„Aus dem Max-Delbrück-Centrum für Molekulare Medizin“

DISSERTATION

Muscarinic receptor 3 modulates microglial response after ischemic stroke

Die Rolle des Muskarinische Rezeptor 3 in der Modulation der Reaktion von Mikroglia nach ischämischem Schlaganfall

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Abbreviations

ACK: Ammonium-Chloride-Potassium
ANOVA: Analysis of Variance
ATP: adenosine triphosphate
BBB: Blood Brain Barrier
Ca^{2+}: calcium
CD11b: Cluster differentiation 11b
CD45: cluster differentiation 45
cm: centimeters
cm/s: centimeters per second
cm^2: squared centimeters
CNS: Central Nervous System
COXII: cytochrome c oxidase subunit 2
EDTA: Ethylenediamine tetra acetic acid
EPM: Elevated Plus Maze
FACS: fluorescence-activated cell sorting
FCS: Fetal Calf Serum
Fig: Figure
Fig S: Supplementary Figure
g: gram
Iba1:Ionized calcium binding adaptor molecule 1
IGF-1: insulin growth factor 1
IL: interleukin
iNOS: inducible nitric oxide synthetase
KO: knockout
L: liter
LPS: lipopolysaccharide
Ly6C: lymphocyte antigen 6 complex
Ly6G: lymphocyte antigen 6 complex locus G6D
m: meter
M3r: muscarinic receptor 3
$M3rKO^{mi}$: $Cx3cr1CreERT^{+/+}m3R^{flox/flox}$ gavaged 3 weeks before MCAo
$M3rKO^{mi/mo}$: $Cx3cr1CreERT^{+/+}m3R_{flox/flox}$ gavaged 1 week before MCAo
MACS: magnetic-activated cell sorting
MCAo: Middle Cerebral Artery Occlusion
mg: milligram
MHC-II: major histocompatibility complex 2
MHz: Megahertz
Min: minutes
ml: milliliters
mm: millimeters
mM: millimolar
mm^3: cubed millimeters
MRI: Magnetic Resonance Imaging
n.s.: non-significant
nAChRs: nicotinic acetylcholine receptors
ng: nanograms
NIH: National Institutes of Health
nm: nanometers
NOR: Novel Object Recognition
NYU: New York University
PFA: paraformaldehyde
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate Buffered Salt
R.E.: Relative Expression
RH: right hind paw
rpm: rotations per minute
RT: Room Temperature
RT-qPCR: Real time quantitative polymerase chain reaction
s: seconds
SEM: standard error of the mean
TNF-α: tumor necrosis factor alpha
WT: Wild type
µm: micrometers

Abstract

English

As the resident immune cells of the brain, microglia are the first cells to get activated after ischemic stroke and they contribute to disease progression in a context-dependent manner. We have previously shown that there is an increase in the microglial population expressing the muscarinic acetylcholine receptor 3 in the lesion of mice after mild ischemia (middle cerebral artery occlusion - MCAo - model). Here, we further study the impact of such population in the outcome after ischemic stroke. First, we induced ischemia in mice depleted of the m3R exclusively in microglia ($M3rKO^{mi}$) and respective m3R-expressing controls. We found that $M3rKO^{mi}$ male (but not female) mice show larger lesions and higher motricity impairments than controls in the acute/subacute phase after stroke (within the first week) and cognitive deficits in a chronic phase after stroke (over 3 weeks after stroke). Additionally, $M3rKO^{mi}$ mice have decreased density of $Iba1^{+}$ cells in the lesion in comparison with controls. We then induced ischemia in male mice depleted of m3R in microglia and in peripheral monocytes ($M3rKO^{mi/mo}$) causing $M3rKO^{mi/mo}$ mice to have lower overall survival rates, motor impairments and decreased density of $Iba1^{+}$ cells in the ischemic lesion in comparison with controls. Collectively, our data show that, after an ischemic event, m3R-expressing microglia and monocytes promote neuroprotection in male mice resulting less severe outcome.

Abstract

Deutsch

Als residente Immunzellen des Gehirns sind Mikroglia die ersten Zellen, die nach einem ischämischen Schlaganfall aktiviert werden und dabei kontextabhängig zum Fortschreiten der Krankheit beitragen. Im Rahmen einer vorangegangenen Studie haben wir in einem Mausmodell für Schlaganfall, bei welchem ein Verschluss der mittlere Hirnarterie eingeleitet wird (MCAo Modell), gezeigt, dass die Läsionen von Mäusen nach einem ischämischen Schlaganfall eine erhöhte Anzahl an Mikroglia, die den muskarinischen Acetylcholinrezeptor 3 (M3R) exprimieren, aufwiesen. In dieser Studie haben wir die Auswirkungen einer solch erhöhten Expression des muskarinischen Acetylcholinrezeptors 3 auf den Ausgang eines ischämischen Schlaganfalls genauer untersucht. Zunächst induzierten wir eine Ischämie in Mäusen, deren Mikroglia spezifisch einen M3R Knockout ($M3RKO^{mi}$) aufwiesen und konnten hierbei zeigen, dass männliche (jedoch nicht weibliche) $M3RKO^{mi}$-Mäuse in der akuten/subakuten Phase nach dem Schlaganfall sowohl größere Läsionen und Bewegungsdefizite als die Kontrollgruppe aufwiesen, als auch kognitive Defizite in der chronischen Phase nach dem Schlaganfall. Zudem fanden wir heraus, dass $M3RKO^{mi}$ Mäuse eine geringere Dichte an Iba1-positiven Zellen in der Läsion im Vergleich zur Kontrollgruppe verzeichneten. Anschließend induzierten wir eine Ischämie in männlichen Mäusen, bei denen die Expression von M3R sowohl in den Mikroglia als auch in zirkulierenden Monozyten depletiert war ($M3RKO^{mi/mo}$), und stellten fest, dass diese Mäuse im Vergleich zur Kontrollgruppe eine verkürzte Gesamtüberlebensrate sowie motorische Beeinträchtigungen aufwiesen. Männliche $M3RKO^{mi/mo}$ wiesen zudem auch eine geringere Dichte von Iba1-positiven Zellen in der ischämischen Läsion auf. Insgesamt zeigen unsere Daten, dass die Expression von M3R in Mikroglia und Monozyten nach ischämischem Schlaganfall neuroprotektiv ist und bei männlichen Mäusen zu einem weniger schweren Verlauf führt.

Synopsis

1. Introduction, state of research, significance of the work for the discipline

1.1 Microglia discovery and function

Over 100 years ago, microglia were first described by Santiago Ramón y Cajal as the third cellular component of the central nervous system (CNS)[1]. Microglia are small, macrophage-like glial cells, and constitute 5-12% of all cells in the adult mouse brain[2] and 0.5-16.6% of all adult human brain cells[3]. These long-lived mitotic cells arise from embryonic yolk sac precursors[4] and, after migrating to the neuroectoderm at embryogenic day 9, become self-renewing[4, 5]. Even though distributed throughout all the CNS, microglia density, morphology and expression of immune receptors vary in different regions both in humans and rodents. It is currently unclear what induces these phenotypical differences in microglia or if these minor differences translate into functional differences[6].

Microglia are paramount in normal and pathologic brain physiology, participating in CNS development, maintenance, and repair[7]. Microglia function was initially determined to be the safeguarding of CNS homeostasis. Homeostatic microglial cells usually comprise small somas with fine processes, being referred to as ramified microglia[6]. These cells are highly dynamic, with their processes constantly scavenging the brain parenchyma for inflammatory cues[8, 9], and eliminate potential threats to the CNS[10]. Apart from their role maintaining CNS homeostasis, microglia are now known to be crucial during CNS development, as, during embryonic neurogenesis, they are responsible for modulating the formation of the neuronal network by eliminating redundant synapses in a process termed synaptic pruning[6]. Additionally, microglia secrete growth factors that support and protect neuronal activity[11].

While having an established role in the conservation of healthy brain function, the role of microglia in brain injury remains controversial. Microglia are the first line of response after insult to the CNS, becoming activated and altering their cell morphology and function. Microglia express pattern recognition receptors that recognize cell wall

components or nucleic acids of pathogens, and express scavenger receptors, purinergic receptors, receptor for advanced glycation end products, and toll-like receptors that recognize components released from stressed or damaged cells, including adenosine triphosphate (ATP), aggregated β-amyloid, and heat shock proteins[12]. Upon activation of these receptors, microglia undergo activation. Microglia activation is characterized by morphological changes such as soma enlargement, and retraction of their thin cytoplasmic processes[8]. Furthermore, activated microglia undergo functional changes such as increased cell mobility and proliferation, and secrete pro-inflammatory cytokines, including Tumor necrosis factor alpha (TNFα), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), Cytochrome c oxidase subunit 1 (COX-II), and Major histocompatibility complex class 2 (MHC-II), the latter enables microglia to present antigens to T cells[6, 12]. These secreted cytokines will then activate other microglia and astrocytes in the vicinity[6, 13]. Finally, this initial microglial response promotes phagocytosis of debris and production of neurotrophic molecules, which result in tissue repair and neuroprotection[14-16].

While this first response is neuroprotective, continuous microglial activation may lead to irreversible brain damage and worsening of the clinical outcome in various CNS diseases[17-19]. Microglia derived radicals and their reactive reaction products, hydrogen peroxide and peroxynitrite, are toxic to healthy cells, leading to oxidative damage and neurodegeneration[17, 19]. Adding to this apparent disparity, contrary to what was initially believed, the expression profile of microglia activation cannot be defined as a dichotomy. Microglial has been shown to present a plethora of activation phenotypes that cannot be characterized by the classical anti- *vs* pro-inflammatory phenotypic transition model. Indeed, some of those activated cells simultaneously express pro- and anti-inflammatory markers[20].

1.2 Microglia in stroke

Stroke is the leading cause of severe disability worldwide and, after coronary heart disease, the second leading cause of death[21-23]. Common sequelae after stroke include paralysis, loss of memory, and impaired language, and movement[24]. Stroke-

related disability often results in an immense emotional and financial burden on survivors, their families and society. Depending on the underlying pathology, stroke can be classified as either ischemic stroke caused by lack of blood flow or hemorrhagic stroke resulting from bleeding[24]. Ischemic stroke accounts for 75-80% of all strokes and is primarily caused by the blockage of cerebrovascular blood flow by a blood clot. Currently mechanical thrombectomy is used to restore blood flow and even though it has improved the outcomes of ischemic stroke patients, only a limited number of patients is eligible to this form of treatment, owing to time constraints. Other therapies, including the use of thrombolytic therapy by tissue-type plasminogen activator, and the use of endothelial progenitor cells have been developed in order to restore the blood flow[25, 26]. However, the time window for their effectiveness is still very narrow, and reperfusion injuries may occur, causing exacerbation of neuroinflammation[26, 27].

Further anti-stroke therapies have emerged aiming at 1) promoting neurogenesis in the damaged core regions to reconstruct and restore normal function and 2) reducing the secondary brain injuries to reduce damage to otherwise healthy brain. To the first point, newborn neurons were found in the ischemic penumbra of stroke patients and rodents, confirming that there is induction of neurogenesis after stroke. However, these newborn cells can display morphological abnormalities, which may result in functional impairments in stroke patients. Therefore, after ischemia, primary neurogenesis by itself is insufficient for functional recovery given that regeneration of the neural network is still required. In this regard, microglia are crucial regulators of neurogenesis considering that they regulate synaptic maturation, and generate trophic factors that promote both axonal growth, and guide cell migration[28]. To the second point, anti-inflammatory microglia modulate a beneficial response[29-31], eliminating cellular debris, and restoring tissue homeostasis[32, 33]. As a matter of fact, microglia are the primary immune cells to respond to ischemia. They are recruited to the injury site, become ameboid[34-36], and initiate and regulate neuroinflammation[18, 37]. However, pro-inflammatory microglia are also the primary executors of post-ischemic inflammation, potentiating cell death and enlargement of the secondary brain damage[38-40]. In this sense, microglia affect post-ischemic outcomes in a context-dependent manner,

mediating both beneficial and detrimental outcomes. Studies show that microglial response after stroke is modulated by age, sex, and additional underlying medical conditions[29-33, 41-51].

Stroke-induced microglial activation is a dynamic process, evolving throughout the pathological stages of disease[41, 42, 52, 53]. Clinical studies of ischemic stroke highlight the presence of activated microglia in all phases of ischemia: acute, subacute, and chronic[14, 15, 54]. During the acute phase of ischemia, neurons in the infarct region become deprived of oxygen and glucose, the lesion expands, and peripheral leukocytes infiltrate the lesion, causing edema[55]. Microglia are the first cells to respond in the ischemic brain, and as they are vulnerable to hypoxia, they rapidly become activated. The speed of microglial activation after ischemia varies depending on the animal model studied, ranging from a few minutes to a few hours[34, 56-63], and activated microglia can be detected in the lesion several weeks after the ischemic event[31, 34, 62, 64, 65]. Subsequently, several days after the ischemic event, the inflammatory response gradually decreases[66]. In this chronic phase, activated microglia secretes neurotrophic factors such as Insulin growth factor 1 (IGF-1) exclusively in the lesion area. Selective ablation of proliferating microglia at this chronic timepoint increases the size of the lesion, the number of apoptotic neurons, the expression of anti-inflammatory cytokines such as of IL-6 and IL-1β, TNF-α and ablates the expression of IGF-1. These observations led to the conclusion that microglia activation in a chronic time point after ischemia promotes tissue repair[28, 44].

Targeting microglia activation after ischemic stroke has therapeutic potential. For instance, microglia are known to be the main potentiators of oxidative stress and inflammation after stroke and treatment with Caffeic Acid Phenethyl Ester has been shown to reduce oxidative stress and decrease inflammation[24, 67]. Our lab has also shown that inhibition of inducible nitric oxide synthetase (iNOS) secretion by microglia has therapeutic value after a mild ischemic event[68]. Moreover, modulation of microglia after ischemia using of MicroRNA has been shown as a successful therapeutical approach to decreasing inflammation[69-71]. Likewise, others have focused on

modulating neuroinflammation using stem cells to drive microglia polarization towards an anti-inflammatory phenotype after ischemia[72].

Another nefarious contribution of microglia after stroke is associated with the damaging of the blood brain barrier (BBB). The BBB prevents neurotoxic plasma components, blood cells, and pathogens from entering the CNS and regulates the transport of molecules in and out of the CNS to maintain homeostasis. Endothelial cells, connected by tight and adherent junctions, form the first barrier of the BBB. They produce and release vascular regulatory factors to regulate brain microcirculation. The basement membrane forms a second barrier of the BBB. This is a layer of extracellular matrix proteins that supports epithelial and endothelial cells, separating them from the brain tissue[73]. Activated microglia after ischemia phagocytes vascular endothelial cells, increasing their permeability[74, 75], which in turn increases the risk for secondary hemorrhages[47, 76]. Furthermore, activated microglia produce cytokines and chemokines that disrupt BBB integrity by altering cytoskeletal organization, tight junction protein expression, and matrix metalloproteases production[73].

Increased BBB instability promotes infiltration of cells from the periphery, such as lymphocytes and monocytes[73, 77]. Monocytes have a short lifespan and are recruited to the lesion from the periphery at a later timepoint than microglia, during the post-reperfusion phase. Monocytes are thought to be more tolerant to hypoxic conditions, and, thus they populate the core of the lesion and differentiate into macrophages[78]. As classical markers of microglia activation cannot distinguish between microglia and monocytes in the brain[28, 30, 65], monocytes within lesions have often been described as microglia/macrophages[77]. Recent studies with transgenic bone marrow chimeras to distinguish between brain and circulating myeloid populations have made clear that, in the context of ischemia, resident microglia and infiltrating monocytes behave differently and the functional differences between these two myeloid populations in ischemic lesion require further elucidation[77, 79-81]. As to microglia, the role of infiltrating monocytes after ischemia is context-dependent, becoming both biomarkers of inflammation, thus detrimental[49], and potentiators of microglial anti-inflammatory

response, thus beneficial[51, 82]. These potentially disparate roles suggest that drugs designed to modulate microglia may adversely impact the infiltrating monocyte populations.

1.3 M3r, microglia and stroke

Our group has shown that, even though microglia and peripheral monocytes express low levels of muscarinic receptors in naïve brains and in the periphery, after ischemia both monocytic populations overexpressed the acetylcholine muscarinic receptor 3, M3r in the ischemic region[83]. We showed an expansion of the population of microglia/monocytes that is activated by carbachol (a muscarinic receptor agonist) in the ischemic lesion in comparison to naïve brains. Additionally, our lab showed that carbachol acts as a microglial chemoattractant and impairs microglial phagocytosis[83]. Furthermore, others investigated the effects of M3r mediated signaling on microglial function *in vivo* using Cre/lox technology. They found that acute activation of the M3r pathway in microglia increased phagocytic activity and production of pro-inflammatory cytokines in the brain. In contrast, chronic activation of the M3r signaling pathway attenuated the increase in brain cytokine production and the sickness behavior triggered by peripheral administration of a low dose of lipopolysaccharide (LPS)[84]. Despite advances in pre-clinical therapies to promote long-term recovery, none has, so far, succeeded in clinic and these collective observations highlight the potential importance of M3r signaling in microglia as a therapeutic target after ischemic stroke. Herein, using a transgenic mouse model where depletion of *M3r* in microglia and monocytes is possible, we investigated the effects of modulation of *M3r* expression in microglia and monocytes upon ischemia. We administered tamoxifen to *M3r*$^{flox/flox}$; *Cx3cr1*-CreERT mice (and respective controls) at different time points thus modulating in which monocytic population *M3r* was depleted at the time of the ischemic event and found that in male, but not female, mice, the expression of *M3r* in microglia is protective in the acute/subacute and chronic phases after ischemia. Furthermore, we show that *M3r* expression in peripheral monocytes is also protective in the acute/subacute phase after ischemia.

2. Methodology

2.1 Animal model and induction of cerebral ischemia

All governmental and internal regulations were followed for the handling of animals throughout these experiments. We generated a new mouse model by crossbreeding *Cx3cr1*-CreERT mice and *M3r*$^{flox/flox}$ mice. We used tamoxifen gavages to activate the Cre-ERT construct to deplete *M3r*. Mice were gavaged with tamoxifen (500mg/kg/day), as previously described[85], inducing systemic depletion of *M3r* in Cx3cr1^{+} cells (namely microglia and peripheral monocytes). Age- and sex- matched *Cx3cr1*-CreERT$^{-/-}$*M3r*$^{flox/flox}$ littermates were used as controls, following the same tamoxifen regimen.

Ischemia was induced by 30 min MCAo, as previously described[86]. Briefly, mice were anesthetized with 1.5% of isoflurane and a vaporizer was used to maintain anesthesia until the end of the surgery. A monofilament coated with a silicone hardening mixture, engineered to promote complete occlusion of the artery, was inserted into the internal carotid artery up to the anterior cerebral artery. The filament remained in place for 30 minutes causing occlusion of the middle cerebral and anterior choroidal arteries, thus inducing mild ischemia. After 30 minutes, the filament was removed, allowing for blood reperfusion to the region. After the surgery, the mice were kept on a heated cushion and monitored until fully awake. Following governmental regulations, all mice showing obvious signs of suffering after MCAo were sacrificed **(Table 1**).

On a first set of experiments, we induced ischemia 3 weeks after administration of tamoxifen. The interval between administration of tamoxifen and induction of ischemia was enough to guarantee the turnover of the peripheral monocyte population, thus *M3r* was exclusively depleted in microglia (M3rKO^{mi}). Behavioral assessment was performed in both male and female mice at an acute/subacute phase comprised from day 2 to day 7 after MCAo. On a second set of experiments, we evaluated the outcome of ischemia in male mice depleted of M3r in microglia (M3rKO^{mi}) at a chronic time frame from day 20 to day 28 after MCAo. On a third set of experiments, male mice were administered tamoxifen less than a week before MCAo, thus all Cx3cr1^{+} cells were depleted of *M3r*

upon ischemia (M3rKO$^{mi/mo}$). The total numbers of mice used for all experiments are described in **Table 1**. At all times, the experimenters were blinded to the animals' genotypes.

Finally, we performed 30 min MCAo in male *Cx3cr1*$^{-/-}$ mice and age and sex matched wildtype (WT) controls(C57bl6 mice) to determine the isolated effects of depletion of *Cx3cr1* before ischemia. We submitted these mice to the same behavioral protocol previously described for acute/subacute behavior (accelerated rotarod test, pole test and corner test) and performed MRI scans on day 3 after MCAo, as described in **Supplementary Fig. 1A**.

Table 1

Depletion of M3r exclusively in microglia				
	Male		Female	
	Control	M3rKO^{mi}	Control	M3rKO^{mi}
Underwent MCAo	25	36	13	12
No visible lesion on MRI	1	1	1	4
Sacrificed/Died abruptly	11	12	3	1
Used for isolation of CD11b+ cells	5	5		
Acute/Subacute behavior	9	11	9	7
Barnes maze, Open Field test, Novel Object Recognition, Forced Swimming	11	20		
Catwalk and Elevated Plus maze	8	17		

Depletion of M3r in microglia and peripheral monocytes		
	Male	
	Control	M3rKO$^{mi/mo}$
Underwent MCAo	24	34
No visible lesion on MRI	0	3
Sacrificed/Died abruptly	5	13
Used for isolation of CD11b+ cells	13	11
Acute/Subacute behavior	6	7
RNA isolation	5	5

Table 1 (continuation)

Cx3cr1 knockout mice		
	Male	
	Control	Cx3cr1KO
Underwent MCAo	7	6
No visible lesion on MRI	0	2
Acute/subacute behavior	5	4

Adapted from Costa et al.[87]

2.2 Magnetic Resonance imaging (MRI)

3 days after MCAo, the mice were placed under anesthesia and MRI scans were performed. We used a 7 Tesla rodent scanner with a 16cm horizontal bore magnet and a shielded gradient with an inner diameter of 9cm, an H-resonance-frequency of 300MHz, and a maximum gradient strength of 300mT/m. For imaging, we used a 20 mm – 1H-RF quadrature-volume resonator (20 mm inner diameter). Data acquisition and image processing were carried out with the Bruker software Paravision 5.1. While mice were under anesthesia, they were placed on a heated cushion (to avoid hypothermia) and a T2-weighted 2D turbo spin-echo sequence was used (imaging parameters TR / TE = 4200/36ms, rare factor 8, 4 averages, 32 axial slices with a slice thickness of 0.5mm, field of view 2.56x2.56cm, matrix size 256x256).

Leveraging the Analyze 10.0 software, the scans were used to determine the lesion volume. First, we selected the hypertense ischemic areas in axial T2-weighted images with a region of interest tool. After which the software proceeded to perform threshold segmentation by connecting all pixels within a specific threshold range about the selected seed pixel. The software then generated a 3D object map of the whole ischemic region from which the total volume was calculated.

2.3 Acute/subacute behavioral analysis

The acute/subacute analysis of motor impairment was comprised of 3 tests: accelerated rotarod, corner test and pole test **(Fig 1A)**. The accelerated rotarod test measures

motor coordination and balance in rodents. The animal is placed on a horizontal rod that rotates about its long axis being accelerated from 4 to 40 rpm in 300s; the animal must walk forwards to remain upright and not fall off[88]. Mice were trained on day 2 and 3 before MCAo. The average baseline latency was obtained the day before MCAo and the test was repeated on day 2 and 5 after MCAo. On each day, three trials were run on each animal with at least 15 min time interval in between each trial, and the average retention time of three trials was computed.

The pole test is used to access bradykinesia (slowed down movement ability) and extrapyramidal locomotion[89]. For this test, the mouse is placed at the top of an 80 cm-high pole coated with rough material, head held upwards. The time to perform a 180° turn and to reach the platform at the bottom is measured. The latency to turn and to descend the pole was determined as the average of 4 trials in each day. This test followed the same timeline as the accelerated rotarod test and was always performed after the rotarod test.

Finally, the corner test is used to assess sensory-motor function, as sensory and postural asymmetry. It detects contralateral deficit and ipsilateral steering deviation. It is considered a very robust test since it reflects multiple asymmetries, including forelimb, hind limb, posture, and steering bias[88]. For this test, a cardboard forming a diamond-shaped structure with two 30° angle corners is tightly attached to a normal cage. The mice were allowed to explore the inside of the diamond-shaped structure freely. When animals approach the corner, both sides of the whiskers are stimulated simultaneously, causing the animal to rotate 180°. While healthy animals show no side preference, after stroke, unilaterally brain-damaged mice preferentially turn towards their non-impaired side[90], which in our experiments was the left side. As the animals reached one of the narrow corners, we evaluated which side they chose to turn to, for a total of 10 turns each day. The test was performed on day 5 before (baseline assessment) and day 6 after MCAo. On day 7 after MCAo, mice were perfused, and their brains were processed for immunohistochemistry.

2.4 Chronic behavioral analysis

Starting at 20 days after MCAo, during the chronic phase after ischemic stroke, we evaluated the effects of ablation of M3r in microglia on motricity (catwalk test), cognitive impairment (Novel object recognition and Barnes maze tests), anxiety-like (open field and elevated plus maze tests), and depression-like (forced swimming test) behaviors **(Fig 2A)**. The catwalk test is an automated gait analysis system for rodents that assesses motor function and coordination during continuous locomotion along a walkway and was performed as previously described[91]. For this test, the apparatus consists of a one-meter-long walkway with a glass floor and two walls spaced 80 mm apart. As mice are placed on the platform the light (internally reflected) from two fluorescent tubes illuminates the animal's paws only at the points where they touch the glass, producing a bright paw print image. A video camera installed under the glass records the walking. The CatWalk program was then used to analyze this footage. Contact areas were indexed and interactively assigned colored tags (e.g., left/right fore-/hind paw, nose, abdomen and tail), which allowed quantitative analysis of gait. This enabled the measurement of the following locomotive parameters: gait speed and the step cycle of their right hind paw (RH). The step cycle indicates if the mice are limping as it counts the time taken from when a paw is lifted from the platform until it touches the platform again. We chose the right hind paw as this is the paw most likely affected by left-sided MCAo. The mice were trained for two days prior to surgery (by placing an escape box at one end of the walkway), baseline scores were recorded the day before MCAo, and mice were then tested on day 20 after MCAo.

On day 21 after MCAo we performed the open field test. The open field test is influenced by factors such as exploratory drive (curiosity) and fear (or anxiety). Mice have a tendency to spend most of the time in close proximity to the walls, a phenomenon referred to as thigmotaxis[92]. For this test, we used a 3600 cm^2 squared enclosed apparatus with a camera mounted on top. The mice were placed in the center of the platform and allowed to explore freely for 5 minutes while being recorded. To minimize the stress, we used dim lighting and the investigator exited the room where the apparatus was located while the animals were being recorded. We then determined

the amount of time each animal spent in the center of the arena and the total distance walked.

After performing the open field test, still on day 21 after MCAo, we performed the elevated plus maze test (EPM), as previously described[93]. The EPM is generally used to test anxiety[88] During this test mice were placed in the center of an apparatus consisting of a black Plexiglas cross, standing on a tripod, 50cm from the floor. The cross is composed of four arms extending out of a central squared platform. Two of these arms consist of open alleys (not surrounded by walls). Perpendicular to those there are two enclosed arms, in which the alleys the mice can walk through were enclosed by a 15cm tall wall. A camara was mounted on top of the apparatus and the mice were allowed to explore the apparatus for 5 minutes while being recorded. We evaluated the time spent in each arm and in the center of the platform. In this test, the time spent in the open arms reflects a conflict between the animal natural preference for enclosed areas and their innate motivation to explore novel environments. When mice spend longer time in the open arms, they are displaying anti-anxiety behavior. In other words, the more anxious the mice are, the more they will avoid the open arms[93].

On day 22 after MCAo, the same apparatus as in the open field test was used for the novel object recognition test. The test evaluates memory and cognition and is composed of three sessions. In the first session, mice were allowed to explore the apparatus for 5 minutes when it was completely empty. They were recorded and that information was used for the open field test, previously described. 24 hours later, during the second session (familiarization session), we placed two objects diagonally away from each other, towards the center of the apparatus and the mice were allowed to explore for 10 minutes, while being recorded. To reduce induced preference, both objects were the same size and made of the same material. We used two cylindrical blocks of wood. The mice were then returned to their home cage and 3 hours later, for the last session (test session), one of the objects was replaced by a novel, unfamiliar object and the mice were allowed to explore for 5 minutes. The new object was the same size and made of the same material as the previously used object, however it

consisted of a different shape – the new object was a cone. We measured the ratio of time spent exploring the new object by total time spent on both objects. This test is purely based on the innate preference of the rodent to explore the novel object rather than the familiar one. Thus, a rodent that remembers the familiar object will spend more time exploring the novel object[94].

From day 23 to day 27, we used the Barnes maze, a dry land based behavioral test, to study spatial memory. In this test, animals learn the relationship between distal cues in the surrounding environment and a fixed escape location[95]. The Barnes maze setup consists of an elevated circular platform with 20 evenly spaced holes around the perimeter. A dark Plexiglas chamber with an edible reward was mounted underneath one hole, providing the mice with a safe place to hide, while the remaining 19 holes were left empty. There were different clues in the walls surrounding the apparatus. One wall had a large white circle, one wall had a red triangle, one wall had a green square, and one wall had a door. The light was kept very bright as both bright light and open spaces are aversive to rodents, thus serving as motivating factors to induce escape behavior. Additionally, upon activation, the platform would constantly vibrate, and the vibration would stop once the mice entered the escape location. The mice explored the apparatus for 120s per trial in a total of 6 trials per day. The escape box was set at a fixed location for the first 3 days of the test. At the beginning of day 4 (day 26 after MCAo), the escape box was relocated, thus changing the target. Mice were allowed to explore the apparatus for 6 120s trials per day on days 26 and 27 after MCAo. The latency to enter the target box was determined as the average of the 6 trials measured in each day.

Finally, on day 28 after MCAo, we used the forced swimming test, as previously described[96]. This test was developed as a model for predicting the clinical efficacy of antidepressant drugs and is now widely used to analyze depressive-like behavior[88]. In this test, mice are placed in an inescapable transparent tank filled with water and their escape related mobility behavior is measured. The tank consists of a 30cm high, and 20cm diameter cylinder glass jar filled with tepid water up to a 15cm height mark placed

on the glass. After the mice are placed inside the jar, due to the height of the water they are unable to touch the floor and due to the total height of the jar, they are unable to escape. A video camera is placed on top of the apparatus, and, after placing the mice inside the jar, the investigator leaves the room, and the mice are recorded for a total of 300s. During the behavioral analysis, as the mice swim trying to escape from the jar, the total active mobility time is measured. Upon completion of the behavioral test on day 28 after MCAo, the mice were perfused, their brains were dissected and used for immunohistochemistry.

2.5 Immunohistochemistry

After both acute/subacute and chronic behavioral evaluations mice were put under deep anesthesia and perfused with physiologic saline followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. Their brains were dissected, kept in 4% PFA overnight, and transferred for a solution of 30% of sucrose in 0.1M phosphate buffer pH 7.4, until they sank to the bottom of the tube. The brains were sectioned from a dry ice cooled copper block on a sliding microtome, in 40μm-thick coronal sections and stored at -20°C in a cryoprotectant solution (25% ethylene glycol, 25% glycerol and 0.05M phosphate buffer).

Immunohistochemistry was performed in one out of every six sections by free floating immunohistochemistry. The sections were blocked with 10% of donkey serum to avoid unspecific staining. We then used anti-Iba1 primary antibody at a dilution of 1:500 and a biotinylated donkey anti-rabbit secondary antibody at a dilution of 1:125. The signal was developed using 3,3′-diamino-benzidine as a chromogen.

For quantification of the density of Iba1-immunoreactive cells in the lesion area, we leveraged the Stereoinvestigator® software. First, with a 2.5x magnifying objective, we delimited the lesion area in all the serial sections in which it was detectable (**Fig 3A top**). The software then determined in which of the frames(30x30) in the generated grid(750x750) cells should be counted. Then, with a 40x objective, we manually counted the Iba1-positive cells with identifiable cell bodies in all the software-defined counting

frames (all cells inside the red/green square, **Fig 3A bottom**). Per software indication, cells crossing the green lines were included in our quantification and cells crossing the red lines were excluded from our quantification. Finally, we used the volume of the lesion calculated by the stereoinvestigator to normalize the counted number of cells. As an internal control, we quantified the density of $Iba1^+$ cells in the contralateral region of the lesion.

2.6 Brain, spleen and blood cell isolation for gene expression analysis

A different set of animals followed the previously detailed tamoxifen and MCAo regimens but were not used for any behavioral assessment and the mice were perfused 7 or 28 days after MCAo. Using a brain matrix, a 4mm coronal section of fresh brain tissue was dissected from the ischemic and the contralateral hemispheres. The tissue was dissociated mechanically with razor blades and then enzymatically with Adult Brain Dissociation Kit and a gentleMACS Dissociator. We then generated a myelin-free cell suspension, using the Debris Removal Solution from the kit, which was filtered using a 70µm cell strainer. Spleens were maintained in dissociation buffer (PBS containing 5.6% glucose and 15mM Hepes) mechanically dissociated using a razor blade. They were then filtered through a 70µm cell strainer to obtain a single cell suspension. Red blood cells were lysed using ACK buffer. Using magnetically charged MicroBeads we isolated CD11b cells from the ischemic area, the contralateral area, and the spleen of the same mouse using magnetic sorting (MACS). The cells were then frozen in Trizol and used for RNA extraction. Blood was collected in EDTA-covered microtubes, diluted in PBS, and overlaid on a Ficoll solution to isolate peripheral blood mononuclear cells (PBMCs). The cells were stored in RNA lysis buffer from the ReliaPrep, RNA Miniprep systems kit and used for RNA extraction.

2.7 RNA isolation and RT-qPCR

For RNA isolation, 0.2ml of chloroform was added to 1ml of Trizol reagent, and the tubes were vigorously shaken. After centrifugation, the mixture separates into three phases: a lower red, phenol-chloroform phase, an interphase, and a colorless upper phase where RNA exclusively remains. We transferred the upper phase to a fresh tube

with 0.5µl of GlycoBlue to make the pellet more visible. The RNA was precipitated using 1-1.5x volume of isopropanol overnight at 4°C. After centrifugation, the pellets containing the precipitated RNA were washed with ethanol, air-dried and dissolved in nuclease free water. PBMCs RNA was isolated following the protocol provided by the ReliaPrep, RNA Miniprep systems kit. Samples were stored at −80 °C until further use.

First-strand cDNA was synthetized using the SuperScript II reverse transcriptase using oligo-dT primers 12–18 according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) reactions were performed in a 7500 Fast Real-Time thermocycler using the SYBR Select Master Mix according to the manufacturer's instructions. The expression of *M3r* was normalized to the expression of the housekeeping gene *Tbp* in the same sample. Primers used are following: *M3r* forward ACCTGTTCACGACCTACATCA, *M3r* reverse AGTGAGTGGCCTGGTAATAGAAA; *Tbp* forward AAGGGAGAATCATGGACCAG, *Tbp* reverse CCGTAAGGCATCATTGGACT.

2.8 Flow cytometry analysis (FACS)

For FACS analysis, a different set of M3rKO^{mi} animals and respective controls was submitted to the previously described tamoxifen regimen and to MCAo. The animals were then perfused with saline solution 7 days after MCAo. A 4µm section of their ipsi- and contralateral areas of the ischemic injury was obtained, and cells were mechanically dissociated in a buffer containing 1M Hepes pH 7.3 and 45% glucose. For removal of myelin and debris, a 75%/25% Percoll gradient was prepared, and cells were recovered from the gradient interphase, washed and used immediately. After blocking for unspecific binding with anti-FcyIII/II receptor antibody, the single cell suspension was incubated with fluorochrome-conjugated antibodies against cell surface markers: CD45, CD11b, Ly6G, and Ly6C in FACS buffer and fixed in 4% PFA. Matched isotype controls were used to set appropriate gates. Cell acquisition was performed on a BD FACS Canto TM II flow cytometer and results were analyzed using FlowJo software.

2.9 Statistical analysis

GraphPad Prism 8 software was used for statistical analysis and data are represented in the figures as mean±SEM. For statistical analysis of lesion volume, open field, novel object recognition and forced swimming tests, quantification of $Iba1^+$ cells and expression of the *m3R* in the blood and in the spleen an unpaired student's t test was performed. For statistical analysis of survival experiments, Gehan-Breslow-Wilcoxon test was performed. For statistical analysis of accelerating rotarod, corner test, pole test, catwalk test, elevated plus maze and barnes maze a two-way ANOVA following Sidak's multiple comparison test was performed. For quantification of the expression of the *m3R* in the brain a one-way ANOVA followed Tukey's multiple comparison test was used. Significance in the figures is indicated as n.s. (not significant) if $p>0.05$; or, if significant, with the calculated p-value. For detailed methodology, please refer to the original article[87]

3. Results

3.1 M3r-associated neuroprotection after stroke is sexually dimorphic

We first induced ischemia in 13 weeks old M3rKO^{mi} mice, where M3r is exclusively depleted in microglia. Depletion of *m3R* was confirmed by RT-qPCR (**Fig S1A-C).** As per our timeline **(Fig 1A)**, 3 days after ischemia, we determined the lesion volume by MRI. Depletion of M3r in microglia in male mice results in significantly larger lesions than controls (**Fig 1B**). Throughout the first 7 days after ischemia, several mice have abruptly perished and/or, per government regulations, had to be sacrificed due to obvious signs of suffering. We found that M3r depletion in microglia exclusively has no impact on the overall survival of male mice (**Fig 1C**). Finally, we found no differences in volume of lesion of female M3rKO^{mi} mice and controls **(Fig 1D)** and M3r-expressing microglia does not provide females a survival advantage after ischemia **(Fig 1E)**.

3.2 M3r-expressing microglia protects from acute motor and chronic cognitive impairments after ischemia

Acute motor impairment after MCAo has been extensively shown. We hereby hypothesized that male mice lacking M3r exclusively in microglia presented impaired motility. We performed 3 motility assessments after MCAo: the rotarod test, the pole test, and the corner test, following a strict timeline (**Fig 1A)**. We observed no differences between the 2 groups of male mice in the corner test (**Fig 1F)**. Compared to controls, male M3rKO^{mi} mice show impairments on the rotarod both at day 2 and 5 after MCAo **(Fig 1G)**. In the pole test male M3rKO^{mi} mice show impairments at day 5 in both parameters tested (**Fig 1H)**. Female mice were also evaluated following **Fig 1A** timeline, and we concluded that the impact of M3r-expressing microglia after ischemia is sexually dimorphic given that M3r-expressing microglia are protective in male, but not in female mice after ischemia (**Fig 1I-K**). In the M3rKO model we used, the CreERT construct is inserted in the *Cx3cr1* gene thus Cx3cr1CreERT$^{+/+}$ mice are also depleted of

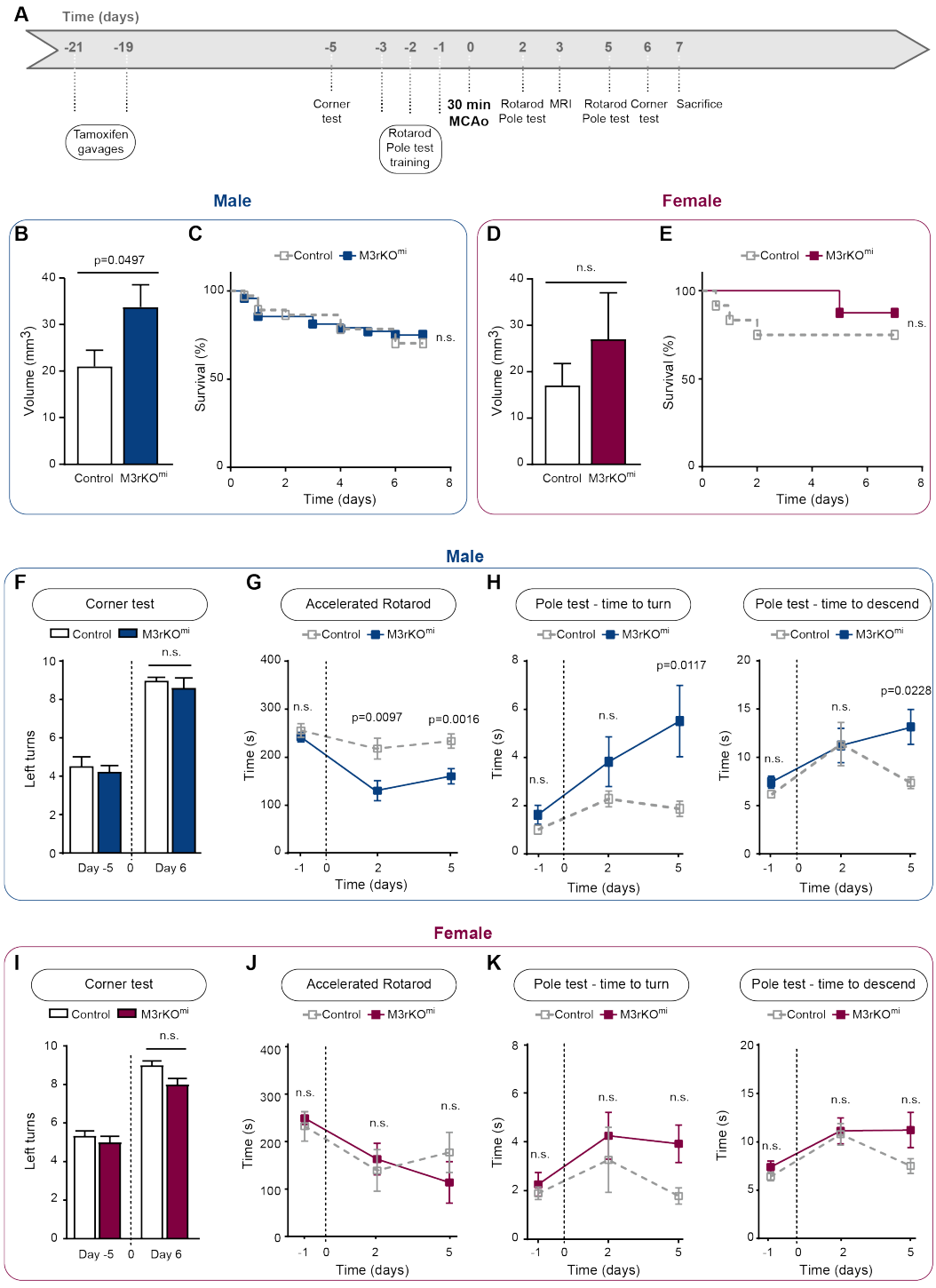

Figure 1: M3r expression in microglia is neuroprotective in male mice in the acute/subacute phase after ischemia. A. Timeline of experiments. Shortly, *Cx3cr*-CreERT$^{+/+}$*M3r*$^{flox/flox}$ mice were given tamoxifen thus depleting M3r in Cx3cr1^{+} cells. 3 weeks after tamoxifen, the peripheral monocytic population was renewed thus only microglia are M3r-depleted (M3rKO^{mi}). *Cx3cr1*-CreERT$^{-/-}$*M3r*$^{flox/flox}$ were used as controls. Ischemia was induced using 30 min of MCAo. 3 days after MCAo mice underwent

magnetic resonance imaging (MRI) and their lesion volume was measured from a T-2-weighted scan. Motricity impairment after MCAo was evaluated using 3 different tests. First, the corner test was performed on day 5 before and day 6 after MCAo. Second, accelerated rotarod was performed. Mice had 2 days training and were tested 1 day before and 2 and 5 days after MCAo. Third, the pole test was performed after the accelerated rotarod. 7 days after MCAo, the mice were sacrificed. The post-ischemia outcome in males (in blue) and females (in red) was studied separately. **B.** Male mice with M3r-depleted microglia have larger lesions than age- and sex-matched controls. **C.** There are no differences in the overall survival of male M3rKO^{mi} mice and controls after ischemia. **D.** There are no differences in the post-ischemic lesion volume between M3rKO^{mi} females and controls. **E.** There are no differences in the overall survival of female M3rKO^{mi} and controls. **F.** There are no differences between male M3rKO^{mi} and controls on the corner test. **G.** M3rKO^{mi} males display impaired motor coordination, measured by the accelerated rotarod at day 2 and day 5 post-MCAo compared to controls. **H.** M3rKO^{mi} male mice show impaired extrapyramidal coordination (pole test) on day 5 after MCAo on both parameters tested (time to turn 180° in the pole -**left**- and time do descend the pole- **right**). Female M3rKO^{mi} show no impaired motricity by the **I.** corner test, **J.** accelerated rotarod and **K.** pole test. Adapted from Costa et al.[87]

Cx3cr1. To determine the impact of depletion of Cx3cr1 in our model, we performed MCAo in Cx3cr1KO and WT mice and found no differences in lesion volume or motricity **(Fig S2)**. We thus conclude that M3r depletion and not Cx3cr1 depletion of microglia after ischemia is the likely the responsible for the poor prognosis observed in male mice.

We next evaluated the long-term effects of M3r-depletion in microglia in male mice upon ischemia, following a strict timeline **(Fig 2A)**. We found no differences between M3rKO^{mi} mice and controls in the catwalk or open field test (**Fig 2B, C),** indicating that the acute motility impairments observed were normalized overtime. We also evaluated depression-like behavior and cognitive impairment 21+ days post-ischemia (**Fig 2A**). We found no differences between M3rKO^{mi} mice and controls at the elevated plus maze **(Fig 2D)** and the forced swim test **(Fig 2E)**. However, we observed impaired novel object recognition skills (**Fig 2G)** and deficits in memory and learning on the fifth day (day 27 after MCAo) of Barnes Maze test (**Fig 2F).**

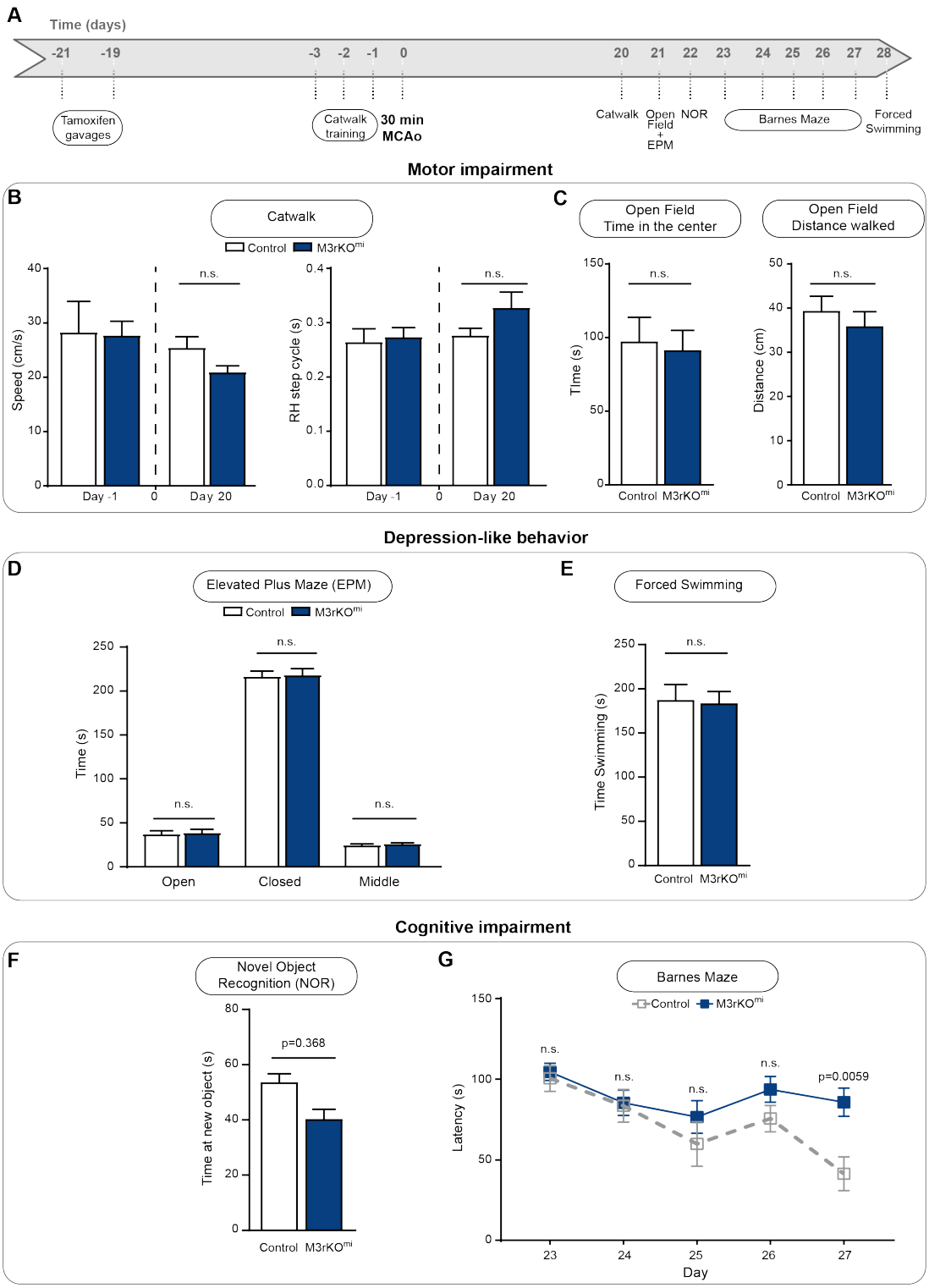

Figure 2: Male M3rKO^{mi} mice show impaired cognition in a chronic phase after MCAo. A. To determine the impact of M3r-depletion exclusively in microglia in a chronic post-ischemia phase, we accessed long-term motricity, depression and anxiety-like behaviors and cognitive impairment after MCAo. First, for the catwalk test, we evaluated gait speed and right hind paw step cycle as indicators of

motility on day 20 after MCAo compared baseline before MCAo. 21 days post-MCAo, motility impairments were determined with the open field test by measuring the total distance walked and the time spent in the center of the apparatus. We performed Elevated Plus Maze (EPM) to measure anxiety-like behavior. To determine cognitive impairment, 22 days after MCAo, we performed the Novel Object Recognition (NOR) test and from days 23-27 we performed the Barnes Maze test. Finally, to analyze depression-like behavior we performed the forced swimming test on day 28 after MCAo. The mice were sacrificed on day 28 post-MCAo. **B.** We found no differences in the speed **(left)** or right hind paw step cycle **(right)** in the catwalk test between the 2 groups. **C.** We found no differences in the total distance walked **(left)** or time spent in the center **(right)** between the 2 groups in the open field test. **D.** We found no differences between the 2 groups in the EPM, **E** or the forced swimming test. **F.** On day 22, M3rKO^{mi} mice show impaired recognition of the novel object in comparison with controls. **G.** After relocation of the target hole (on day 26) M3rKO^{mi} male mice show decreased latency in finding the new target hole on day 5 of the Barnes Maze test, 27 days after MCAo. Adapted from Costa et al.[87].

Collectively, male mice with M3r-depleted microglia have larger lesions, acute motility impairment, and chronic cognitive deficits. Thus far, we have determined M3r-microglia signaling is protective in male, but not female mice after ischemia.

3.3 M3r expression facilitates microglia infiltration in the ischemic lesion

Additionally, we found decreased density of Iba1^{+} cells in the lesion of M3rKO^{mi} mice in comparison with controls both in males (**Fig 3B)** and females **(Fig 3C)**. After isolating CD11b^{+} cells in the lesion and contralateral brains of M3rKO^{mi} mice and controls we further characterized the monocyte cell composition of their lesions by FACS. We found increased density of CD11b^{+}CD45^{lo} cells in the lesions of M3rKO^{mi} mice in comparison with the contralateral side of those brains and increased CD11b^{+}CD45^{hi} cell density in the lesion *vs*. the contralateral side of the brain in both M3rKO^{mi} and control mice (**Fig 3D)**.

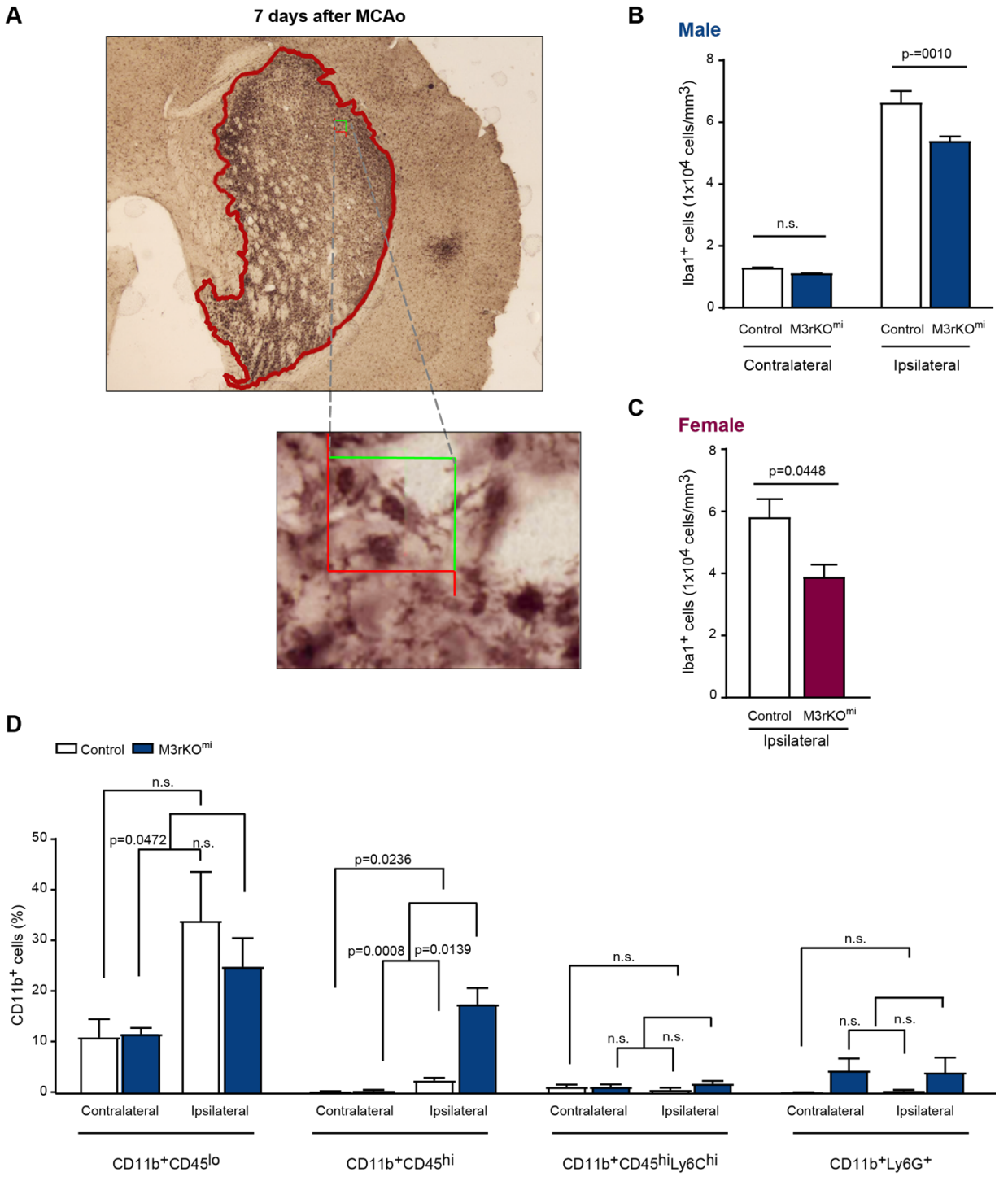

Figure 3: Depletion of M3r in microglia impairs infiltration of microglia in the ischemic lesion. A. Leveraging a stereoinvestigator® we determined the density of Iba1$^+$ cells in randomized counting frames **(bottom** red and green square) in the previously delimited ischemic lesion **(top)** in coronal sections of brains, 7 days post-MCAo. **B.** Male (blue) M3rKO^{mi} and control mice show no differences in density of microglia in the contralateral side of the lesion. However, male M3rKO^{mi} mice have fewer Iba1$^+$ cells in

the lesion than controls. **C.** Female (red) M3rKO^{mi} mice have fewer Iba1^{+} cells in the lesion than controls. **D.** Male M3rKO^{mi} mice have more CD11b^{+}CD45^{lo} microglia in the lesion compared to their contralateral brain. There is a trend for fewer CD11b^{+}CD45^{lo} cells in the lesion of M3rKO^{mi} mice than in controls however, due to low experimental n, this difference does not reach statistical significance. There are more CD11b^{+}CD45^{high} cells in the lesion of M3rKO^{mi} mice then in their contralateral brain and then in controls. No differences were found in the density of Cd11b^{+}CD45^{high}Ly6C^{high} and CD11b^{+}Ly6G^{+} cells in the lesions of M3rKO^{mi} or controls and to contralateral brain. Adapted from Costa et al[87].

3.4 M3r expression in peripheral and CNS monocytes is protective after stroke

To further elucidate M3r-mediated modulation of ischemic outcomes, we induced ischemia in mice lacking M3r simultaneously in microglia and peripheral monocytes. Mice were given tamoxifen 6 and 4 days before MCAo (**Fig 4A)** not allowing the turnover of the peripheral monocytes thus depleting M3r both in microglia and peripheral monocytes (M3rKO$^{mi/mo}$). We confirmed depletion in the CNS and spleen by RT-qPCR **(Fig S1D-F).**

We observed no differences in the lesion volume **(Fig 4B)** but found that M3rKO$^{mi/mo}$ mice have decreased overall survival compared to controls **(Fig 4C)**. Additionally, depletion of M3r in microglia and monocytes before ischemia results in decreased microglia density in their lesions compared to controls **(Fig 4D)**. When analyzing acute/subacute motricity in these mice (**Fig 4A)**, we found no differences between the two groups on the rotarod test (**Fig 4E)**. However, M3rKO$^{mi/mo}$ mice show impaired motricity both in the corner test (**Fig 4F)** and in the pole test at day 5 after MCAo (**Fig 4 G)**.

We then investigated what motricity assessment test would be the best indicator of size of lesion, by correlating the lesion volume with performance in the test for the acute/subacute phase **(Fig S3A-C)**. We found a significant correlation between day 5 of the pole test and lesion volume (**Fig S3B and C right)**, however we believe that the pole test alone would not be a strong indicator of stroke severity and advocate for a more extensive behavioral assessment.

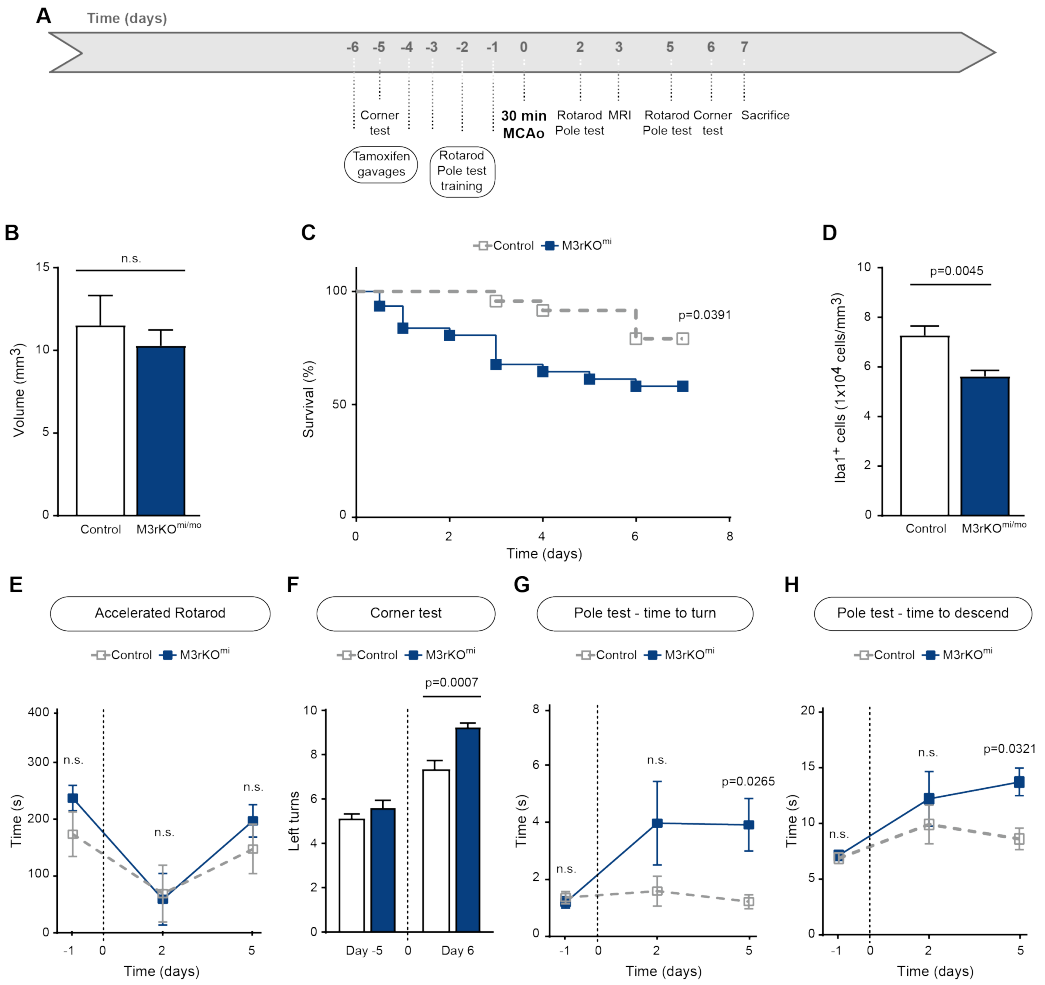

Figure 4: Expression of M3r in microglia and peripheral monocytes is neuroprotective in mice after stroke. A. We followed the experimental timeline from **Fig 1** except here male mice were given tamoxifen 6 and 4 days before MCAo. With this paradigm there was no turnover of the peripheral population thus M3rKO$^{mi/mo}$ mice are depleted of the M3r in both microglia and peripheral monocytes upon ischemia. **B.** We found no differences in the lesion volume by MRI. **C.** M3rKO$^{mi/mo}$ mice have worsened overall survival in comparison to controls. **D.** M3rKO$^{mi/mo}$ mice have decreased density of Iba1^{+} cells in their lesion than controls. **E.** We found no differences in the accelerated rotarod between both groups. **F.** M3rKO$^{mi/mo}$ mice perform significantly fewer left turns than controls. **G.** M3rKO$^{mi/mo}$ mice show impairments in both parameters by the pole test on day 5 post-MCAo in comparison with controls. Adapted from Costa et al.[87].

Finally, we sought to determine if tamoxifen ingestion influenced post-stroke outcomes. We compared lesion volumes and overall survival rates of control *vs*. control and knockout *vs*. knockout mice administered tamoxifen 3 weeks or 1 week before MCAo. Both control and KO mice which ingested tamoxifen one week before MCAo have

smaller lesions in comparison with the mice gavaged 3 weeks prior to MCAo (**Fig S4A-B)**, but no differences in survival **(Fig S4C-D)**. We thus conclude that the ingestion of tamoxifen right before MCAo might be slightly neuroprotective.

4. Clinical applications, limitations of the work, further questions

Following our previous observation that, after mild ischemia, there is an increased density of M3r-expressing microglia/monocytes infiltrating the ischemic lesion[83], we hereby uncovered that, after a mild ischemic event, these M3r-expressing microglia and monocyte populations are neuroprotective in male mice.

Neuroinflammatory response to brain damage is partially regulated microglial cholinergic signaling[97]. Microglial cells express nicotinic acetylcholinergic receptors (nAChRs)[98] and α7-nAChRs, in particular, are known suppressors of the CNS inflammation[97, 99-109], including after stroke[110, 111]. Accordingly, using acetylcholinesterase inhibitors before ischemia results in attenuated lesion size and improves the outcome both in mice[112, 113] and humans[114, 115]. Microglia also express cholinergic muscarinic receptors[83, 116], but modulation of microglia's muscarinic response after stroke has been far less studied. As naïve microglia hardly express muscarinic receptors and their expression is only detected in diseased microglia, modulation of the microglial muscarinic response upon disease is relevant[83]. Particularly, M3r-expressing microglia have dimorphic effects in the brain: while acute microglial M3r-activation *in vivo* induces phagocytosis and a pro-inflammatory response, chronic M3r-activation promotes an anti-inflammatory response[84].

In the initial set of experiments, we induced mild ischemia (30 min MCAo) in mice depleted of M3r exclusively in microglia. We determined overall survival, ischemic lesion volume and performed a series of behavioral assessments at both acute/subacute and chronic phases post-stroke. First, we observed that, in the acute/subacute phase after ischemia, mice lacking M3r in microglia have larger lesions, and impaired motility compared to controls in two (pole test, accelerating rod) out of the three behavioral tests (with no differences on the corner test). We thus concluded that $M3r^{+}$ microglia are protective post-stroke. Second, muscarinic-cholinergic modulation of naïve microglia potentiates directed motility[83], and decreases phagocytosis *in vitro* and *in vivo[83, 84]* and after stroke there is a larger population of microglia responding to carbachol-

stimulation (a structural, more stable, analogue of acetylcholine). Accordingly, M3rKO^{mi} mice have worse outcomes after stroke and their lesions have fewer Iba1^{+} cells. Previous reports correlate decreased density of microglia/macrophages in the lesion with worsened post-ischemic outcomes[44, 117]. Based on our discovery, we hypothesize that, depletion of M3r in microglia halted the chemoattraction of neuroprotective monocytes to the lesion, potentiating the negative outcome observed. The relative contribution of microglia *versus* macrophages is difficult to discern since these cells express similar activation markers. We postulate that the observed decrease in the density of Iba1^{+} cells in the lesion might be either explained by decreased infiltration of peripheral monocytes, decreased infiltration/proliferation of microglia or a combination of both scenarios. We found that lesions of M3rKO^{mi} mice have increased density of CD11b^{+}CD45^{hi} cells but the densities of CD11b^{+}CD45^{hi}Ly6C^{hi} and CD11b^{+}CD45^{+}Ly6G^{+} cells are similar to controls, thus concluding that depleting M3r in microglia induced a new phenotype, with increased expression of the otherwise low CD45 marker, rather than it induced increased infiltration of monocytes. However, confirmation of this conclusion requires further experiments. Third, to completely deplete M3r, we used Cx3cr1-CreERT$^{-/-}$M3r$^{flox/flox}$ mice, which are also depleted of Cx3cr1. To determine the effect of Cx3cr1 depletion in our model, we leveraged Cx3cr1KO and WT mice and followed the same experimental timeline as for the M3rKO^{mi} mice. Depletion of Cx3cr1 had no impact on lesion volumes or motricity impairment when compared to WT mice post-stroke. Our results are in accordance with previous reports showing that depletion of Cx3cr1 had no negative impact or even caused a less detrimental response after stroke[118, 119]. However, considering that some of the behavioral tests had low statistical power, and that depletion of Cx3cr1 was reported to impair the performance in the rotarod test[120], we cannot completely exclude the possibility that, in our model, depletion of Cx3cr1 could have been detrimental to motricity after ischemia (especially in the accelerated rotarod test). Fourth, we found effect of M3r-depletion in microglia in female mice. There is sexual dimorphism in modulation of microglia phenotype and function[121-123], including after stroke[77, 124, 125], with female microglia being protective in male mice after ischemia[126]. Additionally, the outcome of stroke both in rodent and human females is

age- and hormone-dependent with older post-menopause females being more at risk than age-matched males and younger pre-menopause females[124, 127, 128]. At a younger age, female mice are less negatively affected than males after MCAo[129]. As our experiments were performed in young mice, intrinsic, sex- and age-related microglia phenotype could potentially explain the differences observed between males and females. To further characterize the phenotype of $M3r^{+}$ microglia after stroke, older, post-menopause females and age-matched male mice depleted of the receptor in microglia should be studied. Considering that M3r activation decreases phagocytosis and promotes chemoattraction, we hypothesize that 1) expression of M3r in male microglia might be age-dependent and thus only younger mice might have a larger population of M3r-expressing microglia after ischemia; 2) as female mice become more susceptible to stroke after menopause and as the expression of M3r does not affect the post-stroke outcome in young females, we hypothesize that M3r-mediated protection by microglia might be modulated by sexual hormones.

The second set of behavioral analysis we determined the impact of M3r-depletion in microglia in a chronic post-stroke phase. Approximately 30% of stroke survivors suffer from depression after stroke[130]. Depression is associated with poor long-term functional outcome after stroke and can halt recovery by impairing willingness to participate in rehabilitation and cognition in general[131]. Cognitive impairment is amongst stroke patients, and 10% of them develop dementia after the first stroke, and over 30% after recurrence of stroke[132]. We thus accessed motricity (catwalk), anxiety-like (open field and elevated plus maze), depression-like (forced swimming) and cognition behaviors (Barnes maze and novel object recognition) 20 to 28 days after MCAo. Male $M3rKO^{mi}$ mice show impaired cognition in a chronic post-stroke phase. The cholinergic system modulates cognition[133-136] and glial cholinergic deficiency has been linked to impairment of cognition[137-139]. Microglia respond directly to acetylcholine via α7-nAChRs and influence both short-term and long-term synaptic function and plasticity, contributing to cognition[140]. More experiments are required to further elucidate how microglia and cholinergic neurons communicate and to further elucidate how $M3r^{+}$ microglia modulate cognition after stroke.

In the third set of behavioral tests, we determined the effects of concomitant depletion of M3r in microglia and peripheral monocytes upon ischemia. Here, depletion of M3r in CNS and periphery caused impaired motricity in two out of three tests performed (corner test and pole test). Controls and M3rKO$^{mi/mo}$ mice have similar lesion sizes, but M3rKO$^{mi/mo}$ mice show lower overall survival after ischemia. The fact that there are non-CNS resident cells responding to brain damage is not surprising. After brain injury, peripheral immune cells, some even expressing cholinergic receptors[141], infiltrate the brain parenchyma and shape the local inflammatory response[18, 142, 143]. α7-nAChRs modulate macrophage-mediated protection in the CNS after ischemia[144]. With the recent advances in technology, allowing for instance for single cell analysis, we believe we will soon have a much better understanding of the contribution of different subpopulations of microglia and/or monocytes modulating on the post-stroke outcome.

Finally, we observed that mice not treated with tamoxifen (WT and Cx3cr1KO) have larger lesions than controls treated with tamoxifen. Also, we observed that controls given tamoxifen 1 week before MCAo have smaller lesions than controls given tamoxifen 3 weeks before MCAo **(Fig S4A)**. In this sense, tamoxifen is transiently protective during MCAo and, consequently, we cannot entirely distinguish the effect of simultaneous depletion of M3r in microglia and monocytes from the effect of tamoxifen. However, tamoxifen was not protective of the overall survival of controls **(Fig S4B)**. Therefore, we can conclude that depletion of M3r in peripheral monocytes and microglia results in higher stroke-related deaths. Overall, we hereby report that motricity is impaired in M3rKO^{mi} and M3rKO$^{mi/mo}$ male mice shortly after MCAo and cognition is impaired in M3rKO^{mi} three weeks after MCAo.

5. Conclusions

We show that depletion of M3r exclusively in microglia and in microglia and monocytes resulted in detrimental post-stroke outcome in young male mice. We thus conclude that M3r-expressing microglia is protective after ischemia in male mice. Further studies on how to promote expression of M3r in microglia and macrophages, without compromising M3r-signaling in other organ systems would be necessary to leverage the potential therapeutical benefits of M3r modulation after ischemia. Thus far, our work has highlighted a completely novel mechanism through which microglia/monocytes modulate brain disease and has highlighted the importance of sexually dimorphic analysis of disease modulation and progression.

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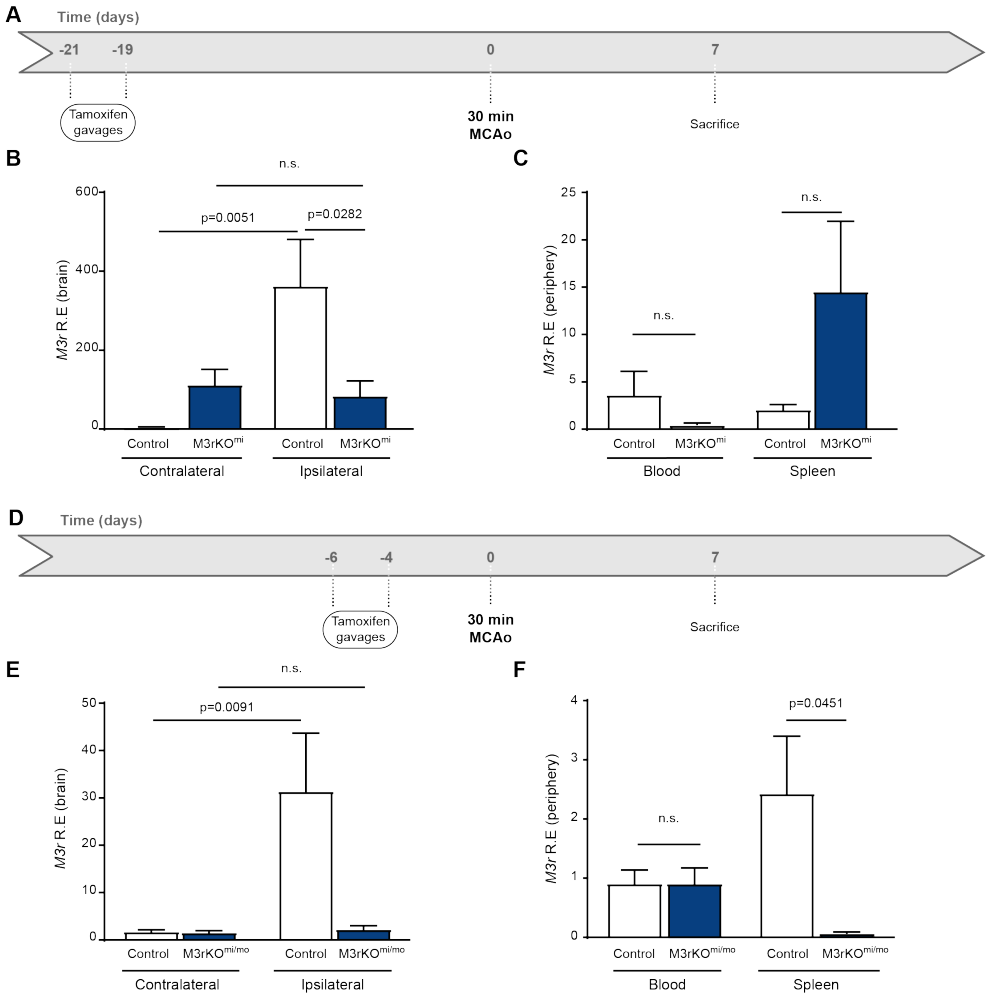
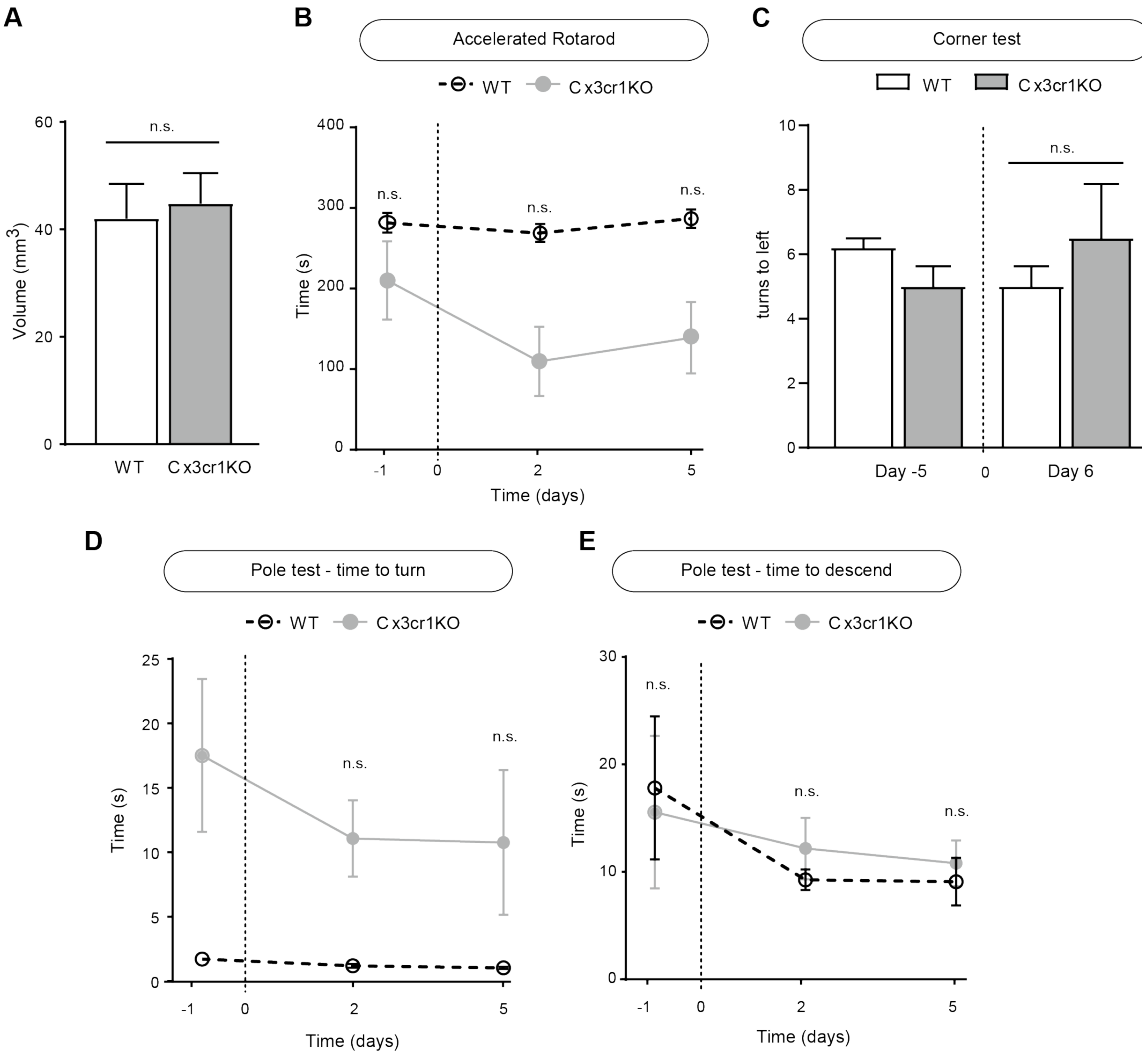
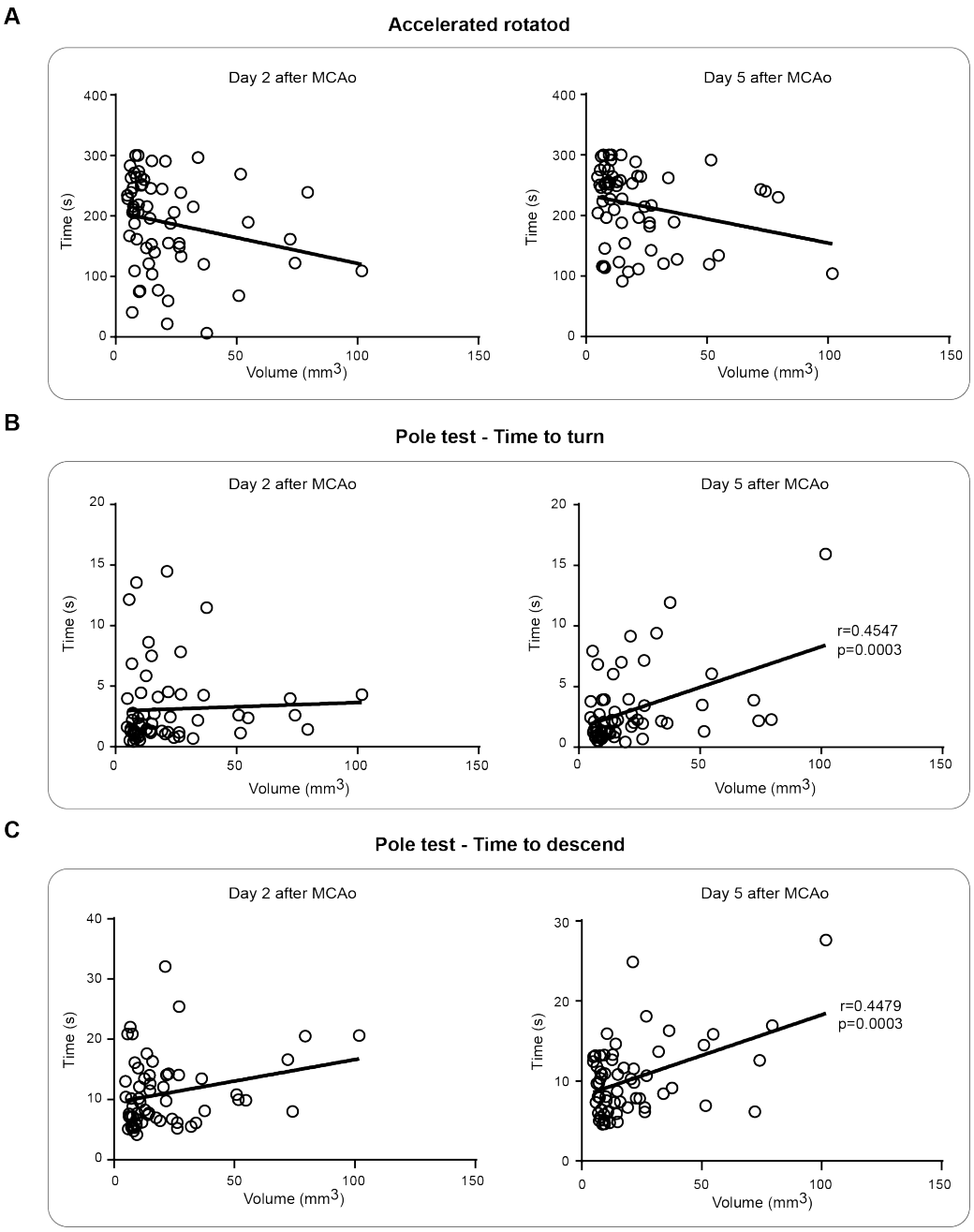
7. Supplementary figures



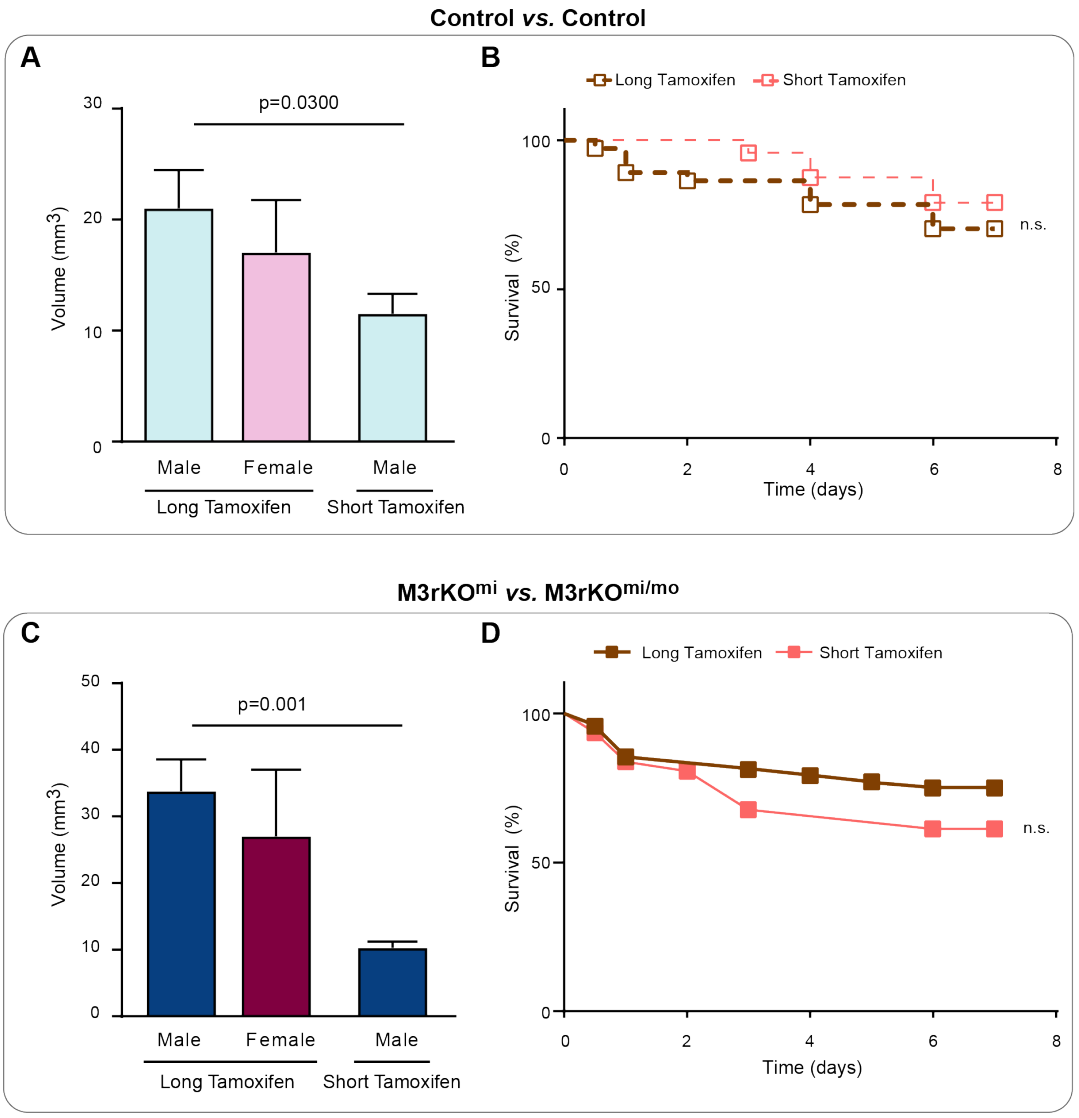
Supplementary Figure S1: We generated a mouse model that makes possible depletion of M3r exclusively in microglia or simultaneously in microglia and peripheral macrophages. A. When mice were given tamoxifen 3 weeks before MCAo, **B** we found that increased *M3r* expression in controls but not M3rKO^{mi} mice. **C.** Also, we found no difference in *M3r*-expression in the spleen or blood of M3rKO^{mi} mice compared to controls. **D.** When mice were given tamoxifen the week before MCAo, **E** we found that increased *M3r* expression in the lesion of controls compared to their contralateral side and to M3rKO$^{mi/mo}$ mice lesions. **F.** We found lower splenic expression of *M3r* in M3rKO$^{mi/mo}$ mice than in controls. Adapted from Costa et al.[87].



Supplementary Figure S2: Depletion of C3xcr1 does not cause worsening of the outcome after stroke. A. We found no differences in the volume of Cx3cr1KO mice lesions compared to controls. We found no differences between both groups in the **B.** accelerated rotarod, **C.** corner test and **D.** on both parameters measured by the pole test. Adapted from Costa et al.[87].



Supplementary Figure S3: Lesion volume correlates with motricity impairment on the pole test. A. We found no correlation between lesion volume and performance on the rotarod on day 2 **(left)** and day 5 **(right). B.** We found no correlation between lesion volume and impairment on performing the 180° turn on day 2 but found a correlation on day 5. **C.** As in **B.** the lesion volume did not correlate with performance on descending the pole on day 2 but correlated on day 5 after MCAo. Adapted from Costa et al.[87]



Supplementary Figure S4: Administration of tamoxifen before ischemia is protective. A. Control mice given tamoxifen less than a week before MCAo (short tamoxifen) show smaller lesions than mice given tamoxifen 3 weeks before MCAo (long tamoxifen). **B.** Tamoxifen gavages shortly before MCAo do not give mice an advantageous overall survival. **C.** M3rKO mice given tamoxifen shortly before MCAo ($M3rKO^{mi/mo}$ mice) show smaller lesions than mice given tamoxifen 3 weeks before MCAo ($M3rKO^{mi}$ mice). Adapted from Costa et al.[87].

Statutory Declaration

“I, Amanda Luisa de Andrade Costa, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “Muscarinic receptor 3 modulates microglial response after ischemic stroke”/ ‘‘Die Rolle des Muskarinische Rezeptor 3 in der Modulation der Reaktion von Mikroglia nach ischämischem Schlaganfall” independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of contribution to the top-journal publication for a PhD degree

Authors: Amanda Costa, Verena Haage, Seulkee Yang, Stephanie Wegner, Burcu Ersoy, Bilge Ugursu, Andre Rex, Golo Kronenberg, Karen Gertz Matthias Endres Susanne A. Wolf, Helmut Kettenmann

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Author contribution in details:

- **Amanda Costa:** Formal Writing (original draft and review & editing). Performed experiments and analysis for Figure 1, 2, 3, 4 (except counting cells in the contralateral side of Figure 4B and FlowJo analysis of Figure 4D), 5, Supplementary Figure 2 (except isolation of blood cells), 3, 4 and 5.
- **Verena Haage:** Writing (original draft). Isolation of blood cells - Supplementary Figure 3B and D
- **Seulkee Yang:** Analysis of contralateral side in Figure 4B
- **Stephanie Wegner:** Trained Amanda Costa to perform the behavioral analysis
- **Burcu Ersoy:** Performed analysis of Supplementary Figure 2A
- **Bilge Ugursu:** Performed analysis for Supplementary Figure 2 B-E
- **Andre Rex:** MCAo surgeries
- **Golo Kronenberg:** Conceptualization, Supervision.
- **Karen Gertz:** Conceptualization, MCAo surgeries, Investigation, Supervision.
- **Matthias Endres:** Conceptualization, Funding acquisition.

- **Susanne A. Wolf:** Shared senior author. Conceptualization, Analysis of Figure 4D data, Writing – (original draft, review & editing), Supervision, Project administration, Funding acquisition.
- **Helmut Kettenmann:** Shared senior author. Conceptualization, Investigation, Writing (original draft, review & editing), Supervision, Project administration, Funding acquisition.

Berlin,

_____ Signature of the Student

_____ Signature, date and stamp of the Supervisor

Extract from the Journal Summary List “Neurosciences”

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **“NEUROSCIENCES”** Selected Category Scheme: WoS
Gesamtanzahl: 267 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	43,107	33.162	0.068480
2	NATURE NEUROSCIENCE	63,390	21.126	0.164700
3	ACTA NEUROPATHOLOGICA	20,206	18.174	0.041660
4	BEHAVIORAL AND BRAIN SCIENCES	9,377	17.194	0.010240
5	TRENDS IN COGNITIVE SCIENCES	27,095	16.173	0.040040
6	JOURNAL OF PINEAL RESEARCH	10,695	15.221	0.010560
7	NEURON	95,348	14.403	0.218680
8	TRENDS IN NEUROSCIENCES	20,163	12.314	0.024480
9	Annual Review of Neuroscience	14,042	12.043	0.015020
10	MOLECULAR PSYCHIATRY	20,353	11.973	0.049290
11	BRAIN	52,970	11.814	0.074030
12	BIOLOGICAL PSYCHIATRY	43,122	11.501	0.053320
13	PROGRESS IN NEUROBIOLOGY	12,929	10.658	0.013230
14	Nature Human Behaviour	1,230	10.575	0.006550
15	SLEEP MEDICINE REVIEWS	6,920	10.517	0.010920
16	ANNALS OF NEUROLOGY	37,336	9.496	0.048630
17	Molecular Neurodegeneration	4,248	8.274	0.011350
18	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	26,724	8.002	0.051580
19	FRONTIERS IN NEUROENDOCRINOLOGY	4,196	7.852	0.005490
20	Neurology-Neuroimmunology & Neuroinflammation	1,996	7.353	0.008220
21	NEUROPSYCHOPHARMACOLOGY	25,672	7.160	0.039090

Selected JCR Year: 2018; Selected Categories: "NEUROSCIENCES"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Brain Stimulation	5,457	6.919	0.014470
23	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,876	6.878	0.006420
24	NEUROENDOCRINOLOGY	5,046	6.804	0.005690
25	NEUROSCIENTIST	4,986	6.791	0.008520
26	BRAIN BEHAVIOR AND IMMUNITY	14,533	6.170	0.025700
27	BRAIN PATHOLOGY	5,263	6.155	0.007880
28	Alzheimers Research & Therapy	3,160	6.142	0.010700
29	JOURNAL OF NEUROSCIENCE	175,046	6.074	0.233460

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Curriculum Vitae

"My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection."

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