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Blocking of bradykinin receptor B1 protects from focal closed head injury in mice by reducing axonal damage and astroglia activation

Christiane Albert-Weissenberger[1], Christian Stetter[2], Sven G Meuth[3,4], Kerstin Göbel[3], Michael Bader[5], Anna-Leena Sirén[2,6] and Christoph Kleinschnitz[1,6]

[1]*Department of Neurology, University Clinic of Würzburg, Würzburg, Germany;* [2]*Department of Neurosurgery, University Clinic of Würzburg, Würzburg, Germany;* [3]*Institute of Physiology – Neuropathophysiology, University of Münster, Münster, Germany;* [4]*Department of Neurology – Inflammatory Disorders of the Nervous System and Neurooncology, University of Münster, Münster, Germany;* [5]*Max-Delbrück-Center for Molecular Medicine, Berlin, Germany*

The two bradykinin receptors B1R and B2R are central components of the kallikrein–kinin system with different expression kinetics and binding characteristics. Activation of these receptors by kinins triggers inflammatory responses in the target organ and in most situations enhances tissue damage. We could recently show that blocking of B1R, but not B2R, protects from cortical cryolesion by reducing inflammation and edema formation. In the present study, we investigated the role of B1R and B2R in a closed head model of focal traumatic brain injury (TBI; weight drop). Increased expression of B1R in the injured hemispheres of wild-type mice was restricted to the later stages after brain trauma, i.e. day 7 ($P < 0.05$), whereas no significant induction could be observed for the B2R ($P > 0.05$). Mice lacking the B1R, but not the B2R, showed less functional deficits on day 3 ($P < 0.001$) and day 7 ($P < 0.001$) compared with controls. Pharmacological blocking of B1R in wild-type mice had similar effects. Reduced axonal injury and astroglia activation could be identified as underlying mechanisms, while inhibition of B1R had only little influence on the local inflammatory response in this model. Inhibition of B1R may become a novel strategy to counteract trauma-induced neurodegeneration.

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Introduction

Traumatic brain injury (TBI) is a major cause of death and disability worldwide but in spite of extensive research efforts specific therapies are still lacking (Tagliaferri *et al*, 2006). This is mainly due to the complex and heterogeneous pathophysiology of TBI which in the early phase (hours to days) depends on the extent and type of the primary impact, whereas at more advanced stages (weeks to months) secondary degenerative mechanisms become relevant and determine the long-term functional outcome, e.g., motor impairment and cognitive decline. An acute focal brain trauma results in cortical contusion, rhexis of blood vessels (hemorrhages), and impaired blood flow in the surrounding areas (ischemia) and is accompanied by a strong and immediate inflammatory response and massive edema formation (Albert-Weissenberger *et al*, 2012; Raslan *et al*, 2010). By contrast, generalized brain trauma or the later stages after focal injury are characterized by diffuse axonal damage, leading to secondary neurodegeneration and atrophy. In addition, a glial scar is often formed that hampers tissue regeneration and repair (Sargin *et al*, 2009; Sirén *et al*, 2006). To date, these secondary injury processes are only poorly understood and the

Correspondence: Dr C Kleinschnitz, University of Würzburg, Department of Neurology, Josef-Schneider-Str. 11, 97080 Würzburg, Germany or Dr A-L Sirén, University of Würzburg, Department of Neurosurgery, Josef-Schneider-Str. 11, 97080 Würzburg, Germany. E-mail: christoph.kleinschnitz@uni-wuerzburg.de or Siren.A@nch.uni-wuerzburg.de

[6]These authors contributed equally to this work.
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cell types and molecular pathways involved therein await clarification.

The kallikrein–kinin system constitutes a framework of serially connected serine proteases, namely coagulation factor XII, kininogen, and plasma kallikrein. All components of the kallikrein–kinin system have been identified in the brain (Camargo *et al*, 1973; Kariya *et al*, 1985; Kizuki *et al*, 1994) and kinins (bradykinin and kallidin) constitute the end products of this enzymatic cascade. Kinins are highly active peptide hormones that mediate their proinflammatory effects via two G-protein-coupled receptors, bradykinin receptor B1 (B1R) and B2R (Marceau and Regoli, 2004). B2R is constitutively expressed in many cell types and mediates the physiologic effects of bradykinin, whereas B1R is induced after tissue damage (Marceau and Regoli, 2004). After activation, both B1R and B2R trigger classical inflammatory cascades such as cytokine release, immune cell invasion, and increased vascular permeability (Marceau and Regoli, 2004). However, preliminary evidence suggests that kinins and their receptors might also be involved in degenerative processes, especially in the central nervous system (Lemos *et al*, 2010).

It is meanwhile well established that the kallikrein–kinin system has a critical role in TBI and other acute disorders of the central nervous system such as ischemic stroke (Gröger *et al*, 2005; Austinat *et al*, 2009). Many of these previous studies named B2R as the prime culprit responsible for inflammation and tissue damage (Gorlach *et al*, 2001; Plesnila *et al*, 2001; Thal *et al*, 2009; Trabold *et al*, 2010; Zweckberger and Plesnila, 2009). However, using mouse models for ischemic stroke, cortical brain injury (cortical cryolesion), and multiple sclerosis (Austinat *et al*, 2009; Göbel *et al*, 2011; Raslan *et al*, 2010) we recently identified B1R as the promising therapeutic target, whereas no beneficial effects could be observed after either acute pharmacological inhibition or constitutive genetic deletion of B2R.

In the present study, we analyzed in parallel the consequences of B1R and B2R deficiency or therapeutic blockade on functional outcome, neurodegeneration, and inflammatory processes in a mouse model of focal closed head injury.

Materials and methods

Animals

A total of 244 male, 10 to 16 weeks old mice weighing 25 to 30 g were included in the study, which was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 83-123, revised 1996). All protocols were approved by governmental authorities (Regierung von Unterfranken, Würzburg, Germany; file number: 55.2-2831.01-3/09). $B1r^{-/-}$ mice were described previously (Austinat *et al*, 2009; Göbel *et al*, 2011; Pesquero *et al*, 2000; Raslan *et al*, 2010), $B2r^{-/-}$ mice were purchased from the Jackson Laboratories (Maine, USA). $B1r^{-/-}$ mice and $B2r^{-/-}$ mice were backcrossed for more than 10 generations into a C57Bl/6 background. Age- and sex-matched C57Bl/6 mice (Charles River, Sulzfeld, Germany) served as controls.

Experimental Conditions

Focal closed head injury was performed as previously described (Albert-Weissenberger *et al*, 2012; Flierl *et al*, 2009). Briefly, after induction of isoflurane anesthesia, the spontaneously breathing mouse was placed in a stereotactic frame and a midline longitudinal scalp incision was made and the skull was exposed. After identification of the right anterior frontal area (1 mm lateral to the midline in the mid-coronal plane) as impact area, a weight with a silicone-covered blunt tip of 2-mm diameter was dropped with a final impact of 0.01 J, resulting in TBI. After trauma, mice received 95% oxygen for 2 minutes and were returned to their cages. Operation time per animal did not exceed 10 minutes. Sham operation included anesthesia and exposure of the skull but without weight-drop injury.

To block the B1R in C57Bl/6 wild-type mice, the selective B1R inhibitor R-715 (Ac-Lys-[D-βNal^7, Ile^8]desArg^9-BK; Tocris Bioscience, UK; Gobeil *et al*, 1996) dissolved in physiologic saline (Braun, Melsungen, Germany) was administered intravenously into the retroorbital vein plexus at a total volume of 200 μl 1 hour after weight drop and then once daily between day 2 and day 7 (the end of the study) at a dosage of 1 mg/kg body weight. Age- and sex-matched control C57Bl/6 mice received an equal volume (200 μl intravenously) of physiologic saline (vehicle).

All animal experiments were performed in accordance with the recently published ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Mice were randomly assigned to the operators (CAW) by an independent person not involved in data acquisition and analysis. We performed surgery and evaluation of all read-out parameters while being blinded to the experimental groups.

The following conditions excluded mice from end-point analyses (exclusion criteria):

1. Death within 24 hours after trauma
2. Visible skull fracture
3. Neurological severity score (NSS) <2 1 hour after trauma

Of the 275 mice subjected to weight-drop injury, 31 mice (11.3%) met at least one exclusion criterion and were withdrawn from the study, resulting in a total of 244 included mice. The number of excluded animals (drop-out rate) was evenly distributed between the experimental groups ($P > 0.05$).

Neurobehavioral Evaluation

The neurobehavioral status of mice was assessed by the NSS (Albert-Weissenberger *et al*, 2012; Flierl *et al*, 2009), a composite score of 10 different items, including tasks on motor function, alertness, and physiological behavior with

Table 1 Neurological severity score (NSS)

Task	*Points (success/failure)*
Exit circle	0/1
Interest in environment	0/1
Mono-/hemiparesis	0/1
Straight walk	0/1
Startle reflex	0/1
Beam balancing	0/1
Beam walk, 3 cm	0/1
Beam walk, 2 cm	0/1
Beam walk, 1 cm	0/1
Round stick balancing	0/1
Maximal Score	**10**

lower scores indicating less deficits (Table 1). One point is awarded for the absence of a tested item or for the inability to perform the tasks, and no point is given when succeeding the task. Mice were evaluated after 1 hour, 3 days, and 7 days after weight-drop injury.

Real-Time PCR with Reverse Transcription

RNA was isolated from the ipsilateral hemispheres at day 1 and day 7 after injury. Tissue from sham-operated mice served as controls. Tissue homogenization, RNA isolation, and real-time PCR with reverse transcription were performed as described (Austinat *et al*, 2009; Raslan *et al*, 2010). Briefly, total RNA was prepared with a Miccra D-8 power homogenizer (ART, Mühlheim, Germany) using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) and was quantified by a photometer. Then, 250 μg of total RNA was reversely transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol using random hexamers. Relative mRNA levels of cytokines and B1R and B2R were quantified with the fluorescent TaqMan technology. PCR primers and probes specific for murine B1R (assay ID: Mm00432059_s1), B2R (assay ID: Mm00437788_s1), β-amyloid precursor protein (β-APP; assay ID: Mm01344172_m1), interleukin-1β (assay ID: Mm004344228_m1), transforming growth factor β-1 (assay ID: Mm00441724_m1), tumor necrosis factor (TNF)α (assay ID: Mm00443258_m1), glial fibrillary acidic protein (GFAP) (assay ID: Mm00443258_m1), nestin (assay ID: Mm00450205_m1), and vimentin (assay ID: Mm01333430_m1) were obtained as TaqMan Gene Expression Arrays (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase and β-actin (TaqMan Predeveloped Assay Reagents for gene expression, part number: 4352339E and 4352341E; Applied Biosystems) were used as endogenous controls to normalize the amount of sample RNA. PCR was performed with equal amounts of cDNA in the GeneAmp 7700 sequence detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were incubated at 50°C for 2 minutes, at 95°C for 10 minutes followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Water controls were included to ensure specificity. Each sample was measured in triplicate and data points were examined for integrity by analysis of the amplification plot. The $\Delta\Delta$Ct method was used for relative quantification of gene expression (Livak and Schmittgen, 2001).

Immunohistochemistry

Immunohistochemistry from injured brain tissue was performed as previously described (Austinat *et al*, 2009; Raslan *et al*, 2010). Briefly, 10-μm thin brain cryosections were prepared using a cryostat (Leica, Bensheim, Germany). Immunohistochemical staining with antibodies for the detection of GFAP-expressing astrocytes (rabbit, 1:500, anti-GFAP; Ab7260; Abcam, Cambridge, UK), activated microglia/macrophages (rat, 1:100, anti-CD11b; MCA7; Serotec, Raleigh, USA), and damaged axons (mouse, 1:2000, non-phosphorylated neurofilament (anti-SMI32), Covance, Muenster, Germany) was performed following standard protocols using an avidin–biotin system (Vector Laboratories, Burlingame, USA) and 0.02% diaminobenzidine as chromogen (Kem-En-Tec Diagnostics, Taastrup, Denmark). Negative controls included omission of either the primary or secondary antibody and gave no signals (not shown). For the quantification of immune cells or neurofilament-positive retraction bulbs indicative for axonal damage, identical brain sections (thickness 10 μm) at the level of the basal ganglia (0.5 mm anterior from bregma) were selected at day 1 and day 7 after trauma. Numbers of activated astrocytes, microglia/macrophages, or neurofilament-positive retraction bulbs were determined from 4 cortical fields at a 40-fold magnification from 4 subsequent slices (distance 10 μm) from 4 to 6 different animals under a Nikon microscope Eclipse 50i (Nikon, Düsseldorf, Germany; Austinat *et al*, 2009). Immunofluorescence staining was performed as described previously (Kleinschnitz *et al*, 2010). Antibodies used in this study were anti-NeuN (1:1000, Chemicon, Billerica, USA) and Alexa Fluor 488-coupled goat antibodies recognizing mouse IgG (1:200, Invitrogen, Karlsruhe, Germany). For detection of apoptosis, a TUNEL *in situ* Cell Death Detection Kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions.

Statistical Analysis

All values were expressed as mean ± standard deviation (s.d.) except for the NSS scale, which is depicted as scatter plots, including median with the 25% percentile and the 75% percentile given in the brackets in the text. Data were analyzed by 1-way analysis of variance (ANOVA) or in case of measuring the effects of two factors simultaneously 2-way ANOVA with *post hoc* Bonferroni adjustment for P values. Functional outcome score was compared by nonparametric Kruskal–Wallis test with *post hoc* Dunn's multiple comparison test. If only two groups were compared, unpaired, two-tailed Student's *t*-test was applied. A $P < 0.05$ was considered statistically significant. For statistical analysis, PrismGraph 5.0 software package (GraphPad Software, La Jolla, CA, USA) was used.

Numbers of animals ($n = 18$) necessary to detect a standardized effect size on functional outcome (NSS scale,

primary endpoint) ≥0.20 in *B1r*$^{-/-}$ mice or *B2r*$^{-/-}$ mice compared with wild-type mice were calculated via *a priori* power analysis with the following assumptions: $\alpha = 0.05$, $\beta = 0.2$, mean, standard deviation 20% of the mean (StatMate 2.0, GraphPad Software).

Results

Expression of B1R and B2R after Focal Closed Head Trauma

In the first set of experiments, we analyzed the mRNA expression of B1R and B2R in the ipsilateral hemispheres of C57Bl/6 wild-type mice after traumatic weight-drop injury over time (Figure 1). Constitutive expression of both kinin receptors was low in sham-operated animals, which is in line with our previous results in cortical brain injury or ischemic stroke (Austinat *et al*, 2009; Raslan *et al*, 2010). B1R and B2R transcripts were only slightly induced on day 1 after trauma ($P < 0.05$). After 1 week however, expression levels of both receptors were higher but the difference compared with sham-operated mice was significant only for the B1R (B1R: 3.1 ± 1.3-fold induction, $P < 0.05$; B2R: 3.8 ± 2.8-fold induction, $P > 0.05$).

Taken together, these data indicate that both kinin receptors are expressed in the murine brain and—just like their ligand bradykinin (Trabold *et al*, 2010)—undergo induction after cerebral trauma, suggesting a functional role of the kallikrein–kinin system after focal closed head injury.

Blocking of B1R, but not B2R, Improves Functional Recovery after Focal Closed Head Injury

To investigate the functional role of B1R and B2R in focal closed head trauma, *B1r*$^{-/-}$ and *B2r*$^{-/-}$ mice were used. At a very early stage after weight drop, i.e. after 1 hour, neurological deficits as assessed by the NSS were comparable between wild-type mice, *B1r*$^{-/-}$ mice, and *B2r*$^{-/-}$ mice ($P > 0.05$), indicating that baseline trauma occurred to a similar extent in all the groups in this model (Figure 2A). After 3 days however, B1R-deficient mice performed significantly better than wild-type controls (median NSS: 2.5 (2.0, 4.0) (wild-type) versus 1.0 (0.0, 2.0) (*B1r*$^{-/-}$ mice); $P < 0.001$). Importantly, protection from TBI was not only transient in *B1r*$^{-/-}$ mice but persisted until day 7 (median NSS: 3.0 (2.0, 4.0) (wild-type) versus 1.0 (1.0, 2.0) (*B1r*$^{-/-}$ mice); $P < 0.001$). By contrast, deletion of B2R had no beneficial effect either on day 3 (median NSS: 2.5 (2.0, 4.0) (wild-type) versus 2.0 (1.0, 3.25) (*B2r*$^{-/-}$ mice); $P > 0.05$) or day 7 (median NSS: 3.0 (2.0, 4.0) (wild-type) versus 2.0 (1.0, 2.25) (*B2r*$^{-/-}$ mice); $P > 0.05$).

As congenital B1R deficiency protected from experimental brain trauma, we next tested whether pharmacological targeting of B1R is equally effective. Indeed, wild-type mice treated with the selective B1R blocker R-715 (1 mg/kg body weight) starting from 1 hour after weight drop had a significantly reduced total NSS score on day 3 (median NSS: 3.0 (1.0, 3.0) (controls) versus 1.0 (1.0, 1.0) (R-715); $P < 0.05$) and day 7 (median NSS: 2.0 (1.0, 3.0) (controls) versus 1.0 (0.0, 1.0) (R-715); $P < 0.05$) compared with vehicle-treated controls (Figure 2B). Again, no differences were observed at an early stage after TBI, i.e. after 1 hour ($P > 0.05$).

Blocking of B1R Reduces Axonal Damage, Neuronal Apoptosis, and Astroglia Activation after Focal Closed Head Injury

Next we sought to elucidate the underlying mechanisms of this B1R-specific neurotoxicity in brain trauma. One pathophysiological hallmark of severe TBI is diffuse axonal injury (Buki and Povlishock, 2006). Hence, we followed the expression of β-APP, a well-established marker of axonal injury (Gentleman *et al*, 1993), over time in our weight-drop model (Figure 3A). On day 1 after TBI, we did not observe induction of β-APP mRNA in the injured hemispheres of *B1r*$^{-/-}$ mice and wild-type mice compared with sham-operated controls ($P > 0.05$) and the

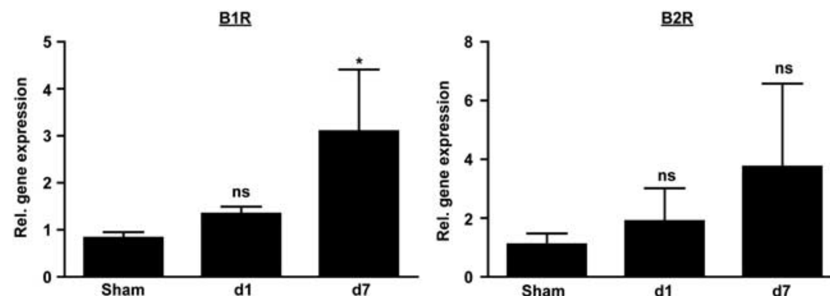

Figure 1 Induction of B1R and B2R is restricted to the later phase after focal closed head injury. Relative (Rel.) gene expression of B1R (left) and B2R (right) in the lesioned hemispheres of wild-type mice on day 1 (d1) or day 7 (d7) after weight-drop injury or sham operation ($n = 4$ per group and time point). *$P < 0.05$, 1-way analysis of variance (ANOVA), Bonferroni's *post hoc* test compared with sham-operated mice. ns, not significant.

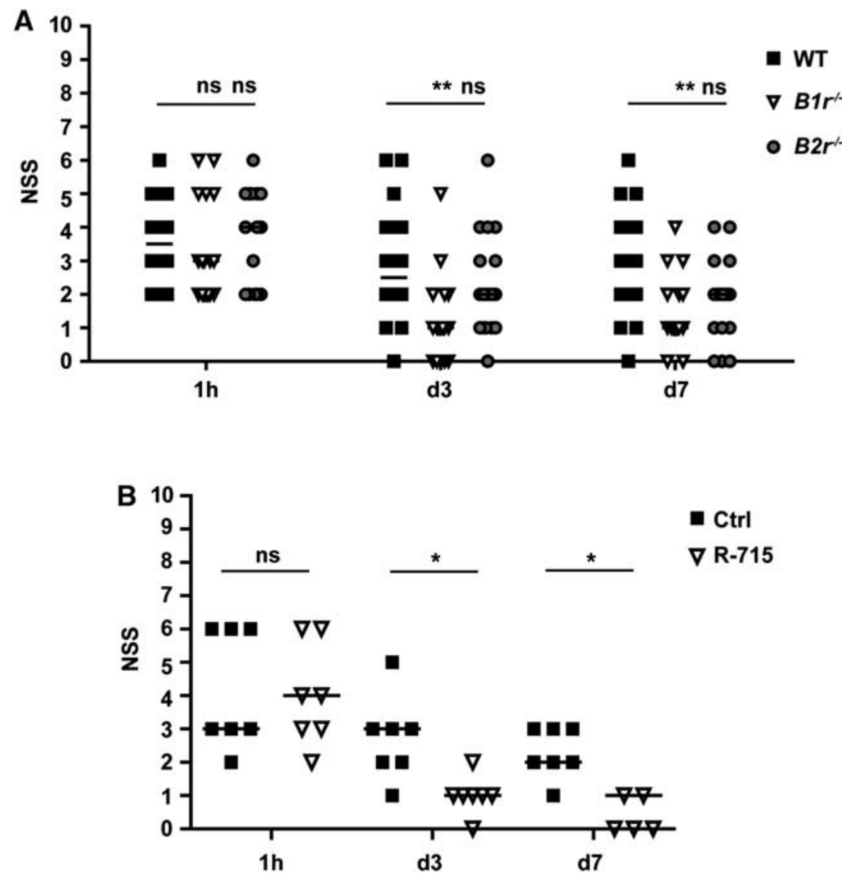

Figure 2 Blocking of B1R, but not B2R, improves functional outcome after weight-drop injury. (**A**) $B1r^{-/-}$ mice had a significantly lower neurological severity score (NSS) on day 3 (d3) and day 7 (d7) after focal closed head trauma compared with wild-type (WT) mice while the early functional deficits after 1 hour were similar. No protection was observed in $B2r^{-/-}$ mice (n = 18 per group). (**B**) WT mice treated with the specific B1R blocker R-715 (1 mg/kg body weight) starting from 1 hour after trauma also developed less severe neurological deficits on day 3 (d3) and day 7 (d7) compared with vehicle-treated mice (Ctrl), thereby confirming our results in transgenic mice (n = 7 per group). $^{*}P < 0.05$, $^{**}P < 0.001$, Kruskal–Wallis test with *post hoc* Dunn's multiple comparison test compared with WT mice or untreated mice, respectively. ns, not significant.

amount of β-APP transcripts was comparable between both groups ($P > 0.05$). Until day 7 however, there was a significant rise in β-APP gene expression only in wild-type mice (mean: 1.0 ± 0.2-fold induction (wild-type, sham) versus 2.0 ± 0.2-fold induction (wild-type, day 7); $P < 0.0001$) while β-APP remained low in B1R-deficient mice (mean: 0.9 ± 0.2-fold induction ($B1r^{-/-}$, sham) versus 1.4 ± 0.2-fold induction ($B1r^{-/-}$, day 7); $P > 0.05$; mean: 2.0 ± 0.2-fold induction (wild-type, day 7) versus 1.4 ± 0.2-fold induction ($B1r^{-/-}$, day 7); $P < 0.001$), indicating less traumatic axonal injury in these animals. This was also confirmed on the protein level by immunohistochemistry against non-phosphorylated neurofilament (anti-SMI32 antibody), a marker for acute axonal damge (Figure 3B). On day 7 after TBI, significantly more neurofilament-positive axonal retraction bulbs were counted in the cortices of wild-type mice compared with control mice (mean: 248.3 ± 19.3 neurofilament-positive retraction bulbs (wild-type, day 7) versus 73.3 ± 39.0 neurofilament-positive retraction bulbs ($B1r^{-/-}$, day 7); $P < 0.05$). We also quantified the amount of neurons undergoing apoptosis 1 week after weight-drop injury by TUNEL assay (Figure 3C). At this time point, mice lacking B1R also showed significantly less apoptosis-induced neuronal loss than wild-type mice (mean: 37.8 ± 4.7 TUNEL-positive neurons (wild-type, day 7) versus 21.8 ± 5.7 TUNEL-positive neurons ($B1r^{-/-}$, day 7); $P < 0.05$).

Reactive astrocyte activation and gliosis after brain injury can inhibit tissue regeneration and repair and contribute to permanent disability and cognitive decline (Frugier *et al*, 2012). Therefore, we investigated the astroglia response in wild-type mice and $B1r^{-/-}$ mice after weight drop. Seven days after traumatic challenge, mRNA expression of the astrocyte marker GFAP (mean: 2.4 ± 0.6-fold induction (wild-type, day 7) versus 1.0 ± 0.2-fold induction ($B1r^{-/-}$, day 7); $P < 0.05$) and that of the intermediate filaments nestin (mean: 4.1 ± 1.1-fold induction (wild-type, day 7) versus 2.3 ± 0.2-fold induction ($B1r^{-/-}$, day 7); $P < 0.05$) and vimentin (mean: 2.1 ± 0.3-fold induction (wild-type, day 7) versus

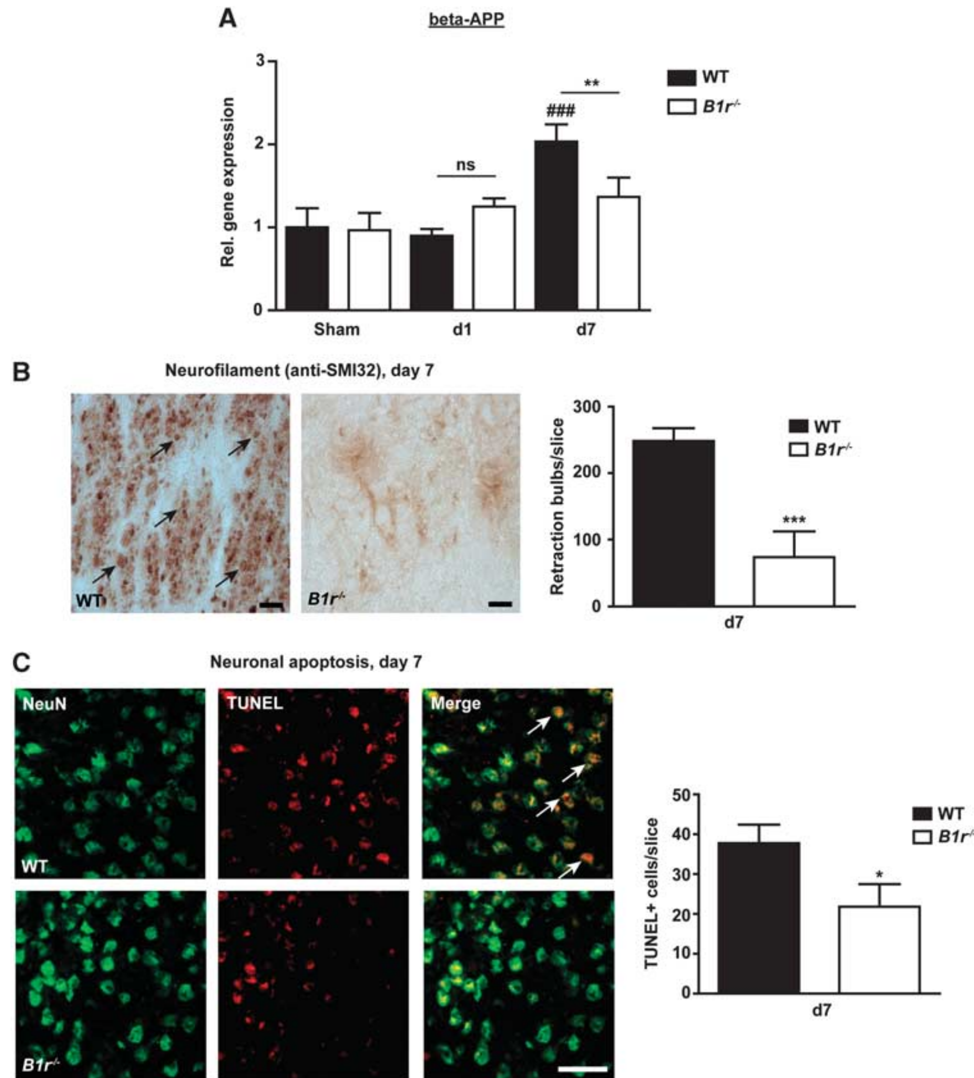

Figure 3 B1R deficiency reduces delayed axonal damage and neuronal apoptosis after weight-drop injury. (**A**) The injury-induced rise of β-amyloid precursor protein (β-APP) mRNA expression as an indicator of diffuse axonal injury seen in wild-type (WT) mice on day 7 (d7) after head trauma was significantly attenuated in $B1r^{-/-}$ mice ($n = 4$ per group and time point). $^{###}P < 0.0001$, 2-way analysis of variance (ANOVA), Bonferroni *post hoc* test compared with sham-operated mice; $^{**}P < 0.001$, 2-way ANOVA, Bonferroni *post hoc* test compared with WT mice. (**B**) (left) Representative brain sections from WT and $B1r^{-/-}$ mice 7 days after weight-drop injury. Apparently more axons stained positive for non-phosphorylated neurofilament (anti-SMI32, arrows), a marker for axonal damage, in the cortices of WT mice compared with $B1r^{-/-}$ mice on day 7 (d7) after weight-drop injury and increased traumatic axonal damage could be confirmed by counting the numbers of neurofilament-positive retraction bulbs (right; $n = 4$ per group). $^{***}P < 0.0001$, unpaired, two-tailed Student's t-test compared with WT mice. Bar = 10 μm. (**C**) Left panels show representative brain sections from WT and $B1r^{-/-}$ mice 7 days after weight-drop injury, immunolabeled for the neuronal marker NeuN and subjected to TUNEL assay to detect apoptosis. Right panels show the number of TUNEL-positive neurons per brain slice in the injured hemispheres of both groups on day 7 (d7) ($n = 4$ per group). $^{*}P < 0.05$, unpaired, two-tailed Student's t-test compared with WT mice. Bar = 100 μm. ns, not significant; Rel., relative.

1.1 ± 0.1-fold induction ($B1r^{-/-}$, day 7); $P < 0.05$) was significantly lower in $B1r^{-/-}$ mice compared with wild-type mice, while no differences were observed at earlier stages, i.e. day 1 ($P > 0.05$) (Figure 4A).

We also quantified the numbers of GFAP-positive cells (astrocytes) in the damaged hemispheres of $B1r^{-/-}$ mice and controls over time (Figure 4B). In accordance with our gene expression data, significantly less cells stained positive for GFAP in mice lacking B1R on day 7 (mean: 73.1 ± 36.9 GFAP-positive cells (wild-type, day 7) versus 19.8 ± 12.9 GFAP-positive cells ($B1r^{-/-}$, day 7); $P < 0.05$), but not on day 1 ($P > 0.05$), after weight drop compared with wild-type mice.

Taken together, these data suggest that improved functional outcome in $B1r^{-/-}$ mice after brain trauma is due to less astroglia activation and reduced axonal injury.

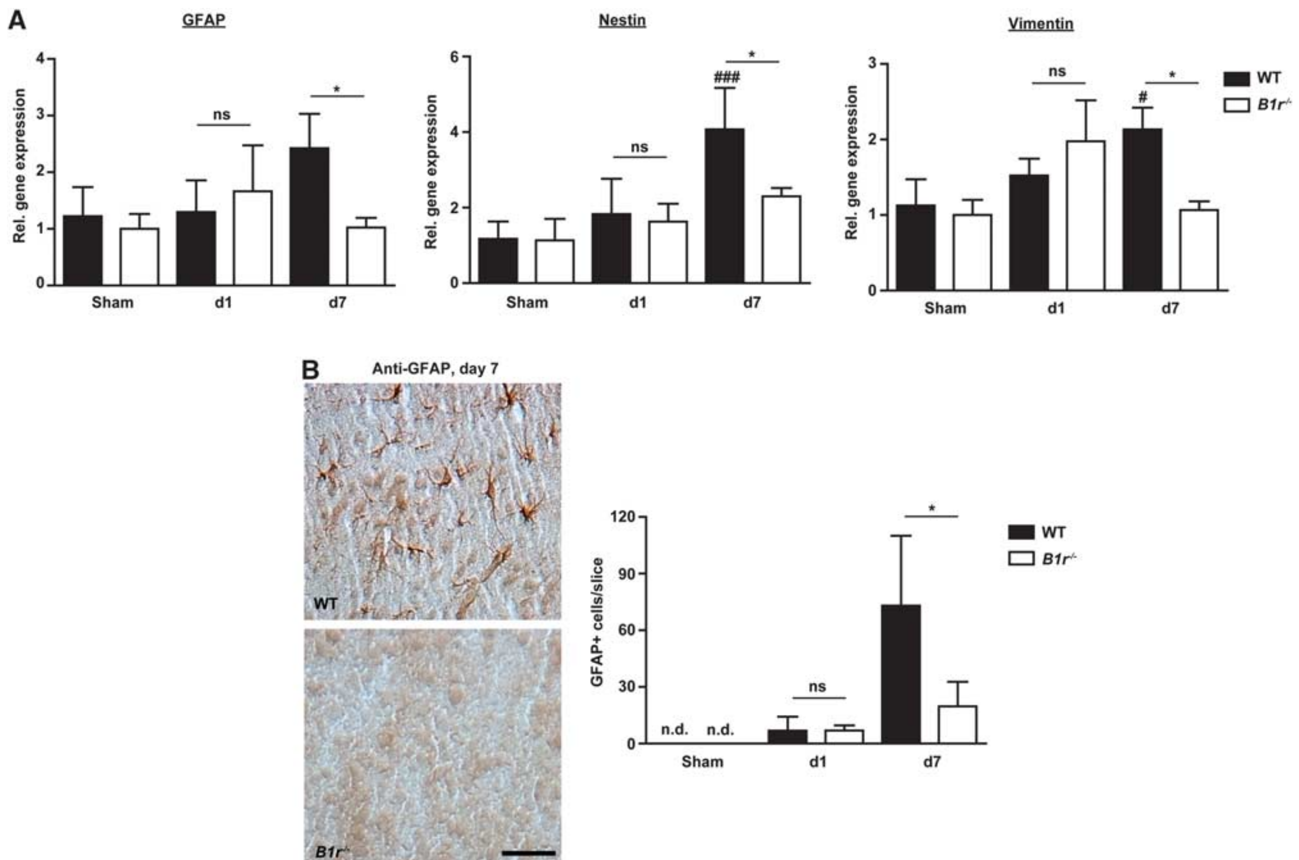

Figure 4 B1R deficiency reduces delayed astrogliosis after weight drop injury. (**A**) Relative (Rel.) gene expression of the astrocyte activation markers glial fibrillary acidic protein (GFAP), nestin, and vimentin in the lesioned hemispheres of wild-type (WT) mice or $B1r^{-/-}$ mice on day 1 (d1) or day 7 (d7) after weight-drop injury or sham operation. Note that all astrocyte markers were significantly lower expressed in the brains of $B1r^{-/-}$ mice on day 7 ($n=4$ per group and time point). (**B**) In line with our mRNA expression data, significantly less cells stained positive for GFAP in the injured brains of $B1r^{-/-}$ mice compared with WT on day 7 (d7) after focal closed head trauma ($n=6$ per group and time point). $^{\#}P<0.05$, $^{\#\#\#}P<0.0001$, 2-way analysis of variance (ANOVA), Bonferroni *post hoc* test compared with sham-operated mice; $^{*}P<0.05$, 2-way ANOVA, Bonferroni *post hoc* test compared with WT mice. n.d., not detectable; ns, not significant.

Blocking of B1R does not Alter the Inflammatory Response after Focal Closed Head Injury

Our previous studies demonstrated that B1R is critically involved in the inflammatory response evolving after cortical cryolesion or focal cerebral ischemia (Austinat *et al*, 2009; Raslan *et al*, 2010). To test whether this also applies for focal closed TBI, the mRNA expression levels of several prototypic pro- and antiinflammatory cytokines were measured in the injured hemispheres by real-time PCR with reverse transcription (Figure 5A). There was no significant difference in the amount of interleukin-1β or transforming growth factor β transcripts between $B1r^{-/-}$ mice and wild-type controls on day 1 or day 7 after weight drop ($P>0.05$). Only TNFα was expressed at significantly lower levels 1 week after trauma in mice lacking B1R (mean: 4.0 ± 0.5-fold induction (wild-type, day 7) versus 1.3 ± 0.4-fold induction ($B1r^{-/-}$, day 7); $P<0.001$).

In addition, the cellular inflammatory response as represented by the numbers of CD11b+ macrophages/microglia cells invading the traumatic tissue was also similar in mice with or without B1R ($P>0.05$) (Figure 5B).

This indicates that B1R is obviously not of major importance for mediating inflammation after focal closed head trauma.

Discussion

Here we show that genetic depletion of B1R improves functional outcome after focal closed head injury in mice. This protective effect persisted at later stages after trauma and could be reproduced by a pharmacological approach using a selective B1R inhibitor (R-715) in wild-type animals. Importantly, R-715 was even effective when applied in a 'clinically' relevant scenario, i.e. 1 hour after trauma. By contrast, blocking of B2R had no effect. Mechanistically, the inhibition of B1R mitigated axonal damage, apoptosis of neurons, and astrocyte activation, while the impact on post-traumatic inflammation was small.

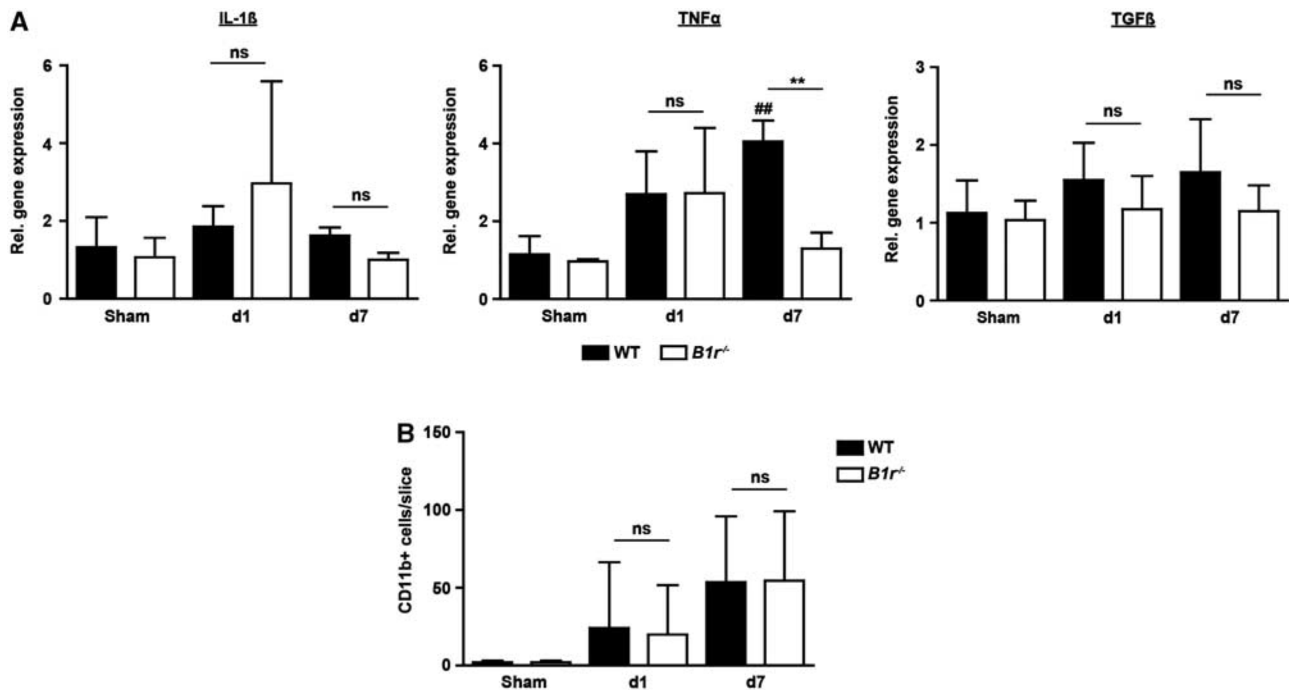

Figure 5 B1R deficiency has only little effects on the local inflammatory response after weight-drop injury. (**A**) Relative (Rel.) gene expression of several prototypic pro- and antiinflammatory cytokines in the lesioned hemispheres of wild-type (WT) mice or $B1r^{-/-}$ mice on day 1 (d1) or day 7 (d7) after weight-drop injury or sham operation. Note that only tumor necrosis factor (TNF)α levels were significantly lower in $B1r^{-/-}$ mice at the later phase after brain trauma ($n = 4$ per group and time point). (**B**) In line with these findings, the numbers of CD11b+ microglia/macrophages invading the injured brain were similar between both groups ($n = 6$ per group and time point). $^{\#\#}P < 0.001$, 2-way analysis of variance (ANOVA), Bonferroni *post hoc* test compared with sham-operated mice; $^{**}P < 0.001$, 2-way ANOVA, Bonferroni *post hoc* test compared with WT mice. IL, interleukin; ns, not significant; TGF, transforming growth factor.

PCR analysis revealed that the induction of both kinin receptors, especially B1R, after weight drop-induced head trauma arises with a delay of several days (day 7) but is absent during the early phase (day 1). These findings suggest that in this trauma model the kallikrein–kinin system is pathophysiologically relevant especially at more advanced stages when degenerative processes prevail (Albert-Weissenberger *et al*, 2012; Tashlykov *et al*, 2007). Indeed, blocking of B1R was able to reduce axonal injury, neuronal cell death, and astrocyte activation, while the antiinflammatory effect was modest and restricted to less TNFα formation, which is in contrast to more acute and circumscribed trauma models such as cryogenic cortical lesion (Raslan *et al*, 2010). Interestingly, TNFα can induce GFAP expression in astrocytes *in vitro* (Zhang *et al*, 2000) as well as after brain trauma *in vivo* (Rostworowski *et al*, 1997) and previous studies using experimental closed head injury in rats demonstrated that post-traumatic recovery from day 1 to day 14 after injury is improved by pharmacological inhibition of TNFα (Shohami *et al*, 1996, 1997).

Although it is well accepted that both kinin receptors are present on neurons (Raidoo and Bhoola, 1997), the situation is less clear for astrocytes. In the majority of astrocytes cultured from rats, the selective B1R agonist des-Arg9-BK elicited physiological responses with much lower potency than the B2R agonist bradykinin, supporting the fact that the kinin receptors are predominantly of the B2R subtype. However, a population of astrocytes exists in the rat brain that only responds to des-Arg9-BK (Cholewinski *et al*, 1991; Gimpl *et al*, 1992). The mechanisms by which astrocyte activation contribute to tissue damage and subsequent neurological impairment after focal closed head injury need to be further established but might include excessive glutamate release (Parpura *et al*, 1994) or enhanced expression of matrix metalloproteinases (MMPs), especially MMP-9 (Hsieh *et al*, 2004).

In contrast to our results, a number of previous reports have stressed the importance of B2R rather than B1R in the pathophysiology of TBI (Gorlach *et al*, 2001; Hellal *et al*, 2003; Plesnila *et al*, 2001; Trabold *et al*, 2010; Zweckberger and Plesnila, 2009). Accordingly, blocking of B2R either genetically or pharmacologically improved neurological deficits and reduced tissue damage in these studies. The reasons for this discrepancy are not exactly clear at present. Certainly, the different trauma models (e.g., weight drop versus controlled cortical impact and animal species (e.g., rats versus mice) used as well as the pharmacological properties and route of BR-antagonist administration have a role here. Another contributing factor may also be the differential

pattern of B1R and B2R expression in the various experimental settings. In the model of controlled cortical impact, the expression peak of B1R and B2R was reached as early as 6 hours and the maximum of bradykinin formation even occurred after 2 hours (Trabold *et al*, 2010). Similarly, cortical cryolesion induced a very rapid increase of B1R and B2R already after 12 hours and expression levels again approximated control values after 24 hours (Raslan *et al*, 2010). Notably, despite a vast amount of experimental data describing a beneficial effect when blocking B2R in TBI, translation into the clinic was unsuccessful so far. The B2R blocker anatibant (LF 16-0687) tended to increase mortality in a phase II trial in trauma patients (Shakur *et al*, 2009) and another B2R antagonist, CP-0127 (deltibant), also failed to prove efficacy in humans (Marmarou *et al*, 2005). As a consequence, a recent Cochrane analysis concluded that the benefit of B2R antagonists in TBI remains unproven (Ker and Blackhall, 2008). Although no B1R blockers have been tested in clinical trials of TBI so far, this approach could be promising (Rodi *et al*, 2005). The specific B1R-antagonist R-715 reduced brain damage and improved neurological outcome after cortical cryolesion (Raslan *et al*, 2010) and ischemic stroke (Austinat *et al*, 2009) even hours after the initial insult, which is in line with the present findings in the weight-drop model.

Taken together, we could show that inhibition of B1R, but not B2R, improves neurological outcome after focal closed head injury by reducing axonal damage and astroglia activation. Blocking of B1R may become a novel strategy to combat the detrimental effects of the kallikrein–kinin system after brain trauma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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