synaptic fraction of the adult and aged compared to neonatal hippocampus. (D) GluN2A/2B ratios were similar among different age groups. *P < 0.05, **P < 0.005. Data reported as the mean ± SEM.*

Western blotting in PC showed that the expression of GluN1 ($F(2, 32) = 0.01$, $p = 0.98$), GluN2A ($F(2, 27) = 0.1$, $p = 0.9$) and GluN2B ($F(2, 27) = 0.56$, $p = 0.57$) subunits were not altered in synaptic fractions (Fig. 3.3A, 3.3B, 3.3C). However, GluN2B extrasynaptic ($F(2, 27) = 4.26$, $p = 0.02$) displayed a higher expression in neonates compared to adult brains ($p = 0.007$) (Fig. 3.3C). Synaptic:extrasynaptic ratios in GluN1 ($F(2, 32) = 4.61$, $p = 0.01$) and GluN2A ($F(2, 27) = 3.47$, $p = 0.04$) subunits showed statistically higher levels in adults compared to neonates ($p = 0.01$ & $p = 0.01$) (Fig. 3.3A, 3.3B). PC synaptic GluN2A/2B ratio ($F(2, 28) = 5.17$, $p = 0.01$) was significantly higher in adult brains compared to neonate ($p = 0.004$) and aging brains ($p = 0.02$) (Fig. 3.3D).

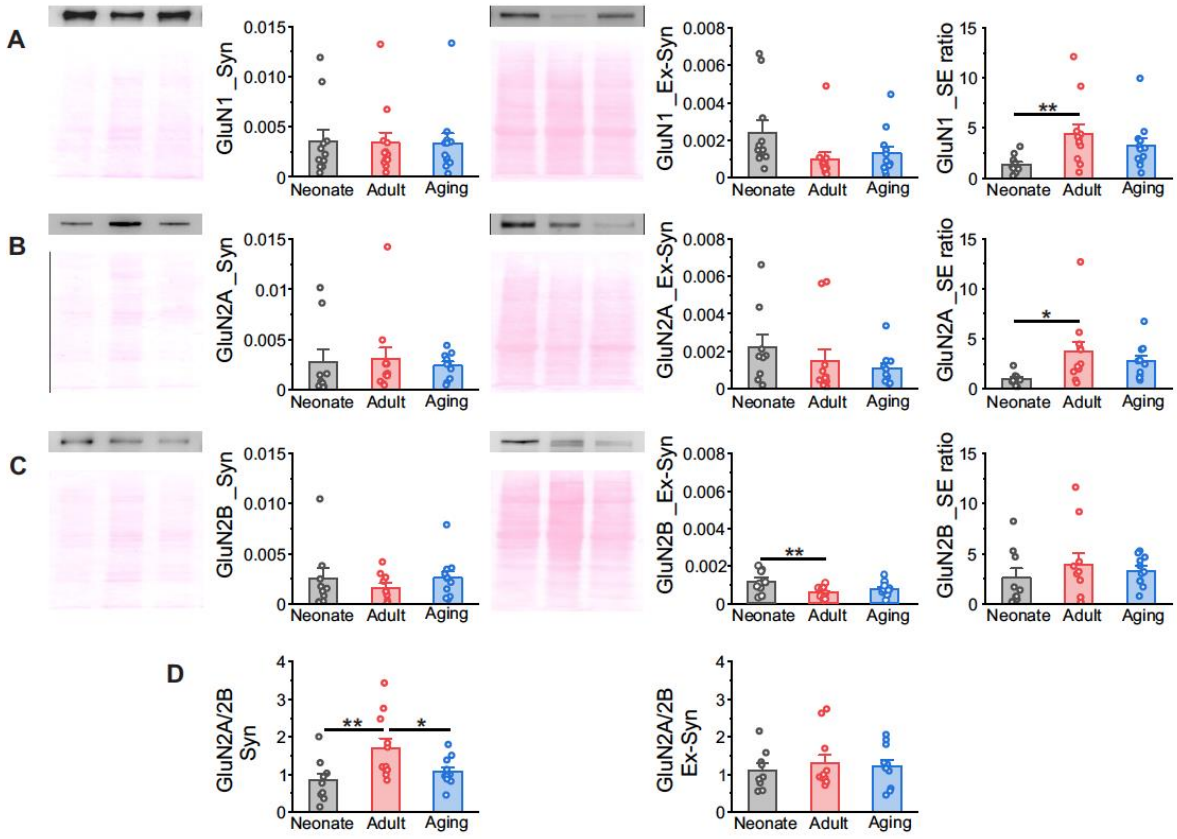


Fig. 3.3. PC GluN1, GluN2A and GluN2B expression at different ages. *(A, B) Comparing expression of synaptic and extrasynaptic GluN1 and GluN2A proteins in the PC of neonate, adult and aged rats (n=8). The synaptic:extrasynaptic ratio of GluN1 and GluN2A subunits showed higher expression in adults than neonates. (C) No age-related changes were found in the GluN2B synaptic expression while the extrasynaptic expression was higher in the PC of neonates than adults. (D) GluN2A/2B ratios was significantly higher in adult brains compared to neonate and aged brains. *P < 0.05, **P < 0.005. Data reported as the mean ± SEM.*

We also investigated the expression of Cav1.2 in the synaptic and extrasynaptic fractions of neonate, adult and aging rats. Hippocampal results showed no age-related alteration of Cav1.2 protein in the synaptic ($F(2, 20) = 3.081$, $p = 068$), and extrasynaptic fractions ($F(2,20) = 0.27$, $p = 0.76$) (Fig. 3.4A).

Also, no age-related changes of Cav1.2 protein was observed in the synaptic ($F(2,9) =1.48$, $p = 0.27$) and extrasynaptic fractions ($F(2,9) = 0.71$, $p = 0.51$) of PC (Fig. 3.4B). In order to obtain more robust data, more experiments should be performed on PC.

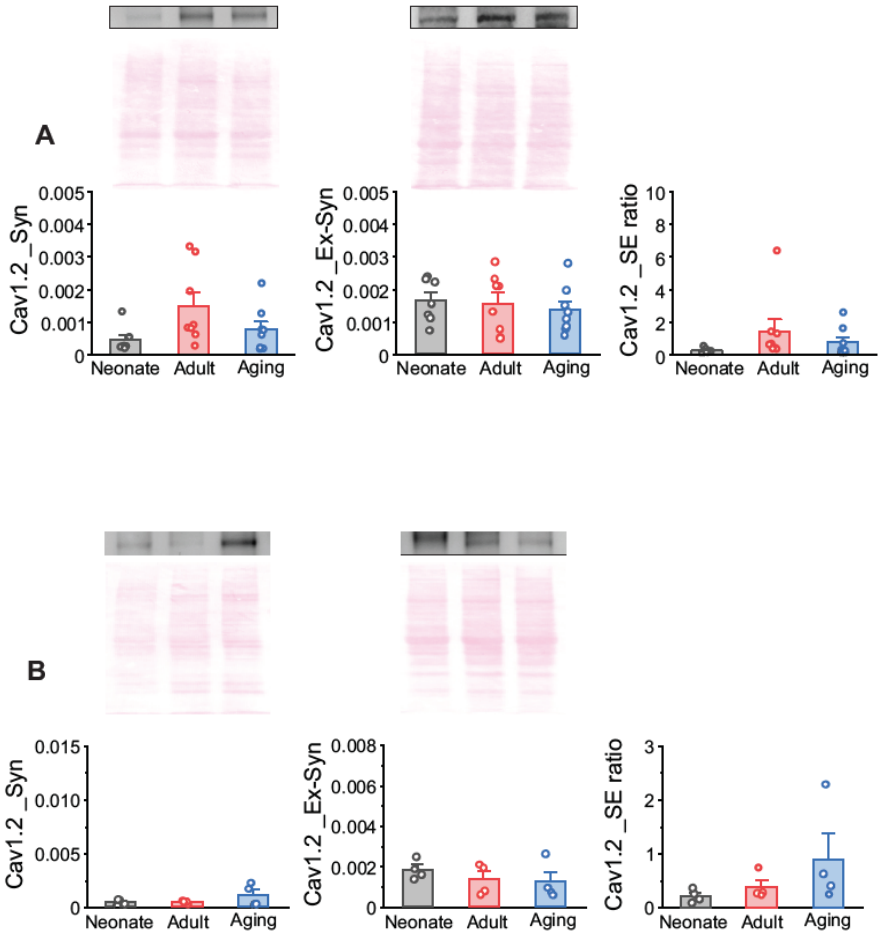


Fig. 3.4. In hippocampus and PC there was no age-related alteration of Cav1.2 protein in the synaptic and extrasynaptic fractions. *(A) Quantitation of Cav1.2 expression did not differ between groups in the hippocampus (n=7). (B) Quantitation of Cav1.2 expression did not differ between groups in the PC (n=4). Data reported as the mean ± SEM.*

With western blotting, the entire structure is homogenized, potential alterations in different hippocampal subregions may be undetected. Therefore we performed immunohistochemical analysis to detect distinct dendritic subregions.

Immunoreactivity against Cav1.2 subunit in synaptic (PSD95+) and extrasynaptic (PSD95-) regions of adult and aging dorsal hippocampus (CA1, CA3 and DG) and PC (L1a and L1b) was tested (Fig. 3.5). Confocal analysis of all three hippocampal subregions, revealed no difference in levels of synaptic or extrasynaptic Cav1.2 between adult and aged brains (Fig. 3.5B, 3.5C, 3.5D). Also, we found no age-related changes in Cav1.2 expression in synaptic and extrasynaptic areas of LIa and LIb despite the fact that PSD95 showed an age-related decline in LIa neurons (n = 7, t = 3.41, p = 0.005) (Fig. 3.5E, 3.5F). Specifically, we observed that the relative expression of Cav1.2 extrasynaptically, but not synaptically, was enhanced when we averaged layer Ia and layer Ib of PC (n = 7, t = 2.41, p = 0.03) (Fig. 3.5G).

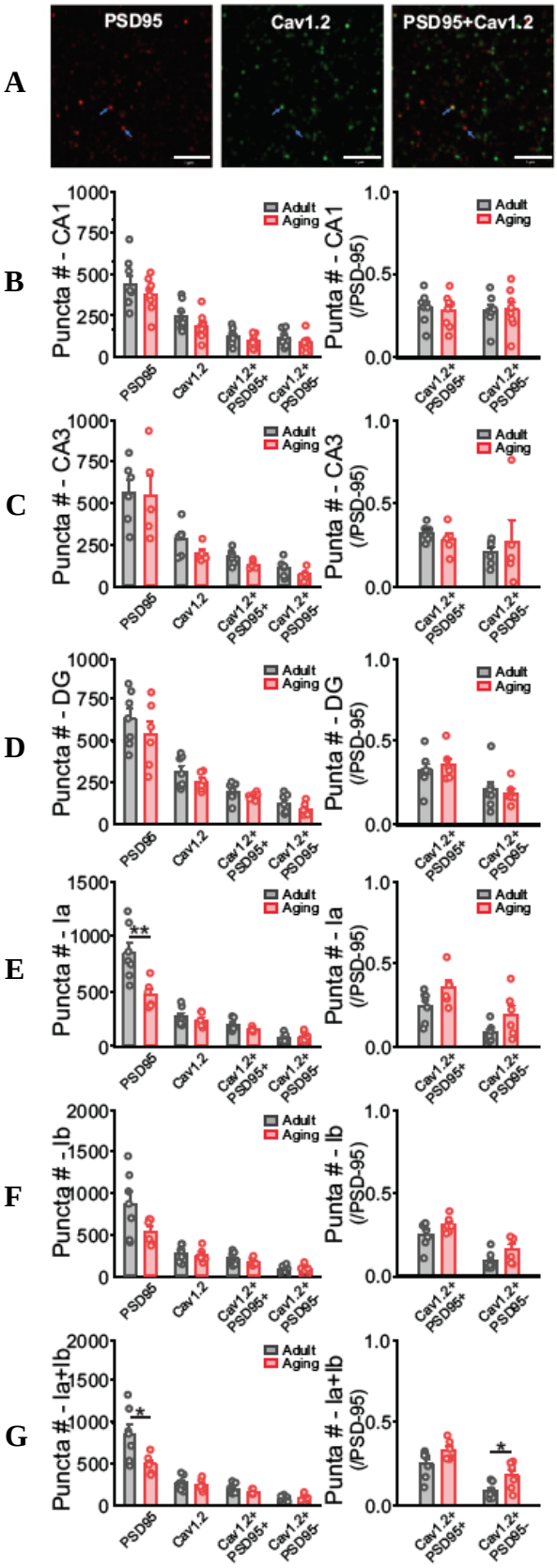

A
PSD95
Cav1.2
PSD95+Cav1.2
B
Puncta # - CA1
Punta # - CA1 (/PSD-95)
C
Puncta # - CA3
Punta # - CA3 (/PSD-95)
D
Puncta # - DG
Punta # - DG (/PSD-95)
E
Puncta # - Ia
Punta # - Ia (/PSD-95)
F
Puncta # - Ib
Punta # - Ib (/PSD-95)
G
Puncta # - Ia+Ib
Punta # - Ia+Ib (/PSD-95)
Adult
Aging
PSD95
Cav1.2
Cav1.2+ PSD95+
Cav1.2+ PSD95-

Fig. 3.5. Expression of Cav1.2 subtype in synaptic (PSD95+) and extrasynaptic (PSD95-) of adult and aging dorsal hippocampus (CA1, CA3 and DG) and PC (L1a and L1b). *(A) Representative confocal images showing immunohistochemical labeling for PSD95, Cav1.2 and co-localized PSD-95 and Cav1.2 in adult (n=7) and aging (n=6) rats. Immunofluorescent puncta depicted in red represent PSD95, while green immunofluorescent puncta demonstrate Cav1.2. (B, C, D) Across all three dorsal hippocampal subregions (CA1, CA3 and DG) no difference was identified between synaptic (PSD95+) and extrasynaptic (PSD95-) expression of Cav1.2 protein. (E) PC dendritic layer 1a does not show age-related synaptic and extrasynaptic changes however, compared to adult rats, aging rats showed a decline in the expression of PSD95. (F) In PC dendritic layer 1b there is no age-related synaptic and extrasynaptic alteration. (G) Averaging 1a and 1b of PC revealed that aging increase the expression level of the extrasynaptic Cav1.2, but not the synaptic. *P < 0.05. Data reported as the mean ± SEM.*

We next investigated the expression of LTCC subunits in the somatic region of adult and aging rats (Fig. 3.6 & 3.7). Significant increases in Cav1.2 expression was observed in the somatic regions of aging CA1 (n = 5, $t = 2.88$, $p = 0.02$) (Fig. 3.6B2). The soma:dendritic ratio of Cav1.2 also increased with aging in hippocampal CA1 (n = 5, $t = 6.81$, $p = 0.001$) (Fig. 3.6B4). However, no significant changes were observed in CA3 pyramidal neurons (Fig. 3.6C2, 3.6C3, 3.6C4) or DG granule cells (Fig. 3.6D2, 3.6D3, 3.6D4). Although adult and aging rats had a similar pattern and intensity of Cav1.2 labeling in the somatic (Fig. 3.6E2) and dendritic layers (Fig. 3.6E3) of PC, the soma to dendritic ratio of Cav1.2 expression increases with aging in PC (n = 5, $t = 4.08$, $p = 0.003$) (Fig. 3.6E4).

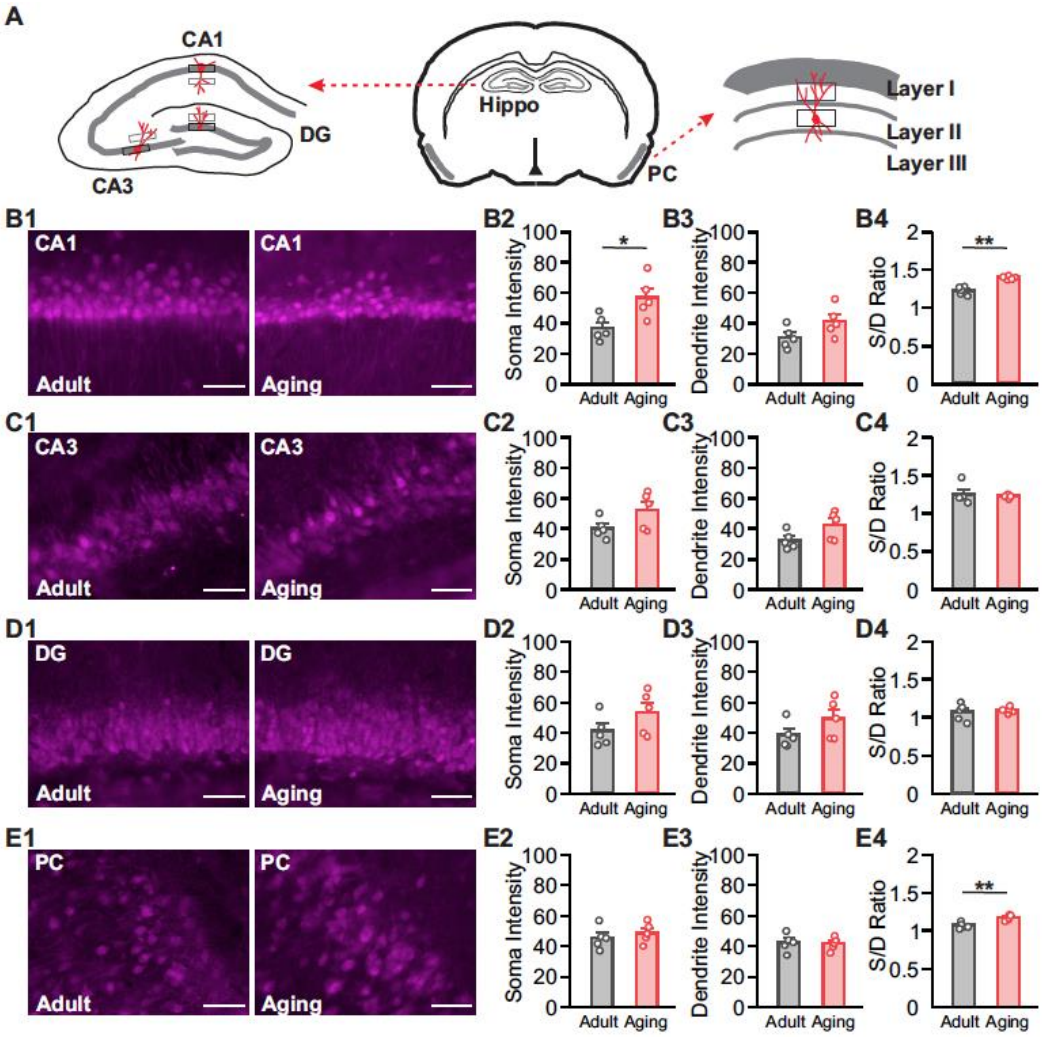


Fig. 3.6. The soma:dendritic ratio of Cav1.2 expression increased with aging in hippocampal CA1 and PC. *(A) In each hippocampal CA1, CA3 and DG, and PC section, 2 rectangular regions of interest (ROI) with equal dimensions were quantified in both dendritic and somatic layers of adult (n=5) and aging (n=5) brains. (B1, C1, D1, E1) Representative sections showing immunohistochemical labeling for Cav1.2 in CA1, CA3, DG and PC respectively, of adult and aging rats. (B2) Quantitative analysis of integrated fluorescence intensity in the soma of CA1 pyramidal neurons of adult and aging rats showed increased expression of Cav1.2 with aging. (B3) No differences in Cav1.2 expression in CA1 were detected between dendritic layers of adult and aging rats. (B4) The soma:dendritic ratio of Cav1.2 expression increases with aging in CA1. (C2, D2, E2) Adult and aging rats showed a similar pattern and intensity of Cav1.2 labeling in somatic layers of CA3, DG, and PC. (C3, D3, E3) Adult and aging rats showed a similar pattern and intensity of Cav1.2 labeling in dendritic layers of CA3, DG, and PC. (C4, D4) The soma:dendritic ratio in CA3 and DG showed no differences in Cav1.2 expression. (E4) The soma:dendritic ratio of Cav1.2 expression increases with aging in PC. Fluorescence intensities and analyses were performed using raw, unmodified images. *P < 0.05, **P < 0.005. Data reported as the mean ± SEM.*

In addition, we examined whether the pattern of expression in the somatic region of adult and aging rats is restricted to only the Cav1.2 subunit of LTCCs or if the Cav.1.3 subunit may also be affected. The result from the immunohistochemical analysis revealed that the expression of Cav1.3 in the soma of CA1 pyramidal neurons and DG granule cells of adult and aging rats had no differences with age (Fig. 3.7B2, 3.7D2). In contrast, Cav1.3 dendritic intensity in CA1 ($n=5$, $t=2.6$, $p=0.03$) and DG ($n=5$, $t=3.67$, $p=0.006$) increased with aging (Fig. 3.7B3, 3.7D3), thus the Cav1.3 soma:dendritic ratio in both CA1 ($n=5$, $t=2.75$, $p=0.02$) and DG ($n=5$, $t=2.33$, $p=0.04$) of hippocampus decreased with aging (Fig. 3.7B4, 3.7D4). In CA3 there were age-related elevations of Cav1.3 protein in both somatic ($n=5$, $t=3.45$, $p=0.008$) (Fig. 3.7C2) and dendritic layers ($n=5$, $t=3.67$, $p=0.006$) (Fig. 3.7C3) while the soma:dendritic ratio did not change (Fig. 3.7C4). Cav1.3 expression in pyramidal cell layer II of PC of aging rats was lower than that in adult rats ($n=5$, $t=3.05$, $p=0.01$) (Fig. 3.7D2), while dendritic layer I did not statistically differ across age groups (Fig. 3.7D3). Consequently, the soma: dendritic ratio of PC neurons significantly decreased with age ($n=5$, $t=2.47$, $p=0.03$) (Fig. 3.7D4).

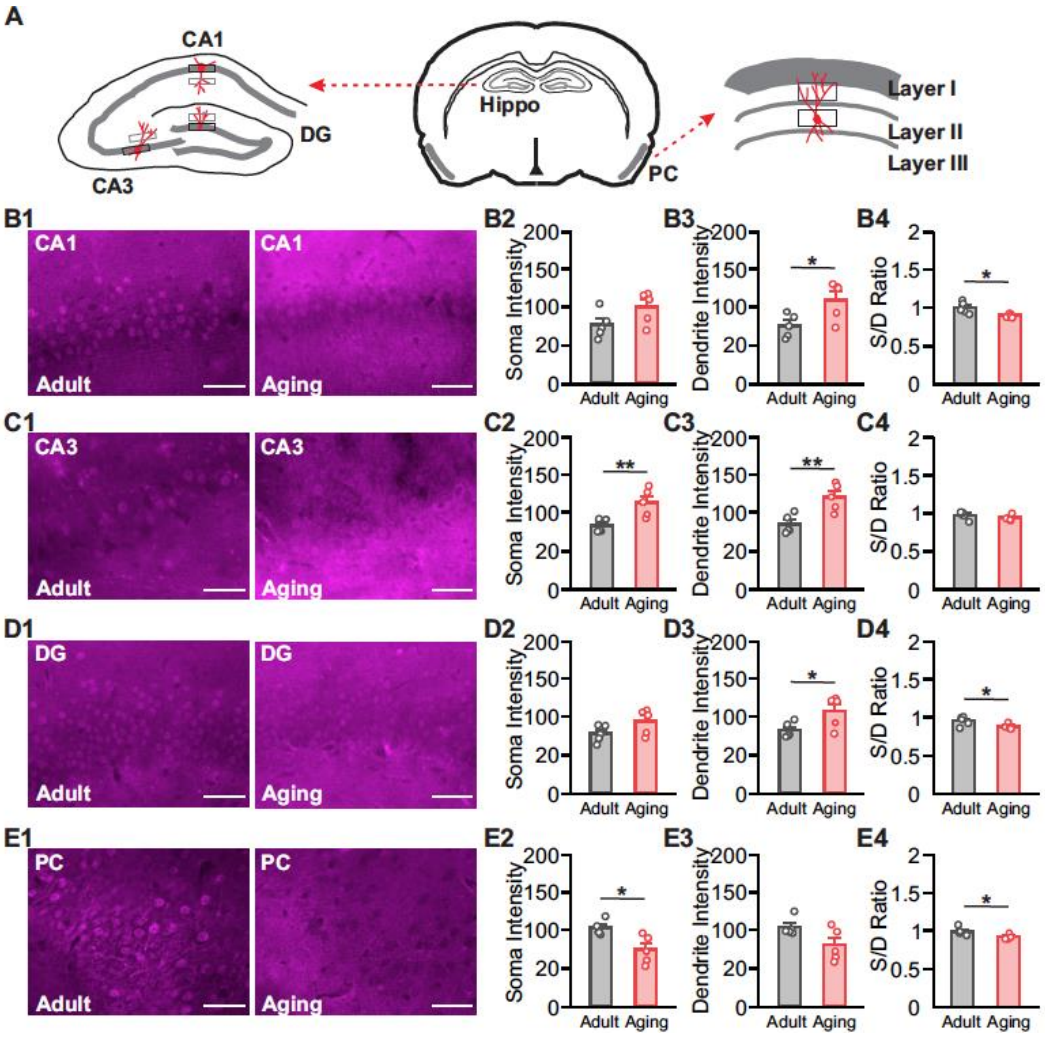


Fig. 3.7. The soma:dendritic ratio of Cav1.3 expression decreased with aging in hippocampal CA1, DG and PC. *(A) In each hippocampal CA1, CA3 and DG, and PC section, 2 rectangular regions of interest (ROI) with equal dimensions were quantified in both dendritic and somatic layers of adult (n=5) and aging (n=5) brains. (B1, C1, D1, E1) Representative sections showing immunohistochemical labeling for Cav1.3 in CA1, CA3, DG and PC respectively, of adult and aging rats. (B2) Quantitative analysis of integrated fluorescence intensity in the soma of CA1 pyramidal neurons of adult and aging rats showed no difference in the expression of Cav1.3 with aging. (B3) Dendritic intensity of Cav1.3 increased in CA1 with aging. (B4) Cav1.3 soma:dendritic ratio decreased with aging in CA1. (C2, C3) Both somatic and dendritic layers of CA3 exhibited age-related elevation of Cav1.3 protein. (C4) The soma:dendritic ratio in CA3 showed no differences in Cav1.3 expression. (D2) Adult and aging rats showed a similar intensity of Cav1.3 labeling in the somatic layer of DG. (D3) In aging rats, the dendritic layer of DG granular neurons expressed higher levels of Cav1.3 than in adult rats, which (D4) resulted in a significant decline in the soma to dendritic ratio of DG neurons with aging. (E2) In aging rats, pyramidal cell layer 2 of PC expressed lower levels of Cav1.3 than adult rats, while (E3) dendritic layer 1 exhibited no change in age groups. consequently, (E4) there is a significant decline in the soma:dendritic ratio of PC neurons with aging. Fluorescence intensities and analyses were performed using raw, unmodified images. *P < 0.05, **P < 0.005. Data reported as the mean ± SEM.*

4. Discussion

Ca^{2+} permeable channels are membrane-spanning proteins that control the concentration of intracellular Ca^{2+} within the neuron. Within the brain the most studied Ca^{2+} permeable channels are NMDARs and LTCCs. The current study is the first to study these critical Ca^{2+} permeable channels simultaneously in different age groups.

A functional NMDAR is composed of three main subunits GluN1, GluN2A and GluN2B whose expression levels determine properties of the receptors such as glutamate affinity, open probability, deactivation kinetics, channel conductance and subcellular localization (Erreger et al., 2007; Furukawa et al., 2005; Papadia et al., 2008; Sanz-Clemente et al., 2013; Vieira et al., 2020). Depending on the age of the animal, these subunits may express differently in synaptic or extrasynaptic dendritic spines. In this study we performed immunohistochemistry and western blot techniques to investigate the distribution of these subunits in neonate, adult and aging rats.

Memory, learning, and spatial orientation are all fundamental cognitive functions associated with the hippocampus (Akhondzadeh, 1999; Olton et al., 1978; W. A. Suzuki & Clayton, 2000), while, in rats, olfactory learning is associated with the piriform cortex. NMDARs play a pivotal role in all these processes (T. V. P. Bliss & Collingridge, 1993).

Neonatal hippocampus expresses NMDAR subunits in a complex pattern that varies with age (Cull-Candy et al., 2001). GluN1, GluN2A and GluN2B expression all change notably after birth. Expression of GluN1 mRNA and protein is abundant at birth, and

continue to increase to adult levels about the third postnatal week (Monyer et al., 1994; Sheng et al., 1994; Wenzel et al., 1997; Zhong et al., 1995). Although GluN1 increases throughout the developmental stages and into adulthood, NMDARs are not static and they do not just remain in synaptic sites. It is possible for them to move laterally from synaptic sites into the extrasynaptic membrane (Tovar & Westbrook, 2002). In our study, we have found that GluN1 is statistically lower in neonatal hippocampal synaptic fractions compared to adult and aging rats, while, GluN1 extrasynaptic expression was higher in neonates (Fig. 3.2A). In agreement with the literature even though there are fewer GluN1s in hippocampal neonates comparing to adult rats, we found that the majority are located in extrasynaptic regions. Neonatal extrasynaptic NMDARs play a different role and can be advantageous for learning (Schmidt-Salzmann et al., 2014). As opposed to adult extrasynaptic NMDARs, neonatal extrasynaptic NMDARs support effective depolarization and promote synapse formation (Schmidt-Salzmann et al., 2014).

As a result of aging, some studies report that the expression of GluN1 decreases (P. Liu et al., 2008) or remains consistent (Zhao et al., 2009) in the hippocampus. Corresponding to the Liu study, we found no age-related GluN1 alterations between adult and aging hippocampus (Fig. 3.2A).

In PC, as the main olfactory cortex in rats, there were no age-related changes in the expression of the GluN1 subunit in synaptic and extrasynaptic fractions. Nonetheless, the GluN1 subunit showed a higher synaptic to extrasynaptic ratio in adults compared to neonates (Fig. 3.3A). GluN1 being an obligatory subunit of NMDARs, we can generalize that adult NMDARs occupy more synaptic than extrasynaptic membranes in PC.

NR2 subunits determine the functional properties of NMDARs (Traynelis et al., 2010). GluN2A-containing receptors have the lowest sensitivity to glutamate; however, they have a higher open probability than GluN2B-containing receptors (Brimecombe et al., 1997; Sanz-Clemente et al., 2013; Vieira et al., 2020). Deactivation kinetics is measured by the excitatory post-synaptic current decay. GluN2A containing NMDARs have a faster decay rate (Paoletti et al., 2013), that consequently means GluN2Bs stay open for a longer time and make a larger contribution to the total Ca^{2+} transfer into the cell. As for their subcellular localization, they can exist both synaptically and extrasynaptically within the dendrites, which is dependent on age (Groc et al., 2006; Hardingham & Bading, 2010). After birth, synapses are almost exclusively composed of GluN2B-containing NMDARs. With maturation, GluN2A would occupy synapses mostly, while GluN2B would reside primarily in the extrasynaptic area (Dumas, 2005). Additionally, aging causes GluN2B-containing NMDARs to move from the synaptic site to the extrasynaptic area, which promotes neuronal death (Avila et al., 2017; Potier et al., 2010). In agreement with previous studies (Ge et al., 2019; Monyer et al., 1994; Wenzel et al., 1997), our results showed GluN2A expression increase in hippocampal synapses from neonatal to adult and aging brains (Fig. 3.2B), however in contrast to other studies (Dumas, 2005; Ge et al., 2019; Wenzel et al., 1997; Zhao et al., 2009), an age-related increase was also observed in synaptic GluN2B but not extrasynaptic GluN2B (Fig. 3.2C). In synaptic fractions of PC, in contrast to the hippocampus, neither the GluN2A nor GluN2B subunits were altered. Neonatal brains, however, demonstrated higher levels of extrasynaptic GluN2B than adult brains (Fig. 3.3B, Fig. 3.3C) suggesting that neonatal brains are more susceptible to activate CREB shut off pathway (Hardingham & Bading, 2002).

Developmental changes in NMDAR-dependent synaptic plasticity are thought to be caused by an increase in the GluN2A/2B ratio (Castellani et al., 2001; Gambrill & Barria, 2011; Quinlan, Philpot, et al., 1999). As opposed to literature that reports an increase in the ratio of GluN2A/GluN2B in the hippocampus as development progresses into adulthood (Ge et al., 2019), our results showed that in hippocampus this ratio remained consistent among different age groups (Fig. 3.2D). Meanwhile, in the PC, the synaptic GluN2A/2B ratio was significantly higher in adult brains as compared to neonatal and aging rats (Fig. 3.3D). A higher level of GluN2A/GluN2B is associated with an increased threshold for induction of LTP (Franks & Isaacson, 2005; Kirkwood et al., 1995) which cause learning and memory retention to be less facilitated. Our findings suggest that PC is less plastic and more stable than the hippocampus in adults. This may indicate that sensory structures during development have an early plastic critical period, followed by a stable and less dramatic plastic phase in maturity, while with aging the structure in terms of plasticity becomes more fragile again. Our study was the first that demonstrated these findings in the piriform cortex.

Some types of olfactory learning are also mediated by the L-type calcium channels (Berger & Bartsch, 2014; Ghosh et al., 2017; Jerome et al., 2012). LTCCs are important Ca^{2+} entry gates and are thought to be responsible for excessive Ca^{2+} influx in aged brains according to the Ca^{2+} hypothesis of aging (Khachaturian, 1987; Landfield, 1987). Based on numerous studies, LTCC current increases with aging in the hippocampus, which explains age-related cognitive decline (Campbell et al., 1996; Disterhoft & Oh, 2006; Foster, 2007; Landfield, 1994; Shankar et al., 1998; Thibault et al., 2001; Thibault & Landfield, 1996). Whether the density of LTCCs increases with aging is still unknown. We demonstrated with western blot analysis that in the whole hippocampus there was no aging-related LTCC

alteration in the synaptic and extrasynaptic fractions (Fig 3.4A, 3.4B). Thus, even if there were changes in the channel density within the hippocampal structure, as we took the whole structure as one, it was difficult to detect potential variations within different subareas.

One possible caveat of the current study is the technique that we used to separate synaptic and extrasynaptic fractions for western blot analysis. In this study, we used triton-X to separate insoluble membrane fractions that have been "PSD-enriched" from "non-PSD-enriched" membrane fractions. The “PSD-enriched” synaptic fraction included post synaptic membrane while "non-PSD-enriched" membrane fraction included not only extrasynaptic membrane, but also perisynaptic and presynaptic membrane. Therefore, extrasynaptic fractions for western blotting were suboptimal.

Hippocampal CA1 displays the age-related LTCC current elevation most prominently (Campbell et al., 1996; Moyer & Disterhoft, 1994; Thibault & Landfield, 1996). Studies show that the surface expression of Cav1.2 subunits increases in CA1 and CA3 hippocampus of aged F344xBN rats (Núñez-Santana et al., 2014), along with the current changes. Therefore, we systematically examined Cav1.2 expression in the dorsal hippocampus (CA1, CA3 and DG), and in the piriform cortex dendrites (L1a and L1b), in order to elucidate the sites of Cav1.2 upregulation with age. Our double staining immunohistochemical results of the synaptic (colocalized with PSD95) and extrasynaptic (non-colocalized with PSD95) Cav1.2 in the hippocampus showed no age-related changes in CA1, CA3 and DG of adult and aging hippocampus (Fig. 3.5B, 3.5C, 3.5D) suggesting that age-related cognitive deficits cannot be attributed to the upregulation of Cav1.2 in the

dendritic membrane of hippocampal neurons of SD rats. This contradiction could be explained by the age and strain of rats used in each study. Núñez-Santana has used 3–4 month and 30–32-month-old hybrid Fischer 344 X Brown Norway aged rats (Núñez-Santana et al., 2014), while Veng has used 4-month and 24-month-old Fischer 344 rats (Veng & Browning, 2002) and our study was conducted on 3-6 month and 20-24 month-old Sprague Dawley rats.

An earlier study has been shown that aging has no effect on synaptic density of dendritic layers LIa and LIb in PC of 29 month-old SD rats (Curcio et al., 1985). However, we observed loss of synapses of LIa as PSD95 declined significantly with aging (Fig. 3.5E). As a result of merging the two PC dendritic sublayers (LI1+LIb), we observed an increase in extrasynaptic (not colocalized with PSD95) Cav1.2 in aging brains, suggesting that Cav1.2 were more likely dissociated from PSD95.

By looking into the somatic expression of LTCC subunits, our immunohistochemical data demonstrated that with aging, the soma to dendritic layer of CA1 expressed higher Cav1.2 and lower Cav1.3 subunits (Fig 3.6B, 3.7.B), suggesting that age-related LTCC current elevation may be only limited to Cav1.2 subunit in the CA1 soma of SD rats but not Cav1.3. Cav1.2 as the dominant LTCC subunit, mediates activity-dependent gene transcription (Gomez-Ospina et al., 2006; Zhang et al., 2006) and exhibits a different Ca^{2+} dependent coupling to hippocampal neurons than Cav1.3 (Hasreiter et al., 2014). Cav1.3 subunits are most likely associated with inhibitory neurons since activation of $GABA_BR2$ increases Cav1.3 currents and intracellular Ca^{2+} through Cav1.3, but not through Cav1.2

(Park et al., 2010). Thus Cav1.3 downregulation in somatodendritic CA1 cannot compensate for Cav1.2 rise associated with aging since they have completely different effects.

Although it has been reported that with aging Cav1.2 subunits increase in the soma of both CA1 and CA3 pyramidal neurons (Núñez-Santana et al., 2014), in our analysis, no alteration of Cav1.2 in CA3 was observed (Fig. 3.6C). However, notably there was an age-related upregulation of Cav1.3 in the soma of aging CA3 (Fig. 3.7C).

In PC, the results of our study demonstrated elevated Cav1.2 expression in the soma to dendritic ratio (Fig. 3.6E), whereas Cav1.3 expression has decreased in the soma to dendritic regions (Fig. 3.7E). According to these data, excessive Cav1.2 somatic regions of PC neurons may be associated with age-related olfactory impairment.

In summary, the current study supported the Ca^{2+} hypothesis of aging (Khachaturian, 1987; Landfield, 1987) in that there were higher Cav1.2 expression levels in pyramidal neuron CA1 and higher somatodendritic and extrasynaptic Cav1.2 in PC of aging SD rats. This imbalance may be correlated to the disruption of Ca^{2+} homeostasis and cognitive decline seen in normal aging subjects (Gibson & Peterson, 1987; Thibault et al., 2007). This study shed light on mechanisms that may underlie age-related memory loss. Understanding factors that lead to Ca^{2+} dysregulation is crucial for developing novel therapeutics that help maintain normal Ca^{2+} homeostasis, restore plasticity, and retain memory.

In the future, we will conduct a behavioural study to investigate the role of LTCCs and NMDARs in associative learning in SD rats. Furthermore, to examine current changes in Ca^{2+} permeable channels in adult and aging SD rats, an electrophysiological study will be conducted.

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Appendix 1

Animal protocol approval

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
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