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The effects of sympathetic nerve damage on satellite glial cells in the mouse superior cervical ganglion

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ABSTRACT

Neurons in sensory, sympathetic, and parasympathetic ganglia are surrounded by satellite glial cell (SGCs). There is little information on the effects of nerve damage on SGCs in autonomic ganglia. We studied the consequences of damage to sympathetic nerve terminals by 6-hydroxydopamine (6-OHDA) on SGCs in the mouse superior cervical ganglia (Sup-CG). Immunostaining revealed that at 1–30 d post-6-OHDA injection, SGCs in Sup-CG were activated, as assayed by upregulation of glial fibrillary acidic protein. Intracellular labeling showed that dye coupling between SGCs around different neurons increased 4-6-fold 1-14 d after 6-OHDA injection. Behavioral testing 1–7 d post-6-OHDA showed that withdrawal threshold to tactile stimulation of the hind paws was reduced by 65–85%, consistent with hypersensitivity. A single intraperitoneal injection of the gap junction blocker carbenoxolone restored normal tactile thresholds in 6-OHDA-treated mice, suggesting a contribution of SGC gap junctions to pain. Using calcium imaging we found that after 6-OHDA treatment responses of SGCs to ATP were increased by about 30% compared with controls, but responses to ACh were reduced by 48%. The same experiments for SGCs in trigeminal ganglia from 6-OHDA injected mice showed no difference from controls, confirming that 6-OHDA acted selectively on sympathetic nerves. However, systemic inflammation induced by lipopolysaccharide did not affect SGCs of Sup-CG, but did influence SGCs in trigeminal ganglia in the same manner as 6-OHDA did on SGCs in Sup-CG. We conclude that even though SGCs in sympathetic and sensory ganglia are morphologically similar, they are quite different functionally, particularly after damage.

1. Introduction

The peripheral sympathetic nervous system (SNS) consists of paravertebral and prevertebral ganglia, which innervate the circulatory system and numerous other organs, such as the gastrointestinal and genitourinary tracts (Fasano and Niel, 2009). Neurons in these ganglia participate in a variety of reflexes that are highly important in homeostasis. Dysfunction in the activity of sympathetic nerves can result in numerous clinical disorders; for example sympathetic overactivity is associated with cardiovascular disease (Malpas, 2010). There is evidence that the SNS plays a role in pain mechanisms (Bosscher, 2001; Walters, 2018) and it has been reported that sympathetic nerves contribute to neuropathic pain by interacting with sensory neurons (Minett et al., 2012). Complex regional pain syndrome (CRPS), a highly painful condition that usually arises after a trauma, is partially associated with sympathetic dysregulation, but its underlying mechanisms are obscure (Casale et al., 2015). In addition, various sympathectomy procedures are used to treat chronic pain (Fujimoto et al., 2012; Gunduz and Kenis-Coskun, 2017) including sympathectomy for CRPS therapy (Li et al., 2018; Melis et al., 2002).

Neurons in sympathetic ganglia, like those in sensory ones, are surrounded by satellite glial cells (SGCs), but research on SGCs in sympathetic ganglia has lagged behind that on SGCs in sensory ganglia (see Hanani, 2010). There is considerable evidence that injury to peripheral nerves induces pathological changes in SGCs in sensory ganglia, which may contribute to chronic pain (Dublin and Hanani, 2007; Durham and Garrett, 2010; Hanani, 2015; Huang et al., 2013; Jasmin et al., 2010; Liu et al., 2012; Takeda et al., 2009). In response to nerve injury, several morphological and molecular changes take place in SGCs of sympathetic ganglia, but the functional significance of these changes

Abbreviations: 6-OHDA, 6-hydroxydopamine; CRPS, complex regional pain syndrome; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; ET-1, endothelin 1; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; IR, immunoreactive; LPS, lipopolysaccharide; P2R, purinergic P2 receptors; SGC, satellite glial cell; Sup-CG, superior cervical ganglia; SNS, sympathetic nervous system; TG, trigeminal ganglion

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is still missing (Hanani, 2010; Hu and McLachlan, 2004). After sciatic nerve transection, the glial marker glial fibrillary acidic protein (GFAP) was upregulated in SGCs of rat lumbar sympathetic ganglia (Hu and McLachlan, 2004). Elfvin et al. (1987) showed a marked increase in GFAP immunoreactivity in the superior cervical ganglia (Sup-CG, which are part of the paravertebral chain) of rats and guinea pigs after perturbation, but there is no information on similar changes in mouse Sup-CG. Neurons in sympathetic ganglia, unlike those is sensory ones, are endowed with synapses, and it was found that following nerve injury, SGCs in sympathetic ganglia can separate the pre- and post-synaptic membranes – synaptic stripping (De Stefano et al., 2007; Hanani, 2010).

Another functional change in sensory ganglia following peripheral injury is an increase in gap junction-mediated coupling among SGCs. It was found that this coupling is enhanced in sensory ganglia following nerve section or inflammation (Blum et al., 2014; Cherkas et al., 2004; Dublin and Hanani, 2007; Hanani et al., 2002; Huang et al., 2010; Ohara et al., 2008). Similarly, in a study on mouse paravertebral and prevertebral sympathetic ganglia it was found that injury increased SGC coupling (Hanani et al., 2010). Augmented glial coupling may contribute to the symptoms of neuropathic pain (Hanani, 2015; Spray and Hanani, 2019; Wu et al., 2012) as it can facilitate the spread of excitatory signals by allowing movement of ions and signaling molecules between the glial envelopes of adjacent neurons (Hanani, 2015; Hanani et al., 2002). Furthermore, it was reported that administration of gap junction blockers prevented the inflammation-induced decrease in the pain threshold (Dublin and Hanani, 2007; Hanstein et al., 2010; Huang et al., 2010).

The pharmacology of SGCs in sympathetic ganglia is largely unexplored. There is evidence for functional purinergic P2 receptors (P2R) in SGCs of Sup-CG (Calvert et al., 2004; Kumagai and Saino, 2001), and it has been reported that following cardiac ischemia the expression of P2X7 receptors is elevated in SGCs of rat Sup-CG (Liu et al., 2013). We have found that SGCs in Sup-CG are highly sensitive to endothelin 1(ET-1) (Feldman-Goriachnik and Hanani, 2017). It was reported that SGCs in rat Sup-CG respond to another peptide, pituitary adenylyl cyclase-activating polypeptide (PACAP) (Isobe et al., 2017), but the sensitivity to this peptide was much lower than that described for ET-1. In a recent calcium imaging study we have shown that SGCs in Sup-CG respond to acetylcholine (ACh), which is the main neurotransmitter in these ganglia (Feldman-Goriachnik et al., 2018). Using transgenic mice expressing Gq-coupled designer receptors exclusively activated by designer drugs, Xie et al. (2017) found that selective stimulation of SGCs in sympathetic ganglia elevated heart rate. They concluded that activation of SGCs in these ganglia induced excitation of sympathetic neurons, which led to norepinephrine release from them onto the heart, causing tachycardia.

Much is known on how nerve damage alters SGCs in sensory ganglia, but there is very little information on such effects on SGCs in sympathetic ganglia. One method for studying the consequences of damage to sympathetic nerves is chemical sympathectomy by 6-hydroxydopamine (6-OHDA), a neurotoxin that destroys sympathetic nerve terminals selectively (Ferrari et al., 1991; Mundinger et al., 2008). When injected peripherally, 6-OHDA enters noradrenergic sympathetic nerves terminals via norepinephrine reuptake transporters, where it is oxidized to generate free radicals that destroy the terminals (Qi et al., 2012). This treatment spares cholinergic neurons, Schwann cells, non-myelinating glia including ganglionic SGCs and endothelial cells (Xie et al., 2017).

In view of the important role of sympathetic ganglia in pathological conditions, we studied in the present work the influence of sympathetic nerve terminals damage by 6-OHDA on SGCs in the mouse Sup-CG. We investigated SGCs in trigeminal ganglia (TG) for comparison.

2. Materials and methods

2.1. Animals

Balb/c mice 2–5 months old of either sex (males:females about 1:1), weighing 19–23 g, were used (N = 117). Preliminary experiments showed that the results for females and males were not different. The procedures were approved by the Animal Care and Use Committee of the Hebrew University-Hadassah Medical School and conform to the National Institutes of Health standards for the care and use of laboratory animals. Mice were injected intraperitoneally (i.p.) with 6-hydroxydopamine (6-OHDA, Sigma-Aldrich, St. Louis, MO, USA) (50 mg/kg) dissolved in saline containing 1% ascorbic acid. Control animals (N = 38) were injected with vehicle. The doses were selected on the basis of previous work (Mundinger et al., 2008), and on preliminary tests. In some of the experiments in 6-OHDA-treated mice, the gap junction blocker carbenoxolone (CBX; Sigma-Aldrich, St. Louis, MO) was injected i.p. (100 mg/kg) 1 h before behavioral testing. At 1, 3, 7, 14 and 30 days after the injection the animals were euthanized by CO_2 inhalation and the Sup-CG and TG were removed and placed in a bath filled with Krebs solution; composition (in mM): NaCl (118), KCl (4.7), NaHCO3 (14.4), MgSO4 (1.2), NaH2PO4 (1.2), CaCl2 (2.5), and glucose (11.5); saturated with 95% O_2 and 5% CO_2; pH 7.3. Other mice were injected i.p. with lipopolysaccharide (LPS) from *E. coli* (L-2630, Sigma-Aldrich), dissolved in saline, 2.5 mg/kg. We found that the injection of saline alone had no effect on the results and therefore used naïve animals as controls (N = 12).

2.2. Immunohistochemistry

Ganglia were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 90 min at room temperature. They were then washed in 0.1 M PBS and incubated overnight at 4 °C in PBS with 20% sucrose before freezing in Tissue-Tek embedding medium (Sakura Finetek, Torrance, CA). Sections were cut 10 μm thick using a cryostat (Jung CM3000, Leica Microsystems, Wetzlar, Germany) and thaw mounted on SuperFrostPlus slides (Menzel, Braunschweig, Germany). Sections were washed in PBS and incubated with 50 mM ammonium chloride for 30 min to reduce autofluorescence, then washed in PBS and incubating in a blocking solution containing 3% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100 for 2 h at room temperature. Primary antibody against glial fibrillary acidic protein) rabbit anti-GFAP; Dako, Copenhagen, Denmark), was diluted 1:400 in PBS containing 1% BSA, and incubated overnight at 4 °C. Controls omitted the primary antibody. Primary antibody against glutamine synthetase (goat anti-GS; Santa Cruz, CA, USA) was diluted 1:100 in PBS containing 1% BSA, and incubated overnight at 4 °C. Primary antibody against toll-like receptor 4 (rabbit anti-TLR4; Abcam, www.abcam.com) was diluted 1:250 in PBS containing 1% BSA, and incubated overnight at 4 °C. Sections were washed in PBS and incubated with secondary antibody, donkey anti-rabbit conjugated to Alexa Fluor 594 (Abcam, www.abcam.com) diluted 1:400 in PBS containing 1% BSA and 10 μM 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) to stain the nuclei for 2 h at room temperature. For GS staining, sections incubated with secondary antibody, donkey anti-goat conjugated to DyLight 549-TFP ester (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:200 in PBS containing 1% BSA and DAPI. Finally, sections were washed in PBS and visualized using an upright microscope (Axioskop, Zeiss, Jena, Germany), equipped with fluorescent illumination and a digital camera (Penguin 600CL, Pixera Corp., Los Gatos, USA), connected to a personal computer. All the images were taken under identical conditions. Neurons that were surrounded by GFAP-positive SGCs by > 50% of their circumference were counted and expressed as a percentage of the total number of neurons present in the fields analyzed (see Warwick and Hanani, 2013). Four fields from different, non-adjacent sections were analyzed from each ganglion and

then averaged. A total of about 80–150 neurons was analyzed from each ganglion.

2.3. Intracellular labeling

For the dye injection and calcium imaging, the capsule around the ganglia was removed. Ganglia were fixed to the bottom of a silicon rubber-coated dish using fine pins. The dish was placed on the stage of an upright microscope, equipped with fluorescent illumination and a digital camera connected to a personal computer. The dish was superfused with Krebs solution. Individual SGCs were injected with the fluorescent dye Lucifer yellow (LY, Sigma-Aldrich), 3% in 0.5 M LiCl solution from sharp glass microelectrodes, connected to a preamplifier (NeuroData Instrument Corp., New York, NY, USA). The dye was passed by hyperpolarizing current pulses, 100 ms in duration; 0.5 nA in amplitude at 10 Hz for 3–5 min. The dye injections were made under visual inspection to allow cell identification. After the injection of each cell, we checked whether labeled SGCs (dye-coupled cells) were present around neighboring neurons as a result of dye passage from the injected cell. The coupling incidence was calculated as the ratio between the total number of injected cells to the number of dye-coupled ones.

2.4. Ca^{2+} imaging

For Ca^{2+} microfluorimetry SGCs in intact ganglia were loaded with the Ca^{2+} indicator Fluo-3 AM [10 μM; Invitrogen (www.invitrogen.com)] in minimum essential medium-α for 70 min in an incubator at 37 °C. The dye is preferentially taken up by SGCs (Weick et al., 2003). Ganglia were mounted in a recording chamber on the stage of an Axioskop FS microscope and superfused at 4 mL/min with Krebs solution saturated with 95% O_2 and 5% CO_2. Test substances were applied by rapidly changing the bath solution. Adenosine-triphosphate (ATP) or Acetylcholine (ACh; all from Sigma-Aldrich) were applied in the bath solution. Images were acquired with cooled CCD camera (PCO, Kelheim, Germany), using Imaging Workbench 5 software (www.imagingworkbench.com). Fluorescence was excited at 450–490 nm, and emitted fluorescence (above 520 nm) was increased by elevated $[Ca^{2+}]_{in}$. Images were recorded at 0.3 Hz. The fluorescence ratio F/F_0, where F_0 is the baseline, was used to describe relative changes in $[Ca^{2+}]_{in}$ concentration. About 40 SGCs were imaged simultaneously in each field. Doses and timing were chosen according to our previous works (Blum et al., 2014; Feldman-Goriachnik et al., 2018).

2.5. Behavioral testing

Mice were placed in a clear plastic box on a wire mesh floor and were allowed to accustom to their new environment for at least 20 min before behavioral testing. Pain thresholds were assessed by observing withdrawal responses to mechanical stimulation, using von Frey hairs (Stoelting, Wood Dale, IL, USA). Hairs of 0.07–6 g were applied 10 times at intervals of 5–20 s in ascending order. The von Frey hairs were pressed against the plantar skin of the hind paw until the hair buckled. Sharp retraction of the stimulated hind paw was considered as a response in the paw. The threshold response was defined when 6 out of 10 responses occurred. The right and left hind paws were both tested and results were averaged.

2.6. Statistical analysis

Unpaired Two-tailed *t*-test or One-way ANOVA with Dunnett's Multiple Comparison Test was used to analyze data obtained in the immunohistochemical experiments. Data are from experiments on at least 6 ganglia (3 mice) for each of the data points. The number of cells for each of the data point was about 1300.

Dye coupling data were pooled for each time point from multiple experiments, this was done because in different dye coupling experiments, different numbers of cells were injected and relatively small numbers of cells were injected per experiment. When an LY-injected cell was found to be dye-coupled it was marked as 100, and when it was not coupled, as 0. These data were analyzed using Unpaired Two-tailed *t*-test or One-way ANOVA with Tukey's Multiple Comparison Test (this procedure was suggested by a professional biostatistician). Data are from experiments on 6 ganglia (3 mice) at least for each of the data points. The number of cells for each of the data points was between 40 and 118.

Unpaired Two-tailed *t*-test or One-way ANOVA with Dunnett's Multiple Comparison Test was used in Ca^{2+} imaging experiments. Each of the data points is based on recordings of the responses from 4 to 7 ganglia (3–4 mice). A total of about 30–100 cells were recorded from each ganglion. Behavioral data were analyzed using Unpaired Two-tailed *t*-test. Data are from experiments on 9 mice for each of the data points. $P < 0.05$ was considered as statistically significant. Values are expressed as mean ± SEM.

3. Results

3.1. Glutamine synthetase (GS) expression in SGCs

The glial marker GS is expressed in SGCs in sensory ganglia (Miller et al., 2002; Weick et al., 2003). We examined whether this is the case for SGCs in the mouse Sup-CG. For comparison, we examined TG from the same mice. Immunostaining for GS in Sup-SG revealed very low expression of this protein in SGCs compared with the staining of TG (Fig. 1A, B).

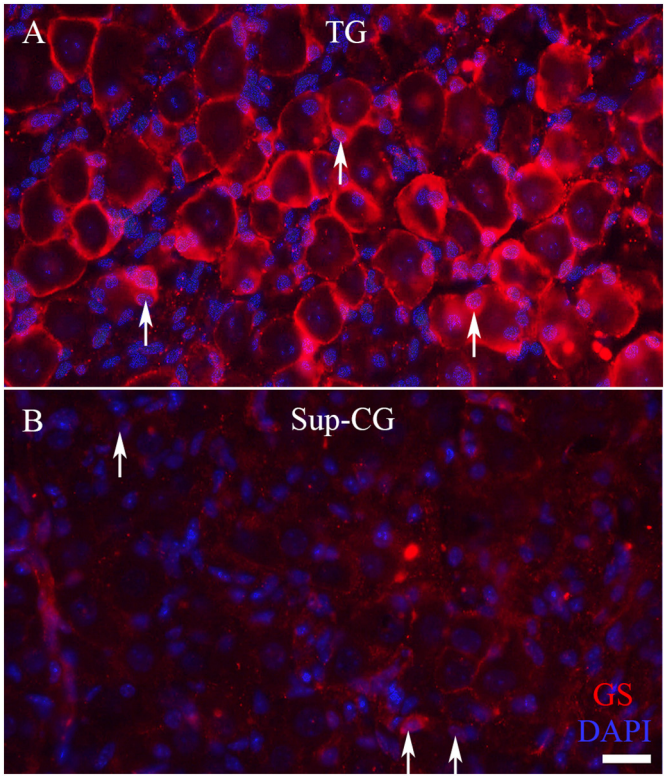

Fig. 1. Glutamine synthetase (GS) expression in normal SGCs of mouse TG and Sup-CG. The images show examples of GS immunoreactive (IR) SGCs. (A) High expression of GS in SGCs in a TG. (B,) In Sup-CG there is very low GS expression in SGCs. Arrows indicate several GS-IR SGCs. Scale bar, 20 μm. The tissues were processed and photographed under the same conditions.

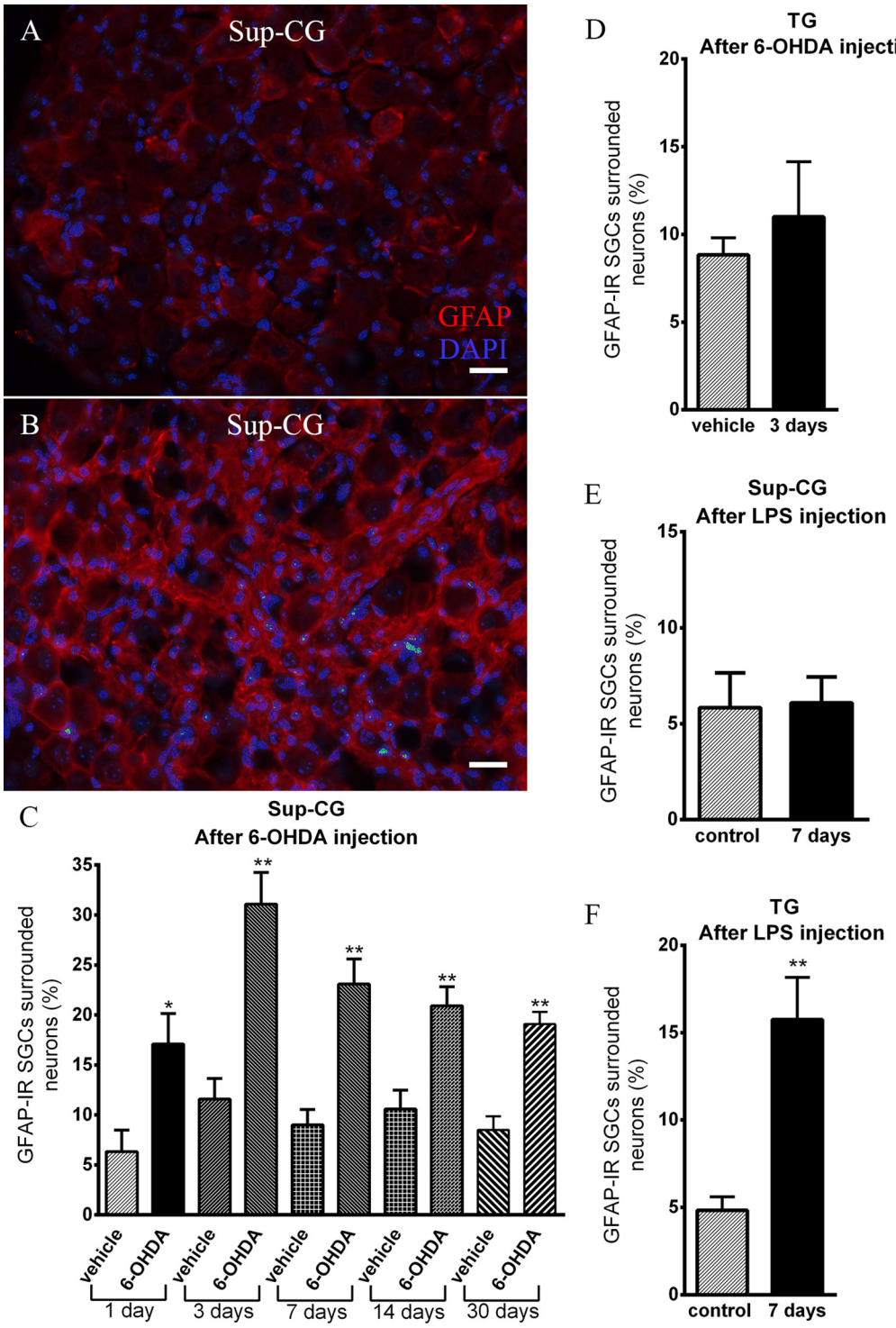

Fig. 2. Changes in GFAP expression in SGCs of Sup-CG and TG after 6-OHDA and LPS injections. The images show two examples of GFAP-IR SGCs in Sup-CG sections: (A) GFAP-IR SGCs in vehicle-treated mouse and (B), 7 d after 6-OHDA injection. Scale bars 20 μm. (C) Histograms showing the augmented GFAP expression in Sup-CG 1–30 d post-6-OHDA. (D) GFAP expression in TG after 6-OHDA injection. (E) GFAP expression in Sup-CG after LPS injection. (F) Augmented GFAP expression in TG after LPS injection. Error bars indicate S.E. *P < 0.05, **P < 0.01 compared with vehicle. Data are from experiments on at least 5 ganglia for each of the data points. Unpaired Two-tailed *t*-test was used for comparison.

3.2. Activation of SGCs following 6-OHDA injection

Increased expression of GFAP serves as a marker for glial activation (Warwick and Hanani, 2013). To determine whether specific injury to sympathetic nerves alters GFAP expression in Sup-CG, we used a single i.p. injection of 6-OHDA, which destroys sympathetic nerve terminals selectively. Immunohistochemical analysis revealed a large increase in GFAP expression in SGCs of Sup-CG at 1, 3, 7, 14 and 30 days post-6-OHDA injection (Fig. 2A–C), indicating glial activation. In vehicle-injected mice, about 9% of neurons were surrounded by GFAP immunopositive (IR) SGCs, whereas in ganglia from 6-OHDA-treated mice the value was 17–31%, depending on the time after 6-OHDA injection. To examine the influence of 6-OHDA outside of the sympathetic nervous system, we carried out the same experiment on TG and observed no influence of 6-OHDA on GFAP expression (Fig. 2D). These experiments were done 3 days post 6-OHDA injection on the basis of the large glial activation in Sup-CG at that time.

We have found previously that systemic inflammation induced by LPS activated SGCs in sensory ganglia (Blum et al., 2014; Feldman-Goriachnik et al., 2015) and now tested whether SGCs in Sup-CG are also activated by LPS. The results did not show increased GFAP expression in SGCs 7 d post-LPS injection compared with control (Fig. 2E).

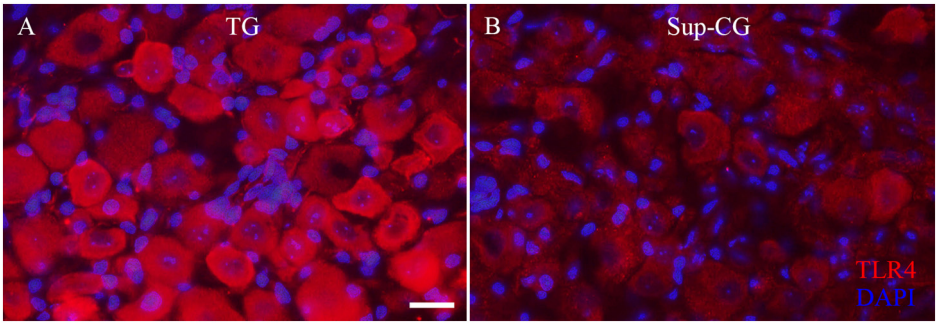

Fig. 3. Comparison of TLR4 expression in TG and Sup-CG. The images show examples of TLR4-IR neurons in TG and Sup-CG sections from control mice. (A) TLR4-IR is high in neurons in TG. (B), TLR4-IR is low in neurons in Sup-CG. Scale bar 20 μm.

For comparison we carried out the same experiment on TG and observed a 3-fold increase in GFAP expression in SGCs 7 d post-LPS injection (Fig. 2F), confirming that LPS induced glial activation in TG.

3.3. Toll-like receptor (TLR4) expression

Neurons in sensory ganglia contain TLR4, which is the LPS receptor (Barajon et al., 2009; Diogenes et al., 2011; Hosoi et al., 2005). To explain the results above it can be suggested that TLR4 is absent in the Sup-CG. Immunostaining for TLR4 in TG (Fig. 3A) showed strong expression of TLR4 in the neurons. In contrast, TLR4 expression was considerably lower in neurons of Sup-CG neurons (Fig. 3B). These findings are consistent with the lack of the LPS-induced effects in Sup-CG. TLR4 immunoreactivity was absent in SGCs in both ganglion types.

3.4. Dye coupling following 6-OHDA injection

It has been proposed that augmentation of gap junction-mediated coupling is associated with SGC activation in sensory ganglia following nerve injury (Huang et al., 2010; Feldman-Goriachnik et al., 2015). To determine whether 6-OHDA-induced nerve injury influences SGC coupling in sympathetic ganglia, we injected LY into single SGCs in ganglia from 6-OHDA and vehicle-injected mice. We observed 4–6-fold greater incidence of dye coupling between SGCs around different neurons in Sup-CG from mice 1, 3, 7 and 14 days after 6-OHDA injection, depending on the time after the injection. At day 30 dye coupling incidence returned to control level (Fig. 4C). In contrast, in TG we observed no influence of 6-OHDA on SGC coupling (Fig. 4D). Systemic LPS augments SGC coupling in sensory ganglia (Blum et al., 2014; Feldman-Goriachnik et al., 2015), and next we tested the effect of LPS on SGC coupling in Sup-CG. In Sup-CG from mice 7 d post-LPS injection we found no difference in dye coupling compared with control (Fig. 4E). This is consistent with the GFAP results described above, suggesting that SGCs in Sup-CG were not influenced by systemic LPS. Similar experiments on TG showed that in control ganglia 50% of SGCs were coupled to other SGCs around different neurons, and in ganglia from mice 7 d after LPS injection dye coupling increased to 77% (Fig. 4F), in concert with the GFAP results on LPS injected mice described above.

3.5. Pain behavior

There is evidence that the SNS contributes to neuropathic pain (Minett et al., 2012). To determine whether damage of sympathetic nerves by 6-OHDA induces mechanical hypersensitivity we performed behavioral testing, using von Frey hairs 1, 3 and 7 d after 6-OHDA injection. Withdrawal threshold to tactile stimulation of the hind paws was reduced by 65–85%, depending on the time after 6-OHDA injection, consistent with tactile hypersensitivity (Fig. 5). Next we investigated the analgesic effects of the gap junction blocker carbenoxolone (CBX) in 6-OHDA-treated mice. A single intraperitoneal injection of 100 mg/kg CBX restored normal tactile thresholds at day 7 after 6-OHDA (Fig. 5), suggesting that SGC coupling contributes to the mechanical hypersensitivity.

3.6. The effect of systemic 6-OHDA on SGC responses to ATP and ACh - Ca^{2+} imaging studies

3.6.1. Responses of SGCs to ATP

It has been reported previously that SGCs in Sup-CG are endowed with purinergic P2 receptors (P2R, Calvert et al., 2004; Kumagai and Saino, 2001). We used intact, freshly isolated Sup-CG, to learn by Ca^{2+} imaging about the function of these receptors in SGCs following 6-OHDA injection.

Application of ATP caused a clear increase in $[Ca^{2+}]_{in}$ in SGCs (Fig. 6A). Various types of inflammation were found to augment the sensitivity of SGCs in sensory ganglia to ATP (Blum et al., 2014; Feldman-Goriachnik et al., 2015; Kushnir et al., 2011). To assess whether the treatment with 6-OHDA has a similar effect in the Sup-CG, we measured responses to 5 μM ATP 7 d post-6-OHDA, and found that the responses were increased by about 30% in Sup-CG compared with vehicle (Fig. 6B,C). The same experiment for the TG showed no difference in responses obtained on 6-OHDA injected mice and on control mice (Fig. 6D), indicating that 6-OHDA acted selectively on sympathetic nerves.

To find out whether the change in the response to ATP in Sup-CG is specific for 6-OHDA, we carried out the Ca^{2+} imaging 7 d after inducing a systemic inflammation by LPS, which was reported to increase the sensitivity of SGCs to ATP in sensory ganglia (Blum et al., 2014; Feldman-Goriachnik et al., 2015). LPS injection augmented the response to ATP in TG (Fig. 6F), but not in Sup-CG (Fig. 6E), again in accord with results described above.

3.6.2. Responses of SGCs to ACh

Using Ca^{2+} imaging, we have shown that SGCs in Sup-CG respond with $[Ca^{2+}]_{in}$ elevation to ACh, (Feldman-Goriachnik et al., 2018). We now studied responses of SGCs to ACh 7 d after 6-OHDA injections and found that they were reduced by 48% compared with controls (Fig. 7), in contrast to the augmented responses to ATP.

4. Discussion

The basic organization of SGCs in sensory and sympathetic ganglia is similar; these cells make an envelope around the neurons, and are mutually coupled within the envelope. However, apparently because neurons in these two types of ganglion are affected in a different manner under pathological conditions, SGCs are also altered differently. Following 6-OHDA injection, which selectively injures sympathetic nerves, SGCs in the Sup-CG, but not in TG, were affected. Conversely, in the TG, where neurons are endowed with TLR4, SGCs are altered by LPS. Neurons in Sup-CG do not display TLR4, and according

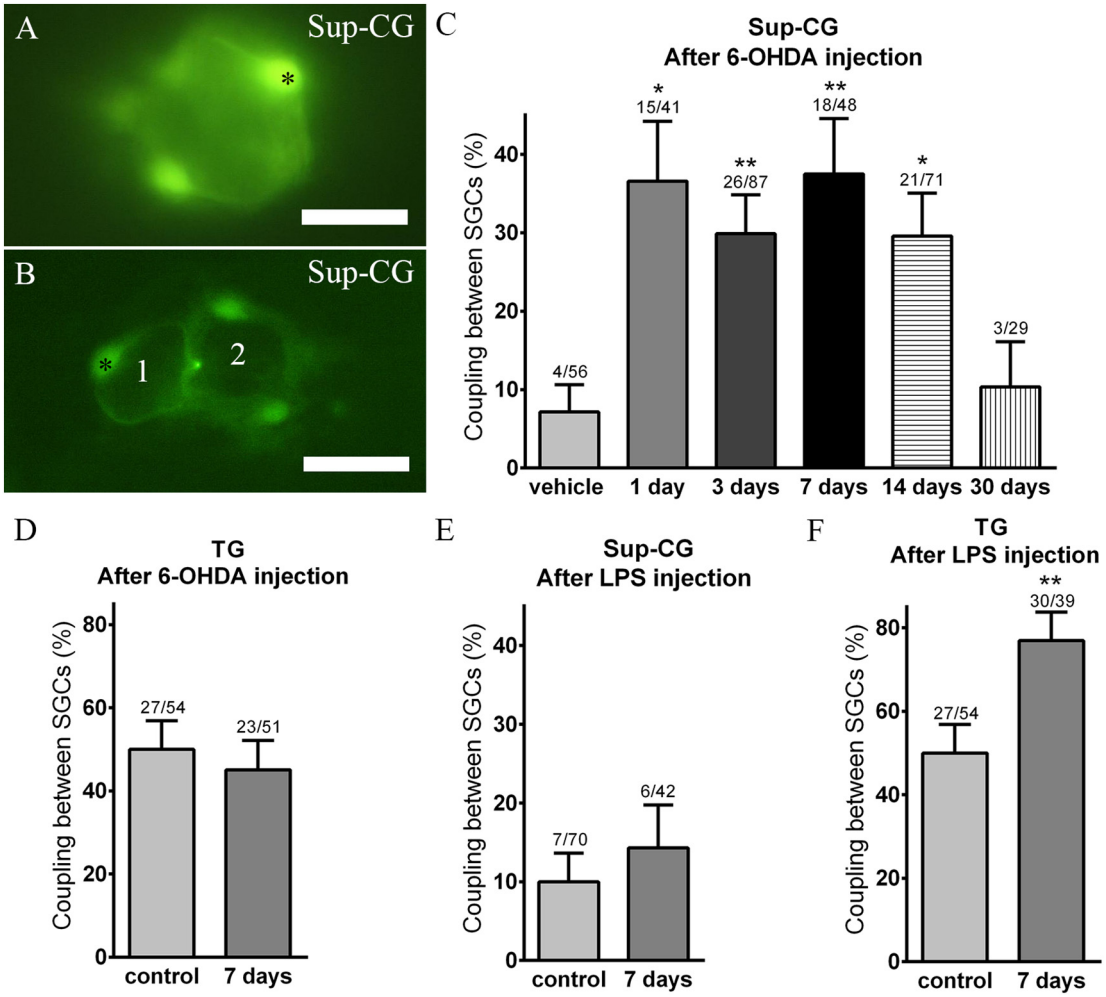

Fig. 4. Changes in dye coupling between SGCs in Sup-CG and TG after 6-OHDA and LPS injections. The micrographs show examples of dye coupling between SGCs in live, isolated ganglia. (A) In controls, LY-injected SGCs are usually coupled to other SGCs only around the same neuron. (B) Dye coupling between SGCs around different neurons in 6-OHDA-treated mouse. An SGC around neuron 1 was injected, and the dye passed into SGCs around neuron 2. Asterisks indicate the LY-injected SGCs. Scale bars, 20 μm. (C) The histogram shows augmented coupling among SGCs in Sup-CG at times after a single 6-OHDA injection. The numbers above each bar indicate (the number of coupled cells)/(the total number of injected cells). (D) Coupling among SGCs in TG after 6-OHDA injection. (E) Coupling among SGCs in Sup-CG after LPS injection. (F) Augmented coupling among SGCs in TG after LPS injection. Error bars indicate S.E. *P < 0.05, **P < 0.01 compared with vehicle. Data are from experiments on 4–5 ganglia for each of the data points. One-way ANOVA with Dunnett's Multiple Comparison Test or Unpaired Two-tailed *t*-test was used for comparison.

to the assays used here, SGCs in this ganglion are not influenced by LPS.

4.1. Glutamine synthetase expression

Glutamine synthetase (GS) is a marker of glial cells in both CNS and sensory ganglia, and we tested whether it is expressed in SGCs in Sup-CG. Somata of sensory neurons (Kung et al., 2013) and of SGCs Laursen et al., 2014; Wagner et al., 2014) release glutamate, which is transported into SGCs, and is converted to glutamine by GS (Berger and Hediger, 2000; Miller et al., 2002). In sensory ganglia neuronal somata as well as SGCs express glutamate receptors (Carlton and Hargett, 2007; Huettner, 1990; Lovinger and Weight, 1988; Sato et al., 1993). Thus, glutamate can serve as a bidirectional mediator between neurons and SGCs in sensory ganglia and therefore GS is likely to be essential for the proper function of these ganglia. There is evidence for the presence and function of a large variety of neurotransmitters in the Sup-CG (Klimaschewski et al., 1996), e.g., ACh, GABA, substance P, but reports on glutamate receptors in this ganglion have been sporadic (Kiyama et al., 1993; Shigemoto et al., 1992). Moreover, in a functional study glutamate failed to evoke calcium responses in the neurons (Kammermeier and Ikeda, 2002). Therefore, although a role for glutamate in the Sup-CG cannot be ruled out entirely, its functional contribution is probably minor, which correlates with the low GS expression glial cells in Sup-CG compared with TG (Fig. 1).

4.2. Activation of SGCs

It was found that GFAP was upregulated in SGCs in sympathetic ganglia following injuries such as preganglionic or postganglionic denervation of these ganglia (Elfvin et al., 1987), or sciatic nerve transection (Hu and McLachlan, 2004). We observed a 3-fold increase in GFAP expression in SGCs of Sup-SG following 6-OHDA injection. Mundinger et al. (2008) observed that following 6-OHDA injection, *cfos* mRNA (a marker for physiological activity) was elevated in both SGCs and neurons in sympathetic ganglia and that Fos protein was elevated only in SGCs. This indicates that 6-OHDA induced a profound effect on SGCs, which supports our results. The mechanisms by which the stimulation of Sup-CG neurons leads to glial activation is not known, but it may be suggested that the neurons release chemical messengers such as nitric oxide, which act on the SGCs, as found in sensory ganglia (Belzer and Hanani, 2019).

The elevation in GFAP peaked at day 3 post-6-OHDA, and slowly

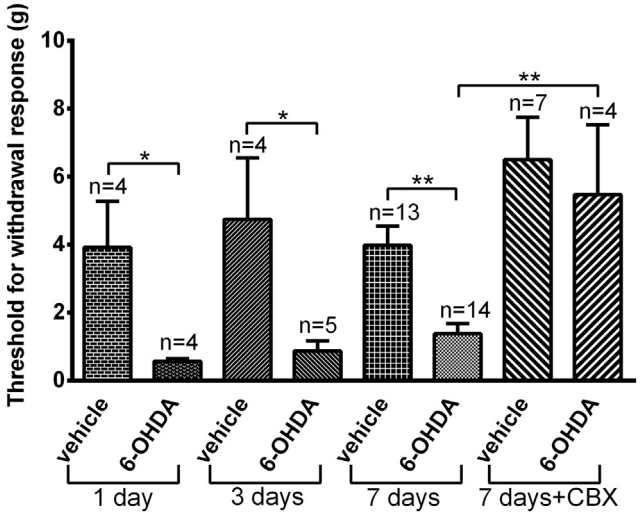

Fig. 5. Systemic injection of 6-OHDA causes tactile hypersensitivity in mice, measured in the hind paws with von Frey hairs. At 1, 3 and 7 d after 6-OHDA injection, threshold for withdrawal response is significantly lower than in vehicle injected animals. In 6-OHDA injected mice, 1 h post carbenoxolone (CBX) injection, pain threshold is elevated. Numbers above bars indicate the number of animals tested. Error bars indicate S.E. **P < 0.01 compared with vehicle, unpaired Two-tailed *t*-test.

declined afterwards, but was still greater than control at day 30 (Fig. 2). This is in agreement with Qi et al. (2012) who reported that nerve regeneration after sympathectomy with 6-OHDA required at least 1 month. It can be concluded that the persistence of GFAP upregulation reflects the incomplete recovery of Sup-CG neurons. LPS did not influence GFAP expression in SGCs of Sup-CG, apparently because neurons in this ganglion do not possess TLR4 (Fig. 3B) and are therefore not affected by LPS. In the TG, neurons express TLR4 (Fig. 3A), and SGCs in TG show GFAP upregulation following LPS injection. These findings indicate that injured neurons in both ganglion types can communicate with the surrounding SGCs and activate them. The mechanisms underlying this communication are currently under investigation.

4.3. Dye coupling

Dye coupling among SGCs was not increased in Sup-SG from LPS-injected mice (Fig. 4E), in agreement with the GFAP results described above; this is in contrast to the increase induced by LPS in TG (Fig. 4F) and other sensory ganglia (Blum et al., 2014; Feldman-Goriachnik et al., 2015). This again indicates that, according to the present assays, SGCs in Sup-CG are not influenced by LPS. However, under selective damage to sympathetic nerve terminals by 6-OHDA, SGC dye coupling in the Sup-CG was augmented by 6-fold (Fig. 4C), in correlation with the GFAP results.

It has been proposed that augmentation in gap junctions could be part of the SGC activation process in sensory ganglia (Huang et al.,

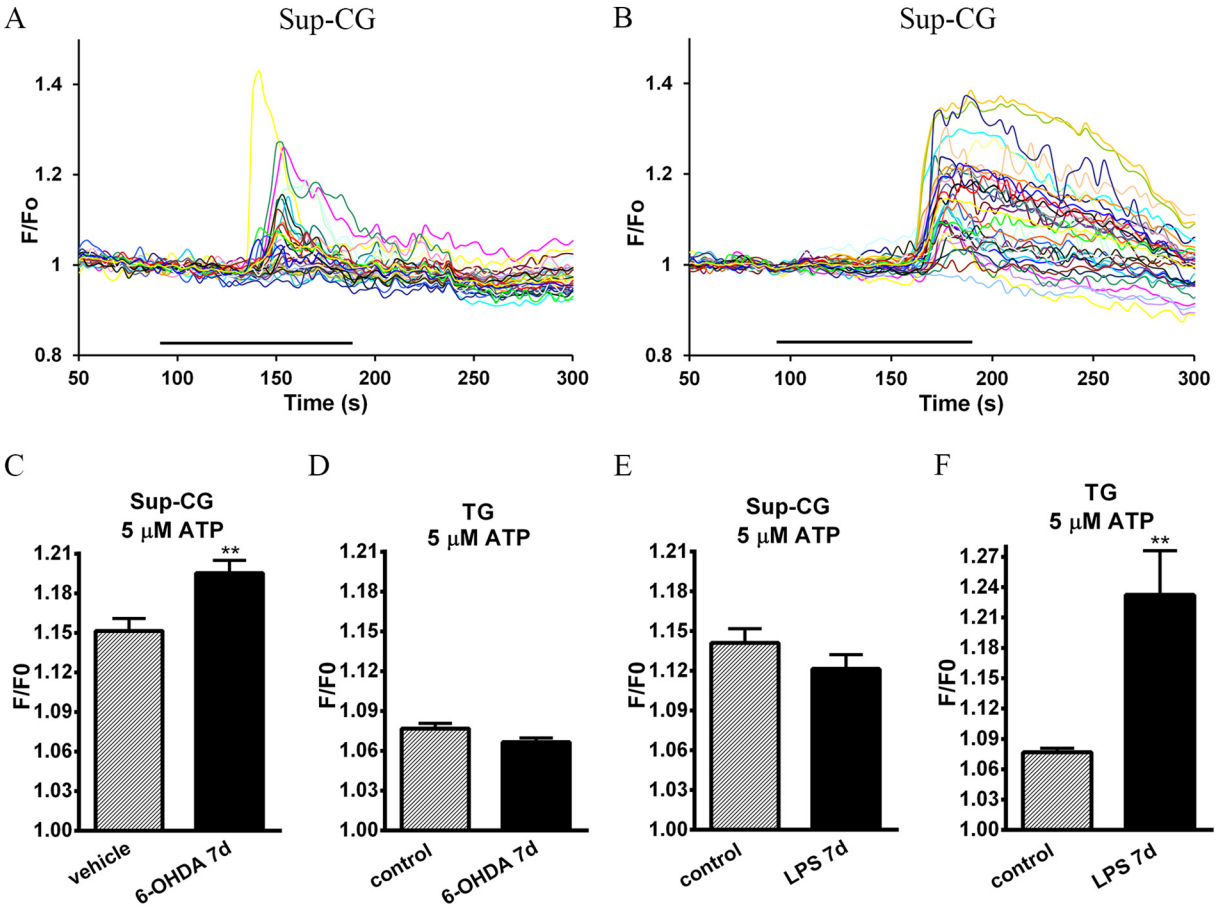

Fig. 6. Responses to ATP in SGCs of Sup-CG and TG from 6-OHDA- and LPS-injected mice, as assayed by Ca^{2+} imaging. (A) Example of responses to 5 μM ATP in Sup-CG from vehicle-treated mouse. (B) Example of responses to 5 μM ATP in Sup-CG 7 d post 6-OHDA injection. The recordings are from a total of 33 cells for (A) and 35 cells for (B). The bar under the tracings indicates the time of ATP application. (C) Augmented responses to ATP in Sup-CG 7 d post 6-OHDA injection. (D) Responses to ATP in TG 7 d post 6-OHDA injection. (E) Responses to ATP in Sup-CG 7 d post LPS injection. (F) Augmented responses to ATP in TG 7 d post LPS injection. Data are from experiments on 5–7 ganglia for each of the data points. The number of cells for each bar is between 151 and 357. Error bars indicate S.E. **P < 0.01 compared with vehicle control. Unpaired Two-tailed *t*-test was used for comparison.

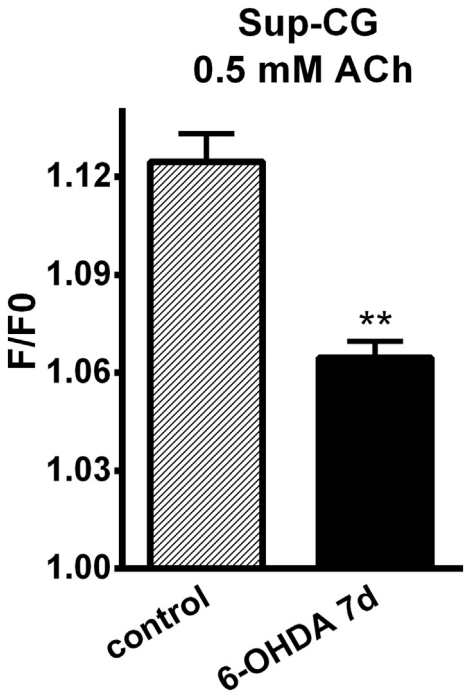

Fig. 7. Reduced responses to 0.5 mM ACh in Sup-CG 7 d post 6-OHDA injection. Data are from Ca^{2+} imaging experiments on 4–7 ganglia for each point. The number of cells for each bar is 160–246. Error bars indicate S.E. **P < 0.01 compared with control. Unpaired Two-tailed *t*-test was used for comparison.

2010), and it is conceivable that this holds for sympathetic ganglia as well. Augmented SGC coupling in sensory ganglia leads to neuronal hyperexcitability and pain (Huang et al., 2010; Ohara et al., 2008), and it can be hypothesized that the increased SGC coupling reported here can similarly lead to abnormal neuronal activity in sympathetic ganglia.

4.4. Pain behavior

We demonstrated that 6-OHDA induced tactile hypersensitivity (Fig. 5). In rats, 6-OHDA did not cause pain (Coderre et al., 1984; Li et al., 2013; Wei et al., 2002), but a report on mice agrees with our observations (Fasmer et al., 1986). The disagreement may be explained by the different species (rats vs. mice) and different experimental conditions. Our results are consistent with reports that sympathetic nerves participate in pain (Kim and Chung, 1991; Minett et al., 2012) and in injury-induced neuroinflammation (McLachlan and Hu, 2014), but the mechanisms underlying these observations are not known. Apparently abnormal SNS activity contributes to pain, consistent with studies where sympathectomy significantly alleviated mechanical allodynia in pain models (Nascimento et al., 2015; Fujimoto et al., 2012), and was used to treat CRPS in humans (Melis et al., 2002). Obviously, the role of the SNS in pain requires further study, but our results reaffirm the connection between SNS injury and pain.

We tested the possibility that the 6-OHDA-induced tactile hypersensitivity might be associated with the augmented SGC coupling by injecting treated mice with the gap junction blocker CBX. As can be seen in Fig. 5, 1 h after CBX injection withdrawal threshold returned to control level, suggesting a role for SGC coupling in Sup-CG in pain sensation. This effect is reminiscent of the analgesic actions of blocking gap junctions in various pain models (Ohara et al., 2008; Warwick and Hanani, 2013). It could be proposed that stronger SGC coupling leads to enhanced neuronal activity in sympathetic ganglia, causing hypersensitivity, and blocking the coupling reduces nerve activity, which alleviates pain. This idea was supported in studies on sensory ganglia, but needs to be explored for sympathetic ones. The augmented pain behavior would require hyperexcitability of neurons in the pain pathway – dorsal root ganglia and/or the dorsal horn of the spinal cord. Clarifying this issue will require further study.

4.5. Effect of ATP on SGCs

It has been reported that SGCs in Sup-CG are endowed with P2R (Calvert et al., 2004; Kumagai and Saino, 2001). Calvert et al. (2004) used Ca^{2+} imaging in cultures of mouse Sup-CG, and concluded that glial cells responded via the activation of P2YR. It should be noted that glial cells were identified visually, and because these cells may change their phenotype in culture (Belzer et al., 2010), their identity (SGCs or Schwann cells) is uncertain. Kumagai and Saino (2001) used Ca^{2+} imaging in rat Sup-CG, and obtained somewhat different results, identifying P2XR in SGCs. These authors described their tissue as intact ganglion, but they incubated the ganglia in collagenase for 12 h; still, this preparation is closer to the physiological situation than cultures. Here we also demonstrated that ATP caused a clear increase in $[Ca^{2+}]_{in}$ in SGCs in intact, freshly isolated Sup-CG (Fig. 6A,C). Therefore this is the first report showing P2R-mediated responses in SGCs of Sup-CG under conditions that are close to physiological. There is evidence that ATP is released in Sup-CG (e.g. McCaman and McAfee, 1986; Vizi et al., 1997), apparently from preganglionic nerve terminals, and SGCs can thus be a target for this neurotransmitter.

Inflammation (local or systemic) augments responses of SGCs to ATP in sensory ganglia (Kushnir et al., 2011; Blum et al., 2014). Similar information for sympathetic ganglia was absent. We showed here for the first time that under damage to sympathetic nerve terminals, SGCs in Sup-CG displayed enhanced responses to ATP (Fig. 6B,C), as observed for sensory ganglia under inflammation. However, systemic inflammation did not induce changes in responses to ATP in Sup-CG (Fig. 6E). The functional consequences of the increased sensitivity of SGCs to ATP in the Sup-CG are not clear. It was proposed that in sensory ganglia ATP participate in intercellular signaling in the form of calcium waves (Hanani, 2015), and a similar function may be suggested for the Sup-CG. Responses to breakdown products of ATP, such as ADP, which acts on P2YR, are also conceivable.

4.6. Responses to ACh

We have shown recently using calcium imaging that SGCs in mouse Sup-CG are sensitive to ACh and that this neurotransmitter activates SGCs (Feldman-Goriachnik et al., 2018). Here we found that damage to sympathetic nerve terminals reduced the responses of SGCs to ACh (Fig. 7), which correlates with the results of a study showing that 6-OHDA suppressed the expression of functional neuronal nicotinic receptors in rat celiac ganglia (Mundinger et al., 2008). This effect was attributed to the reduction of supply of nerve growth factor (NGF) from the periphery. It can be suggested that as in the neurons, the lower expression of ACh receptors in SGCs is due to NGF deficiency. It is interesting to note that damage increased the sensitivity of SGCs to ATP but reduced the sensitivity to ACh. The functional implications of these findings are currently obscure, and further study of the pharmacology of SGCs in both systems is required.

4.7. Limitations in this work

Several limitations in this work need to be mentioned: 1. The systemic administration of 6-OHDA ablates all the peripheral sympathetic nerves and might influence cardiovascular and other body functions. However, it is very likely that under the conditions of the present work such influence is minimal because we used a small dose of 6-OHDA (50 mg/kg). Moreover, the SNS is not essential to life in a controlled environment, and its dysfunction becomes evident under emergency or stress (Hilal-Dandan and Brunton, 2014). It was reported that several days after 6-OHDA administration cardiovascular changes were minor,

and cardiac output was not altered (Nichols et al., 1985; Laycock and Lightman, 1989) Indeed, the 6-OHDA–treated mice had a normal appearance, consistent with normal cardiac function. 2. Local application 6-OHDA was found to have considerable effects on various central nervous system regions (Bové and Perier, 2012). We applied it systemically, but no central actions are expected because 6-OHDA does not cross the blood brain barrier (Delrue-Perollet et al., 1995). Still, Kawa et al. (1979) observed that after i.p. 6-OHDA injection (150 mg/kg) in rats, there was an accelerated turnover of noradrenaline in the hypothalamus (but not in the cortex), and therefore some central effects may not be ruled out at high 6-OHDA doses. 3. We used CBX as a gap junction blocker, but it may have other actions. However, we expect minimal side effects of this drug, because it does not cross the blood brain barrier, and it binds to cells in sensory ganglia (see Warwick and Hanani, 2013). We argue that CBX has a significant effect mainly when gap junction function is augmented. As shown is Fig. 5, CBX did not have a significant effect on pain behavior in control mice, but had a clear action in 6-OHDA treated mice, suggesting that it acted on the augmented gap junctions in these animals.

5. Conclusions

The present findings support the idea that even though SGCs in sympathetic ganglia are morphologically similar to those in sensory ones, they are affected differently after insults to peripheral nerves. This correlates with the differences between neurons in sensory and sympathetic ganglia, most prominently the presence of synapses in sympathetic ones. Following a specific damage to sympathetic nerve terminals, SGCs in Sup-CG changed their properties in the same manner as SGCs in TG under systemic inflammation, but systemic inflammation did not affect SGCs of Sup-CG.

Declaration of competing interest

None.

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