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BRIEF COMMUNICATION

Effects of LPS on P2X3 receptors of trigeminal sensory neurons and macrophages from mice expressing the R192Q *Cacna1a* gene mutation of familial hemiplegic migraine-1

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Abstract A knockin (KI) mouse model with the R192Q missense mutation in the *Cacna1a* gene commonly detected in familial hemiplegic migraine was used to study whether trigeminal ganglia showed a basal inflammatory profile that could be further enhanced by the lipopolysaccharide (LPS) toxin. Adenosine-5′-triphosphate (ATP)-gated purinergic ionotropic receptor 3 (P2X3) currents expressed by the large majority of trigeminal sensory neurons were taken as functional readout. Cultured R192Q KI trigeminal ganglia showed higher number of active macrophages, basal release of tumor necrosis factor alpha (TNFα), and larger P2X3 receptor currents with respect to wild type (WT) cells. After 5 h application of LPS in vitro, both WT and R192Q KI cultures demonstrated significant increase in macrophage activation, very large rise in TNFα mRNA content, and ambient protein levels together with fall in TNFα precursor, suggesting potent release of this inflammatory mediator. Notwithstanding the unchanged expression of P2X3 receptor protein in WT or R192Q KI cultures, LPS evoked a large rise in WT neuronal currents that recovered faster from desensitization. Basal R192Q KI currents were larger than WT ones and could not be further augmented by LPS. These data suggest that KI cultures had a basal neuroinflammatory profile that might facilitate the release of endogenous mediators (including ATP) to activate constitutively hyperfunctional P2X3 receptors and amplify nociceptive signaling by trigeminal sensory neurons.

Keywords Purinergic receptors · Pain · Neuroinflammation · TNFα · Macrophage · ATP

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Abbreviations

α,β-meATP	α,β-methyleneadenosine 5′-triphosphate
ATP	Adenosine-5′-triphosphate
AU	Arbitrary unit
BCA	Bicinchoninic acid
$Ca_V2.1$	Voltage-activated calcium channel 2.1
DRG	Dorsal root ganglion
FHM-1	Familial hemiplegic migraine type 1
Iba1	Ionized calcium binding adaptor molecule 1
KI	Knockin
LPS	Lipopolysaccharide
P2X3	Purinergic ionotropic receptor 3
PCR	Polymerase chain reaction
SEM	Standard error of the mean
TNFα	Tumor necrosis factor alpha
WT	Wild type

Introduction

Migraine is a chronic neurovascular brain disorder characterized by recurrent attacks of intense headache (typically lasting 4–72 h) often accompanied by central symptoms [1]. Although a central origin of migraine attacks has been proposed, it seems likely that the acute onset of headache is at least partly due to sensitization of trigeminal sensory neurons [1–3], the large majority of which are activated by extracellular adenosine-5′-triphosphate (ATP) via purinergic ionotropic receptor 3 (P2X3)-sensitive receptors [4–6]. Trigeminal ganglion sensory neurons of a transgenic mouse model (i.e., R192Q knockin (KI)) expressing the R192Q missense mutation in the α1 subunit of the P/Q-type Ca^{2+} channel that is encoded by the *Cacna1a* gene [7], and which causes human familial migraine type-1 (FHM-1), show functional upregulation of P2X3 receptors under basal conditions consistent with their constitutive sensitization [8]. Nevertheless, the mechanisms that produce the P2X3 receptor gain-of-function remain unclear. One theory to account for migraine acute attacks suggests that, at the meningeal level, there are early biochemical and cellular alterations responsible for a "sterile inflammation" (with infiltration of macrophages and other immune cells) that facilitates painful signaling to the brain [9–11]. The present report investigated whether an inflammatory stimulus could modulate P2X3 receptor function in association with increased immune cell activity. Previous studies have shown that primary cultures of trigeminal ganglia recapitulate the main characteristics of in situ ganglia and can be used as a model to explore the molecular mechanisms of nociception [4, 8]. Hence, we exploited the use of lipopolysaccharide (LPS), a canonical inflammatory agent [12] that rapidly activates ionized calcium binding adaptor molecule 1 (Iba1)-immunopositive macrophages [13] with production of their major inflammatory cytokine TNFα in dorsal root ganglia (DRG) in vivo and in vitro [14] and in basal ganglia [15]. The aim was to clarify if LPS application could differentially affect macrophage presence, TNFα release, and P2X3 receptor-mediated currents in trigeminal ganglion cultures from wild type (WT) and R192Q KI ganglia.

Methods

Animals and tissue culture

$Ca_V2.1$ R192Q KI and WT littermates were used. This colony was first supplied by the Leiden University Medical Centre [7] and subsequently expanded locally. Mice were maintained in accordance with the Italian Animal Welfare Act and their use was approved by the Local Authority Veterinary Service. Genotyping was performed by polymerase chain reaction (PCR) as previously reported [7]. Trigeminal ganglion cultures were obtained from animals at the age of P12–14 as described before [4, 8] and employed after 24 h from plating. To evoke acute inflammation, in parallel experiments, WT or R192Q KI trigeminal ganglia cultures were treated with LPS (0.5 μg/mL, from *Escherichia coli* 0111:B4; Sigma, Milan, Italy) for 5 h [14] or kept for similar length of time in culture medium (sham condition). For cell biology and immunochemistry experiments, cultures were washed after 5 h and processed for data collection, while for patch clamp recording, the LPS medium was washed out with standard extracellular solution and cells were recorded over the next 2 h. Similar length of culturing conditions without LPS application did not change electrophysiological cell responses.

Immunofluorescence microscopy

Immunocytochemistry of trigeminal ganglia in culture from WT or KI mice was performed as already described [4]. The following antibodies were used: anti-P2X3 (1:300; Neuromics, Edina, MN, USA) and anti-Iba1 (1:300; Wako, Osaka, Japan) with secondary immunostaining based on Alexa-Fluor 488- or 594-conjugated antibodies (1:500; Invitrogen, S. Giuliano Milanese, Milan, Italy). Nuclei were counterstained with DAPI (1:1,000; Sigma). For quantitative analysis, we used a standard region of interest (ROI) of 640×480 μm. Images from cultures were visualized with a Zeiss Axioskop fluorescence microscope (Zeiss, Zurich, Switzerland) and analyzed with MetaMorph software for cell counting and area measurement (Molecular Devices, Downingtown, PA, USA).

Real-time PCR and protein analysis

Total mRNA was extracted from primary cultures as described before [4, 8]. Reactions were run in duplicate in an iQ5 thermocycler using IQ SyBr Green Supermix Reactions (Bio-Rad Hercules, CA, USA). Calculations for relative mRNA transcript levels were performed using the comparative method between cycle thresholds of different reactions [4]. Quantitative PCR was performed following the MIQE guidelines [16]. All primer sequences were designed using Beacon designer (PREMIER Biosoft, CA, USA). Primers for TNFα (locus: NM_013693) are forward: 5′-GTGGAACTGGCAGAAGAG-3′ and reverse: 5′-CCA TAGAACTGATGAGAGG-3′. Data normalization was carried out against the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, locus NM_008084, forward primer: 5′-AGAAGGTGGTGAAG CAGGCATC-3′ and reverse primer: 5′-CGAAGGTGGAA GAGTGGGAGTTG-3′). LPS-treated mRNA levels were expressed as fold increase in comparison to mRNA levels of their own control cultures considering them equal to 1.

Western blotting was performed as described earlier [4, 8], using antibodies against anti-pro-TNFα (1:200; Abcam, Cambridge, UK), anti-P2X3 (1:200; Alomone, Jerusalem, Israel), and anti-β-tubulin III (1:2,000; Sigma). Grey values were quantified with UVI band software (Uvitec, Cambridge, UK). Total protein content of ganglia cultures was measured with the bicinchoninic acid (BCA) kit purchased from Sigma.

ELISA analysis

TNFα was quantified in the supernatant of trigeminal ganglia with a custom-made mouse TNFα ELISA kit (Thermo Scientific, Rockford, IL, USA) and SearchLight Mouse Cytokine Array I (Aushon Biosystem, Billerica, MA, USA). Medium was collected in accordance with the manufacturer's instructions. Data normalized on protein content of each sample were evaluated with BCA test. Each sample was run in duplicate. All data were expressed as fold increase of WT control samples.

Patch clamp recording

Full details of the electrophysiological methods have been previously reported [4, 8]. In brief, trigeminal neurons were superfused continuously (2 mL/min) with physiological solution containing (in millimolar): 152 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH adjusted to 7.4 with NaOH). Cells were patch-clamped in the whole-cell configuration using pipettes with a resistance of 3–5 MΩ when filled with the following solution (in millimolar): 140 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 2 Mg_2ATP_3, 2 GTP, 10 HEPES, and 10 EGTA (pH adjusted to 7.2 with KOH). Cells were held at −60 mV, data were filtered at 1 kHz, and acquired by means of a DigiData 132XInterface and pClamp 8.2 software (Molecular Devices, Sunnyvale, CA, USA). To obtain stable and reproducible P2X3 receptor currents, its synthetic and specific agonist α,β-methylene-ATP (α,β-meATP) was applied with a fast superfusion system (Rapid Solution Changer RSC-200; Bio-Logic Science Instruments, Claix, France). We measured current peak amplitudes, current rise time (for 10–90 % of amplitude), onset of desensitization (estimated by calculating the first time constant of current decay, τ_{fast}, in accordance with our previous reports), and recovery from desensitization (with paired-pulse experiments in which α,β-meATP applications were spaced at 30-s interval). Recovery was expressed as percent of the first response in each pair [4, 8].

Statistics

Data are expressed as mean ± SEM, where *n* indicates the number of independent experiments or the number of investigated cells. Statistical analysis was performed using the Student's *t* test or the Mann–Whitney rank sum test after the software-directed choice of parametric or nonparametric data, respectively (Sigma Plot and Sigma Stat, Systat Software Inc., San Jose, CA, USA). A p value of ≤ 0.05 was accepted as indicative of a statistically significant difference.

Results

Effect of LPS pretreatment on primary cultures of WT or R192Q KI trigeminal ganglia

In analogy with studies investigating the LPS-mediated inflammatory reaction of sensory ganglia in culture [14], we applied LPS (0.5 μg/mL) for 5 h to WT or R192Q KI trigeminal cultures. Figure 1a compares representative images of trigeminal ganglion cultures from WT or R192Q KI mice under sham conditions or after LPS application. In basal control conditions, the number of Iba1-immunoreactive cells was significantly higher in R192Q KI than in WT cultures (Fig. 1b). The average area of Iba1-positive macrophages was also larger for R192Q KI cultures in basal conditions (Fig. 1c) and was significantly increased by LPS (Fig. 1c) even if this treatment did not change the number of these cells (Fig. 1b). The number of P2X3-immunopositive neurons was similar in WT and R192Q KI cultures [8] and was not affected by LPS (Fig. 1d). This result suggests that the inflammatory stimulus by LPS could affect Iba1-positive cells without a neurotoxic action on trigeminal ganglion neurons.

The rapid inflammatory response evoked by LPS is typically characterized by an early rise of tumor necrosis factor alpha (TNFα) mRNA levels of DRGs in vitro [14, 17]. In the present experiments, LPS treatment largely increased the TNFα mRNA levels (normalized with respect to their controls), an effect particularly strong for R192Q KI cultures (Fig. 1e). Alongside these changes in mRNA signal, we could also detect a similar pattern of increase in the TNFα protein content of the culture medium (Fig. 1f). Such a strong rise in TNFα medium levels was mirrored by an LPS-induced drop in the expression of the TNFα a precursor protein in WT and R192Q KI cultures (Fig. 1g, h). Hence, these results are consistent with a large stimulation of TNFα synthesis and release by LPS, especially intense in cultures of R192Q KI trigeminal ganglia.

Effect of LPS on P2X3 receptor function

Since most (approximately 80 %) trigeminal ganglion neurons express P2X3 receptors [4, 8] whose enhanced activity is implicated in pain transducing mechanisms of migraine models [2, 8, 18], we investigated whether LPS could differentially affect P2X3 receptor expression and function in R192Q KI neurons as compared to WT neurons. In

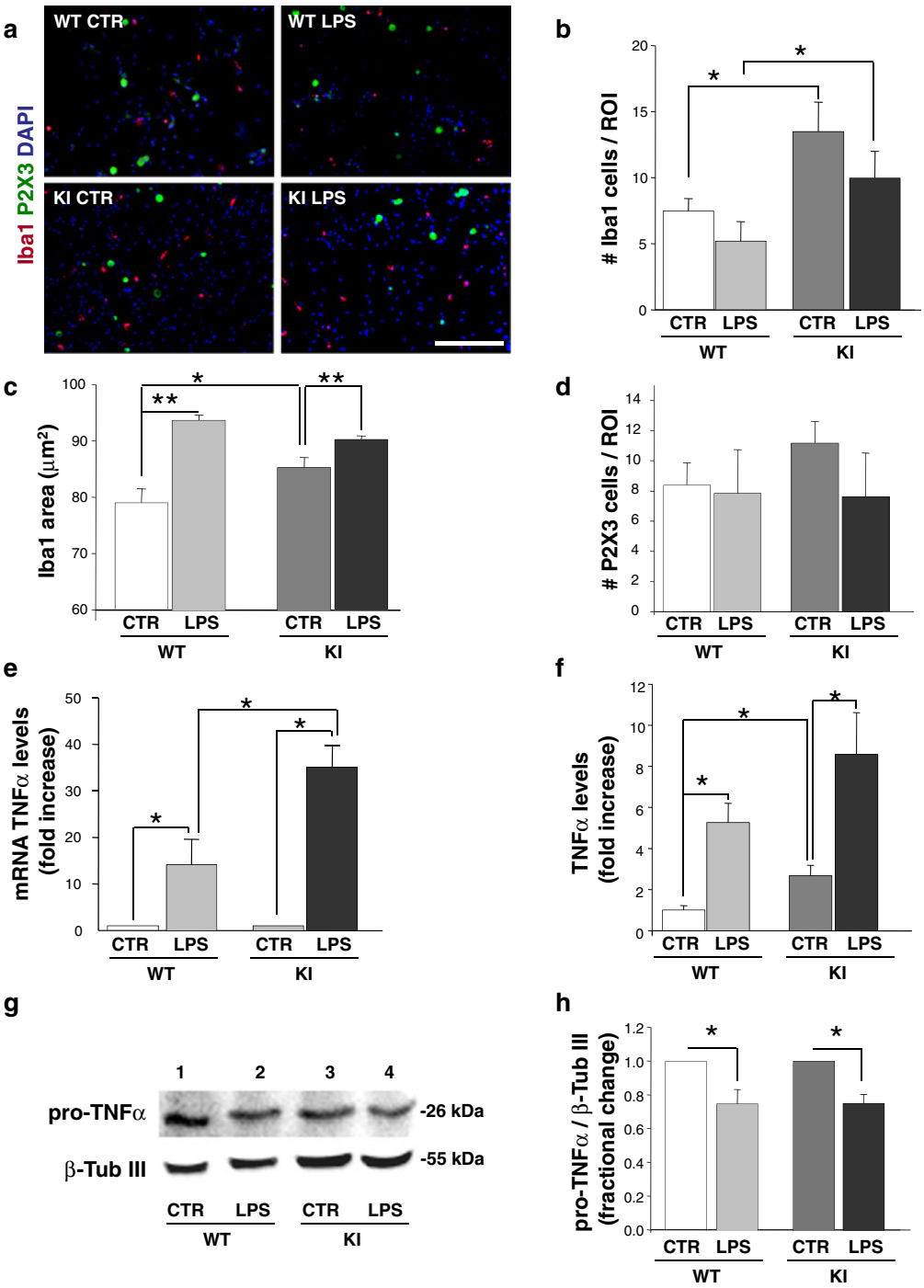

Fig. 1 Effect of LPS on trigeminal ganglion cultures. **a** Examples of fluorescence microscopy images of WT or R192Q KI trigeminal ganglion cultures in control conditions (*left*) or after 5 h of LPS treatment (*right*; 0.5 μg/mL) immunostained with antibodies against Iba1 (*red*), P2X3 (*green*), and counterstained with DAPI (*blue*). *Scale bar*, 100 μm. **b** Histograms quantify the average number of Iba1-positive cells per ROI (640×480 μm) in cultures from WT or R192Q KI mice, in control conditions or after 5 h of LPS treatment (n=4 experiments, *p<0.05). **c** Average area of Iba1-positive cells in WT or R192Q KI cultures in control conditions or after 5 h of LPS treatment (n=4 experiments, *p<0.05, **p<0.01). **d** Average number of P2X3-positive cells per ROI (640×480 μm) in WT or R192Q KI cultures, in control conditions or after 5 h of LPS treatment (n=5 experiments, data were not statistically different). **e** TNFα mRNA levels of WT or R192Q KI cultures after 5 h of LPS and are expressed as fold increase in comparison to their controls and normalized versus GAPDH levels (n=3; *p<0.05). **f** Levels of TNFα in culture medium from WT or R192Q KI cultures expressed as fold increase in comparison to WT controls (n=3, *p<0.05). **g** Example of Western blots shows different levels of pro-TNFα expression in lysates from WT or R192Q KI cultures in control conditions (*lanes 1* and *3*) and after 5 h of LPS (WT LPS and KI LPS, *lanes 2* and *4*). β-TubulinIII is shown as loading control (*bottom panels*). **h** Histograms show mean values of pro-TNFα expression (optical density; AUs) normalized with respect on β-tubulin III (n=4, *p<0.05)

accordance with data by Nair et al. [8], we confirmed that the R192Q mutation did not change the P2X3 protein expression (Fig. 2a, b), which is in line with the data on immunopositive neurons (Fig. 1d).

Figure 2c shows examples of membrane currents induced by a 2-s application of the selective P2X3 receptor agonist α,β-meATP (10 μM; a concentration eliciting near-maximal response; [8]) to WT or R192Q KI neurons under sham conditions or 5 h after LPS. In all cases, the agonist application elicited a fast-developing inward current (Fig. 2c) that rapidly decayed because of receptor desensitization, a characteristic typical of currents mediated by P2X3 receptors [19]. As previously reported [8], R192Q KI neuronal currents under control conditions were larger than those recorded from WT neurons (Fig. 2c, d). When WT ganglia were treated for 5 h with LPS, a significant potentiation of P2X3 receptor-mediated currents was observed in WT neurons only (Fig. 2c, d). Indeed, the increment in WT current average amplitude brought their values to the level observed in KI neurons. Current rise time (Fig. 2e, left) and onset of desensitization (Fig. 2e, middle) remained the same in WT and R192Q KI after LPS. Nevertheless, the percentage of recovery from desensitization (Fig. 2e, right) was significantly higher in LPS-treated WT neurons as compared to untreated WT, while it remained unaffected after the LPS treatment in R192Q KI.

Discussion

The principal novel finding of the present report is the demonstration of a constitutive neuroinflammatory phenotype of R192Q KI cultures associated with enhanced P2X3 receptor responses which showed maximal efficacy already under basal conditions. Simulating acute inflammation with LPS triggered intense TNFα release especially in R192Q KI cultures and might have liberated endogenous mediators acting on a background of facilitated P2X3 receptors to express neuronal sensitization. Thus, in a mouse genetic model of migraine, an inflammatory challenge would operate through non-neuronal cells to exploit the heightened function of P2X3 receptors. Future studies will be necessary to identify the precise molecular mechanisms linking immune cells to sensory neuron activity, and why the immune system had become more reactive to LPS.

Purinergic receptors have been considered crucial in pain transduction as ATP has been demonstrated to be an important player in neuroinflammatory disorders as well as in migraine pain [6, 20]. Our previous studies have shown that our mouse model with the *Cacna1a* gene R192Q mutation that causes FHM-1 showed enhanced P2X3 function without a corresponding change in protein expression [8]. We have attributed this phenotype to an altered receptor phosphorylation state caused by potentiated Ca^{2+} influx [8].

The present study sought to find out whether, in our FHM-1 mouse model, an association between inflammatory condition and trigeminal sensory neuron function could be observed. A canonical inflammatory stimulus such as LPS is reported to act on neurons and non-neuronal cells, inducing a reactive cascade with release of pro-inflammatory cytokines [21, 22], amongst which TNFα appears to play a prominent role [14]. Interestingly, TNFα has been found to potentiate P2X3 receptors in DRG sensory neurons [23], even if it is unclear whether this action is mediated through release of other endogenous mediators like for example ATP.

The average macrophage population in trigeminal ganglion cultures, identified with the Iba1 antibody [13, 24, 25], was larger in R192Q KI rather than WT ones and showed wider cell area consistent with a stronger basal activation state of these cells. LPS treatment increased average macrophage area (an index of inflammatory state) [26], demonstrating that neither WT nor R192Q KI cultures in basal conditions had attained their full inflammatory potential that could be further modulated by LPS application. The macrophage morphological change was associated with higher levels of TNFα in the culture medium together with a drop of its precursor and a rise in TNFα mRNA, a set of observations best explained by enhanced release of this inflammatory mediator especially by R192Q KI cultures.

The next question we addressed was whether this morphological and cellular background had been translated into a change in P2X3 receptor function of trigeminal sensory neurons [4, 8]. P2X3 receptor expression was similar in WT and R192Q KI neurons and was unaltered by LPS application. Nonetheless, the significant potentiation of WT P2X3 currents (with improved recovery from desensitization) mimicked the basal P2X3 function of R192Q KI neurons. LPS, however, did not further enhance P2X3 receptor function of R192Q KI trigeminal ganglion neurons. This observation raises a number of interesting implications. First, R192Q KI P2X3 receptors probably had reached, already under basal conditions, their maximal level of responsiveness. Second, it seems likely that this constitutive sensitization of R192Q KI neurons was contributed by the abundant, active macrophages. In fact, by inducing stronger macrophage activation in WT cultures, larger P2X3 receptor currents with faster desensitization recovery emerged.

Since LPS can trigger ATP release [27, 28] that, in turn, stimulates TNFα synthesis and release [29] and further ATP release [30, 31], it seems plausible that, in R192Q KI cultures, LPS might have facilitated release of ATP that, via activation of hyper-responsive P2X3 receptors, could amplify nociceptive signaling, while TNFα expressed the increased immune cell activity. This notion is consistent with

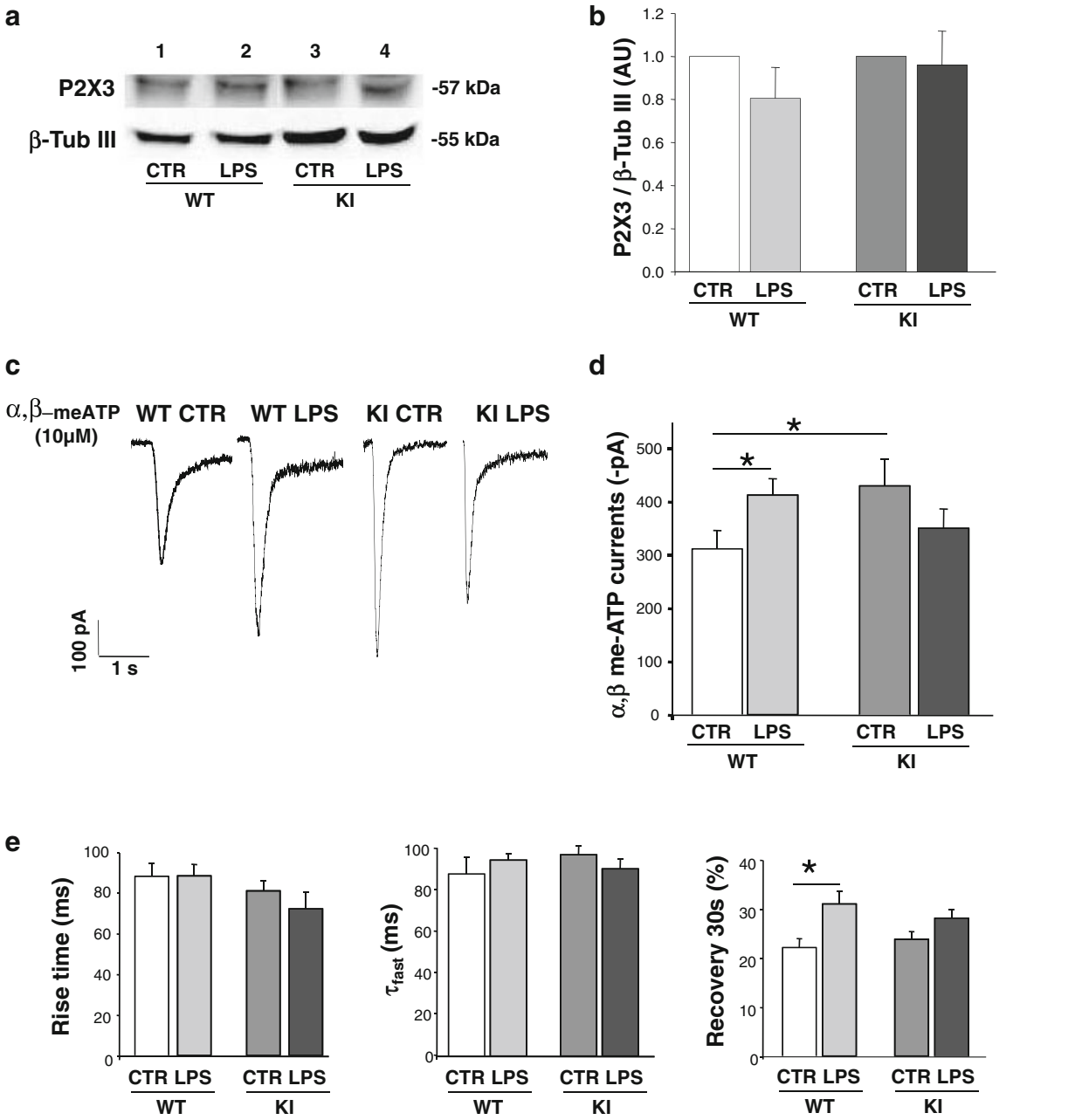

Fig. 2 Neuronal P2X3 receptor-mediated responses in control conditions and after LPS. **a** Example of Western blots shows similar levels of P2X3 receptor expression in lysates from WT or R192Q KI trigeminal cultures (*lanes 1* and *3*), and from LPS (5 h)-treated cultures (WT LPS and KI LPS, *lanes 2* and *4*). β-TubulinIII levels are shown as loading control (*bottom panel*). **b** Histograms show mean values (optical density; AUs) of P2X3 subunit expression normalized versus β-tubulinIII; $n=4$; data are not significantly different. **c** Representative examples of currents induced by application of α,β-meATP (10 μM, 2 s) to WT or R192Q KI neurons in control conditions or after 5 h LPS application. **d** Histograms show average peak amplitudes of P2X3-mediated currents (WT CTR, $n=30$; WT LPS, $n=38$; KI CTR, $n=34$; KI LPS, $n=34$). Note that LPS increases responses from WT neurons only; $*p<0.05$. **e** Average data for rise time (*left*; calculated on the 10–90 % current rise; WT CTR, $n=10$; WT LPS, $n=17$; KI CTR, $n=22$; KI LPS, $n=14$), and for desensitization onset (*middle*; expressed as the first time constant, τ_{fast}, of current decay; WT CTR, $n=8$; WT LPS, $n=18$; KI CTR, $n=35$; KI LPS, $n=20$) of P2X3 receptor currents. Data are not significantly different for WT, R192Q KI, and LPS-treated neurons. Recovery from desensitization (*right*; expressed as percent of control amplitude in a paired-pulse agonist application); $*p<0.05$; WT CTR, $n=21$; WT LPS, $n=40$; KI CTR, $n=33$; KI LPS, $n=40$

previous observations that ATP (via P2Y receptors) can stimulate release of TNFα from primary cultures of rat cortical astrocytes [32] and of rat brain microglia [33]. These results, therefore, support the role of non-neuronal cells in controlling the sensory ganglion phenotype and their potential role in trigeminal pain [34, 35].

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Conflict of interests The authors declare that they have no competing interests.

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