Nanobody-Mediated Inhibition of P2X7 on Microglia Improves Stroke Outcome

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Research

Keywords: P2X7, Nanobodies, Ischemic stroke, Nanomedicine, Cerebrovascular disease

Posted Date: November 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1050647/v1

Abstract

Background

Previous studies have demonstrated that purinergic receptors could be therapeutic targets to modulate the inflammatory response in multiple brain disease models. However, tools for the selective and efficient targeting of these receptors are scarce. The new development of P2X7-specific nanobodies (nbs) enables us to effectively block the P2X7-channel.

Methods

Temporary middle cerebral artery occlusion (tMCAO) in wildtype and P2X7-transgenic mice was used as a model for ischemic stroke. ATP release was assessed in transgenic ATP sensor mice. Stroke size was measured without treatment and after injection of P2X7-specific nbs i.v. and i.c.v. directly before tMCAO-surgery. P2X7-GFP expressing transgenic mice were used to show immunhistochemically P2X7 distribution in the brain. In vitro cultured microglia were used to investigate calcium-influx, pore-formation via DAPI uptake, caspase 1 activation and IL-1b release after incubation with P2X7-specific nbs.

Results

ATP sensor mice showed an increase of ATP-release in the ischemic hemisphere compared to the contralateral hemisphere or sham mice up to 24 h after stroke. We could further verify the role of the ATP-P2X7 axis in P2X7-overexpressing mice, which showed significantly greater stroke volumes after 24 h. *In vitro* experiments with primary microglia cells showed that P2X7-specific nanobodies were capable of dampening the ATP-trigged calcium-influx and formation of membrane pores measured by Fluo4 fluorescence or DAPI uptake. We found a lower caspase 1 activity and a subsequently lower IL-1b release. However, the intravenous (i.v.) injection of P2X7-specific nanobodies compared to isotype controls before the tMCAO-surgery did not result in smaller stroke size compared to isotype controls. As demonstrated by FACS, nbs had only reached brain infiltrating macrophages but not microglia. To reach microglia, we injected the P2X7-spezific nbs or the isotype directly intraventricularly (icv). 30 mg of P2X7-specific nbs proved efficient for microglial targeting, reducing post-stroke microglia activation and stroke size significantly.

Conclusion

Here, we demonstrate the importance of locally produced ATP for the tissue damage observed in ischemic stroke and we show the potential of icv injected P2X7-specific nbs to reduce ischemic tissue damage.

Introduction

Ischemic stroke is the second leading cause of death and the leading cause of disability in the western world [1]. Currently the only available therapy is reperfusion of the ischemic brain area by either an i.v.

lysis with recombinant tissue plasminogen activator (r-tPA) or a mechanical thrombectomy. Because of the limited time window for applying these therapies and several contraindications, only a minority of patients is eligible to receive these therapies. Since all attempts at direct pharmacological neuroprotection have failed, new therapeutic opportunities combating ischemic brain damage are needed.

Stroke induces a sterile inflammation, which worsens the initial brain damage and neurological outcome [2]. The hypoxic brain tissue releases a vast amount of molecules, which can activate cells like microglia in the surrounding tissue and lead to an infiltration of other immune cells like neutrophils, amplyfing the inflammatory cascade [3]. These molecules include adenosine triphosphate (ATP) as wells as nicotinamide adenine dinucleotide (NAD), heat shock protein (HSP), and high-mobility group box 1 protein (HMGB1). These molecules can activate the inflammasome and induce secretion of pro-inflammatory cytokines by innate immune cells [4, 5]. They activate several pathways such as the ATP/P2X7 or the NFκB pathway [6]. The P2X7 receptor is a homotrimeric, ligand gated non-selective-cation channel, which is expressed in the central nervous system as well as on immune cells [7]. Several studies have shown that experimental stroke size in $P2X7^{-/-}$ mice is smaller compared to wildtypes [8]. In addition, blocking of the P2X7-channel by brilliant blue G (BBG) attenuates the ischemic damage [8]. However, systemic BBG cannot be used in humans since it is unspecific and toxic.

Nanobodies (nbs) are single domain antibodies (sdAbs) derived from camelid heavy chain antibodies. With their long complementarity determining region 3 (CDR3), they can access cavities or clefts on membrane proteins that are often inaccessible to antibodies [9]. Nbs are highly specific and show a low immunogenicity and toxicity. In contrast to small molecule inhibitors, which often bind to several receptors, nanobodies are highly specific.

In this study, we used P2X7 specific nbs to treat mice directly before tMCAO surgery. We find that these nbs need to be injected intracerebrally to properly reach microglia cells and protect from ischemic stroke. Our nbs can substantially reduce the activation of microglia including the secretion of IL-1β.

Methods

Animals

All animal experiments were approved by the local animal care committees (Behörde für Justiz und Veterinärwesen Hamburg, Nr 006/18; Regierung von Oberbayern) and conducted following the “Guide of the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 83-123, revised 1996). All mice were kept at a constant temperature of 22±2°C with a 12 hours light-dark cycle and ad libitum access to food and water. Only 12 to 18-weeks-old male mice were used for this study. C57BL/6 mice were bought from Charles River (Bar Harbor, ME 04609, USA), whereas generation of pmeLUC transgenic and P2X7-EGFP transgenic mice (line 17 in C57BL/6) was described previously [10, 11].

Production of P2X7-nanobodies

P2X7-antagonizing nanobodies 1c81 and 13A7 were selected and cloned into the pCSE2.5 expression vector (kindly provided by Thomas Schirrmann, Braunschweig, Germany)[12] as described previously [10, 13]. 13A7 was fused to the hinge, CH2, and CH3 domains of mouse IgG2c resulting in a heavy chain format (13A7-Fc; nb A), whereas 1c81 was dimerized and fused to the albumin-specific Nb Alb8 [14] resulting in a bispecific heterotrimeric nb with an extended half-life (1c81-dim-alb, nb B). HEK-6E cells were transfected with the constructs and 6 days after the transfection the nanobodies were purified from the cell supernatant by affinity chromatography on a protein-G sepharose column. Buffer was exchanged by gel filtration on a PD-10 column. Concentration and purity was monitored by SDS-Page and BCA™ Protein Assay Kit (Pierce).

tMCAO-Surgery and stroke size analysis

Transient middle cerebral artery occlusion (tMCAO) was performed as previously described in two independent laboratories (Hamburg and Munich) [15–17]. Mice were anesthetized with 1.5% isoflurane in 100% O_2 and intraperitoneal injection of 0.05 mg/kg body weight buprenorphine in saline. A midline skin incision in the neck was made before ligating the proximal common carotid artery (CCA) and the external carotid artery (ECA) without disrupting the venous vessels. Vital parameters were continuously monitored with the PhysioSuite (Kent Scientific Corporation, USA). Occlusion was confirmed by a laser Doppler monitor (moorVMS-LDF; Moor Instruments UK) and persisted for 40 (Hamburg) or 60 minutes (Munich). Mice with an occlusion rate of less than 80% were excluded.

Stroke size was measured by TTC-staining and MRI (Hamburg), or by cresyl violet staining (Munich). We used a 7T MR (magnetic resonance) small animal imaging system (ClinScan, Bruker, Ettlingen, Germany). The image protocol comprised T2-weighted imaging MRI. Calculation of corrected stroke volumes were performed as described previously [18].

Infarct volumes and total areas of the treated hemisphere were calculated with the use of Fiji software.

IV- and ICV-Injections of Nanobodies

P2X7-specific Nanobodies (Nb A, 13A7-Fc) was injected (100 µg in 100 µl PBS) i.v into the retroorbital plexus. P2X7-specific Nanobodies (Nb B, 1c81-dim-HLE) or isotype nb against a human CD38 were injected (30 µg in 2 µl PBS (phosphate buffered saline) containing 60mg/ml trehalose and 0.4mg/ml Tween-20) directly into the ventricles of the brain by using a stereotaxic apparatus. Mice were anesthetized with tramadol 1 mg/kg bodyweight one day before surgery. Directly prior to the surgery mice were anesthetized with isoflurane (4% for induction, 2,5% for maintenance) in 100% oxygen. After placing the mice in a stereotactic frame (Stoelting, 51615) a 1-cm-long incision was made above the midline. A cranial burr hole (0,9 mm) was drilled 1.1 mm lateral and 0.5 mm posterior to the bregma. Nanobodies were drawn into a 10-µl Hamilton syringe (Hamilton, 1701RN) connected to a 26-gauge needle (Hamilton, 26G, Point Style 4, 12°) controlled by a motorized stereotaxic injector (Stoelting, integrated stereotaxic injector [ISI]).

The needle was slowly introduced 2,3 mm deep into the left ventricle (**Suppl. Fig. 3**). Following a period of 5 minutes to let the ventricular system re-expand, 2 µl of solved nbs at a concentration of 15 µg/µl were injected 1 µl/min. This was proceeded by another 10 minute break and slow removal of the needle. Vital parameters were monitored by an animal support unit (Minerve, Esternay, France). Body temperature was maintained throughout the procedure at 37°C using a feedback-controlled heating device.

In vivo ATP measurement after tMCAO using pmeLUC-TG

3 hours before tMCAO surgery 150 mg/kg luciferin (Promega) was injected i.p.. *In vivo* ATP release was monitored by whole body luminometry performed using the IVIS-Perkin Elmer *in vivo* imaging system. In vitro calibration was performed in brain homogenates from the pmeLUC-TG mice.

Microglia and macrophages preparation and FACS

Animals were euthanized and perfused with phosphate-buffer saline. Brains were dissected and digested in 1 mg/ml collagenase and 0.1 mg/ml DNAse. Separation from myelin and debris was performed by density centrifugation with Percoll (GE Helthcare). The following antibodies and detection systems were used: anti-mIgG1-BV421 (Biolegend), anti-mIgG2-BV421 (Biolegend), Fc blocking anti-CD16/CD32 (BioXcell) mAb77 (Alb8-specific mouse monoclonal antibody kindly provided by Ablynx). Microglia were gated as mentioned in the supplementary (Suppl. Fig. 1). In the first step cells were incubated (30 min on ice). Ex vivo samples were incubated with an addition of 0,5 µg of P2X7 specific nanobody in presence of Fc blocking anti-CD16/32. For detection of cell-bound P2X7 nanobodies, cells were incubated either with anti-mIgG2 (Nb A) or with mAb77 (Nb B) followed by fluorochrome-conjugated antibodies in the presence of Fc blocking anti-CD16/CD32. Calcium influx was measured by Fam-FLICA detection system. DAPI-uptake and IL-1β release was monitored by commercial flow-cytometry. IL-1b ELISA.

Immunostaining

For immunostaining, mice were sacrificed and their brain were fixed in paraffin. Staining was performed on 50µm free floating sections using IbA1 (FUJIFILM Wako Pure Chemical Corporation), DAPI (ThermoFisher Scientific), NeuN (ThermoFisher Scientific).

Results

ATP is released rapidly after ischemic stroke

We analyzed ATP release after tMCAO by using an ATP-sensing pmeLUC transgenic (pmeLUC-TG) mice ubiquitously expressing a firefly-derived luciferase on the outer layer of the plasma membrane [11, 19], which is activated by extracellular ATP (eATP). These pmeLUC-mice are able to detect changes of the eATP concentration in the micromolar range in a strictly ATP-selective fashion since they are insensitive to all other nucleotides.

Directly after tMCAO, a base image was taken (Fig. 1). At 90 min after artery occlusion, eATP release/luminescence substantially increased in the ischemic hemisphere. After 24h, we could still detect

a strong signal in the ischemic hemisphere. Rough estimations of the in vivo eATP concentration were done by an in vitro concentration gradient (**Suppl. Fig. 1**).

These findings indicate that ATP is rapidly and continuously released after stroke.

P2X7 overexpression exacerbates the resulting stroke volume

P2X7 is the main pro-inflammatory receptor for eATP in the CNS. Immunostaining of P2X7-EGFP transgenic mice revealed that P2X7 is expressed mainly on IbA1-positiv cells (Fig. 2A). Merged stainings of IbA1 and GFP (green fluorescent protein) showed for most part a congruent symmetry, where neurons, stained with NeuN, did not show any GFP expression. In order to evaluate the relevance of P2X7 for ischemic stroke, we used P2X7-overexpressing mice [10]. Littermate mice and P2X7-overexpressing mice were subjected to tMCAO and their stroke size determined by TTC. After 24h, we found that stroke sizes between WT-cohort and P2X7-overexpressing mice were significantly different. The P2X7-overexpressing mice had a mean ischemic volume of 52.50 mm^3 ± 8.52 mm^3 compared to littermates with a mean ischemic volume of 36.66 mm^3 ± 13.64 mm^3 (**; p< 0.01, Fig. 2B). Also the percentage of the ischemic hemisphere differed significantly between the two cohorts with 49.6 % ± 876 % in the P2X7-overexpressing cohort and 3415 % ± 1.56 % (**; p<0,01) in the control group (Fig. 2B). In a second cohort subjected to tMCAO in a different laboratory (Munich) after 60 min of occlusion, we saw a trend towards greater infarct sizes, which was not significant (**Suppl. Fig. 4**)

P2X7 **specific nbs inhibits the ATP signal in vitro**

Binding of extracellular ATP to the P2X7 receptor leads to an opening of the P2X7 channel and an influx of Ca^{2+} and Na^{+} and an efflux of K^{+}. Together, these events result in the assembly of the NLRP3 inflammasome, Caspase-1 activation followed by IL-1β release through pores formed by GasderminD [4].

To verify that our P2X7-specific nanobodies can modulate these effects in microglia, we tested their effects on ATP stimulated primary microglia. 100 µg of P2X7 specific nbs decreased ATP-evoked calcium influx compared to isotype control nbs (Fig. 3A). In addition, targeting P2X7 by nbs led to a dampened ATP-evoked pore formation monitored by DAPI uptake (Fig. 3B). Caspase-1 activation, measured by the FAM-FLICA detection system, was significantly reduced in the presence of P2X7-blocking nbs (Fig. 3C). ATP- and LPS-evoked IL-1β release was also significantly reduced after preincubation with P2X7-specific nanobodies (Fig. 3D; ***; p<0.001). Further investigation showed that even low doses of nbs were sufficient to suppress IL-1β release (Fig. 3E).

The data shows that P2X7-specific nbs inhibit microglial activation in response to an ATP challenge.

Intravenous injection of P2X7-specific nanobodies did not affect stroke size

Next, we investigated the effect of i.v. injected P2X7-specific nbs on stroke size in wildtype mice. We injected 100 µg of a P2X7-specific nbs prior to tMCAO. Stroke size was analyzed 24h after surgery in two independent cohorts by histology or MRI, respectively. We did not find any significant reduction in stroke size compared to isotype control nbs. Isotype-treated wild-type mice showed a parenchyma loss of 60.9 % ± 12.3 % in the ischemic hemisphere in TTC-cohort and 62.92% ±1.68% in the MRI cohort. Nb-treated mice showed an almost identical loss of parenchymal tissue with 59.41% ± 15.13% in the TTC-cohort and 63.72% ± 3.73% in the MRI-cohort (Fig. 4A).

To detect if our nbs cross the blood brain barrier (BBB), we injected fluorophore labeled P2X7-specific nbs i.v. 1h after tMCAO. After 24h, we analyzed the MFI of P2X7-specific nb bound to either microglia or infiltrating macrophages. We found that 100µg of i.v. injected nbs did not label brain microglia but could be detected on infiltrating macrophages (Fig. 4B).

These findings indicate that high concentrations of i.v. injected P2X7-specific nbs do not cross the BBB in relevant amounts to reach microglia even though stroke disrupts the BBB [20].

ICV injection of P2X7-specific nbs reduces stroke size

In order to circumvent the BBB, we performed direct icv injection of nbs to the brain and examined the effect of icv injected P2X7-specific nbs on stroke size. We injected 30 µg of P2X7-specific nbs or an isotype control nbs into C57/B6 mice. 24h after tMCAO, we analyzed stroke size and found that the P2X7-specific nbs had significantly decreased stroke sizes (26.16 mm^3 ± 10.29 mm^3 compared to isotype 42.02 mm^3 ± 8.49 mm^3 ***; $p<0.01$, Fig. 5A). This effect was also reflected by the loss of viable tissue (22.78 % ± 8.84 % compared to isotype 38.00 % ± 8.32 %; ***; $p<0,01$, Fig. 5A). In addition, flow cytometry of brain resident microglia of these mice showed a strong signal of P2X7-specific nbs in contrast to the control isotype cohort (Fig. 5B), which could not be further increased by ex vivo addition of the P2X7-specific nbs. Functional P2X7 activation was tested by analysis of ATP-induced DAPI-uptake (Fig. 5C). 160 min after icv injection of P2X7-specific nbs, microglia showed lower DAPI uptake.

Thus, icv injected nbs bound to P2X7 on microglia and reduce ischemic stroke sizes.

Discussion

Here, we show that eATP (extracellular ATP) is present in substantial amounts early after cerebral ischemia and that the cells responding to the eATP challenge are microglia rather than infiltrating macrophages. Blocking P2X7 on microglia diminishes the tissue damage caused by the ischemia. Intracerebroventricular injection is necessary to bypass the BBB and render microglia accessible for P2X7-specific nanobodies.

Mounting evidence indicates that stroke triggers a strong inflammatory response. The injured tissue releases a myriad of molecules that can activate the surrounding or infiltrating immune cells. Potent activators of local immune responses are danger-associated molecular patterns (DAMPs). These include

for example eATP, NAD, HSP, and HMGB1. Some of these endogenous danger signals can induce activation of the inflammasome and the secretion of proinflammatory cytokines by innate immune cells [4, 21]. Using transgenic mice that express luciferase on the outer layer of the cell membrane, we could show that similar to traumatic brain injury [22], eATP is released very early during ischemic tissue damage. In addition, the signal is sustained over 24 hours clearly indicating an ongoing release of eATP in the ischemic tissue. Therefore, it is likely that eATP and its cognate receptors play an important role for the initiation of the inflammatory reaction following stroke. eATP activates purinergic receptors. While the microglial P2Y12 receptor is important for the microglia neuron interaction, the pro-inflammatory response by microglial is likely triggered by P2X7, which is highly expressed by microglia (Fig. 2A)[23–25]. Three polypeptide subunits, each with two transmembrane domains, form an ion-permeable channel upon eATP activation. Opening of the channel induces Na^+ and Ca^{2+} influx and K^+ efflux, resulting in plasma membrane depolarization and initiation of Ca^{2+} signaling cascades. The K^+ efflux through the P2X7 receptor is upstream of the NLRP3-mediated inflammasome complex, which cleaves pro-caspase 1 and leads to a subsequent cleavage of pro-IL-1β and pro-IL-18 into their biologically active forms [5]. The amount of accessible intracellular pro-IL-1β and pro-IL-18, however, depends on a first signal transmitted by receptors such as the toll like receptors (TLR), particularly TLR4, or TNF-receptors and subsequent NFκB.

After ischemic stroke, expression of P2X7 is increased on microglia [26] and can induce cell death in ischemic microglia [27]. We and others have shown that experimental stroke in P2X7-/- mice results in smaller infarcts and that blockade of P2X7 with brilliant blue G (BBG) reduces cerebral ischemic damage [8, 28, 29]. In addition, the inhibition of pannexin 1 decreases the amount of damage after cerebral ischemia, but there is no additional benefit if P2X7 is also blocked [30].These data are still controversial [31]. Yanagisawa and colleagues saw an exacerbation of ischemic brain damage when P2X7 was blocked. Similar findings were also reported by Kang et al. [32], who observed an effect on CNTF production but no effect on lesion size.

One explanation for these discrepancies is the use of BBG. Small molecule inhibitors are often only semi-specific and toxic. Particularly, BBG is not specific for P2X7 and is known to have dose dependent off target effects. On the other hand, nanobodies, recombinant single domain antibodies (sdAbs) derived from camelid heavy chain antibodies, are a promising new technology platform. The first nanobody-based reagents developed by Ablynx-Sanofi have entered clinical trials and achieved FDA approval (targeting TNF-α, von Willebrand Factor, RANKligand, and IL-6 receptor [33]. Nanobodies, named for their small size (3 nm, 12 kDa), offer several key advantages compared to small molecule inhibitor. These include low toxicity, no off target effects and in the case of P2X7R a more potent inhibition [13]. Even compared to conventional antibodies, nanobodies tend to be advantageous. The advantages of nbs over conventional abs include a higher propensity for binding to functional epitopes on proteins, high stability, better solubility, lower immunogenicity, rapid and targetable in vivo biodistribution. In addition, the possibility of assembling nanobody multimers, and the low costs and easiness of production makes them ideal candidates for treatment [34]. Fusion of a nanobody (monomer or multimer) to the Fc domain

of a conventional antibody yields a heavy chain antibody with reconstituted Fc-mediated effector functions, including binding to Fc receptors, extended half-life, and complement activation. This allows a much broader tailoring of nanobodies than of conventional antibodies to the particularities of different pathophysiologies [35].

The BBB is a major obstacle for the treatment of brain disease with biologicals. Under healthy conditions, it is only permeable for lipophilic molecules of up to 400 kDa of size [36]. In addition, the delivery of conventional antibodies to the brain is further hampered by the Fc-receptor mediated efflux to the blood [37]. Therefore, nanobodies lacking an Fc-part represent a promising alternative to reach targets behind the BBB. Yet, under non-pathological conditions monovalent nbs do not reach sufficient concentrations for in vivo brain imaging [38] or therapeutic purposes [39]. In stroke, a biphasic BBB breakdown is caused by activated matrix metalloproteinase -2 (MMP-2), MMP-3 and MMP-9 [40, 41]. The breakdown of the BBB is initially reversible but becomes permanent with the mounting release of MMP-3 and MMP-9 [42]. These findings suggest that antibodies or nanobodies would have an easier access to the brain. However, as we can show here, only a minor portion of the intravenously injected nanobodies reached the brain. While macrophages from the blood stream were quickly covered with nanobodies, when they reached the brain, microglia did not carry any nanobodies and their function was unimpaired (Fig. 4). These observations are similar to observations in antibody crossing of the BBB where usually a direct shuttle system such as the transferrin receptor is needed in order to enter the brain [43].

In stroke, microglia are the first immune cell to respond while macrophages enter the brain at later stages [15]. Therefore, it is not surprising that there was no difference in ischemic lesion size after iv nanobody injection. In contrast, after an intra-ventricular injection of P2X7 nanobody, we could reach an up to 95% of the microglia. This level of P2X7R blockage was sufficient to inhibit microglial IL-1β secretion and improved outcome. Our study shows that an inhibition of signaling by eATP is only effective if it is done early and reaches the microglia population. In humans, microglial P2X7R blockage could be accomplished by lumbar injection into the cerebral spinal fluid. This way of direct injection in the CNS is already used as a therapeutic option for other neurological diseases like neuronal ceroid lipofuscinosis [44].

Conclusion

Taken together, we can demonstrate the importance of locally produced eATP for the damage in ischemic stroke and the potential of intra-ventricularly injected P2X7 nanobodies to reduce this damage.

Abbreviations

(e)ATP: (extracellular) adenosine triphosphate; tMCAO: temporary middle cerebral artery occlusion; h: hours; DAPI: 4,6-diamidino-2-phenylindole; i.v.: intravenous; i.p.: intraperitoneal; icv: intracerebroventricular; r-tPa: recombinant tissue plasminogen activator; TLR; toll-like receptors; NAD: nicotinamide adenine dinucleotide; HSP: heat shock protein; HMGB1: high-mobility group box 1 protein;

DAMPs: damage-associated molecular patterns; NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells; BBG: brilliant blue G; nbs: Nanobodies; sdAbs: single domain antibodies; CDR3: complementarity determining region 3; CH: constant domain heavy chain; IgG: immunoglobulin G; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CCA: common carotid artery; ECA: external carotid artery; MRI: magnetic resonance imaging;; TTC: triphenyl tetrazolium chloride; PBS: phosphate-buffered saline; FLICA: Fluorochrome-labeled inhibitors of caspases; FACS: fluorescence-activated cell sorting; BBB: blood brain barrier; IL: interleukin; RANK: receptor activator of nuclear factor k-B); MMP: matrix metalloproteinases; CNS: central nervous system; GFP: green fluorescent protein

Declarations

Disclosure

None.

Ethics approval and consent to participate

All animal experiments were approved by local animal care committees (Behörde für Justiz und Verbraucherschutz der Freien und Hansestadt Hamburg; Lebensmittelsicherheit und Veterinärwesen, Hamburg Germany, permission number: 006/18). This was a purely animal study with no involvement of patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Research was supported by the DFG Research Unit FOR 2879 Immunostroke (projects A1 [4248778651] to TM, A3 [428663564] to TM and AL), SFB 1328 (project A13 to TM and ET, A15 to ET), the ‘Hermann und Lilly Schilling Stiftung’ to TM and Else-Kröner Stiftung to MW, and by the Italian Association for Cancer Research (AIRC) grant n. IG 22883 to FDV.

Author Contributions

TM, FN, BR, MG, CP and MW designed the study. MW, CP, PL, AL, AN, MG, SF, FDV and BR carried out various experiments, acquired and analyzed the data. MW, TM, MG, CG, ET, FDV, BR, AL, FN wrote the manuscript and edited the manuscript. All authors have given approval to the final version of the manuscript.

Acknowledgements

We thank Lennart Pöls and Oliver Schnapauff for excellent technical assistance. We also thank the departments of Radiology and of Neuroradiology of the UKE for assistance with the MRI measurements.

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Figures

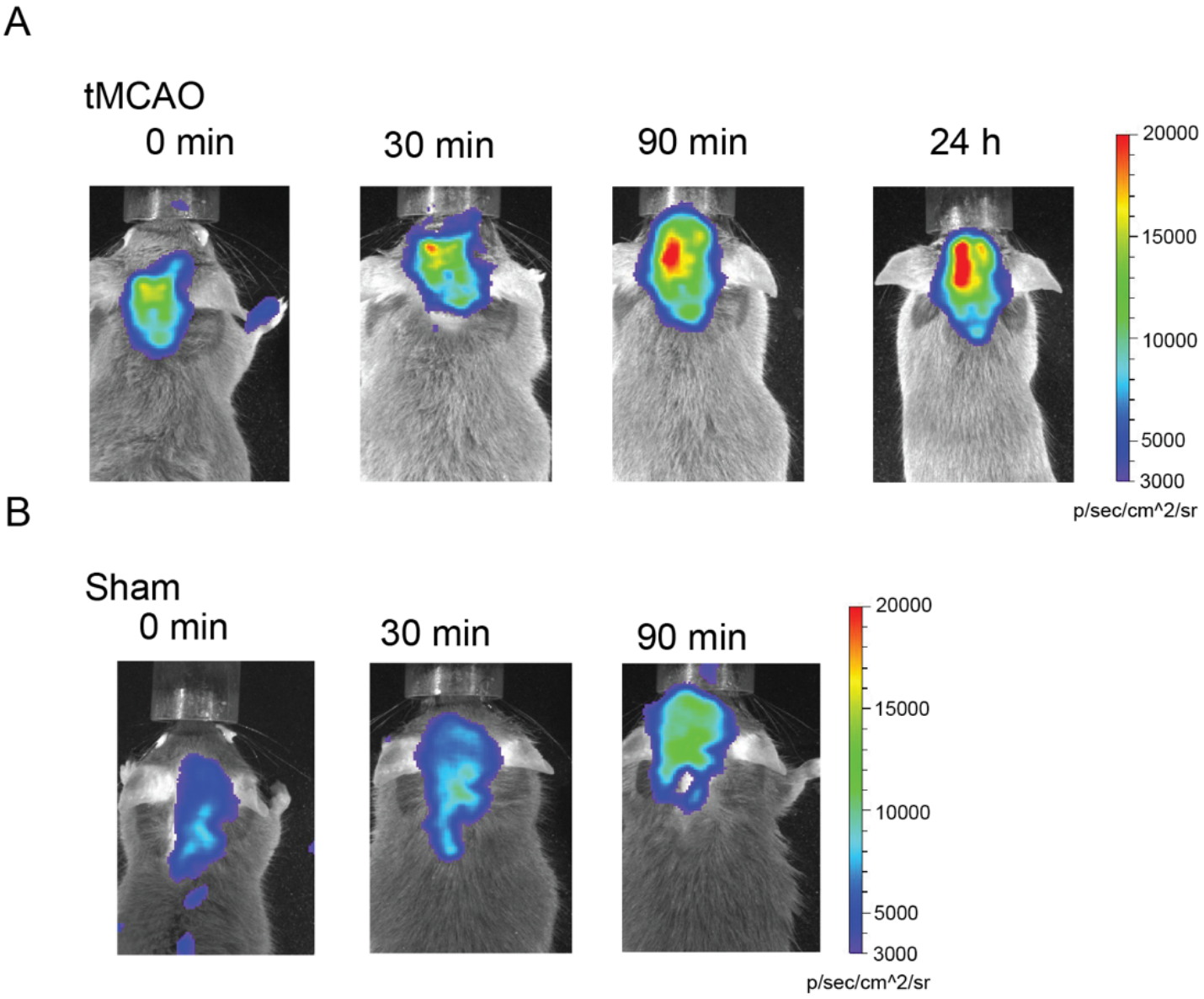

Figure 1

ATP is released after stroke ATP-sensor mice (pmeLuc-mice) show extracellular ATP in the brain (A) After tMCAO, ATP is rapidly released in the ischemic hemisphere starting after 30 min and still prominent after 24h compared to the contralateral hemisphere. (B) Sham mice do not show substantial ATP-level difference between both hemispheres. Six different mice were stroked with similar results. These are representative images.

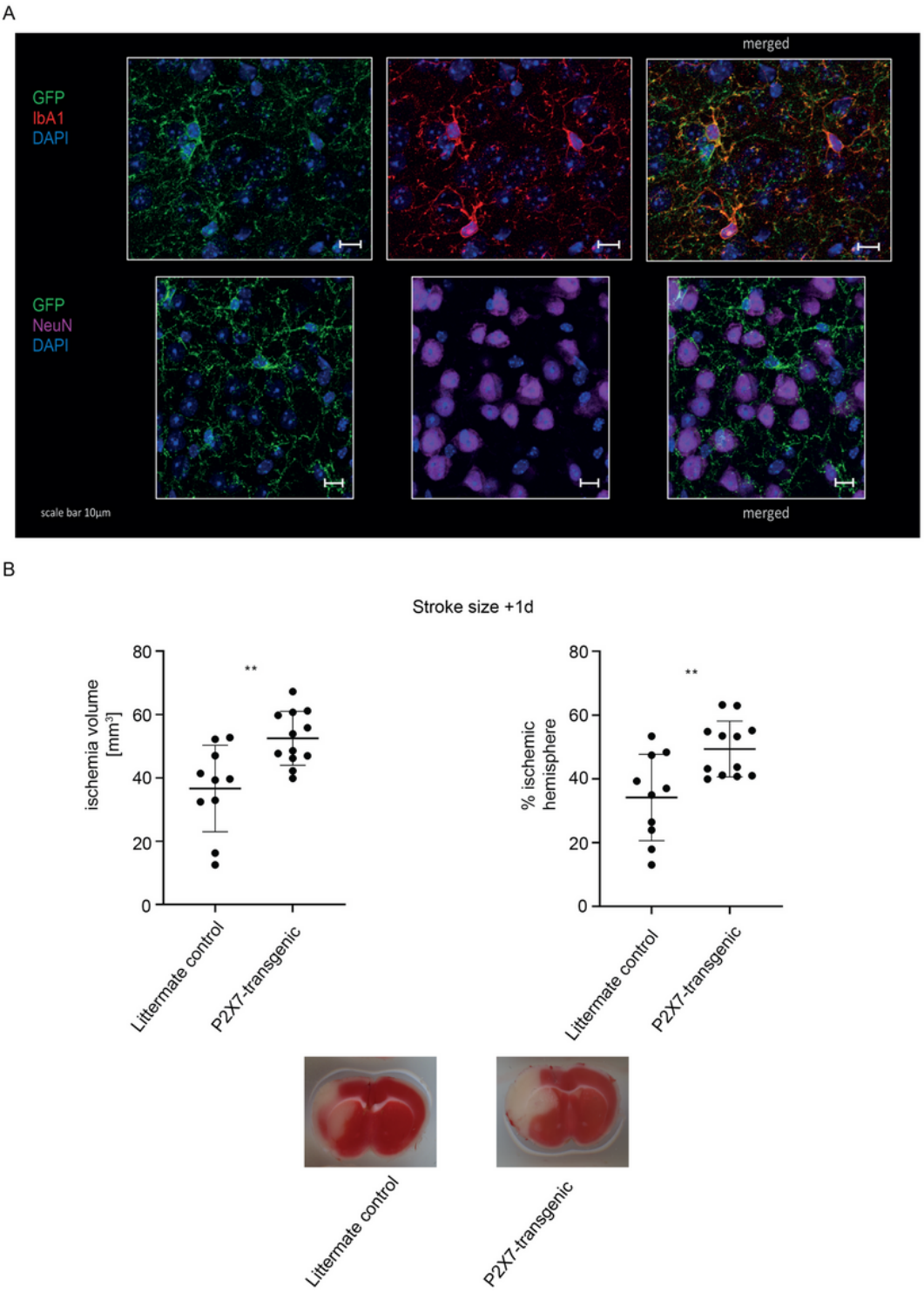

Figure 2

P2X7 is mainly expressed on microglia and P2X7-overexpressing transgenic mice have greater strokes Immunostaining on 50µm free floating sections of P2X7-EGFP (A) transgenic mice illustrated that P2X7 is highly expressed on IbA1-positive (red) cells, compared to NeuN positive (purple) neurons. DAPI (blue) was used to stain the nucleus. Negative control see suppl. Fig. 5. (B) One day after tMCAO-surgery, P2X7-overexpressing mice have significant higher infarct volume (52.50 mm3 ± 8.52 mm3 vs 36.66 mm3 ±

13.64 mm3; **; p<0.01) and a significant higher %-loss of parenchyma in the ischemia hemisphere (49.36% ± 8.76% vs 34.15% ± 13.56%; **; p<0.01). Statistical significances analyzed by Student t test. Data are presented as median ± range of 12 P2X7-overexpressing mice and 10 wildtype littermate controls. Representative triphenyltetrazolium chloride (TTC) staining at day 1 following tMCAO treatment.

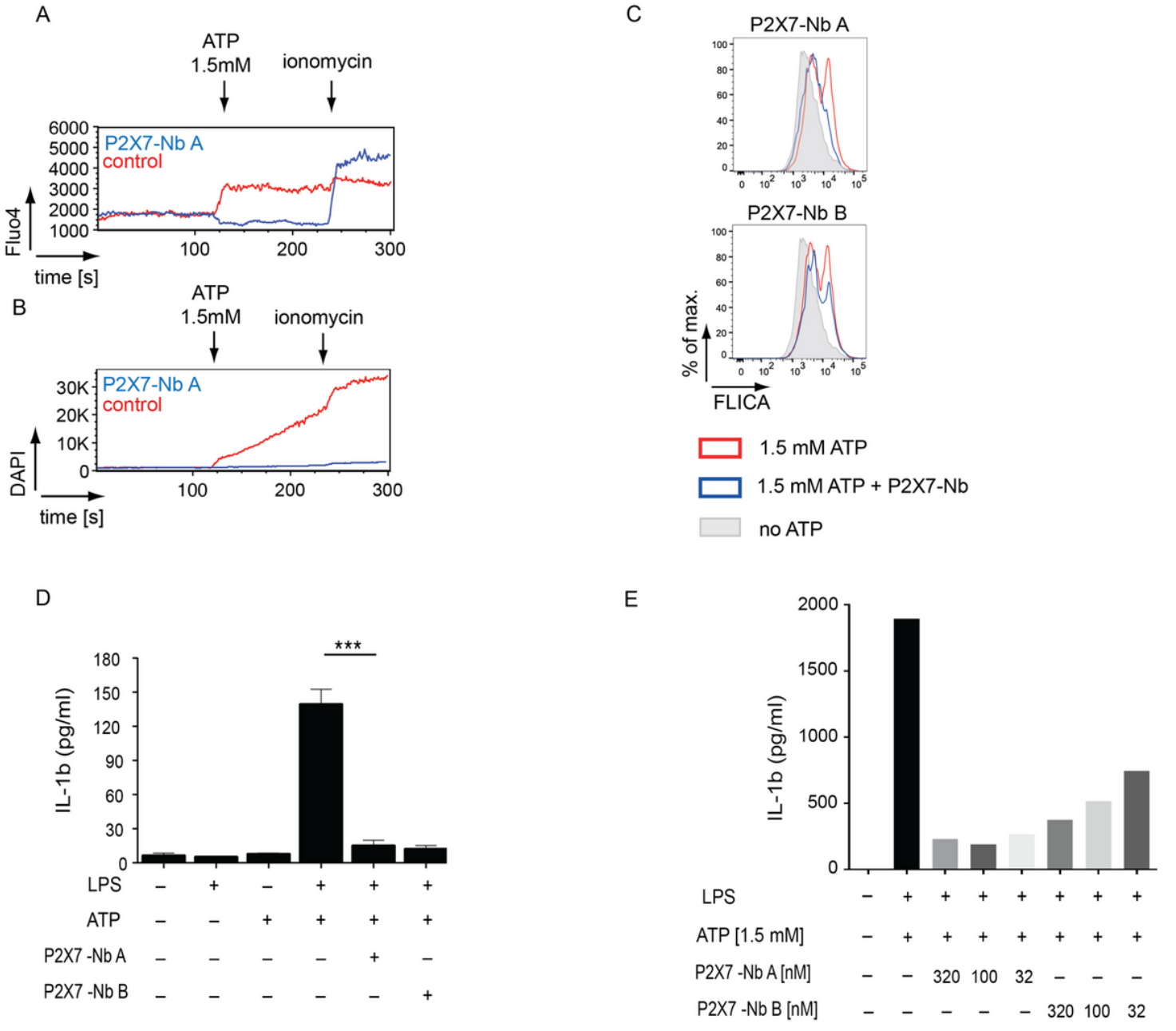

Figure 3

P2X7-specific nanobodies influenced the P2X7-pathway in vitro Primary microglia preincubated with P2X7-specific nbs showed a substantially lower calcium influx, (monitored by Fluo4 (A)), and DAPI-uptake (B) than the isotype control group after ATP (1.5 mM) challenge. As a positive control ionomycin showed a facilitating calcium-influx across the plasma membrane. (C) In the absence of P2X7-specific

nbs stimulated microglia showed a higher caspase 1 activation (measured by appearance of FAM-FLICA) compared to ATP activated microglia in presence of these specific nbs. (D) Blockade of P2X7 by specific nbs dampened IL-1β release in cultured microglia after ATP and LPS-stimulation. Both types of P2X7-specific nbs used in this investigation decreased caspase 1 activation significantly (***; p<0,001). Findings in D were specified in E, showing that IL-1β release can be reduced by using even low doses of nbs.

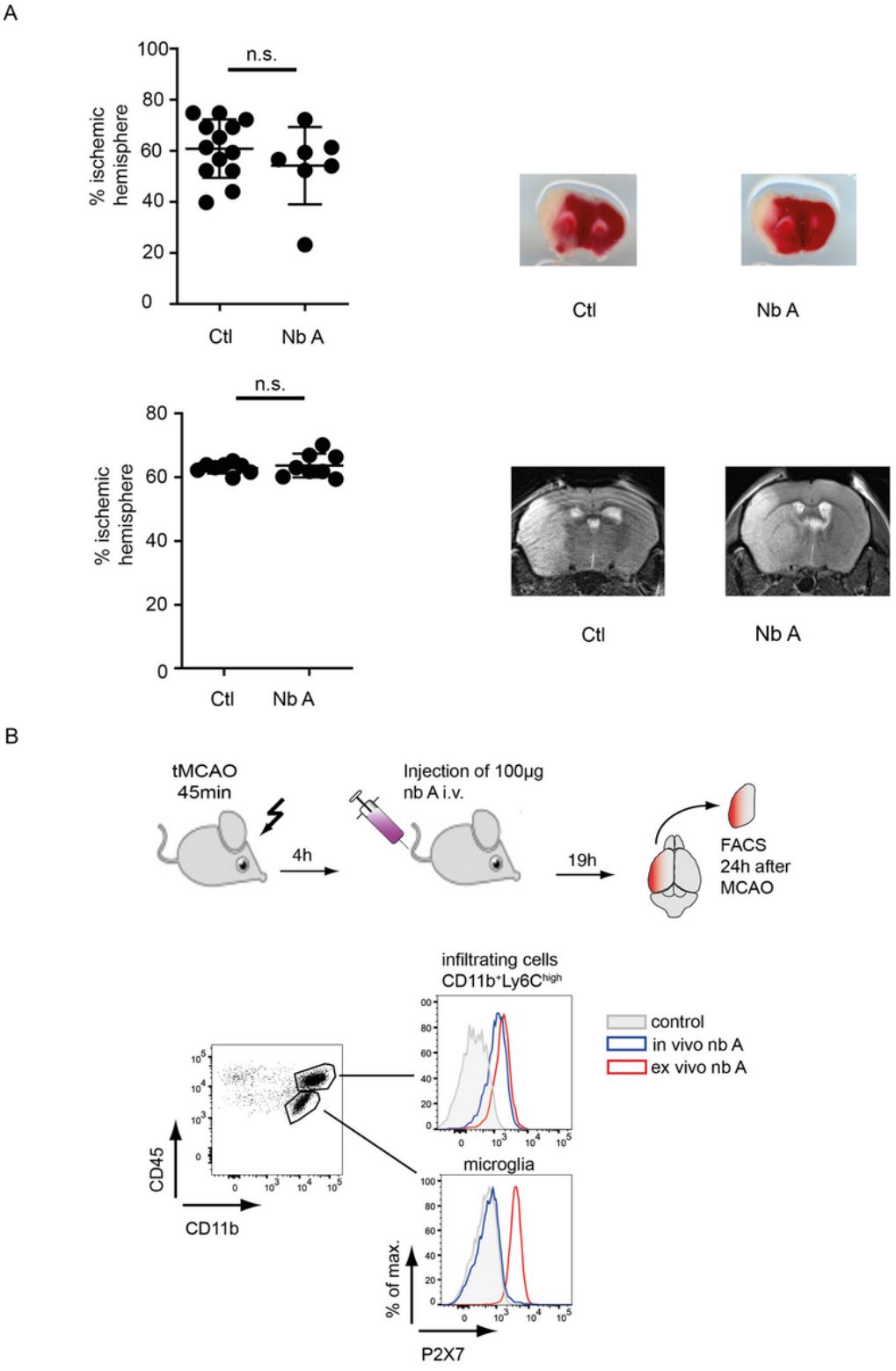

Figure 4

Intravenous injected P2X7-blocking Nbs did not cross the BBB and did not influence stroke size C57BL6J mice received 100 µg of P2X7-specific nbs or control nbs intravenously. 24h after stroke, lesion size was measured via MRI and TTC. Between both groups % of parenchymal loss in the ischemic hemisphere did not differ significantly (A; TTC: Ctl-isotype: 58.65% ± 11.98% vs P2X7-secific nb: 52.21% ± 15.13%; MRI: Ctl-isotype: 62.92% ± 1.68% vs P2X7-specific nb: 63.72% ± 3.73%). (B) To examine if nbs cross the BBB, brains of i.v. nb treated mice were analyzed 24h after the treatment and stroke. After injecting 100 µg of nbs, infiltrating macrophages (C; CD45+CD11b+Ly6Chigh) were occupied by P2X7-specific nbs, whereas brain resident microglia (C; CD45+CD11intermed) did not show any P2X7-specific nb on their surface in flow cytometry. As a positive control 0.5 g of nb A was added ex vivo to illustrate full microglial occupancy.

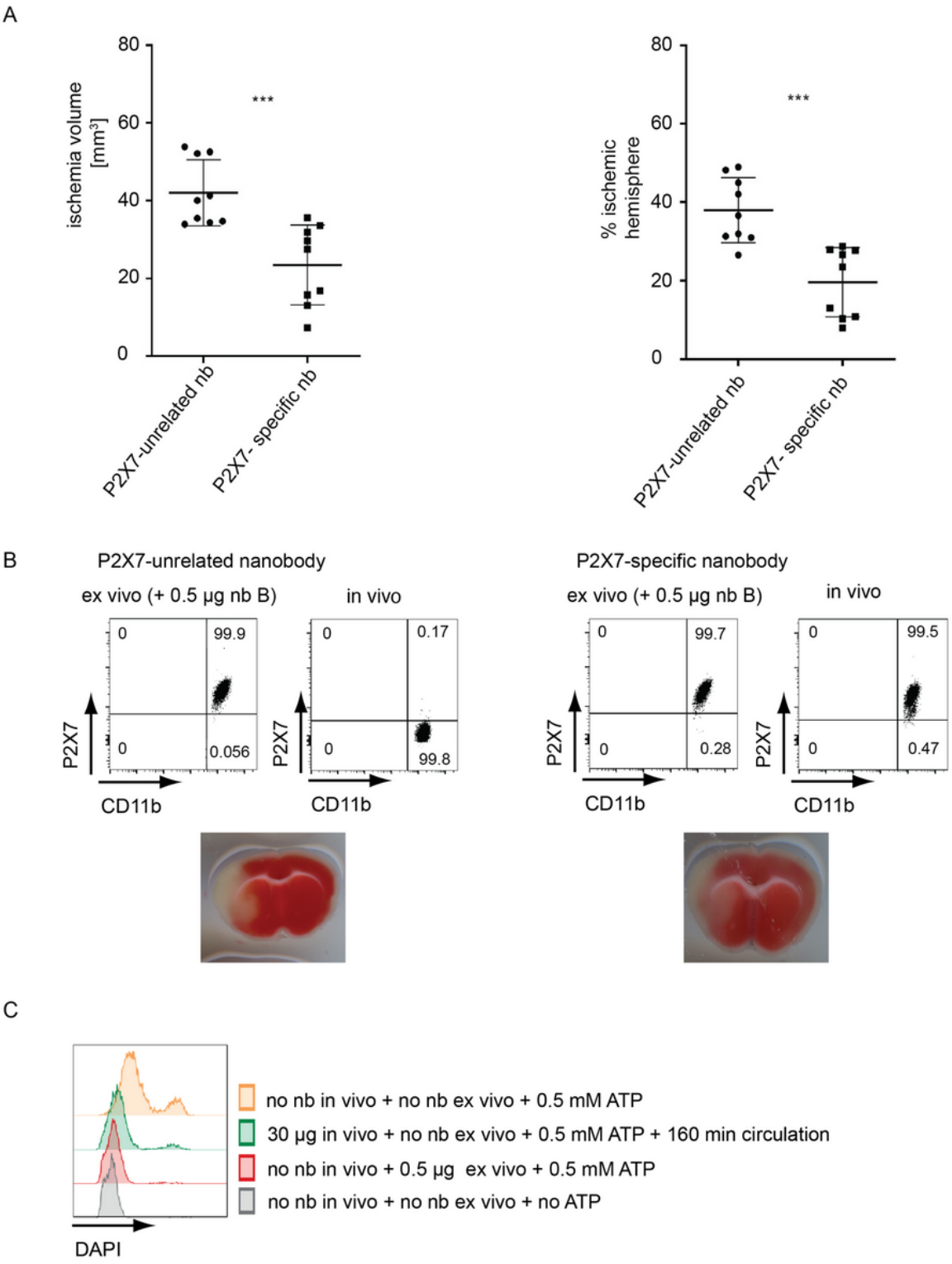

Figure 5

ICV injection of P2X7-nbs significantly reduced stroke size 30µg of P2X7-blocking nbs were injected icv directly before tMCAO surgery to C57BL6J mice. 24h after tMCAO mice were sacrificed. Mice treated with P2X7-blocking nbs showed significantly smaller ischemia volumes and a significant smaller %- of parenchyma loss in the ischemic hemisphere compared to mice treated with a control isotype nb (26.16 mm3 ± 10.29 mm3 vs 42.02 mm3 ± 8.49 mm3; ***; p<0.01; 22.78 % ± 8.84 % vs 38.00 % ± 8.32 %; ***;

p<0.01). (A) Flow cytometry of brain resident microglia showed a full occupancy of P2X7-specific nbs in difference to the control cohort (B; gating strategy Suppl. Fig 2). Statistical significances were analyzed by Student t-test. Data are presented as median ± SD of 9 P2X7-specific nb injected mice and 9 isotype control injected mice. (B) Representative triphenyltetrazolium chloride (TTC) staining at 24h following tMCAO. In addition, (C) 160 min after icv-injection or ex vivo administration of 30 µg of P2X7.-specific nbs, isolated microglia showed substantially lower DAPI-uptake. Microglia in absence of nbs and ATP did not show any DAPI-uptake.

Supplementary Files

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- SupplementaryFigures.docx