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Aggrecan directs extracellular matrix mediated neuronal plasticity

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ABSTRACT: In the adult brain, the extracellular matrix (ECM) influences recovery after injury, susceptibility to mental disorders, and is in general a strong regulator of neuronal plasticity. The proteoglycan aggrecan is a core component of the condensed ECM structures termed perineuronal nets (PNNs), and the specific role of PNNs on neural plasticity remains elusive. Here, we genetically targeted the *Acan* gene encoding for aggrecan utilizing a novel animal model. This allowed for conditional and targeted loss of aggrecan *in vivo*, which ablated the PNN structure and caused a shift in the population of parvalbumin expressing inhibitory interneurons towards a high plasticity state. Selective deletion of the *Acan* gene in the visual cortex of male adult mice reinstated juvenile ocular dominance plasticity, which was mechanistically identical to critical period plasticity. Brain-wide targeting improved object recognition memory.

SIGNIFICANCE STATEMENT: The study provides the first direct evidence of aggrecan as the main functional constituent and orchestrator of PNNs, and that loss of PNNs by aggrecan removal induces a permanent state of critical period-like plasticity. Loss of aggrecan ablates the PNN structure, resulting in invoked juvenile plasticity in the visual cortex and enhanced object recognition memory.

INTRODUCTION

The capability of the brain to adapt and change alters during the lifespan, decreasing with age. This limited adult brain plasticity is advantageous to stabilize functional neuronal circuits but limits recovery of function after injury. In juvenile animals, immature cortical circuits are particularly malleable at certain stages of development termed critical periods (CPs) when adequate sensory stimuli are necessary to refine cortical circuits into functional units. Closure of the CPs and concomitant loss of juvenile plasticity coincide with maturation of inhibitory circuits (Hensch, 2004) and condensation of extracellular matrix around subtypes of neurons. These perineuronal nets (PNNs) are enriched in chondroitin sulfate proteoglycans (CSPGs) and associated matrix molecules (Hartig et al., 1994; Deepa et al., 2006). Remarkably, enzymatically degrading the CS glycosaminoglycan (GAG) component (Pizzorusso et al., 2002) or ablating the cross-linking cartilage link protein 1 (Crtl1) (Carulli et al., 2010) restores juvenile plasticity in the adult animal and affects memory and learning, as revealed by behavioral tasks such as fear memory extinction (Gogolla et al., 2009) and spontaneous object recognition (Romberg et al., 2013). However, the identity of the CSPG responsible for these effects has not been determined. Here we investigate the role of the CSPG aggrecan, a major constituent of PNNs, encoded by *Acan* (Matthews et

al., 2002). Aggrecan in PNN formation has only been studied *in vitro* (Giamanco et al., 2010; Kwok et al., 2010), as mutation in the *Acan* gene results in embryonic lethality due to the lack of cartilage in the trachea and other critical structures (Rittenhouse et al., 1978). In this study we developed a conditionally gene-targeted *Acan* mouse model to address aggrecan's role in PNN formation, plasticity, and memory function in the brain.

MATERIALS AND METHODS

Animal housing and ethics

All animal work was carried out in accordance with national law and regulation, in Cambridge in accordance with the UK Animals Scientific Procedures Act (1986) and in Oslo by the approval of the Norwegian Animal Research Committee. Animals used in this study were housed in standard housing conditions with a 12 hour light/dark cycle. Animals were housed in groups of 2-5 per cage, with the exception of one individual. Animals were fed and watered *ad libitum*.

Generation of Acan-loxP and breeding strategy

Two JM8A3.N1 embryonic stem (ES) cell lines of agouti C57BL6/N origin (Pettitt et al., 2009); #HFP0602_5_G11 and #HFP0602_5_F12, carrying the targeted *Acan* gene were purchased from the European Mouse Mutant Cell Repository (EuMMCR). The two ES cell lines are clones containing the same transgenic cassette surrounding exon 4 of the mouse *Acan* gene (Fig. 1a) a general "knock-out first" design used by the EUCOMM project, termed transgenic allele *tm1a* (Skarnes et al., 2011). Genomic DNA from the ES cell lines **was** analyzed by southern blotting to confirm correct genetic targeting. ES cells were plated and prepared according to the supplier's guidelines before microinjection into 50 blastocysts of albino C57BL/6J tyr-/- origin and subsequent transfer to 3 pseudo pregnant females per line. The chimeric offspring, hybrid F_1 generation, was selected by appearance of coat color spots originating from transgenic cells. The F_1 animals were then crossed with albino C57BL/6J tyr-/- , and transmission of genotype into G_1 generation was detected by coat color and verified by PCR genotype analysis using GT3 and WT primers (Table 1) confirming the heterozygote *tmc1a* transgenic animal. The *tm1a* allele is not viable in a homozygous state, as the transgenic cassette interferes with *Acan* expression. The mouse was further crossed with the ROSA26::FLPe strain expressing FLP-*FRT* recombinase removing the majority of the transgenic cassette leading to off spring carrying the Cre-*lox* conditional knock-out allele *tm1c* (Fig. 1a), designated as the conditional knock out of *Acan*; B6(Cg)-

ACAN$^{tm1c(EUCOMM)Hmgu>/Jwfa}$, hereafter referred to as Acan-loxP. PCR genotype analysis using primer pairs FLP, tmc1, and GT5, confirmed presence of FLP recombinase and presence of *tm1c*. The *tm1c* allele is viable as a homozygote. Conditional knock-out of Acan is achieved by removing exon 4 of the *Acan* gene by Cre-lox recombination resulting in the *tm1d* allele (Fig. 1a). Loss of exon 4 induces a shift in the reading frame, and thereby prevents protein translation.

Brain-wide and developmental Cre-lox recombination by Nestin-CRE

To achieve brain-wide knock-out of *Acan,* Acan-loxP$^{+/+}$ was crossed with Nestin-CRE (CreNes), where Cre recombinase is expressed under the control of the promoter and the nervous system-specific enhancer present in the second intron of the rat Nes gene (Tronche et al., 1999). The offspring of Acan-loxP$^{+/-}$ CreNes mice were then back crossed for one at least one generation. The offspring of this cross; WT littermates, Acan-loxP$^{+/+}$, Acan-loxP$^{+/-}$ Cre, and Acan-loxP$^{+/+}$ Cre, were used for histological analysis and behavioral testing. Again, PCR genotype analysis was conducted using WT, GT3, GT3WT and CRE primer pairs (Table 1). All of the resulting offspring had a normal phenotype and body size (data not shown). Juvenile animals explored and developed normally and were able to rear and feed themselves (experimenter observations). All genotypes were established using PCR on genomic DNA extracted from an ear punch.

Table 1: Primers used for genotyping

Primer Set	Forward	Reverse	Annealing temp. (cycles)	Expected band size
CRE	5'-GCAGAACCTGAAGATGTTCGCGAT-3'	5'-AGGTATCTCTGACCAGAGTCATCC-3'	62°C (34 cycles)	733bp
FLP	5'-GTCCACTCCCAGGTCCAACTGCAGCCCAAG-3’	5'-CGCTAAAGAAGTATATGTGCCTACTAACGC-3’	64°C (35 cycles)	725bp
Tm1c	5’-AAGGCGCATAACGA	5’-ACTGATGGCGAGCTCA	58°C (34 cycles)	1055bp

	TACCAC-3’	GACC-3’		
GT3	5’-GAGGGCATACAGTCCACCAT-3’	5’-GTGGTATCGTTATGCGCCTT-3’	60°C (30 cycles)	613bp
GT3 WT	5’-GAGGGCATACAGTCCACCAT-3’	5’-CCATCATCAGGCATTCTCCTT-3’	61°C (30 cycles)	613bp
GT5	5’-AAGGCGCATAACGATACCAC-3’	5’-CCGCCTACTGCGACTATAGAGA-3’	58°C (30 cycles)	7092bp (pre FLP cross) 218bp (post FLP cross)

Local and acute Cre-lox recombination by virus and retrograde tracer injection

AAV9.hSyn.HI.eGFP-Cre.WPRE.SV40 (Catalog number AV-9-PV1848) was purchased from University of Pennsylvania Vector Core, and stored at -80˚C. Prior to surgery, aliquots were thawed on ice and diluted in filtered 1X PBS to a concentration of 10^{13} GC/mL. Adult mice (3-4 months old) of either Acan-loxP$^{+/+}$ or Acan-loxP$^{+/-}$ genotype were anesthetized with isoflurane mixed with air (5% induction, 1.0-1.8% maintenance) using a Somnosuit vaporizer (Kent Scientific, TX, USA) and a custom made mask, and placed on a controllable heating pad in a stereotaxic frame. They were injected subcutaneously with buprenorphine (0.04mg/kg) and carprofen (5 mg/kg), and bupivacaine (13 mg/kg) locally. The scalp was shaved and cleaned with 70% ethanol and chlorhexidine, and a small incision made in the skin. A craniotomy (1-1.5 mm diameter) was performed above the binocular zone of primary visual cortex using a hand-held dental drill. The virus solution was loaded into a glass pipette (opening diameter approximately 15 µm) and mounted in a Nanoject 3 micro injector (Drummond Scientific, PA, USA). A total of 500 nL was injected in a stepwise manner over 10 minutes, and the pipette left in the tissue for 10 minutes before retraction (injection coordinates relative to lambda were AP 0.0 mm, ML 3.0 mm, DV 0.5 mm). The wound was rinsed repeatedly with sterile 0.9% NaCl, and closed by suture. The animals were observed for at least one hour before they were placed back in the colony. Animals were monitored closely for a week following surgery, and given subcutaneous injections of buprenorphine and carprofen for the first three days after surgery. For retrograde tracing between V1 and the dorsal part of the lateral geniculate nucleus, Cholera toxin subunit B conjugated to AlexaFluor 488/594 was purchased from Life Technologies (CA, USA) and a 1% solution made by reconstitution in 1x PBS. Surgical procedures were as described above. The tracer injections were intentionally targeted to a more medial part of V1 to

prevent leakage into the neighboring V2L region. A total of 200nL was injected for each of the tracers at AP 0.0 mm, ML 2.7.0±0.1 mm DV 0.5mm, the injections of each conjugate separated by 0.2 mm. The wound was cleaned with sterile 0.9% NaCl and closed by suture. Medication was performed as described above.

Histology

At the end of experiment or appropriate time-point, animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused with either 0.9% NaCl (ocular dominance experiment) or $Na_2HPO_4.2H_2O$ (Acan-loxP CreNes) buffer, followed by 4% paraformaldehyde (PFA). Brains were dissected out and post fixed in PFA solution overnight and cryoprotected in 30% sucrose in 1x PBS for 24 hours. Coronal sections of 30 or 40µm were collected from the cryostat or freezing microtome and transferred to 1X PBS. Sections were rinsed in 1x PBS and blocked for 1 hour in 2-10% animal serum (species dependent on secondary antibody host) before primary antibody incubation overnight in blocking solution (Table 2). The following day, sections were rinsed and incubated with secondary antibodies for 2 hours. Sections were rinsed and mounted on Superfrost slides with FluorSave™ Reagent (Merck Millipore, Germany).

Following ocular dominance testing of AAV9.Cre injected Acan-loxP$^{+/+}$ mice wide-field fluorescence images were acquired with an AxioPlan 2 (Zeiss, Germany) microscope through a 10x objective, and high-resolution overview images stitched using the Mosaix extension in the AxioVision software. Confocal images were acquired using a FluoView FV1000, software version 1.7 (Olympus), through a 20x or 60x PlanApo objective. Image analysis was performed using ImageJ (NIH) and Photoshop CS4/6 (Adobe).

For histological quantification of 3-4 month-old Acan-loxP$^{+/+}$ x CreNes crosses images were captured using a Leica SPE confocal microscope using either x10 or x63 objectives with a 1024 x 1024 image resolution (n=3 per group). For PNN quantification at least 5 images per section (3 sections per brain, approximately 300µm apart) were taken on a single confocal plane in layers 4 and 5 of the mouse barrel cortex (Fig. 2). Images contained at least one WFA labelled PNN (if detected). For Acan-loxP$^{+/+}$ CreNes, where no PNNs were detected, representative images were captured randomly in the same region. For PV cell analysis by immunohistochemistry at least 5 z-stack images (10 stacks, ~3µm apart) were taken per section with at least 5 sections analyzed per animal (approx. 300µm apart). Images were then analyzed with an automated custom script using the EBImage package from R statistical software (Pau et al.,

2010). To reduce variance in antibody binding all groups used for quantification were histologically stained in batches using the same conditions and antibody solutions.

Protein and RNA analysis

For aggrecan protein and gene expression analysis, snap frozen Acan-loxP$^{+/+}$ and CreNes Acan-loxP$^{+/+}$ brains (n = 3 per group) were homogenized over ice and both protein and RNA was extracted using an AllPrep DNA/RNA/protein Mini Kit (Qiagen, NL). Prior to aggrecan protein quantification 20µg brain homogenate from each sample was de-glycosylated with chondroitinase ABC in PBS with acetic acid (50U/ml, ph7.8) for 24hrs at 37°C to remove GAG side chains from the aggrecan core protein. Following de-glycosylation samples were subjected to a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method using a 4-12% Bis-Tris Mini Gel (NuPAGE, Invitrogen, USA) and Western blotting protein transfer to a PVDF membrane. Membranes were left to air dry before being washed three times with 2% Triton X-100 in Tris buffer solution (TBS-T). After the final wash, membranes were incubated in a blocking buffer (10% skimmed milk made in TBS-T) for 1 hour. After blocking, membranes were then incubated in primary antibodies overnight at 4°C with 5% skimmed milk in TBS-T. The following morning, membranes were washed in triplicate in TBS-T and incubated in secondary antibody for 1 hour, after which they were washed again. Proteins were then visualized using the electrochemiluminescence (ECL) method. Protein quantification was achieved using ImageJ comparing aggrecan signal intensity relative to β-actin. For aggrecan all bands >250kd were included for analysis similar to previous studies (Miyata and Kitagawa, 2016) while β-actin produced a clear band at 37kd.

For gene expression analysis mRNA was converted to single stranded cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA). Samples were then analyzed using quantitative PCR with inventoried TaqMan probes (Applied Biosystems, USA) for *Acan* and *Actb*. A final concentration of 14ng of cDNA per reaction was used. All samples were run in triplicate and averaged prior to analysis. Measurements for the expression of *Acan* were then presented relative to *Actb* for graphing and statistical analysis.

Table 2: Antibodies used to label PNN components and PV+ cells

Primary antibodies				
Reactivity	**Host species**	**Supplier**	**cat #**	**RRID**
N-acetylgalactosamine	n.a. (lectin)	Sigma	L-1516	AB_2620171
Aggrecan	Rabbit	Millipore	ab1031	AB_90460
Crtl-1	Mouse	Millipore	MABT85	AB_11203324

Crtl-1	Goat	R&D	af2608	AB_2116135
GFP	Chicken	Life	ab13970	AB_300798
Neurocan	Sheep	Thermo	PA5-47779	AB_2608508
Versican	Rabbit	BosterBio	PB9453	not registered
Brevican	Mouse	BioLegend	820101	AB_2564837
tenascinR	Mouse	R&D	MAB1624	AB_2207001
Parvalbumin	Goat	Swant	PVG-214	AB_2313848
Parvalbumin	Rabbit	Swant	PV27	AB_2631173
Secondary used in biotin-tagging				
anti-mouse	Goat	Life	A-24522	AB_2535991
anti-goat	Donkey	abcam	ab6884	AB_954842
anti-rabbit	Goat	Life	31820	AB_228340

Quantification and statistics of the histology signal

Prior to image processing a 30% threshold was applied to the image to eliminate non-specific binding. Cells whose soma were in the Z-stack image were extracted and combined intensity values for all pixels in a cell were averaged and cropped images of PV positive cells were saved for manual verification. The AU scale was derived as before based on mean PV values extracted from the WT group.

Assessing OD plasticity in visual cortex using optical imaging of intrinsic signal

Visual stimulus was generated in Matlab using the Psychophysics toolbox, and presented on a 27.5 x 34 cm computer monitor (Dell Computers, 60 Hz refresh rate) placed 25 cm from the mouse. The stimulus consisted of a thin drifting bar (2˚ wide) moving up or down with temporal frequency of 0.17 Hz and spatial frequency of 0.05 cycles per degree. The stimulus was repeated 20 times, each presentation interleaved with a grey blank screen. The duration of each recording was 120 seconds. Stimulus was restricted to the binocular field of vision (20˚), with an offset of 5˚ towards the respective open eye.

Eight to ten weeks after virus injection the animals were prepared for imaging. The experimenter performing imaging experiments and analysis was blind to treatment group and genotype. A custom steel plate for head fixation was attached to the skull using cyanoacrylate and dental acrylic, leaving the area above the visual cortex open. This was covered by a thin layer of nitrocellulose (New-Skin) to prevent desiccation and reactive cell growth.

After head plate implantation, the first imaging of intrinsic signals was performed to measure baseline responses through each eye. Repeated optical imaging of intrinsic signals was performed as described (Kaneko et al., 2008). The mouse was anesthetized with isoflurane (2% for induction and 0.5% during recording) supplemented with an intramuscular injection of chlorprothixene (2mg/kg body weight). The mice were head fixed and placed on a heating pad in front of the computer monitor and images were recorded transcranially by a Teledyne Dalsa M30P camera (Teledyne, CA, USA) using two adjacently mounted Nikon 50mm 1.2 lenses, and custom Matlab software. Light was provided by LED lights mounted in a ring surrounding the camera lens. Two 525nm LEDs were used to focus on the surface blood vessels before focus was shifted 600 µm below the surface. The light source was switched to three 615 nm LED lights (XPEBRO-L1-R250-00B02 from Cree Inc., NC, USA) and emission light was filtered with a red interference filter (610 ± 10 nm). Images were acquired with a frame rate of 30 fps. The phase and amplitude of cortical responses at the stimulus frequency were extracted by Fourier analysis as described (Kalatsky and Stryker, 2003). The responses to stimulation of each of the eyes were recorded for 4-6 trials, alternating between which eye was covered. The median of the peak response amplitude (R) of all trials for each eye were used to calculate an ocular dominance index (ODI) by ODI = (R^{contra} - R^{ipsi} / R^{contra} + R^{ipsi}).

Immediately after baseline imaging, the eyelids to the contralateral eye was sutured shut. Lidocaine was applied to the sutured area and the animal returned to its home cage. After four days, the mice were again anesthetized, the closed eyelid was re-opened and the imaging procedure repeated as described above.

Assessing memory by spontaneous object recognition behavioral task

In order to gauge memory ability of Acan-loxP$^{+/+}$ Cre mice, a Spontaneous Object Recognition (SOR) behavioral task was used. During testing, male mice (n = >7 per group, approximately 3 months old) were placed individually in a Y-shaped maze (30cm high, all arms 16cm in length and 8cm wide) as previously described (Romberg et al., 2013). Briefly, animals were habituated to the testing arena for two consecutive days prior to testing where animals were allowed to explore freely for 5 minutes each day. Each testing session occurred during the dark phase and consisted of a sample phase and a choice phase, using the same objects as in Romberg et al. 2013. During the sample phase, a mouse was placed in the start arm of the maze with sample objects (two identical objects) placed in the two remaining arms and left to explore the objects freely for 5 minutes. After a 3 hours or 24 hour delay animals were returned to the maze for the choice phase. During the choice phase two new objects were placed in the sample arms,

one that is the same as the sample objects (familiar object) and one that was new to the mouse (novel object). For each delay time, mice underwent at least one testing session. All testing sessions were separated by a minimum of 48hrs to allow recuperation. The presentation of the object pairs and their appearance in either the left or right sample arm were counterbalanced so that a particular object in a set or a particular side might be novel for one animal and not for another to avoid unforeseen bias. The maze and all objects were wiped down with 70% EtOH in between each testing session in order to avoid any olfactory bias that could have been introduced during the previous testing session. Time spent exploring each object during the sample and choice phase was assessed and scored at a later date using the video recordings. A discrimination score was calculated for the choice phase by subtracting the amount of time exploring the familiar object from the amount of time exploring the novel object and then dividing by the total exploration time:

$$\frac{Time\ exploring\ novel - Time\ exploring\ familiar}{Time\ exploring\ novel + Time\ exploring\ familiar}$$

A positive score indicates recognition of the novel object, while a score of 0 or below indicates the mouse was unable to recognize the novel object. An average was taken for the total sample times per object and the discrimination scores for the choice per time delay.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Statistical analysis was performed using R (R Foundation) or GraphPad Prism 7 (Graphpad Software, CA, USA). Datasets were tested for normality using Shapiro-Wilk's test or Fisher's F test. Comparisons between two groups were tested by Welch's t-test or paired t-test. Multiple group analysis was achieved using a type 1 or type 3 ANOVA with the appropriate follow up test, either post hoc Tukey HSD or comparing two groups by Welch's t-test.

Biochemical quantification of aggrecan protein and the Acan transcript were conducted as described in the *Protein and RNA analysis* paragraph above. Separate brain homogenates from 3 male animals, 3-4 months of age from litters originating from the same breeding pair, belonging to each of the 2 experimental groups; Acan-loxP$^{(+/+)}$ and Acan-loxP$^{(+/+)}$Cre, were analyzed. For protein quantification ImageJ was used to quantify aggrecan signal which was normalized to β-actin signal, each lane corresponding to an individual animal (Fig. 1d). For mRNA quantification triplicates of 14 ng cDNA derived from each of the individual animals were run with TaqMan probes against *Acan* and *Actb*, and the relative signal was quantified by the accompanying software (Fig. 1e). The 2 experimental groups were compared and tested in a Welch's t-test.

Table 3: Statistical analysis biochemical quantification

Welch Two Sample t-test				
	T	Df	p-value	mean of x, mean of y
Ab1031 WB	11.365	2.7346	**0.002216** **	1.7059754. 0.7572072
qPCR	5.501	2.0556	**0.02963** *	0.7260774, 0.1914709
Signif. codes: '***' 0.001 '**' 0.01 '*' 0.05				

Quantification of PNN intensity by WFA and Aggrecan immunohistochemistry were conducted as described in the *Histology* paragraph above. There were 3 male animals of 3-4 months of age originating from the same lines of breeding in each of the 3 experimental groups; WT, Acan-loxP$^{(+/+)}$ and Acan-loxP$^{(+/-)}$Cre. At least 5 images from each of 3 sections from each brain of the individual animals were analyzed. Images were analyzed with an automated custom script using the EBImage package from R statistical software (Pau et al., 2010). For each experimental group, more than 150 and 50 PNN structures where quantified respectively by WFA and aggrecan histochemistry. Each PNN structure is represented as a dot in Figure 2b and 2c. The 3 experimental groups were tested in One-way ANOVA with a TukeyHSD post hoc test.

Table 4: Statistical analysis quantification of PNN intensity

Anova - WFA					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Group	2	0.234	0.1170	8.419	0.000244 ***
Residuals	695	9.660	0.0139		
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
Tukey multiple comparisons of means - WFA 95% family-wise confidence level					
	Diff	Lwr	upr	p adj	

tghetcre-tg	-0.04293423	-0.06804163	-0.01782683	0.0001934
wt-tg	-0.01396332	-0.04019866	0.01227202	0.4241323
wt-tghetcre	0.02897091	0.00310826	0.05483356	0.0236190

Anova - Aggrecan

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Group	2	0.082	0.04100	4.676	0.0104 *
Residuals	192	1.683	0.00877		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tukey multiple comparisons of means – Aggrecan 95% family-wise confidence level

	Diff	lwr	upr	p adj
tghetcre-tg	-0.041990928	-0.08357968	-0.0004021726	0.0472421*
wt-tg	0.009533334	-0.02685560	0.0459222690	0.8100014
wt-tghetcre	0.051524262	0.01058598	0.0924625400	0.0092913**

Quantification of PV intensity by PV immunohistochemistry was conducted as described in the *Histology* paragraph above. There were 3 male animals of 3-4 months of age originating from same lines of breeding, in each of the 4 experimental groups; WT, Acan-loxP$^{(+/+)}$, Acan-loxP$^{(+/-)}$Cre, and Acan-loxP$^{(+/+)}$Cre. 5 z-stack images, each consisting of 10 individual images 3 µm apart, were taken from each of 5 sections from each brain of the individual animals and analyzed. Images were analyzed with an automated custom script using the EBImage package from R statistical software (Pau et al., 2010). More than 150 PV positive cells were quantified for each experimental group and represented as a dot in Figure 2f. The 4 experimental groups were tested in One-way ANOVA with a TukeyHSD post hoc test.

Table 5: Statistical analysis quantification of PV

Anova - PV

	Df	Sum Sq	Mean Sq	F value	Pr(>F)

Group	3	6.65	2.2169	39.53	<2e-16 ***
Residuals	1160	65.05	0.0561		
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
Tukey multiple comparisons of means – PV 95% family-wise confidence level					
	diff	lwr	upr	p adj	
tgcre-tg	-0.09178599	-0.149596189	-0.033975795	0.0002750***	
tghetcre-tg	-0.16498059	-0.228473244	-0.101487941	0.0000000****	
wt-tg	0.04478937	-0.002047122	0.091625869	0.0668269	
tghetcre-tgcre	-0.07319460	-0.138715560	-0.007673641	0.0214535*	
wt-tgcre	0.13657537	0.087024012	0.186126719	0.0000000****	
wt-tghetcre	0.20976997	0.153692925	0.265847008	0.0000000****	

Quantification of PNN intensity by WFA after local removal of Acan was conducted as described in the *Histology* paragraph above. There were 6 male animals (3-4 months) originating from different litters but from the same breeding pair in which each of the two hemispheres contributed to 2 experimental groups; Controlateral control and AAV9.Cre injected hemispheres. 3 sections from each of the individual animals were imaged. Each of the hemispheres of the individual animals is represented as a dot in Figure 3c. The 2 experimental groups were tested in a paired t-test. Results are stated in the legend of figure 3.

Assessing OD plasticity was conducted as described in the *Assessing OD plasticity in visual cortex using optical imaging of intrinsic signal* paragraph above. There were 4, 3, and 7 male animals aged 5-7 months originating from the same line two breeding pairs and some from the same litters in the 3 different experimental groups; Acan-loxP$^{(+/+)}$ untreated, Acan-loxP$^{(+/-)}$ AAV9.Cre injected, and Acan-loxP$^{(+/+)}$ AAV9.Cre injected. Responses to visual stimuli by each of the two eyes were recorded in 4-6 trials per eye. Each trial consisted of 20 repetitions of a visual stimulus of 120 seconds with simultaneous imaging of the cortex. Initially, baseline responses by the two individual eyes and corresponding ODI for all animals were determined (Fig. 1c, d and e). Filled colored circles indicate population means while open grey circles indicate individual animals. After MD of 4 days, the trials were repeated. Eye responses and ODI are represented in similar fashion. A shift in eye responses and ODI induced by MD of 4 day were tested in a paired t-test within the 3 experimental groups, or with Wilcoxon signed rank test if the distribution was not normal. Results are stated in the legend of figure 4.

Assessing memory of spontaneous object recognition was conducted as described in the *Assessing memory by spontaneous object recognition behavioral task* paragraph above. There were 7, 9 (which of one was singly housed), 10 and 7 male animals of ~3 months of age originating same line of breeding in

each of the 4 experimental groups; WT, Acan-loxP$^{(+/+)}$, Acan-loxP$^{(+/-)}$Cre, and Acan-loxP$^{(+/+)}$Cre. Average exploration times for all experiments in each of the experimental groups varied between 40 and 50 seconds (Fig. 5b). To assess differences in the ability to retain memory (Fig. 5c) Anova was used to compare variances between the groups prior to engaging in post hoc analysis. A significant difference was found in the timepoint variable which then justified the exploration of the timepoint differences within groups and reduced the likelihood that significant values were reached through chance.

Table 6: Statistical analysis spontaneous object recognition task

Anova Table (Type III tests) – SOR				
	Sum Sq	Df	F value	Pr(>F)
(Intercept)	1.10135	1	74.7174	3.939e-12 ***
Timepoint	0.45081	1	30.5840	7.362e-07 ***
Residuals	0.88441	60		
Welch Two Sample t-tests – SOR timepoint				
3hr v 24hr	T	Df	p-value	mean of x, mean of y
WT	2.5638	11.555	0.02547 *	0.291830, 0.127394
Homo	4.326	11.201	0.001154 **	0.27991577, 0.07462545
Het	2.7797	12.071	0.01658 *	0.26169284, 0.08900274
HomoCre	0.92565	9.832	0.3768	0.2668251, 0.2013353
Signif. codes: '***' 0.001 '**' 0.01 '*' 0.05				

RESULTS

This mouse model was established by acquiring JM8A3.N1 ES cells (Pettitt et al., 2009) carrying the "knockout-first" conditional *tm1a* (Fig. 1a) from the European Mouse Mutant Cell Repository (EuMMCR) (Skarnes et al., 2011). The initial strain was subsequently converted to B6(Cg)-ACAN$^{tm1c(EUCOMM)Hmgu>/Jwfa}$ hereafter referred to as Acan-loxP, where Cre recombinase induces an exon deletion and a frame-shift mutation in *Acan* (Fig. 1a).

Brain-wide targeting of *Acan* was achieved by crossing Acan-loxP with Nestin-CRE (CreNes) where Cre recombinase is expressed under the control of the promoter and the nervous system-specific enhancer present in the second intron of the rat *Nes* gene (Tronche et al., 1999).

Targeting aggrecan in the brain of Acan-loxP$^{+/+}$ CreNes mice was accompanied by complete loss of histochemical staining of PNNs in the cortex using the PNN marker *Wisteria floribunda agglutinin* (WFA) (Fig. 1b). Ablation of aggrecan was verified by immunohistochemistry and western blotting (Fig. 1c, d) and reduced *Acan* mRNA in the brain (Fig. 1e). In these animals PNNs detected by WFA were absent in the brain. The loss of aggrecan prevented aggregation of other PNN component ECM molecules into PNNs, effectively abolishing PNNs in the adult cortex (Fig. 1f, representative example from > 50 images per PNN component. Fig. 2a, overview of sampling). In contrast, the non-targeted Acan-loxP$^{+/+}$ mice with two intact alleles expressing *Acan,* and the targeted heterozygous Acan-loxP$^{+/-}$ CreNes mice having one intact allele, both had anatomically normal PNNs (Fig. 1b, f), although the intensity of the WFA staining was reduced in the heterozygote (Fig. 2b-e). The crucial dependence of aggrecan for PNN formation is in line with previous results from *in vitro* models demonstrating that aggrecan, link protein and hyaluronic acid are essential components of net formation and stability (Giamanco et al., 2010; Kwok et al., 2010). In contrast, knock-out of the link protein Ctrl1 has been shown to give only a partial reduction of WFA-positive PNNs (Carulli et al., 2010).

A shift in the distribution of WFA histochemical labeling intensity has been suggested to correlate with the degree of neuronal plasticity in learning and memory (Balmer et al., 2009). Recently, parvalbumin (PV) expression on GABAergic interneurons has been shown to be influenced by PNNs in the hippocampus (Yamada et al., 2015), and the differentiation state and activity of PV+ interneurons relate to learning and memory formation (Donato et al., 2013). To assess if the reduction or complete lack of aggrecan affects the local PV expression, z-stacked images were acquired, and mean PV intensities of neurons in somatosensory cortex were extracted (> 150 cells per group, 3- 6 sections per group). In both the heterozygous Acan-loxP$^{+/-}$ CreNes mice and homozygous Acan-loxP$^{+/+}$ CreNes mice there was a shift towards lower PV expression by PV+ interneurons in the cortex compared to wild type and Acan-loxP$^{+/+}$

354 controls (Fig 2f-h), indicating that even partial attenuation of the *Acan* gene induces a low-PV state. This
355 suggests that Acan-loxP$^{+/+}$-CreNes mice are in a state of high network plasticity.

356

357 To investigate the effect of locally removing aggrecan in the adult mouse brain, *Acan* was targeted by
358 injecting the viral vector AAV9.hSyn.HI.eGFP.WPRE.SV40 (AAV9.Cre) carrying Cre recombinase into
359 the primary visual cortex (V1) (Fig. 3a). The synapsin promoter restricted expression to neurons. Local
360 injections of AAV9.Cre in adult Acan-loxP$^{+/+}$ mice caused a complete loss of aggrecan and WFA labeling
361 in V1 eight weeks after virus injection (Fig. 3b, c). The combined expression of GFP and Cre by the virus
362 revealed loss of PNNs surrounding infected neurons, demonstrating that expression of aggrecan by
363 neurons is needed to form the PNN structure. Again, loss of aggrecan disrupted aggregation of the other
364 PNN components (Fig. 3e), suggesting that aggrecan is essential for sustaining PNNs in the visual cortex.
365 Together, these targeting experiments demonstrate an essential role for aggrecan in PNN formation.

366

367 PNNs have been implicated in limiting adult brain plasticity due to their; CS content (Pizzorusso et al.,
368 2002; Miyata et al., 2012), enclosing mesh structure (Carulli et al., 2010), ability to bind and sequester
369 active molecules (Beurdeley et al., 2012; Dick et al., 2013), and role in controlling (Kwok et al., 2011)
370 the activity of fast spiking PV positive neurons (Balmer, 2016; Lensjo et al., 2017). Although several
371 PNN CSPGs have been linked to plasticity regulation, the role of aggrecan, the backbone of PNN remains
372 to be investigated, and a full picture of plasticity regulation is lacking. Activity-dependent plasticity was
373 assessed by inducing ocular dominance (OD) plasticity in the binocular region of primary visual cortex
374 (V1) of adult mice using monocular deprivation (MD). Tracing of the connections between the lateral
375 geniculate nucleus and V1 revealed normal retinotopic connectivity between V1 and lateral geniculate
376 nucleus in the thalamus in the non-targeted Acan-loxP$^{+/+}$ animal (Fig. 4a). Visual responses before and
377 after MD were recorded using optical imaging of intrinsic signals (Fig. 4b), and all groups displayed
378 robust responses to visual stimuli (more than 2x the response compared to during grey screen
379 presentations) before MD (Fig 4d, "baseline"). Strikingly, four days of MD were sufficient to produce a
380 strong and significant shift in OD in adult AAV9.Cre injected Acan-loxP$^{+/+}$ mice, while no effect was
381 observed in AAV9.Cre injected Acan-loxP$^{+/-}$ mice or non-injected Acan-loxP$^{+/+}$ mice (Fig. 4c). Longer
382 lasting sensory deprivation has been shown to induce some OD plasticity also in adult mice, but the
383 mechanism differs from juvenile plasticity (Sawtell et al., 2003; Ranson et al., 2012). To determine if
384 PNN removal caused enhanced adult plasticity or instead reinstated juvenile plasticity, the responses to
385 stimulation of each eye before and after MD were investigated. We did not see the adult OD plasticity

pattern, characterized by strengthening of ipsilateral input to V1. Instead, the shift in OD was driven both by a potentiation of ipsilateral responses and a reduction in contralateral responses, indicating plasticity mechanisms similar to juvenile animals (Fig. 4d). No significant effects were observed in non-injected Acan-loxP$^{+/+}$ mice or AAV9.Cre injected Acan-loxP$^{+/-}$ mice (Fig. 4d).

Several interventions affecting PNNs have effects on learning and memory (Gogolla et al., 2009; Carulli et al., 2010; Romberg et al., 2013). The effect of brain-wide knockdown of aggrecan in learning and memory behavior was tested in the spontaneous object recognition paradigm (Romberg et al., 2013). In this paradigm, depicted in figure 5a, animals (> 7 per group) identify novel from familiar objects (choice phase) following a delay from familiar object presentation (sample phase). Animals were tested after three and 24 hour delays, at which times ChABC-treated and Crtl1 knockout animals show enhanced memory retention (Romberg et al., 2013). Following the three hours delay between sample and choice phases, all groups displayed a good ability to recognize the novel from familiar objects (discrimination ratio > 0.25). At 24 hours of the sample phase, the wild type, Acan-loxP$^{+/+}$ and Acan-loxP$^{+/-}$ CreNes groups (Fig. 5c) showed little remaining memory. Remarkably, the object recognition memory in the Acan-loxP$^{+/+}$ CreNes group was still sustained at 24 hours after object exposure (Fig. 5c), indicating a stronger recognition memory in mice lacking aggrecan. These results are very similar to those obtained after ChABC treatment and in Crtl1 knockouts, suggesting that the Acan knockout affects memory through effects on glycan chains in PNNs. Although heterozygous Acan-loxP$^{+/-}$ CreNes have reduced PNNs intensity and altered PV expression, alterations in their behavior were not detected.

DISCUSSION

It has been suggested that aggrecan-containing PNNs play an essential role in regulation of brain plasticity. However, the hypothesis has been difficult to test directly due to technical limitations such as the off-target effects of the PNN-degrading enzyme Chondroitinase ABC and the dependence on intact aggrecan for cartilage formation. In the current work, we established a conditional knock-out model for aggrecan and demonstrate that aggrecan is indeed required for the aggregation of PNNs and that its removal reopens juvenile plasticity in visual cortex and affects memory processing. This work reveals that aggrecan is essential for PNN formation *in vivo*, and specific loss of PNNs increase adult brain plasticity.

PNNs probably control synaptic plasticity and dynamics through several mechanisms. Most of these

417 depend on the actions of the sulfated CSPG glycosaminoglycan chains attached to the several CSPGs that
418 populate PNNs (Sorg et al., 2016), selective binding and facilitated internalization of the homeobox
419 transcription factor OTX2 which affects maturation of PV+ interneurons (Beurdeley et al., 2012),
420 binding and presentation of chemorepulsive semaphorin3A (Dick et al., 2013), and regulation of AMPA
421 receptor mobility (Frischknecht et al., 2009). These mechanisms are based on the distinctive PNN
422 structure; the tight cross-linking structures of matrix proteins, CS and hyaluronic acid polysaccharides
423 forming a condensed compact matrix surrounding the cell soma and proximal dendrites, and the ability of
424 the negatively charged sulfation patterns of the CS to allow specific interactions with positively charged
425 domains in PNN interacting proteins. Indeed, the ratio of 4- and 6-sulfated CS influences neuronal
426 plasticity (Miyata et al., 2012). Our data suggest that aggrecan plays a key role for the PNN structure,
427 possibly through the large number of glycan chains that it carries, but clearly by affecting formation.
428 Removal of aggrecan abolishes aggregation of PNNs, and essentially also incorporation of other ECM
429 components into the structure (Fig 1 and 3). Aggrecan is an efficient cross-linker interacting with
430 hyaluronic acid and link protein by its N-terminal G1 domain and the extensively CS GAG modified
431 central domain (Morawski et al., 2012). The three globular domains; G1, G2 and G3 interact with several
432 matrix molecules aiding cross-linking, which could explain the failure of other components to aggregate
433 in its absence. The increased plasticity after PNN removal could also be a direct consequence of neuronal
434 activity changes in PV^{+} cells. This has been shown to be an important factor in regulation of CP plasticity
435 (Hensch, 2004), and previous work has shown that PNN removal reduces the activity of PV+ neurons
436 (Balmer, 2016; Lensjo et al., 2017). The changes in activity could be a result of reduced ion buffering,
437 changes to the synaptic inputs onto PV+ cells, alteration within the PV cells due to changed interaction
438 with external molecular cues, or a combination of these.

439

440 Condensation of PNNs in sensory cortices correlates with closure of periods of heightened neuronal
441 plasticity in sensory systems. Our results show that monocular deprivation induces a strong and rapid
442 change in cortical activation in V1 after just four days of MD. The timeframe of this shift is faster than
443 what has been observed in CP aged mice (Sato and Stryker, 2008), but is in line with previous work on
444 adult mice with induced plasticity (Fu et al., 2015). While monocular deprivation in adults normally
445 effects only responses to ipsilateral eye stimulation, we observed changes in responsiveness to both contra
446 –and ipsilateral inputs to V1 (Fig. 4), similar to how this shift occurs in CP aged animals. This finding is
447 supported by our recent work which showed that brief MD in adult rats following enzymatic degradation
448 of PNNs caused a similar effect on recorded single units in V1, in both hemispheres relative to the

449 deprived eye (Lensjo et al., 2017). These effects differ from other manipulations to activate adult cortical
450 plasticity such as Lynx1 deletion (Morishita et al., 2010), which arises as a result of potentiated responses
451 to ipsilateral stimuli. Taken together, this suggest that the PNN is a determinant factor in closure of the
452 CPs in juvenile development, through its effect on PV+ neurons and action as a structural barrier.
453 Removal of aggrecan collapses the PNN structure and leads to a persistent state of juvenile-like plasticity.

454 Our data also show that populations of PV+ interneurons in both homozygous and heterozygous Acan-
455 loxP$^{+/+}$-CreNes mice are shifted to a state where they express less PV (Fig. 2). Others have suggested that
456 the level of PV in PV+ interneurons determine if the network is in a state of high or low plasticity
457 (Donato et al., 2013) through a mechanism involving gene expression of PV and the GABA-synthesizing
458 enzyme GAD67 and regulation of the activity of the neurons through changes in dendritic synaptic
459 contacts. Decreased PV expression and synapses are associated with hippocampal learning, while fear
460 conditioning leads to a high-PV state. PV+ neuron-controlled shifts in network state are suggested as an
461 integrated mechanism of learning and memory acquisition. ChABC treatment to degrade PNNs shifted
462 the network to a low-PV state, indicating that the glycans on CSPGs are a key component in controlling
463 PV interneuron influence on network state. Mounting evidence from other brain structures supports a
464 broader role for PNNs where they affect behavior, memory and learning processes. We show that PNN
465 removal by aggrecan knock-out improves object recognition memory (Fig. 5), in line with previous work
466 (Romberg et al., 2013). Recently, aggrecan and PNNs have been implied in various psychiatric disorders
467 (Pantazopoulos et al., 2015), and in the progression of neurodegenerative diseases (Suttkus et al., 2016).
468 Subtle differences in matrix and PNN structure might impact the balance between a resilient and a
469 susceptible and vulnerable state.

470 In the current study, the Acan-loxP mouse line has established a role for this CSPG in the control of
471 plasticity in visual cortex and in memory. Aggrecan is the key component for assembling and sustaining
472 the PNNs structure and function in the adult brain and may play a central role in regulating adult brain
473 plasticity.

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Author contributions:

DR, KKL, TD, SY, MRA, TH, MF, JWF and GD designed and planned the experiments. MRA, JWF and GD initiated the work and together with MF developed the Acan-loxP$^{+/+}$ mouse model. DR, KKL, MRA and GD performed breeding and genotyping. DR performed w.blot, qPCR, histology experiments and quantification of developmental and brain-wide knock-out of *Acan*. KKL performed histology and all surgical procedures in acute and local knock-out of *Acan*. TD performed monocular deprivation experiments assisted by TH and KKL. DR performed testing and analysis for behavioral experiments together with SY. MRA, MF, TH, JWF, and GD contributed with analysis and writing. The resulting manuscript was written by DR, KKL, MRA, TH, MF, JWF and GD.

FIGURES:

Fig. 1: Brain-wide targeting of the *Acan* gene results in abolition of WFA labelled PNNs and individual PNN components.

a: Schematic representation of the transgenic cassette designed by EuCOMMM; tm1a allele, containing exon 4 of the *Acan* gene, the subsequent conversion to tm1c by Flp-*FRT* recombination and the knock-out

allele tm1d by Cre-*lox* recombination. **b:** Representative image of cortical layers 1 – 6 from barrel cortex in wild type (WT), transgenic controls (Acan-loxP$^{+/+}$), heterozygous *Acan* knockout (Acan-loxP$^{+/-}$Cre) and homozygous *Acan* knockout (Acan-loxP$^{+/+}$Cre). Sections were stained with WFA (PNN marker, red) and DAPI (nuclear marker, blue), n = 3 per group and scale bar = 100µm. WT, Acan-loxP$^{+/+}$ and Acan-loxP$^{+/-}$Cre mice appear to have similar numbers and disposition of PNNs, while a complete abolition of PNN staining was observed in Acan-loxP$^{+/+}$Cre brains. **c:** Representative examples of western blot of brain homogenates from Acan-loxP$^{+/+}$ and Acan-loxP$^{+/+}$Cre mice using the polyclonal aggrecan antibody ab1031. **d:** Quantification of aggrecan protein. **e:** Quantification of *Acan* mRNA expression from Acan-loxP$^{+/+}$ and Acan-loxP$^{+/+}$Cre mouse brains. **f:** Representative images of immunohistochemistry of aggrecan, brevican, neurocan, phosphacan, versican, tenascin-R (Tn-R), link protein 1 (Crtl1), and brain link protein 2 (Bral2) from WT, Acan-loxP$^{+/+}$, Acan-loxP$^{+/-}$Cre and Acan-loxP$^{+/+}$Cre mouse brains. For antibodies, see table in Materials and methods. ** = $p < 0.01$, * = $p < 0.05$, error bars are ± SEM.

Fig. 2: Partial and total removal of aggrecan affects PNN and PV interneuron properties. a: Coronal brain section from a wild type mouse showing the approximate locations in the barrel cortex used for histological sampling (labeled by *). Section stained the perineuronal net marker *Wisteria floribunda* agglutinin (WFA, red) and aggrecan (ab1031, green). Scale bar = 500µm. **b-e:** Mean intensity values of WFA-positive (**b)** or aggrecan-positive (**c**) PNNs in the cortex of WT (purple), Acan-loxP$^{+/+}$ (blue) and Acan-loxP$^{+/-}$Cre (red) were extracted and analyzed. Acan-loxP$^{+/-}$Cre displayed a significant reduction in average WFA intensity and aggrecan intensity compared to WT and Acan-loxP$^{+/+}$ controls. **f:** Mean intensity values of PV+ neurons in the cortex of WT (purple), Acan-loxP$^{+/+}$ (blue), Acan-loxP$^{+/-}$Cre (red) and Acan-loxP$^{+/+}$Cre (green) were extracted and analyzed. Both Acan-loxP$^{+/-}$Cre and Acan-loxP$^{+/+}$Cre groups displayed a significant reduction in average mean intensity per PV+ cell compared to WT and Acan-loxP$^{+/+}$ controls. Same cortical locations were used for PV histological sampling as depicted for WFA and Aggrecan in a. **g:** Examples of high, medium and low expressing PV neurons. **h:** Categorization of individual cells based on mean PV intensity indicated a shift towards a low PV network configuration in the Acan-loxP$^{+/-}$Cre and Acan-loxP$^{+/+}$Cre groups compared to controls.

Fig. 3: Targeted knock-out of *Acan* in visual cortex of adult mice disintegrates PNNs.
a: Experimental time line and schematic illustration indicating injection of AAV9.CreGFP (AAV9.Cre) in V1 of Acan-loxP$^{+/+}$ mice. **b** and **c**: WFA labeled PNNs gradually disappear over time within the

AAV9.Cre treated area. Sections were co-stained for GFP to identify transfected neurons. Three weeks after injection the transfected neurons produced the GFP-Cre fusion protein. After 8 weeks all PNNs in the injected area in V1 were abolished. Fluorescence intensity measurements of WFA staining were significantly lower in the AAV9.Cre treated area compared to the corresponding area in the contralateral untreated hemisphere, p=0.0006 (paired t-test, n=6 mice). Measurements were performed across all cortical layers and averaged from 3 sections for each animal. **d** and **e**: Representative confocal images from AAV9.Cre treated area within V1 and the corresponding area in the contralateral untreated hemisphere from the same section. Local knock-out of *Acan* in V1 removed staining for aggrecan as well as Crtl-1, tenascin-R, versican and neurocan.

Fig. 4: Local removal of *Acan* in V1 reactivates juvenile ocular dominance plasticity.

a: Injection of the retrograde tracer Cholera toxin subunit B (CtxB) conjugated to different flurophores, at two closely aligned sites in V1 of non-targeted Acan-loxP$^{+/+}$ mice. Representative wide-field image from V1 (upper) and confocal images from the dorsal part of the lateral geniculate nucleus (dLGN) of the thalamus. Labeled projections stem from adjacent patches of neurons in dLGN, indicating a normal connectivity pattern with retinotopic organization. Sections were counter-stained with DAPI to distinguish brain areas, and data was reproduced in two mice. **b:** Experimental time line for optical imaging of intrinsic signals before and after monocular deprivation (MD) of adult mice. **c:** Ocular dominance index (ODI) calculated for responses to stimulating the ipsi –and contralateral eye separately, before and after four days of MD. Positive ODI indicates contralateral bias while negative ODI indicates ipsilateral bias. Four days of MD did not affect ocular dominance in non-injected Acan-loxP$^{+/+}$ mice (p=0.21, paired t-test, n=4 mice) or in heterozygous AAV9.Cre injected Acan-loxP$^{+/-}$ mice (p=0.32, paired t-test, n=3 mice). In AAV9.Cre injected Acan-loxP$^{+/+}$ mice, MD caused a significant shift in activation towards the open eye (p=0.003, paired t-test, n=7 mice). **d:** Stimulus evoked responses of intrinsic signals through the open or closed eye before and after four days of MD. The MD in AAV9.Cre injected Acan-loxP$^{+/+}$ caused both increased responses to ipsilateral stimulation (p=0.02, paired t-test, n=7 mice) and reduced responses to contralateral stimulation (p=0.009, paired-test, n=7 mice), while no effect was observed in controls. Acan-loxP$^{+/+}$, deprived eye responses: p=0.73 (Wilcoxon signed rank test), open eye responses: p=0.48 (paired t-test), Acan-loxP$^{+/-}$ AAV9.Cre injected, deprived eye responses: p=0.46

636 (paired t-test), open eye responses: p=0.70 (paired t-test). Open circles and grey lines indicates individual
637 animals, solid circles and thick lines indicate population mean±standard deviation.

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640 **Fig. 5: Targeting of *Acan* enhances object recognition memory.**

641 **a:** Diagram of the experimental paradigm. **b:** Mean sample times of WT, Acan-loxP$^{+/+}$, Acan-loxP$^{+/-}$Cre
642 and Acan-loxP$^{+/+}$Cre for 3 hour and 24 hour sample phases during spontaneous object recognition testing.
643 All animals participated well in the test with the average exploration time about 40 seconds. **c:** Mean
644 object recognition scores following 3 hour and 24 hour delays. A significant decrease in object
645 recognition ability was detected in the WT, Acan-loxP$^{+/+}$ and Acan-loxP$^{+/-}$Cre groups following a 24
646 delay between sample and choice phases while Acan-loxP$^{+/+}$Cre did not. ** = $p < 0.01$, * = $p < 0.05$, ns =
647 not significant.

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