

Charles University

Faculty of Science

Study programme: Physiology

Branch of study: Neurobiology



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Geneticky snížená produkce CS-4 typu sulfatace perineuronálních sítí jako zdroj juvenilní plasticity v procesech učení a paměti

Genetic knockout of CS-4 sulfation of perineuronal nets as a source of juvenile like plasticity in processes of memory and learning

Master's thesis

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Prague, 2022

Prehlásenie

Prehlasujem, že som záverečnú prácu spracovala samostatne a že som uviedla všetky použité informačné zdroje a literatúru. Táto práca, ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe, dňa 5.9.2021

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Pod'akovanie

V prvom rade by som sa chcela pod'akovať svojmu školiteľovi RNDr. Jiřímu Růžičkovi, Ph.D. za veľmi dobre premyslenú tému diplomovej práce, za konzultácie pri písaní, za podporu na mojej ceste vysokoškolským štúdiom a v poslednom rade za všetky techniky, ktoré ma naučil. Takisto ďakujem svojej rodine, ktorá mi bola oporou pri písaní tejto práce.

Abstract:

Extracellular matrix affects the functioning of the brain. Among others, learning and memory can be affected by the changes in the composition and properties of the extracellular matrix. Different sulfation patterns on the chondroitin sulfate proteoglycans, one of the extracellular matrix components, can either enhance or reduce synaptic plasticity and neuronal growth. This leads to the changes in the formation of memory. In my thesis I defined the role of 4-O-sulfation of the chondroitin sulfate proteoglycans in the learning and memory in the young and early adult animals. To do so I used Chondroitin sulfotransferase 11 knockout mice strain, which has decreased amount of 4-O-sulfation. I tested their long-term, short-term, and working memory using behavioural tests. I also monitored the morphological changes in the perineuronal nets and changes in the number of synapses using immunohistochemical methods and confocal microscopy.

Keywords: extracellular matrix, learning, memory, chondroitin sulfate proteoglycans, sulfation pattern

Abstrakt:

Medzibunková hmota ovplyvňuje mozgové funkcie. Medzi funkcie, ktoré sú ovplyvnené zmenami v zložení a vlastnostiach medzibunkovej hmoty patrí aj učenie a pamäť. Rôzne sulfatačné vzorce na chondroitín sulfát proteoglykánach, jednej zo zložiek medzibunkovej hmoty, môžu buď zvýšiť alebo znížiť synaptickú plasticosť a rast neurónov. To potom vedie k zmenám vo fungovaní pamäte. V mojej diplomovej práci som definovala úlohu 4-O-sulfatace na chondroitín sulfát proteoglykánach v učení a pamäti u mladých a mladých dospelých myší. To som robila pomocou myšej línie s vyradeným génom pre Chondroitín sulfát transferázu 11, u ktorej je zníženú množstvo 4-O-sulfatace. U týchto myší som testovala dlhodobú pamäť, krátkodobú pamäť a pracovnú pamäť pomocou rôznych behaviorálnych testov. Okrem toho som sledovala morfológické zmeny v perineuronálnych sieťach a zmeny v počte synapsí pomocou imunohistológie a mikroskopie.

Kľúčové slová: medzibunková hmota, učenie, pamäť, chondroitín sulfát proteoglykány, sulfatačný vzorec

List of Abbreviations

ADAMTS – a disintegrin and metalloproteinase with thrombospondin motifs

AGC – aggrecan

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

C4S - chondroitin 4-O-sulfate

C6S - chondroitin 6-O-sulfate

ChABC – chondroitinase ABC

Chst11 - carbohydrate sulfotransferase-11

CNS – central nervous system

CS – chondroitin sulfate

CS4ST - 4-O-sulfotransferase

CS6ST - 6-O-sulfotransferase

CSPG – chondroitin sulfate proteoglycan

ECM – extracellular matrix

EGF – epidermal growth factor

GAG – glycosaminoglycans

GalNAc - N-acetylgalactosamine

GlcNAc – N-acetylglucosamine

GPI – glycosylphosphatidylinositol

HAPLN – hyaluronan and proteoglycan link protein

HAS – hyaluronan synthase

HS – heparan sulfate

LAR - leukocyte common antigen-related phosphatase

LTD – long-term depression

LTP – long-term potentiation

MEC – medial entorhinal cortex

MMP – matrix metalloproteinase

NgR1 – Nogo receptor 1

NgR3 – Nogo receptor 3

NMDA - N-methyl-D-aspartate

Otx2 - Orthodenticle homeobox 2

PNN – perineuronal net

PTP σ - protein tyrosine phosphatase σ

PV+ – parvalbumine positive

Sema3A – semaphorin 3A

Sema5A – semaphorin 5A

WFA – *Wisteria floribunda* agglutinin

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1 Introduction

Extracellular matrix of the brain has a wide range of roles in the brain functioning. It is a structural support for the cells, mediates their signalling, maintains a gradient of growth factors during development, inhibits or supports neuronal growth and synaptic plasticity. It can carry out all those functions thanks to its intricate composition. It is composed by various proteoglycans and glycoproteins, which are connected by tenascins and link proteins.

Function of proteoglycans, and therefore extracellular matrix, which is composed of them, differs based on their sulfation pattern. Sulfation pattern is managed by enzymes chondroitin sulfotransferases.

Most important in this thesis are 4-O-sulfation and 6-O-sulfation of the chondroitin sulfate proteoglycans. Chondroitin sulfate proteoglycans work both directly as a signalling molecules and by binding another signalling molecules. Mediated effect is sulfation dependent. 6-O-sulfation, which is most prevalent in the young animals is more permissive for neuronal growth and synaptic plasticity. On the other hand, 4-O-sulfation, which ratio increases with aging, is less permissive for neuronal growth and synaptic plasticity. Changes in the 6-O-sulfation and 4-O-sulfation very well coincide with the decrease in the plasticity.

Presence of the various chondroitin sulfate proteoglycans differs between brain areas and developmental stages. Changes in the composition of extracellular matrix have effect on the synaptic plasticity. They can modulate long-term potentiation and depression, which are the prerequisites for learning. Thus, chondroitin sulfate proteoglycans can influence learning by modulating them.

Hippocampus is important region in learning and memory. Many types of memory are associated with hippocampus, such as fear memory, spatial memory, object recognition memory or social memory. Disruption of the perineuronal nets or change in their sulfation pattern affects learning and memory.

In my master's thesis I researched the impact of the changes in the extracellular matrix, namely decrease of 4-O-sulfation, mediated by knockout of Chondroitin sulfotransferase-11 in the adult brain on the perineuronal net structure, then consequently on the synaptic changes and finally

the effect on the memory. I tested novel object recognition memory, social memory as well as spatial long-term memory, reversal learning and short-term working memory.

2 Brain extracellular matrix

Extracellular matrix (ECM) in brain fills space in between neurons and glia and it takes up 10-20% of the brain matter (Cragg, 1979). It constitutes mainly of hyaluronan, proteoglycans and link proteins. Collagenous and non-collagenous glycoproteins are present in the brain in much lower abundance than in other tissues (Rutka *et al.*, 1988). ECM provides structural support for the cells and modulates cell-matrix interactions. In addition to that brain ECM has special function in regulation of neuronal plasticity and neurite growth (Sethi and Zaia, 2017). Components of ECM are produced both by neurons and glial cells. As hyaluronan and link proteins are produced exclusively by neurons, production of different types of proteoglycans can take place either in neurons, glial cells or both of them. (Yamaguchi, 2000; Carulli *et al.*, 2006). Brain extracellular matrix can be distinguished to the three types, namely basement membrane, which covers blood-vessels, condensed matrix of perineuronal nets, which are enveloping certain types of neurons and loose matrix (interstitial ECM) in between (Lau *et al.*, 2013). In addition to that also perisynaptic matrix and perinodal extracellular matrix are distinguished by some authors (Fawcett, Ohashi and Pizzorusso, 2019).

2.1 Brain ECM composition

Composition of brain ECM differs from the extracellular matrix in the rest of the body. There is lower abundance of collagens and higher abundance of proteoglycans and hyaluronan than in other types of ECM (Novak and Kaye, 2000).

2.1.1 Proteoglycans

Proteoglycans are formed by core protein and bound glycosaminoglycans (GAG). Glycosaminoglycans are made of unbranched polysaccharide chains consisting of repeated disaccharides. N-acetylgalactosamine (GalNAc) and glucuronic acid form chondroitin and dermatan sulfate chains. Keratan sulfate chain is formed by N-acetylglucosamine (GlcNAc) and galactose (Ruoslahti, 1988). Amino sugars in the GAG are often sulfated. Glycosaminoglycans can be divided in the several groups based on their sugar composition, sulfation, and bonds: heparin, heparan sulfate (HS), keratan sulfate, dermatan sulfate and chondroitin sulfate (CS) (Novak and Kaye, 2000). There is a big difference in the number of GAG chains attached on the core protein between different proteoglycans. Proteoglycan aggrecan can have up to hundred bound chondroitin sulfate chains and up to sixty keratan

sulfate chains (Hardingham, 1981), while another proteoglycan versican has only five to fifteen GAG binding sites depending on its splicing variant (Dours-Zimmermann and Zimmermann, 1994).

Core structure

Core protein is different in each proteoglycan. They can be put together in groups based on same structural motifs. Most abundant group of proteoglycans with the similar protein core in brain ECM are lecticans, or hyalectans, which are chondroitin sulfate proteoglycans. They are mainly secreted ECM molecules (Yamaguchi, 2000). Lecticans have similar -N and -C end globular domains (Morgelin *et al.*, 1989). They contain hyaluronan binding site at the -N end (Watanabe *et al.*, 1997) and epidermal growth factor (EGF)-like, CRP element and lectin domain at the -C end (Ujita *et al.*, 1994).

Sulfation pattern

Chondroitin chains can have different types of sulfation. Unit A is 4-O-sulfated (C4S) by 4-O-sulfotransferases (CS4ST) at the N-acetylgalactosamine, C unit is 6-O-sulfated (C6S) by 6-O-sulfotransferases (CS6ST) at the GalNAc as well. There are also oversulfated disaccharides with two sulfated sites at once. D unit is 6-O-sulfated at the GalNAc and 2-O-sulfated at the glucuronic acid. E unit is 4-O-sulfated at glucuronic acid and 6-O-sulfated at N-acetylgalactosamine (Maeda, 2010). Sulfation pattern of brain chondroitin sulfate changes with the aging. C-6 sulfation decreases jointly with the decreased expression of chondroitin 6-sulfotransferase, on the other hand C-4 sulfation is increased in the developing brain (Kitagawa *et al.*, 1997). Sulfation pattern in the loose extracellular matrix of brain does not change anymore after the end of critical period. On the contrary, in the perineuronal nets C6S concentration continues to decrease, which changes the C4S/C6S ratio towards more restrictive C4S. PNNs from the 18-month-old rat are more inhibitory for neuronal growth than PNNs from 3-month-old rat, because they have higher C4S/C6S ratio (Foscarin *et al.*, 2017).

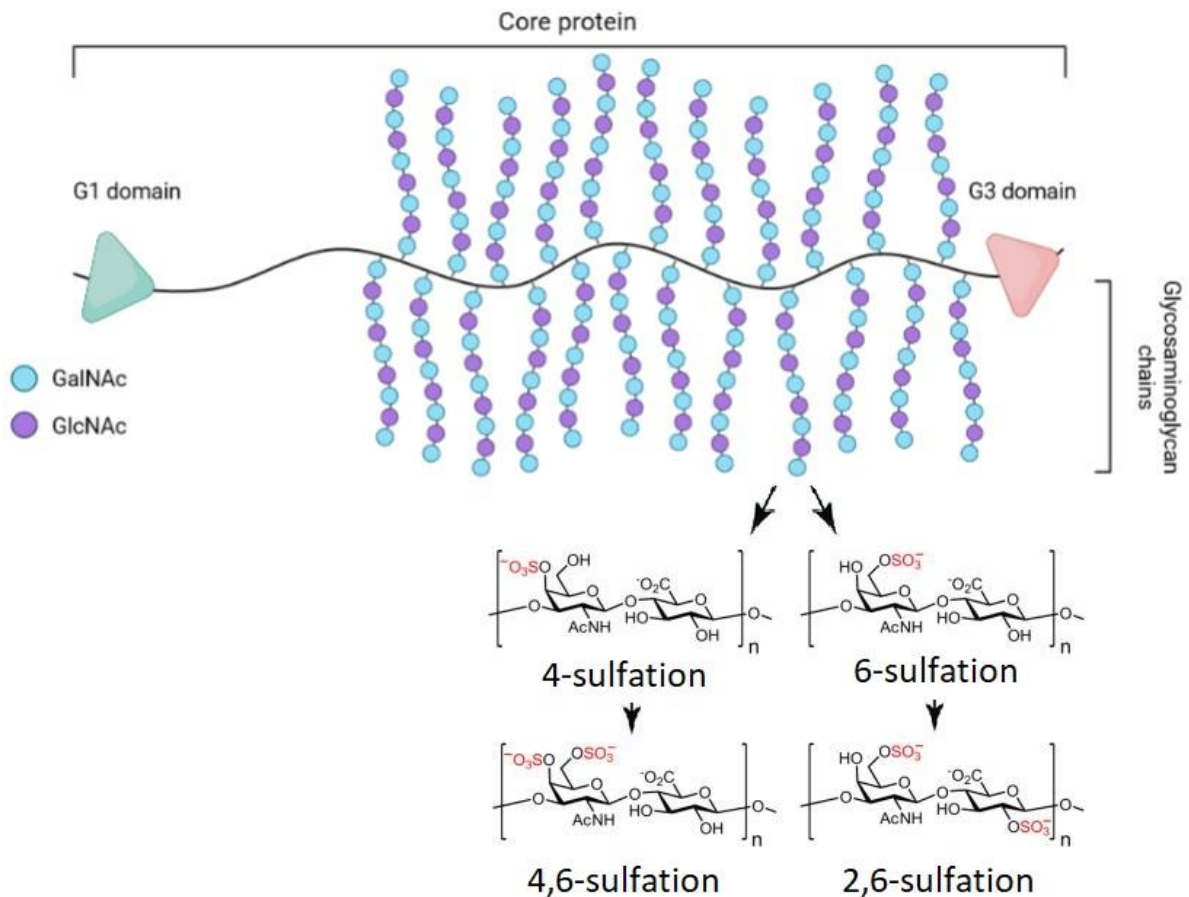


Figure 1 Chondroitin sulfate proteoglycan scheme and its sulfation patterns. Created with BioRender.com

2.1.1.1 Carbohydrate sulfotransferase-11

Carbohydrate sulfotransferase-11 (Chst11) also called chondroitin 4-sulfotransferase 1 is enzyme responsible for transfer of sulfate group to the 4th position of N-acetylgalactosamine (Yamauchi *et al.*, 2000). It sulfates non-sulfated chondroitin to form A unit but it does not transfer sulfate group to the already 6-O-sulfated chondroitin to form 4,6-O-disulfated E unit (Hiraoka *et al.*, 2000) or does so only to the limited extent (Yamauchi *et al.*, 1999). In addition to the chondroitin, carbohydrate sulfotransferase-11 also sulfates dermatan to form dermatan sulfate (Hiraoka *et al.*, 2000). Dermatan sulfate is not present in the brain (Trowbridge and Gallo, 2002).

Mice with homozygous knockout of *Chst11* gene in whole body are not viable. Cartilage growth plate has disrupted embryonal development in the absence of C-4 sulfation (Kluppel *et al.*, 2005). Decreased expression of Chst11 leads to the decreased amount of chondroitin sulfate A and E units and also to the shortened chondroitin sulfate chains, which implicates role of Chst11 in the chain elongation (Uyama *et al.*, 2006). Chst11 mRNA was observed mainly in the

cerebral cortex, hippocampus, thalamus, olfactory bulb, caudate putamen, midbrain and cerebellum regions of postnatal mice brain (Mitsunaga *et al.*, 2006). Overall level of expression of the *Chst11* in the adult brain remains stable (Foscarin *et al.*, 2017).

2.1.1.2 Aggrecan

Aggrecan (AGC) is a type of proteoglycan – lectican. It is dominantly present in the cartilage, but it also has important role in the brain extracellular matrix. Aggrecan protein core can bind both chondroitin sulfate and keratan sulfate chains, number of bound CS chains is higher than KS chains (Hardingham, 1981; Novak and Kaye, 2000). Brain aggrecan can exist in the keratan sulfate free form, in contrast to the cartilage AGC (Li *et al.*, 1996). In the rats, aggrecan concentration starts increasing postnatally and reaches plateau concentration at 5 months of age (Milev *et al.*, 1998). Adult concentrations of aggrecan in mice are reached at the 3 months of age (Carulli *et al.*, 2010). Fully functional perineuronal nets can be formed only in the presence of aggrecan (Rowlands *et al.*, 2018). Formation of perineuronal nets is synchronized with the increase in the aggrecan concentration (Ye and Miao, 2013).

2.1.1.3 Neurocan

Neurocan is proteoglycan with bound chondroitin sulfate chains. This proteoglycan is present exclusively in the central nervous system. It is connected to the hyaluronan via link proteins. Neurocan is expressed mostly in embryonic state, in the adult brain it is cleaved by matrix metalloproteinases (MMP) (Rauch *et al.*, 1991; Engel *et al.*, 1996). Neurocan binds also to the cell adhesion molecules N-CAM and Ng-CAM. This binding inhibits neurite outgrowth (Friedlander *et al.*, 1994).

2.1.1.4 Versican

Versican is another chondroitin sulfate proteoglycan, which is present in the brain ECM. There are four isoforms of versican V0, V1, V3 and V4. Each of them have different amount of bound chondroitin sulfate chains (Dours-Zimmermann and Zimmermann, 1994). Versican inhibits neurite outgrowth similarly to the neurocan (Schmalfeldt *et al.*, 2000). Versican is present in the brain already in the embryonic state. In the prenatal brain V1 isoform is prevailing. Postnatally its concentration lowers, which is similar to the neurocan. In the maturing brain versican concentration again starts to rise similarly to the aggrecan. In this case it is the V2 isoform of versican. This isoform is most prevalent in the mature brain (Milev *et al.*, 1998).

2.1.1.5 *Brevican*

Brevican is chondroitin sulfate proteoglycan present exclusively in the brain ECM. There are two isoforms of brevican. Excreted in the extracellular space and membrane-bound by the glycosylphosphatidylinositol (GPI) anchor (Seidenbecher *et al.*, 1995). Glial cells are expressing predominantly GPI-anchored form of brevican (Seidenbecher *et al.*, 1998). Neurons express brevican as well (John *et al.*, 2006). Levels of brevican starts rising postnatally and they reach their maximum in the adult brain (Seidenbecher *et al.*, 1998).

2.1.1.6 *Hyaluronan*

Hyaluronan (HA) is only glycosaminoglycan, which is not bound on the protein core, therefore it is not a proteoglycan. It is also only GAG which is not sulfated (Novak and Kaye, 2000). HA is composed of repeating glucuronic acid and N-acetylglucosamine subunit (Meyer, 1951). Hyaluronan serves as a scaffold for the binding of other ECM molecules and formation of extracellular matrix in the brain (Kwok, Carulli and Fawcett, 2010). Hyaluronan is produced by hyaluronan synthases (HAS), which are bound to the cytoplasmatic membrane. They are secreting hyaluronan to the extracellular space. Three isoforms of HAS exists – HAS1, HAS2 and HAS3 (Itano *et al.*, 1999). HAS isoforms are expressed in various quantities in different parts of the brain (Carulli *et al.*, 2006).

2.1.1.7 *Heparan sulfate proteoglycans*

Heparan sulfate proteoglycans (HSPG) constitutes of protein glycosylated by heparan sulfate GAG chains (Lindahl *et al.*, 1989). They can be either membrane-bound proteins, such as syndecans and glypicans or secreted, such as perlecan, agrin and collagen XVIII (George and Geller, 2018). Heparan sulfate can have different sulfation patterns similarly to the chondroitin sulfate chains (Kreuger and Kjellén, 2012). HSPG share common PTP σ cell receptor with CSPG (Aricescu *et al.*, 2002; Shen *et al.*, 2009).

2.1.2 **Glycoproteins**

2.1.2.1 *Collagens*

Collagenous glycoproteins are formed by three polypeptide chains, which form triple-helix. Different collagens are distinguished by amino-acid sequence of the polypeptide chains. Collagens are synthesized and secreted as procollagens. Once they are in the extracellular space

they are cleaved by proteases, which enables cross-linking between them (Shoulders and Raines, 2009). Expression of collagen in the brain parenchyma was unclear up until recently. Initial studies did not find type VI collagen (Marvulli, Volpin and Bressan, 1996) and later experiments in which collagen VI was found in brain tissue offered alternative explanations for their results (Braghetta *et al.*, 1996). Recent study by Zech *et al.* confirmed presence of type VI collagen mRNA in the various parts of the brain (Zech *et al.*, 2015).

2.1.2.2 Fibronectin

Fibronectin is non-collagenous glycoprotein present in brain ECM. It is formed by two subunits bound by disulfide bond. Each subunit contains multiple domains, which enables binding to the collagen, integrins, heparin and glycosaminoglycans (Wierzbicka-Patynowski and Schwarzbauer, 2003).

2.1.2.3 Link proteins and tenascins

Hyaluronan and proteoglycan link proteins (HAPLN) also called link proteins in short are glycoproteins which connects hyaluronan with proteoglycans. There are four types of them, HAPLN1, -2, -3 and -4 (Spicer, Joo and Bowling, 2003). They vary in the binding of different proteoglycans (Shi *et al.*, 2004).

HAPLN1 (also called cartilage-linking protein 1 - CRTLN1) is link protein, which is present in the perineuronal nets. It is produced by neurons covered in PNN. Increase in the mRNA expression coincides with the beginning of PNNs formation and in the adult it decreases (Galtrey *et al.*, 2008). HAPLN1 is necessary for the correct perineuronal nets formation, in the knockout mice lacking HAPLN1 perineuronal nets were attenuated in the adult brain (Carulli *et al.*, 2010).

HAPLN4 (also called brain-specific link protein 2 - BRAL2) is predominantly present in the brain. It is produced by the PNN positive neurons (Bekku *et al.*, 2003). HAPLN4 mRNA levels increase coincides with the PNNs formation similarly to the HAPLN1 and its levels remain stable up to the adulthood (Galtrey *et al.*, 2008). HAPLN4 colocalizes with the brevican in the PNNs. HAPLN4 deficient mice does not form proper perineuronal nets, brevican is absent in them, but other components are only slightly changed in contrast to HAPLN1 deficient mice, whose PNN had marked decrease in all of the components (Bekku *et al.*, 2011).

HAPLN2 (also called brain-specific link protein 1 - BRAL1) colocalizes with the V2 versican at the Ranvier nodes. It is produced mainly by the neurons (Oohashi *et al.*, 2002). Versican and brevican were absent in the HAPLN2 deficient mice Ranvier nodes. Morphological changes were not observed but nerve conduction was decreased in the *Hapln2* knockout mice (Bekku *et al.*, 2010).

Tenascins are another type of glycoprotein present in the brain ECM which are connecting proteoglycans to the cell surface (Kato *et al.*, 2013). There are four types of tenascins but only two of them are present in brain, namely tenascin-C and tenascin-R. Tenascin-R is present exclusively in the brain extracellular matrix (Bourdon *et al.*, 1983; Wolff, Rathjen and Chiquet-Ehrismann, 1991). It is essential in the formation of perineuronal net similarly to the HAPLN proteins (Weber *et al.*, 1999). Apart from perineuronal nets, tenascin-R is localized predominantly at the nodes of Ranvier (Bartsch *et al.*, 1993).

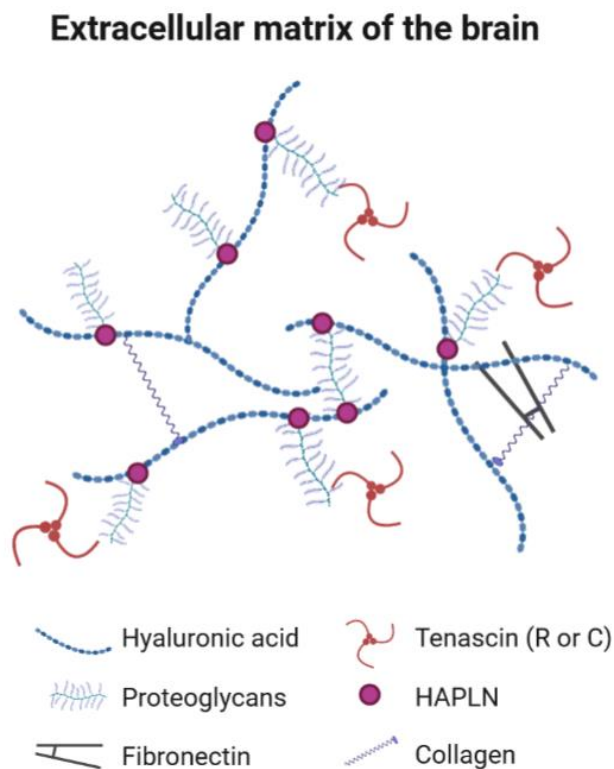


Figure 2 Extracellular matrix of the brain. Created with BioRender.com

2.2 Function of chondroitin sulfate proteoglycans in ECM

2.2.1 Mechanisms of CSPG signalisation

Chondroitin sulfate proteoglycans can either directly bind to the cell receptors and mediate effect themselves or bind other signalling molecules and increase their concentration near the cell (Schwartz and Domowicz, 2018). Only currently known chondroitin sulfate proteoglycan receptors on the cell are protein tyrosine phosphatase σ (PTP σ), which binds the CS part of CSPG (Shen *et al.*, 2009) and LAR (Leukocyte common antigen-related phosphatase) (Fisher *et al.*, 2011) and Nogo receptors NgR1 (Nogo receptor 1) and NgR3 (Nogo receptor 3) (Dickendesher *et al.*, 2012).

PTP σ

PTP σ is not just receptor for CSPGs, but also for heparan sulfate proteoglycans (Aricescu *et al.*, 2002). Differently sulfated CS chains stimulates different processes in the cell. Chondroitin-4,6-disulfate (E unit) inhibits neuronal growth via PTP σ receptor and epidermal growth factor receptor (EGFR) (Kaneko *et al.*, 2007) and Rho/Rho-kinase (ROCK) pathways (Sivasankaran *et al.*, 2004). 6-O-sulfated chondroitin sulfates did not inhibit growth of the neuron, also 4-O-sulfated CS did so only in the much higher concentration than oversulfated E unit (Brown *et al.*, 2012). There is some concern about validity of such experiments because used CSPG are obtained by extraction and their composition and sulfation pattern may differ between labs.

LAR

LAR receptor inhibits neuronal growth after CSPG binding. Its function is mediated through Akt and RhoA pathways (Monnier *et al.*, 2003; Fisher *et al.*, 2011). LAR and PTP σ signalling pathways are partially overlapping, but they are not the same. In PTP σ signalling Rho activates ROCK, which inactivates neuronal growth through microtubules. In LAR signalling Rho inactivates actin-binding protein cofilin. LAR inhibits neuronal growth through actin filaments (Ohtake *et al.*, 2016).

Nogo receptors

Two of the Nogo receptors are binding CSPG - NgR1 and NgR3. Their binding is sulfation pattern dependent. They bind oversulfated chondroitin sulfates – CS-D and CS-E and also

dermatan sulfate. They do not bind 4-O-sulfated CS-A or 6-O-sulfated CS-C. Activation of Nogo receptors causes growth inhibition of the neuron (Dickendesher *et al.*, 2012).

Protein core interaction

Also, protein core can have direct effect on the cell. Receptors and signalling pathways involved in such signalling are not known. But there are experiments, which showed effect of deglycosylated proteoglycans on the cell growth and morphology (Lemons *et al.*, 2003; Beller *et al.*, 2013).

Signalling molecules

Different sulfation pattern of CSPG can also change their ability to bind signalling molecules (Deepa *et al.*, 2002; Maeda *et al.*, 2003). 4-O-sulfation is most prevalent form in the loose matrix, perineuronal nets and cytoplasmically bound chondroitin and heparan sulfates. Amount of 6-O-sulfated, 2,6-O-disulfated and non-sulfated CSPG/HSPG is higher in the perineuronal nets than in the loose matrix. Amount of 2,4-O-disulfated proteoglycans is same in all types of ECM (Deepa *et al.*, 2006).

Signalling molecules, which bind to the CSPG of extracellular matrix in the sulfation dependent manner include chemokines, morphogens, neurotrophic and growth factors (Schwartz and Domowicz, 2018). Oversulfated CS-E showed the best binding ability for majority of the CSPG-binding signalling molecules (Deepa *et al.*, 2002; Maeda, Fukazawa and Hata, 2006). Low sulfated CS-A, CS-B and CS-C interacts with wide range of signalling molecules as well even though their binding is weaker than with oversulfated CS-E (Mizumoto, Fongmoon and Sugahara, 2013). CS-D which is also disulfated has weaker binding ability than CS-E, it is comparable to the low sulfated chondroitin sulfates. This indicates that binding is pattern-specific, not just higher with the higher amount of sulfation (Shipp and Hsieh-Wilson, 2007).

2.2.2 Neurodevelopment

Proteoglycans are involved in neurodevelopment. HSPG are binding growth factors and morphogens in the site-specific manner (Inatani *et al.*, 2003). CSPG are involved mainly in axon-guidance (Wilson and Snow, 2000) and synaptogenesis (Favuzzi *et al.*, 2017). Their specificity is retained by the various sulfation patterns, which are changing during development. Those changes continue throughout the adult life and in the aging (Wang and Fawcett, 2012).

2.2.3 Perineuronal nets

Perineuronal net (PNN) is specialized type of dense extracellular matrix in the brain, which is present around synapses and soma of some neurons (Lau *et al.*, 2013). Majority of the neurons covered in the perineuronal nets are GABAergic parvalbumine positive (PV+) interneurons and pyramidal cells in hippocampus (Lensjø *et al.*, 2017). PNNs consist of chondroitin sulfate proteoglycans, hyaluronan, link proteins and tenascins (Yamaguchi, 2000). Only 1,3% of the brain CSPG are part of the perineuronal nets, majority is in the loose ECM (Deepa *et al.*, 2006).

Perineuronal nets have multiple functions. They are capturing ions secreted during action potential and prohibit their diffusion away from the synapse, they also work as an ion exchanger (Morawski *et al.*, 2015). Another of PNNs features is protection of cells against oxidative stress (Morawski *et al.*, 2004). Perineuronal nets prohibit neuronal growth and formation of new synapses since they contain growth inhibitory CSPG and bind additional diffuse inhibitory signalling molecules. In addition to the growth inhibitory signalling molecules, PNNs can bind more signalling molecules, which can have wide range of functions (Schwartz and Domowicz, 2018).

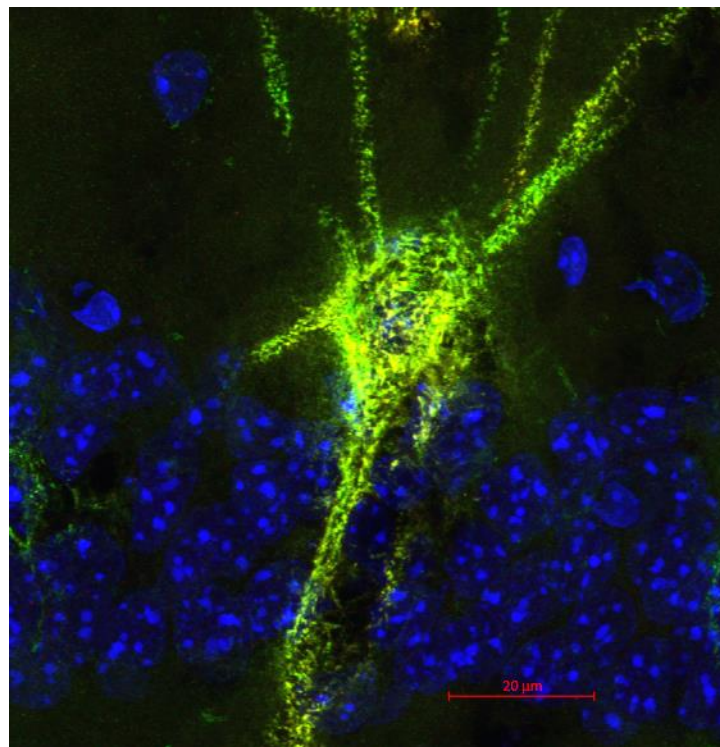


Figure 3 Perineuronal net covered neuron. Blue – DAPI, cell nucleus; Green – Wisteria floribunda agglutinin, Red – anti-aggrecan antibody, both perineuronal nets

2.2.4 Semaphorin 3A

Semaphorin 3A (Sema3A) is chemorepulsive axon-guidance molecule (Luo, Raible and Raper, 1993; Chédotal *et al.*, 1998). It is present in the dense matrix of perineuronal nets. Sema3A binds with high affinity to the GAG of the CSPG. Presence of semaphorin 3A in the perineuronal nets is one of the mechanisms by which PNNs inhibits neuronal growth and plasticity (Vo *et al.*, 2013). It binds specifically to the oversulfated CS-E chain (Dick *et al.*, 2013). Semaphorin is present only in some brain parts. Pyramidal cells in the CA2 area of hippocampal system have high Sema3A concentration in their perineuronal nets as well as in the loose matrix in between. In contrast with that only few neurons in the CA1 and CA3 areas of hippocampus have semaphorin 3A containing perineuronal nets. In the CA3 area loose matrix contains Sema3A (Vo *et al.*, 2013).

2.2.5 Orthodenticle homeobox 2

Orthodenticle homeobox 2 (Otx2) is transcription factor, which has a role in brain development. It binds to the chondroitin sulfate part of the CSPG. Binding to the perineuronal nets increases its concentration around the cell and therefore intake to the cell. Its binding depends on the sulfation pattern, C4S is more potent in binding Otx2 than C6S. With the aging 4-O-sulfation increases which causes increased concentration of Otx2 (Miyata *et al.*, 2012). Otx2 is involved in the maturation of electrophysiological properties of the neurons (Sugiyama *et al.*, 2008).

3 Learning and memory

3.1 Role of sulfated proteoglycans in neuronal plasticity and synaptogenesis

3.1.1 Synapse

Synapse is composed of presynaptic terminal, synaptic cleft and postsynaptic terminal (Luo, 2010). Different way of describing a synapse is a tetrapartite synapse, it constitutes of presynaptic terminal, postsynaptic terminal, extracellular matrix and glial cells (Dityatev and Rusakov, 2011). Synapse is formed at the place where two adjacent cells signalize to each other by growth supporting signals. Neuronal activity is condition for the synapse formation and its prevailing. New synapses can be formed and old ones lost during the life (Caroni, Donato and Muller, 2012). Synapses can also change their transmission efficacy. Certain activity can lead to the short-term potentiation, short-term depression, long-term potentiation or long-term depression. All those processes are part of synaptic plasticity (Dityatev and Schachner, 2003).

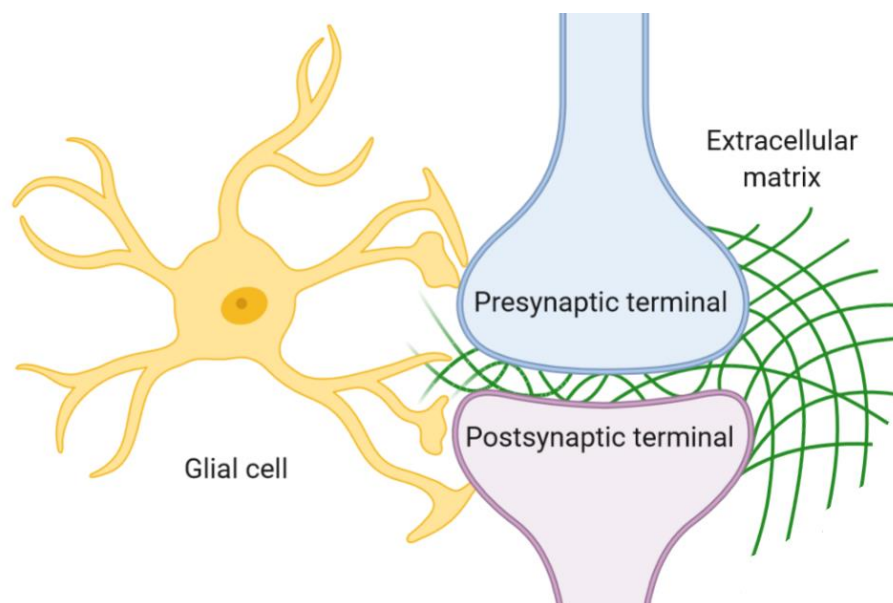


Figure 4 Tetrapartite synapse. Created with BioRender.com

There is an interplay between extracellular matrix and synaptic terminals during whole life – in the development, maintenance, strengthening or restriction of their growth, those mechanisms are called homeostatic plasticity (Pyka *et al.*, 2011; Miyata *et al.*, 2012; Favuzzi *et al.*, 2017). ECM interacts with the other synaptic components either by binding signalling molecules or by working as a signalling molecule themselves (Dityatev and Rusakov, 2011). Chondroitin sulfate

proteoglycans are part of the synapses mainly in the form of perineuronal nets in the ECM part of tetrapartite synapse. Semaphorin 3A is present in the synapses bound to the CSPG and HSPG (Vo *et al.*, 2013).

There are two types of synapses, excitatory which are activating postsynaptic neuron are mainly glutamatergic synapses (Meldrum, 2000) and inhibitory which are inhibiting postsynaptic neuron are mainly GABAergic synapses (Fritschy and Brünig, 2003). Number of excitatory and inhibitory synapses on each neuron is responsible for its final excitability (Zhou and Yu, 2018).

3.1.2 Synaptic plasticity

Short-term plasticity

Short-term potentiation lasts from milliseconds to the minutes. It is induced by short bursts of neuronal activity, which leads to accumulation of calcium in presynaptic terminal. Increased calcium concentration changes the probability of neurotransmitter release. Short-term depression can be caused by the depletion of the neurotransmitter after the first activation. These processes are regulated by many additional mechanisms within the presynaptic neuron, by postsynaptic neuron and neighbouring glial cells (Citri and Malenka, 2008).

Long-term plasticity

Long-term potentiation (LTP) lasts from hours to the months. Long-term potentiation is based on both pre- and postsynaptic modifications which are later stabilised by changes in the protein synthesis. Most studied is LTP in the neurons of CA1 region of hippocampus, which is based on the activation of NMDA (N-methyl-D-aspartate) receptors on the postsynaptic membrane. They can be activated only when presynaptic neuron releases glutamate coincidentally with the depolarization of postsynaptic neuron, which releases Mg^{2+} blocker from the NMDA receptor. Long-term potentiation is then carried out by increasing the number of postsynaptic glutamatergic AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and later by changes in protein synthesis. LTP involves morphological changes of the dendritic spines, and they might involve also changes in the synapse number. Long-term depression (LTD) is realized through modest activation of postsynaptic NMDA receptors which then induce endocytosis of the AMPA receptors (Dityatev and Schachner, 2003; Citri and Malenka, 2008).

Role of CSPG in synaptic plasticity

Enzymatic removal of CSPG lead to reduction of both LTP and LTD in the CA1 hippocampal region. It did not have effect on the short-term potentiation. In the knockout mice without tenascin-R LTP was also decreased, and it did not decrease anymore after enzymatical degradation of chondroitin sulfate proteoglycans in PNNs. That indicates that mechanism by which LTP was reduced in both cases is same. But even though reduction of LTP was same in tenascin-R and CSPG deficient mice, they differed in short-term potentiation. Tenascin-R knockout caused reduction in short-term potentiation, while degradation of CSPG did not have any effect on it (Bukalo, Schachner and Dityatev, 2001). In the another PNN component, brevican knockout mice LTP was deficient as well, but signal transmission, learning and memory, as tested in Morris water maze, were functional (Brakebusch *et al.*, 2002). In the quadruple knockout of tenascin-R, tenascin-C, brevican and neurocan early-LTP (<75 min) was absent, while late-LTP (>24 hr) was present (Jansen *et al.*, 2017).

Mice which had decreased 6-O-sulfation of CSPG, which is growth permissive type of sulfation, normally present in the younger mice had premature memory loss accompanied by loss of LTP in the hippocampus and cortex (Yang *et al.*, 2021).

3.1.3 Long-term potentiation and learning

Hippocampus plays a key role in the formation of declarative memory (Squire, Stark and Clark, 2004). Working memory is dependent on both hippocampus and prefrontal cortex (Barbas and Blatt, 1995). In the rodents one of the most studied forms of declarative memory is spatial memory tested primarily by the Morris water maze test (Morris *et al.*, 1982). Role of long-term potentiation in learning is supported by various types of evidence. Pharmacological inhibition of hippocampal LTP or knockout animal lacking some key component of LTP disabled hippocampal learning (Morris, 1986; Tsien, Huerta and Tonegawa, 1996; Balschun and Wetzell, 2002). Hippocampal long-term potentiation was also recorded *in vivo* during learning (Whitlock *et al.*, 2006). Site-specific enzymatic reduction of dendritic spines which are remodelled after learning caused the disruption of the memory (Hayashi-Takagi *et al.*, 2015). In the experiment by Nabavi *et al.* they paired fear conditioning with optogenetic activation of auditory inputs in amygdala. Then they optogenetically simulated LTD in the given area of amygdala. Afterwards there was no fear reaction after conditioned stimulus. In the next step

they induced LTP and by that reactivated induction of fear memory by conditioned stimulus (Nabavi *et al.*, 2014).

3.1.4 Neuronal plasticity

In this thesis I use the definition of neuronal plasticity as morphological and functional changes. Changes in the brain during life do not happen only on the synaptic level by changing the amount of the receptors or size of the dendritic spines but also by increasing or decreasing the general amount of the synapses or by the neuronal growth. Neuronal plasticity is on highest levels in the developing brain before closure of critical period. But it can also be reactivated in the adult brain. For example, environmental enrichment causes enhanced turnover and increase in the number of synapses (Bednarek and Caroni, 2011). Stress decreases number of synapses in the hippocampus and increases their number in amygdala (McEwen and Gianaros, 2010). Neuronal growth can be artificially reactivated after injury, which damaged the axon or neuronal soma. CSPG plays a inhibitory role in the axonal growth and their guidance (Pizzorusso *et al.*, 2002; Lemons *et al.*, 2003; Loers *et al.*, 2019).

Increased density of the PNNs leaves smaller space for synaptic proteins, thereby inhibiting their plasticity (Sigal *et al.*, 2019). Quadruple knockout mice without PNN components – tenascin-R, tenascin-C, brevican and neurocan, with reduced PNNs in hippocampus had increased number of excitatory synapses and decreased number of inhibitory synapses in hippocampal neurons (Gottschling *et al.*, 2019). On the other hand, CSPG can also have a specific signalization function with activating effect on plasticity. Brevican presence in the perisynaptic space of PV+ interneurons induced excitatory input by upregulating density of AMPA receptors in excitatory synapses. Brevican levels were changing in response to the neuronal activity. Loss of brevican lead to the impairment in the spatial and working memory (Favuzzi *et al.*, 2017). While brevican regulates excitatory synapses, neurocan influences the amount of inhibitory synapses (Schmidt *et al.*, 2020). Number of inhibitory synapses on the PV+ inhibitory interneurons decreases with the age. Which means that PV+ interneurons are more inhibitory in the older animals and that consequently impairs learning. In the old mice which had experimentally increased C6S in contrast to C4S, perineuronal nets were more permissive, number of inhibitory synapses on PV+ interneurons increased to the adult state and learning was improved (Yang *et al.*, 2021).

3.1.5 Critical period

Critical period is a timespan with high neuronal plasticity. Neurons are growing, new synapses are being formed and strengthened or redundant synapses are reduced. Only those synapses which are being activated based on the external stimuli are preserved. Critical period starts after birth and ends before adulthood in the different brain parts at different time. Closure of critical period is regulated by the increased concentration of chondroitin sulfate proteoglycans in the PNNs. Experimental degradation of CSPG by chondroitinase ABC (ChABC) leads to reinstatement of juvenile-like plasticity (Pizzorusso *et al.*, 2002; Carulli *et al.*, 2010; Hylin *et al.*, 2013). In addition to the changes in the total CSPG concentration, changes in their sulfation pattern are involved in the closure of critical period as well. There is an increase in C4S/C6S ratio (Foscarin *et al.*, 2017). Upregulation of C6S can reinstate the plastic state after the closure of critical period similarly to the enzymatical degradation of CSPG (Miyata *et al.*, 2012).

3.1.6 Metalloproteinases

Matrix metalloproteinases (MMP) are proteolytic enzymes which are acting mainly in the extracellular space. MMP9, which is required for the LTP is present in the perisynaptic space and is elevated after the LTP induction. It is a key enzyme in the synaptic remodelling (Nagy *et al.*, 2006; Bozdagi *et al.*, 2007). MMP9 and MMP2 are cleaving chondroitin sulfate proteoglycans (Zuo *et al.*, 1998; Mercuri *et al.*, 2000). In the experiment, which induces synaptic plasticity in the visual cortex by monocular deprivation both MMP9 and MMP2 increased their activity in the perisynaptic space and induced PNN degradation (Murase *et al.*, 2019).

ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) are secreted proteolytic enzymes. ADAMTS-4 degrades various brain CSPGs (Tauchi *et al.*, 2012). In the mice with increased C6S, aggrecan, one of the PNNs components, was degraded by the increased activity of ADAMTS. This indicates that CSPG with different sulfation patterns can mediate their effect on plasticity through proteolytic enzymes (Miyata and Kitagawa, 2016).

Expression of both ADAMTS and MMPs is highest during development coincidently with the critical period (Wen *et al.*, 2018).

3.2 Adult brain is stabilised by perineuronal nets

3.2.1 Perineuronal nets in the visual cortex

Classical model in research of the critical period in the neuronal development is monocular deprivation of animal, which leads to the enhancement in the function of non-deprived eye and in case that deprived eye is not used at all from the birth to the loss of function of given eye (Wiesel and Hubel, 1965). Eye deprivation does not have notable effect on the adults. CSPG, which form perineuronal nets appear in the visual cortex at the end of the critical period (Lander *et al.*, 1997; Pizzorusso *et al.*, 2002). Perineuronal nets in the dark reared animals did not form properly, neurocan was not concentrated in the PNN as it is in animals with properly formed visual cortex. The animals which were put at the light still during critical period were able to form PNN with correctly distributed neurocan (Pizzorusso *et al.*, 2002). Since inhibitory effect of CSPG on the neuronal growth is caused mostly by chondroitin sulfate chains, their enzymatical degradation can re-new axonal growth (Moon *et al.*, 2001). In the experiment performed by Pizzorusso *et al* monocular deprivation of adult rats combined with injection of chondroitin sulfate degrading enzyme – chondroitinase ABC – into the visual cortex led to the change in the ocular dominance as if they were still in the critical period (Pizzorusso *et al.*, 2002). Similarly targeted *in vivo* loss of the aggrecan, which is a necessary component for the PNN formation, in the visual cortex led to the reinstatement of the juvenile plasticity in the monocular deprivation experiment (Rowlands *et al.*, 2018).

In another experiment by Pizzorusso *et al* they went even further and recovered the ocular function in the adult rat, which was monocularly deprived during critical period by applying chondroitinase ABC into the visual cortex (Pizzorusso *et al.*, 2006).

Since Crtl-1 link protein is necessary for the proper formation of PNNs, its knockout mice retain neuronal plasticity into the adulthood. It was showed in the experiment by the Carulli *et al* in which mice lacking link protein retained ocular dominance plasticity at the adulthood. Concentrations of the other PNN components remained unchanged. They also observed changes in the sulfation pattern of CS in the visual cortex. Changes started at the postnatal day 21, up to the adult mice in 3 months 6-sulfation decreased and was replaced by 4-sulfation. Concentration of di-sulfated forms was decreased as well (Carulli *et al.*, 2010).

End of the critical period coincides with the changes in the sulfation pattern of CS to the higher ratio of C4S/C6S. Transgenic mice with overexpressed enzyme for the 6-sulfation had increased amount of C6S in the perineuronal nets and PNNs were reduced. Those mice had preserved juvenile-like plasticity in the experiment with monocular deprivation (Miyata *et al.*, 2012).

3.2.2 Enriched environment results in decrease of PNNs in cerebellar nuclei

In the mice reared in the enriched environment axonal buttons of Purkinje cells in the cerebellar nuclei are enlarged in the comparison with mice reared in the standard conditions, which shows that environment causes the changes in the cerebellar nuclei. Expression of extracellular matrix components is decreased, and expression of metalloproteinases increased in the cerebellar nuclei of mice from enriched environment. Number of perineuronal net enveloped neurons was decreased. Also in the knockout mice, which were lacking Ctrl-1 link protein and therefore did not have properly formed perineuronal nets axonal buttons of Purkinje cells were enlarged similarly to the state found in the mice from enriched environment. This study showed that external stimulus can induce changes in the extracellular matrix of the adult and thereby increase plasticity (Foscarin *et al.*, 2011).

3.3 Role of perineuronal nets in the memory

3.3.1 Fear memories

Fear memories are based on pairing of neutral stimulus with aversive stimulus. Such memories cannot be erased in adult rats (Myers and Davis, 2007), but in rats younger than 3 weeks they can be permanently erased by conditioning to the neutral stimulus without pairing it with the aversive one (Kim and Richardson, 2007). Change in the formation of extinguishable and non-extinguishable fear memories after postnatal day 21 coincides with the perineuronal net formation. In the experiment by Gogolla *et al* they degraded chondroitin sulfate chains in the basolateral amygdala, which stores fear memories, by chondroitinase ABC in the 3 months old mice. Subjects which were injected with the enzyme before fear-conditioning exhibited erasure of the fear memories similar to the juvenile subjects. This shows that degradation of perineuronal nets reinstates juvenile-like state in the brain. But there was also a difference between induced and physiological state, erasure of fear memories in the ChABC treated mice was faster than in the untreated young mice. They hypothesized that it might be related to the

diffuse CSPG which are present in the wild type mice but are dissolved together with the PNN in the ChABC treated mice (Gogolla *et al.*, 2009a).

In the study by Yang *et al* they transplanted progenitors of inhibitory interneurons from the embryonic mice into the amygdala of 5- to 7-week-old mice. Transplanted cells induced juvenile-like plasticity in the amygdala of recipient mice for the short period of time. Erasure of fear memories was possible in those mice at the age of 8- to 9-weeks, when critical period is already finished in the untreated mice. At the time of induced neuronal plasticity there was a decrease in the number of perineuronal net positive interneurons (Yang *et al.*, 2016).

In another experiment by Hylin *et al* they investigated the role of perineuronal nets in the acquisition and retention of fear memories. PNNs were dissolved after fear conditioning by combination of ChABC and another PNN dissolving enzyme – hyaluronidase. Acquisition of fear memories was tested 48 hours after conditioning. Disruption of hippocampal PNNs impaired the acquisition of the contextual fear memory but not of the cue-based fear memory. This is in agreement with the hypothesis that hippocampus is responsible for the spatial learning and memory. Dissolved PNNs in hippocampus impaired trace fear memory as well. Trace fear conditioning is based on separation of unconditioned and conditioned stimuli by few seconds. Acquisition of the fear memory was possible in the mice with disrupted hippocampal PNNs, but connection to the place, where it took place or how long is the time between unconditioned and conditioned stimuli was impaired (Hylin *et al.*, 2013).

3.3.2 Object recognition memory

Novel object recognition (NOR) test is a rodent test for long-term or short-term explicit memory (Ennaceur and Delacour, 1988) which depends on the perirhinal cortex, prefrontal cortex and hippocampus (Meunier *et al.*, 1993; Tanimizu, Kono and Kida, 2018). In the experiment by Romberg *et al* they found out, that Crtl-1 knockout mice and mice treated with the ChABC in the perirhinal cortex had decreased amount of PNN-positive neurons, in that area and improved object recognition memory (Romberg *et al.*, 2013). Object recognition was enhanced also in the knockout mice without aggrecan in which brain perineuronal nets are not formed properly (Rowlands *et al.*, 2018).

Object recognition memory in the tauopathy mice model of Alzheimer disease is significantly reduced. After injection of ChABC into the perirhinal cortex object recognition was reinstated to the normal levels (Yang *et al.*, 2015).

In the brevicin knockout mice long-term object recognition memory (after 24 hours) was improved similarly to the Ctrl-1 knockout or ChABC treated mice, but they displayed the impairment in the short-term protocol with 5 minutes interval (Favuzzi *et al.*, 2017).

Blocking of C4S by antibody was sufficient to enhance long-term object recognition memory to the 24 hours in the wild-type mice and in the tauopathy model it managed to restore normal object recognition (Yang *et al.*, 2017). With the aging, ratio of 6-O-sulfation and 4-O-sulfation changes to the more C4S, this change is expected to play a role in the worsening of memory. Reduction of the C6S in the transgenic mice leads to the premature memory loss in the NOR test. On the other hand, increased C6S levels in the aged mice restored NOR discrimination ratio almost to the levels of young mice. It also enhanced long-term memory up to 24 hours like the ChABC digestion or blocking of C4S by antibody did (Yang *et al.*, 2021).

3.3.3 Spatial memory

Areas responsible for spatial orientation are hippocampus and medial entorhinal cortex (MEC). Position of the animal in the space is encoded in the grid cells. Grid cells firing is based on stable regular pattern, which is virtually present in the space. This pattern is stable within multiple visits of one place, distance in which once cell is activated stays the same (Fyhn *et al.*, 2004). Onset of the grid cells activity coincides with the perineuronal net formation in the medial entorhinal cortex (Lensjø *et al.*, 2017). In the experiment by Christensen *et al* they digested perineuronal nets in the MEC. Disruption of perineuronal nets caused reduction in the spiking of the PV⁺ inhibitory interneurons. This resulted in the decreased specificity of the grid cell firing both in familiar and novel arena (Christensen *et al.*, 2021).

Persistent stress led to the increase in the perineuronal nets in the hippocampus and impairment in the spatial memory (Riga *et al.*, 2017). That was supported by the finding that knockout mice for the signalling molecule neuropeptide Y, which is involved in the stress signalling, had increased expression of PNNs in the hippocampus and impaired spatial learning (Bertocchi *et al.*, 2021). On the other hand, digestion of PNNs in hippocampus by ChABC improved the re-learning in the long-term Morris water maze (Ruzicka *et al*, unpublished results). Tenascin-R

deficient mice have improved reversal learning, similar to the ChABC treated mice. They have improved spatial working memory as well (Morellini *et al.*, 2010).

Brevican knockout mice, which had impaired short-term memory in the NOR test had deficits also in the spatial working memory as tested in T-maze (Favuzzi *et al.*, 2017).

3.3.4 Social memory

Areas responsible for social recognition memory are hippocampus, medial prefrontal cortex, amygdala and anterior cingulate cortex (Tanimizu *et al.*, 2017). CA2 area of hippocampus is responsible for the social memory – ability to remember a given individual. Mice with lesioned CA2 area or transgenic mice with inactivated CA2 pyramidal neurons were not able to differentiate between their littermate and novel mouse, while their sociability was not affected (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014). Pyramidal neurons in CA2 area are covered in perineuronal nets, which are suppressing synaptic plasticity (Carstens *et al.*, 2016). In the study by Yoshioka *et al* they found out that mice with decreased concentration of chondroitin sulfate, which led to the reduction of perineuronal nets had improved social recognition memory (Yoshioka *et al.*, 2017).

4 Aims

In this thesis we aimed to characterize heterozygous nestin-Cre *Chondroitin sulfotransferase-11* knockout mice strain following the changes in morphology of PNNs and synaptic connectivity of PV+ interneurons. Additionally, using battery of behavioural tests, we are describing for the first time the explicit role of 4-O-sulfation levels in learning and memory of young and early adult animals.

Aim 1 was to determine the impact of decreased chondroitin 4-O-sulfation in ECM on various aspects of learning and memory. We tested object recognition memory, spatial memory, and social memory. In those tests we focused on the differences in the short-term memory, long-term memory and working memory.

Aim 2 was to address developmental character of *Chst11* knockout. We monitored the long-term changes using repetition of our battery of behavioural test in the 3 months and 6 months.

Aim 3 was to confirm the impact of *Chst11* knockout on the extracellular matrix. We monitored the morphological changes in perineuronal nets on PV+ interneurons in hippocampus. To follow the morphological changes in the PNNs we have monitored alternations in the synaptic connectivity.

5 Materials and methods

5.1 Experimental design scheme

Experiment consisted of two sets of behavioural testing periods at 3 months and 6 months, measured on the same mice. Immunohistochemistry was done afterwards. First behavioural testing started at the approximately 3 months of age, at least 48 hours of leisure in the home cage were between each test. Same subjects were sequentially used for all tests.

First performed test was open field test, to assess whether there is any difference in the anxiety between experimental animals and controls. Second test was spontaneous alternation test using the Y-maze. It also served as the habituation phase for the next – spontaneous object recognition test. There were four sets of novel object recognition test – with 3-hour, 24-hour, 48-hour and 96-hour time span between sample and choice phase. Elevated zero maze, an anxiety test, was done afterwards. In the next three weeks Morris water maze tests were performed, spatial acquisition test for long-term memory during first week, spatial reversal test in the beginning of the second week and test for short-term working memory in the third week.

When the tested mice reached 6 months of age same set of tests was performed. The mice used for the testing at 6 months were same individuals as ones tested at 3 months. One additional test was done at the beginning of the 6-month old testing. It was three-chamber sociability test, which measures both their sociability and their ability to recognize novel individual.

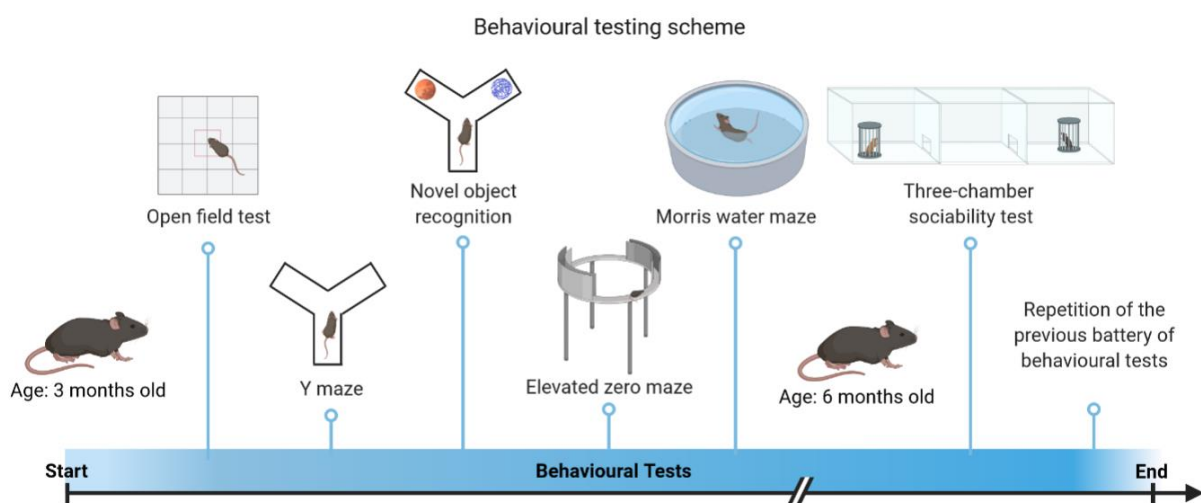


Figure 5 Scheme of the behavioural tests. Created with BioRender.com

Immunohistochemistry was done at the samples from the tested subjects after they finished second set of the tests. Three different types of staining were used. One for inhibitory synapses at the parvalbumin positive neurons, one for excitatory synapses at the PV+ neurons as well and third for perineuronal nets. Number of inhibitory and excitatory synapses was counted and changes in the length, arborization and somatic area of perineuronal nets were measured.

5.1.1 Mice and housing

For purpose of the study nestin-Cre *ChST11KO* mice (Nestin-CreErt2 x (Gt(Rosa)26Sor(CAG-Flpo,-EYFP) x C57BL/6N-A<tm1Brd>Chst11<tm1a(KOMP)Wtsi>/WtsiFlmg)) (M, n=12; F, n=14) and their littermate controls FloxP (C57BL/6N-A<tm1Brd>Chst11<tm1a(KOMP)Wtsi>/WtsiFlmg) (M, n=12; F, n=4) and nestin-Cre (Nestin-CreErt2 x (Gt(Rosa)26Sor(CAG-Flpo,-EYFP)) (M, n=10; F, n=10) were used. Males and females were housed and tested separately in their own set of behavioural arenas. The animals were bred at BIOCEV and maintained at the animal facility at the Institute of Experimental Medicine, Czech Academy of Science. The animals were housed one to five together, with a 12/12 h light/dark cycle (lights off at 7:00 P.M.) and food and water *ad libitum*. All behavioural tests were conducted during light phase of cycle. All animal procedures were approved by the ethical committee of the Institute of Experimental medicine, Czech Academy of Sciences.

5.1.2 Open field test

Open field test was used to measure level of anxiety by comparing time spent in the central and peripheral parts of the arena. It is based on spontaneous mice behaviour; on the one hand they want to explore the new space on the other hand they want to stay hidden near the walls. In all performed behavioural test mice were placed in the testing room at least 30 minutes prior to the testing. Arena for the open field test is open square box made of white plastics with the base of 39x39 cm and height of 20 cm. Each mouse was put in the centre of the arena and left to explore for 5 minutes. Trajectory and average speed of the mouse was recorded using VideoMot2 software. Arena was virtually divided into the central square region and peripheral region around it. Percentage of the time spent in each region was calculated by the software.

5.1.3 Spontaneous alternation

Basis of the spontaneous alternation test is natural behaviour – animal preferentially visits the arm, in which it was latest, because there is highest change, that something changed there.

Spontaneous alternation test was performed in the Y-maze. Y-maze consists of three arms with 120° angle, it was made from white plastics with each arm being 8 cm wide, 15 cm long and walls are 30 cm high. There were not visual cues within the maze. It was surrounded by the curtains which obscured the visual clues from the room and secured dim light. Mice was placed in the central part of the Y-maze and left to freely explore it for 5 minutes. Sequence of the visited arms was recorded. Visit is counted only if all four limbs are within the given arm. Re-visit of the same arm is counted only if the mice left it with all four limbs previously. Maze was cleaned between the testing of different mice. Number of entries and number of correct triads was counted in order to calculate spontaneous alternation. Error was calculated by assigning values 1 to 3 to the different types of errors and dividing their sum by the total number of entries. Value one was assigned to the entry to arm, which was different from the previous entry. Value two was assigned to the entry to the same arm as two entries beforehand and value three to the re-entry of the same arm right afterwards.

5.1.4 Novel object recognition test

Novel object recognition test is memory test based on the spontaneous mice explorative behaviour. In the sample phase they explore two identical objects, in the choice phase they should spent more time exploring novel object. But, even in healthy animals, if too much time passed since the sample phase, they do not remember original object and they will explore them equally. Object recognition test was performed in the Y-maze. Mice were habituated to it by the previously performed spontaneous alternation test. Each test session consisted of a sample phase and a choice phase. In the sample phase two identical objects were placed in two arms of Y-maze. Mice was placed in the empty start arm and left to explore objects for 5 min. Maze and objects were cleaned between the testing of different mice. Time between sample and choice phase was spent in the home cage. The choice phase followed after either 3 hours, 24 hours, 48 hours, or 96 hours. In the choice phase one arm contained novel object and one clean unused copy of original object. In each time period different pair of objects was used (Figure 6) and side of novel one was alternating in the respect to the start arm. The object exploration time was assessed from video recordings independently by two people. The direct nasal and head contacts and sniffing of the object were regarded as an exploratory behaviour. A discrimination ratio was calculated by dividing the difference in exploration of the novel and familiar objects by the total object exploration time. Discrimination ratio varies from 0 (equal exploration for novel and original objects or longer exploration of the original object than novel

one) to 1 (exploration of the novel object only). The test sessions were separated by a minimum of 48 hr.



Figure 6 Pairs of objects used in Novel object recognition test

5.1.5 Elevated zero maze

Elevated zero maze is a variant of classical elevated plus maze. It is an anxiety test, based on two contradictory spontaneous behaviours in mice – exploration of new place and hiding in the enclosed part. Elevated zero maze is circular platform 5 cm wide. It is divided into four regions, two of which have walls and two are open. Open and closed parts are alternating. Maze was surrounded by the curtains which obscured the visual clues from the room and secured dim light. Mouse was put in the centre of the open part and left for 5 minutes to explore. It was recorded on the video. Time spent in the open/closed parts was measured from the video, also rearing was assessed. Platform was cleaned between testing of each mouse.

5.1.6 Morris water maze

Morris water maze was performed in the circular pool, half-filled with the white dyed water. Four spots S, W, N and E, dividing the pool into four sections were marked on the outside side of the pool, hidden from experimental animal. Circular platform was hidden 0,5 cm below the water surface at the middle of quadrant. Pool was closed within the curtains reducing the availability of distal cues. Three types of tests were performed during three consecutive weeks.

First was a spatial acquisition test for long-term memory. Two marks were hanged on the inside of the pool wall at the S and W side. At the beginning of the first day a probe test was done. Underwater platform was not in the pool during probe test. Trajectory of the mice was recorded by the VideoMot2 software. Pool was imaginary divided into the four quadrants, one of them containing the place where platform should be. Probe test was done to set a base number of crossings through the platform area while swimming without it as a goal. Each mouse spent 1 minute in the pool. The test itself took place from Monday, when first probe test was performed to Friday, when second probe test was performed at least 3 hours after learning phase was finished. This probe test measured how precisely did the animal learned the position of the platform. Learning part of the Morris water maze was performed with the platform in the middle of SW quadrant of the pool. Mouse was released to the pool at the water level facing the wall. Each mouse was put into the pool four times within one day, from each position – S, W, N, E. Order of positions from which mice started at each day was semi-random. Trajectory of the mouse was recorded by VideoMot2, recording started at the point when mouse was released and ended either when mouse reached the platform or after one minute. Mouse was then retrieved and returned to the home cage.

Second test was a spatial reversal test. Platform was located at the middle of NE quadrant, opposite from the original position. Test was done for three consecutive days. Each mouse was put into the pool four times within one day, from all positions – S, W, N, E. Order of the positions from which mice started changed each day. Probe test was done at least three hours after testing was finished on the last day.

Third test was aimed at the spatial working memory or short-term memory. Four visual cues were hanged at the S, W, N and E position of the pool wall. Each day was a short learning session, consisting of sample trial and recall trial. Mouse was released four times from the same position and had to reach the platform which position stayed same during that day. The first

trial was a sample in which mouse should learn the path to the platform. Other three are recall trials in which it should remember the platforms position and get there faster. This test was performed five times during five consecutive days. The quadrant in which platform was present and position from which mice started were different each day.

5.1.7 Three-chamber sociability test

Three-chamber sociability test is aimed at the study of social memory. Arena consists of the three boxes with area of 40x20 cm connected by the small closable gates, middle one is empty, and lateral ones contain cages for mice put in the centre of the given chamber. Arena was made of grey metal. Mouse was put at the centre of the middle chamber while the gates were closed. It was left there for 5 minutes to habituate. Afterwards gates were opened. One of the cages was empty while other one contained a mouse from different home cage than the tested mouse. Mouse was let to explore all three chambers for 10 minutes. Then another mouse was put in the empty cage and tested mouse explored chambers for another 10 minutes. Arena, cages, and gates were cleaned with soap water, water, and ethanol between different tested animals. Whole session was recorded on the video. Times spent exploring cage with each mouse and empty cage were measured from the recording. Time spent exploring the cage with the mouse and time spent exploring empty cage were used to measure sociability. Social novelty is defined as preference to spent time with the new mouse rather than original one.

5.1.8 Brain sectioning

The animals were anesthetized in an induction chamber with isoflurane mixed with air. They were then deeply anesthetized with an intraperitoneal injection of Rometar (xylazinum 20 mg/ml) and Narketan (ketaminum 100 mg/ml) and intracardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. The brains were dissected out and postfixed in 4% PFA overnight. After postfixation, the brains were gently washed in PBS for 8 h. Then in 20% saccharose for another 24 hours and kept in 30% saccharose with added azide until sectioning. Just before cutting, brains were put in the cryoprotective (OCT Mounting media, VWR Chemicals) and frozen. Frozen brains were cut on the cryotome to the 20µm sections.

5.1.9 Immunohistochemistry

Immunohistochemistry protocol for visualization of perineuronal nets and synaptic connectivity on parvalbumin inhibitory interneurons was used. Namely for visualization of PNNs we used

antibodies for aggrecan (Anti-aggrecan polyclonal antibody from rabbit, Merck Millipore, dilution 1:500) and *Wisteria floribunda* (WFA) staining (WFA biotin conjugate, Sigma-Aldrich, dilution 1:150). Additionally for collocation of pre- and postsynaptic components of excitatory and inhibitory synapses on PV+ soma we have used these antibodies; for inhibitory synapses VGAT (Polyclonal rabbit antibody, Synaptic Systems, dilution 1:100), GEPH (Monoclonal mouse antibody, Synaptic Systems, 1:100), PV (Parvalbumin monoclonal mouse antibody, Synaptic Systems, dilution 1:50); for excitatory synapses VGLUT (Polyclonal chicken antibody, Synaptic Systems, dilution 1:100), PSD95 (Monoclonal mouse antibody, Synaptic Systems, dilution 1:100) and PV.

Marker for aggrecan was visualized by secondary antibody Gar594 (Goat-anti rabbit, Thermo Fisher, dilution 1:200) and WFA by Streptavidin Alexa Fluor 488 (biotin binding protein, Thermo Fisher, dilution 1:300). Secondary antibody for inhibitory synapse VGAT was Gar594 as well, for GEPH it was Gam488 (Goat anti-mouse, Thermo Fisher, dilution 1:300) and for PV it was Gam405 (Goat anti-mouse, Thermo Fisher, dilution 1:300). Secondary antibodies used for excitatory synapses were Gar594 for VGLUT, Gam488 for PSD95 and Gam405 for PV.

5.1.10 Microscopy

Images were captured using Confocal Microscope Zeiss LSM 880 Airyscan. Synapses were captured by 100x objective, with 512x512 resolution using Airyscan mode. Around 50-60 Z-stack images of each neuron, through its whole soma were taken. Images of 15 neurons per each animal were taken.

Perineuronal nets were captured by 20x objective, with 1024x1024 resolution. All pictures were captured using same settings. Length of pixel side is 0,52 μm . Z-stack spanned through whole section of brain, 20 to 30 slices. Images of at least 20 neurons per animal were taken.

5.1.11 Image analysis

Analysis of the number of inhibitory and excitatory synapses and morphometric analysis of perineuronal nets was performed in Fiji ImageJ. Colocalization of the pre-synaptic and post-synaptic marker next to each other was used for identification of the synapses. They were counted manually and divided by the number of sections.

5.1.12 Statistics

One-way ANOVA was used to analyse behavioural tests with one variable and Two-way ANOVA was used for the analysis of the behavioural tests with two variables. Two-way repeated measurement ANOVA was used in repetitive measures. Tukey or Bonferroni post hoc multiple comparison tests were used. Analysis was performed using GraphPad Prism 9.3.1 software. Results in the graphs are displayed as arithmetic means with standard error of mean and individual values, except for Morris water maze learning curve, where only arithmetic means are displayed for better clarity. P-value $\leq 0,05$ was considered statistically significant and was marked by *, $p \leq 0,01$ was marked by ** and $p \leq 0,001$ was marked by ***.

6 Results

6.1 Open field test

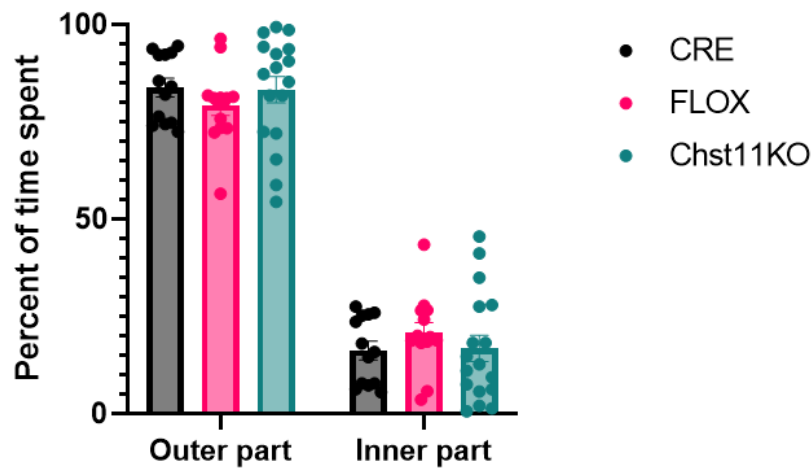


Figure 7 Time spent in outer or inner part of arena in open field test

There was not significant difference between groups in the time they spent exploring outer and inner parts of the arena (Figure 7). This test was used to confirm, that *Chst11* knockout mice have normal exploratory behaviour. Since the knockout mice behaved similar to the controls, they could be compared in the memory test. If they had impaired for example exploratory behaviour, they could not be tested in the novel object recognition test.

6.2 Spontaneous alternation

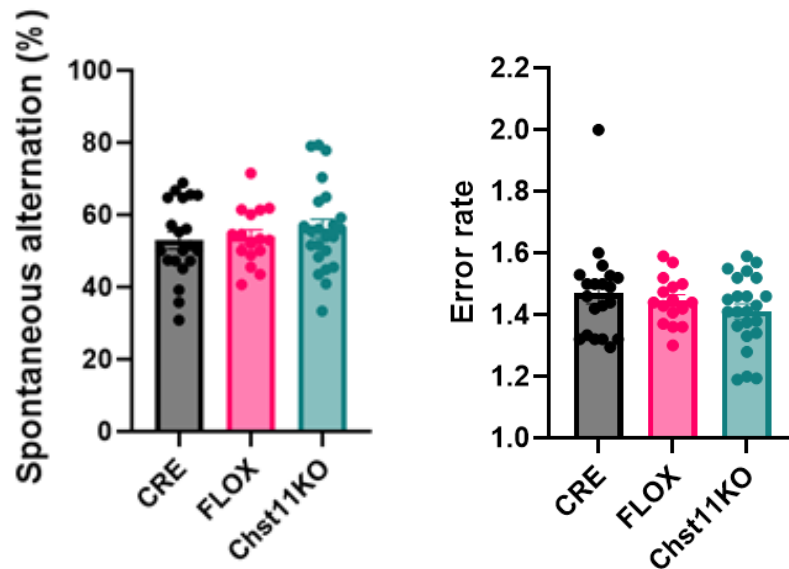


Figure 8 Spontaneous alternation and error rate

There was not significant difference in their spontaneous alternation rate neither in 3 months nor in 6 months. There was not difference in their error rates either (Figure 8). This could also be taken as a confirmation that the spatial memory and exploratory behaviour of the *Chst11* knockout mice is same as memory and behaviour of the controls.

6.3 Novel object recognition test

6.3.1 3 months

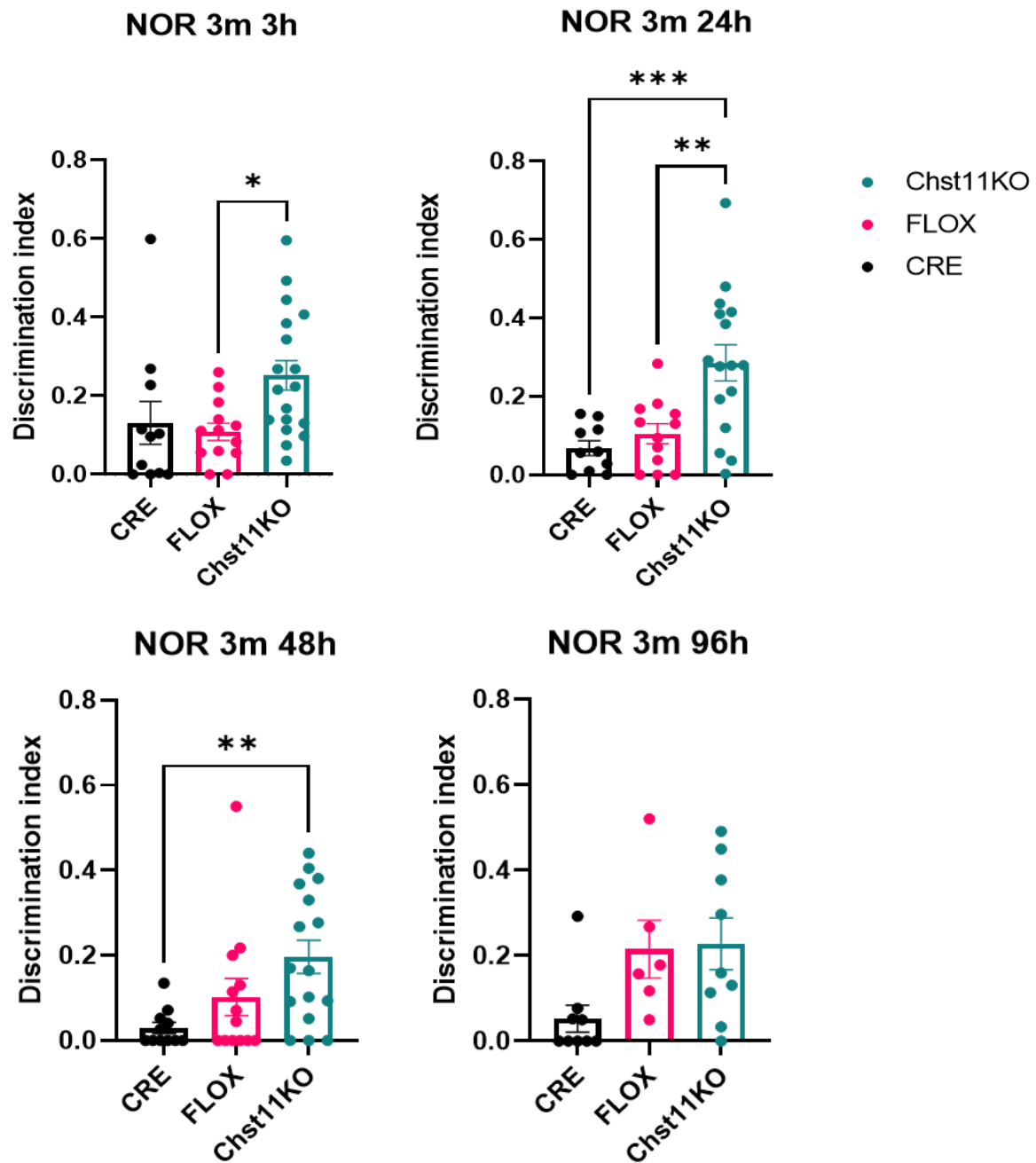


Figure 9 Novel object recognition test, performed in the 3 months of age with the span of 3, 24, 48 and 96 hours

In the novel object recognition test performed in the 3 months of age *Chst11* knockout mice showed better memory than controls in all four-time intervals (Figure 9). In accordance with the previous studies loosened perineuronal nets in the perirhinal cortex should improve explicit memory. Both control groups were able to distinguish between old and new object as well even though not as good knockout mice. There was a significant difference between groups in the 3

hours FLOX vs Chst11KO ($p = 0,0272$ *; $q = 3,813$), in the 24 hours CRE vs Chst11KO ($p = 0,0008$ ***; $q = 5,716$) and FLOX vs Chst11KO ($p = 0,0031$ **; $q = 5,021$), in the 48 hours CRE vs Chst11KO ($p = 0,0088$ **; $q = 4,458$).

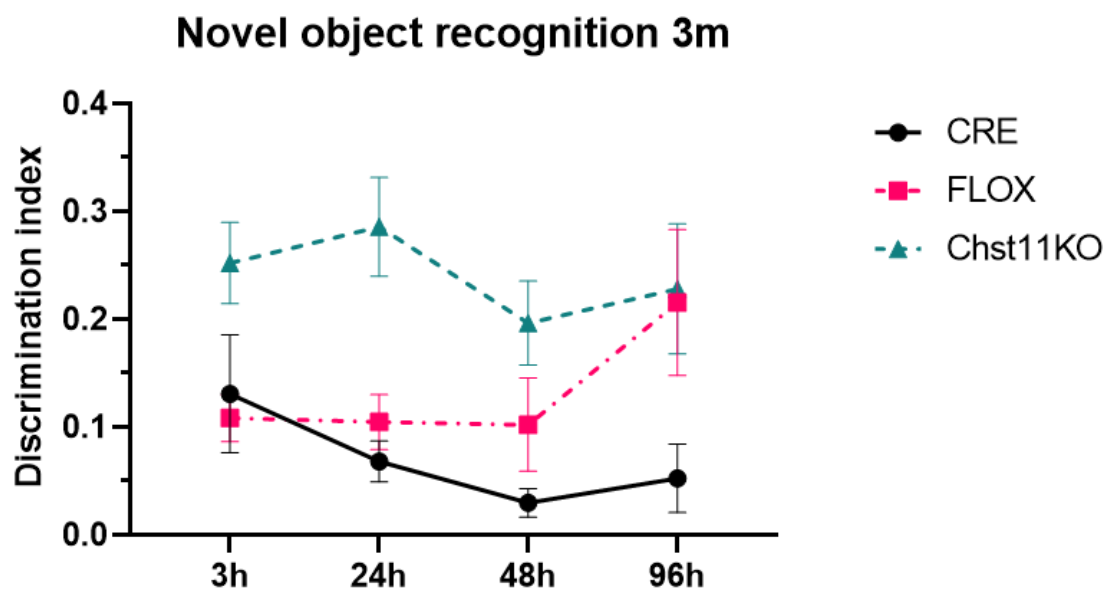


Figure 10 Development in the discrimination ratio from 3 hours to 96 hours in 3 months old mice

6.3.2 6 months

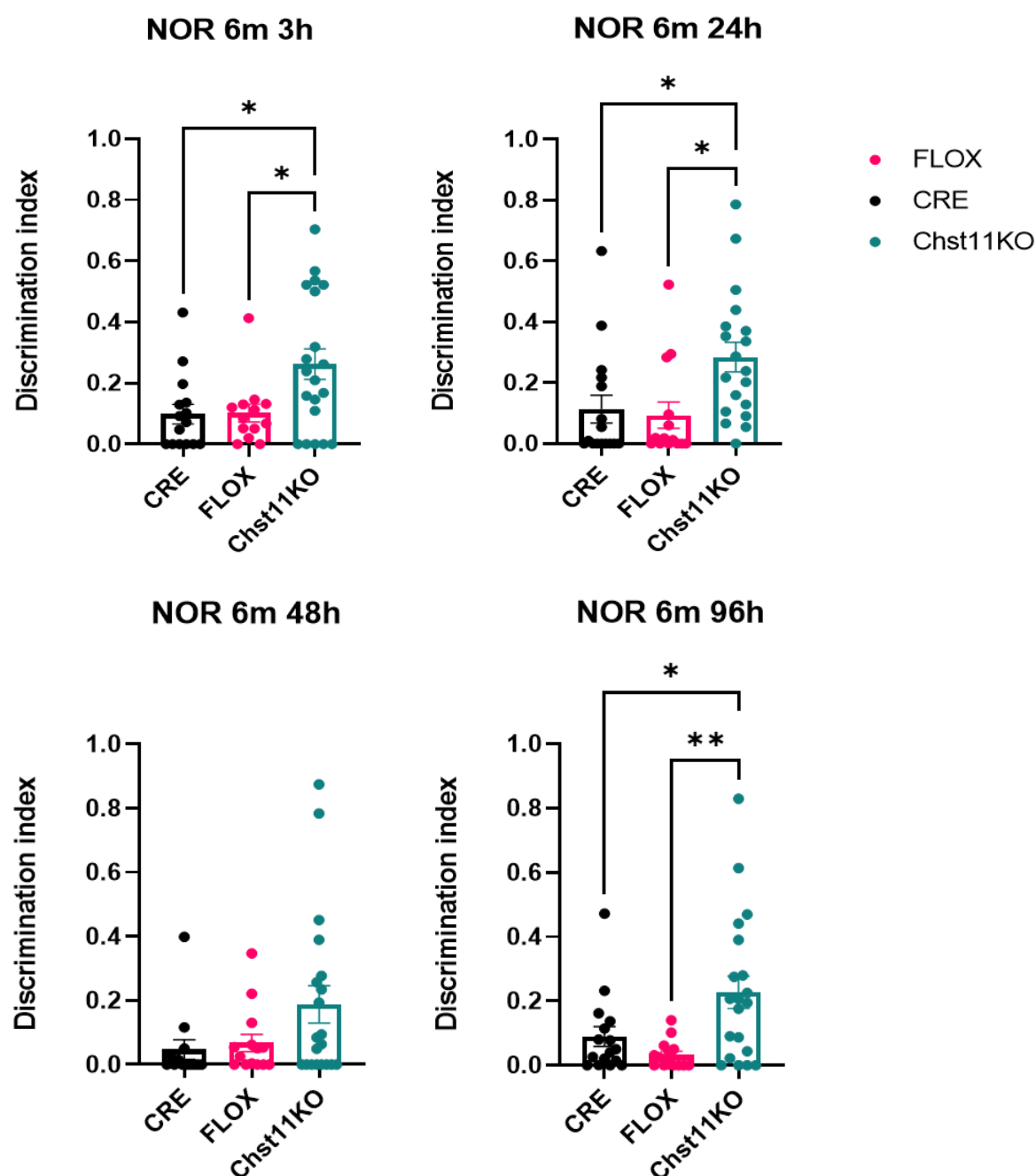


Figure 11 Novel object recognition test, performed in the 6 months of age with the span of 3, 24, 48 and 96 hours

In the novel object recognition test performed at the 6 months of age *Chst11* knockout mice showed better memory than controls in the all time spans, even though there was not a significant difference in the 48 hours (Figure 11). Ability of knockout mice to discriminate between object was same as in 3 months, it shows that enhanced memory in the mice with decreased C4S persists long into the adulthood. Both CRE and FLOX had decreased discrimination ratio from the state in the 3 months. There was a significant difference in the 3

hours CRE vs Chst11KO ($p = 0,0198 *$; $q = 3,968$) and FLOX vs Chst11KO ($p = 0,0307 *$; $q = 3,719$), in the 24 hours CRE vs Chst11KO ($p = 0,0277 *$; $q = 3,776$) and FLOX vs Chst11KO ($p = 0,0165 *$; $q = 4,065$) and in the 96 hours CRE vs Chst11KO ($p = 0,0366 *$; $q = 3,61$) and FLOX vs Chst11KO ($p = 0,0031 **$; $q = 4,912$).

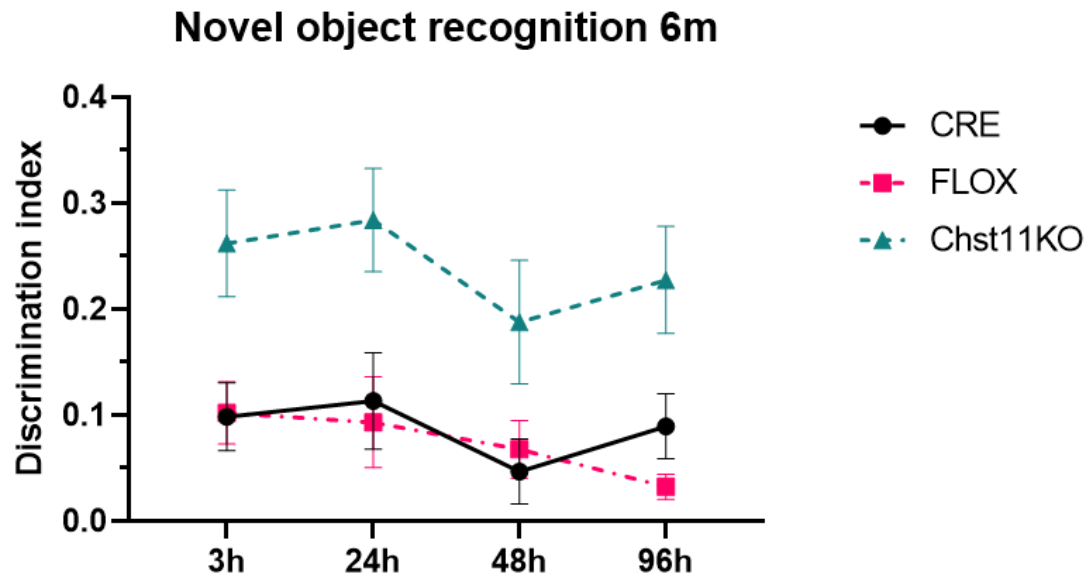


Figure 12 Development in the discrimination ratio from 3 hours to 96 hours in 6 months old mice

6.5 Elevated zero maze

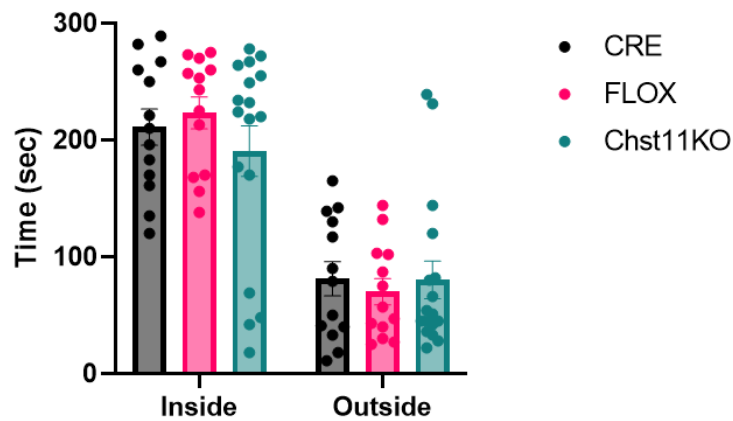


Figure 13 Time spent in the open and closed part of the elevated zero maze

In the elevated zero maze there was not difference between groups in the time they spent in the open or enclosed part of maze (Figure 13). This similarly to the open field test and Y-maze spontaneous alternation test indicates that the behaviour of *Chst11* knockout mice is same as of the control mice.

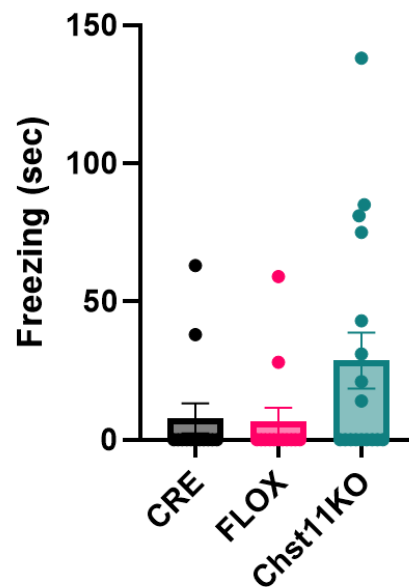


Figure 14 Freezing behaviour of the mice in the open part of the elevated zero maze

Even though there was not a difference in the time spent in the outer and inner part of the open field test (Figure 7) or in the time spent in the outer or inner part of elevated zero maze (Figure 13), *Chst11* knockout mice were freezing more in the open part of the elevated zero maze, which indicates increased anxiety in them (Figure 14). This observation was however not statistically significant.

6.6 Morris water maze

6.6.1 3 months acquisition

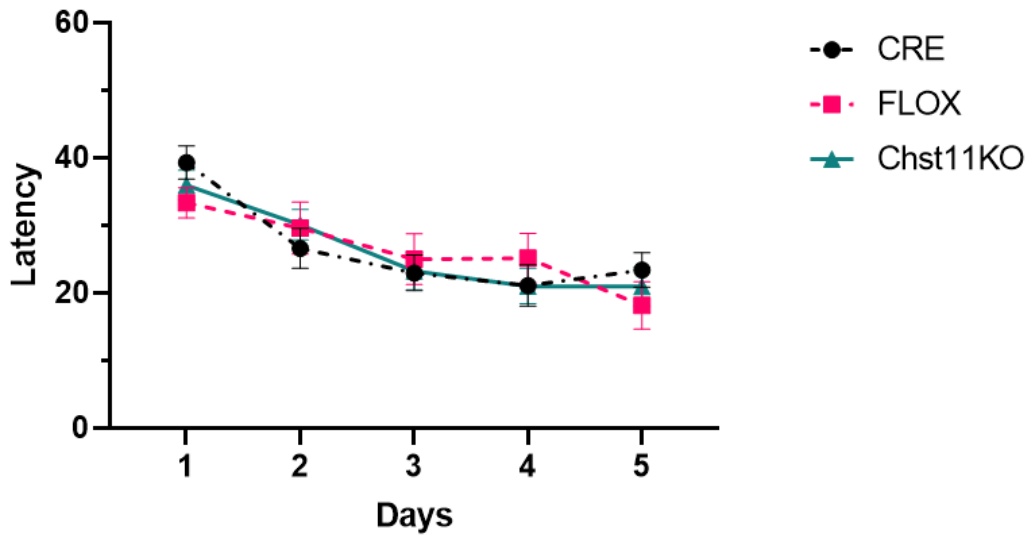


Figure 15 Learning curve in Morris water maze at 3 months

All three groups decreased their latency in the finding of platform between day one and day five and there was not a difference between groups in the learning curve (Figure 15). There was a significant difference in CRE mice day 1 vs day 2 ($p = 0,0013$ **; $q = 5,485$), day 1 vs day 3 ($p < 0,0001$ ***; $q = 7,059$), day 1 vs day 4 ($p < 0,0001$ ***; $q = 7,866$) and day 1 vs day 5 ($p < 0,0001$ ***; $q = 6,867$). In FLOX group day 1 vs day 5 ($p = 0,0004$ ***; $q = 5,87$) and day 2 vs day 5 ($p = 0,0168$ *; $q = 4,424$). In ChST11KO group day 1 vs day 3 ($p = 0,0001$ ***; $q = 6,257$), day 1 vs day 4 ($p < 0,0001$ ***; $q = 7,364$), day 1 vs day 5 ($p < 0,0001$ ***; $q = 7,39$), day 2 vs day 4 ($p = 0,0152$ *; $q = 4,47$) and day 2 vs day 5 ($p = 0,0143$ *; $q = 4,496$).

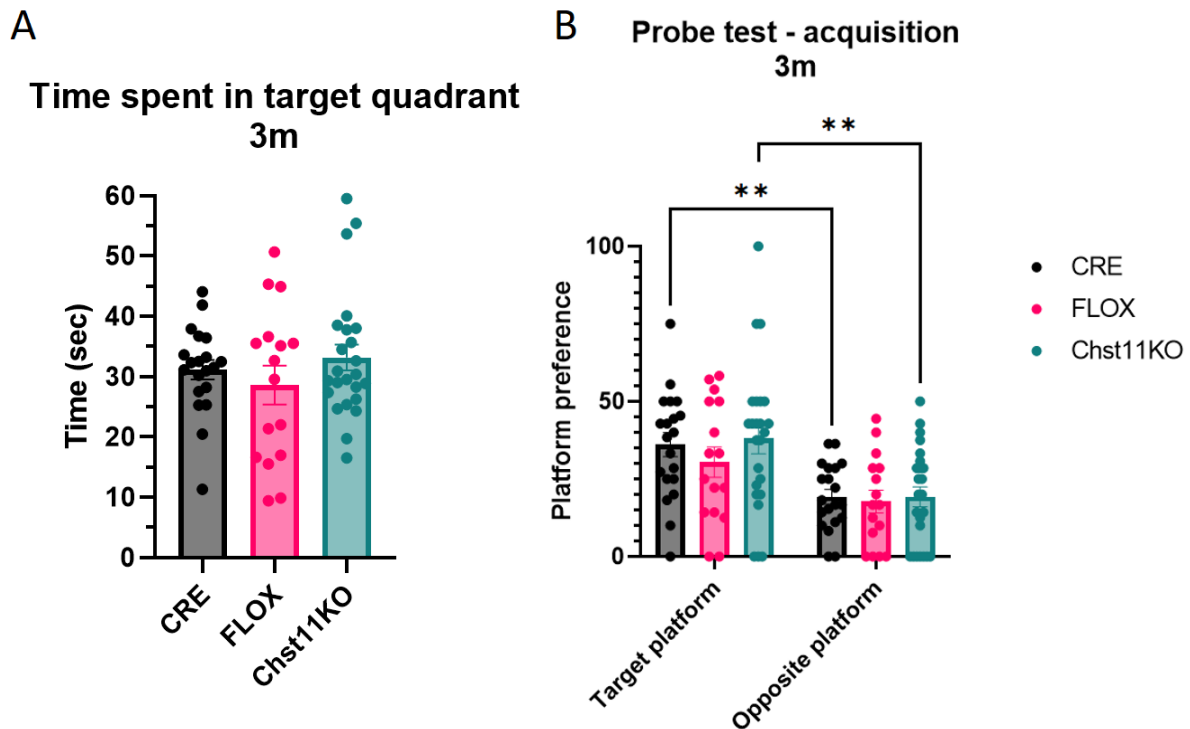


Figure 16 A – Time spent in the target quadrant during probe test after acquisition phase in 3 months; B – Preference of the place, where target platform was vs preference of the opposite platform

There was not a significant difference in the time spent in target quadrant between groups. It shows, that neither of them learned it better than other groups (Figure 16 A). Another parameter that was investigated was a preference of the target platform, in contrast to the opposite platform it was calculated from the number of times mice swam during probe test through virtual target platform from the total crossings of all four platforms. It shows that mice learned the position of the target platform during acquisition phase. All three groups crossed target platform more times than opposite platform and both CRE and Chst11KO had a significant difference (Figure 16 B). CRE preference for Target platform vs Opposite platform ($p = 0,0097$ **; $t = 3,008$) and Chst11KO Target platform vs Opposite platform ($p = 0,0011$ **; $t = 3,68$).

6.6.3 3 months reversal

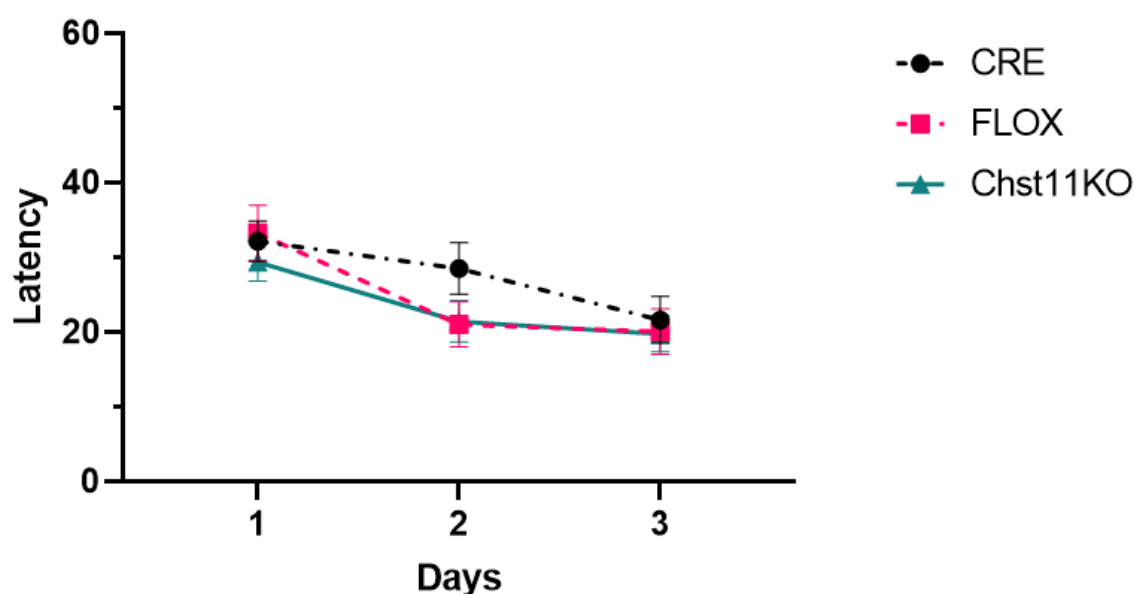


Figure 17 Morris water maze reversal learning in 3 months

In the next week of reversal learning all three group were able to learn the new position of the platform (Figure 17). And there was not a significant difference in the latency between groups. There was a significant difference in CRE group in day 1 vs day 3 ($p = 0,0317$ *; $q = 3,597$), in the FLOX group both in day 1 vs day 2 ($p = 0,0247$ *; $q = 3,729$) and day 1 vs day 3 ($p = 0,0137$ *; $q = 4,026$). In the Chst11KO group in day 1 vs day 3 ($p = 0,0377$ *; $q = 3,503$).

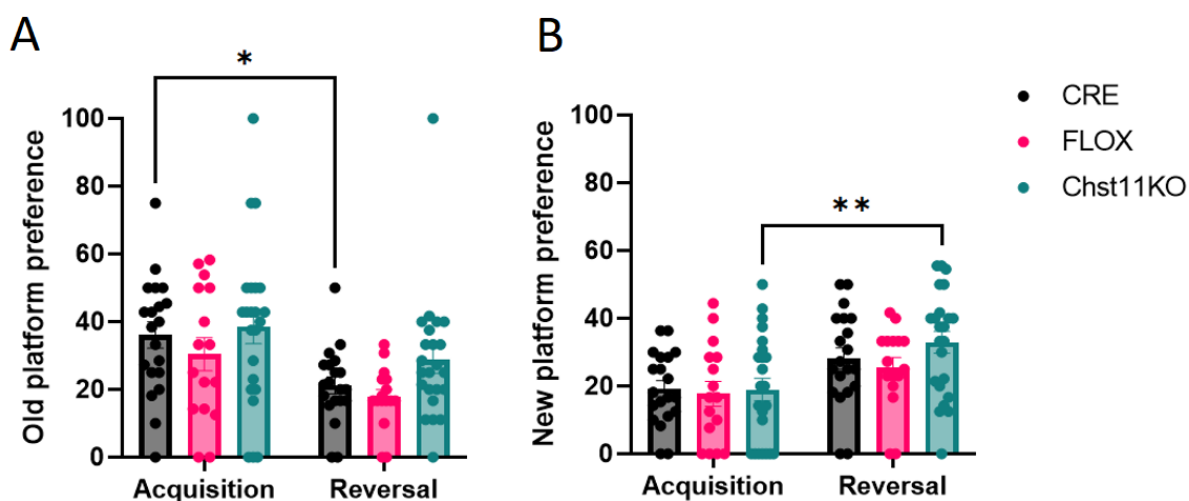


Figure 18 Probe test after reversal learning in 3 months. A – Preference of the original platform after acquisition week and after re-learning week; B – Preference of the new platform after acquisition week and after re-learning week

Figure 11-A shows how well did mice forgot the original platform and did not try to search for it after they learned the position of new platform in reversal phase. Significant was only difference in the CRE group, acquisition vs reversal ($p = 0,0299$ *; $t = 2,622$), FLOX group did

not searched for the platform at the old place more than is random chance for crossing any platform. Mice from Chst11KO group had problem forgetting the original platform. They crossed the place of original platform more often than control CRE and FLOX groups and also more than is a random chance (Figure 18 A). On the other hand, all three groups were able to learn the position of the new platform in the reversal phase, including Chst11KO, which spent there significantly more time than during probe test after acquisition phase. Chst11KO acquisition vs reversal ($p = 0,0032$ **; $t = 3,364$).

6.6.4 3 months short-term memory

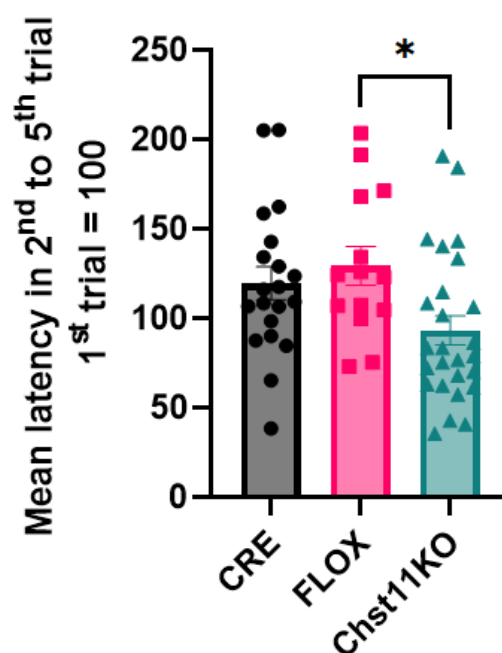


Figure 19 Morris water maze test for short-term memory in 3 months

In the Morris water maze test for short-term memory latency to the platform in first trial is base value – 100. Another three consecutive trials are compared to the first value. Results smaller than hundred means that mice were able to remember the position of the platform and get there faster than in the first trial. Only Chst11KO group showed mean latency in the 2nd to 5th trial smaller than 100 (Figure 19). *Chst11* knockout mice have better short-term memory than controls. There was a significant difference between the main control FLOX vs Chst11KO ($p = 0,0292$ *; $q = 3,716$).

6.6.5 6 months acquisition

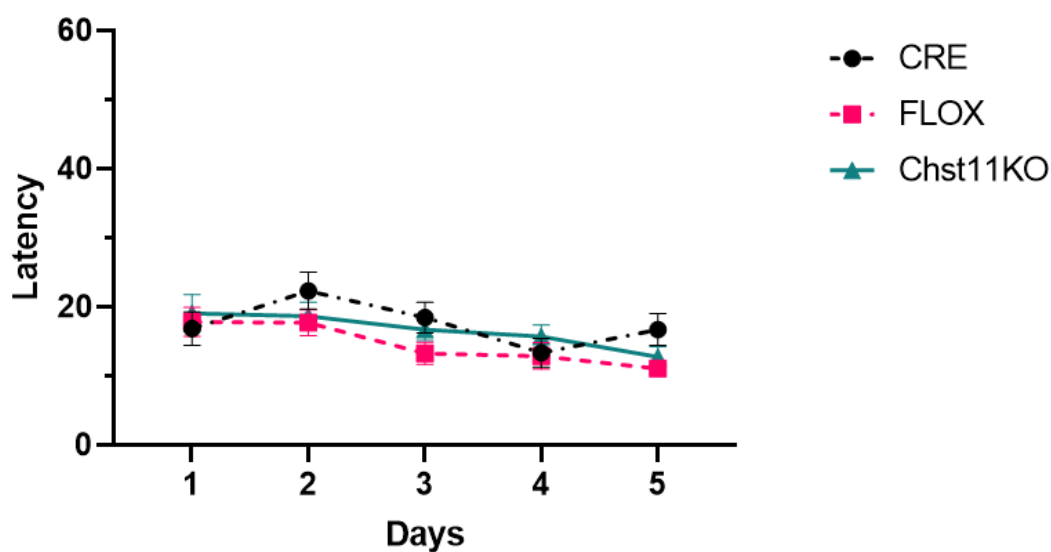
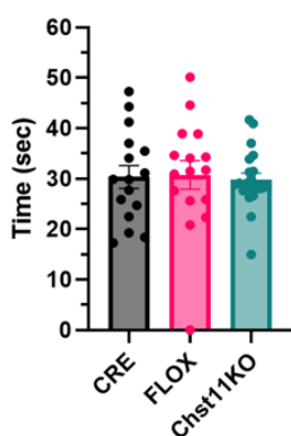


Figure 20 Learning curve in Morris water maze in 6 months

Mice in the 6 months were not naïve. They have already learned the principle of finding the platform, such as swimming in the given length from the pool wall. Their latency of the first day was same as the latency at the end of the first acquisition week in 3 months of age and it stayed almost same during whole 5 days of the test (Figure 20).

A

Time spent in target quadrant
6m



B Probe test - acquisition
6m

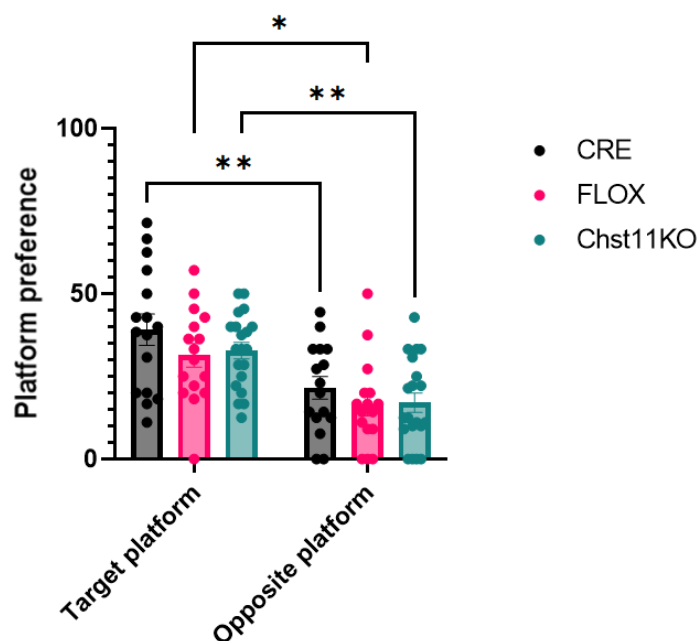


Figure 21 A – Time spent in the target quadrant during probe test after acquisition phase in 6 months; B – Preference of the place, where target platform was vs preference of the opposite platform

Same as in the 3 months (Figure 16 A) there was not a significant difference in the time spent in target quadrant between groups (Figure 21 A). All three groups were able to learn the position of the platform during acquisition phase (Figure 21 B). There was a significant difference in the preference for the target platform in all three groups. CRE target platform vs opposite platform ($p = 0,0019^{**}$; $t = 3,532$), FLOX target platform vs opposite platform ($p = 0,0102^{*}$; $t = 3,002$) and Chst11KO target platform vs opposite platform ($p = 0,0019^{**}$; $t = 3,537$).

6.6.6 6 months reversal

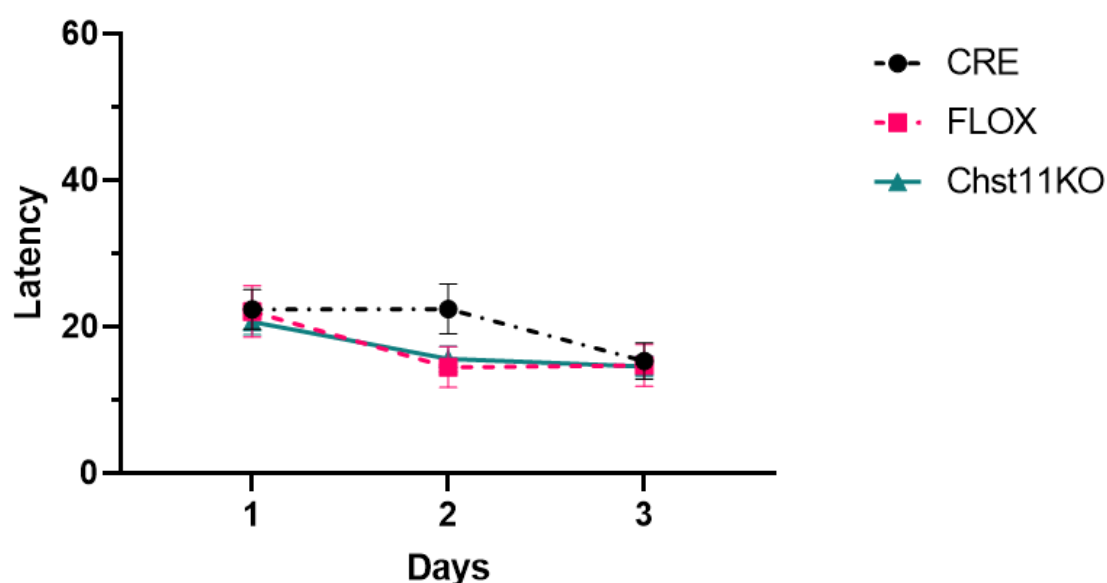


Figure 22 Morris water maze reversal learning in 6 months

Similar to the acquisition phase latency started at the 20 seconds and it did not get significantly smaller (Figure 22). It was caused by the mice not being naïve.

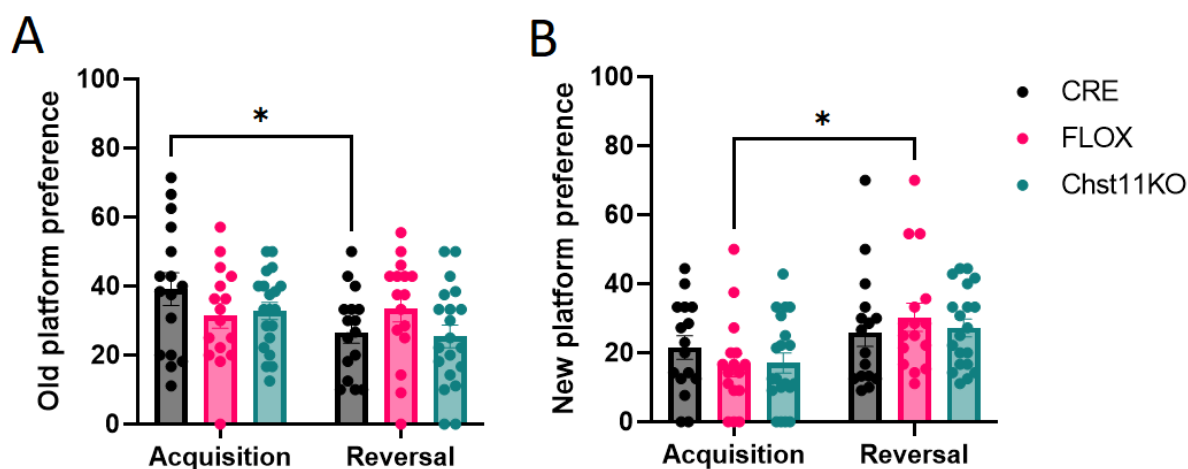


Figure 23 Probe test after reversal learning in 6 months. A – Preference of the original platform after acquisition week and after re-learning week; B – Preference of the new platform after acquisition week and after re-learning week

Only CRE group had significantly smaller preference after reversal phase for the old platform than after acquisition phase, CRE acquisition vs reversal ($p = 0,0489$ *; $t = 2,444$). FLOX and Chst11KO had almost same preference for the old platform both after acquisition and reversal phase (Figure 23 A). All three groups were able to learn the position of the new platform (Figure 23 B). Only FLOX showed significant difference in the preference of new platform after reversal phase, FLOX acquisition vs reversal ($p = 0,0186$ *; $t = 2,798$).

6.6.7 6 months short-term memory

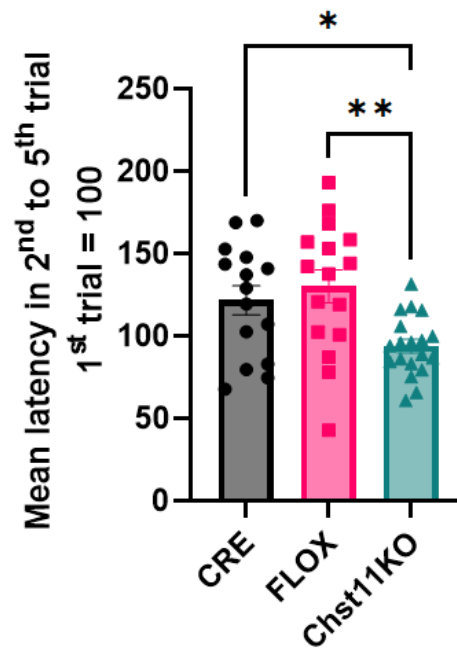


Figure 24 Morris water maze test for short-term memory in 6 months

Same as in the 3 months only *Chst11* knockout mice had mean latency smaller than 100, which means that knockout mice have better short-term memory than controls. There was a significant difference between CRE vs Chst11KO ($p = 0,0348$ *; $q = 3,64$) and FLOX vs Chst11KO ($p = 0,0039$ **; $q = 4,811$).

6.8 Three-chamber sociability test

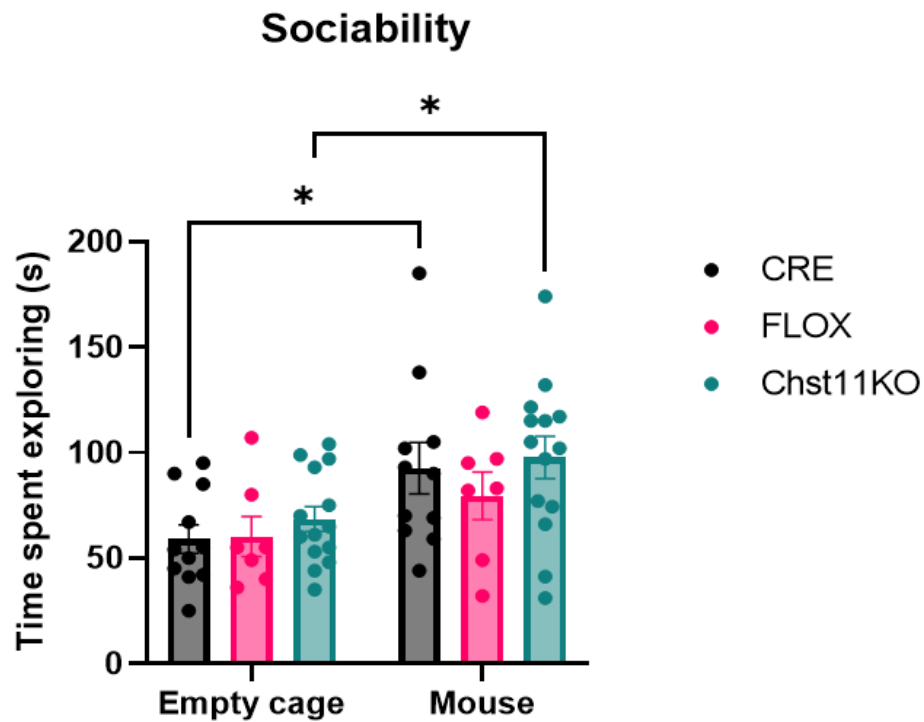


Figure 25 Sociability in three-chamber sociability test

Sociability shows preference of the mouse for another unknown mouse over other environment. All three groups showed preference for the introduced mouse over empty cage (Figure 25). CRE empty cage vs mouse ($p = 0,0398$ *; $t = 2,555$) and Chst11KO empty cage vs mouse ($p = 0,0444$ *; $t = 2,512$).

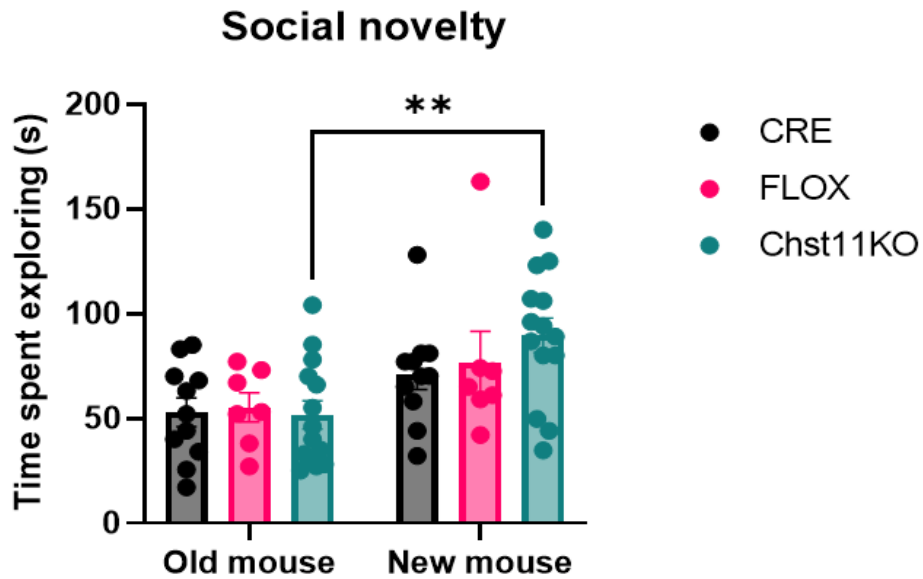


Figure 26 Social novelty in three-chamber sociability test

Social novelty shows the hippocampus-dependent social recognition memory of the mice. *Chst11* knockout have better social memory than knockout, even though there is not a significant difference between groups. *Chst11* knockout group is also only which had a significant difference in the preference of the old mouse vs new mouse ($p = 0,0015$ **; $q = 3,685$) even though both CRE and FLOX showed preference for the new mouse (Figure 26).

6.10 Morphometric analysis of perineuronal nets

6.10.1 Length of the axons and dendrites

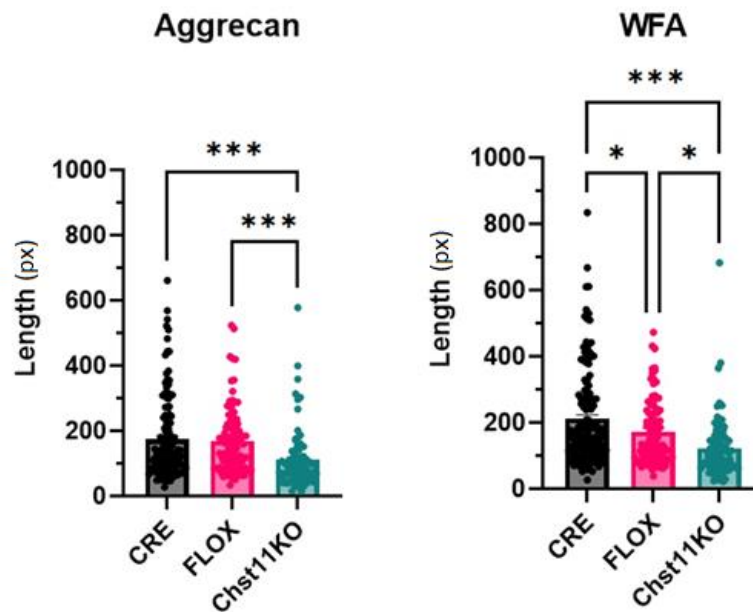


Figure 27 Sum of the length of axons and dendrites visualized either by anti-aggrecan antibody or by WFA

Neurons from knockout mice had shorter protrusion parts covered in perineuronal nets as visualised by aggrecan and WFA than neurons from both control groups (Figure 27). There was a significant difference in anti-aggrecan antibody stained samples in CRE vs Chst11KO ($p < 0,0001$ ***; $q = 6,113$) and FLOX vs Chst11KO ($p = 0,0008$ ***; $q = 5,205$). And WFA stained CRE vs FLOX ($p = 0,0377$ *; $q = 3,487$), CRE vs Chst11KO ($p < 0,0001$ ***; $q = 7,927$) and FLOX vs Chst11KO ($p = 0,0111$ *; $q = 4,103$). Length of one pixel is $0,52 \mu\text{m}$.

6.10.2 Arborization of axons and dendrites

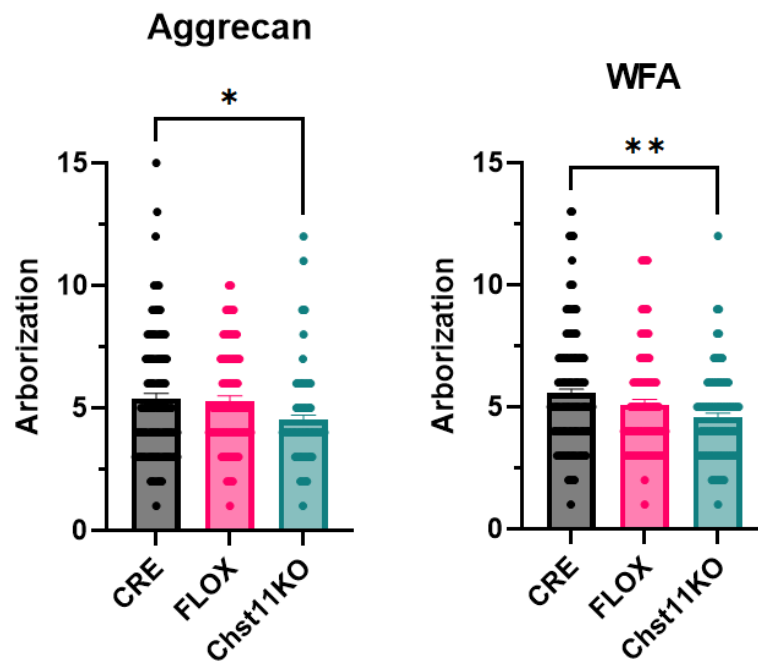


Figure 28 Number of branches visualized by anti-aggrecan antibody or by WFA

Neurons from Chst11KO mice had smaller number of branching of the anti-aggrecan antibody and WFA visualized parts than controls. In both staining there was a significant difference between CRE group and Chst11KO group. FLOX had a bit higher number of branches even though there was not a significant difference (Figure 28). Aggrecan CRE vs Chst11KO ($p = 0,0144$ *; $q = 3,979$) and WFA CRE vs Chst11KO ($p = 0,0018$ **; $q = 4,878$).

6.10.3 Somatic area

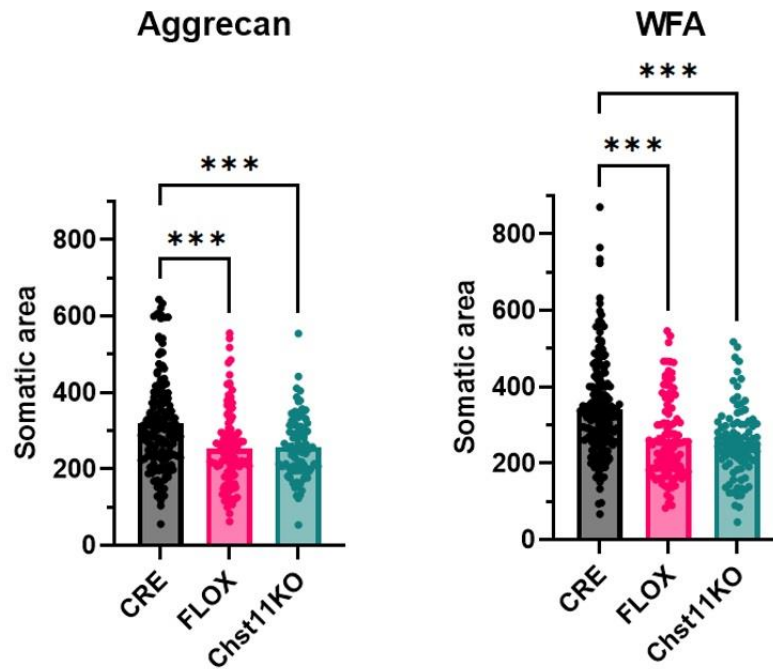


Figure 29 Area of the neuronal soma stained either by anti-aggreCAN antibody or WFA

There was a significant difference in the somatic area between CRE and other two group (Figure 29). It can not be said that knockout mice have smaller neurons since FLOX is a main littermate control and they were same. In the anti-aggreCAN antibody stained CRE vs FLOX ($p < 0,0001$ ***; $q = 6,803$), CRE vs Chst11KO ($p = 0,0001$ ***; $q = 5,92$). And in WFA stained CRE vs FLOX ($p < 0,0001$ ***; $q = 8,181$) and CRE vs Chst11KO ($p < 0,0001$ ***; $q = 8,165$).

6.11 Morphometric analysis of synapses

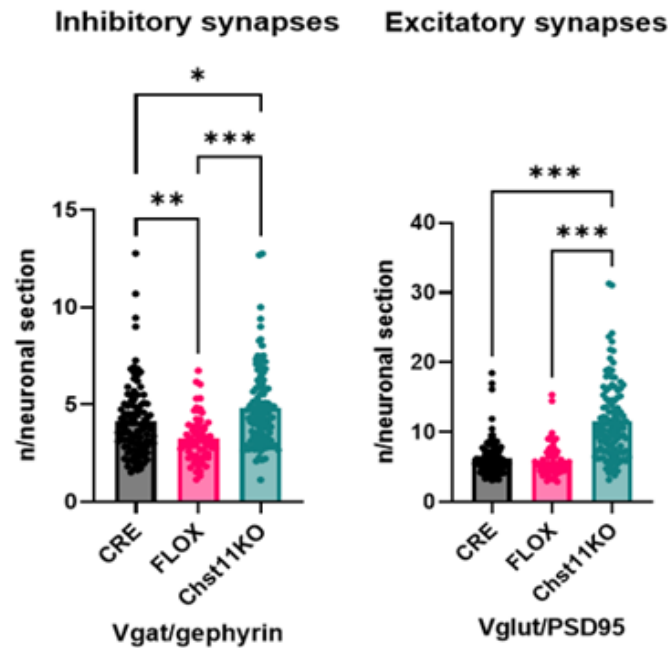


Figure 30 Number of new inhibitory and excitatory synapses per neuronal section

Number of both inhibitory and excitatory synapses on the neuronal soma of PV+ neurons in CA1 region of hippocampus was increased (Figure 30). There was also an increase in excitatory to inhibitory ratio, making neurons more excited than ones in the control groups. There was significant difference in inhibitory synapses CRE vs FLOX ($p = 0,0053$ **; $q = 4,44$), CRE vs Chst11KO ($p = 0,0118$ *; $q = 4,078$) and FLOX vs Chst11KO ($p < 0,001$ ***; $q = 8,206$). And significant difference in the excitatory synapses CRE vs Chst11KO ($p < 0,001$ ***; $q = 13,38$) and FLOX vs Chst11KO ($p < 0,001$ ***; $q = 12,12$).

7 Discussion

Changes in the epitopes of extracellular matrix of brain, specifically changes in the sulfation pattern during the lifetime and their effect on the functioning of the brain have recently become a topic of interest (Miyata *et al.*, 2012; Foscarin *et al.*, 2017; Yang *et al.*, 2017, 2021). We have explored the effect of changes in the sulfation ratio on chondroitin sulfate proteoglycans in extracellular matrix of brain. In our study we used heterozygous nestin-Cre *Chondroitin sulfotransferase-11* knockout mice strain, which has decreased amount of 4-O-sulfation. That causes a shift in the sulfation ratio towards 6-O-sulfation. Nestin promoter is active both in neurons and glial cells, therefore all extracellular matrix in the brain is affected.

In the wild-type mice 4-O-sulfation is gradually increasing after birth up to the adulthood in the 3 months when it reaches plateau concentration. On the other hand 6-O-sulfation is gradually decreasing in adult animal, especially after one year of age, which leads to the shift towards 4-O-sulfation (Foscarin *et al.*, 2017). In our study we aimed to characterize a mice strain which will retain sulfation pattern of the young individual, with prevailing C6S, during adulthood and aging.

Reduction of perineuronal nets either enzymatically or in the knockout strains missing components of the perineuronal nets has the effect on the brain functioning in the fear memory (Gogolla *et al.*, 2009b; Hylén *et al.*, 2013), object recognition memory (Romberg *et al.*, 2013; Favuzzi *et al.*, 2017; Rowlands *et al.*, 2018), spatial memory (Morellini *et al.*, 2010; Favuzzi *et al.*, 2017; Christensen *et al.*, 2021) and social memory (Yoshioka *et al.*, 2017).

This artificial disruption of perineuronal nets have natural counterpart in the metalloproteinases, which are degrading extracellular matrix (Mercuri *et al.*, 2000; Tauchi *et al.*, 2012). Activity of matrix metalloproteinases is sulfation dependent. Changes in the sulfation pattern have effect on the stability of extracellular matrix, C-4 sulfated matrix is more stable than C-6 sulfated (Miyata and Kitagawa, 2016).

We examined the effect of decreased C4S on general behaviour, both short-term and long-term memory, specifically object recognition memory, spatial memory, and social memory.

At first, we assessed their exploratory behaviour and anxiety. In the open field test all three groups spent more time in the outer part, near walls but they were also exploring the central part (Figure 7). In the elevated zero maze, they spent more time in the enclosed part than in the

open part (Figure 13). They all showed normal exploratory behaviour and there was not a significant difference between groups. *Chst11* knockout mice were freezing more while in the open part of the elevated zero maze than control groups, which is a sign of increased anxiety. But there was not a significant difference between them and controls (Figure 14).

In the Y-maze spontaneous alternation test we did not find any difference between groups neither in spontaneous alternation nor in error rate (Figure 8). It shows that there are not differences in the spatial working memory between tested groups. It is not easy to detect improved results than in healthy control mice, since they have only their natural explorative behaviour as a motivation. In continuation of the study, we will explore impact of our knockout at aging animals where working spatial memory is already affected.

In the novel object recognition memory test, we found out, that *Chst11* knockout mice were better than controls at discriminating the novel object from the sample in all four time periods both in 3 months (Figure 9) and in 6 months (Figure 11). Adult wild-type mice are not able to discriminate sample and novel objects at the 24- hour period anymore (Şık *et al.*, 2003). This shows that *Chst11KO* has enhanced object recognition memory. This result is in accordance with previous experiments in which enzymatic degradation of PNNs (Romberg *et al.*, 2013) or brevican knockout (Favuzzi *et al.*, 2017) and aggrecan knockout (Rowlands *et al.*, 2018) led to the improvement in the long-term object recognition memory. Interestingly, both control groups had higher discrimination ratio in the 3 months (Figure 9) than in 6 months (Figure 11) even though at 3 months it was still lower than in knockout mice. It might be explained by higher plasticity still present from the juvenile brain at the 3 months, but not at the 6 months. Wild-type mice from study by Şık *et al* were 4 months old and they were not able to discriminate novel object at the 24-hour period anymore same as our controls in 6 months.

In the long-term spatial memory, as tested by Morris water maze, there was no difference between the groups neither in the learning curve (Figure 15; Figure 20) nor in the preference for platform site in probe test (Figure 16; Figure 21). All three group were able to learn the position of the platform both in 3 months and in 6 months. Those results are in agreement with study of tenascin-R knockout strain (Morellini *et al.*, 2010) where was not observed any change in the long-term spatial learning either. On the other hand, enzymatical degradation of PNNs led to the improvement in the long-term spatial memory (Ruzicka *et al*, unpublished results).

In our results there was no difference in the reversal learning curve between groups neither in 3 months (Figure 17) nor in 6 months (Figure 22). In the tenascin-R knockout mice strain they observed the changes during the reversal learning phase, where knockout mice learned the position of the new platform faster than control mice (Morellini *et al.*, 2010). We did not observe enhanced learning during reversal phase in our strain, but we observed changes in the probe test, which contradictory were not observed by Morellini *et al.* Our results from 3 months showed that *Chst11KO* mice had problem forgetting the old position of the platform (Figure 18) while it was best in learning the position of the new platform. This indicates attention problems. Explanation for the difference between our results might be in the different analysis of results. We compared preference for the given platform in the first probe test and second probe test, while Morellini *et al* compared the preference between two platforms from the second probe test. If we analysed it the same way as they did there would not be significant difference between two platform sites since knockout mice had increased preference for both old and new site.

Results from 6 months reversal learning were not same as from 3 months. Only CRE group was able to forget the old platform, both FLOX and *Chst11KO* showed the similar preference in both probe tests and knockout mice did not have higher preference for the old platform than FLOX. All three groups were able to learn the position of the new platform, but *Chst11KO* was not better than control groups as it was in 3 months (Figure 23).

Chst11 knockout mice showed better short-term memory in the Morris water maze both in 3 months (Figure 19) and in 6 months (Figure 24) than control groups.

Results from three-chamber sociability test shows that knockout strain as well as controls are healthy sociable animals, since all of them preferred cage with another mouse over empty cage (Figure 25). Sociability is prerequisite for measuring social memory, which is measured in social novelty test. While all three groups preferred novel mouse over old one, *Chst11KO* showed higher preference for the novel mouse than controls (Figure 26). In the used set-up we are measuring short-term memory. Enhanced short-term memory in the knockout mice is in agreement with our result from Morris water maze test for short-term memory, where knockout mice showed improvement as well. Social novelty was increased also in the mice with decreased chondroitin sulfate and therefore impaired PNNs (Yoshioka *et al.*, 2017), which supports our results that impairment in the perineuronal nets leads to the enhanced short-term memory.

Hippocampus is involved in all three types of memory, which we studied (Ennaceur and Delacour, 1988; Fyhn *et al.*, 2004; Tanimizu *et al.*, 2017). That's why we chose to use PNN covered PV+ interneurons from CA1 area of hippocampus for the morphometric analysis.

There are various theories how perineuronal nets can affect synaptic plasticity. Such as by mechanically obstructing formation of synapse (Sigal *et al.*, 2019) or by activating signalling pathways in the neuron (Favuzzi *et al.*, 2017; Schmidt *et al.*, 2020). While the mechanism is not clear, we know that decreased amount of PNNs or change in its structure are associated with increased synaptic and neuronal plasticity (Pizzorusso *et al.*, 2002; Rowlands *et al.*, 2018).

In our study we used two antibodies for the visualization of PNNs - Wisteria floribunda agglutinin (WFA) and anti-aggrecan antibody. Results from both types of staining were similar. Added up length of the PNN-covered axons and dendrites was shorter in the *Chst11KO* mice than in controls (Figure 27). *Chst11KO* mice also had decreased arborization as compared to the controls (Figure 28). Both of those properties show decreased amount of perineuronal nets. Third measured property was the somatic area. *Chst11KO* group had same area of the soma as FLOX control group. CRE mice had bigger somatic area (Figure 29). Since there was not difference between knockout mice and FLOX mice we cannot say that there are changes in the size of neuronal soma in our studied strain. Observed reduction of perineuronal nets and especially aggrecan components is in agreement with studies focused on high C6S/C4S ratio, leading to rather unstable structure of PNNs (Miyata and Kitagawa, 2016).

Changes in the brain functioning are dependent on the electrophysiological changes, such as short-term depression and potentiation and long-term depression and potentiation – synaptic plasticity. We expect that change in the sulfation pattern affects synaptic plasticity as it was showed in the previous studies with enzymatically removed PNNs or knockout strains of certain PNN component (Bukalo, Schachner and Dityatev, 2001; Jansen *et al.*, 2017). In the brevican-deficient mice they observed changes in the LTP but not in the learning and memory (Brakebusch *et al.*, 2002). They tested only long-term memory and reversal learning in the Morris water maze. We did not find any change in the long-term memory either, while results from reversal learning are unclear so it does not make this study contradictory to ours. Most relevant study for us is the one by Yang *et al.*, in which they tested mice with decreased 6-O-sulfation in the young mice, which led to the loss of LTP in the hippocampus and impairment in the learning and memory (Yang *et al.*, 2021).

Another morphometric analysis we performed was quantification of the inhibitory and excitatory synapses on the neuronal soma of PV+ neurons. There was a significant increase in the number of inhibitory synapses in the *Chst11* knockout mice, number of excitatory synapses in knockout mice was doubled as compared to controls (Figure 30). In the study by Yang *et al* in which they increased C6S in the aged mice, which could be considered similar to our decrease in C4S they observed the increase in number of inhibitory synapses on the soma as well (Yang *et al.*, 2021). They hypothesized that increased inhibition of PV+ inhibitory interneurons in hippocampus leads to the activation of hippocampus and therefore improvement in the memory. Unlike us they did not count the excitatory synapses. Even though our increase in the number of inhibitory synapses is similar we cannot say that PV+ neurons are inhibited since we know that number of excitatory synapses is increased more significantly. Which means that PV+ interneurons will be more excited. Our hypothesis is that more excited inhibitory interneurons are working better at blocking the noise from the signal, which helps the memory.

8 Conclusion

In this study we characterized heterozygous nestin-Cre *Chondroitin sulfotransferase-11* knockout mice strain in the young and early adult mice. Our tests were aimed mainly at the changes in the learning and memory. We also examined correspondent morphometric changes on the neurons. We demonstrated that knockout mice with decreased C4S to C6S ratio had better short-term memory and long-term object recognition memory both as young and early adult mice. In the morphometric analysis of PNNs there was a significant reduction in length and arborization of WFA and aggrecan positive nets. In the colocalization analysis of presynaptic and postsynaptic markers there was an increased amount of both inhibitory and excitatory synapses on parvalbumin positive neurons, with increased excitatory/inhibitory ratio.

In the continuation of this study same battery of behavioural test will be performed at the one year of age and 20 months of age. Goal is to monitor changes in the learning and memory caused by changed sulfation ratio throughout whole adult life. In those time periods it will be possible to monitor the effect of sulfation during aging. Also, biochemical analysis of CS-GAG fractions level and their sulfation patterns will be performed to confirm our results. Another study using homozygous parvalbumin-cre *Chst11* knockout, which is following up our results is aimed to assess whether *Chst11* knockout mice could be used as a model for schizophrenia. Since animals with reduced C4S epitope had increased anxious behaviour in the elevated zero maze as compared to controls.

It is important to understand the effect of changes the brain on the learning and memory during one's life to develop strategies, which enhance memory in the elderly or slow down its deterioration. Our study is helping to understand the role of extracellular matrix in those processes.

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