Ankersmit Laboratory

for Diagnosis & Regeneration in Thoracic Diseases & Applied Immunology

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Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcomes in a traumatic brain injury mouse model

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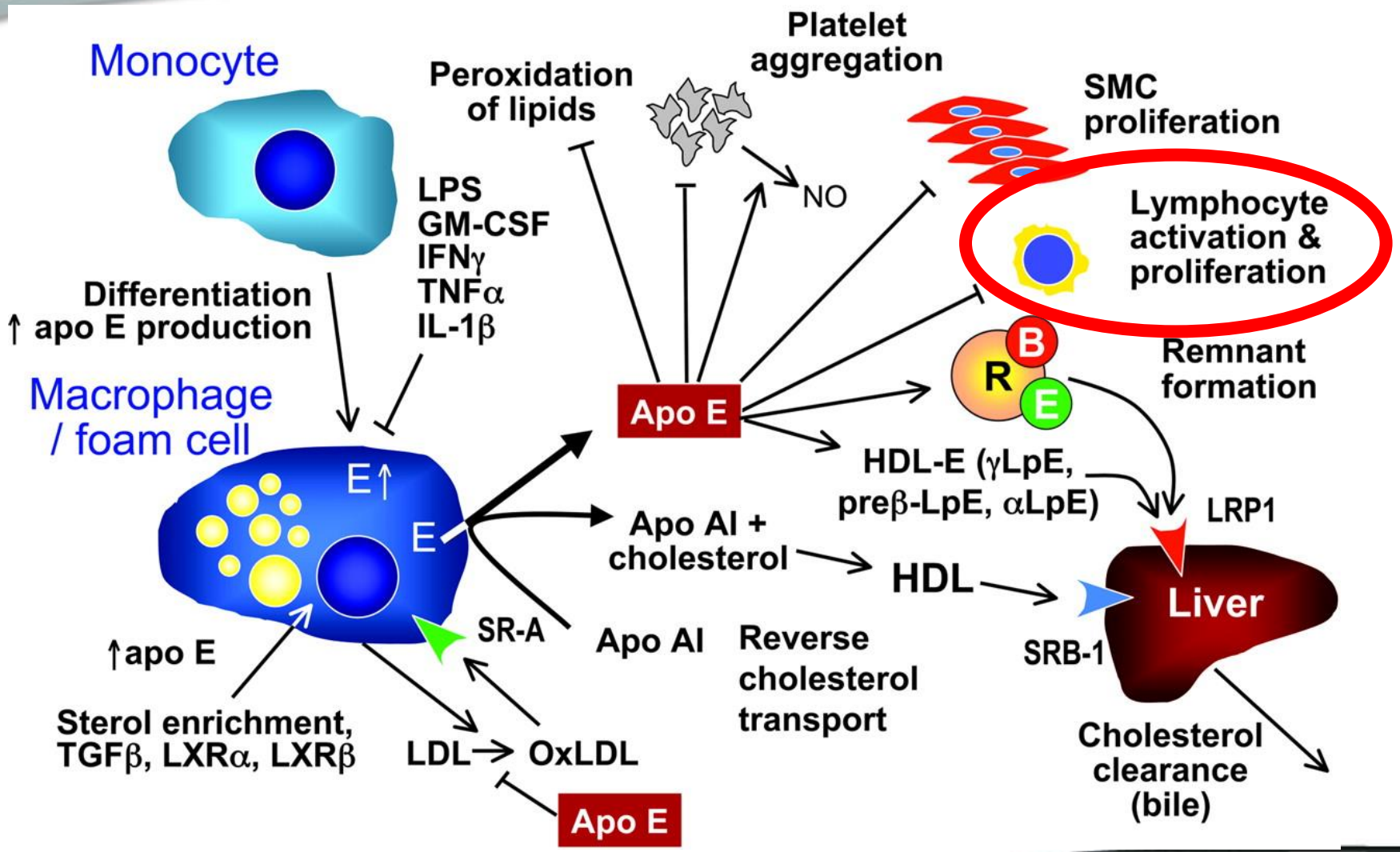
Apo E


Monocyte
Peroxidation of lipids
Platelet aggregation
SMC proliferation
NO
LPS
GM-CSF
IFNγ
TNFα
IL-1β
Lymphocyte activation & proliferation
Differentiation
↑ apo E production
Macrophage / foam cell
Apo E
B
R
E
Remnant formation
HDL-E (γLpE, preβ-LpE, αLpE)
LRP1
E↑
E
Apo AI + cholesterol
HDL
Liver
SR-A
Apo AI
Reverse cholesterol transport
SRB-1
↑apo E
Sterol enrichment, TGFβ, LXRα, LXRβ
LDL → OxLDL
Cholesterol clearance (bile)
Apo E

Apo E

- Apolipoprotein E
  - Lipoprotein: transport of triglyceride acids and cholesterol in blood
- Polymorphism on Chr. 19
  - apoE2: reduced binding affinity to LDL-receptor, atherosclerosis,
  - apoE3: the „neutral“ Apo E genotype
  - apoE4: associated with atherosclerosis, alzheimers disease, cerebrovascular disease
- Function:
  - found to reduce glial activation and inflammatory cytokine release in vitro
  - → extend to mouse model,
  - but: Apo E holoprotein does not cross the blood brain barrier and would not be suitable for peripheral administration
- CN-105:
  - smaller peptide
  - Designed modeling the polar receptor binding face of the helical apoE receptor binding region (Ac-VSRRR-amide)

Aim of the study

- → to investigate the therapeutic potential of CN-105 in a murine model of closed head injury

- Hypothesis: intravenous administration of CN-105 damphens neuroinflammatory responses and thus possibly improves the functional ourcomes

Material and Methods

- **Closed head injury model**
  - 12-14 weeks old male mice
  - anesthesia induction, tracheal intubation and lungs were mechanically ventilated
  - secured in a stereotactic device on acrylic cast to allow 3mm of space below the head for acceleration/deceleration; no ear bars to avoid basilar skull fracture
  - metallic disc was adhered at the skull, immediately caudal to bregma
  - 2.0-mm diameter pneumatic impactor→ single midline impact
  - sham mice were treated identically except for absence of impact
- **Drug administration**
  - Animals were placed in restrainer
  - A) single i.v. dose of 100µL drug was administered by tail vein
  - B) vehicle treated animals received 100µL of normal saline

Material and Methods

- **Immunohistochemistry**
  - to assess effect of CN-105 on inflammation, neuronal injury etc.
  - immunohistochemical staining performed using the F4/80 antibody and Fluoro-Jade B stain on days 10 and 1, respectively, after TBI
  - IHC was performed on separate cohorts of mice from those used in neurobehavioral tests
  - for histological assessment: secondary antibody, biotinylated goat anti-mouse IgG (1:3000), ABC, DAB (all from vector laboratories)

- **Cell quantification and image analysis**
  - A) F4/80 quantification:
  - brains of 5 TBI treated and 6 TBI vehicle treated mice were counted
  - sections of dorsal hippocampus was analyzed by stereoinvestigator software
  - immunopositive microglia identified with 20x objective and total number estimated by optical fractionator method

Material and Methods

- B) Fluoro-Jade B:
- Brain sections of 6 TBI treated and 6 TBI vehicle treated mice were counted
- dorsal hippocampus was examined for degenerating neurons using epifluorescence microscope

- **Testing for functional deficits**
  - Automated Rotarod to assess vestibulomotor function
  - one day before TBI: clinical training trial at accelerating rotational speed for 200 seconds and then three additional test trials (n=11-12 per group)
  - → average time to fall from cylinder was recorded as baseline latency
  - Mice were tested on consecutive days post-injury and received three consecutive daily trials with accelerating rotational speed

Material and Methods

- Morris Water Maze to assess spatial learning and memory
- submerged platform placed in a pool with 105cm diameter, opaque water
- Four trials /day for 4 consecutive days: days 28-31 after TBI (n=11-12 per group)
- Mice were introduced to varying quadrants of the pool
- Probe trial on day 4 (=last day) of the experiment: platform was removed and mice were allowed to swim freely for 60 sec → percent of time the mice spent in the platform quadrant was quantified

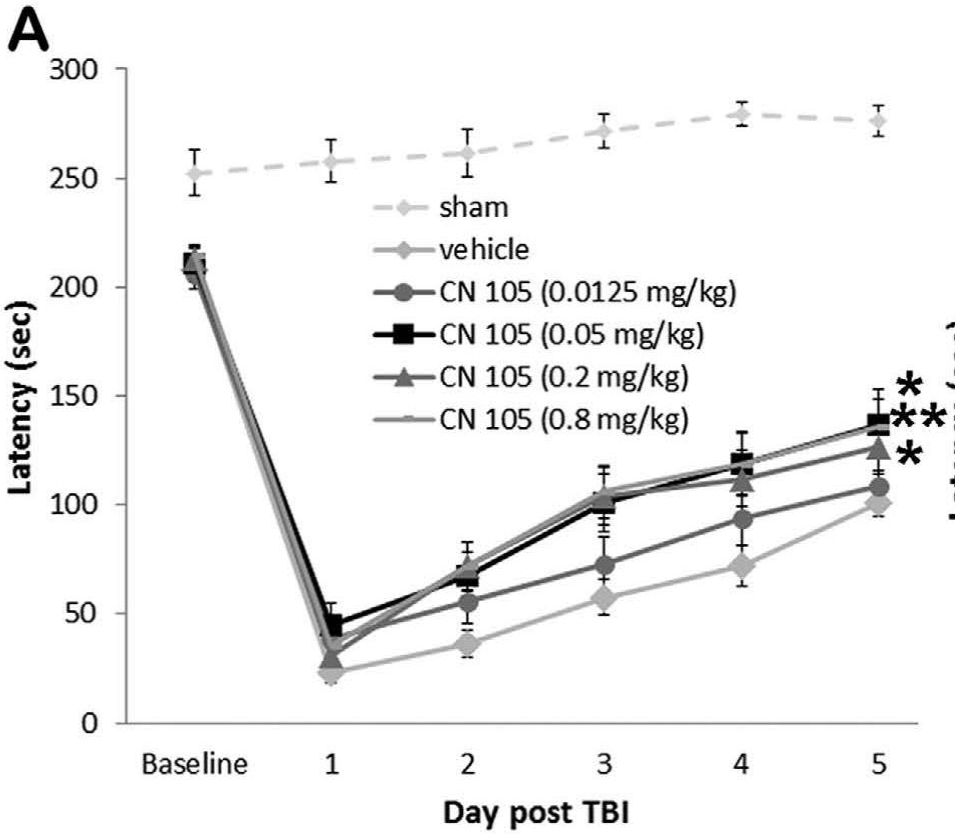
- **RNA extraction and RT-PCR**
  - brain tissue was processed for RNA extraction from a separate cohort of treated and untreated mice on day 1 post injury (CN-105, n=4; vehicle, n=3; control (sham-operated), n=3)
  - gene expression was measured using the Mouse Inflammatory Response and Autoimmunity PCR Array (profiles the expression of 84 inflammation and autoimmunity related genes)

Material and Methods

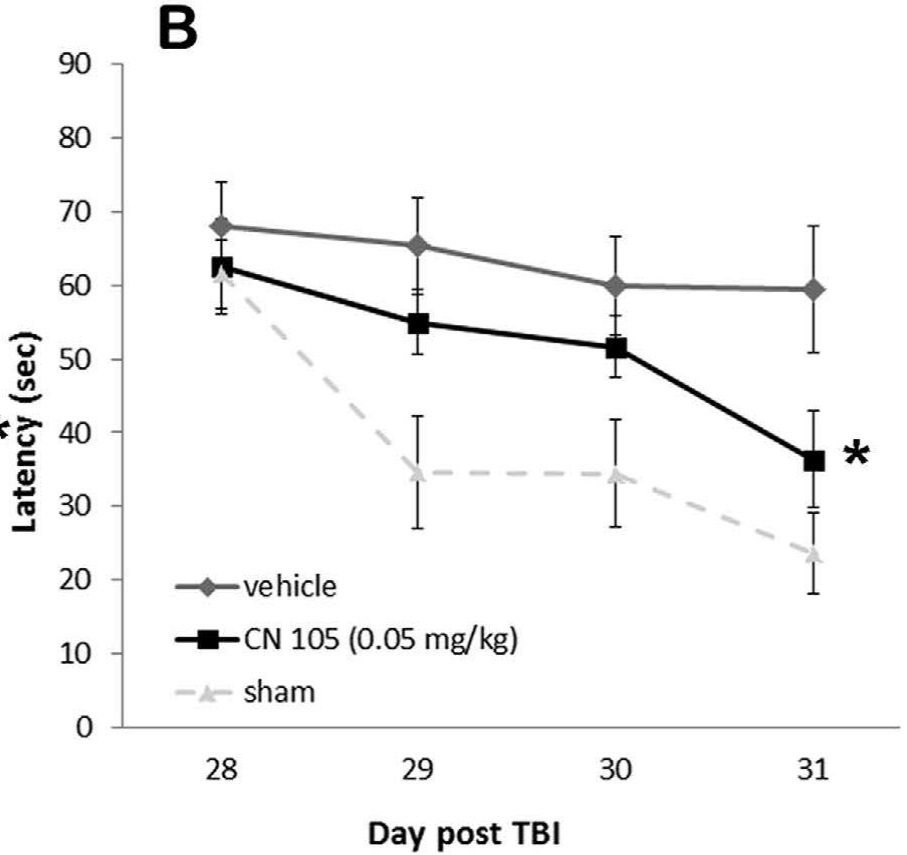
- **Gene expression data analysis:**
  - inflammatory and autoimmunity related genes (84 genes)
- **Pharmacokinetic analysis:**
  - to assess CNS penetration: quantitative whole body autoradiography analysis (QWBA) was performed and areas of interst in blood and brain were compared

Results

Assessment by Rotarod (CN-105: 2h post TBI)



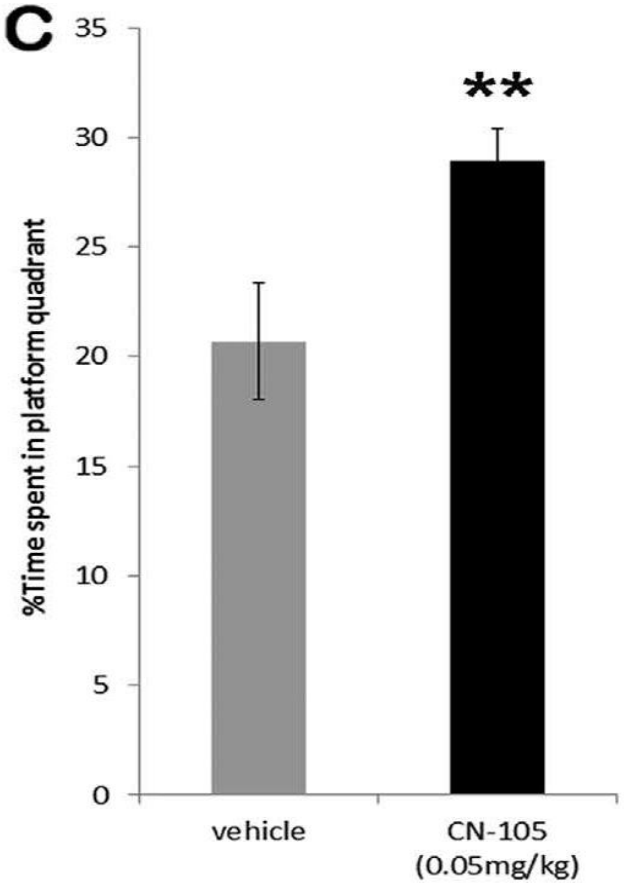
Assessment by MWM (CN-105: 2h post TBI)



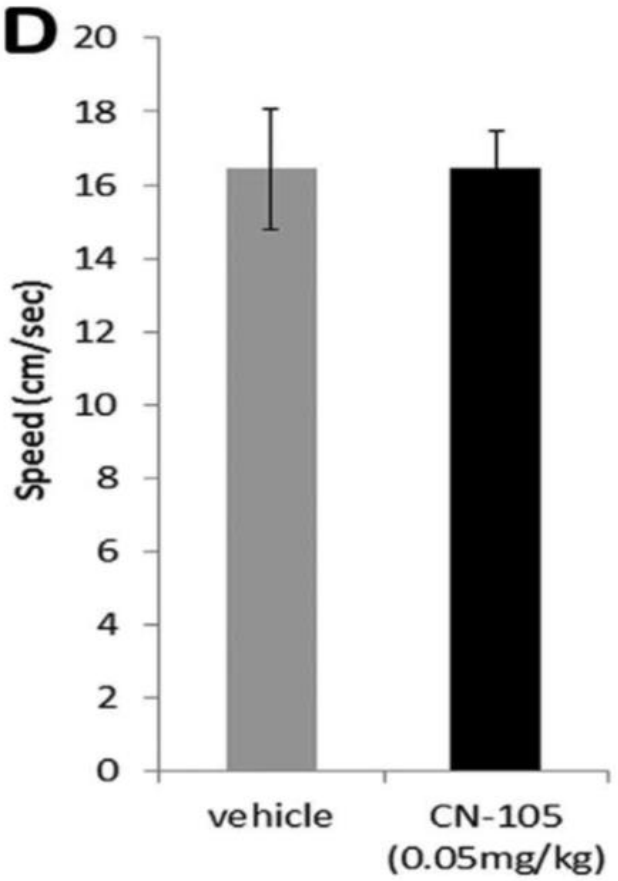
*$p < 0.05$ and ** $p < 0.01$

Results

Probe trial on day 4 of MWM experiment



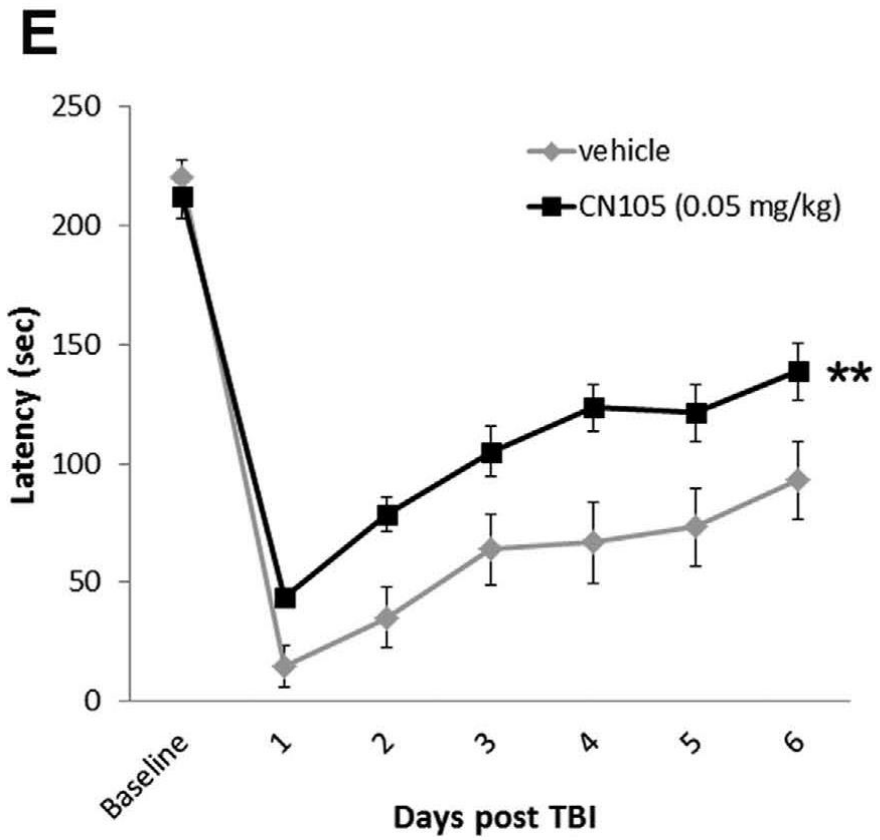
Swim speed during whole MWM experiment



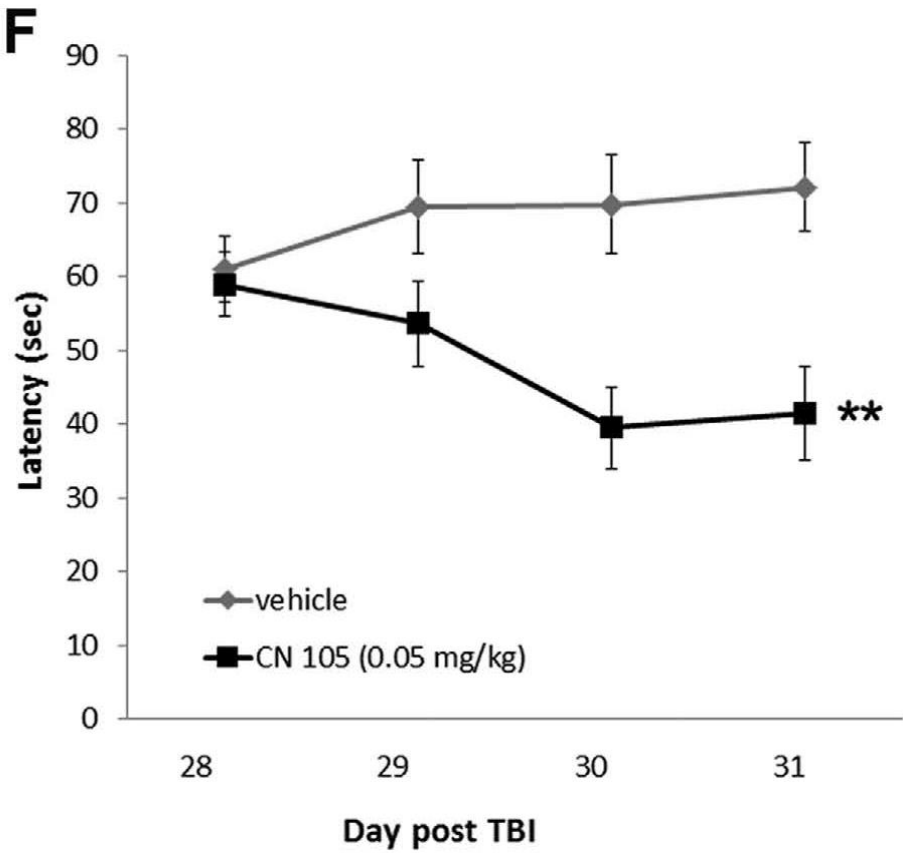
*p < 0.05 and ** p < 0.01

Results

Assessment by Rotarod (CN-105: 4h post TBI)



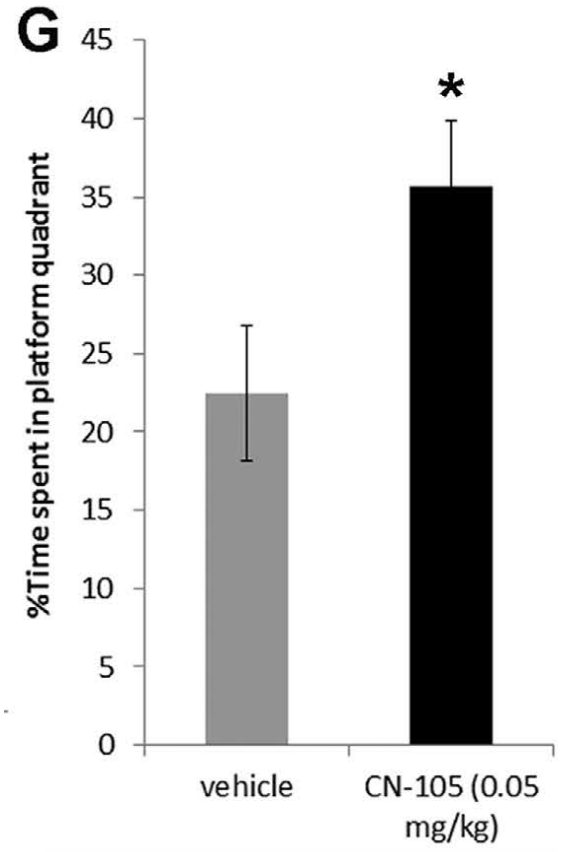
Assessment by MWM (CN-105: 4h post TBI)



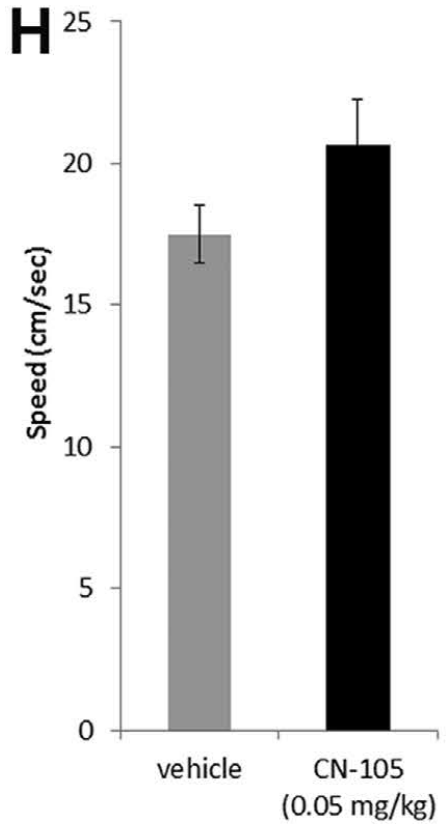
*$p < 0.05$ and ** $p < 0.01$

Results

Probe trial on day 4 of MWM experiment (CN-105: 4h post TBI)



Swim speed during whole MWM experiment (CN-105: 4h post TBI)



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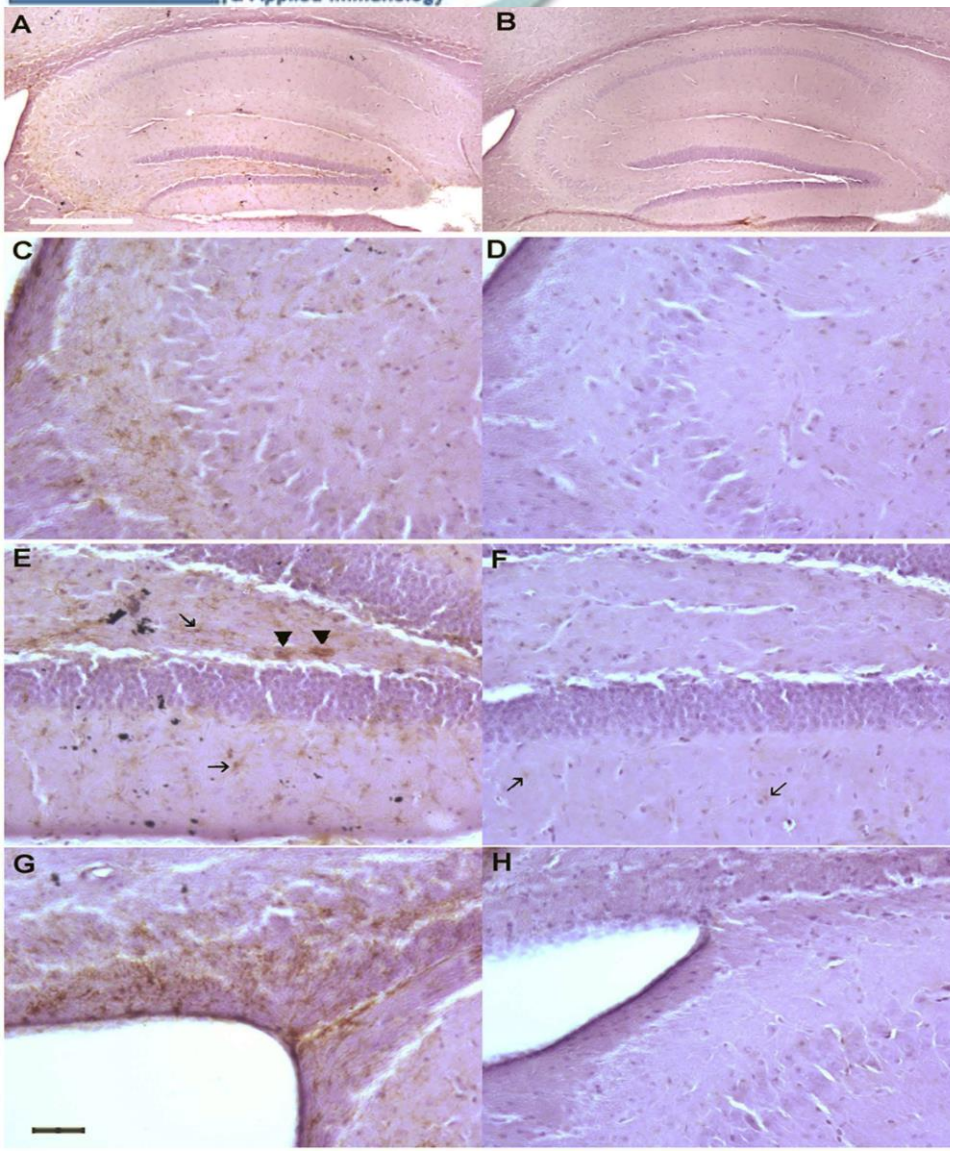
Results



Comparison of activated F4/80 immunostained microglia in hippocampus (10 days post TBI)

A, C, E, G → treated by vehicle

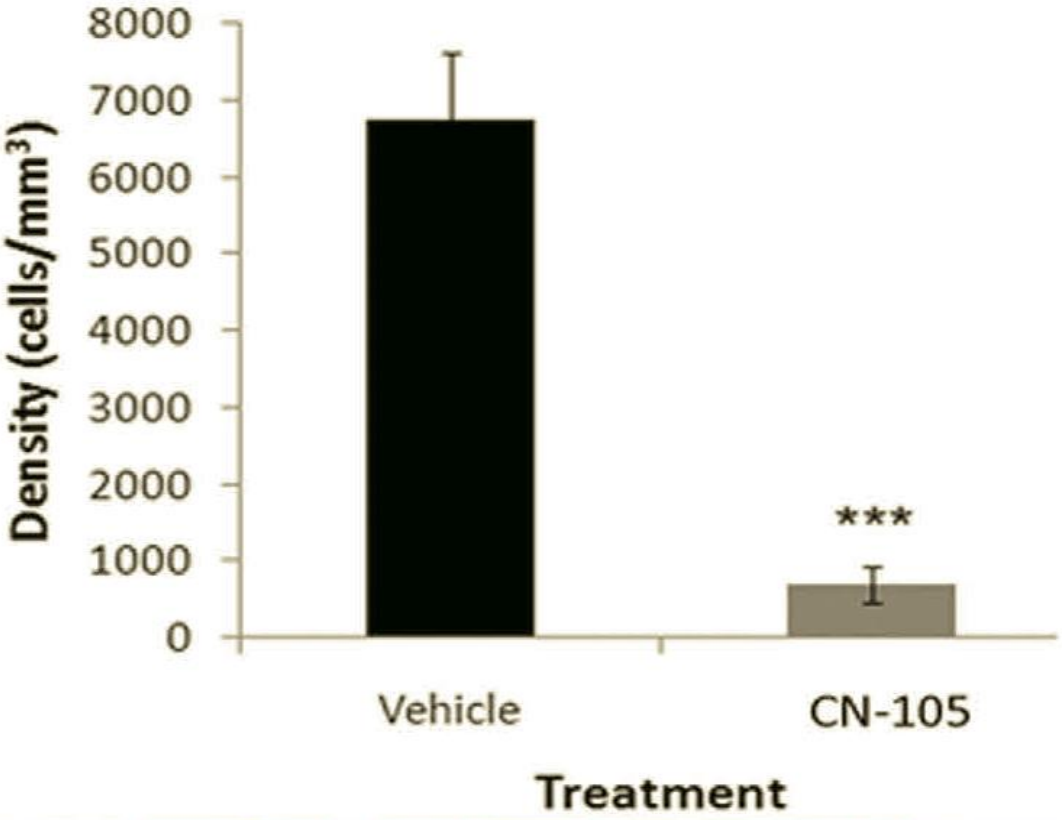
B, D, F, H → treated by CN-105

C+D: CA3 region (cornu ammonis mit Pyramidenzellen)

E+F: polymorphic region (with marked microglia cells)

G+H: periventricular region (corpus callosum and fimbria)

Results



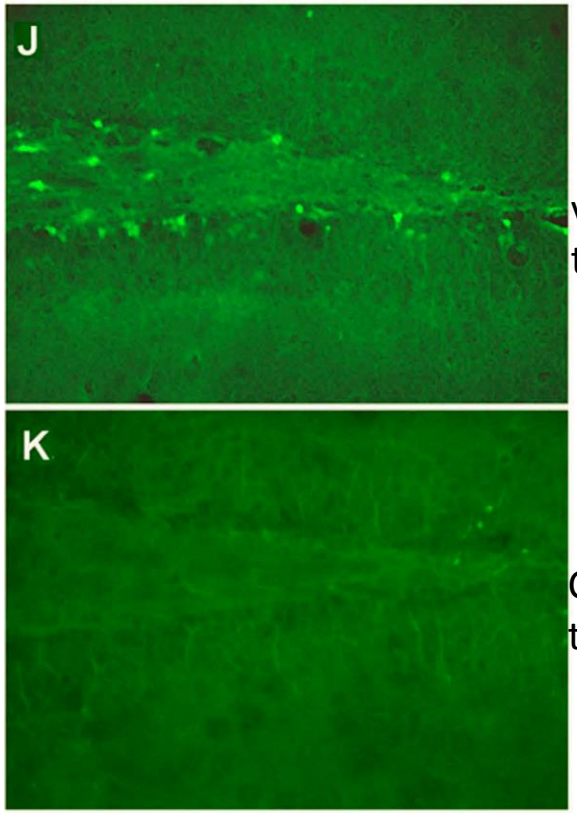
Comparison of activated F4/80 immunostained microglia in hippocampus (10 days post TBI)

→ significant reduction in microgliosis

****p* =0.0002

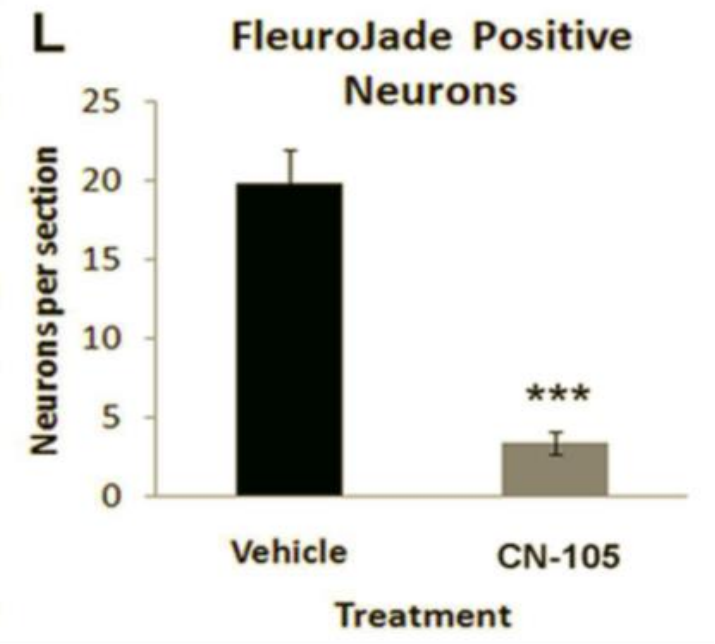
Results

Fluoro-Jade B stained brain slices 24h post-TBI: degenerating neurons in dorsal dentate gyrus



vehicle treated

CN-105 treated



***p <0.0001

→ significant reduction of neuron degeneration

Results

Gene	S-v	S-CN-105	TBI-v	TBI-CN-105	S-CN-105/S-v	TBI-v/S-v	TBI-CN-105/S-v
Cxcl3	3.0E−05	2.3E−05	6.7E−01	1.6E+00	0.8	22171.7	54032.0
Ccl11	7.8E−05	2.5E−04	6.4E−01	1.8E−01	3.2	8199.7	2253.1
Ccr4	3.9E−06	6.0E−06	2.6E−02	3.1E−02	1.5	6537.5	7945.9
Ccl20	2.6E−05	1.1E−04	1.2E−01	7.9E−02	4.4	4574.0	3086.8
Tlr6	4.7E−04	6.2E−04	1.2E+00	7.0E−03	1.3	2528.8	14.9
Ccl24	3.2E−05	9.7E−05	7.6E−02	5.1E−03	3.0	2356.9	159.1
Myd88	7.8E−04	1.1E−03	1.8E+00	6.6E−03	1.5	2327.8	8.5
Cxcl9	6.8E−05	1.0E−04	1.5E−01	2.2E−03	1.5	2265.1	32.2
Lta	1.2E−04	2.6E−04	1.3E−01	1.1E−01	2.3	1133.5	917.0
Tnfsf14	2.5E−05	1.3E−04	2.5E−02	9.0E−03	5.2	1027.7	365.9
Il9	1.2E−05	*	1.2E−02	1.4E−02	**	957.7	1155.5
Ifng	7.3E−05	5.8E−05	5.6E−02	4.3E−03	0.8	767.1	59.7
Il10	1.9E−05	1.1E−04	1.5E−02	1.6E−03	5.7	766.1	82.2
Il1b	1.1E−03	2.0E−03	7.5E−01	4.6E−01	1.8	683.8	416.6
Il8rb	8.3E−06	6.1E−05	5.2E−03	5.4E−03	7.3	623.4	658.4
Il6	1.4E−04	1.1E−04	5.4E−02	1.8E−03	−1.3	395.1	13.2
Il1a	1.4E−03	1.9E−03	5.5E−01	5.4E−02	1.4	382.3	37.7
Cd40	5.4E−04	8.3E−04	1.6E−01	2.1E−02	1.5	301.4	38.8
Ccr2	2.6E−04	5.5E−04	7.1E−02	1.5E−03	2.1	271.6	5.6
Cxcl1	6.0E−05	1.6E−04	1.5E−02	1.8E−03	2.7	244.8	29.4
Ccr7	1.2E−04	2.1E−04	2.7E−02	3.4E−02	1.8	228.3	283.2
Il18rap	1.1E−04	4.5E−05	2.4E−02	6.6E−03	0.4	211.3	58.6
Tnf	2.8E−05	3.5E−05	5.5E−03	3.8E−02	1.3	197.3	1367.7
C4b	3.6E−03	7.0E−03	6.3E−01	5.9E−01	2.0	176.5	163.3
Il8ra	1.3E−05	*	2.2E−03	2.6E−03	**	168.5	196.8
Cxcr4	4.3E−03	6.6E−03	6.6E−01	9.0E−02	1.5	153.8	21.0
Cxcl11	2.4E−05	9.0E−05	3.4E−03	8.8E−03	3.8	141.3	372.3
Il6ra	3.4E−03	4.9E−03	4.8E−01	1.6E−01	1.5	140.3	47.9
Il22	4.3E−05	4.7E−04	5.6E−03	3.4E−03	10.8	129.4	77.8
Il1rn	2.3E−05	*	2.8E−03	1.7E−03	**	121.0	74.2
Ccl2	5.8E−04	8.3E−04	6.5E−02	2.5E−02	1.4	110.8	42.3
C3ar1	3.5E−03	4.1E−03	3.7E−01	1.2E−01	1.2	108.2	34.9

Assessment of gene expression

(24h post sham or TBI injury)

→ using a pathway array specific for inflammatory and immune responses (84 genes)

4 cohorts:

- Sham + vehicle (S-v)
- Sham + CN-105 (S-CN-105)
- TBI + vehicle (TBI-v)
- TBI + CN-105 (TBI-CN-105)

→ <u>57 genes were upregulated, 12 were downregulated, 10 were unchanged</u>

*mRNA expression was not detectable
**relative values could not be calculated because one of the components was not detectable

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Results

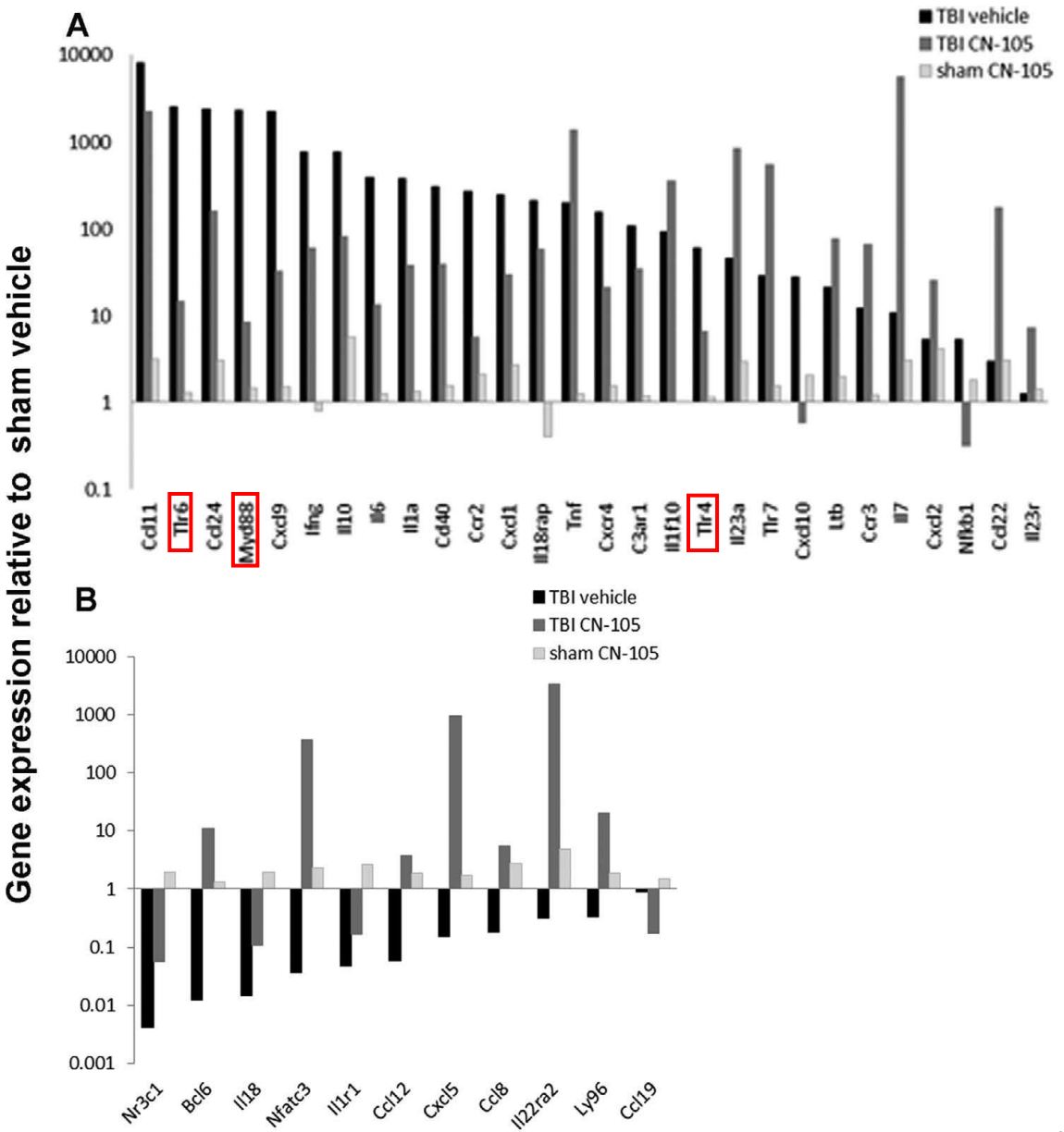
Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
Il22ra2	10895	Tlr3	1.3	*Fasl*	−2.0
Nfatc3	10044	Ccr7	1.2	*Csf1*	−2.0
Cxcl5	6257	Ccr4	1.2	*Il1rap*	−2.2
Bcl6	921	Il9	1.2	*Ccl4*	−2.6
Il7	515	Crp	1.2	*Ccl2*	−2.6
Ccl12	64.2	Il8ra	1.2	*Tnfsf14*	−2.8
Ly96	61.2	Il8rb	1.1	*Tollip*	−2.9
Ccl22	60.2	Tlr2	1.0	*Il6ra*	−2.9
Ccl8	31.2	Ripk2	−1.1	*C3ar1*	−3.1
Tlr7	19.0	Kng1	−1.1	*Il18rap*	−3.6
Il23a	18.5	Hdac4	−1.1	*Ccl11*	−3.6
Nr3c1	13.7	C4b	−1.1	*Ccl19*	−5.2
Il18	7.2	Fos	−1.2	*Cxcr4*	−7.3
Tnf	6.9	Cd40lg	−1.2	*Cd40*	−7.8
Il23r	5.8	Ccl7	−1.2	*Cxcl1*	−8.3
Ccr3	5.4	Cd25	−1.2	*Tlr4*	−9.0
Cxcl2	4.8	Lta	−1.2	*Il10*	−9.3
Il1f10	3.8	Flt3l	−1.4	*Il1a*	−10.1
Ltb	3.6	Tlr1	−1.4	*Ifng*	−12.9
Il1r1	3.5	Cd20	−1.5	*Ccl24*	−14.8
Cxcl11	2.6	Cebpb	−1.5	*Nfkb1*	−16.6
Il10rb	2.6	Tirap	−1.5	*Il6*	−29.8
Tlr5	2.5	Itgb2	−1.5	*Cxcl10*	−47.9
Cxcl3	2.4	Il1rn	−1.6	*Ccr2*	−48.3
Ccr1	2.3	Ccl5	−1.6	*Cxcl9*	−70.3
C3	1.5	Il1b	−1.6	*Tlr6*	−170
Ccl3	1.5	Il22	−1.7	*Myd88*	−273
Ccl1	1.5	Ccl17	−1.7	*Nos2*	*

Assessment of gene expression
(24h post TBI with CN-105)

→ inflammatory gene expression of CN-105 relative to vehicle treated group

*mRNA expression was not detectable

Results



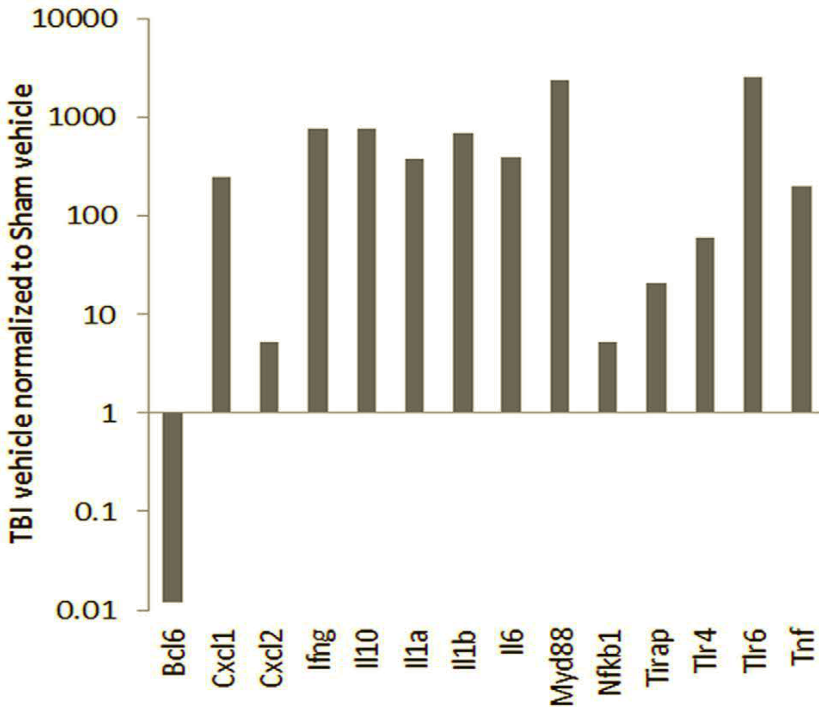
CN-105 ameliorates changes in inflammatory gene expression
(24h post TBI)

→ upregulated/downregulated expression of genes relative to sham vehicle

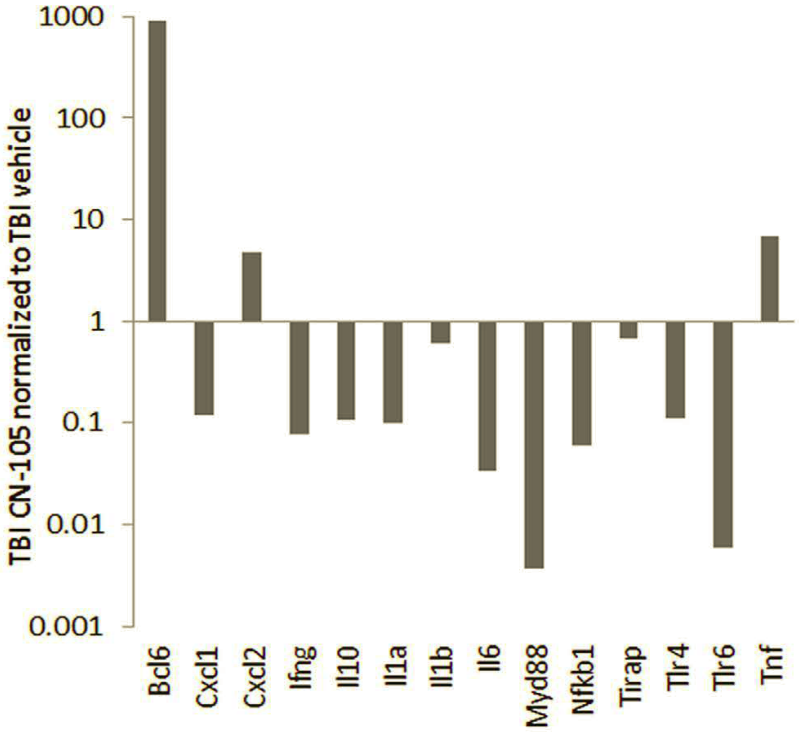
Results

TLR signalling is downregulated by CN-105
(24h post TBI)

B

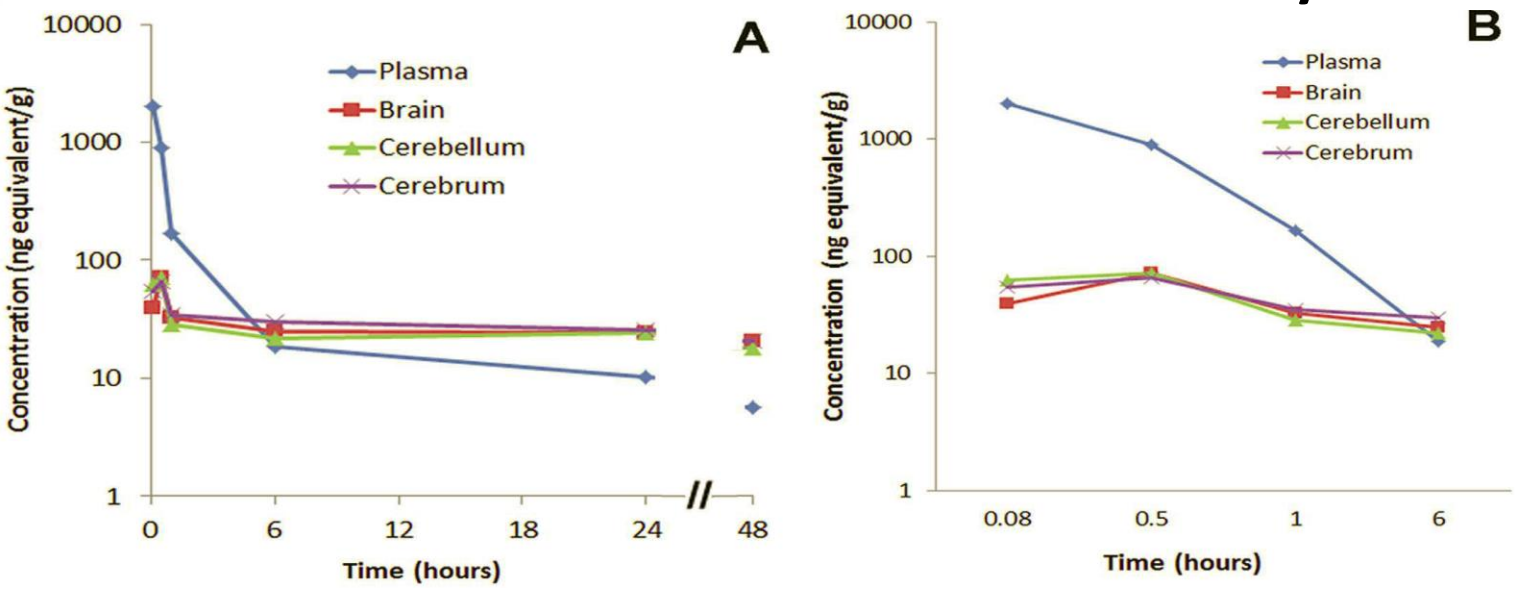


D



Results

Pharmacokinetic studies of CN-105 demonstrate CNS bioavailability



concentration of C14-radioactivity in plasma and CNS following intravenous dose of radiolabelede CN-105 peptide

	Radioactivity (ng equiv/g) in tissue following bolus (hours = h)					
	0.08 h	0.5 h	1 h	6 h	24 h	48 h
Blood (Cardiac)	1102	500	95	15	14.1	15.5
Brain	39.5	71.2	32.5	24.9	24.1	20.9
% Brain/Blood	3.58%	14%	34%	167%	170%	135%

Percent of radioactivity contributed to blood in the brain

Results

CN-105 is associated with:

- A) improved behavioral function
  - by Rotarod (better vestibulomotor performance)
  - by MWM (better preserved spatial learning memory)
  - no significant motor differences
  - pretreatment of mice 30min prior to TBI had no additional effects („data not shown“)
- B) reduced microgliosis and neuronal injury following TBI
  - esp. in CA3 und polymorphic region of hippocampus
  - degenerating neurons are significantly reduced
- C) changes in inflammatory gene expression patterns following TBI
  - 57 of 84 inflammatory genes were upregulated in TBI mice (esp. TLR-signalling pathway)
  - TBI-CN-105 lead to reduced inflammatory gene upregulation compared to TBI-v
- D) penetration into the CNS compartment
  - progressive increase of radioactivity in brain as compared to blood (3,6% at 5 minutes, 170% at 24h)

Discussion

- ApoE and ApoE-mimetic peptides decrease neuroinflammatory responses and secondary cell death
  → Already shown in several animal studies of acute brain injury
- mRNA levels of inflammatory cytokines and chemokines return to pre-injury levels by 24 hours or more?
- Difficulty to characterize endogenous microglia
  - Primitive macrophage entering embryonic brain vs. hematogenous macrophage that are recruited into brain following to injury
  - But number and activation status is decreased after CN-105 treatment
- Not all indicators are decreased by CN-105 treatment at 24 hours post injury
  - TNF alpha increases

Discussion

- MyD88 has recently shown to be significantly increased after TBI in several mouse model experiments
- Mechanisms by which CN-105 (ApoE) modulate inflammatory rsponse is completely indefined
  - Via specific receptor interaction
  - Connection to LRP-1 receptor? NMDA-receptor via PSD-95?
- Gene expression suggests key role of BCL6 in inflammation process
  - Early changes of NF-kB, chemokines and cytokines
  - Central key repressor in TLR signalling pathway?
- CN-105 may directly effect the blood brain barrier (tight junctions)

Discussion

- Administerd up to 4 hours following injury
  → Temporal window should be further extended
- Limitations of study:
  - ApoE polymorphism → modulate receptor binding via allosteric effects
  - Differential gene assay only focused on expression of inflammatory markers
- Rodent models:
  - Brains have reduced ration of white: grey matter
    → Not ideal to model the diffuse axonal injury
  - Early changes of NF-kB, chemokines and cytokines
  - Central key repressor in TLR signalling pathway?
- CN-105 may directly effect the blood brain barrier (tight junctions)

→ promising therapeutic strategy in treatment of acute brain injury

Thank you for your attention!
Any questions?