Interplay of enriched housing and brevican/neurocan deficiency in modulating brain structure and cognition in aged mice

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Summary

The brain hyaluronan-based extracellular matrix (ECM) is associated with many neuronal processes that affect cognitive function. One major constituent of the ECM are chondroitin sulfate proteoglycans among which neurocan and brevican are of particular interest, as both are involved in processes of neuronal plasticity, they are expressed differently during neurodevelopment and maturation and both are increasingly expressed in hippocampal synaptosomes of aging mice. Although neurocan peak expression is during late embryonal development and early postnatal life, its upregulation in adult brevican knockout mice suggests that it is able to partially compensate the lack of brevican. Both components are important constituents of condensed as well as diffuse ECM and can be found in perineuronal nets and in the perisynaptic and perinodal ECM. As part of these structures, they are involved in processes of diffusion, neuroprotection and neuroplasticity.

Brain aging goes along with an increase of oxidative damage, molecular waste accumulation, increase in inflammation, decline in neuroplasticity and loss of grey matter. This decline in cellular functionality leads to deficits in cognitive abilities. The cognitive decline also goes along with age-related changes to the ECM. An enriched environment can ameliorate cognitive aging. Especially exercise is shown to have beneficial effects. On the cellular level, these effects are manifested, among others, in an increase in neurogenesis and neuroplasticity. Possibly those effects could be mediated by changes of the brain ECM, as environment and exercise have been shown to affect ECM composition.

This project aims to elucidate the role of brevican and neurocan in the brain of aged mice and to shed light on their contribution to aging-related modulation of the ECM, brain structure and cognitive abilities. Another aim was to explore, if the effects of enriched environment on cognitive abilities could be mediated via the ECM.

Brevican/neurocan double knockout and wildtype mice were reared their whole life in cages containing a running wheel and mild sensory enrichment. Mice reared in standard environment were used as control. After approximately 20 months, aged mice were submitted to cognitive tasks, to test their spatial and fear learning capabilities and cognitive flexibility. For analysis of the ECM composition and brain structure, the brains were analyzed using immunohistochemistry, immunoblots and proteomics. Brain anatomy was furthermore assessed via magnetic resonance imaging.

Indeed, the deficiency of brevican and neurocan affects the ECM composition during aging, as aggrecan and HAPLN1 show a double knockout specific increase in aged, respectively young mice. In addition, more ECM components and interaction partners were found affected by the lack of both lecticans in a proteome analysis of aged mice. Proteomics and immunohistochemical analysis revealed a reduced inflammatory load in aged double knockout mice, compared to wildtype. Furthermore, double knockout mice display a decrease in volumes of cortex and hippocampus that can be partly rescued in aged mice by voluntary exercise, hinting towards a complex interaction of ECM composition and rearing environment throughout the lifespan. The hippocampus of brevican/neurocan deficient mice was characterized by a dysplasia of the pyramidal cell layer of the CA1 region. Despite this dysplasia, the mice show normal spatial learning. Deficiencies in trace fear learning could still hint towards a deficit in CA1 function, although more experiments are needed to confirm this correlation.

Generally, double knockout of brevican and neurocan lead to better cognitive flexibility. However, enriched rearing impeded this effect. So, while brevican/neurocan deficiency can have pro-cognitive effects in aged mice, environmental factors modulate those effects on cognitive abilities.

Taken together, it could be shown, that brevican and neurocan play a vital part for brain anatomy and that this relation is also linked to environmental factors such as exercise. The role of brevican and neurocan in cognition of aged mice is multifaceted and depend on the cognitive task, possibly as different brain areas are differently affected by the knockout.

Zusammenfassung

Die auf Hyaluronan basierende extrazelluläre Matrix (EZM) des Gehirns wird mit vielen neuronalen Prozessen in Verbindung gebracht, die kognitive Fähigkeiten beeinflussen. Ein Hauptbestandteil der EZM sind Chondroitinsulfat-Proteoglykane, von denen Neurocan und Brevican von besonderem Interesse sind, da beide in Prozessen der neuronalen Plastizität involviert sind, sie während der neuronalen Entwicklung unterschiedlich exprimiert werden und beide in Synaptosomen aus dem Hippocampus alternder Mäuse verstärkt exprimiert werden. Neurocan wird während der späten Embryonalentwicklung und direkt nach der Geburt am stärksten exprimiert, eine erhöhte Expression in erwachsenen Brevican-Knockout-Mäusen deutet aber darauf hin, dass Neurocan das Fehlen von Brevican kompensieren kann. Beide Komponenten sind wichtige Bestandteile sowohl der diffusen, als auch der kondensierten EZM und sind unter anderem in perineuronalen Netzen sowie in der perisynaptischen und perinodalen EZM zu finden. Als Teil dieser Strukturen sind sie an Prozessen der Diffusion, Neuroprotektion und Neuroplastizität beteiligt.

Die Alterung des Gehirns geht mit einer Zunahme an oxidativen Schäden, entzündlichen Prozessen, Anhäufung von molekularem Abfall, einer Abnahme der Neuroplastizität und Verlust grauer Substanz einher. Dieser Rückgang der zellulären Funktionalität führt zu Defiziten in kognitiven Fähigkeiten. Kognitive Einbußen gehen auch mit altersbedingten Veränderungen der ECM einher. Eine angereicherte Umwelt kann den kognitiven Verfall abmildern. Insbesondere Sport und mehr Bewegung haben nachweislich positive Auswirkungen. Auf zellulärer Ebene äußert sich dies unter anderem durch eine Zunahme der Neurogenese und Neuroplastizität. Möglicherweise werden positive Effekte auch durch Veränderungen der EZM im Gehirn vermittelt, denn die Umwelt und körperliche Bewegung haben nachweislich auch einen Effekt auf die Zusammensetzung der EZM.

Dieses Projekt zielt darauf ab, die Beteiligung von Brevican und Neurocan an altersbedingter Modulation der EZM, an Veränderungen der Gehirnstruktur und kognitiven Fähigkeiten im Gehirn alter Mäuse zu untersuchen. Ein weiteres Ziel war es zu ermitteln, ob die Auswirkungen einer angereicherten Umwelt auf kognitive Fähigkeiten durch die EZM-Zusammensetzung moduliert werden können.

Mäuse mit Brevican/Neurocan-Doppelknockout und Wildtyp-Mäuse wurden lebenslang in Käfigen mit Laufrad und sensorisch angereicherter Umwelt aufgezogen. Mäuse, die in Käfigen mit Standardausstattung aufgezogen wurden, dienten als

Kontrolle. Nach etwa 20 Monaten wurde räumliches Lernen, Angstlernen sowie die kognitive Flexibilität der Mäuse getestet. Um die EZM-Zusammensetzung und die Gehirnstruktur zu untersuchen, wurden die Gehirne mittels Immunhistochemie, Immunoblots und Proteomanalyse untersucht. Die Anatomie des Gehirns wurde darüber hinaus mittels Magnetresonanztomographie analysiert.

Das Fehlen von Brevican und Neurocan wirkt sich auf die EZM-Zusammensetzung während des Alterns aus. Aggrecan und HAPLN1 wiesen in alten bzw. bereits in jungen knockout Mäusen einen Anstieg auf. Auch andere EZM-Komponenten und Interaktionspartner sind durch das Fehlen beider Lecticane beeinträchtigt, wie sich in der Proteomanalyse alter Mäuse zeigte. Analyse des Proteoms und Immunhistochemie zeigten außerdem eine Verringerung der Entzündung im Gehirn von alten Doppelknockout-Mäusen im Vergleich zu Wildtyp-Tieren. Darüber hinaus zeigen Doppelknockout-Mäuse ein geringeres Volumen im Kortex und Hippocampus. Hierbei spielte die Interaktion zwischen Alter und Umwelt eine Rolle, was auf eine komplexe Interaktion zwischen EZM-Zusammensetzung und Umwelt während der gesamten Lebensspanne hindeutet. Der Hippocampus von Mäusen mit Brevican/Neurocan-Defizienz wies eine Dysplasie in der Pyramidenzellschicht der CA1-Region auf. Trotz dieser Dysplasie zeigten die Mäuse normales räumliches Lernen. Defizite beim Angstlernen in einem Paradigma mit zeitlicher Assoziation könnten allerdings auf ein Defizit in der CA1-Funktion hindeuten. Es sind aber weitere Experimente erforderlich, um diesen Zusammenhang zu bestätigen.

Im Allgemeinen führte der Doppelknockout von Brevican und Neurocan zu einer besseren kognitiven Flexibilität. Diese Wirkung wurde jedoch durch eine angereicherte Umwelt beeinträchtigt. Während also eine Reduktion von Brevican und Neurocan positive Auswirkungen auf die kognitiven Fähigkeiten haben kann, besteht eine Interaktion zwischen Umwelt und EZM-Zusammensetzung, die sich auf die kognitiven Fähigkeiten auswirkt.

Zusammengenommen konnte gezeigt werden, dass Brevican und Neurocan für den Aufbau des Gehirns eine wichtige Stellung einnehmen und dass diese Rolle auch mit Umweltfaktoren und Bewegung zusammenhängt. Die Rolle von Brevican und Neurocan bei der Kognition gealterter Mäuse ist komplex und hängt von der kognitiven Aufgabe ab, möglicherweise, weil verschiedene Hirnareale durch den Knockout unterschiedlich betroffen sind.

List of abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ASST	Attentional set shifting task
Bcan	Brevican
BDNF	Brain derived neurotrophic factor
BRIX1	Ribosome biogenesis protein BRX1 homolog
ChABC	Chondroitinase ABC
CSPG	Chondroitin sulfate proteoglycane
DCLK2	Doublecortin-like kinase 2
DCX	Doublecortin
DDT	D-dopachrome tautomerase
DG	Dentate gyrus
DKO	Double-knockout
ECM	Extracellular matrix
EE	Enrichment with exercise
FDR	False discovery rate
FLII	Flightless 1
GBP1	Guanylate−binding protein 1
GFAP	Glial fibrillary acidic protein
GPI	Glycosylphosphatidylinositol
HA	Hyaluronan
HAPLN	Hyualuronan and proteoglycan link protein
ICAM5	Intercellular adhesion molecule 5
IGKC	Immunoglobulin kappa C
IHC	Immunohistochemistry
IL-6	Interleukin-6
LTP	Long-term potentiation
MMP	Matrix metalloproteinase
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
Ncan	Neurocan
PB	Phosphate buffer
PBS	Phosphate buffered saline

PNN	Perineuronal net
PV+	Parvalbumin containing (interneuron)
SDC2	Syndecan-2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHIRPA	SmithKline, Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
ST	Standard (rearing)
TBS-T	TRIS-buffered saline with Tween-20
WB	Western blot
WFA	*Wisteria floribunda* agglutinin
WT	Wildtype

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1 Introduction

1.1 The brain extracellular matrix (ECM)

The extracellular space of the brain is filled with molecules, which together form the extracellular matrix, surrounding and embedding all cells and structures of the brain. With up to 20%, a substantial part of the brain volume constitutes of matrix components and interstitial fluid (Nicholson et al., 2012). But the composition of the brain matrix is highly variable during development (Rauch, 2004), in different brain regions (Brückner et al., 2003; Jäger et al., 2013), between different cell types (Lensjø et al., 2017; Seeger et al., 1994) and even between different cellular structures (Hedstrom et al., 2007). As diverse as its composition are the functions the brain ECM is involved in, including neuroprotection (Brückner et al., 1999), neuroplasticity (Brakebusch et al., 2002; Frischknecht et al., 2009; Pizzorusso et al., 2002) and tissue diffusivity (Bekku et al., 2010).

Generally, the brain ECM is of different constitution, compared to non-neuronal peripheral ECM, as instead of collagens and fibronectin it consists mainly of proteoglycans and their binding partners (Zimmermann & Dours-Zimmermann, 2008). Within the central nervous system, the composition differs between the ECM of the basement membrane, which is bordering blood vessels and the pial surface and ECM of the brain parenchyma. While the ECM of the basement membrane consists in large parts of laminin, fibronectin, collagen and heparan sulfate proteoglycans (Reed et al., 2019), the ECM of the brain parenchyma contains hyaluronan (HA), chondroitin sulfate proteoglycans (CSPG), link proteins and tenascins, amongst other components (Frischknecht & Seidenbecher, 2008).

1.1.1 Composition and properties of the hyaluronan-based ECM

Hyaluronan is a main brain ECM constituent and binding partner to a diverse set of glycoproteins and proteoglycans. HA gets synthesized by the transmembrane proteins hyaluronic acid synthases 1, 2 and 3 (Itano & Kimata, 2002) and degraded to fragments of differing size by six different hyaluronidases (Csoka et al., 2001).

Among the main binding partners of HA are CSPGs. Lecticans are a group of CSPGs that take on an important role in the nervous system. Their name is derived from the lectin domain at the C-terminus. The N-terminus is comprised of an HA-binding domain and the central domain contains multiple covalently bound chondroitin sulfate chains

(Yamaguchi, 2000). The length of the central domain and number of chondroitin sulfate chains differs between the lecticans. All four lecticans, aggrecan, brevican (Bcan), neurocan (Ncan) and versican are secreted into the extracellular space, although Bcan also appears as an isoform with a glycosylphosphatidylinositol (GPI) -anchor that is linked with the cell membrane (Seidenbecher et al., 1995; Yamaguchi, 2000). While aggrecan and versican also get expressed in non-neuronal tissue, Bcan and Ncan are expressed in the nervous system (Rauch et al., 2005). Lecticans bind with their C-terminal lectin domain to tenascin-R, another constituent of the HA-based ECM (Anlar & Gunel-Ozcan, 2012; Yamaguchi, 2000). Tenascin-R is ubiquitously expressed in the adult brain, while tenascin-C, another member of the tenascin family, is mostly expressed during brain development (Ferhat et al., 1996). While tenascins crosslink multiple lecticans, hyualuronan and proteoglycan link proteins (HAPLN) attach to CSPGs and HA, to stabilize the binding. HAPLN3 is expressed ubiquitous in multiple different tissues, while HAPLN2 and HAPLN4 are predominantly expressed in the nervous system (Oohashi et al., 2015). The latter is genomically closely localized to the Ncan gene, while the HAPLN2 gene is neighboring the Bcan coding region (Bekku et al., 2003; Nomoto et al., 2002). Besides the mentioned main components of the ECM, many other different molecules and interaction partners are part of the ECM and involved in its many functions. The HA-based ECM can be differentiated into structurally and functionally distinct structures that can present as particularly condensed. One being the perinodal ECM. This special form of ECM can be found along axons, surrounding the nodes of Ranvier. It consists mainly of Bcan, Ncan, versican and HAPLN2 besides having HA as a backbone (Bekku & Oohashi, 2010; Fawcett et al., 2019). It is proposed, that the perinodal ECM serves as a diffusion barrier, as a sink for positively charged sodium ions or that it affects voltage gated sodium channel localization (Bekku et al., 2009; Lopreore et al., 2008; Oohashi et al., 2002; Susuki et al., 2013). The axon initial segment also contains its own unique ECM structure with Bcan as an important component, that may also aid in axonal function (Hedstrom et al., 2007).

Similar to the perinodal ECM, a dense ECM structure of equal composition can be found surrounding mostly inhibitory, but also excitatory neurons. The so called perineuronal nets (PNN) consist of HA and varying kinds of CSPGs (these include Bcan, Ncan, aggrecan, phosphacan and versican), link proteins and tenascins, though the exact composition can vary between PNNs of different brain areas (Miyata et al.,

2018; Reichelt et al., 2019)(Figure 1). Accordingly, the visualization of PNNs within the brain can be achieved by staining for their main components. Aggrecan for example is a major constituent of all PNNs. *Wisteria floribunda* agglutinin (WFA) is another important marker for PNNs, as it labels the *N*-acetylgalactosaminide residues of matrix glycoproteins (Härtig et al., 2022), although not all PNNs can be labeled by it.

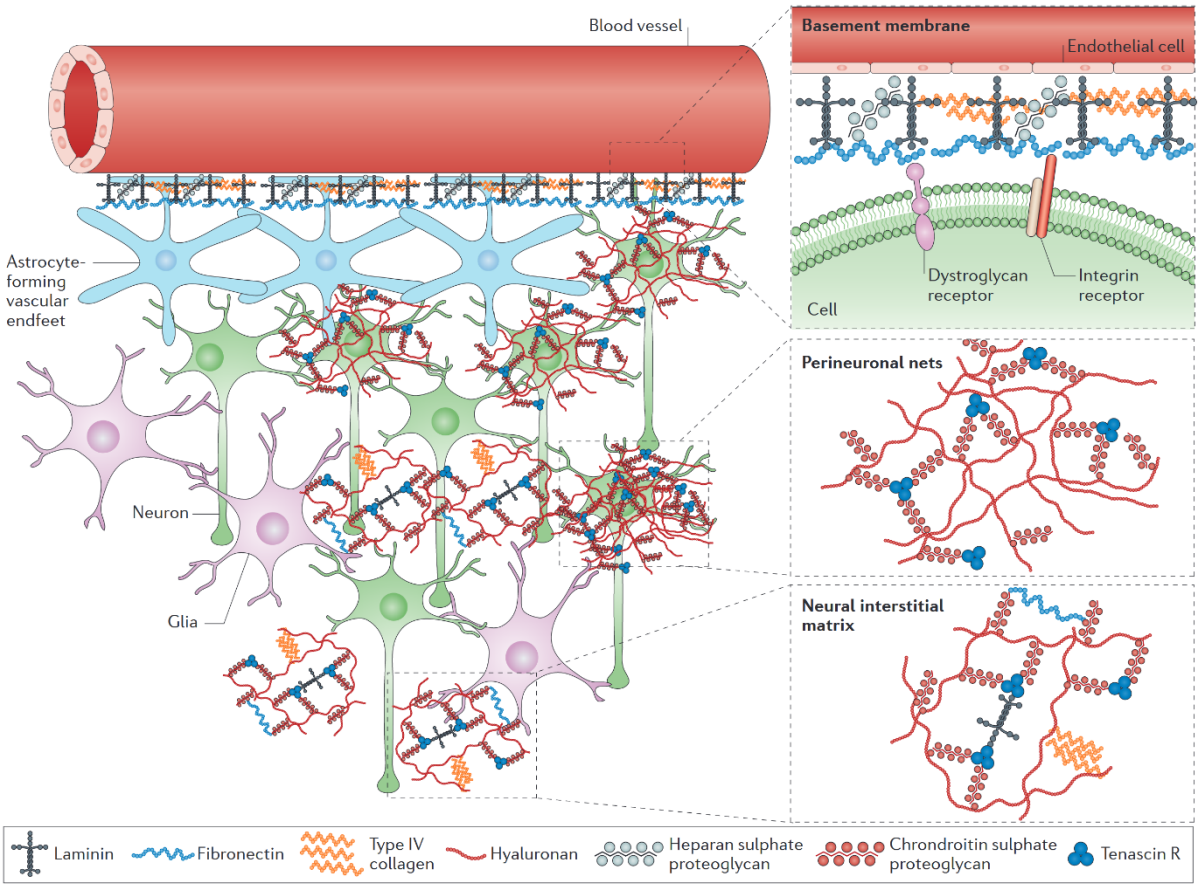


Figure 1: Overview of different brain ECM structures. ECM structures at the basement membrane, surrounding neurons as PNN and in the interstitial space display a difference in composition, that might go along with different functions (from L. W. Lau et al., 2013).

PNNs start to form at the end of the critical periods, marking the end of major reconstructions of neuronal connections happening during development (Oohashi et al., 2015). Knocking out components of the ECM has an effect on PNN development and appearance in varying degrees. In cell cultures hyaluronan, tenascin-R, a lectican and link protein are needed for the formation of PNNs (Fawcett et al., 2019; Giamanco et al., 2010; Kwok et al., 2010; Morawski et al., 2014a). Aggrecan is the lectican mostly involved in PNN formation, while Bcan and Ncan are not as important for formation and PNNs arise even in their absence (Brakebusch et al., 2002; Rowlands et al., 2018;

Zhou et al., 2001). However, lack of Ncan affects the presence of Bcan, aggrecan and HAPLN1 in PNNs in the medial nucleus of the trapezoid body (Schmidt et al., 2020). Knockout of HA synthases, HAPLN1, HAPLN4 or tenascin-R also leads to the formation of attenuated PNNs (Arranz et al., 2014; Bekku et al., 2012; Brückner et al., 2000; Carulli et al., 2010). Altering the PNN composition via knockout or digestion not only gives information about structural composition, it also hints towards their function. ECM glycostructures can be digested by treatment with enzymes targeting specific constituents. Chondroitinase ABC (ChABC) is an often-used enzyme that cleaves away the chondroitin sulfate chains of CSPGs (Takashima et al., 2021), revealing the neuronal plasticity mediating effect of PNNs and ECM in general (Chu et al., 2018). Hyaluronidase on the other hand digests HA chains, which removes the HA-based ECM (Tona & Bignami, 1993). Both enzymes act on a broader scale, digesting not only components of PNNs, but also diffuse ECM.

Those manipulations of PNNs show a strong influence on neuroplasticity in general (Carulli et al., 2010; Favuzzi et al., 2017; Rowlands et al., 2018; Sorg et al., 2016), but they are also implicated to mediate electrophysiological properties of parvalbumin containing GABAergic interneurons. Those fast-spiking interneurons are abundantly present in the cortex and play an important role for neural oscillations (Wingert & Sorg, 2021).

Apart from the condensed, lattice-like perineuronal ECM, there is also the diffuse ECM in the interstitial space and especially surrounding synapses. This kind of ECM generally consists of the same components as PNNs and it may act as diffusion barriers and is involved in neuroplasticity and synapse stabilization (Chelyshev et al., 2020; Faissner et al., 2010).

The brain ECM undergoes dynamic modulations, that can be performed by proteases of the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family. There are 5 members of the ADAMTS family with aggrecanase and proteoglycanase function, of which ADAMTS4 is particularly interesting, as it is very efficient in digesting Bcan (Nakamura et al., 2000; Valenzuela et al., 2014).

Matrix metalloproteinases (MMPs) are another class of proteinases, that is involved in matrix modulation. While 24 different MMPs have been identified, only a couple of them are relevant for ECM digestion. So far, MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -14, -19, -20 have been found to digest ECM components such as laminin, tenascins and

aggrecan. MMP-9 in particular stands out among the MMPs, as it is involved in synaptic plasticity and learning (Ethell & Ethell, 2007).

1.1.2 The lecticans brevican and neurocan

As already mentioned in the previous chapter, Bcan plays a pivotal role in the composition of condensed and diffuse ECM. It is a crucial component of axonal ECM at the axon initial segments in the brainstem (Hedstrom et al., 2007) and nodes of Ranvier (Bekku et al., 2009). While being present in PNNs (Figure 2), Bcan does not largely impact their development (Brakebusch et al., 2002) but is essential for their function. Bcan can modulate the excitatory input to parvalbumin containing (PV+) interneurons that are largely enwrapped in PNNs (Favuzzi et al., 2017). Since PV+ interneurons are involved in modulating network activity that is associated with cognitive functions (Fuchs et al., 2007), Bcan potentially has an impact on those cognitive functions. Indeed, Bcan knockout mice display short-term memory deficits in spatial learning tasks and in the novel object recognition task (Favuzzi et al., 2017). Furthermore, Bcan is implicated in cocaine-induced craving (Lubbers et al., 2016).

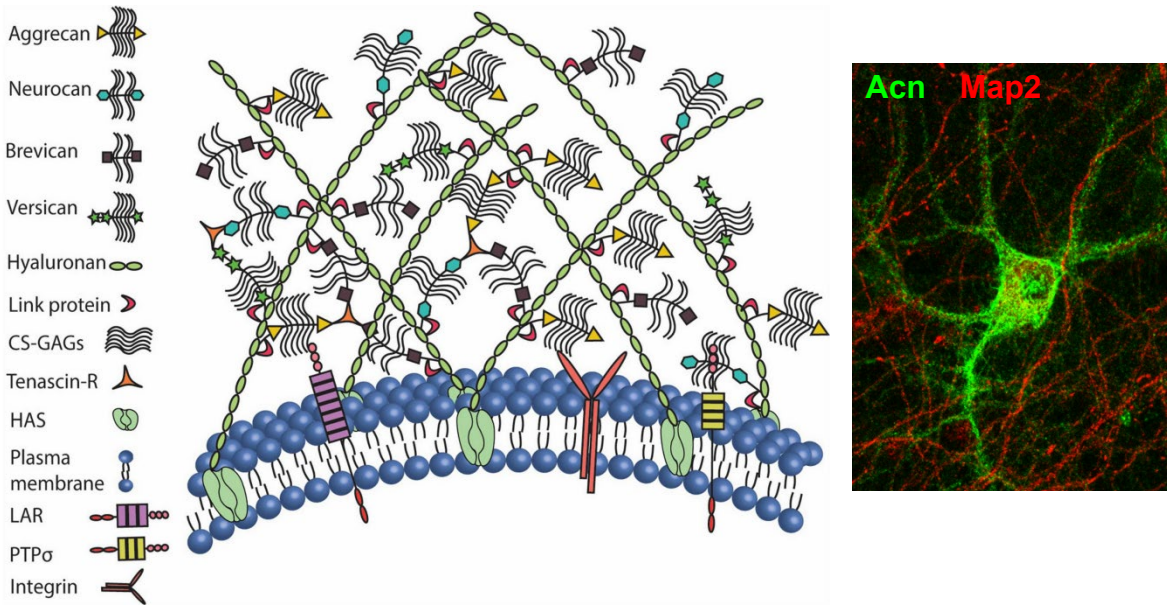


Figure 2: Detailed overview of PNN composition. Left: The binding of brevican and neurocan to hyaluronan is reinforced by link protein and they interconnect with other CSPGs via tenascin-R (from Tewari et al., 2022). Right: PNN staining of a hippocampal neuron from primary cell culture using anti-aggrecan antibody. Dendrites were stained using anti-Map2 antibody.

At excitatory synapses Bcan is target to dopamine D1 receptor-induced cleavage, highlighting its potential role in mechanisms of synaptic plasticity (Mitlöhner et al., 2020). The feasibility of this function gets underlined by defects in maintenance of long-term potentiation (LTP) in Bcan knockout mice (Brakebusch et al., 2002). The Bcan knockout mice were generated by inserting a *lacZ* cassette into the exon 2 and as a consequence eliminating the complete Bcan coding sequence (Figure 3A) (Brakebusch et al., 2002). The Bcan gene is located on mouse chromosome 3 in close vicinity of the coding region of link protein HAPLN2 (Nomoto et al., 2002; Rauch et al., 1997). Interestingly, knockout of Bcan also leads to an upregulation of Ncan in the mouse brain, suggesting at least a partial compensation (Brakebusch et al., 2002).

Ncan is structurally similar to Bcan, but gets expressed mainly during late embryonal and early postnatal development (Zhou et al., 2001). Via its interactions with neuronal factors for growth, mobility and cell adhesion it has the potential to modulate neurite outgrowth and cell migration in the developing brain (Rauch et al., 2001).

Even though Ncan is only sparsely expressed in the adult brain ECM, the knockout of Ncan leads to LTP maintenance deficits (Zhou et al., 2001). Knockout of Ncan is associated with a manic phenotype, as knockout mice show increased activity, lower anxiety and compulsivity in a diverse set of behavioral tests (Miró et al., 2012). The Ncan knockout was done in the past in three different ways. In the present study the knockout via the *Cre-loxP* system will be of interest (Zhou et al., 2001)(Figure 3B).

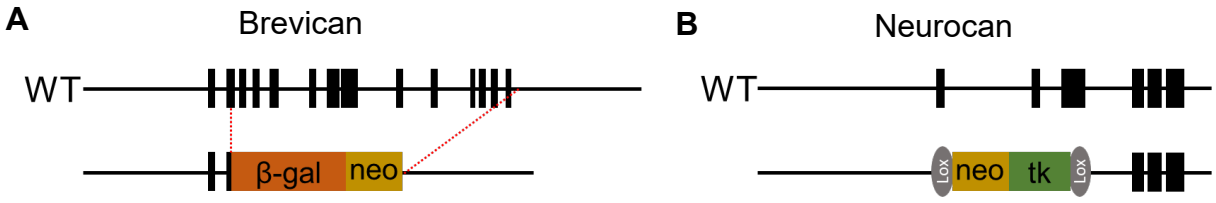


Figure 3: Generation of Bcan and Ncan knockout mice. Black boxes indicate exons. **(A)** A *lacZ* gene cassette was introduced into the coding sequence of the Bcan gene (β-gal = β-galactosidase, neo = neomycin)(modified from Brakebusch et al., 2002). **(B)** The Ncan knockout mice were generated via *Cre-loxP* system. The neomycin and thymidine kinase (tk) cassette was eliminated via transient *Cre* transfection (modified from Zhou et al., 2001).

1.1.3 The ECM during maturation and aging

The ECM undergoes major restructuring during brain development. Multiple ECM components are involved in neurite outgrowth, axon guidance and cell development. Hyaluronan is expressed early during embryonal development (Spicer & McDonald,

1998), also tenascin-C and Ncan are predominantly expressed during development, indicating a major role for proper formation of the central nervous system (Jones & Jones, 2000; Tan et al., 1987; Zhou et al., 2001). A switch in ECM composition during maturation and especially the emergence of PNNs are hallmarks of the closure of critical periods. Instead of high functional and structural plasticity, that allows the environment to shape neuronal networks, the adult ECM rigidifies neuronal connections and networks (Gundelfinger et al., 2010). One illustrative example of how juvenile plasticity can be restored in adults by modifying the ECM is given by Pizzorusso et al., 2006: Deprivation of one eye during mouse development changes the ocular dominance, as long as the critical period is not yet closed. This shift is highly inhibited in adult animals, but digesting the ECM of the visual cortex with chondroitinase ABC allows a shift in ocular dominance also in adults. This shift is accompanied by an increase of dendritic spine density.

So, while the juvenile ECM plays a distinct role for neurodevelopment, the adult ECM is associated with other functions besides strengthening neuronal connections. It is also involved in neuroprotection (Morawski et al., 2004), diffusion of neurotransmitter and cations and lateral diffusion of receptors on the cell surface (Gundelfinger et al., 2010). Both, neuroprotection and diffusion are relevant for brain aging, as for example oxidative stress is of high relevance for brain aging (Mariani et al., 2005) and also diffusion parameters change throughout the brain (Zavaliangos-Petropulu et al., 2019). Changes to the ECM during aging could affect those functions and could be an explanation for progressing cognitive decline in healthy aging. Likewise, the manipulation of the ECM and specialized ECM structures such as PNNs have the potential to increase neuroplasticity and memory function during aging (Sorg et al., 2016).

Végh et al., 2014 found that during aging the ECM components Bcan, Ncan and HAPLN1 increase in synaptosomal fractions from the hippocampus of mice. As the hippocampus is a central hub for learning and memory function, those changes could be associated with the decline in hippocampal function and in hippocampus-based learning task during aging (Végh et al., 2014). Also, in the aged hippocampus plaque-like aggregations of the large extracellular glycoprotein reelin appear (Knuesel et al., 2009), while reelin shows an age-related decline in the enthorinal cortex, that can be associated with deteriorating cognition (Stranahan et al., 2011). Reelin is highly involved in cortical development and might interact with ECM components (Tissir &

Goffinet, 2003). Also HA shows an upregulation during aging with uncertain consequences (Peters & Sherman, 2020).
ECM alterations not only are important for age-related pathologies, they also appear during healthy aging and potentially affect cognitive decline. But due to the involvement in many different functions, it is not completely clear if aging-related ECM changes are beneficial or disadvantageous.

1.2 The aging brain

Aging is a process of progressive decline in functionality. Disturbed cellular processes also occur in the absence of age-related neurodegenerative diseases. On the molecular level, brain aging comprises an increase in oxidative damage and accumulation of molecular waste, as the function of lysosomal and proteasomal pathways decline. Together with dysfunction of cellular metabolism, this can lead to cellular stress. Furthermore, the level of inflammation increases, which may contribute to decreased neurogenesis and disturbances of neuronal networks (Mattson & Arumugam, 2018). These malfunctioning result in loss of mental acuity, memory function and even sensory and motoric decline.
Aging-related changes of the brain are also quantified on a broader scale. Grey matter volume is found to be reduced in aged humans (Taki et al., 2011) and mice (Taylor et al., 2020). Rodents show a reduction in volume especially in areas of the isocortex, whereas other areas for example in the brainstem and cerebellum remain unaffected (Clifford et al., 2023). Multiple brain areas are also affected by changes in diffusion. Aged mice display loss of fractional anisotropy in the thalamus and cortex, indicating a reduction in directional diffusion, while overall diffusivity increases (Taylor et al., 2020). A reduction of fractional anisotropy can also be observed in the hippocampus of aged rats, where it is associated with poor learning abilities and reduction in CSPGs (Syková et al., 2002). Changes to the ECM could be one reason for a change to diffusion parameters during aging. Altered diffusion could lead to deranged neuromodulator or neurotransmitter distribution. Dysfunctional modulatory inputs in brain aging have been observed for acetylcholine (Jacobson et al., 2013), dopamine (Abdulrahman et al., 2017) and noradrenaline (Stemmelin et al., 2000).
As the life expectancy rises around the world, it is increasingly important to determine the molecular and functional background of brain aging and to identify possible effective countermeasures.

1.2.1 Molecular and cellular brain aging

As neurons are postmitotic cells, damage of the central nervous system can be hardly recovered. Multiple aberrant processes intertwine and accumulate, making it increasingly difficult for neurons to maintain their complex structure and functionality during aging.

Among the accumulating negative contributors of aging are reactive oxygen species. Those highly reactive derivatives of oxygen are able to alter other molecules such as proteins and lipids, leading to cellular defects (Poon et al., 2004). Cellular mechanisms protecting from reactive oxygen species are associated with higher cognitive abilities in aging. For example overexpression of the extracellular superoxidase dismutase, which is mostly found in the ECM, is associated with enhanced LTP and spatial learning (D. Hu et al., 2006).

Similarly, the inflammatory load increases in brain aging. This includes an increase of astroglia and microglia that enter an activated state (Norden & Godbout, 2013; Rodríguez et al., 2014), accompanied by an increase of proinflammatory cytokines like interleukin-1β, interleukin-6 and tumor necrosis factor α (Cribbs et al., 2012). While properly regulated mechanisms of the immune system are important for neuroplasticity, a dysregulation of pathways during aging can have detrimental effects on synapses (Shi et al., 2015).

The strain on neurons and glia cells from reactive oxygen species and inflammation can negatively influence neuronal plasticity. The progressive loss of neuronal plasticity is a fundamental mechanism leading to cognitive decline during aging. On the structural side, this can be observed as a region-specific change in dendritic spine density, loss of synapses (Peters et al., 2008) and aberrant dendritic branching, especially in the prefrontal cortex. Compared to pathological aging, there is no significant hippocampal and neocortical cell death in physiological aging (Burke & Barnes, 2006). Changes can also be found in functional plasticity. The calcium conductance is higher in aged neurons, caused for example by an increase of L-type calcium channels (Thibault & Landfield, 1996). This could be the reason for changes in hippocampal LTP and long-term depression that were often observed in aged animals (Barnes et al., 2000; Norris et al., 1996).

As the hippocampus and prefrontal cortex are particularly affected by aging-related defects in neuroplasticity, cognitive abilities that rely on those regions are affected by aging, as it will be discussed in the next chapter.

PNNs possibly have a protective effect on neurons during physiological and pathological aging. Not only do they protect against reactive oxygen species, they are also implied to protect against toxic amyloid beta species, which arise during Alzheimer's disease (Morawski et al., 2014b).

1.2.2 Cognitive aging

One hallmark of aging is the progressive loss in memory function and thus decline in learning ability. Diminished function of the hippocampus and other regions of the medial temporal lobe, which are important for learning and memory, drive this decline. Thus, hippocampus-based cognitive abilities are severely impacted in aging. This manifests in impairments of acquisition and retention of spatial memory (Gawel et al., 2018; Yanai & Endo, 2021). Also other regions of the temporal lobe are affected: Aging leads to a decline in perirhinal function which results in deficits in object recognition (Burke et al., 2011). In general, age-related loss of function in the medial temporal lobe, including the hippocampus, leads to a decline in episodic memory (Köhncke et al., 2021). Intact episodic memory enables recollection of spatial and temporal information. Many studies show a decline in aged humans (Hayes et al., 2015; Nyberg et al., 2012) but there are also correlates of episodic memory declining in rodents (Fordyce & Whner, 1993; Kishimoto et al., 2001).

Besides the temporal lobe, functional decline of the prefrontal cortex during aging is associated with decreased cognitive flexibility (McQuail et al., 2021). This higher order cognitive function is important for adjusting learned strategies based on changing conditions.

While the decline in neuronal function in brain areas underlying cognitive function directly contribute to the intellectual decline, areas of sensory processing are also affected by neuronal dysfunction and may aggravate the decline in mental acuity during aging. In mice, a decline in olfaction (Moreno et al., 2014) as well as visual, acoustic (Tremblay et al., 2012) and somatosensory (Voglewede et al., 2019) processing can be observed. Central nervous system-based causes of this deterioration are found in synaptic perturbations (Richard et al., 2010; Voglewede et al., 2019), aberrant neuronal activity (Ding et al., 2018) and loss of PV+ inhibitory neurons (Martin del Campo et al., 2012).

In rodents, aging is associated with an increase of anxiety (Lamberty & Gower, 1993) that goes along with but does not arise from a decrease of motor activity (Boguszewski

& Zagrodzka, 2002). High anxiety can be alleviated by environmental enrichment. Furthermore, other behavioral parameters and cognitive functions were found to benefit from enrichment (Bhagya et al., 2017).

1.3 The impact of the environment on brain function

The environment can modulate cognitive abilities. This modulation occurs via negative or positive influences.

One negative influencing factor on cognition is stress. Stressors used in behavioral experiments are early life stress by maternal separation (Francis et al., 2002), restrain stress which can be applied chronically (Bhagya et al., 2017) and social stress (Mitra et al., 2006). Applying those stressors on mice leads to a decline in learning and memory, that can be attributed to changes in dendritic arborization (McLaughlin et al., 2007), reduced hippocampal neurogenesis (Mitra et al., 2006), reduced neurotrophic factors and impairment in LTP induction in the hippocampus (Sterlemann et al., 2009). Maternal separation also compromises social behavior (Kambali et al., 2019). Those negative effects of stress can be alleviated by positive environmental factors. Positive factors used for enrichment are sensory stimulation and exercise. Sensory enrichment often consists of larger cages, nest building material, different objects that get replaced regularly, shelters and diverse smells (Bayne, 2018; Blount & Coppola, 2020). This kind of enrichment has a robust anxiolytic effect (Hendershott et al., 2016; Kimura et al., 2019), it increases neurogenesis, neurotrophic factors, synaptogenesis and enhances memory function (Birch et al., 2013). Environmental enrichment of rodent housing often incorporate both, sensory enrichment and exercise, however exercise alone is able to elicit many positive effects of enrichment (Clemenson et al., 2015).

Exercise treatment can be achieved by placing a running wheel in the cage, to allow for voluntary running. Or it can be applied by forced treadmill running. The latter was shown to produce positive cognitive effects (Ang et al., 2006), but other studies made contradictory observations, that possibly arise from an increased stress level from forced exercise. Yuede et al., 2009 found, that in a mouse model of Alzheimer's disease, voluntary running leads to better memory performance and lower plaque formation, while forced running could not produce such positive effects. Indeed, forced running can lead to an increase of corticosterone levels and anxiety in mice (Svensson et al., 2016).

In general, exercise boosts brain angiogenesis, neurogenesis, neurotrophic factors, neuroplasticity and consequently improves cognition, as discussed in the following chapter.

1.3.1 Exercise improves cognition during aging

Several molecular processes are affected by exercise. Aging-related changes in angiogenesis-related gene expression can be reversed and are associated with an increase of microvessel branching and density in the hippocampus (Murugesan et al., 2012). Better blood perfusion and thus better metabolic support of neurons as a result of exercise could explain the observed association between increased angiogenesis and cognitive function (Anderson et al., 2010; Clark et al., 2009). Blood factors generated during physical activity could also positively impact neurogenesis (Horowitz et al., 2020). Those effects not only improve cognition during healthy aging, they also attenuate negative effects of ischemic stroke (Zhang et al., 2022), the incidence of which increases with age (Yousufuddin & Young, 2019).

Besides increased metabolic support from improved vascularization, exercise can increase hippocampal neurogenesis during aging also via the increase of growth factors like brain derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (Trejo et al., 2008; Vaynman & Gomez-Pinilla, 2016). Especially elevated levels of BDNF from exercise are implicated to improve cognitive function via its positive effects on synaptic function and its potential neuroprotective effects (Vaynman & Gomez-Pinilla, 2016). Neuroprotective effects of exercise are also facilitated by reduction of pro-inflammatory factors like nuclear factor κB and tumor necrosis factor α and reduction of reactive oxygen species, if exercise is performed long-term (Wahl et al., 2021).

The robust positive effects of physical activity on spatial learning are mediated by changes to the hippocampus. The hippocampal area benefits from aforementioned effects of exercise in particular. An improvement of spatial learning after running exercise in aged rats is associated with an increase of BDNF and nerve growth factor mRNA, as well as with rescue of age-related LTP impairment in the dentate gyrus (DG) (O'Callaghan et al., 2009). Improvements of LTP in aged animals via running could be mediated by changes to synapses, as the dendritic spine density increases on neurons in the hippocampal CA1 and DG and entorhinal cortex in exercised rats. The increased number of spines is paralleled by changes in spine morphology, especially in the CA1

region (Stranahan et al., 2007). Cognitive effects of exercise include, besides improvement of spatial learning, also an increase in fear learning (Falls et al., 2010; Mäkinen et al., 2023). This suggests that apart from the hippocampus, other brain areas like the amygdala are affected by exercise as well.

Physical activity ameliorates many effects of aging in the healthy animal and additionally, it can improve cognitive outcomes in models of aging-related neuropathological diseases. Hence, voluntary wheel running improves spatial learning in models of Alzheimer's disease (García-Mesa et al., 2011; Parachikova et al., 2008) and mitigated loss of dopaminergic neurons alongside better motor function in a model of Parkinson's disease (Y.-S. Lau et al., 2011).

Effects of exercise not only depend on motivational, potentially stress-inducing factors (forced vs. voluntary), they might also depend on duration. While some benefits are visible after a brief duration of exercise, some other effects require longer periods of physical activity. Although there are not many studies investigating the differences between short-term and long-term exercise, there is evidence, that some benefits arise only after a longer duration of exercise. In human studies, the benefits increased, when exercise was continued after a 6 months period (Voelcker-Rehage et al., 2011). In the cerebellum lifelong running decreases oxidation of lipids and DNA in aged rats, while 3 months of running starting at 18 months of age only attenuates lipid oxidation (Cui et al., 2009). Apart from this, it is confirmed, that lifelong exercise is at least able to impart the same positive effects as short-term exercise. In rodents, lifelong exercise with or without sensory enrichment leads to better metabolism and body composition (McMullan et al., 2016), physical fitness, increase in BDNF (Garcia-Valles et al., 2013), improved recognition memory, spatial learning (Morgan et al., 2018) and neuroplasticity (Leal-Galicia et al., 2008).

1.3.2 Interactions of ECM and environment

The observed effects of environmental enrichment on plasticity and other pro-cognitive processes could be in part mediated by modulation of the ECM. A clear impact of the environment on ECM composition has been observed for stress exposure. Chronic social defeat stress leads to a significant increase of Bcan, Ncan, phosphacan and HAPLN1 in the dorsal hippocampus, alongside an increase of PNNs in the CA1 region. This ECM modulation is associated with impaired object location memory that can be rescued by ChABC application. The cognitive impairment could result from a reduction

of inhibitory transmission in the hippocampus (Koskinen et al., 2020; Riga et al., 2017). The stress-related increase of CSPGs and PNNs in rodents could arise from an increase of corticosterone (Alaiyed et al., 2020).

Positive environmental stimulation on the other hand is able to improve cognitive and physiological abilities via ECM modulation. In mouse models of stroke, especially paw placement is enhanced by enrichment, which goes along with a reduction of PNNs in the somatosensory cortex (Madinier et al., 2014). Beneficial effects of exercise on stroke outcomes could be also mediated via modulation of the ECM located at the basal lamina (X. Wang et al., 2014). In addition, in a mouse model of Alzheimer's disease the benefits of short-term enrichment could be linked to alteration of the ECM. Here, enrichment helps alleviate the loss of PNNs in the hippocampus, that occurs during Alzheimer's disease (Cattaud et al., 2018).

In healthy animals, exposure to enrichment along with running wheels reduces Bcan expression in the hippocampus. Especially the GPI-anchored Bcan isoform shows a persistent downregulation after 30 days of enrichment (Favuzzi et al., 2017). Apart from ECM remodulation via changes in gene expression, the observed effects could likewise be conferred via MMPs. Enrichment leads to an increase of MMP-9 in the hippocampus (Cao et al., 2014). However, in the cerebellum no such increase of MMP-9 could be observed, even though cerebellar PNNs are reduced by 8 weeks of environmental enrichment (Stamenkovic et al., 2016), suggesting different modes of modulation, dependent on brain region and possibly also ECM composition. In old healthy rats, environmental enrichment including running wheels is able to reinstate ocular dominance plasticity in the visual cortex. Likely, the reduced intensity of PNNs in the visual cortex contributes to the observed increase of plasticity (Scali et al., 2012).

1.4 Aims of the study

The ECM composition and integrity is heavily involved in cellular processes that affect cognitive abilities. Especially Bcan, as a major component of PNNs and diffuse ECM, is a significant factor for ECM-based regulation of neuroplasticity (Brakebusch et al., 2002; Mitlöhner et al., 2020). While Ncan is only sparsely expressed in the adult nervous system, it is still involved in LTP maintenance and it shows a compensatory upregulation in Bcan-knockout mice (Brakebusch et al., 2002; Zhou et al., 2001). The analysis of double-knockout (DKO) mice of Bcan and Ncan could help unveil the

function of the two similar lecticans in the ECM meshwork, in neuroplasticity and cognitive functions.

Both, Bcan and Ncan are increased in the hippocampus of aging mice. While an association with cognitive decline is observed, the details of this correlation are not clear (Végh et al., 2014). Changes of the brain ECM during aging might be involved in age-related cognitive decline via the impact on neuronal plasticity, PNN functionality and tissue diffusivity. Filling a large part of the brain parenchyma, the ECM might even affect aging-related loss of brain volume. This project aims to elucidate the role of Bcan and Ncan in the brain of aged mice and to shed light on their contribution to aging-related modulation of the ECM and brain function.

Additionally, it shall be explored, if the effects of enriched environment on cognitive abilities could be mediated via the ECM. The observed effects of the environment on the ECM could underlie the pro-cognitive effect of enrichment. Since a large effect of enrichment can be achieved with exercise, the focus here was put on effects from lifelong enrichment with voluntary exercise (EE).

Taken together, this project aims to explore, how aging and EE affect the ECM of mice with a DKO of Bcan and Ncan, as compared to wildtype (WT) and how those effects could mediate differences in cognitive abilities (Figure 4).

This project addresses the following research questions:

1. How does the ECM composition change during aging in mice lacking Bcan and Ncan?
2. Does the lack of Bcan and Ncan affect brain anatomy during aging?
3. Are cognitive abilities of aged mice affected by Bcan and Ncan DKO?
4. Is the effect of environmental enrichment via exercise modulated by Bcan and Ncan knockout?

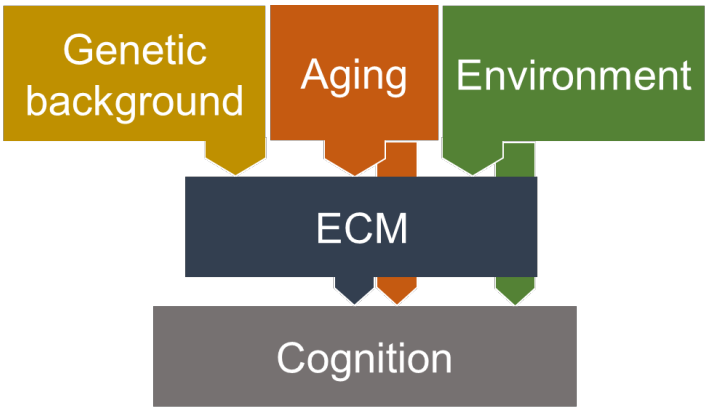


Figure 4: The hypothesis of the study summarized. Both aging and the environment can affect cognitive abilities via modulating the composition and/or integrity of the neural ECM. Genetic manipulation of ECM composition via knockout of Bcan and Ncan helps understand the relevance of both lecticans for aging-related cognitive decline.

2 Material and Methods

2.1 Generation of Bcan and Ncan double knockout mice

DKO mice were generated by heterozygous breeding of Bcan and Ncan single knockout mice. Resulting homozygous DKO and WT mice were used in homozygous breedings, to generate the mice needed in the experiments. It was not feasible to generate DKO mice via heterozygous breeding, as it produced too many mice with the wrong genotype.

Bcan knockout is described in Brakebusch et al., 2002. Here, the whole coding sequence of the Bcan gene was omitted, introducing a *lacZ* gene and neomycin expression cassette (Figure 3A). Ncan knock out was conducted as described by Zhou et al., 2001: In short, the TATA box including the whole transcription starting site and the first two exons were replaced by a cassette containing neomycin and thymidine kinase. This cassette was flanked by *loxP* sites that were used to omit the whole cassette by transient *Cre* transfection. Both knockouts were generated in 129Sv mouse line that was subsequently backcrossed with C57BL/6 for at least five generations.

As mentioned, WT and DKO mice with C57BL/6NCrl background were bred from homozygous parents or in exceptional cases with one parent being heterozygous in one gene. As breeding was not successful for the WT mice of the old group used in spatial and fear learning tasks, additional mice with similar age and same genetic background were purchased from Charles River. Breeding was conducted at the animal facilities of the Leibniz Institute for Neurobiology.

2.2 Animal rearing

Experiments were approved and authorized by the animal care committee at the Landesverwaltungsamt Sachsen-Anhalt and ran under the licenses 42502-2-1610 LIN, 42502-2-1705 DZNE and TWZ.

All experiments were conducted with male mice. Behavioral experiments in young mice were conducted at age 4-5 months (elevated plus maze and marble burying), old mice were tested at age 19-20 months (open field, novel object location recognition, water cross maze, trace fear conditioning) or at a later age, as indicated. Mice were reared in individually ventilated cages and changed every week with 3+ mice and every two weeks for cages with 1-2 mice. The light-dark cycle was inverted at least two weeks

before the start of experiments and all experiments were conducted during the dark phase. Mice were habituated to handling at least a week before the start of experiments.

The enriched environment consisted of a red plastic house with running wheel attachment, a cotton stick for nest building and in certain cases a cardboard tunnel (Figure 5). The cardboard tunnels were not included for most groups, as they elicited increased territorial aggressiveness. All cages contained cotton tissue paper for nest building.

The experimenter was blinded to the genotypes while conducting the experiments. Whenever possible, rearing type was blinded by removing the enrichment before conducting the experiment.

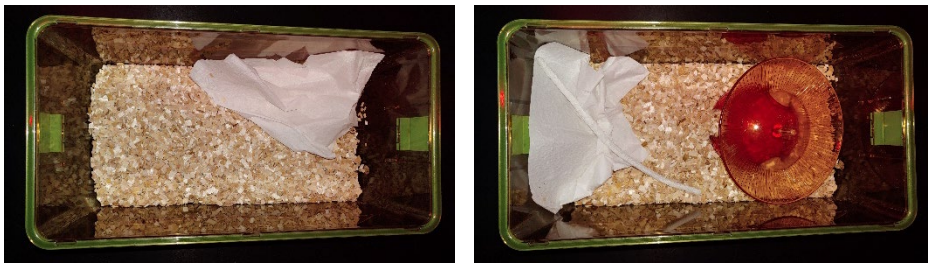


Figure 5: Rearing conditions. Left: Standard reared mice had tissue paper available. Right: Enrichment consisted of a running wheel on top of a house and a cotton stick.

2.3 SHIRPA

A shortened version of the modified SHIRPA (SmithKline, Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment), as it is used by the Taiwan mouse clinic (http://tmc.sinica.edu.tw/sop_shirpa.html) was used for a basic phenotyping. The full SHIRPA protocol was originally developed by D. C. Rogers et al., 1997. No comprehensive health phenotyping was needed here, as it had been already performed after the generation of the mouse line.

First, the mouse was transferred gently into a viewing jar (15 cm height x 11 cm diameter) on top a metal plate and observed for 5 min. Data on respiration rate, tremor, body position, spontaneous activity, defecation and urination was taken.

Next, the mouse was dropped from the metal plate ~30 cm into an arena (20 x 33.5 x 57 cm) and the transfer arousal was assessed. Inside the arena, palpebral closure, gait, pelvic elevation, tail elevation and touch escape were evaluated. To check the general hearing ability, a ~90 dB click sound was generated, using a clicker (TRIXIE

Basic Clicker) and the startle response was observed. Trunk curl and limb grasping were analyzed by holding the mouse up by the tail.
While still holding the mouse by the tail, visual placing was assessed by slowly bringing the mouse closer to a metal grid above the arena. The distance was noted when the mouse reached for the grid. For grip strength testing, the mouse was allowed to grab the metal grid with the forelimbs. The Pinna reflex was tested by stimulating the base of the pinna with a single brush fiber and the corneal reflex was tested with a piece of soft fiber. Finally, the negative geotaxis test was done by putting the mouse onto a vertically raised metal grid and turning it around 90°, so that the mouse was facing down. It was investigated if the mouse was able to turn around and climb the grid.
During the whole procedure data on fear behavior, aggression and vocalization was noted.

2.4 Elevated plus maze

The task was conducted in dim white light conditions. The light source was mounted directly above the maze to avoid shadows within the maze. The whole maze was mounted in ~50 cm height. Mice were brought to the testing room in their home cage and stayed in the home cage until the start of the experiment. Mice were put into the maze facing the closed arm opposite to the experimenter and were allowed to explore for 5 minutes. It was counted how often the mice would enter the open arm and how much time they would spend there. The mouse had to have the whole body (excluding tail) in the arm to be counted as entered. Between mice the maze was cleaned with 70% Ethanol.

2.5 Marble burying

Cages (22 x 38 x 19 cm) were filled with 5 cm bedding and 18 glass marbles (Ø 15-16 mm) evenly distributed on top of the bedding. Mice were allowed to explore the cage for 30 minutes in red light and the number of exposed marbles was counted every 5 minutes. A marble counted as buried when 2/3 of the marble was not visible.

2.6 Novel object location recognition

The open field arena used here had dimensions of 50 x 50 cm and 50 cm high walls. Mice were traced in the arena via ANY-maze Software (Stoelting Co.) and time spent

in the different parts and running speed were measured for analysis. All mice were habituated to the open field one day before the novel object/location recognition task. This habituation step consisted of a 20 min period the mice spent in the empty arena with red light. 2h later, mice spent another 20 min in the arena in dim white light conditions, with visual cues attached to the walls of the arena. This first part of the habituation was analyzed to evaluate general anxiety. The next day, mice were habituated to the objects, before conducting novel location and novel object recognition tests.

A lamp directly above the arena provided dim white light without casting shadows in the arena. Four different visual cues were hung on the sidewalls. Familiar objects consisted of 50 ml tubes, filled with toy bricks and scented with cumin. Novel objects were interlocking toy brick towers of similar height as the tubes, that were scented with cinnamon. None of the objects were climbable for the mice. The protocol consisted of three parts: Mice underwent a habituation phase of 20 minutes, the familiar objects were placed in location 1 and 2. In the novel location recognition part the object in location 2 was moved to location 3 and the mice could explore for 10 min. In the final novel object recognition part, the object in location 3 was exchanged for a novel object that the mice could again explore for 10 min. Between mice the arena and objects were cleaned with 10 % ethanol. Intertrial time was 2 hours. Interaction time and number of head pokes were recorded with ANY-maze software. Discrimination ratios of exploration time and head pokes were calculated as followed: Discrimination ratio = [time(novel) – time(familiar)] / time(total). Mice with a total object interaction time of less than 10s were excluded from the analysis.

2.7 Water cross maze

The water cross maze protocol was based on the place learning protocol in the water cross maze by Kleinknecht et al., 2012. The maze consisted of 4 arms (north = N, east = E, south = S, west = W) with a diameter of 150 cm. Visual cues were placed outside the maze. The maze was filled with 22°C (+/-1°C) water so that the transparent target platform was submerged just underneath the water surface. For the training phase the platform was placed into the western arm, for reversal learning it was placed into the eastern arm. Training and reversal learning were each performed in 5 days, with 6 trials per mouse. Mice were grouped into batches of 6-7, resulting in an intertrial time of 8-10 minutes. The mice spent the intertrial time in their cage, which was partly

warmed up by an infrared lamp. A trial started by placing the mouse in one of three starting positions, with the head facing away from the wall. The mouse was allowed to explore the maze and find the platform within 30s. After that time the mouse was led to the platform and spent there another 20s. If the mouse succeeded in finding the platform, it was allowed to remain there for 10s. Then the mouse was removed from the maze with a cooking spoon, quickly dried on tissue paper and put back into the cage.

Starting positions were changed in a semi random manner, with the start being in NSSEEN on odd and SEENNS on even days during the training and NSSWWN on odd and SWWNNS on even days during reversal. A correct trial was counted when the mouse entered the target arm and found the platform. Latency of entering the arm and finding the platform was recorded. The whole task was done in dim white light.

2.8 Trace fear conditioning

The trace fear conditioning was conducted with the TSE Fear Conditioning System (TSE Systems). The mice received an auditory cue of 2.4 kHz and a mild 2 seconds foot shock of 0.3 mA. The protocol consisted of 4 experimental days: On day 1 the mice were habituated to the test chamber for 5 minutes. On day 2 the training took place. The training started and ended with a 2 min pause and it consisted of five repetitions of the following procedure: 10 s auditory cue, 20 s trace period, 2 s foot shock, 2 min pause. On day 3, the contextual fear learning was assessed by subjecting the mice for 5 minutes to the same test chamber as during the training. On day 4 the cue learning was investigated by performing the training protocol without giving the aversive stimulus. The cue retrieval was performed in cages with bedding material. For each mouse a fresh cage was used. During the habituation, training and context retrieval, the test chambers were cleaned with 70% ethanol between mice.

For analysis relative freezing time was compared between the groups.

2.9 Attentional set shifting task (ASST)

The following protocol is modified from Heisler et al., 2015.

The test setup consisted of an opaque plastic box with dimensions of 43x22x21 cm. Inside the box a larger waiting area and two choice areas were divided. The choice areas could each be closed with doors that could be operated from outside the box. Three identical ceramic bowls were used in the task, one was filled with water and put

in the waiting area. The other two were put in the choice areas and filled with material, so that the mice had to bury to retrieve the reward. Although only one bowl contained the reward during the experiment, the unrewarded bowl was also scented with the reward. Outside those bowls a piece of filter paper was attached that was used to apply different odors during the task. The whole experiment was conducted in dim white light and the setup was cleaned with water after each round. Alcohol was not used for cleaning here, to avoid disturbing the olfaction of the mice.

The procedure consisted of 10 experimental days over a period of 12 days. From day 1-3 mice were habituated to the experimenter. On day 3, mice were weighted and starting on day 4, mice where food-restricted until the end of the experiment. The weight of mice was kept at ~85% of their baseline. On day 4, mice were also habituated to the bowls and rewards (choco rice crispies) inside their homecage. On day 5 mice were group-habituated to the test setup. For this, all mice from a cage were put into one box and allowed to dig in the bowls to retrieve rewards. Both bowls were unscented and filled with bedding material. The mice spent day 6 and 7 in their homecage.

Day 8 consisted of another round of habituation. This time, individual mice were introduced to the setup. Only one bowl contained rewards. Each mouse underwent 3x4 trials, with each round the rewards were put deeper into the bedding material within the bowl, so that the mouse learned to dig in the material.

Day 9 was the first day of testing. In the first phase of the ASST called “simple discrimination”, mice needed to learn to discriminate either odor or material to retrieve a reward. Half of the animals from each group started with either dimension. Each mouse was given 4 free trials, where both bowls could be freely searched for a reward. From trial 5 on, the door to the other bowl was closed, if the mouse started digging in one bowl. A sequence of 6 correct trials qualified a mouse for the next phase, however if the 6^{th} trial after a sequence of 5 correct trials was done wrong, the mouse was allowed one more trial for correction. If this trial was done correct, the mouse was qualified for the next phase.

On day 10, the second dimension was introduced. During this compound discrimination phase a different set of odors and materials were used. Mice that started simple discrimination with medium as relevant dimension had to find the reward, irrespective of the odor of the bowls. Only one of the two different media was rewarded. During reversal, the relevance was switched to the formerly unrewarded medium.

On day 11, mice had to find the reward after an intra-dimensional shift: a new set of odors and media were introduced and mice formerly trained to recognize the correct medium had to relearn the correct medium irrespective of the odor. Again, during reversal the relevant medium was changed to the previously unrewarded one.
On day 12, during the extra-dimensional shift phase, mice had to learn to focus on the previously irrelevant dimension to find the reward. Also, in this phase a reversal trial was conducted.

2.10 Mouse sacrificing, brain extraction and perfusion

For protein analysis via western blot, mice were shortly anesthetized with isoflurane and then killed by cervical dislocation. The brain was promptly removed. Either, the brain was directly frozen in liquid nitrogen and stored at -80°C for later dissection or the dissection was directly performed after brain removal. Dissection was performed in ice-cold homogenization buffer 1 (Table 1), containing protease inhibitor. All samples were frozen at -80°C until further use.
For perfusion of dead animals, mice were sacrificed with CO^2, then the body was pinned down and the heart was exposed by cutting away the ribcage. A 16 mm needle was inserted into the left heart chamber and phosphate buffered saline (PBS)(Table 1) flushed through the circulating system. PBS and blood exited through a cut made in the right ventricle. During PBS flush the tail tip was collected for potential genotyping. The flush continued for approximately 10 minutes until the liver and other tissue turned lighter. Then the body was flushed with 4% paraformaldehyde (Table 1) for another 10-15 min, until the body became stiff. After extraction the brain was post-fixed in 4% paraformaldehyde over night at 4°C, the paraformaldehyde was washed off and the brain was incubated for another 2 days in 1M sucrose. The final step consisted of freezing the brain in 2-methyl-butane that was cooled down using liquid nitrogen. Brains were stored at -80°C until further use.

2.11 SDS-PAGE and Western Blot

Homogenization buffer (1 ml per 100 mg tissue) was added to each sample before homogenization. Samples were then homogenized using an electric tissue grinder with 1000x rpm torque (K-Control TLC, Kaltenbach & Voigt GmbH). The samples were centrifuged at 1000x g for 10 min and the supernatant was used for further procedures.

The next steps were a protein quantification via Pierce BCA Kit (Thermo Fisher Scientific) and dilution of samples to 2 µg/µl.

20 µg protein per sample were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Before denaturation for 5 min at 95°C, 100 mM DTT and SDS sample buffer (Table 1) were added to the samples. They were then spun down in a final centrifugation step. Gels were run for 20 min at 8 mA per gel (E865, Consort), by then the proteins reached the separation gel and current was increased to 12 mA per gel. After adequate separation of proteins, the gels were examined under UV light. Proteins were then blotted onto a 45-µm pore polyvinylidene fluoride membrane (Immobilon, Millipore). First step involved membrane activation with methanol, the membrane was subsequently packed up together with the gel, filter paper and sponges into the blotting chamber that was filled with blotting buffer (Table 1). Western blots for aggrecan quantification were prepared with 2.5-10% acrylamide (Table 1) and blotted for 2 h and 15 min, all other proteins were quantified via gels with 5-20% acrylamide (Table 1) that were blotted for 1 h and 45 min. Blotting current was 200 mA. Afterwards, each membrane was imaged via UV exposure and the resulting image was used for normalization to protein quantity.

Previous to antibody incubation, blots were blocked with western blot (WB) blocking solution (Table 1) for 1 h at room temperature. Antibody incubation was performed on a shaker at 4°C over night, using the same solution for dilution of antibodies. Primary antibodies were diluted according to Table 2. On the next day, blots were washed 3x 12 min with TRIS-buffered saline with Tween-20 (TBS-T)(Table 1), then incubated with secondary antibody diluted in blocking solution and again washed 3x 12 min with TBS-T. All peroxidase-coupled secondary antibodies used for the immunoblots were purchased from Jackson Immuno Research and diluted 1:5,000-1:10,000 (Table 3). Finally, imaging was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) via an ECL Chemocam Imager (INTAS Science Imaging Instruments GmbH).

Fluorescence values were gathered with ImageJ Fiji and values were normalized to blot baseline fluorescence.

2.12 Cryosectioning

The cryostat (CM3050s, Leica) was set to -21°C object temperature and -20°C ambient temperature in the cutting chamber. Brains were equilibrated to -20°C over night. The

cerebellum and olfactory bulbs were cut away with a scalpel and the brain was attached to the object holder using Tissue-Tek O.C.T compound (Sakura Finetek). Starting from the front, tissue was trimmed away until the medial prefrontal cortex could be identified based on anatomical structures using a mouse brain atlas. The amount of trimmed tissue and every collected slice was tracked to control for the anterior-posterior location of each slice within the brain. Hippocampal brain slices were collected the same way. The slices were 40 µm thick and collected in PBS, which was later exchanged for cryoprotection solution, before storing the slices at -20°C.

2.13 Immunohistochemistry (IHC)

Slices were taken from cryoprotection solution and washed 3x 10 minutes in PBS, followed by blocking for 1 hour at room temperature. Primary antibodies were diluted in antibody solution (Table 2); incubation was performed for 48-72h at 4°C on shaker. Slices were washed 3x 10 min in PBS and secondary antibody was again diluted in antibody solution and incubation was done overnight. All secondary antibodies used for IHC were diluted 1:500 (Table 3).

On the next day the secondary antibody was washed away 3x10 min in PBS. Slices were mounted on glass slides (76 x 26 mm, Knittel glass) with mounting medium with or without Dapi (Fluoromount-G, Invitrogen) and coverslips (24 x 32 mm and 24 x 60 mm from Roth, 24 x 50 mm from epredia). Slides were kept at 4°C until analysis and for long-term storage.

2.14 Image acquisition and analysis

Microscopic images were taken via confocal imaging with Leica TCS SP8 STED 3X, using the software LAS X. Image resolution was set to 1024x1024. Additionally, images were acquired using bidirectional X scanning and a 4-line averaging. All other settings were set to default. Z-stack size was 5-6 x 6µm.

ImageJ/Fiji was used for image analysis. For cell counting, the area of interest was selected manually or by using the ROI manager and cells were counted manually with the cell counter tool.

For intensity measurements, Z-stacks were projected by maximum intensity. Hippocampal region intensity measurements were done by Shaobo Jia by manually selecting hippocampal subregions and applying the filters “de-noise” and “Gaussian

blur” with a radius of 2 pixels. The regional intensities were always normalized to the WT ST group.
PNN intensity measurements were also done from manually selected PNNs. Here, the background was subtracted by selecting three adjacent areas without PNNs, and subtracting the mean intensity of those areas from the measured intensity level of the PNNs.

2.15 High-field magnetic resonance imaging (MRI) of mice

The MRI measurements were carried out by Cornelia Hesse (DZNE).
Mice were brought to the scanning room, transferred into a box and then anesthetized with Isoflurane (1.5 % Isoflurane in Oxygen/Nitrogen). During the procedure Isoflurane was delivered according to heartbeat and breathing rate that were measured via oximeter. The scanner bed of the 9.4 tesla MRI scanner (Bruker Corporation) was heated during the whole procedure. The used software was ParaVision 6.0.1 (Bruker Corporation).
First, functional properties of the brain tissue were recorded by diffusion-weighted echo planar imaging with a repetition time of 3,200 ms and echo time of 21.5 ms in 6 directions. 25x 0.5 mm slices were taken in a field of view of 16.9 x 15 mm with 24 averages.
For anatomical scans T1 weighted fast low angle shot with repetition time of 358.52 ms and echo time of 2.911 ms was performed. 34 slices of 0.5 mm thickness were taken with 6 averages.
After approximately 50 min recording time, mice were allowed to wake up under red light and then brought back to the animal room.

2.16 Brain fractionation and Proteomics

Mice were sacrificed using CO_2 and the brain was directly removed and dissected on ice. Hippocampus and cortex (including striatum) were pooled, shock frozen in liquid nitrogen and stored at -80°C until further use.
Using a Potter S (Brain Biotech International), thawed brain tissue was homogenized in ice cooled buffer 2 (10 ml per 1 g tissue) (Table 1) with 12 strokes at 900 rpm. The resulting homogenate was centrifuged for 10 min at 1000x g, at 4°C. The pellet contained nuclei and debris. Supernatant was saved and the pellet was once again

dissolved in the same amount of homogenization buffer 2 and again centrifuged. The resulting supernatant was pooled and used for further procedures.
For separation into soluble “S2” and membrane fraction “P2”, the supernatant was centrifuged at 100,000x g at 4°C for one hour (Optima MAX-XP, Beckman Coulter). The resulting supernatant was saved as S2 fraction, whereas the pellet was dissolved in 9 M Urea at room temperature for 1h under constant rotation. Then, the dissolved pellet was again centrifuged at 100.000x g at 21°C for 30 min. This step removed insoluble debris which was discarded, while the supernatant P2 fraction was used for proteome analysis.
S2 and P2 fractions were shipped to the EMBL Proteomics Core Facility in Heidelberg and processed further for mass spectrometry.

2.17 Statistics

Statistical analysis was done with GraphPad Prism 9.0. Data was checked for normal distribution using Anderson-Darling, D’Agostino & Pearson, Shapiro-Wilk test or QQ plots. Where applicable, outlier testing was performed using ROUT method with Q=1. Group analysis was done with 2-way ANOVA or 3-way ANOVA and 3-way ANOVA with repeated measurements, unless otherwise specified. Post-hoc analysis was performed in cases of significant differences in main factors. Tukey’s multiple comparisons test was used as standard post-hoc test, Fisher’s LSD was used in one case for marble burying analysis, as stated in chapter 3.1.535. Tukey’s multiple comparisons test might be too conservative to detect differences, when the comparison is carried out between all groups even though only certain comparisons are sensible. To avoid missing important observations because of conservative testing, trends in difference are also considered in the analysis and discussion. The mean (M) and standard deviation (SD) are indicated in the text body of the results chapter. Significant differences in the figures are marked as followed, if not indicated otherwise: $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.
For survival analysis data from genotyped and weaned mice were used as extracted from the PyRAT animal facility software (Scionics) used inhouse. The dataset comprised all mice born between December 2018 and February 2023. Only “spontaneous deaths” were considered in the survival analysis, all other reasons of death were removed from the dataset by censoring at the respective time.

2.18 Solutions and buffers

Table 1: Solution and buffers

Solution	Application	Composition
Homogenization buffer 1	Brain dissection & Homogenization	0.32 M Sucrose, 5 mM HEPES, 1 tablet cOmplete EDTA-free Protease Inhibitor, 5 mM EDTA, pH 7.4
Homogenization buffer 2	Homogenization for Proteomics	50 mM PBS, pH 7.4, cOmplete protease inhibitor cocktail
4% Paraform-aldehyde	Perfusion	4% (w/v) Paraformaldehyde in PBS, pH 7.4
Antibody solution	IHC	2% (w/v) BSA, 0.1% Triton X-100 (v/v), PBS
IHC Blocking solution	IHC	5% BSA (w/v), 0.3% Triton X-100 (v/v), in PBS
Phosphate buffered saline (PBS)	IHC, perfusion	2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, 8 mM Na2HPO4, pH 7.4
Cryoprotection solution	IHC	10% PBS, 30% Ethylene Glycol, 30% Glycerin
TRIS buffered saline (TBS)	WB	50 mM Tris, 150mM NaCl, pH 7.6
TBS-T	WB	0.1% Tween-20 (v/v) in TBS
WB Blocking solution	WB	5% (w/v) non-fat dried milk in TBS-T
4x SDS sample buffer	WB	250 mM Tris, 1% SDS (w/v), 40% Glycerol, 0.02% Bromophenol blue, pH 6.8
Electrophoresis buffer	WB	25 mM Tris, 192 mM Glycin, 0.1% SDS, pH 8.3
Blotting buffer	WB	25 mM Tris, 192 mM Glycin, 0.2% SDS, 10% Methanol, pH 8.3
Separation gel (20 %, pH 8.8)	SDS-PAGE	8.25 ml 1.5 M Tris/HCl pH 8.8, 7.5 ml 87 % glycerol, 16.5 ml 40 % acrylamide, 330 µl 10 % SDS, 330 µl 0.2 M EDTA, 22 µl TEMED, 120 µl 0.5 % bromophenol blue, 75 µl 10 % APS, 1.2 % (v/v) 2,2,2-trichlorethanol (TCE)
Separation gel (5 %, pH 8.8)	SDS-PAGE	8.25 ml 1.5 M Tris/HCl pH 8.8, 17.94 ml dH2O, 1.89 ml 87% glycerol, 4.12 ml 40 % acrylamide, 330 µl 0.2 M EDTA, 330 µl 10 % SDS, 22 µl TEMED, 118 µl 10 % APS, 1.2 % (v/v) TCE
Separation gel (10 %, pH 8.8)	SDS-PAGE	8.25 ml 1.5M Tris/HCl pH 8.8, 7.5 ml 87 % glycerol, 8.25 ml 40 % acrylamide, 8.25 ml dH2O, 330 µl 10 % SDS, 330 µl 0.2 M EDTA,

		22 µl TEMED, 120 µl 0.5 % bromophenol blue, 75 µl 10 % APS, 1.2% (v/v) TCE
Separation gel (2.5 %, pH 8.8)	SDS-PAGE	8.25 ml 1.5M Tris/HCl pH 8.8, 20 ml dH2O, 1.89 ml 87% glycerol, 2.06 ml 40 % acrylamide, 330 µl 0.2 M EDTA, 330 µl 10 % SDS, 22 µl TEMED, 118 µl 10% APS, 1.2% (v/v) TCE
Stacking gel (5 %, pH 6.8)	SDS-PAGE	6 ml 0.5 M Tris/HCl pH 6.8, 7.84 ml dH2O, 5.52 ml 87 % glycerol, 3.90 ml 30 % acrylamide, 240 µl 0.2 M EDTA, 240 µl 10 % SDS, 17.2 µl TEMED, 140 µl phenol red, 137 µl 10% APS
9 M Urea	Proteomics	9 M Urea, 4% (w/v) CHAPS, 0.5% Triton X-100, 20 mM Tris, 64 mM DTT

2.19 List of primary antibodies

Table 2: Primary antibodies

Target protein	Host species	Distributor	Article number	Application/ dilution
NeuN	Guinea pig	Synaptic Systems	266004	IHC 1:400
Doublecortin	Rabbit	abcam	ab18723	IHC 1:500
HAPLN1	goat	R&D Systems	AF2608	WB 1:200
GFAP	Rabbit	Synaptic Systems	173002	IHC 1:500
Aggrecan	Rabbit	Millipore	Ab1031	WB 1:500
IL-6	Rat	Thermofisher	AMC0864	IHC 1:250
Iba-1	Guinea pig	Synaptic Systems	234004	IHC 1:500

Table 3: Secondary antibodies

Host & Target species	Conjugate	Distributor	Application/dilution
Donkey anti goat	POD	Dianova	WB 1:5000-10,000
Donkey anti rabbit	POD	Dianova	WB 1:5000-10,000
Donkey anti rabbit	488	Invitrogen	IHC 1:500
Donkey anti rabbit	Cy5	Dianova	IHC 1:500
Donkey anti guinea pig	Cy3	Dianova	IHC 1:500
Donkey anti guinea pig	405	Sigma-Aldrich	IHC 1:500
Donkey anti rat	488	Invitrogen	IHC 1:500

3 Results

3.1 Impact of Bcan/Ncan double-KO and voluntary running on survival, physical fitness and anxiety

Exercise is known to have beneficial effects during aging. It can improve general health via positive effects on the immune system, motor coordination, endurance, muscle strength and on the vascular system (Garcia-Valles et al., 2013; Golbidi & Laher, 2012; Simpson et al., 2012). It is not known yet, if Bcan and Ncan have an impact on the motor function and physiological health in aged mice. Thus, first the parameters of life expectancy, weight and motor function in aged mice of both genotypes, kept with or without lifelong exercise were explored here.

3.1.1 Survival probability over lifespan

Survival probability of all groups was analyzed via Kaplan-Meier Plot and log-rank test. First analysis was done grouping male and female mice together (Figure 6A).

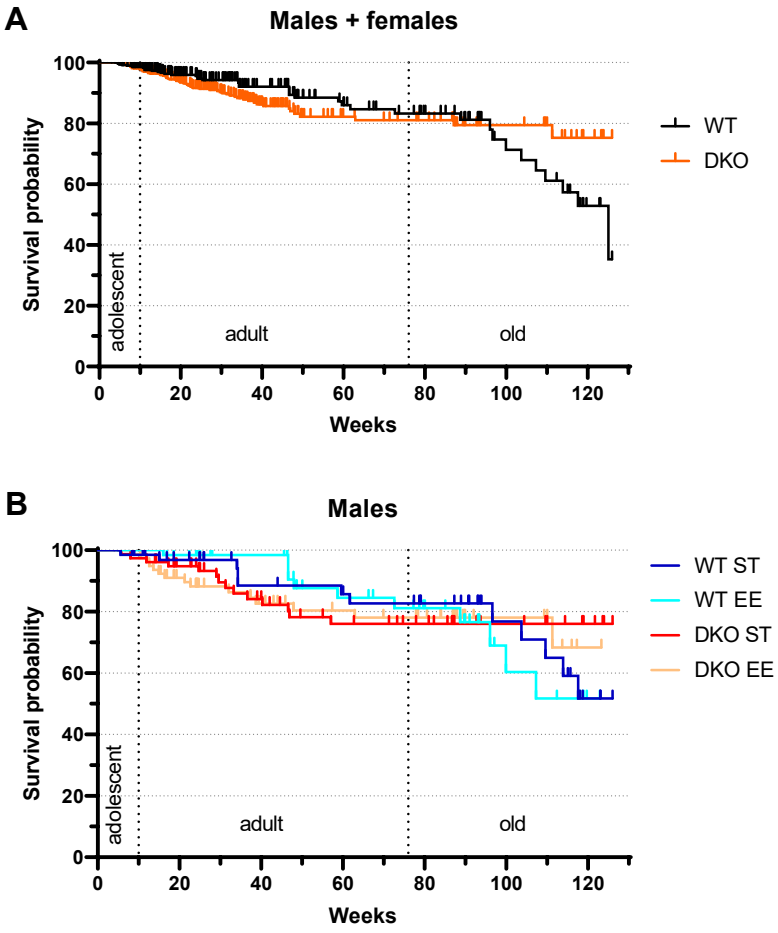


Figure 6: Survival probability of WT and DKO mice visualized in Kaplan-Meier plot. (A) DKO mice have a reduced survival probability during adulthood (X^2 (1) = 6.44, p = 0.011), whereas WT animals exhibit a trendwise reduction in survival probability during old age (X^2 (1) = 3.82, p = 0.051). **(B)** In male mice exercise did not have an effect on life expectancy.

To analyze survival probability during different stages of aging, the data was grouped into adolescent (0-9 weeks old), adult (10-75 weeks old) and old (>76 weeks old) life phases, based on life phase range categories by Flurkey et al., 2007.

Survivability in young animals is not significantly different (X^2 (1) = 3.13, p = 0.077), whereas adult DKO mice show a significantly lower survival probability during adulthood (X^2 (1) = 6.44, p = 0.011). Survival in old mice is not significantly different (X^2 (1) = 3.82, p = 0.051), but WT animals tend to show lower survival probability in that life phase. Survival probability of >96 weeks old WT mice is worse than that of DKO mice. Survival of DKO mice remains at 75% after week 111 until the end of records at week 125, whereas survivability drops to 61% at week 111 for WT mice, down to 35% at week 125.

To investigate the effect of lifelong exercise on survival, data from all male mice that were kept under exercise or standard conditions was plotted and analyzed (Figure 6B). Equally, no significant effect could be shown here for young (X^2 (3) = 1.76, p = 0.62) adult (X^2 (3) = 1.43, p = 0.7) or old mice (X^2 (3) = 6.53, p = 0.09). It appears, that lifelong exercise has no significant elongating effect on life expectancy in either genotype.

The effect of exercise on female mice was not investigated, as the data on housing type was not available for females.

3.1.2 Lifelong running can lead to gait abnormalities

A modified SHIRPA primary screen was conducted to get an overview of the general physical health of aged mice and also to assure proper sensory abilities for the following behavioral tests. An overview of all results can be found in the appendix (Appendix 1).

The startle response test verified that all groups kept a similar hearing ability in old age (Appendix 1). Furthermore, visual placing indicated no differences in sight between groups (X^2 (2) = 1.9, p = 0.39 for genotype and X^2 (2) = 1.0, p = 0.6 for rearing) (Figure 7A). Chi-square statistics also suggest no significant impact of neither genotype (X^2 (3) = 0.96, p = 0.81) nor rearing (X^2 (3) = 1.97, p = 0.58) on spontaneous activity in the viewing jar (Figure 7B). Defecation gives a hint towards the anxiety level of mice. Since defecation data failed in normality tests, statistical analysis was performed via Mann-Whitney test, using genotype and rearing as factors. Genotype did not have a

significant effect on defecation (U = 162, p = 0.577) and neither did rearing (U = 176.5, p = 0.92), thus similar anxiety levels during the test can be assumed (Figure 7C).
Gait on the other hand was found to be affected by rearing environment (X^2 (1) = 5.29, p = 0.02) (Figure 7D). Mice reared with lifelong exercise more often presented a fluid but abnormal gait. In some of those mice, stiff hindlegs could be observed. Lifelong moderate exercise is known to be able to increase the incidence of osteoarthritis in the knee of 18 months old mice as has been proven by Lapveteläinen et al., 1995. Osteoarthritis is an inflammatory disease that presents as joint stiffness.
Another motor function that was assessed within the primary screening is grip strength (Figure 7E). There is no effect on grip strength from genotype (X^2 (2) = 4.13, p = 0.13) nor rearing type (X^2 (2) = 2.35, p = 0.31), although more than 60% of DKO mice from either rearing group show increased grip strength, as compared to only 40% of WT mice. Grip strength was found to be significantly increased in Bcan knockout mice (Brakebusch et al., 2002), so the mild increase found in DKOs likely stems from lack of Bcan. One reason why no significant difference was found in DKOs could be rather methodological: While Brakebusch et al., 2002 used a high precision force sensor, observational assessment was used in this study, which tends to be less accurate.

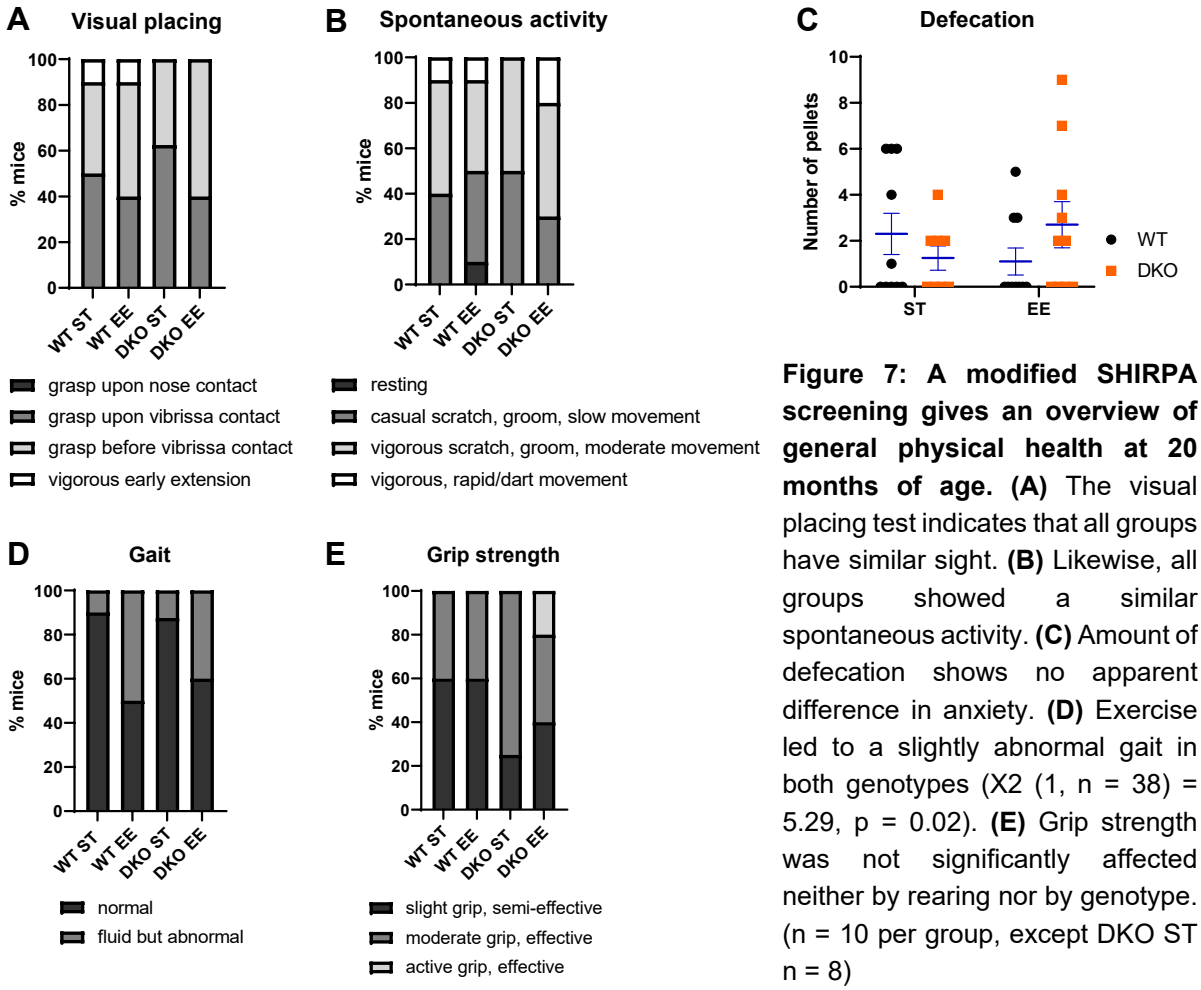


Figure 7: A modified SHIRPA screening gives an overview of general physical health at 20 months of age. (A) The visual placing test indicates that all groups have similar sight. **(B)** Likewise, all groups showed a similar spontaneous activity. **(C)** Amount of defecation shows no apparent difference in anxiety. **(D)** Exercise led to a slightly abnormal gait in both genotypes (X2 (1, n = 38) = 5.29, p = 0.02). **(E)** Grip strength was not significantly affected neither by rearing nor by genotype. (n = 10 per group, except DKO ST n = 8)

3.1.3 Lifelong exercise differently affects running speed in DKO and WT mice

Running and swimming speed were further analyzed to assess the physical abilities of aged mice. It was important to ensure that the abnormalities found in the gait analysis would not negatively affect performance in spatial learning tasks that demand a higher level of physical abilities, such as the water maze.

Running speed was assessed during the red light phase of the open field task. There was no significant effect from rearing (F (1, 35) = 0.3854, p = 0.5388), which would correspond to the effect in the gait analysis (Figure 8A). However, genotype significantly affected running speed (F (1, 35) = 5.878, p = 0.0206). There was also an interaction effect of genotype and rearing (F (1, 35) = 5.411, p= 0.0259), indicating that DKO mice tend to have increased running speed after lifelong exercise, while WT animals tend to have attenuated running speed in the open field arena. Tukey's post hoc test also brings up a significant difference between the exercised DKO (M = 0.06, SD = 0.005 m/s) and WT groups (M = 0.041 SD = 0.02 m/s, p = 0.011), underpinning

further the supposition that both genotypes respond differently to lifelong exercise. Running speed data from the white light phase shows similar differences (Appendix 2).

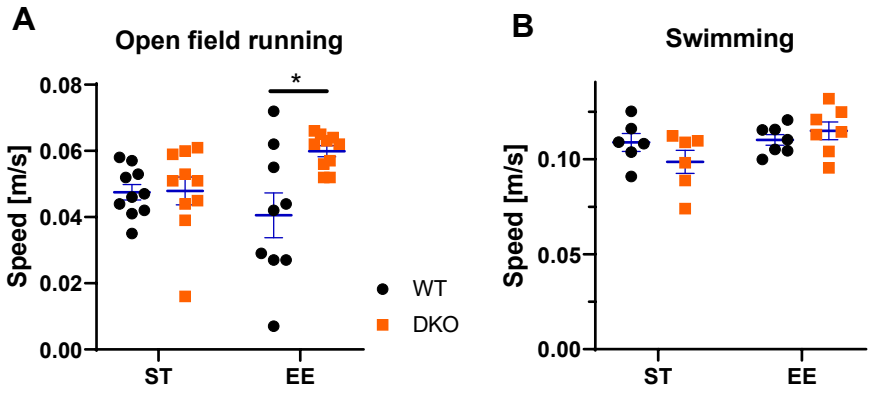


Figure 8: Running and swimming speed data shed light on physical abilities. **(A)** DKO move at higher running speed in the open field arena, when reared with running wheels, as compared to exercised WT mice ($p = 0.011$)($n = 10$ mice per group except WT EE $n = 9$). **(B)** There are no significant group differences in swimming speed. ($n = 6$ in ST groups, $n = 7$ in EE groups).

Swimming speed was similar in all groups ($F(1, 22) = 3.65$, $p = 0.0694$ for rearing and $F(1, 22) = 0.35$, $p = 0.56$ for genotype) (Figure 8B). Although rearing tends to affect swimming speed, indicating that both genotypes benefit from lifelong exercise by showing higher swimming speed.

Altogether, the abnormal gait observed in the primary screening does not interfere with physical abilities that are needed for spatial learning tasks.

3.1.4 Lifelong exercise facilitates body weight maintenance during aging

Voluntary exercise is reported to have a clear weight-reducing effect in young and old mice, especially in models of obesity (McMullan et al., 2016; Mercken et al., 2012). Increase in body weight and body fat percentage are hallmarks of aging in mice (Binyamin et al., 2020; McMullan et al., 2016), that can be attenuated by exercise. Here, it was observed that lifelong voluntary exercise helps aging mice to maintain their body weight (Figure 9). Because of randomly missing weight data from some mice, statistical analysis was done using a mixed-effects model (via restricted maximum likelihood fitting), with Geisser-Greenhouse correction.

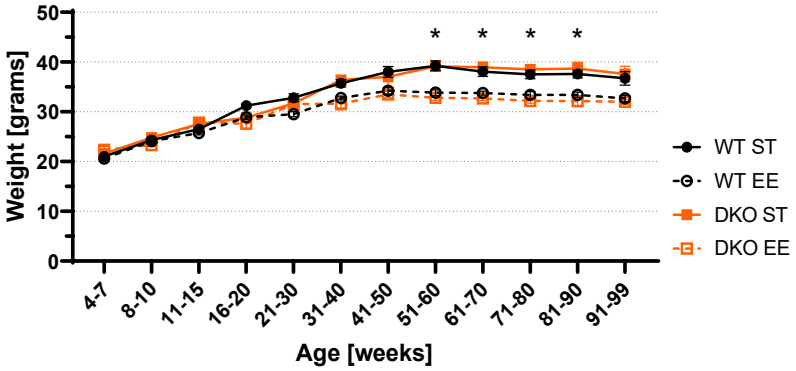


Figure 9: Development of body weight in WT and DKO mice over the lifespan. Postnatal weeks were binned as indicated. Exercise rearing had a significant effect on weight development ($F(11, 873) = 14.93$, $p < 0.0001$), suggesting consistent use of running wheels. Asterisks (*) mark time points with a significant difference in factor rearing appearing in both genotypes ($p<0.01$). The analysis also shows a significant effect of the factor genotype ($F(11, 873) = 2.66$, $p = 0.002$).

Rearing conditions had a significant effect on weight development during aging ($F(11, 873) = 14.93$, $p < 0.0001$). Both standard reared WT and DKO mice showed a continuous significantly higher weight from weeks 51-60 onward ($p < 0.0001$ in weeks 51-60 for both genotypes). Furthermore, genotypes significantly differed in their body weight development over time ($F(11, 873) = 2.66$, $p = 0.002$), but there was no interaction between genotypes and rearing conditions ($F(1, 140) = 1.193$, $p = 0.28$). This is suggesting, that in both genotypes exercise has a similar effect on body weight, while both genotypes have a different weight development, independent from exercise. Although the amount of wheel running was not tracked in this study, the collected body weight data suggests that both genotypes used the running wheel consistently.

3.1.5 Exercise decreases anxiety in old mice

Exercise was shown to exert anxiolytic effects in various anxiety tests, when applied short term (Kim & Han, 2016; Salam et al., 2009) or for longer periods of time in different age groups (Morgan et al., 2018; Zielinski et al., 2013). A similar effect was suspected here for whole life voluntary exercise.

A preliminary open field test revealed, that time spent in the center area is significantly different between genotypes ($F(1, 33) = 8.322$, $p = 0.0068$) (Figure 10B). Furthermore, center time was affected by age x genotype interaction ($F(1, 33) = 10.33$, $p = 0.0029$). The post hoc Tukey's multiple comparisons test reveals a striking difference in anxiety levels between young (4-5 months old) exercised WT ($M = 29.95\%$, $SD = 8.72$) and

DKO mice (M = 11.83%, SD = 5.58, p = 0.0029). When this test was repeated in a larger group of old animals (18-19 months old) as part of habituation to the open field, data showed a significant effect from the rearing environment (F (1, 35) = 5.042, p = 0.0312) (Figure 10C). Data on time spend in the open field rim and corner can be found in the supplements (Appendix 3).

Based on these findings and considering, that a study by Miró et al., 2012 found a manic phenotype in Ncan knockout mice, young (4-5 months old) DKO mice were tested for anxiety and compulsivity in the elevated plus maze and marble burying test. The percentage of time mice spent in the open arm of the elevated plus maze was calculated. Due to right skewness, data on open arm time was transformed (ln(y+1)) before analysis with 2-way ANOVA (Figure 10E).

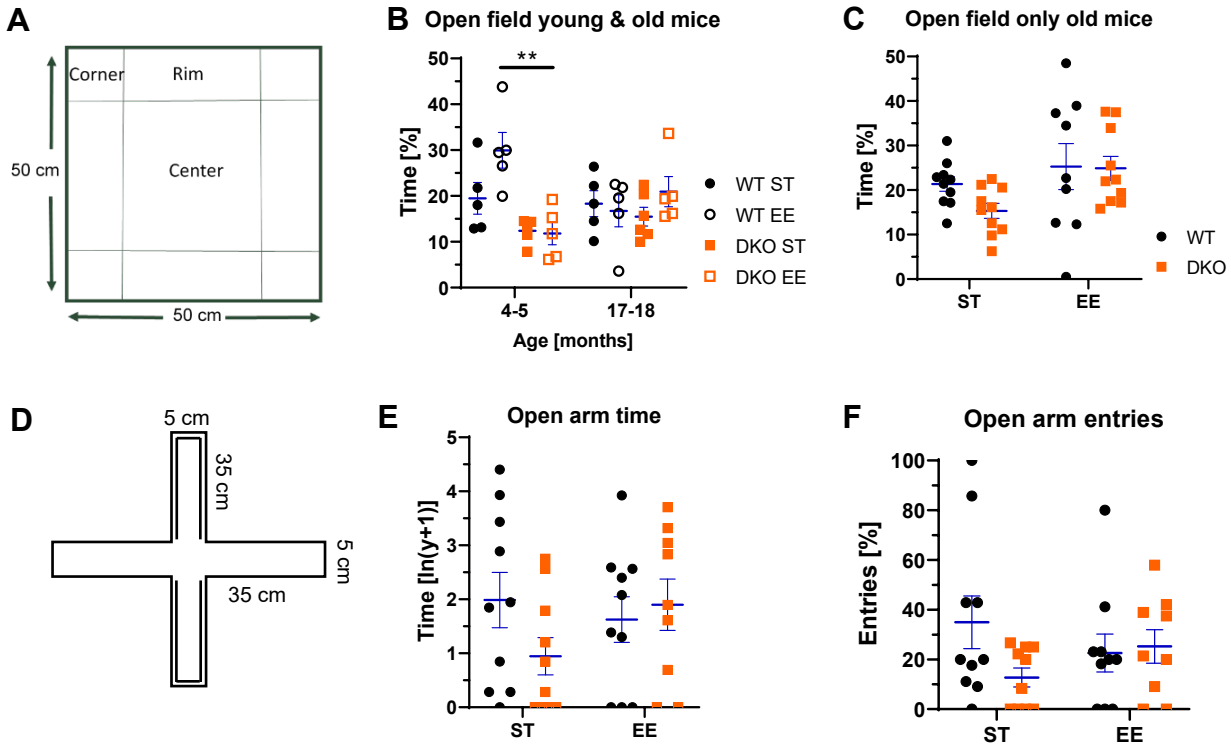


Figure 10: Anxiety tests in open field (A-C) and elevated plus maze (D-F) shows no clear differences. **(A)** Dimensions and setup of the open field arena **(B)** Open field data from preliminary testing shows, that the anxiolytic effect of exercise is not present in young (4-5 months old) DKO mice, while there is no difference in old (17-18 months old) DKO animals (n = 5 in all groups, except old DKO ST n = 6). **(C)** Larger group size of old mice (18-19 months old) shows an anxiolytic effect of exercise in open field (F (1, 35) = 5.042, p = 0.0312, n = 10 in all groups, except WT EE n = 9). **(D)** Dimensions of the elevated plus maze.**(E)** Anxiety testing of young (4-5 months old) mice in elevated plus maze shows no group differences in open arm time and **(F)** the percentage of open arm entries. (n = 10 in all groups).

No significant differences were found between groups in the elevated plus maze. Neither genotype nor rearing condition had an impact on the time mice spent in the open arm and on open arm entries (Figure 10F).

The marble burying task can be interpreted as a measure of compulsive behavior. In this task, there is a trend for interaction of genotype and rearing condition ($F(1, 35) = 3.919$, $p = 0.0557$). Young (4-5 months old) exercised WT mice tend to bury more marbles than standard reared, while this effect does not appear in DKO animals (Figure 11). While there is a significant difference after 10 minutes between the WT standard reared ($M = 5.9$ buried marbles, $SD = 1.472$) and WT exercised group ($M = 10.9$ buried marbles, $SD = 3.48$, $p = 0.0147$) when using uncorrected Fisher's LSD post-hoc analysis in the same way as Miro et al., the difference vanishes when alpha error correction is implemented via Tukey's multiple comparisons test ($p = 0.5363$). This difference is not only observable at the 10 minute time point, but it also appears at 15 minutes of the test period, when using Fisher's LSD ($p = 0.0382$). Thus, there is at least a trend for WT mice to show more marble burying when exercised, that is not present in DKO.

Together with the results from open field and elevated plus maze, the data collected hint towards a special role of Bcan and Ncan in anxiety and compulsivity and their modulation during different age phases.

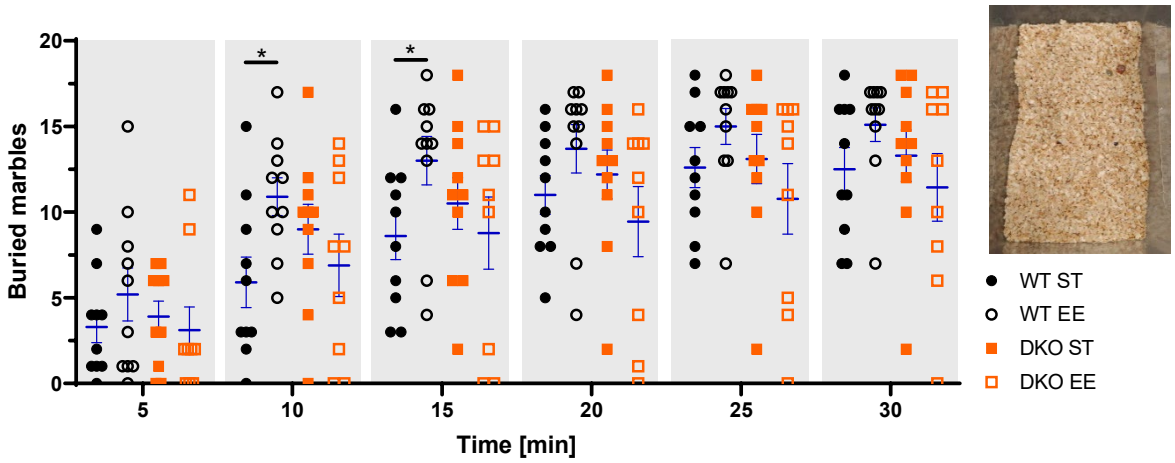


Figure 11: Compulsivity of WT mice tends to be affected by exercise. In young (4-5 months old) mice there is a trend for interaction of genotype and rearing ($F(1, 35) = 3.919$, $p = 0.0557$) and exercised WT mice bury significantly more marbles than standard reared WT mice at 10 and 15 minutes (left)(via Fisher's LSD)($n = 10$ in all groups, except DKO EE $n = 9$). Example image of buried marbles after 30 minutes (right).

3.2 Brain anatomy and tissue diffusivity are affected by ECM composition and environmental enrichment in aged mice

Aging leads to a change in brain structure (Schippling et al., 2017; Taylor et al., 2020) and changes diffusion parameters in the brain parenchyma (Damoiseaux, 2017; Yassa et al., 2010). Structural changes can be attenuated by enriched rearing (Yang et al., 2012). The ECM composition is assumed to be one of the factors to control brain diffusion properties (Cicanic et al., 2018; Syková et al., 2005).

In cooperation with the Angenstein lab at the DZNE Magdeburg, brain structure and diffusion parameters were therefore measured in high-field MRI in 20-21 months old WT and DKO mice reared with or without voluntary running.

3.2.1 Hippocampal Apparent Diffusion Coefficient (ADC) slightly affected by Bcan/Ncan deficiency

While there were no effects from genotype or rearing on the ADC in the frontal cortex (Figure 12A), a main effect of genotype can be observed for hippocampal measurements ($F (1, 28) = 4.438$, $p= 0.0442$). Although with rather low significance, DKO mice show a higher ADC in the hippocampus, independent from rearing condition (Figure 12B). In the cerebellum on the other hand, there is a trend for exercise having an impact on brain tissue diffusion ($F (1, 27) = 3.81$, $p = 0.0613$), this specifically comes to effect in a higher diffusion in the exercised DKO group ($M = 1013.99$ $\mu m^2/s$, $SD = 200.9$) compared to the exercised WT group ($M = 831.076$ $\mu m^2/s$, $SD = 105.9$, $p = 0.0689$)(Figure 12C). However, a single exercised DKO mouse, having an unusual high ADC of 1470.16 $\mu m^2/s$, likely carries this effect. No differences could be found in the ventricles, which were measured as reference (Figure 12D).

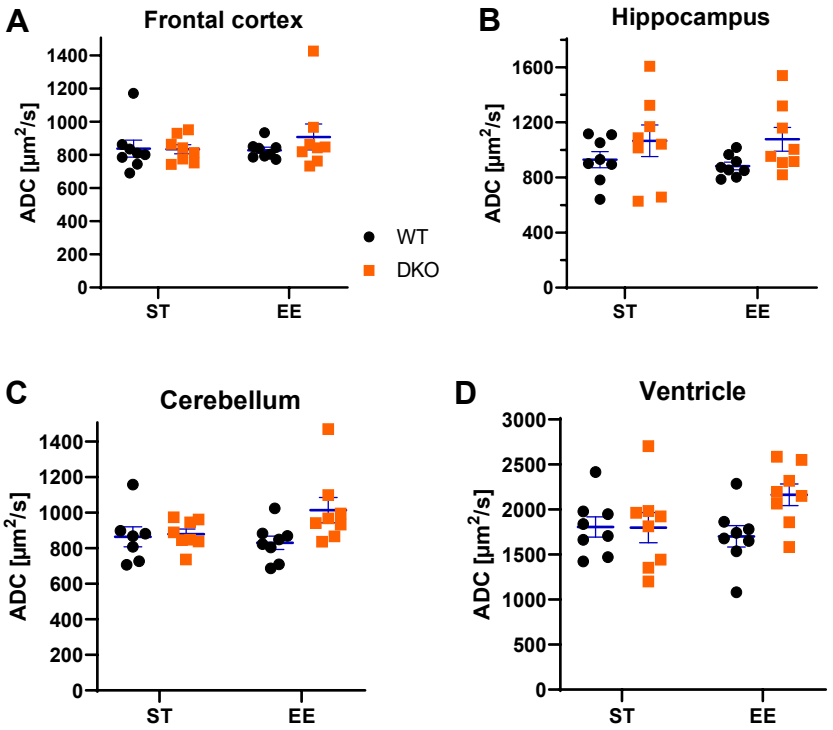


Figure 12: Higher ADC in the hippocampus of old DKO mice. **(A)** While there is no group difference in ADC in frontal cortex of 20-21 months old mice, **(B)** tissue diffusivity is higher in the hippocampus of aged DKO as compared to WT mice ($F(1, 28) = 4.438$, $p = 0.0442$). **(C)** In the cerebellum the DKO tends to increase diffusivity ($F(1, 27) = 3.81$, $p = 0.0613$), with a group effect in exercised DKO mice compared to WT ($p = 0.0689$). **(D)** No significant differences were found in the ventricle. ($n = 8$ in all groups).

3.2.2 Lack of Bcan and Ncan correlates with significantly reduced brain volume

Further, there were clear structural differences found in the brains of DKO, as compared to WT animals. The whole brain volumes differed between young 4-5 months old and aged 20-21 months old mice ($F(1, 58) = 36.63$, $p < 0.0001$)(Figure 13A), as there was an increase of volume during aging in WT and DKO mice from standard rearing ($p = 0.0371$ for WT, $p = 0.0202$ for DKO) as well as for exercised DKO mice ($p < 0.0001$). Furthermore, whole brain volume was generally lower in DKO mice, compared to WT animals. This difference was particularly strong in young mice from both standard ($p = 0.0463$) and exercise ($p = 0.0008$) rearing. The interaction of factors genotype and aging ($F(1, 58) = 14.13$, $p = 0.0004$) was reflected in the reduced volume difference between DKO and WT mice in the old cohort. There was only a trend for a difference in whole brain volume between old standard reared WT and DKO mice ($p = 0.0501$). While exercise had no effect on brain volume in young DKO mice, it lead to an alignment of volume data with WT mice in aged animals.

To get a more detailed impression, specific brain regions of interest were analyzed for structural differences. In cortical volumes DKO mice showed a pronounced difference (Figure 13B), compared to WT ($F (1, 62) = 22.72$, $p < 0.0001$). Especially young DKO mice showed a significantly lower cortical volume in comparison to WT ($p = 0.0463$ for standard, $p = 0.0008$ for exercise reared mice). Similar to whole brain volume, aging lead to an alignment of cortical volumes, as seen by the interaction of factors genotype and age ($F (1, 62) = 8.468$, $p = 0.005$). Old exercised DKO mice had a similar cortical volume as old exercised WT ($M = 117,46$ mm^3, $SD = 5.49$ in WT, $M = 119.75$ mm^3, $SD = 5.86$ in DKO). Cortical volume tended to be lower in old DKO mice from standard rearing, compared to exercised old DKO mice ($p = 0.0692$).

Hippocampal volume was generally affected by aging ($F (1, 58) = 7.15$, $p = 0.0097$) (Figure 13C). Exercised DKO mice showed an increase in hippocampal volume during aging ($p = 0.0154$). There was a stronger effect of genotype on hippocampal volume ($F (1, 58) = 15.51$, $p = 0.0002$). This could be seen particularly in the significant difference in volume between young exercised WT and DKO mice ($p = 0.0033$). This difference between the genotypes was lost in the aged cohort.

Overall, brain volume is clearly affected by lack of Bcan and Ncan, as seen in whole brain volume and specifically in cortex and hippocampus. Interestingly, DKO mice display lower volumes especially at a young age and exercise seems to rescue this lack of brain volume in DKO mice during aging.

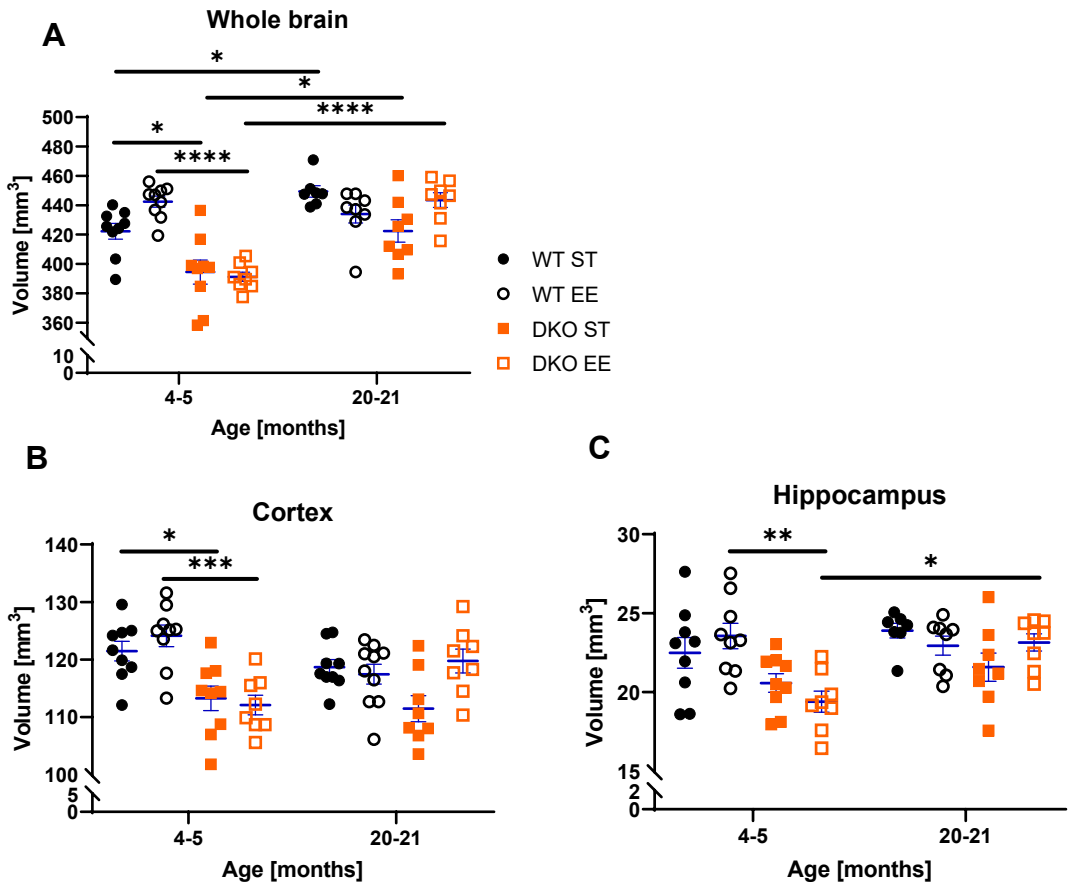


Figure 13: Brain volume differences in young (4-5 months old) and old (20-21 months old) WT and DKO mice. (A) Whole brain volume was significantly affected not only by factors age ($F(1, 58) = 36.63$, $p < 0.0001$) and genotype ($F(1, 58) = 34.68$, $p < 0.0001$), but also by an interaction of genotype and age ($F(1, 58) = 14.13$, $p = 0.0004$). Mice from standard rearing show brain volume increase during aging ($p = 0.0371$ for WT, $p = 0.0202$ for DKO), while only exercised DKO also show an increase ($p < 0.0001$). **(B)** Specifically, the cortical volume is affected by lack of Bcan and Ncan. Here, the genotype ($F(1, 62) = 22.72$, $p < 0.0001$) and the interaction of genotype and age significantly affected cortical volume ($F(1, 62) = 8.468$, $p = 0.005$). Young DKO mice from both rearing conditions have a lower cortical volume than WT from the respective rearing group ($p = 0.0463$ for standard, $p = 0.0008$ for exercise rearing). **(C)** Hippocampal volume is not affected by interaction of factors genotype and rearing, but still both age ($F(1, 58) = 7.15$, $p = 0.0097$) and genotype ($F(1, 58) = 15.51$, $p = 0.0002$) affect hippocampal volume. (n = 9 in young groups, except young DKO EE n = 8; n = 8 in old groups).

3.3 Hippocampal anatomy and cell populations

MRI data disclosed a difference in total and regional brain volumes between old WT and DKO mice. To get a more detailed insight into the brain architecture in Bcan and Ncan DKO mice, coronal brain slices at the level of dorsal hippocampus (at around Bregma -2 mm) were stained with the neuronal marker NeuN (Figure 14). This overview reveals a structural discrepancy in the hippocampal CA1 region that was subsequently analyzed in detail.

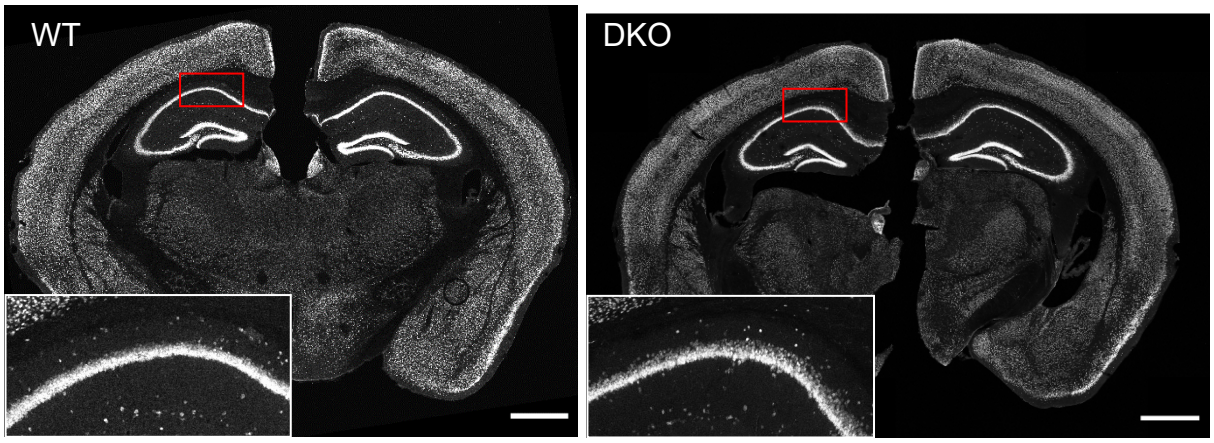


Figure 14: Coronal slices of WT and DKO mouse brains stained with NeuN. Close up of hippocampal CA1 region shows a dysplasia in the pyramidal cell layer. Scale bar: 1 mm.

3.3.1 Dysplasia in hippocampal CA1 region

The pyramidal cell layer of the CA1 region of DKO mice shows a strikingly abnormal formation that presents as diffuse layer (Figure 15A) along the entire dorsoventral axis of the hippocampus (Figure 15B). Pyramidal cell layer width was hence quantified in the dorsal hippocampus (Figure 15C).

The difference in width was only affected by genotype ($F(1, 14) = 14.93$, $p = 0.0017$). Especially within the group of exercised mice, the DKO mice show a remarkably higher CA1 width, compared to WT mice ($M = 27{,}364.01\ \mu m^2$, $SD = 4{,}039.94$ in DKO EE group, $M = 20{,}220.94\ \mu m^2$, $SD = 3{,}079.92$ in WT EE group, $p = 0.0283$). Although not significant, there is a trend towards a difference between WT and DKO mice from standard rearing ($M = 19{,}078.22\ \mu m^2$, $SD = 3771.27$ in WT mice, $M = 24{,}080.6\ \mu m^2$, $SD = 1816.27$ in DKO mice, $p = 0.1575$). Unlike whole brain volume, this dysplasia in the CA1 cannot be rescued by lifelong voluntary running. While NeuN staining shows a clear difference in cell layer structure, staining of astrocytes with anti-glial fibrillary acidic protein (GFAP) antibody does not show a difference (Figure 15A). Thus, neurons seem to be the affected cell type here. Furthermore, the dysplasia is distinguished only by abnormal organization of the pyramidal cell layer, as the number of CA1 neurons is unaltered between genotypes (Figure 15D).

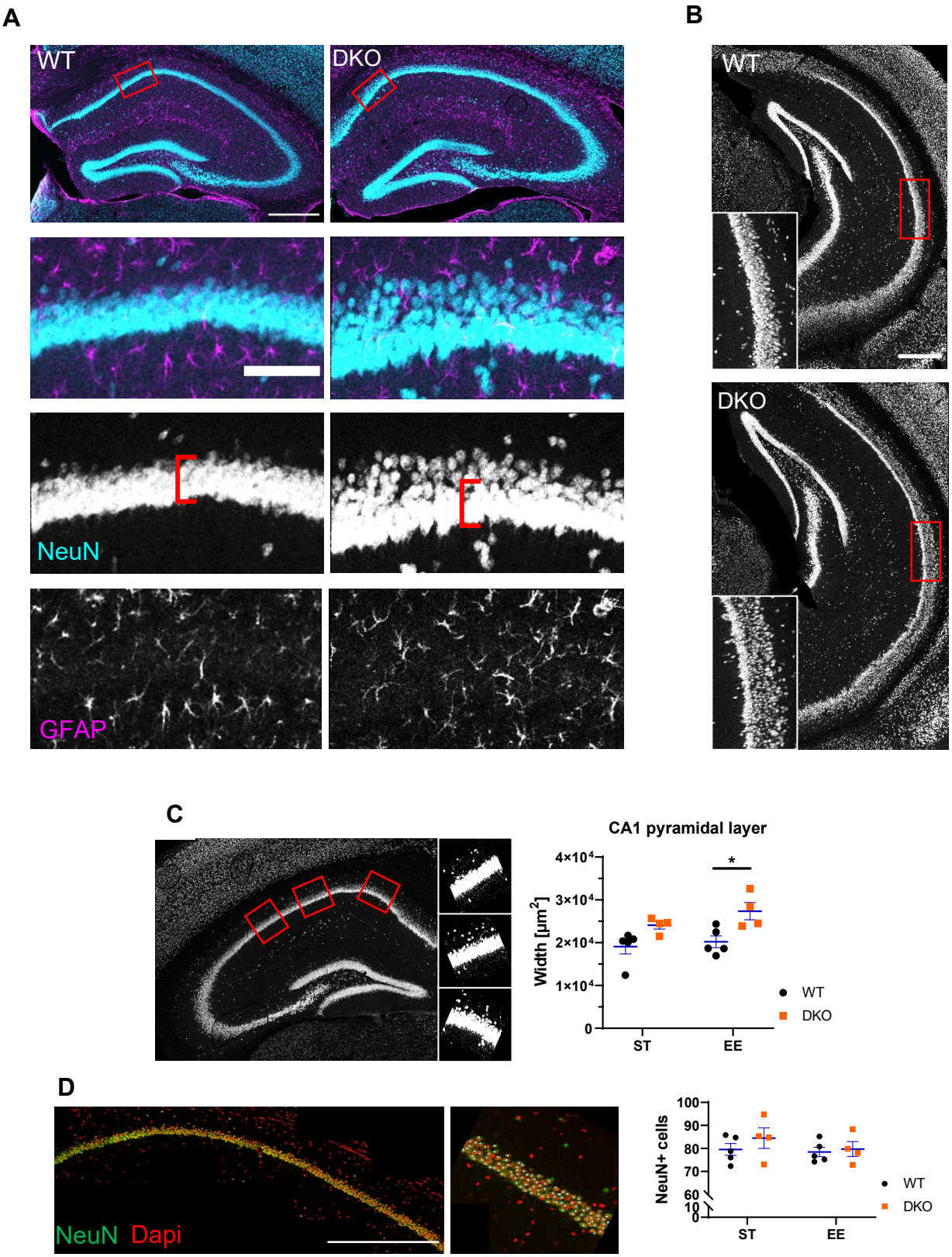


Figure 15: Image annotation on page 44.

Figure 15: DKO mice have a dysplasia in the hippocampal CA1 region. (A) Comparison of hippocampal CA1 layer from 20-21 months old WT and DKO mice showed a clear dysplasia in NeuN staining, that did not manifest in astrocyte distribution (scale bar: 500 µm in overview, 100 µm in close-up). **(B)** The dysplasia extended also into the ventral hippocampus of DKO mice (NeuN staining, scale bar: 500 µm). **(C)** The width of CA1 pyramidal cell layer was quantified in 3x 300x300 µm areas per hippocampus. DKO mice show a significantly broader CA1 layer ($F(1, 14) = 14.93$, $p = 0.0017$), independent of rearing condition. **(D)** No differences could be found in the number of NeuN-positive cells (3x 300x300 µm areas taken from one focal plane. NeuN and DAPI staining were used to identify neurons for counting (cyan dots). Scale bar = 500 µm). (n = 5 mice in WT groups, n = 4 in DKO groups; one slice with 2 hippocampi per mouse were analyzed).

3.3.2 No differences in hippocampal neurogenesis

The hippocampal dentate gyrus is among the few brain areas where adult neurogenesis takes place. Furthermore, the presence of newly generated neurons was observed in the CA1 as well. Those neurons originate from the subventricular zone and appear after ischemic stroke (Bendel et al., 2005), but generally not under normal physiological conditions. Additionally, exercise plays a well-established role in hippocampal adult neurogenesis in the dentate gyrus (Connolly et al., 2022; Mustroph et al., 2012; Van der Borght et al., 2007).

Hence, to explore the possibility of aberrant neurogenesis in the CA1 region of DKO mice and the impact of exercise on dentate gyrus neurogenesis, immature neurons were quantified in WT and DKO animals from both rearing conditions using doublecortin (DCX) staining (Figure 16). The CA1 region of either genotype was devoid of immature neurons, thus analysis was limited to DCX+ cell populations of the dentate gyrus. No significant effect from exercise on the amount of DCX+ cells in the dentate gyrus could be found in any of the groups. Only a subtle tendency for interaction between genotype and rearing could be found via 2-way ANOVA ($F(1, 18) = 1.938$, $p = 0.181$). Possibly, the high variation among the exercised WT mice hampered a conclusive statistical analysis.

Thus, neither 20-21 months old WT nor DKO mice showed a clear effect of exercise on the number of immature neurons in dentate gyrus. While this is not consistent with results from literature, there are other potential mechanisms underlying exercise-induced improvement of spatial learning left to be explored here.

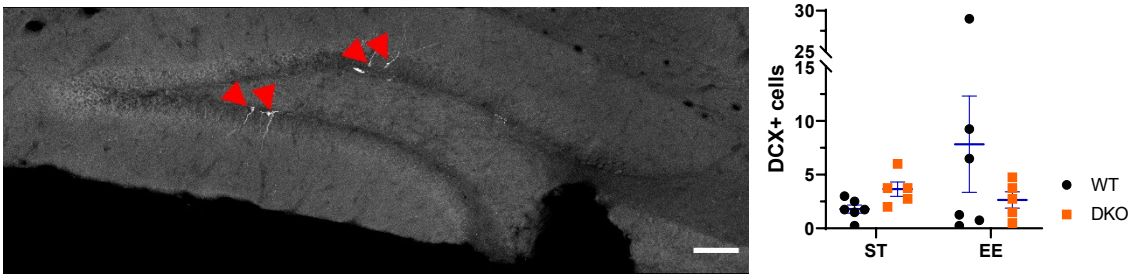


Figure 16: No differences in neurogenesis. Doublecortin positive cells (DCX+)(red arrows) were counted in the dentate gyrus of mice (scale bar: 100 µm). No differences could be found between groups, lifelong exercise did not affect neurogenesis in 20-21 months old WT or DKO. (n = 6 in WT groups, n = 5 in DKO groups; 2 slices with 3-4 hippocampi per mouse were analyzed).

3.3.3 Reduced inflammation in the hippocampus of old DKO mice

Uprise in inflammation is a main feature of aging and is associated with lower brain volumes (Marsland et al., 2015). Additionally, exercise affects inflammation and thus can alleviate detrimental effects of inflammation (Braskie et al., 2014). To explore this relationship, markers of inflammation were investigated in the brains of 18-21 months old WT and DKO mice.

Hippocampal astrogliosis is part of physiological aging (Rodríguez et al., 2014). A significantly higher level of astrocytic marker GFAP in the hippocampus confirmed aggravated gliosis in aged mice ($F(1, 65) = 47.07$, $p < 0.0001$) (Figure 17). However, there are no effects from genotype nor rearing condition.

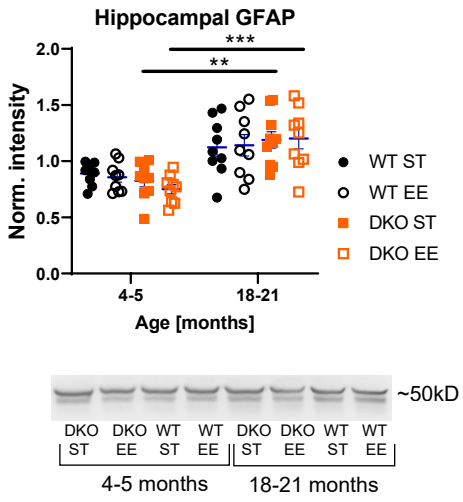


Figure 17: Aging is associated with astrogliosis. Immunoblot data confirms increased levels of GFAP in the hippocampus of aged (18-21 months old) mice ($F(1, 65) = 47.07$, $p < 0.0001$)(top). No effects from genotype and rearing condition were observed. DKO show a significant increase of GFAP during aging ($p = 0.0071$ for standard housed, $p = 0.0005$ for DKO from exercised housing). Anti-GFAP example blot (bottom). (n = 10 in all groups, except old DKO ST n = 11).

The age related increase is specifically strong in DKO mice, as those groups showed a significant increase when reared in standard cages (M = 0.826 A.U., SD = 0.163 in young, M = 1.189 A.U., SD = 0.229 in old mice, p = 0.0071) or with a running wheel (M = 0.751 A.U., SD = 0.124 in young, M = 1.201 A.U., SD = 0.275 in old mice, p = 0.0005). Individual layers and regions of the hippocampus were examined for differences in inflammation markers via immunohistochemical staining of interleukin-6 (IL-6) and microglia marker Iba-1 in 20-21 months old animals (Figure 18). This data was kindly provided by Shaobo Jia (DZNE, Magdeburg).

The analysis revealed that IL-6 and Iba-1 were differently expressed in some hippocampal layers of the DKO mice, while GFAP expression showed no significant difference. In Figure 18 fluorescence intensity of each group was normalized to WT ST and significant differences ($p < 0.05$) were identified via ANOVA and Dunnett's multiple comparisons test.

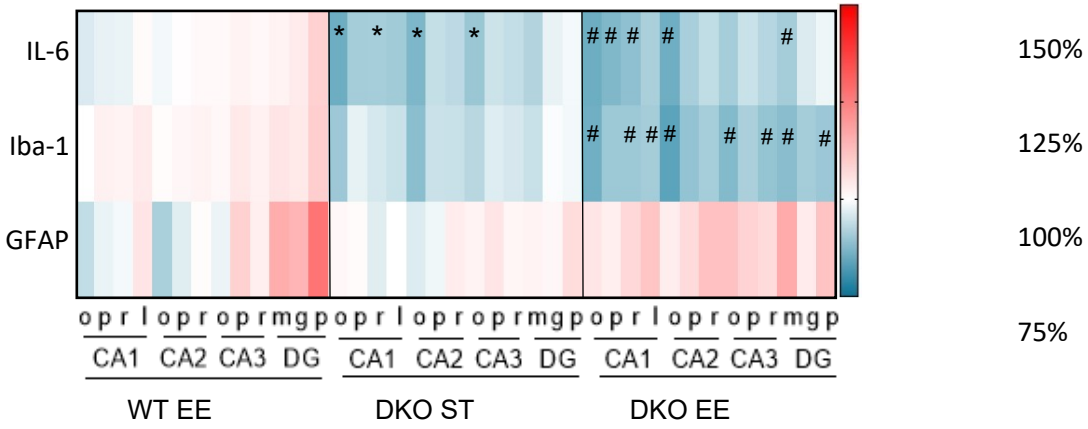


Figure 18: Down-regulation of interleukin-6 and Iba-1 in hippocampal layers of DKO mice. Noticeably, 20-21 months old DKO mice show a reduction of IL-6 and Iba-1 fluorescence intensity, compared to WT mice. No significant differences were found in GFAP fluorescence intensity. Intensity of immunohistochemical staining was measured in different sublayers (o = stratum oriens, p = s. pyramidale, r = s. radiatum, l = s. lacunosum, m = s. moleculare, g = s. granulosum, p = polymorhic layer) of hippocampal regions and normalized to WT ST mice. Asterisks indicate significant differences between WT ST and DKO ST mice. Number signs show significant differences between WT EE and DKO EE mice. Immunostaining was prepared in collaboration with and data was kindly provided by Shaobo Jia. (n = 5 in WT groups, n = 4 in DKO groups; 2 slices with 3-4 hippocampi per mouse were analyzed).

Especially DKO ST mice show a downregulation of IL-6 in multiple layers (stratum oriens, s. radiatum of CA1, s. oriens of CA2 and s. oriens of CA3), compared to WT ST mice. While not significant, Iba-1 also shows a general downregulation in the hippocampus of DKO ST mice. Both IL-6 and Iba-1 are downregulated in multiple

layers of exercised DKO mice, compared to exercised WT. Taken together, the observed results indicate, that aged DKO mice show a lower inflammatory load in the hippocampus. As already shown in western blots (Figure 17), the age-related increase of GFAP on the other hand remains unaffected by Bcan and Ncan knockout.

3.4 ECM composition and PNN characteristics in hippocampus and medial prefrontal cortex (mPFC)

As mentioned previously, Ncan and Bcan are indispensable for correct brain anatomy. But missing ECM components not only lead to abnormal brain structure on a macroscopic level, also neurite outgrowth (Letourneau et al., 1992), synaptic structure (Geissler et al., 2013) and PNN formation (Gottschling et al., 2019) can be affected. Lack of Bcan was found to lead to an upregulation of Ncan in young mice that is accompanied by reduced PNN staining (Brakebusch et al., 2002).
Therefore, the ECM composition was subsequently investigated in the hippocampus and compared among groups.

3.4.1 Exercise slightly increases PNN fluorescence in mPFC

PNNs are thought to play a crucial role in maintaining inhibitory and excitatory balance in neuronal network oscillations (Wingert & Sorg, 2021). This balance is important for proper learning functions.
Here, the PNN numbers in the CA1 were neither affected by DKO of Bcan and Ncan, nor by lifelong exercise in 20-21 months old mice (Figure 19).

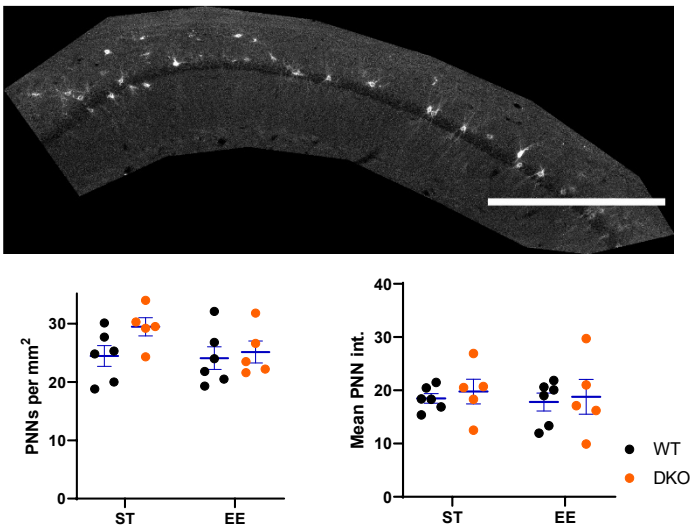


Figure 19: Similar number of PNNs in the CA1 region. WFA+ PNNs were quantified in two adjacent brain slices per mouse. Neither genotype nor rearing condition significantly affected the amount of PNNs in the CA1. (n = 5 in WT groups, 4 in DKO groups; 2 slices with 3-4 hippocampi per mouse were analyzed; scale bar: 500 µm).

Obviously, the number of PNNs does not give much information about effects on ECM composition. Thus, the anatomical analysis was complemented by semiquantitative analysis of protein expression, as presented in the following section.

The whole medial temporal lobe, including the hippocampus, is heavily involved in learning and memory, especially in episodic memory formation (Burgess et al., 2002; Squire & Zola-Morgan, 1991). While the mPFC also is involved in memory functions, it is furthermore an important region for cognitive flexibility (Klune et al., 2021; Logue & Gould, 2014).

The infralimbic and prelimbic area of the mPFC were here the focus of PNN analysis (Figure 20A), as disruption of PNNs in those areas can lead to poorer fear learning abilities (Hylin et al., 2013). While the number of PNNs per mm^2 was not different between groups (Figure 20B), the intensity of WFA+ PNNs was tendentially higher in exercised mice ($F(1, 15) = 2.613$, $p = 0.127$) (Figure 20C). Thus, exercise specifically tends to increase the WFA signal of PNNs in the mPFC of 20-21 months old mice.

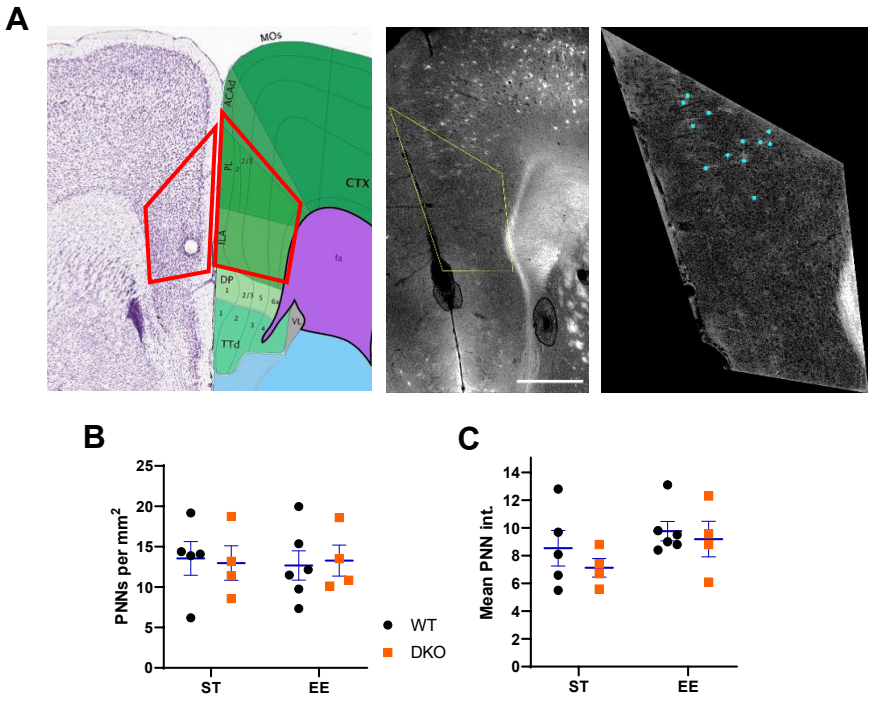


Figure 20: Tendency for higher PNN intensity in mPFC of exercised mice. (A) Areas containing the prelimbic and infralimbic region were selected based on anatomical markers (leftmost image modified from atlas.brain-map.org). PNNs were counted (cyan dots, rightmost image), and mean intensity of PNNs was analyzed. (Scale bar: 500 µm). **(B)** Amount of PNNs per mm^2 did not differ between groups. **(C)** PNN intensity was tendentially higher in exercised mice ($F(1, 15) = 2.613$, $p = 0.127$). (n = 5 in WT ST, n = 6 in WT EE, n = 4 in DKO groups; 2 slices with 2-4 hemispheres per mouse were analyzed).

3.4.2 Abundance of ECM components in the hippocampus is affected by genotype and age

Aggrecan is a major ECM component, playing a substantial role for PNNs (Carulli & Verhaagen, 2021). Comparing the amount of aggrecan between mouse groups in a semiquantitative approach revealed a significant change in expression during aging ($F(1, 57) = 6.452$, $p = 0.0138$) (Figure 21A). A trend in interaction of genotype and age ($F(1, 57) = 3.793$, $p = 0.0564$) suggested, that WT and DKO mice showed different age-related changes in aggrecan levels. Indeed, 18-21 months old WT mice from standard rearing showed a 25,2% reduction in aggrecan in comparison with young (4-5 months old) animals ($M = 1.098$ A.U., $SD = 0.25$ in young mice, $M = 0.821$ A.U., $SD = 0.22$ in old mice). A similar difference could be observed in the exercised group, where aged WT mice showed a 27,1% lower aggrecan level than young animals ($M = 1.042$ A.U., $SD = 0.32$ in young mice, $M = 0.76$ A.U. $SD = 0.26$ in old mice). Those differences could not be found in DKO mice.

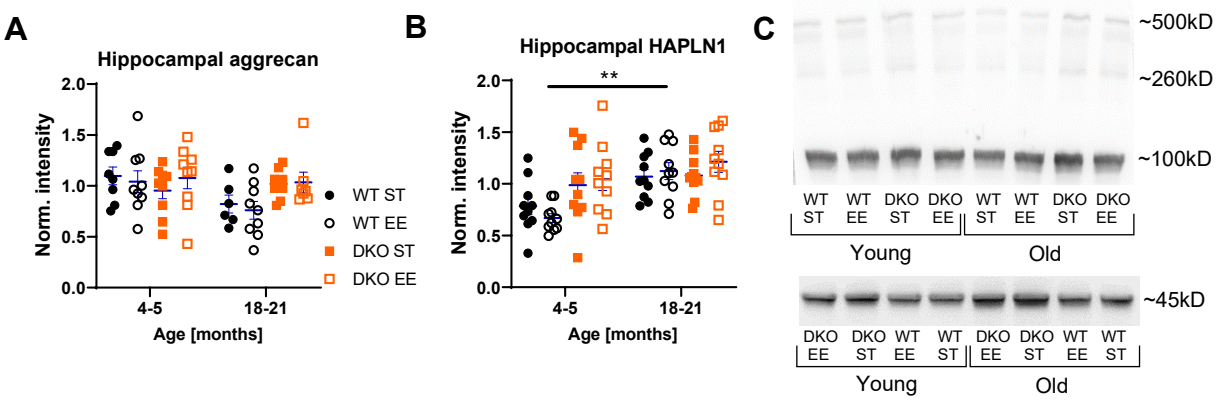


Figure 21: Expression differences in key ECM components in both genotypes during aging. (A) Total aggrecan (Acn) is lower in aged mice ($F(1, 57) = 6.452$, $p = 0.0138$), however genotype tends to affect aggrecan abundance during aging ($F(1, 57) = 3.793$, $p = 0.0564$). (n= 9 in all groups, except young WT ST n = 8, old WT ST n = 6, old DKO EE n = 7) **(B)** Link protein HAPLN1 increases during aging ($F(1, 73) = 16.93$, $p = 0.0001$), but quantity also depends on genotype ($F(1, 73) = 7.842$, $p = 0.0065$). A trend in interacting factors ($F(1, 73) = 3.93$, $p = 0.0512$) suggests, that mostly WT mice are affected by an increase in HAPLN1 during aging. (n = 10 in all groups, except old DKO ST n = 11). **(C)** Examples of anti-aggrecan (top) and anti-HAPLN1 (bottom) immunosignals on blots

A similar observation was made in the analysis of the link protein HAPLN1, where WT and DKO mice showed a different level during aging ($F(1, 73) = 16.93$, $p = 0.0001$)(Figure 21B). But age was not the only main factor affecting HAPLN1 levels in the hippocampus. Genotype also had a high impact ($F(1, 73) = 7.842$, $p = 0.0065$) and there was a trending effect of interaction of both factors ($F(1, 73) = 3.93$, $p = 0.0512$).

Again, DKO mice did not show a major change in HAPLN1 levels during aging, WT animals on the other hand showed a significant increase of 68%, as observed in the exercised animals (M = 0.669 A.U., SD = 0.13 in young mice, M = 1.124 A.U., SD = 0.26 in old mice, p = 0.0088). Although not significant, WT mice from standard rearing also showed an increase of 36.5% (M = 0.783 A.U., SD = 0.26 in young mice, M = 1.069 A.U., SD = 0.22 in old mice).
Taken together, the collected data suggests that Bcan and Ncan play an important role for hippocampal ECM composition during aging.

3.5 Proteome differences between genotypes and housing conditions

To get a more comprehensive and also unbiased insight into molecular differences between groups a proteomics analysis of brain tissue from 25-27 months old mice was performed. Subcellular fractions from collected tissue including cortex, hippocampus and striatum were used in the analysis. In order to investigate differences in soluble and insoluble proteins, different tissue fractions were analyzed. The crude membrane fraction (P2) is enriched in membrane-bound proteins, synaptic proteins, PNN components and also mitochondria and microsomes, whereas the soluble fraction (S2) contains cytosolic and soluble extracellular proteins and soluble proteoglycans. In total, 6827 proteins could be identified, among those, 20 candidate proteins were found to be significantly affected by factors genotype or rearing condition (Figure 22). Proteins were considered a candidate, when at least a 1.5-fold change at a false discovery rate (FDR) of <0.2 was observed. Results of single group comparisons can be found as volcano plots in the supplements (Appendix 4). For the heat-map in Figure 22, the candidate proteins of each group were normalized to the WT ST group. Due to their biochemical properties or intracellular location, some proteins were only detected in a single fraction.

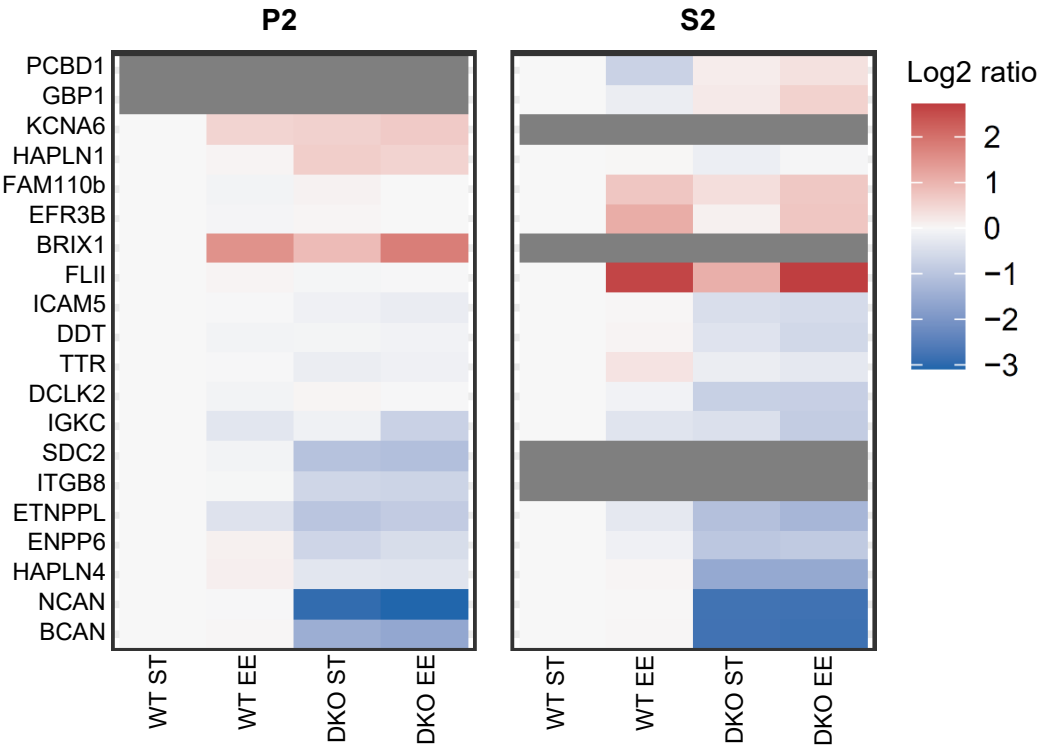


Figure 22: 20 proteins are significantly affected by factors rearing or genotype. The heatmap displays up- or downregulation of proteins, compared to WT ST group. Gray areas indicate that the respective protein was not present in the fraction (P2 = membrane fraction, S2 = soluble fraction). (n = 4 in all groups).

As proof of principle, Bcan and Ncan were found to be significantly downregulated in the DKO. Apart from that, other ECM components and related cell adhesion molecules were affected by the lack of Bcan and Ncan: HAPLN1, HAPLN4, ICAM5 and SDC2. The link protein HAPLN1 was upregulated in the membrane fraction of DKO (1.65-fold, FDR = 0.14 in WT ST x DKO ST), while HAPLN4 was downregulated, mostly in the soluble fraction (0.34-fold, FDR = 2,4e-08 in WT ST x DKO ST). Both proteins are major components of the ECM as they form a link between CSPGs and hyaluronan and thus are important for ECM stability. While HAPLN1 tends to interact more with aggrecan, neurocan and versican isoforms V0 and V1, HAPLN4 was shown to interact with brevican (Bekku et al., 2003; Zimmermann & Dours-Zimmermann, 2008). Syndecane-2 (SDC2), a transmembrane protein of the heparan sulfate proteoglycan family, is also involved in dendritic spine maturation (Ethell & Yamaguchi, 1999). SDC2 was found to be downregulated in DKO mice (0.49-fold, FDR = 0.001 in WT ST x DKO ST).

Among the significantly regulated proteins were some that are functionally linked to the immune system. DDT (0.59-fold, FDR = 0.16 in WT EE x DKO EE), IGKC (0.6-fold,

FDR = 0.04 in WT ST x DKO EE) and again ICAM5 were downregulated, while GBP1 exhibited an upregulation in the soluble fraction of DKO (1.84-fold, FDR = 0.19 in WT EE x DKO EE). The intercellular adhesion molecule 5 (ICAM5) was downregulated especially in its soluble form in the soluble fraction of DKO (0.64-fold, FDR = 0.01 in WT EE x DKO EE). While not directly being a part of the ECM, this protein can be subject to matrix metalloproteinase-9 cleavage, which likely affects dendritic spine maturation (Reinhard et al., 2015). Besides its role in synaptic maturation, ICAM5 is also involved in the regulation of microglia (Paetau et al., 2017) and specifically low levels of soluble ICAM5 in the cerebrospinal fluid are associated with severe forms of multiple sclerosis (Birkner et al., 2019). D-dopachrome tautomerase (DDT) is involved in macrophage migration (Merk et al., 2011) and reduction of DDT attenuates inflammation after spinal cord injury (Ji et al., 2021). Immunoglobulin kappa C (IGKC) was similarly reduced in the soluble fraction of DKO, but as it is expressed on blood cells, it was not further considered for analysis. The varying presence of IGKC in the samples can be explained by variable amounts of blood cells in the brain vessels, as the mice were not perfused before preparation. Last but not least, guanylate−binding protein 1 (GBP1) was another interesting finding in the proteomics analysis, as this protein is majorly involved in inflammatory processes (Tretina et al., 2019).

Apart from those proteins differently regulated in DKO, some protein candidates also appeared to be affected by rearing condition. Most notably, flightless 1 (FLII) was greatly upregulated in exercised WT and DKO (6.63-fold, FDR = 0.02 in WT ST x WT EE and 7.23-fold, FDR = 0.004 in WT ST x DKO EE). This actin-binding protein is involved in many cellular processes (for review see Strudwick and Cowin, 2020). Ribosome biogenesis protein BRX1 homolog (BRIX1) was also mostly upregulated in exercised mice of either genotype (3.05-fold, FDR = 0.01 in WT ST x WT EE and 3.5-fold, FDR = 0.001 in WT ST x DKO EE). This protein is at least in *Xenopus*, involved in ribosome assembly (Kaser et al., 2001).

To get a comprehensive overview of changes to the ECM, a list of ECM components was compiled (Table 4). Existing proteomics data from S2 and P2 fractions were pooled and reanalyzed for this list, to include proteins that had an 1.33-fold increase or 0.75-fold decrease. Most ECM components that were found with proteomics were not differently regulated. Apart from the already mentioned HAPLN1 and HAPLN4, two different kinds of collagen are affected by the DKO.

Table 4: Extensive overview of ECM components that were analyzed in proteomics.

ECM component	Proteomics result
Aggrecan	No change
Versican	No change
HAPLN1	1,4-fold increase in DKO (P2)
HAPLN2	No change
HAPLN3	No change
HAPLN4	0,74-fold decrease in DKO (S2)
Tenascin-C	No change
Tenascin-R	No change
Phosphacan	No change
Collagen alpha-1(I)	No change
Collagen alpha-2(I)	1,9-fold increase in DKO ST/WT ST (S2) 0,5-fold decrease DKO EE/DKO ST (S2)
Collagen alpha-1(XXV)	1,3-fold increase in DKO (S2)
HSPG2	No change
SGSH	No change
HS2ST1	No change
NDST1	No change
HABP4	No change
CEMIP2	No change
CSPG4	No change
CSPG5	No change
Agrin	No change
Fibronectin	No change
TIMP2	No change
MMP17	No change
ADAM9	No change
ADAM10	No change
ADAM11	No change
ADAM15	No change
ADAM17	No change
ADAM22	No change
ADAM23	No change
Testinan-1/SPOCK 1	No change
Testinan-2/SPOCK 2	No change

3.6 Aged DKO mice show normal spatial learning abilities but impaired temporal association learning performance

Lesions of the hippocampus, but also perturbations in network activity or neurotransmitter and -modulator release can lead to an impairment in spatial learning (de Leo et al., 2023; Ego-Stengel & Wilson, 2010; Kempadoo et al., 2016; Moser et al., 1995). Thus, the differences in brain anatomy and ECM composition that were described between the genotypes open up the possibility that genotypes also show a difference in learning abilities. Here, the hippocampus was put into the focus of

investigation, as it plays a key role in age-related cognitive decline (Belblidia et al., 2018; Wimmer et al., 2012) and as it showed highly pronounced dysplasia in the CA1 region of the DKO mice. The hippocampus plays an important role for spatial learning, but it is also involved in temporal association learning, which can e.g. be measured in the trace fear conditioning task.

3.6.1 Normal spatial learning in DKO

Two different learning tasks were used to test the spatial learning abilities of aged mice. A possibly lower physical fitness of aged mice was taken into account when tasks were selected for spatial learning. Both tasks required only a moderate amount of movement and were well doable even for old mice.

The first spatial learning task was the novel location recognition task. This task was combined with the novel object recognition task, which is not a purely hippocampus-dependent learning task, but also involves cortical regions (Antunes & Biala, 2012). While the object recognition task with an inter-trial interval of 24h mostly relies on the hippocampus, learning during a 2h retention as used here relies on the perirhinal cortex among other regions (Reger et al., 2009). Both recognition tasks rely on the animal's intrinsic motivation to explore novelty, as no external reward or reinforcement via negative stimuli is provided.

A two-day comprehensive habituation was done to reduce anxiety caused by an unfamiliar environment, before starting with the experimental protocol (Figure 23A&B). Two mice were excluded from final data analysis (one WT EE and one DKO ST), as those animals showed consistently strong bias towards the familiar object in both tasks. Besides group-wise comparison via 2-Way ANOVA, the learning success of each group was determined via one-sample t-tests.

Almost all groups successfully recognized the novel location, as all groups showed statistical significance in the one-sample t-test ($t(9) = 3.582$, $p = 0.0059$ for standard WT, $t(7) = 3.005$, $p = 0.0198$ for exercised WT, $t(9) = 2.545$, $p = 0.0315$ for exercised DKO)(Figure 23C). Only in DKO mice from standard rearing the discrimination ratio did not reach significance but showed a trend ($t(7) = 2.104$, $p = 0.0735$). No group differences could be recognized in the analysis, as all mice learned similarly well. This is first evidence, that DKO mice have normal spatial learning abilities, despite having a CA1 dysplasia. Surprisingly, exercise did not positively affect learning success neither in the DKO, nor in WT.

The novel object recognition task on the other hand revealed different learning abilities among groups (Figure 23D). While old WT mice from standard housing did not show any learning abilities in this task, lifelong exercised WT mice were able to discriminate between the familiar and novel object (t (7) = 2.788, p = 0.027).

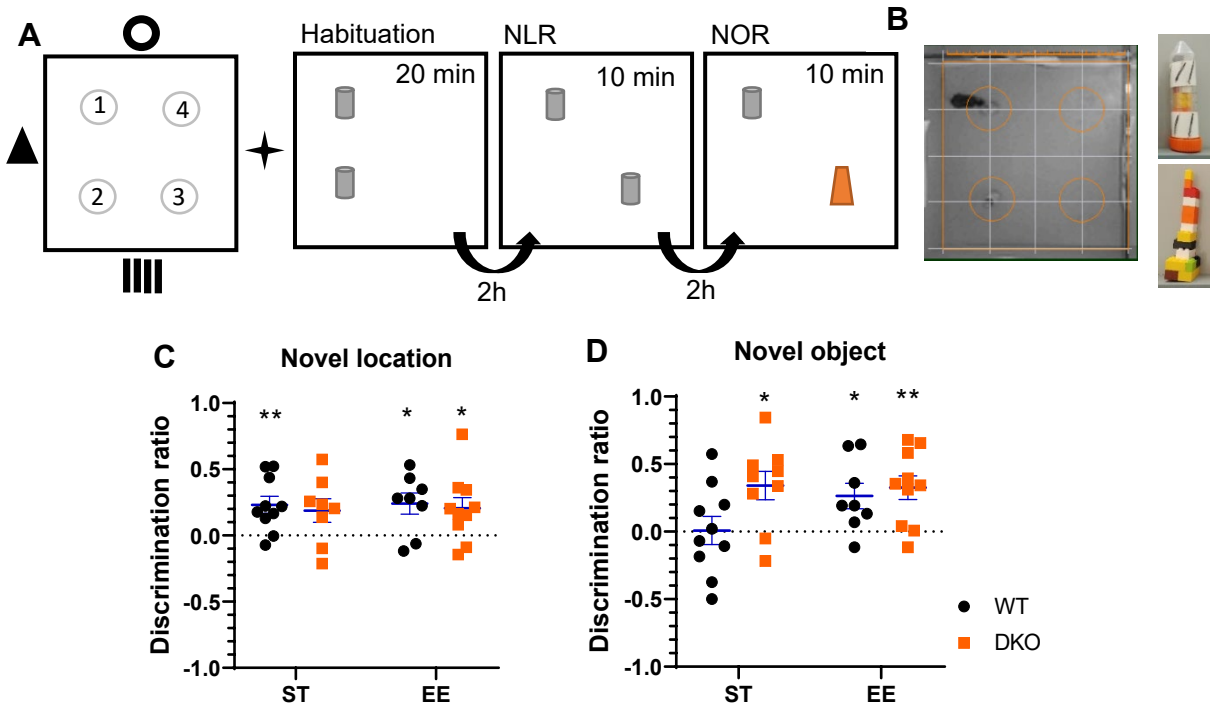


Figure 23: DKO mice mostly show successful spatial learning and improved object recognition. **(A)** Open field was setup with visual cues outside the arena. Circles indicate location and dimension of areas of interest for positions 1-4. Object position, trial time and inter-trial interval during habituation, novel location recognition and novel object recognition are represented schematically (grey object: familiar, orange object: novel). **(B)** Example of experimental setup during habituation (left). A 50 ml tube was used as familiar object, an interlocking brick tower was used as novel object (right). **(C)** All groups of 19-20 months old mice displayed significant spatial learning, except DKO ST, where at least a trend is visible (t (7) = 2.104, p = 0.0735). **(D)** DKO from either housing condition tend to show better object discrimination (F (1, 33) = 3.991, p = 0.054). WT ST fail to learn the task, but WT benefit from lifelong exercise. (n = 10 in all groups, except WT EE n = 9).

So did DKO from standard housing (t (8) = 3.249, p = 0.0117) as well as exercised DKO (t (9) = 3.728, p = 0.0047). Genotype as a main factor showed a trend for influencing the outcome of the novel object recognition task (F (1, 33) = 3.991, p = 0.054), indicating that DKO mice from either housing condition showed superior object recognition performance at old age.

Post-hoc analysis emphasizes this observation, as DKO mice from standard rearing showed an almost significantly increased discrimination ratio (M = 0.341 A.U., SD =

0.32) as compared to WT mice from standard rearing (M = 0.008 A.U., SD = 0.33, p = 0.092). Aged WT mice were not able to discriminate between familiar and novel objects, but benefit from voluntary exercise. DKO mice did not show a clear benefit from exercise, instead, the lack of Bcan and Ncan seemed to be beneficial for this recognition task, at least in old mice. Learning efficacy is slightly more significant for exercised DKO animals, as there is less variation between individual mice (M = 0.325 A.U., SD = 0.28).

Data from novel location and novel object recognition task indicates that Bcan and Ncan deficiency has an impact on cognitive abilities of aged mice. However, the impact seems to depend on the brain regions utilized during the task. Even though DKO mice show a CA1 dysplasia, spatial learning is unaffected and object recognition is improved. This suggests that the function of other brain regions apart from hippocampus could be affected by the double knockout.

Testing spatial learning abilities via novel location recognition task offers the advantage of learning without negative reinforcement, so mostly without negative emotional impacts. On the other hand, this task does not provide much insight into memory acquisition. Considering that and to verify the observations, a maze learning task was carried out subsequently.

The water cross maze (WCM) utilizes water as negative reinforcement to enhance motivation. The protocol used here is derived from the place learning protocol described by Kleinknecht et al., 2012. While the Morris water maze is a popular choice for analysis of spatial learning, the WCM gives the advantage of easier assessment of learning strategies (Kleinknecht et al., 2012). Here, the place learning protocol was used to test spatial learning via allocentric strategy by allowing mice to navigate based on visible cues (Figure 24A). Learning success was evaluated by accuracy of arm entries and latency until animals reached the platform. An accuracy of 83% (5 out of 6 correct arm entries) was considered as criterion of successful learning (Kleinknecht et al., 2012). Apart from that, the relative number of accurate learners that reach or exceed this criterion per day was calculated for each group.

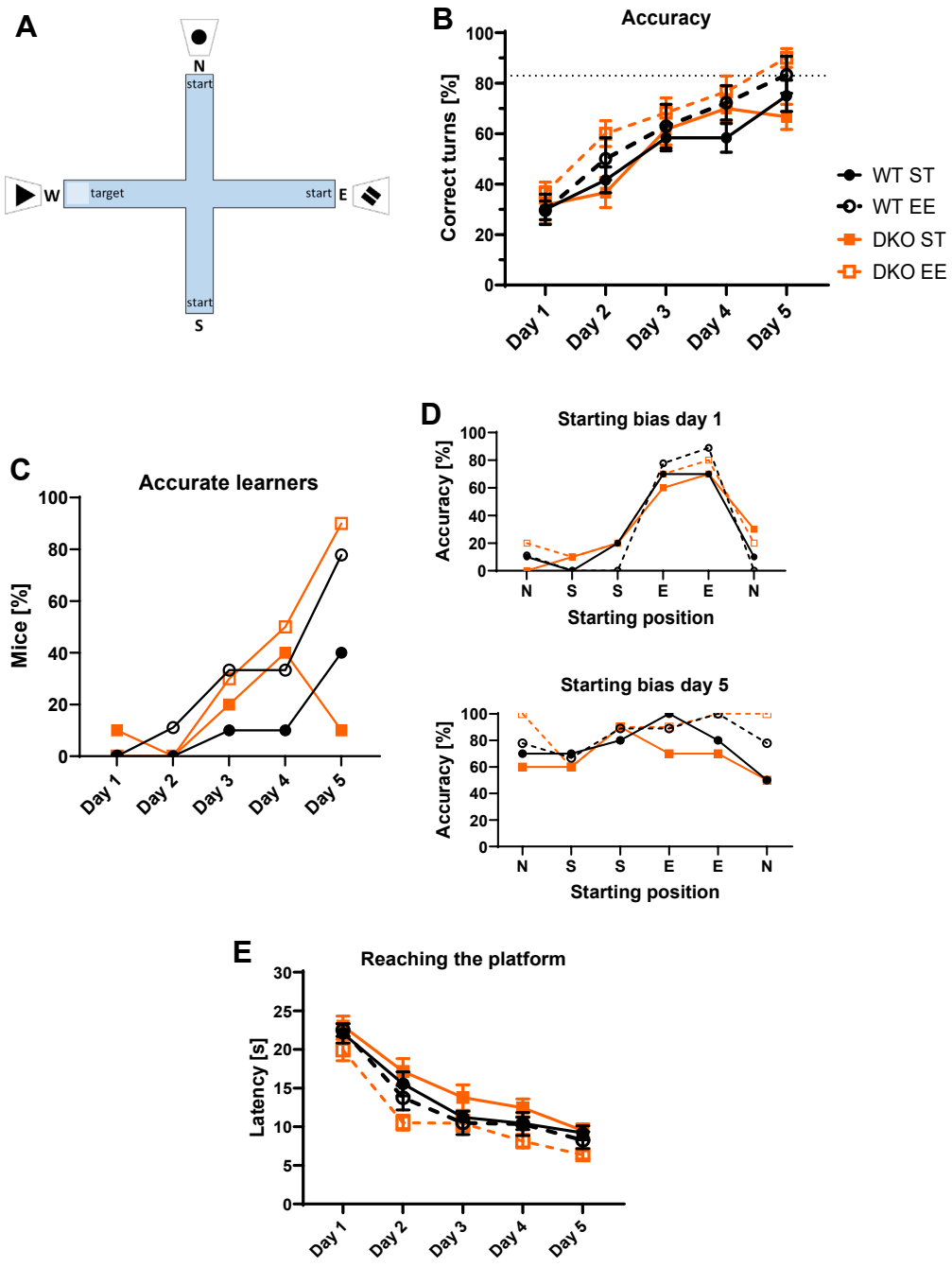


Figure 24: Spatial learning in water cross maze is improved by exercise. (A) Water cross maze set up. **(B)** Accuracy during acquisition phase of water cross maze learning was significantly improved by voluntary exercise (F (1, 35) = 9.05, p = 0.0048). **(C)** This outcome is also reflected in a higher amount of accurate learners in the exercised groups, regardless of genotype. **(D)** The starting bias, present in all groups on day 1, disappears through successful learning. **(E)** Latency to reach the platform is particularly low for exercised mice (F (1, 35) = 8.543, p = 0.006). However, genotypes are affected differently by exercise, as DKO show the highest benefit (F (1, 35) = 4.608, p = 0.0388). (n = 10 in all groups, except WT EE n = 9).

All groups of the 20-21 months old mice showed learning success during the acquisition phase, as seen by increase of accuracy (Figure 24B) over time ($F(3.581, 125.3) = 44.69$, $p < 0.0001$) with no main effects from genotype ($F(1, 35) = 1.234$, $p = 0.2742$). Indeed, the rearing condition did affect the outcome of accuracy ($F(1, 35) = 9.05$, $p = 0.0048$). Exercised mice generally show a higher accuracy, also a higher number of exercised mice reach the criterion on day 5 (Figure 24C). Less than half of standard reared mice reached the criterion during the training, while 80% from the exercised groups reached it on day 5. So, while most mice from the exercised groups reached the criterion set by Kleinknecht et al., 2012 and showed accuracy rates similar to 6-7 weeks old mice that were used in that study, aged non-exercised mice fell behind in their learning abilities.

Besides analysis of mean daily accuracy, also the accuracy rate relative to the starting position was evaluated (Figure 24D). Here, the data revealed a bias for a starting position directly across the target arm. Also, in other starting positions it was observed that mice tended to swim straight ahead when leaving the starting arm. That way they would swim from the southern starting position into the northern arm for example, which lead to a <30% accuracy when starting from either of these arms. But on the final day of training, no starting arm bias was visible anymore (Figure 24D, bottom), showing that mice properly learned the task and abandoned their initial egocentric learning strategy.

As expected, the latency to reach the platform significantly decreased over time ($F(3.151, 110.3) = 113.6$, $p < 0.0001$) and is affected by rearing condition ($F(1, 35) = 8.543$, $p = 0.006$), as exercised mice showed lower latency. While there was a tendency for lifelong exercise to affect swimming speed, as discussed in section 3.1, the effect was not significant ($F(1, 22) = 3.65$, $p = 0.0694$). Thus, the exercise effect on swimming speed was deemed not strong enough to affect latency. Instead, it is more likely, that exercise has a positive effect on spatial learning abilities, as already observed via training accuracy and shown by several other studies (Alomari et al., 2013; Anderson et al., 2000; Ang et al., 2006). Interestingly, an interaction of genotype and rearing affected the latency to reach the platform ($F(1, 35) = 4.608$, $p = 0.0388$). It appears that DKO mice are particularly affected by lifelong exercise, as this group showed the lowest latency during most training days, while DKO mice from standard rearing took the longest time to reach the platform (Figure 24E).

Directly following the 5-day acquisition period, mice underwent a 5-day reversal learning protocol. All mice entered the reversal learning, as all mice learned to reach the platform within 30s (at least 5 out of 6 trials).

Again, all groups successfully learned the reversed platform position, as accuracy significantly increased over time ($F(2.897, 101.4) = 87.28$, $p < 0.0001$) (Figure 25A). Reversal learning was not affected by the main factor rearing ($F(1, 35) = 0.2865$, $p = 0.5959$), instead there was a tendency of genotype affecting the accuracy during reversal learning ($F(1, 35) = 3.965$, $p = 0.0543$).

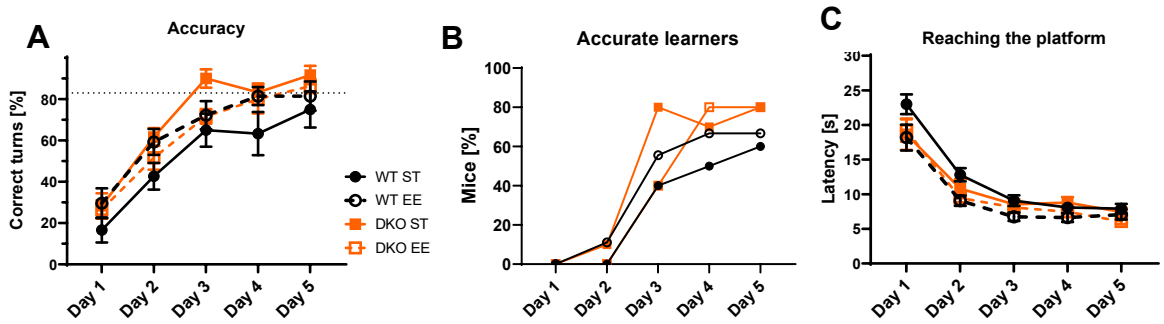


Figure 25: DKO from standard housing have particularly good spatial flexibility. (A) Accuracy during reversal learning is affected by both factors genotype and rearing condition ($F(1, 35) = 5.55$, $p = 0.0242$), with DKO from standard housing showing highest learning abilities. **(B)** This is also reflected in the highest number of accurate learners among the standard reared DKO on day 3. **(C)** Similar to the acquisition phase, all exercised groups have a lower latency during reversal learning ($F(1, 35) = 5.936$, $p = 0.0201$). (n = 10 in all groups, except WT EE n = 9).

The interaction of genotype and rearing environment reached significance ($F(1, 35) = 5.55$, $p = 0.0242$) and the progress of arm entry accuracy suggests, that WT benefitted from lifelong exercise, while DKO mice showed an inverse relationship.

DKO animals from standard rearing were particularly fast learners, as this group reached the criterion already on day 3 of reversal training. Similar to the results from the object recognition task, standard reared DKO mice not only showed proper learning abilities, but instead also seemed to exceed the performance of standard-reared WT mice. Spatial reversal learning relies not only on hippocampal function (Dong et al., 2013), but also on other brain areas e.g., the orbitofrontal cortex (Izquierdo et al., 2017).

Similar to the accuracy rate, also the number of accurate learners increased in each group (Figure 25B), showing that more mice properly acquired spatial memory and increase of accuracy on each day is not solely attributable to percentage increase of accuracy in each mouse.

Latency to reach the platform significantly decreased over time in all groups ($F(2.037, 71.3) = 136.4$, $p < 0.0001$) (Figure 25C). As already seen during the acquisition phase, lifelong exercise affected the latency to reach the platform ($F(1, 35) = 5.936$, $p = 0.0201$), possibly aerobic exercise also positively affected spatial cognitive flexibility.
Overall, the observations from the WCM task indicate, that DKO mice properly acquire spatial memory. Despite the CA1 dysplasia, there was no deficit in spatial learning or spatial cognitive flexibility. Thus, water cross maze data confirms the observation from the novel location task.

3.6.2 DKO mice show impairment in temporal association learning

The hippocampus plays a major role for spatial learning, but it is also involved in other learning paradigms, most notably in contextual fear learning. Classical fear conditioning also critically depends on amygdalar regions, such as the central nucleus and basolateral amygdala (Amano et al., 2011; Barot et al., 2009; Viviani et al., 2011). Trace fear conditioning is a special version of fear conditioning, as it involves a temporal gap between conditioned (CS) and unconditioned (US) stimulus. Classical delay fear conditioning without any time interval between CS and US does not rely on hippocampal function, whereas trace fear conditioning demands a temporal association that cannot be formed after hippocampal lesions (Ahmed et al., 2020; Weiss et al., 1999). Specifically, the CA1 region is of utmost importance for temporal association learning (Ahmed et al., 2020; Huerta & Lisman, 1995; Sellami et al., 2017). It was therefore tested, if the disorganized CA1 pyramidal layer of DKO mice still allows for normal CA1 function in the trace fear conditioning paradigm (Figure 26A).
Increase in freezing time during 10s CS presentation ($F(3.442, 120.5) = 11.79$, $p < 0.0001$) and 20s trace period ($F(3.704, 129.6) = 15.79$, $p < 0.0001$) showed proper acquisition in all groups (Figure 26B). However, there was a different progression in freezing behavior between genotypes during the CS presentation ($F(4, 140) = 3.516$, $p = 0.0091$). As this interaction did not occur during the trace period, it could possibly be a different reaction to the presented sound. All groups showed normal hearing abilities (Appendix 1), therefore hearing impairments were ruled out as a potential cause.

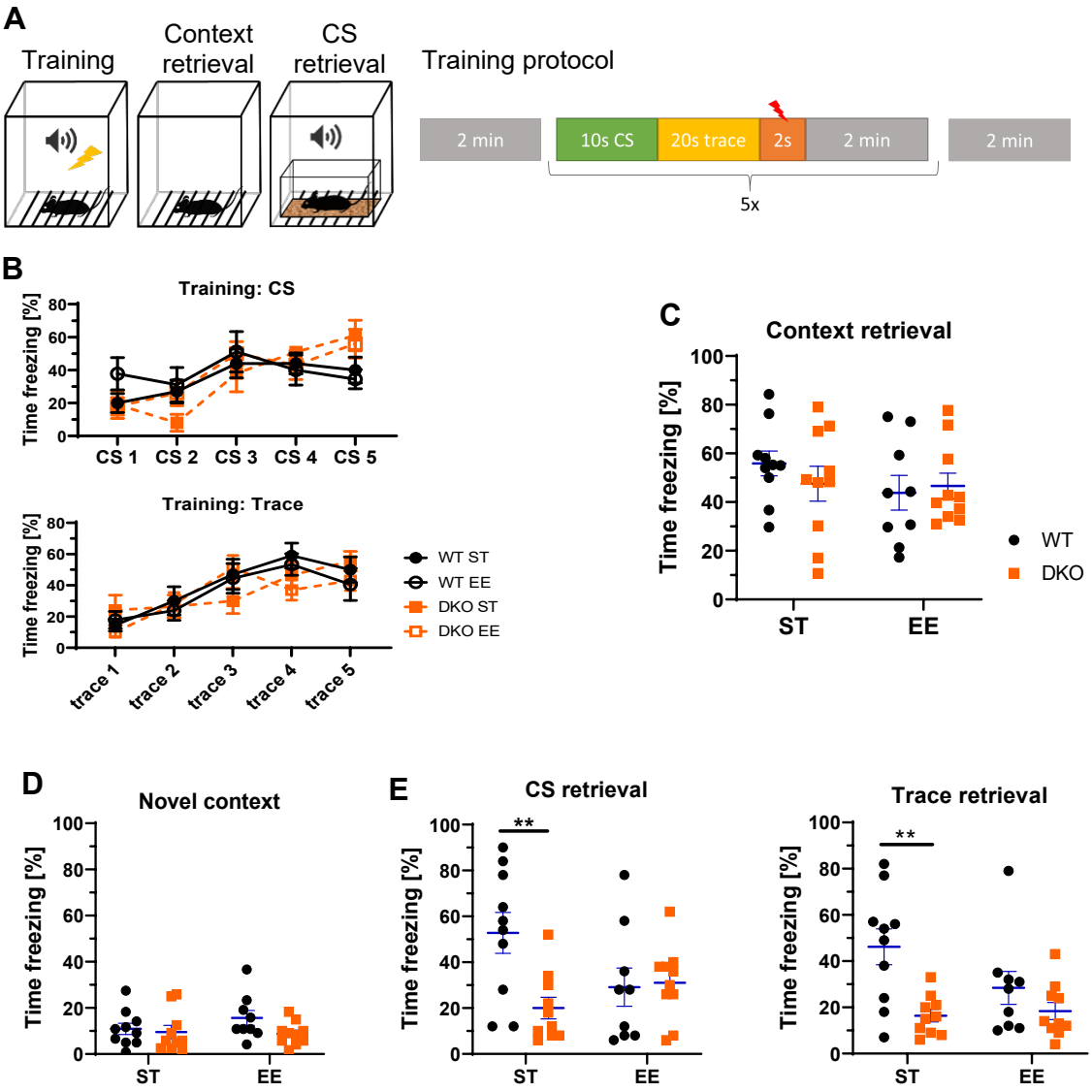


Figure 26: Lack of Bcan and Ncan leads to a temporal association learning deficit. (A) 20-21 Months old mice were subjected to a 4-days protocol consisting of 4 different phases: Habituation (not depicted), training, context retrieval and CS retrieval. During the training a 2.4 kHz tone was presented as CS and 0.3 mA electric stimulus as unconditioned stimulus. **(B)** All groups display an increasing fear response during CS presentation (top) and trace period (bottom) during acquisition. **(C)** No group differences in contextual fear learning. **(D)** Exposing mice to the novel context used during cue retrieval elicits only a minor fear reaction. **(E)** Cue-elicited fear response is reduced in DKO during CS ($F(1, 35) = 4.944$, $p = 0.0327$) and trace period ($F(1, 35) = 12.37$, $p = 0.0012$). Specifically standard-reared DKO mice show a learning deficit, compared to WT ($p = 0.0092$ during CS, $p = 0.0032$ during trace), whereas mice from exercised housing have a similar fear response. ($n = 10$ in all groups, except WT EE $n = 9$).

Proper acquisition of fear memory in all groups could be seen in the context retrieval (Figure 26C). There was no impact from main factors genotype nor rearing condition, proving, that all groups displayed normal contextual fear learning.

Low freezing in novel environment could be observed in all groups before, showing that the fear response was not generalized (Figure 26D). However, freezing to CS presentation was significantly affected by genotype ($F(1, 35) = 4.944$, $p = 0.0327$) and

an interaction of factors genotype and rearing environment (F (1, 35) = 6.227, p = 0.0174) (Figure 26E). A similar observation could be made for the main factor genotype during the trace period of the retrieval phase (F (1, 35) = 12.37, p = 0.0012), whereas the interaction of both factors did not significantly affect trace freezing (F (1, 35) = 3.041, p = 0.09). During both phases, WT mice from standard rearing showed significantly higher freezing than standard reared DKO (M = 52,8%, SD = 28.11 for WT, M = 20%, SD = 14.82 for DKO during CS, p = 0.0092; M = 46%, SD = 24.5 for WT, M = 16.4%, SD = 8.49 for DKO during trace, p = 0.0032). Clearly, DKO mice from standard rearing did not show a proper retrieval of cue elicited fear.

The role of exercise is more obscure in this learning task. 2-Way ANOVA suggested an interaction of genotype and rearing environment and indeed, during the CS presentation exercised WT showed lower freezing time (M = 29.11%, SD = 24.98) than non-exercised WT (M = 52.8%, SD = 28.11), while exercised DKO showed a slightly higher freezing (M = 31%, SD = 16.23) than DKO from standard rearing (M = 20%, SD = 14.82). This effect was not as pronounced during the trace presentation, most likely because exercised DKO did not show a much higher amount of freezing than the sedentary group.

Overall, this data suggests a deficiency in trace fear learning for DKO mice.

3.6.3 Interaction of genotype and rearing environment in ASST reversal learning

The attentional set shifting task (ASST) is generally used to analyze cognitive flexibility and attention. Performance in ASST depends on the function of various prefrontal cortical areas, as lesions in mPFC lead to an impairment in extradimensional shift learning (Birrell & Brown, 2000), while lesions in the orbitofrontal cortex lead to deficits in reversal learning (McAlonan & Brown, 2003).

Most mice were able to learn the task. In cases, where a mouse failed to learn one phase of the ASST, the respective reversal was not conducted. Only two WT from standard rearing and two exercised DKO failed some phases of the ASST. In those cases, 62 trials until criterion were assumed for calculation. No data on errors from those mice was used in the calculation.

3-way ANOVA revealed a significant interaction of factors genotype and rearing condition (F (1, 133) = 6.016, p = 0.0155) with the number of trials until the criterion is reached (Figure 27A). There was a similar although weak trend in the number of errors

(F (1, 123) = 2.629, p = 0.108) (Figure 27B). Moreover, rearing tended to affect the number of errors in the ASST (F (1, 123) = 3.246, p = 0.074).

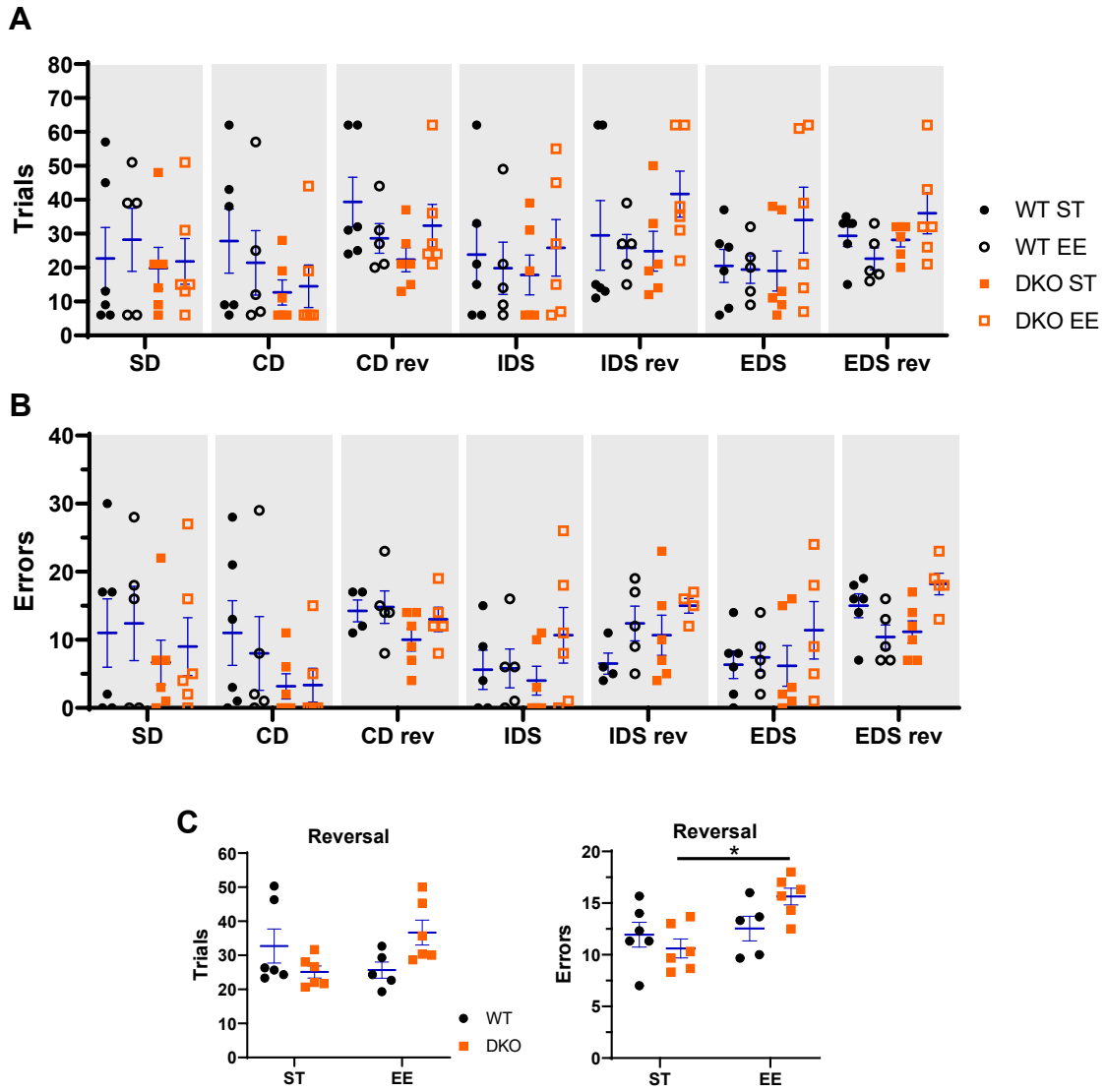


Figure 27: Cognitive flexibility in ASST depends on genotype and rearing type. **(A)** Number of trials until criterion was significantly affected by interaction of genotype and rearing in (F (1, 133) = 6.016, p = 0.0155). **(B)** Number of errors are mostly affected by rearing condition (F (1, 123) = 3.246, p = 0.074). **(C)** Pooled reversal data also presents an interaction of genotype and rearing condition with the number of trials (F (1, 19) = 6.937, p = 0.0164). A similar effect can be seen in the number of errors (F (1, 19) = 4.593, p = 0.0453). Furthermore, the number of errors was affected by rearing condition (F (1, 19) = 7.353, p = 0.0138). Exercise had a strong effect on DKO mice, as there was a significant difference between DKO from standard cages and those reared with running wheel (p = 0.0113). (SD = Simple discrimination, CD = Compound discrimination, IDS = Intra-dimensional shift, EDS = Extra-dimensional shift, rev = reversal)(n = 6 in all groups, except WT EE n = 5).

As reversal learning in the water cross maze showed to be dependent on genotype, the reversal phases of the ASST were considered. For this, data from compound discrimination, intra-dimensional and extra-dimensional shift reversal learning were

pooled and the mean of each mouse was used for statistical analysis. Indeed, an interaction of genotype and rearing environment was also present in the pooled reversal data on number of trials ($F(1, 19) = 6.937$, $p = 0.0164$) (Figure 27C). Similarly, there was an - albeit weak - significant effect of interaction with error numbers ($F(1, 19) = 4.593$, $p = 0.0453$). Interestingly, rearing had a significant effect on errors during reversal ($F(1, 19) = 7.353$, $p = 0.0138$). It seemed that exercised mice have a generally higher error rate. This effect is specifically strong in DKO mice: Here, exercised mice make significantly more errors (M = 15.639 errors, SD = 1,973) than standard reared (M = 10.611 errors, SD = 2.235) in the reversal ($p = 0.0113$).

To control for potential sensory disturbances, mice from each group were tested for age-related loss in olfactory function and showed proper performance (Appendix 5).

Taken together, the data from ASST reinforce the finding that DKO respond differently to lifelong exercise than WT. Furthermore, there was no general effect of genotype on ASST performance.

4 Discussion

This study aimed to investigate the role of the two lecticans Bcan and Ncan in healthy brain aging. The focus was to understand, how double deficiency in Bcan and Ncan impacts brain structure and cognition in aged healthy mice. Although there are studies on age related changes of the brain ECM composition (Morawski et al., 2014b; Reed et al., 2018; Végh et al., 2014), many aspects of those changes and their relevance for cognitive decline remain to be explored. In this study, two main components of the hyaluronan based ECM could be linked to changes in brain anatomy, cognition and inflammation markers in aged mice, shedding light onto the role of Bcan and Ncan for brain aging.

4.1 Bcan/Ncan deficiency affects survival probability and the inflammatory load of the brain

One interesting observation in mice lacking Bcan and Ncan, is a difference in survival probability during different life phases. As of yet, there are no reports that the largely brain-specific proteoglycans Bcan and Ncan can affect survival probability in mice. For Bcan single-KO, Brakebusch et al., 2002 reports a normal lifespan. However, both CSPGs are found to be involved in several different diseases. Both Bcan and Ncan are associated with the progression of different kinds of tumors (Frischknecht & Seidenbecher, 2012; Salmikangas et al., 2023; Su et al., 2017). In the present study, tumors could be observed in both genotypes but all mice developing tumors were excluded from experiments. In general, CSPGs are implied to have adverse effects on repair mechanisms after injury to the central nervous system (Keough et al., 2016; Kwok et al., 2011; L. W. Lau et al., 2012). Based on these studies showing an adverse effect of Bcan and Ncan in disorders, a higher survival would be expected for DKO mice. Instead, both or one of the lecticans might be involved in yet to be explored processes that are vital for health during adulthood and lead to the observed reduction in survival probability in 10-75 weeks old mice (Figure 6). The DKO could also lead to higher masking of existing diseases in mice or to a faster progression, which makes intervention more difficult (Marx et al., 2013). The reason for the observed differences during adulthood were not further explored in this study, as the focus was on aged animals.

In aged mice, a trend for higher survivability in DKO mice could be observed. One reason could lie in the higher death rate among adult DKO mice, compared to WT

mice: Possibly, issues that lead to natural spontaneous death in aged WT mice, already occur in adult DKO mice, leading to a higher survival rate for remaining mice of that genotype. But another reason for better survival in aged DKO mice could lie within the different inflammatory load detected in WT and DKO mice: IL-6 and Iba-1 were found to be reduced in the hippocampus of DKO mice (Figure 18). IL-6 increases in healthy aging and is associated with age-related impairments (Godbout & Johnson, 2004). Similarly, microglia also undergo changes during aging that could be linked to neurodegeneration (Hefendehl et al., 2014). A link of Bcan, Ncan and other CSPGs with neuroinflammatory processes has been found for spinal cord injury and intracerebral hemorrhage via the leukocyte common antigen-related phosphatase (Dyck & Karimi-Abdolrezaee, 2018; Le et al., 2023). Activation of this phosphatase can affect the inflammatory state of microglia via the ROCK pathway and RhoA activation and thus leads to inhibition of repair after injury to the central nervous system (Dyck et al., 2018).

In addition, proteome analysis of certain parts of the forebrain of WT and DKO mice revealed a different level of several proteins involved in inflammatory processes (Figure 22). Increased levels of DDT are a sign of inflammation and reducing DDT levels before lipopolysaccharide administration to induce neuroinflammation, leads to a higher survival in mice (Merk et al., 2011). Hence, lower levels of DDT are another hint towards lower inflammatory load in the DKO, that may improve survival in aged mice. Also, levels of soluble ICAM-5 increase during inflammatory processes (Guo et al., 2000; Lindsberg et al., 2002). Similarly, GBP1 is increased after cytokine stimulation and acts in several inflammatory processes, but overall, it has beneficial effects on cell survival during inflammation (Honkala et al., 2020). The observed differences in immune factors suggest not only a lower level of inflammation in the brain of DKO mice, but also the increase of factors that improve cell survival during inflammatory processes.

However, not all signs of inflammation are attenuated in DKO animals. Hippocampal GFAP increases during aging in both WT and DKO animals (Figure 17) and is not differently regulated in DKO mice (Figure 18). GFAP is a widely used marker for reactive astrogliosis. This increase of astroglia markers is on the one hand a sign of increased inflammation during aging and on the other hand shows that astroglia actively promote cell survival and mitigate damage from inflammation (Rodríguez et

al., 2014). Generally, increase of inflammation during aging is associated with higher frailty and mortality (De Martinis et al., 2006).

4.2 ECM composition during aging in Bcan/Ncan DKO mice

Bcan and Ncan deficiency was found to not only affect the inflammatory load in aged mice, but it also has an effect on other ECM components. Generally, the ECM composition changes during aging, components of the hyaluronan-based ECM such as HAPLN1 but also Bcan and Ncan were shown to increase in hippocampal synaptosomes of aged mice (Végh et al., 2014). But it was also found, that other constituents and associated molecules, such as reelin (Knuesel et al., 2009), MMP12 (Liu et al., 2013) and even hyaluronan (Sugitani et al., 2021) increase during aging.
The composition of the ECM is highly regulated and removing certain components can lead to an altered composition as seen for example in the Bcan single-knockout, where Ncan is reported to be upregulated (Brakebusch et al., 2002).
In the present study, the lack of Bcan and Ncan was found to affect the composition of the ECM during aging. The increase of HAPLN1 in the hippocampus, as reported by Végh et al., 2014 could be replicated here in WT mice, but furthermore, HAPLN1 shows increased expression already in young DKO mice (Figure 21). The proteomic data suggest a higher expression in a broader area of the brain in aged DKO mice, since for this analysis also cortical and striatal tissue was used (Figure 22). As this upregulation appears already in young animals, it could be interpreted as a compensatory effect from the DKO of the two lecticans. HAPLN1 links CSPGs and hyaluronan and thus is thought to stabilize the ECM. It is furthermore essential for proper PNN formation (Carulli et al., 2010) and increased levels could counteract PNN abnormalities, which were observed in Bcan knockout mice (Brakebusch et al., 2002).
Another important constituent of PNNs is the lectican aggrecan. In this study, abundance of aggrecan in the hippocampus during aging is affected by the presence of Bcan and Ncan. Old WT animals tend to have lower levels of aggrecan in the hippocampus, based on immunoblot data, whereas aggrecan levels of DKO mice do not change as much during aging (Figure 21). This difference could not be found in the proteome analysis of merged cortical, striatal and hippocampal tissue samples of aged mice, suggesting that this difference mainly appears in the hippocampus (Table 4). Sugitani et al., 2021 investigated the solubility of aggrecan during aging in a small group of mice and could not find differences in aggrecan abundance, probably because

this study was performed on whole brain tissue. In another study, the amount of aggrecan-positive hippocampal cells increased in aging rats (Tanaka & Mizoguchi, 2009).

Aggrecan takes on a special role in the hippocampus, as it forms dense PNNs in the CA2 region. Removing PNNs in the CA2 region via enzymatic digestions improves neuronal plasticity (Carstens et al., 2016).

As both aggrecan and HAPLN1 are important for neuroprotective properties of PNNs, the higher expression of both components in the DKO mice could be a hint towards improved neuronal health during aging (Suttkus et al., 2014).

HAPLN4 is another link-protein, that is altered by DKO of Bcan and Ncan, as it shows lower levels in DKO mice (Figure 21). A similar effect was already reported for Bcan single-knockout mice (Bekku et al., 2003). In that study, Bcan-deficient mice showed lower HAPLN4 staining at PNNs. But lack of Bcan apparently can have an impact on global HAPLN4 levels. The genes encoding Ncan and HAPLN4 are located adjacent to each other on the same chromosome. But knockout of Ncan does not affect levels of HAPLN4 mRNA (Bekku et al., 2003), thus the elimination of the Ncan coding sequence can be ruled out as a reason. HAPLN4 plays a role for the formation of PNNs and especially the localization of Bcan in PNNs of the brainstem and cerebellum (Bekku et al., 2012). The decrease of HAPLN4 and the lack of its interaction partner Bcan in the DKO mice could affect the function of brainstem areas, which regulate feeding and weight development such as the dorsal raphe nucleus (Nectow et al., 2017; Schneeberger et al., 2022). This could give a hint towards understanding why DKO mice show a different weight development during aging compared to WT animals (Figure 9).

The proteomics analysis also hints towards a downregulation of the ECM component syndecan-2 in the aged DKO mice (Figure 22). No dysregulation of SDC2 has been reported for the Bcan or Ncan single-knockout, as of now. A lower level of SDC2 potentially has positive effects on inflammation-related fibrosis of the brain (Heindryckx & Li, 2018), as SDC2 plays a role in fibrosis (Chen et al., 2004; Tsoyi et al., 2022). Furthermore, SDC2 is involved in dendritic spine formation and lower levels could negatively affect the formation of new dendritic spines (H.-T. Hu et al., 2016).

An adverse effect can also be expected from higher levels of collagen alpha-1 (XXV), as observed in the brain proteome of DKO mice (Table 4). This transmembrane collagen has been linked to pathogenesis of Alzheimer’s disease in mice (Tong et al.,

2009) and was found enriched in amyloid beta plaques of early onset Alzheimer's disease patients (Drummond et al., 2022).

Some of the observed changes in ECM composition could underlie the observed difference in apparent diffusion coefficient particularly in the hippocampus (Figure 12) (Nicholson et al., 2012). The fact that only the hippocampus shows this effect, underlines the importance of Bcan and Ncan to this brain region. The relevance of both lecticans for hippocampal diffusion parameters was also found in young adult mice by Frank Angensteins lab (unpublished data). While WT show an increase of magnitude of diffusion in the hippocampus – measured via apparent diffusion coefficient – from 2 to 6 months of age, this increase could not be observed in brains of Bcan and Ncan single knockout mice, nor in DKO. Possibly, diffusion parameters are affected differently by ECM composition during distinct life phases.

4.3 Brain anatomy in aged mice lacking Bcan and Ncan

Many extracellular matrix components are involved in brain development and thus directly affect brain volume, as shown for example in knockout-models of the heparan sulfate proteoglycans perlecan (Girós et al., 2007) and glypican 1 (Jen et al., 2009) that have a lower brain volume compared to wildtype mice. Also, chondroitin sulfate proteoglycans have the potential to affect brain development, as they affect migration of neural crest cells (Kubota et al., 1999; Perissinotto et al., 2000). The effects of Bcan and Ncan DKO on brain anatomy were explored here, to investigate the importance of those lecticans for brain maturation and potential age-related atrophy.

4.3.1 Lower brain volume in DKO compared to WT mice

The anatomy of the brain changes also in mature and old age, as a progressive loss of volume is reported for several brain areas during aging in humans (Takao et al., 2012).

However, not all brain areas lose volume during aging, as for example can be seen in the brainstem and multiple white matter tracts (Clifford et al., 2023). Also, in mice examined in the present study there was no general decrease in whole brain volume between 6 and 20-21 months old mice (Figure 13A). Rather, both genotypes display an increase in whole brain volume during aging. There is evidence, that even in mature mice, at least until 14 months of age, the corpus callosum, fornix and ventricles increase in volume (Maheswaran et al., 2009). The study by Clifford et al., 2023 shows,

that the increase of volume in adult mice also originates from a volume increase in brainstem, cerebellum and white matter tracts. Indeed, neither cortex nor hippocampus show an increase of volume during aging in standard reared DKO or WT mice, thus other brain regions not investigated here could be accountable for the observed increase in whole brain volume. A slight loss in cortical volume during aging as reported in literature (Clifford et al., 2023) could be confirmed here (Figure 13B).

Lack of Bcan and Ncan results in a reduction not only in whole brain volume, but more specifically in cortical and hippocampal volume, especially in young mice (Figure 13B&C). The same difference was found in striatum and cerebellum (data not shown). Higher levels of Bcan and Ncan were found to be associated with higher brain volume in old humans (Harris et al., 2020), suggesting that this association of both lecticans with brain volume is a cross-species effect.

The difference in volume is mild, if mice are reared in standard environment, but exercised mice show a particularly strong volume difference in young mice. It seems, young WT mice benefit from exercise and show slightly increased volumes, while young DKO mice do not benefit from exercise regarding their brain region volumes. Aerobic exercise is known to increase the volume of several brain regions in old individuals via multiple mechanisms (Colcombe et al., 2006; Erickson et al., 2011) and it is also reported to increase hippocampal volume in adolescent humans and young adult mice (Fuss et al., 2014; Herting & Nagel, 2012). In Bcan and Ncan DKO mice, this positive effect only shows up in aged mice. Increase of hippocampal volume via exercise is attributed to increased neurogenesis in the dentate gyrus (Biedermann et al., 2014). But as there is no effect of exercise on hippocampal neurogenesis in DKO mice (Figure 16), this hypothesis cannot be proven here.

Exercise evidentially has positive impact on brain angiogenesis (Morland et al., 2017), brain-derived neurotrophic factor levels and potentially other neurotrophins (Lippi et al., 2020; Seifert et al., 2010; Szuhany et al., 2015), dendritic arborization (Serra et al., 2019) and also affects the brain ECM (Smith et al., 2015). The exact mechanism, how Bcan and Ncan affect exercise-related brain volume increase during aging still has to be explored. As both lecticans are part of the brain ECM that is involved in many neuronal mechanisms impacting neuroplasticity (Fawcett et al., 2019), tissue diffusivity (Hrabětová et al., 2009) and neuroprotection (Suttkus et al., 2014) amongst others, a complex interaction with many different binding partners and pathways is imaginable. A first clue towards understanding this complex relationship could lie within age-related

changes in the ECM, as observed in this project (Figure 21) and the observation, that Bcan and Ncan knockout leads to an aberrant age-wise expression of other ECM components.

4.3.2 Possible causes and consequences of CA1 dysplasia in DKO mice

The exploration of brain anatomy in the present study revealed a CA1 dysplasia in DKO mice that presents as diffuse pyramidal cell layer and is also present in exercised mice (Figure 15). The tissue examined here stems from 20-21 months old mice – but in fact, the dysplasia can be found already in young adult animals (unpublished data by Nora John from the lab). This indicates, that the dysplasia develops early in life and cannot be explained by age-related brain atrophy. A possible explanation of the dysplasia is offered by the observation that it occurs in Ncan but not in Bcan single-knockout mice (unpublished data by Nora John). Thus, the developmentally expressed Ncan is the actual driver of the CA1 dysplasia in DKO mice.

Ncan expression onset in mice is around day 10 of embryonic development and reaches its peak at birth (Zhou et al., 2001). The window of high Ncan expression coincides with neurogenesis of hippocampal pyramidal cells that form the pyramidal layer in CA1. The final position of pyramidal cells within the layer depends on their time of generation. Pyramidal cells on the side of the CA1 layer closer to the hippocampal fissure form later during development. Malformation of the CA1 pyramidal cell layer can be observed in mice with a non-secreted form of the ECM glycoprotein reelin (Slomianka et al., 2011). Other proteins involved in CA1 layering are doublecortin and doublecortin-like kinase 2 (DCLK2), which are important for correct CA1 lamination during development (Kerjan et al., 2009). While doublecortin was found unchanged in the DKO mice, proteomics analysis reveals lower levels of DCLK2 in Bcan/Ncan DKO mice (Figure 22). Possibly, an interplay of Ncan and DCLK2 during brain development leads to the observed defects. Levels of DCLK2 were only investigated in aged mice though and an attenuated expression in embryonic tissue of DKO mice would have to be confirmed first.

While no anatomical disparity of the hippocampal CA1 region was reported for other ECM knockout models, there is a known effect of ECM component elimination on presence and appearance of PNNs in the hippocampus (Brakebusch et al., 2002; Gottschling et al., 2019). Also, quadruple knockout of tenascin-C, tenascin-R, Bcan and Ncan leads to a loss of PNNs in primary cultures of hippocampal cells (Geissler et

al., 2013). But in the present study, number and intensity of PNNs in the CA1 region were not affected by DKO (Figure 19). This shows, that knockout of Bcan and Ncan does not affect the number of PNNs, but rather the simultaneous knockout together with the binding partners tenascin-C and tenascin-R may drive the loss of PNNs as observed by Geissler et al., 2013.

Hippocampal dysplasia reportedly can result in changes to hippocampal network properties (I.-S. Choi et al., 2004; Kerjan et al., 2009). Aberrant excitability of hippocampal cells, especially in the CA1 region can in turn have consequences for the functionality of this brain region (Wu et al., 2002). In Ncan single knockout mice the long-term potentiation maintenance is evidently disturbed (Zhou et al., 2001), possibly as an effect of the dysplasia. Together with the likewise impaired long-term potentiation maintenance observed in Bcan single-knockout mice (Brakebusch et al., 2002), this could hint towards an impairment of hippocampal network function in the DKO mice.

Thus, the hippocampal function was determined via multiple behavioral tests, to determine a possible attenuation of hippocampal function in DKO mice.

4.4 Differences in learning and memory of aged DKO and WT mice

DKO mice display differences in brain morphology, ECM composition and diffusion during aging and especially in aged animals. Those observed differences led to investigations of cognitive abilities in aged mice. The focus was put on hippocampal learning tasks, to examine a potential malfunction of the hippocampus that might be present in the DKO mice due to the CA1 dysplasia. As the hippocampus plays a central role for episodic memory (Moscovitch et al., 2016), spatial and temporal learning abilities were examined via multiple different learning tasks.

Furthermore, the aim was to investigate effects of exercise on spatial learning abilities in aged mice, as exercise is known to improve spatial learning in old individuals (O'Callaghan et al., 2009).

4.4.1 Spatial learning is generally not attenuated by CA1 dysplasia

Hippocampal function is important for spatial learning, especially when learning involves allocentric strategies (Rinaldi et al., 2020; Suthana et al., 2009). The water cross maze was used to test this learning strategy. Similar accuracy during acquisition hints towards proper function of the hippocampus, even in DKO mice (Figure 24). Mice showed better accuracy, when raised with a running wheel. This confirms the well-

established positive impact of exercise on spatial learning performance especially in old individuals (Cassilhas et al., 2015; Gomes da Silva et al., 2012; S. Wang et al., 2015). The positive effect of exercise on spatial learning could not be found in the novel location recognition task (Figure 23). Only DKO show a slightly better discrimination, when reared with a running wheel, as only this DKO group reaches a significant discrimination ratio. But there is no difference to the standard reared group, thus the improvement is only marginal.

Interestingly, no significant increase of neurogenesis could be detected in exercised mice. Increased neurogenesis is thought to be one mechanism that improves spatial learning abilities. But exercise might improve hippocampal neurogenesis only until a certain age, as another study shows, that 22 months old mice do not exhibit increased neurogenesis after exercise, despite showing improved cognition (Creer et al., 2010). Another possibility is that exercise increases the amount of neuronal progenitor cells that need additional stimulatory input for example via sensory enrichment, for the neurons to survive (Fabel et al., 2009). In the present study, sensory enrichment was only mild. The improved spatial learning in the water cross maze could also be explained by other exercise-mediated pro-cognitive mechanisms, such as an increase of BDNF or general increase of neuroplasticity in the hippocampus (Cassilhas et al., 2015).

Nonetheless, both spatial learning tasks show that DKO mice exhibit normal spatial learning capabilities despite having a dysplastic CA1 region. One possibility could be, that none of the networks that are important for spatial learning are affected by the dysplasia. Alternatively, loss of function may be compensated by other mechanisms. Undeniably, the CA1 region plays an important role for spatial learning (Jeong & Singer, 2022; Okada et al., 2003). But the CA1 region is not only involved in spatial learning. It also plays a role for temporal association learning.

4.4.2 Temporal association learning deficit in DKO mice

Temporal learning takes place, when nonsynchronous events need to be associated over a period of time (Kitamura, 2017). In the present study, a deficit in temporal association learning could be observed in Bcan and Ncan DKO mice (Figure 26E), while context fear learning was unaffected (Figure 26C). Contextual fear learning depends on proper function of hippocampal and amygdalar regions (Kochli et al., 2015; Sanders et al., 2003). Trace fear conditioning on the other hand involves temporal

association that is especially dependent on proper CA1 function (J. L. Rogers et al., 2006; Wilmot et al., 2019). Inhibiting the function of the basolateral amygdala, basal or lateral nuclei of the amygdala during trace fear conditioning leads to deficits in both CS and context retrieval (Kochli et al., 2015). As contextual fear learning was not affected by the DKO in the present study, it was ruled out that amygdalar function was compromised by Bcan/Ncan deficiency. It is likely that the dysplasia of the CA1 contributes to trace fear retrieval deficiencies in DKO mice.

Another possible explanation for the observed deficits in temporal association learning could lie within the medial prefrontal cortex. In a study by Hylin et al., 2013 the ECM, including PNNs, in the hippocampus and mPFC were digested with chondroitinase ABC and hyaluronidase prior to trace fear conditioning. While digestion of the ECM in the hippocampus impaired cue, trace and context retrieval, the digestion in the mPFC only affected cue and trace retrieval – similar to what was observed in the present study. PNNs in the mPFC were unaffected by Bcan/Ncan DKO (Figure 20), hence it was concluded, that the condensed ECM in the mPFC is of minor importance for the observed cognitive impairment. But the effect of the DKO on aggrecan and HAPLN1 (Figure 21) and other ECM components (Figure 22) hints towards changes in ECM composition, that could result in functional deviations, also in other brain areas.

Besides the hippocampal CA1, multiple other brain areas are involved in temporal association learning and retrieval. Apart from the already mentioned mPFC, the enthorinal, perirhinal and retrosplenial cortex (Kholodar-Smith et al., 2008; Kitamura, 2017; Kwapis et al., 2014) and the hippocampal dentate gyrus (Pierson et al., 2015) are important for proper trace fear conditioning. Furthermore, the interconnection of those regions (Kitamura, 2017), the excitability (Gilmartin & Helmstetter, 2010; Wiltgen et al., 2005) and network oscillations (Han et al., 2016) play a role for this complex behavior. The CA1 dysplasia in DKO mice likely plays a disruptive part in the orchestra of temporal association learning and might be the key to understand, why spatial and contextual learning and memory are unaffected by the DKO. But more experiments are needed to test this hypothesis.

4.5 Interaction of brain ECM and environmental factors in aged mice

Nature and nurture, both play a pivotal role for cognition during development and aging. Both, the genotype and environment interact and affect the physiological and cognitive phenotype. The neural ECM is likely involved in the mediation of environmental effects

on the brain structure and behavioral output (Cattaud et al., 2018; Scali et al., 2012; Stamenkovic et al., 2016).

One aim of this project was to elucidate the interactions of brain ECM components Bcan and Ncan and environmental enrichment, mostly via exercise, in aged mice. These investigations lead to a better understanding of age related changes to the ECM composition and how beneficial effects of exercise might be mediated via the ECM.

4.5.1 Impact of exercise on aged mice

The relationship of brain ECM and the environmental factor exercise is characterized by complex interactions throughout an animal's life. In this section, the manifestations of those interactions in different physiological and cognitive parameters shall be discussed.

At the structural level there is the interesting observation in brain (region) volume development from 4-5 to 20-21 months old mice, as discussed in chapter 4.3.1. Bcan and Ncan are indispensable for brain volume increase via exercise in young mice. While lifelong exercise offers a compensatory effect for DKO related brain volume reduction in old mice. Thus, the interaction of Bcan and Ncan DKO with lifelong exercise has no general effect on brain volume, rather it affects brain volume via aging. Aging goes along with several molecular and cellular changes. As especially Bcan but also Ncan are present throughout the whole adult brain in PNNs, loose ECM, at synapses and are involved in several cellular mechanisms, it is not easy to point towards one main mechanism that is accountable for the observations. Rather multiple different mechanisms might play a role here. As mentioned in chapter 4.3.1 the impact on other ECM component levels could be a clue. Alvarez-López et al., 2013 found that long-term exercise has a significant effect on the hippocampal ECM in aged mice. None of the ECM components found in that study were identified as significantly changed in the present study. This could be explained by different age groups and brain areas investigated. A more comprehensive investigation of the role of Bcan and Ncan in exercise-induced effects is needed to shed light on this interaction.

Apart from the differences observed in the two genotypes, summarizing the effects of exercise on WT animals raises some questions: In literature, an increase of hippocampal volume (Cahill et al., 2015), neurogenesis in the DG (Biedermann et al., 2014) and reduction of brain inflammation (Kohman et al., 2013) after exercise in aged mice have been reported. No changes in the mentioned parameters could be observed

in WT mice in the present study. This could be due to different age groups being investigated or different protocols in exercise, as many studies use short-term application of voluntary or forced exercise, instead of long-term or lifelong application. Also, the training intensity has an effect on outcomes (Cabral et al., 2019). Even though exercised mice from either genotype show a lower body weight during aging (Figure 9) – which suggests that running wheels were used consistently – the exercise intensity was not tracked and its inter- and intraindividual variability could be a potential explanation for observations deviating from literature.

No exercise-related effect was observed in ECM composition, neither in immunoblot of hippocampal tissue (Figure 21) nor in proteomics analysis of tissue from forebrain regions (cortex, hippocampus and striatum) (Figure 22). Literature on effects of exercise on ECM composition is sparse, even less is known about effects on aged mice. At least on a shorter time-scale, enrichment with exercise is able to increase matrix modulation in the hippocampus (Nishijima et al., 2015) and decrease PNN number and intensity in the cerebellum (Foscarin et al., 2011). In the present study, there was only a slight tendency for a higher WFA+ PNN intensity in the mPFC of both genotypes (Figure 20). This suggests, that indeed, even long-term exercise can have an effect on the ECM of aged mice and that exercise-related changes to the ECM vary between brain regions.

While the investigated ECM components are not affected by lifelong voluntary exercise, two non-ECM proteins stand out from the proteomics, that are highly upregulated in WT and DKO mice: FLII and BRIX1 (Figure 22). FLII RNA is expressed in basically all tissues and its manifold functions depend on place and degree of its expression (Strudwick & Cowin, 2020). The increased expression in aged exercised mice could be due to its involvement in metabolic regulation (J. S. Choi et al., 2015; Shamsi et al., 2020), as the metabolism is modulated by exercise, also in the brain (Matura et al., 2017). Localizing FLII expression via immunohistochemistry could give more information about its exact role in the brain of exercised mice. BRIX1 levels are probably similarly affected by exercise and subsequent changes in protein biosynthesis, as its homologs in *Xenopus laevis* and yeast are involved in ribosome synthesis (Kaser et al., 2001).

From the behavioral side, lifelong exercised old WT and Bcan/Ncan DKO mice both present some of the well-known effects of exercise, such as a decrease of anxiety (Figure 10C) and improved spatial learning (Figure 24) (Pietrelli et al., 2012; S. Wang

et al., 2015). Although it is remarkable, that the beneficial effect was not present in all cognitive tasks. Spatial learning in the novel location recognition task did not improve by lifelong exercise (Figure 23C), nor did fear memory in the trace fear conditioning task (Figure 26C&E). Cognitive flexibility in the reversal learning tasks was also not improved (Figure 25, Figure 27). No improvement of novel location recognition in contrast to the WCM might derive from differences of spatial learning within the tasks. For maze learning tasks, the positive effect of exercise is well established (Anderson et al., 2000; Mustroph et al., 2012). Those tasks rely on the animal's ability of wayfinding based on distal cues (Vorhees & Williams, 2014). While in the novel location task, the mice discriminate the objects' location based on mostly proximal cues. Spatial navigation based on different kinds of visual cues relies on different brain regions (Chan et al., 2012) and thus can lead to different outcomes in spatial learning tasks.

Although improvements in fear learning by exercise have been reported (Falls et al., 2010), other studies could not confirm those observations (Kohman et al., 2012; O'Leary et al., 2019). As already mentioned, this could be due to differing protocols of exercise and testing, especially since exercise is usually applied short-term. Certain paradigms for enrichment are also reported to induce stress in male mice via unstable social hierarchies (Haemisch et al., 1994) which could result in a reduction of beneficial effects. But stress usually goes along with increased inflammation (Gárate et al., 2013). As no differences in inflammation by rearing condition could be found in the present study (Figure 17, Figure 18, Figure 22), it is unlikely, that the enrichment used in the present study had detrimental effects for overall stress in the aged animals.

One interesting observation could be made when assessing the interaction of rearing environment and genotype on cognitive flexibility: DKO mice from standard rearing show better cognitive flexibility in reversal learning tasks than exercised DKO mice (Figure 25, Figure 27). Similarly, in the novel object recognition task DKO mice from standard rearing show better learning success than WT mice from standard rearing (Figure 23D). This suggests, that cognitive tasks that rely on other brain areas than the hippocampus have an improved function in DKO mice. Potential candidate brain areas and underlying reasons will be highlighted in the next chapter.

4.5.2 Cognitive flexibility is improved in DKO mice from standard rearing

Cognitive flexibility is the ability to adjust previously learned strategies according to unanticipated changes in conditions. The relearning goes along with inhibition of

learned responses, to allow a shift to a formerly irrelevant criterion. Especially the mPFC is involved in cognitive flexibility (Klune et al., 2021).

In the ASST standard reared DKO mice showed better reversal learning performance than exercised DKO, while the performance was not better compared to standard reared WT mice (Figure 27C). However, in the WCM reversal learning, DKO mice from standard rearing seem to outperform every other group (Figure 25). This indicates a good cognitive flexibility in old standard reared DKO mice. But why does the cognitive flexibility not improve by exercise, as it has been shown by other studies (Masley et al., 2009)? Perhaps the answer lies within the development of the mPFC and possible adverse effects from the enrichment. The development of the mPFC does not end until adolescence, especially the balance between excitation and inhibition underlies late developmental changes (Klune et al., 2021). This renders the mPFC particularly susceptible for environmental interventions and indeed, the negative effects of early life stress on the mPFC are well documented (Breach et al., 2019; Ohta et al., 2020; H.-L. Wang et al., 2019). Possibly, the used form of enrichment triggered increased stress by destabilizing social hierarchies specifically during adolescence which led to a long-term misfunctioning mPFC. Additionally, the DKO mice could be specifically affected by this due to the relevance of the CA2 region for social aggression behavior (Leroy et al., 2018). Enrichment increases PNN staining of the CA2 region (Carstens et al., 2016), probably the lack of Bcan and Ncan affects the reorganization of the CA2 PNNs, so that DKO mice are rendered susceptible to social stress during adolescence. To investigate the validity of this hypothesis, the effect of the DKO on social interactions and on mPFC development needs to be explored.

This might explain the rather detrimental effect of exercise on DKO mice during reversal learning. But why do DKO mice from standard rearing show a better performance in the first place? Indeed, there is evidence that the removal of ECM in the auditory cortex improves cognitive flexibility in an auditory learning task (Happel et al., 2014). Both, Bcan and Ncan are involved in mechanisms of plasticity, removing them may affect the excitability of inhibitory interneurons (Favuzzi et al., 2017). The superior performance of ST reared DKO mice in the novel object recognition task suggests that the perirhinal cortex could also be affected by the DKO (Cinalli Jr et al., 2020). Clearly, it is worth investigating other brain areas besides the hippocampus and mPFC for structural and functional changes, to get behind all the observed cognitive differences.

4.6 Conclusion and Outlook

In this study the effects of aging and EE on ECM composition, brain structure and cognition were elucidated.

The DKO of Bcan and Ncan affects the composition of the ECM, specifically in aged mice, but also in young adult animals. With HAPLN1 and 4, direct interaction partners of the lecticans are affected. Aggrecan and HAPLN1 show an upregulation in old, respectively young DKO mouse hippocampus, this shift probably goes along with age-related functional decline. A similar shift can be seen in the hippocampal volume of exercised DKO mice: The DKO differently affects brain area volume in young adult and aged exercised mice. These intriguing results emphasize the need to study the ECM throughout the life span. Also, the CA1 dysplasia indicates that it is well worth investigating the effect of Bcan and Ncan during development and maturation. While the DKO affects brain volume in exercised mice during the course of life, standard reared DKO animals show a reduced brain volume, compared to WT, independent from age.

Interestingly, the CA1 dysplasia in DKO mice does not lead to a general impairment of hippocampal learning. But specifically trace learning, a type of temporal association learning, is impaired in old DKO mice. While this defect could be explained by CA1 dysfunction, further demarcation from other possibly contributing brain areas would help to better understand the observed impairment. Apart from deficits in temporal association learning, aged DKO mice show enhanced cognitive flexibility, compared to WT. Further investigations could help understand the connection between the effects of the DKO on brain volume and ECM composition and the observed cognitive outcome. While PNNs were examined as a first possible link, thorough investigations of other ECM structures like perisynaptic ECM are needed. Furthermore, the observed differences in ECM composition between DKO and WT mice could manifest in alterations in inhibition or excitation of neuronal networks (Dzyubenko et al., 2021), which could affect neuronal oscillations that are involved for example in trace fear learning (Han et al., 2016). This could be explored via measurements of electrophysiological properties.

Last but not least, beneficial effects of exercise on cognition were not mediated by ECM composition, quite the contrary, DKO mice from standard rearing showed better cognitive abilities than their exercised counterparts. While this shows, that the lack of ECM components can have a pro-cognitive effect, it remains to be explored, why the

enrichment seemingly had a detrimental effect on DKO mice that led to a suppression of the improved cognitive abilities. The impact of enrichment on social hierarchies (Haemisch et al., 1994) could be a possible lead worth following.

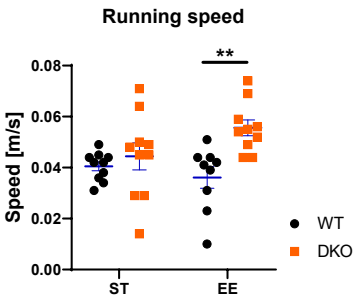
Exercise has complex effects on cognitive abilities and brain volume change during aging in DKO mice that need further investigations.

5 Supplements

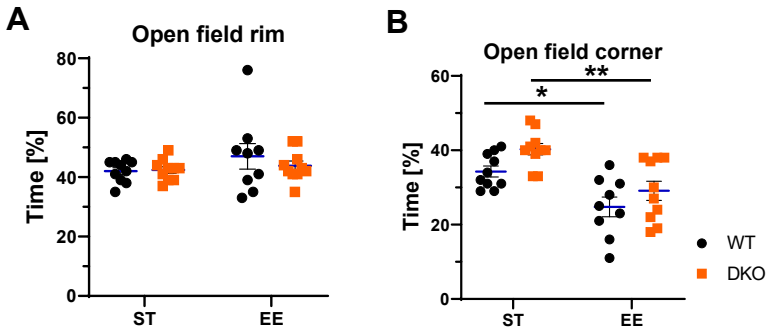
Appendix 1: Table of SHIRPA results.

	Level	Comment	WT ST	WT EE	DKO ST	DKO EE
Respiration rate	0	irregular				
	1	shallow				
	2	normal	10	10	8	10
	3	hyperventilation				
Tremor	0	none	10	10	8	10
	1	mild				
	2	marked				
Body position	0	completely flat				
	1	lying on side				
	2	lying prone				
	3	sitting or standing	10	10	8	10
	4	rearing on hin legs				
	5	repeated vertical leaping				
Spontaneous activity	0	none, resting		1		
	1	casual scratch, groom, slow movement	4	4	4	3
	2	vigorous scratch, groom, moderate movement	5	4	4	5
	3	vigorous, rapid movement	1	1		2
	4	extremely vigorous, rapid movement				
Transfer arousal	0	coma				
	1	prolonged freeze, then slight movement				
	2	extended freeze, then moderate movement				
	3	few seconds freeze, then active movement	10	10	8	10
	4	momentary freeze, then swift movement				
	5	no freeze, immediate movement				
Palpebral closure	0	eyes wide open	10	10	8	10
	1	eyes half closed				
	2	eyes closed				
Startle Response	0	no response			1	2
	1	Preyer's reflex	10	10	7	8
	2	small jump (< 1 cm)				
	3	large jump (> 1 cm)				
Gait	0	normal	9	5	7	6
	1	fluid but abnormal	1	5	1	4
	2	limited movement only				
	3	incapacitated				
Pelvic elevation	0	markedly flattened				
	1	barely touches				
	2	normal (~3 mm elevation)	9	8	8	7
	3	elevated (>3 mm elevation	1	2	1	3
Tail Elevation	0	dragging				
	1	horizontally extended	10	10	8	9

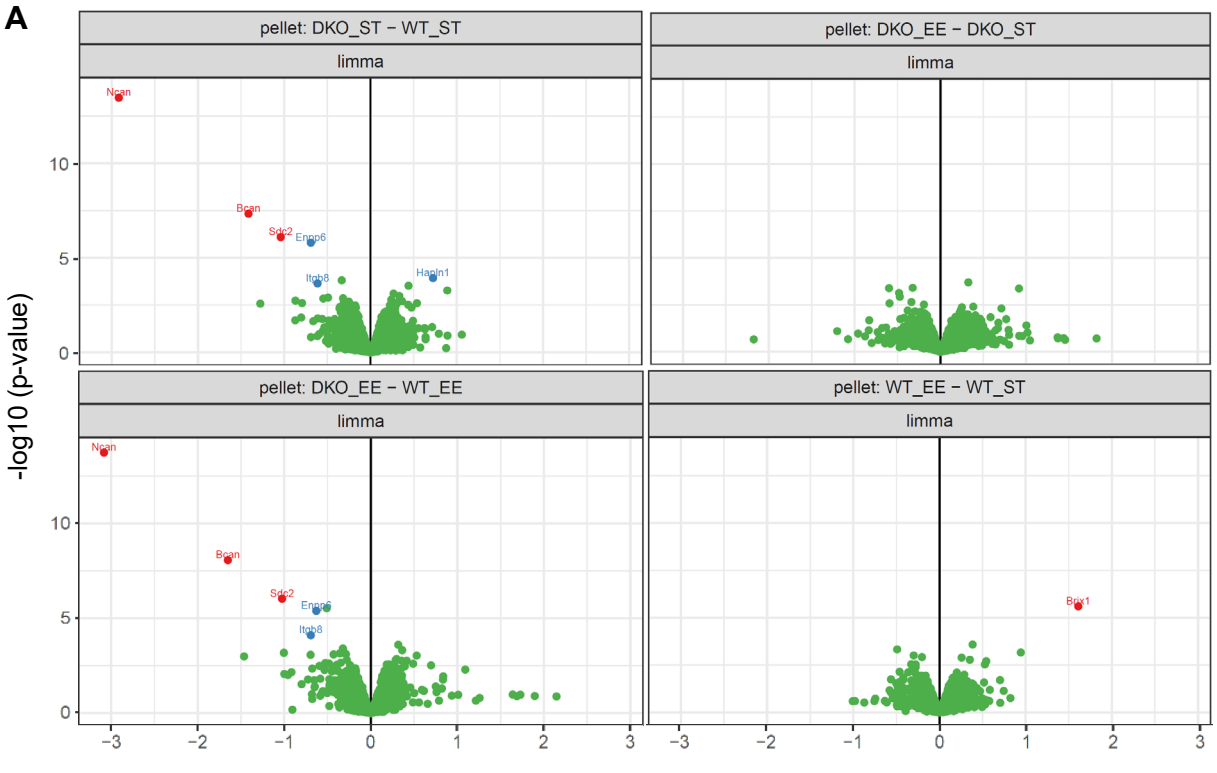
	2	elevated/straub tail				1
Touch Escape	0	no response				
	1	mild (escape to firm stroke)	4	4	2	5
	2	moderate (escape to light stroke)	6	6	6	5
	3	vigorous (escape as finger approaches)				
Trunk Curl	0	absent	10	10	8	10
	1	present				
Limb Grasping	0	absent	10	10	8	10
	1	present				
Visual Placing	0	none				
	1	upon nose contact				
	2	upon vibrissa contact	5	4	5	3
	3	before vibrissa contact (~18 mm)	4	5	3	6
	4	vigorous early extension (~25 mm)	1	1		
Grip strength	0	none				
	1	slight grip, semi-effective	6	6	2	3
	2	moderate grip, effective	4	4	6	4
	3	active grip, effective				2
	4	unusually effective				
Pinna Reflex	0	none	10	10	8	10
	1	moderately brisk flicking				
	2	repetitive flicking				
Corneal Reflex	0	none				
	1	active single eye blink	10	10	8	10
	2	multiple eye blinks				
Negative Geotaxis	0	turns and climbs the grid	9	6	7	9
	1	turns but then freezes				
	2	moves, but fails to turn				
	3	does not moce within 30s				
	4	falls off the grid	1	4	1	1
Aggression	0	none	10	10	8	10
	1	biting or attack				
Fear Behavior	0	none	10	10	8	10
	1	freezes during transfers				
Vocalization	0	none	10	10	8	10
	1	squeaking during handling				
Defecation		Ø	2.556	1.1	1.25	2.7
Urination		no. of mice	5	0	4	2
Time till moving		Ø	4.778	4	3.5	5

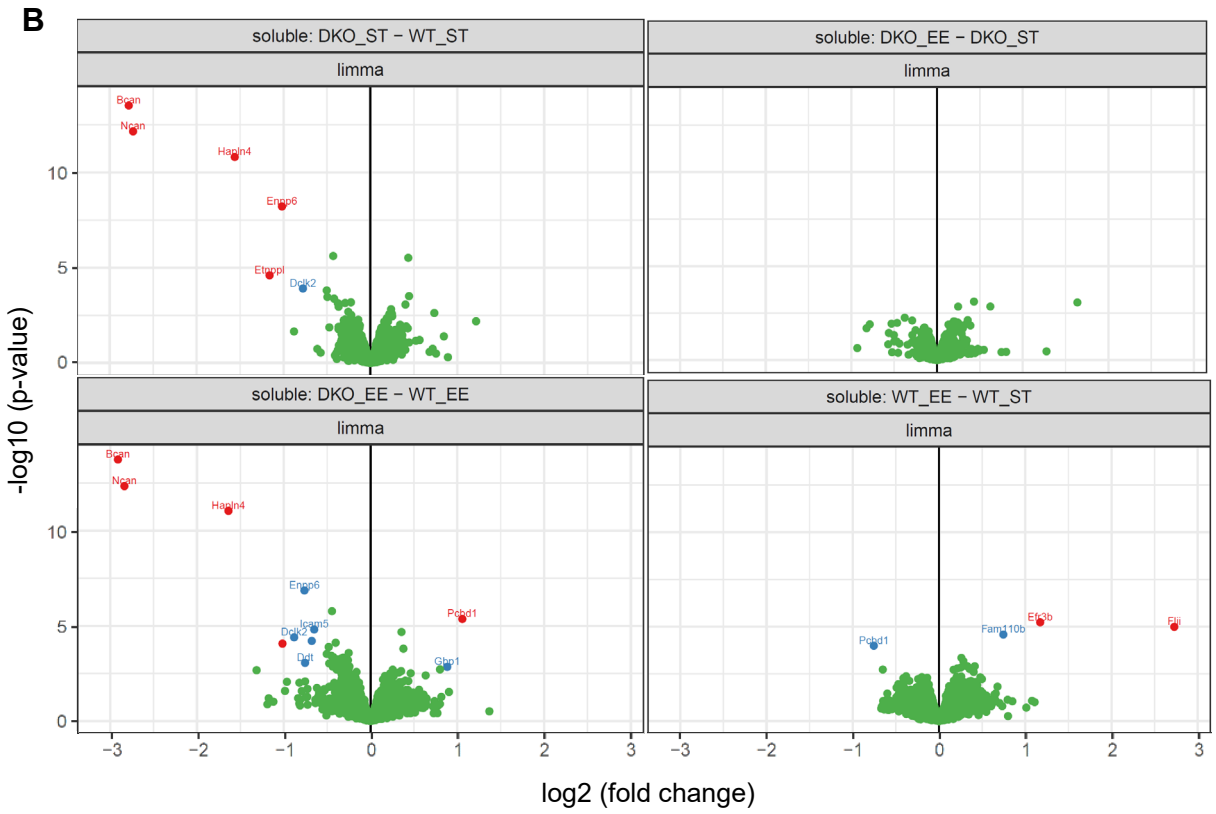


Appendix 2: Running speed in the open field during bright light. Genotype significantly affects running speed in the open field (F (1, 35) = 9.337, p = 0.004). The difference is particularly high in the exercised groups (p = 0.006). This also presents as an interaction of genotype x rearing type (F (1, 35) = 4.148, p = 0.049). (n = 10 per group, except WT EE n = 9).

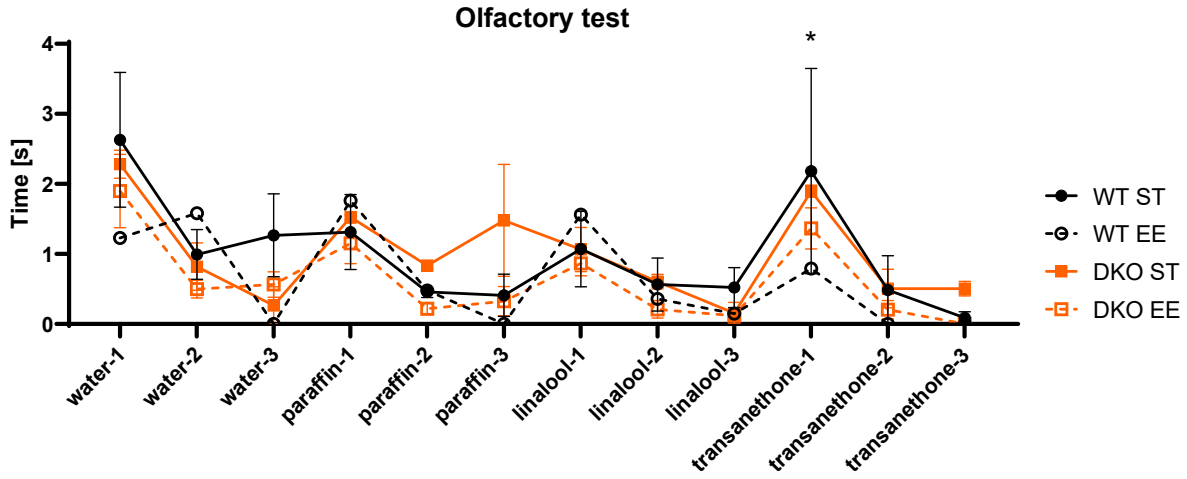


Appendix 3: Anxiety test in the open field. (A) While there are no differences in time spent in the rim of the open field, **(B)** rearing type significantly affects the time spent in the corner (F (1, 35) = 23.98, p < 0.0001). Group comparisons reveal significant differences between WT ST and WT EE (p = 0.0172) and DKO ST and DKO EE (p = 0.003). The factor genotype also affects the time spent in the corner (F (1, 35) = 5.95, p = 0.0199). (n = 10 per group, except WT EE n = 9).





Appendix 4: Proteomics volcano plots. (A) Volcano plots of the pellet fraction. **(B)** Volcano plots of the soluble fraction.



Appendix 5: Olfactory test. Protocol according to Arbuckle et al., 2015. 3-way ANOVA shows a significant dishabituation when transanethone is presented to the mice (asterisk)($F(1, 16) = 6.934$, $p = 0.0181$), indicating proper olfactory function.

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