EVANGELISCHES KRANKENHAUS DÜSSELDORF

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**Elektrophysiologie, Angiologie, Intensivmedizin**

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To
Nam Nguyen, Ph.D.
Manager of Review
JoVE
nam.nguyen@jove.com

27 October 2020

Rebuttal: JoVE submission JoVE62026

Dear Dr Nam Nguyen, dear editorial board,

Thank you very much for the opportunity to submit a revised version of our manuscript.

We are grateful for the valuable and highly constructive feedback we received from the editors and reviewers. We are convinced that integration of the reviewers' comments and revision of the manuscript led to a considerable improvement in quality.

All comments and questions raised from the editors and reviewers are implemented in the revised version of the manuscript and are addressed the following point-by-point response. Changes in the manuscript are highlighted in green, while our suggestion for the video protocol are marked in yellow.

Yours sincerely,

Katharina Scherschel and Christian Meyer

Reviewer #1:

Manuscript Summary: Authors describe a method to collect and process left stellate ganglion in mice. The manuscript is interesting and useful, well written and extremely clear. All the parts are complete and easy-to-use for the researchers needing technical procedures. Authors are well focused on the scientific question and bibliography is exhaustive.

Major Concerns: there are no major concerns.

We thank reviewer 1 for his/her efforts and for agreeing that our manuscript is interesting and useful. All comments by this reviewer are now addressed in the following response and discussed in the manuscript as requested.

Minor Concerns:

Figure 1 is not enough clear for a surgeon new to the technique. My suggestion is to provide an additional figure with a schematic diagram, e.g. add a diagram drawn, made with power point or by freehand, highlighting the main anatomical points useful for orientation during dissection.

We thank reviewer 1 for this suggestion. The reviewer is right that the image was not clear enough. In response to this comment, we now included a schematic figure into the revised version of the manuscript which highlights the main anatomical points useful for orientation during dissection. Additionally, we have now included a new photographic description of the dissection to be clearer for a surgeon new to the technique.

*Figure 1*

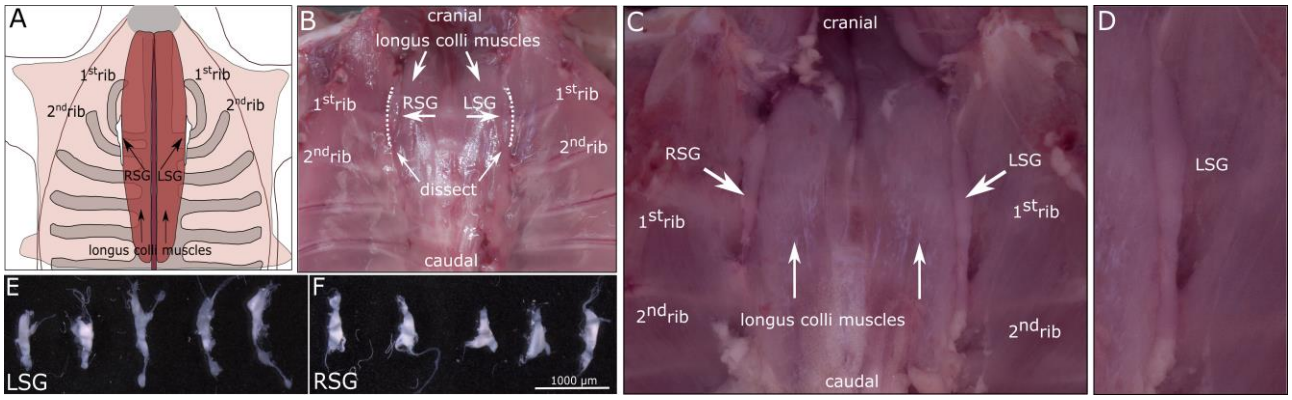


**Figure 1: Location, dissection and morphology of the murine stellate ganglia.** (A) Schematic drawing of the location of the stellate ganglia (SG). (B) View into the thorax after removal of the heart-lung package. It is important to note that the stellate ganglia are not immediately visible most of the time. The longus colli muscles are located lateral from the spine. The SG are located lateral from the muscles at the junction with the first rib. Carefully dissect lateral to the muscles (area marked by dotted line) to uncover the ganglia. After dissection, ganglia (left and right, LSG and RSG respectively) and the sympathetic chain can be made out as white, long structures. (C) An exemplary dissection showing the ganglia and anatomical landmarks. (D) Magnification of the left stellate ganglion. (E) LSG and (F) RSG from wild type, male C57Bl6 mice (16 weeks) were dissected and photographed to show the variations in morphology and size. Scale bar represents 1000 µm.

*Page 9, line 381, Representative Results*

**Figure 1** visualizes how to identify and dissect the SG. **Figure 1A** shows a schematic drawing of the location, while **Figure 1B** presents the view into the thorax after removal of the heart-lung-package. The left and right

longus colli muscles medial from the SG and the rib cage are important landmarks for orientation. Dissection is performed along the dotted lines between muscles and first rib. The SG and the sympathetic chain become visible as white structures (**Figure 1C**). **Figure 1D** shows a magnification of the region between the left longus colli muscle and the first rib, where the left SG is located. […]

**Authors are also encouraged to follow ARRIVE guidelines to provide all information needed in manuscripts describing animal procedures. The guidelines are available at: https://www.nc3rs.org.uk/arrive-guidelines**

We thank the reviewer for raising this important point. In consequence to this encouragement we carefully checked the ARRIVE guidelines and provide additional information to complement the previous version of the manuscript as recommended by this reviewer.

*Page 3, line 102 ff., Protocol*

[…] All procedures involving animals were approved were approved by the Animal Care and Use Committee of the State of Hamburg (ORG870, 959) and conform to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (2011). Studies were performed using male and female (aged 10-24 weeks) C57BL/6 mice (stock number 000664, Jackson Laboratories) and mice homozygous (db/db) or heterozygous (db/het; control) for the diabetes spontaneous mutation (Leprdb; BKS.Cg-Dock7$^{m+/+}$ Leprdb /J, stock number 000642, Jackson Laboratories). […]

*Page 10, line 430-431, Representative Results*

[…] Statistical significance was defined as a P value of <0.05, statistical analysis was performed using Graphpad Prism Version 6. […]

Reviewer #2:

Manuscript Summary:

The authors did a very good job of describing the methodology. The necessary details appear complete.

We thank Reviewer 2 for the positive feedback on our description of methodology and his/her helpful comments which we answer in the following.

Major Concerns:

The major concerns I have with the paper are the following:

1) Our laboratory routinely isolates rat SG neurons for electrophysiological studies. In our approach, we are able to remove the entire connective tissue from the SG. The authors suggest to readers that chemical dissolving may work but it did not work in the authors' hands. I would like to point out that removing the entire connective tissue is not a difficult thing to do. It is akin to removing one's socks. Alternatively, sharp forceps can be used to remove the connective tissue.

We thank the reviewer very much for raising this point and sharing his/her experiences with the isolation of rat SG neurons. As this is an important note, we now address this issue in the revised manuscript and added relevant literature. In addition, we outline that removal of the capsule might vary between species, the type of ganglion and age.

*Page 4, line 155 ff., Protocol: 1. Location and dissection of murine stellate ganglia*

Note: Autonomic ganglia are surrounded by a connective tissue capsule consisting of collagen fibres and fibroblasts [26, 27]. The permeability of these capsules seems to vary amongst species, different kind of ganglia [26] and age [28]. Remove as much connective tissue as possible using Dumont #5/45 forceps and, if necessary, spring scissors.

*Page 13, line 521 ff., Discussion*

[…] Some pitfalls should be kept in mind with the presented methods: we observed inconsistencies in antibody-based staining at some occasions and hypothesized that incomplete removal of the connective tissue capsule ensheathing the SG might be at fault, as they have been described to vary in permeability amongst different types of ganglia.[26] Mechanical removal of the capsule using fine forceps has been described in the superior cervical ganglion of rats up to postnatal day 10 [28] and desheathing is mentioned in literature for adult rat SG [54,55] and mice [56]. Removal of the SG capsule might vary between age [28] and – due to size differences – species. In our experience, fresh dissection, removal of as much connective tissue as possible using fine forceps and thorough permeabilization as described in the protocol at hand, are important factors for successful staining. […]

2) I think it is crucial that the authors inform the reader that SG innervate not only the heart, but they innervate the lungs and sweat glands. Therefore, in their paper, they should avoid making statements that with this approach one is able to study cardiac innervating SG neurons. Normally, the cardiac muscle is injected with a dye that will travel to the SG soma in a retrograded manner to identify cardiac muscle-innervating SG neurons. Unless the SG neurons are labeled, it can't be known with certainty what SG cell subtype is under study.

We thank the reviewer for raising this point. In consequence to this comment, we now inform the reader that the SG also innervates lungs and sweat glands and that it cannot be known with certainty what SG cellular subtype is under study.

*Page 13, line 505 ff., Discussion*

[…] It is important to note that the heart is not the only target of the SG. Amongst others, lungs [47] and sweat glands in the forepaw [48] are also innervated from fibres originating in the SG, the latter are an exception to sympathetic physiology as they express choline acetyltransferase [37]. […]

*Page 13, line 510 ff., Discussion*

[…] When focussing on cardiac disease models, it should be kept in mind for interpretation of results that cardiac neurons cannot be differentiated by morphology or electrophysiological properties from non-cardiac neurons [51]. This can be achieved by retrograde tracing, thereby the location of neurons projecting to the heart was shown to be located in the cranio-medial parts of the SG[52]. […]

**3) Another important fact that authors should point out is that the SG neurons that innervate sweat glands are a unique exception in sympathetic physiology. That is, the main neurotransmitter released is acetylcholine, and these SG neurons are, thus, cholinergic. This further implies that this neuron subtype expresses choline acetyltransferase. Therefore, how did the authors distinguish between Chat expressed in ICN versus SG neurons innervating sweat glands?**

We thank the reviewer for this remark. In consequence, we now point out in the revised manuscript that innervation of sweat glands from the SG is an exception in sympathetic physiology in regard to release of acetylcholine as main neurotransmitter.

*Page 13, line 505 ff., Discussion*

Amongst others, lungs [47] and sweat glands in the forepaw [48] are also innervated from fibres originating in the SG, the latter are an exception to sympathetic physiology as they express choline acetyltransferase [37].

We did not distinguish between *Chat* expressed in ICN and SG neurons on RNA level, but as reviewers 3 and 4 pointed out, fibers in the SG immunoreactive for ChAT on protein level are most likely preganglionic (Morales et al. Proc. Natl. Acad. Sci. USA, 1995, Jiminez et al., Synpase, 2002). Neuronal somata expressing ChAT can thus be accounted to SG neurons innervating sweat glands. In consequence to this comment, we removed the comparison of *Chat* mRNA in ICN versus SG from the revised manuscript to avoid confusion.

**4) In regard to Section 5 (Molecular Analyses), the authors should also point out that the gene expression profile the experimenter will obtain will be made up of glial cells and SG neurons innervating heart, lungs and sweat glands. Therefore, any conclusions drawn from the results with this approach should be done with caution.**

We thank the reviewer for pointing this out. To address this issue appropriately, we have now included new data into the revised manuscript pointing out for the experimenter that the gene expression profile will be made up from glial cells as well as neurons.

The revised version of the manuscript now includes an updated Figure 2 (changes in the Figure are marked with a red box below) with a double in situ hybridization presenting *S100b* (as glial marker) and *Tubb3* mRNA (as neuronal marker) co-stained with an antibody targeting tyrosine hydroxylase showing different kinds of neurons and glial cells.

Additionally, we have included gene expression data of *S100b*, but also *Ki67* as a marker for proliferating cells in Figure 3.

Last, we noted in the discussion that the SG consists of different kinds of cells.

*Page 10, line 400 ff., Representative Results*

[…] Figure 2C-F show exemplary analyses to study the SG on a subcellular level, using whole mount in situ hybridization and immunofluorescent co-staining. The protein TH (Figure 2C, red) and mRNA molecules of *Tubb3* (Figure 2D, white) are expressed in large neuronal cell bodies, while mRNA of *S100b* (Figure 2E, green) is also detectable in surrounding glia cells. In the merge (Figure 2F), it is visible that some neurons are negative for TH but express *Tubb3*, while *S100b* mRNAs can also be detected in surrounding cells, as depicted in the magnification in Figure 2G. […]

*Figure 2*

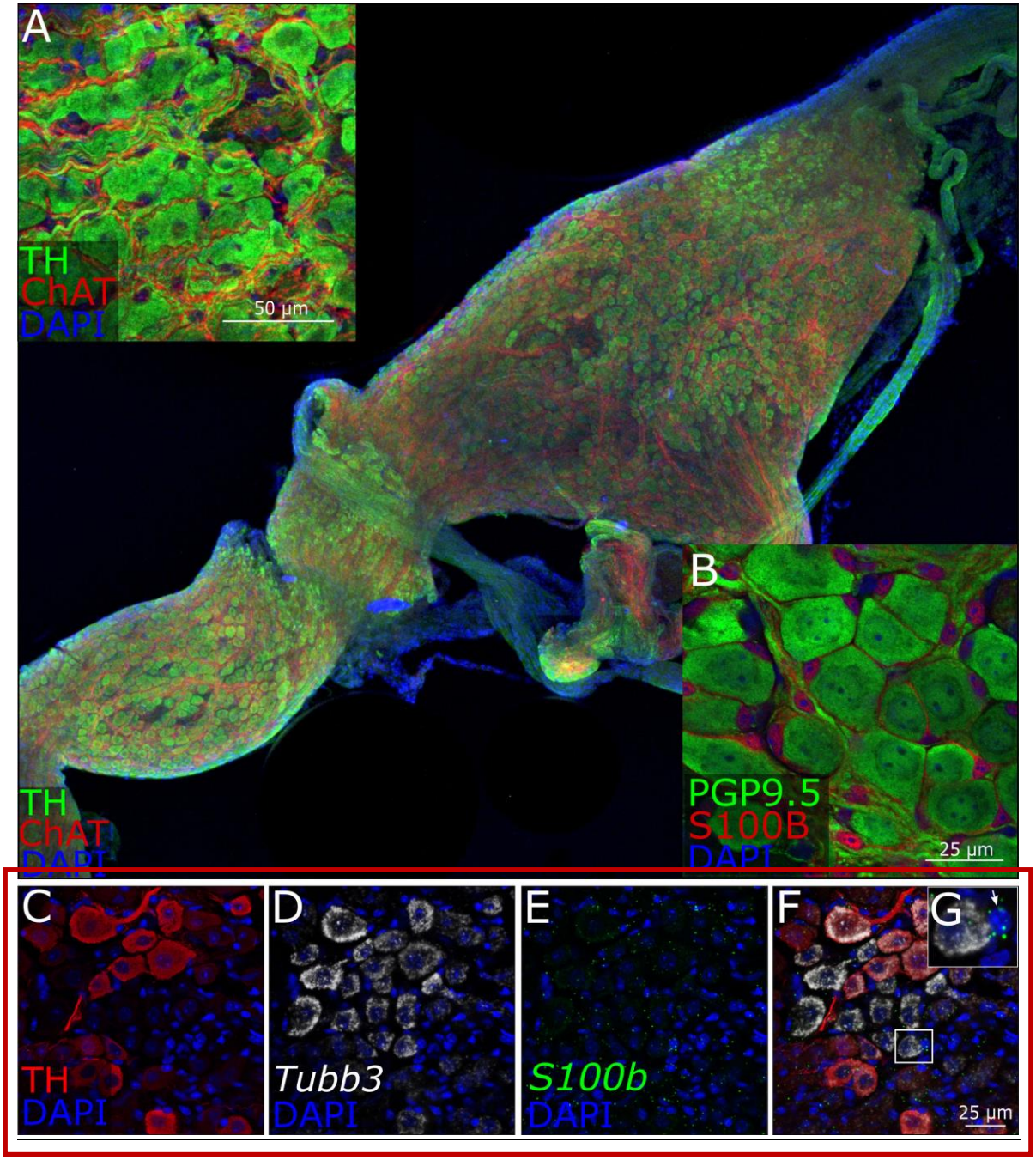

**Figure 2: Visualization of different cell types in murine stellate ganglia via whole mount immunohistochemistry and in situ hybridization.** (A) Gross overview of a murine stellate ganglion stained for the sympathetic marker tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT). The magnification shows strongly TH-positive cell bodies and the presence of ChAT-positive, most likely presynaptic, nerve fibers surrounding neuronal somata. (B) Glial cells ensheathing neuronal cell bodies can be visualized by staining for S100B, here in combination with the neuronal marker PGP9.5 (C-F) Microscopic images from one SG stained whole mount via a combination of immunohistochemistry for TH (red) and in situ hybridization for *Tubb3* (D, white) and *S100b* (E, green). Nuclei are counterstained with DAPI (blue). (F) The merge shows that not all neuronal (*Tubb3*-positive) cells are TH positive. *S100b* mRNAs can be detected within neuronal somata, but also surrounding cells, as marked by an arrow in the magnification in (G).

*Figure 3C*

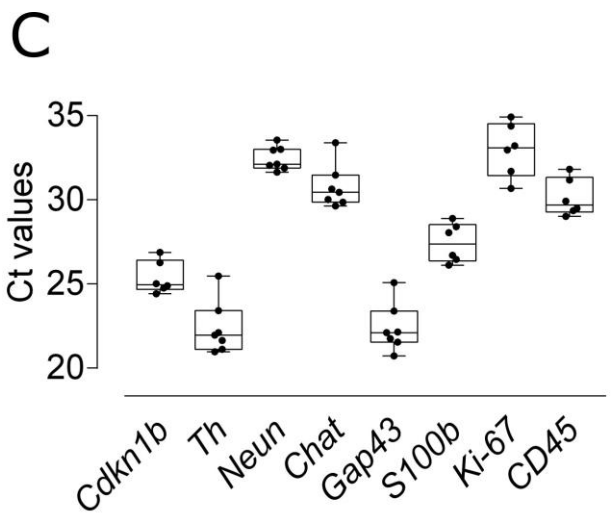


**Figure 3: Potential quantitative analyses and pitfalls**. […] (C) Exemplary genes expressed in the SG that might be useful for characterizing molecular processes. *Cdkn1b* as well as the neuronal marker *Neun/Rbfox3* (to account for the presence of other cell types) can be used for normalization. Tyrosine hydroxylase (*Th*) serves as a sympathetic marker, choline acetyltransferase (*Chat*) for cholinergic transdifferentiation, *Gap43* for neuronal sprouting. Genes expressed in non-neuronal cell types include *S100b* (glial cells), *Ki-67* (proliferating cells) and *Cd45* (immune cells).

*Page 10, line 412 ff., Representative results*

**Figure 3C** shows the expression of genes from different cell types of the SG (n = 6-7). Pooling of both SG from one animal allows gene expression measurements of approximately 24 assays (12 genes in duplicates). We typically normalize samples for *Cdkn1b* (detected at Ct values of 25.4 ± 0.97) as well as the neuronal marker *Neun/Rbfox3* (32.5 ± 0.7) if it is necessary to account for other cell types and neuronal purity of the dissection. Genes that we found useful for characterizing molecular processes in the SG include the sympathetic gene *Th* (22.4 ± 1.6), *Chat*, which could indicate cholinergic transdifferentiation (detectable at Ct values of 30.8 ± 1.3) and *Gap43*, a marker for neuronal sprouting (22.4 ± 1.4). Genes expressed in non-neuronal cell types include *S100b* (for glial cells, 27.3 ± 1.2), *Ki-67* (for proliferating cells, 33.0 ± 1.6) and *Cd45* (for immune cells, 30.2 ± 1.1).

*Page 13, line 515 ff., Discussion*

*[…]* Additionally, it is important to note that besides different types of neurons, sympathetic ganglia are made up of ensheathing glia, so-called satellite glial cells or satellite cells, marked by expression of the glial

marker S100B [53]. While little is known about the role of these cells in cardiovascular pathologies, glial activation and expression of the glial fibrillary acidic protein (GFAP) has been described in SG from patients with arrhythmias [18]. […]

Minor Concerns:

Line 127: "rip" should be "rib"

Thank you, we have corrected this in the revised manuscript.

Line 84: please explain what "IIb" refers to.

We thank the reviewer for this remark. As this was also mentioned by reviewer 4, we now see that guideline-specific language for clinical recommendations is confusing for the target audience. In consequence, we have now excluded this from the revised version of the manuscript.

*Page 2, line 84 ff., Introduction*

[…] Cardiac sympathetic denervation presents an option for patients with therapy-refractory VA with promising results [14, 16, 17]. […]

Reviewer #3:

This very useful piece provides a thorough description of identifying and removing stellate ganglia from mice. The photos included are extremely helpful. This work will be a great training resource for people trying to study SG in mouse models of cardiovascular disease. I have no concerns, just a few minor edits to suggest.

We thank reviewer 3 for this positive appreciation of our work and for agreeing that this will be a great training resource. We answer the minor suggestions by this reviewer in the following.

Line 92. It looks like words are missing after the word "via"

We thank the reviewer for finding this editing mistake. The sentence is now corrected.

*Page 3, line 90 ff., Introduction*

*[…] Experimental studies present novel approaches to treat VA, for example the reduction of sympathetic nerve activity via optogenetics,[22] but in-depth characterization of the SG is still lacking in many cardiac pathologies that go in hand with VA. […]*

Line 127. Last word should be rib instead of rip

Thank you, we have now corrected this in the revised manuscript.

Line 305 section 5.1 - Please consider adding the option that ganglia can be placed in RNAlater on ice if freezing immediately is not an option.

We thank the reviewer for suggesting this option. Our lab has no experiences with RNAlater for the isolation of RNA from SG, but in consequence of this comment, we have now included this as an option for the experimenter in the revised protocol and Table of Materials.

*Page 9, line 341 ff., Protocol*

[…] have liquid nitrogen ready for shock-frosting or consider commercial solutions for protection of RNA (optional, see Table of Materials) [36].

Line 342 suggested edit: "A certain amount of para pre-ganglionic sympathetic fibers is are detectable". These ChAT immunoreactive fibers are probably mostly pre-ganglionic sympathetic fibers.

We thank the reviewer for raising this important point. In consequence of this comment, we have now included the fact that ChAT immunoreactive fibers are probably mostly pre-ganglionic sympathetic fibers in the revised version of the manuscript.

*Page 10, line 395 ff., Representative Results*

[…] TH-expressing neuronal somata are surrounded by nerve fibers staining positive for choline acetyltransferase (ChAT). These are most likely presynaptic fibers [37, 38]. […]

*Page 11, line 448 ff., Figure 2 Subtitle*

[…] Gross overview of a murine stellate ganglion stained for the sympathetic marker tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT). The magnification shows strongly TH-positive cell bodies and the presence of ChAT-positive, most likely presynaptic, nerve fibers surrounding neuronal somata. […]

Reviewer #4:

Manuscript Summary: This methods article describes protocols for the location, dissection, and characterisation of the murine stellate ganglion (SG) at the RNA, protein, and cellular level. The SG contains sympathetic neurons that innervate the heart, and is therefore an important target for the treatment of ventricular arrhythmias. The existence of a video protocol describing the location and dissection of murine SG will be extremely useful for the scientific community, and have a positive general impact. As stated in the article, often the superior cervical ganglion is used instead of the SG to study the sympathetic innervation of the heart in mice, because many researchers do not know how to dissect the SG. Furthermore, the other protocols presented contain useful practical tips on how to characterise the SG and information on how to optimise these techniques for SG neurons.

General comments: The efficacy of the protocol is demonstrated by the experimental results shown in the figures. The clarity of presentation is good. This article is compliant with research standards and has a good technical quality and efficiency. The protocols in this article are detailed and thorough enough such that a researcher in the field could replicate the experiment, except for the protocol for the location and dissection of the SG. This protocol would greatly benefit from the video format. The abstract is appropriate for this methods article. I expect that the steps listed in the procedure would lead to the described outcome. They are clearly explained, and there are no important steps missing. Critical steps are highlighted. The included examples of ways to characterise the SG will be useful to readers.

We thank reviewer 4 for his/her constructive and extensive feedback. We appreciate the time and efforts invested by this reviewer and are convinced that integration of this feedback led to a considerable improvement of the manuscript.

Major Concerns:

Scientific inaccuracy on lines 342 and 375: These ChAT positive fibres are not parasympathetic nerve fibres. These are more likely to be presynaptic sympathetic fibres.

We thank the reviewer for pointing this out. Please excuse. In consequence, we have now corrected in the revised version of the manuscript that ChAT immunoreactive fibers are most likely pre-ganglionic sympathetic fibers and added appropriate citations.

*Page 10, line 395 ff., Representative Results*

[…] TH-expressing neuronal somata are surrounded by nerve fibers staining positive for choline acetyltransferase (ChAT). These are most likely presynaptic fibers [37, 38]. […]

*Page 11, line 450 ff., Figure 2 Subtitle*

[…] (B) The magnification shows sympathetic, strongly TH-positive cell bodies and the presence of presynaptic, ChAT-positive nerve fibers surrounding neuronal somata. […]

Minor Concerns:

The title is not descriptive enough.

We thank the reviewer raising this concern. We have now changed the title of the manuscript to be more descriptive.

*Title:* “The murine stellate ganglion - location, dissection and analysis”

Appropriate controls should be suggested in the protocols.

Thank you for noting this. In consequence of this comment, we now suggest appropriate technical controls at several steps in the protocols.

*Page 5, line 207 ff., Protocol*

Note: Include one SG as antibody control without primary antibody (incubated with antigen-preabsorbed antibody or IgG if available, or blocking buffer).

*Page 7, line 272 ff., Protocol*

Note: Include one SG as negative control, using a probe against a bacterial gene (e.g., dihydro-dipicolinate reductase, Dapb), to check for unspecific binding of amplification reagents in later steps.

*Page 9, line 335 ff., Protocol*

Note: Include controls depending on your experimental design. This could be SGs with different genotypes and disease background and/or other autonomic ganglia, such as the sympathetic superior cervical ganglion (located in the neck area, see detailed description in Ziegler et al. [35]) or parasympathetic ganglia (such as intracardiac ganglia, see Jungen et al.[4]).

*Page 9, line 360 ff., Protocol*

Note: To exclude contamination of the purified RNA with genomic DNA we propose performing a polymerase chain reaction with genomic primers and 1 µl RNA as template, instead minus reverse transcriptase control. This will safe a significant amount of RNA. If RNA is contaminated, use exon-intron boundary primers or intron flanking primers for subsequent quantitative real time polymerase chain reaction.

*Page 9, line 374 ff., Protocol*

Note: Perform non-template control for every gene to exclude false positive results.

The SG is not just involved in cardiac electrophysiology. Other potential applications for the protocols in this **article should be included.**

We thank the reviewer for this important remark. To address it, we have included other potential applications for this protocol in the article.

*Page 13, line 505 ff., Discussion*

[…] It is important to note that the heart is not the only target of the SG. Amongst others, lungs [47] and sweat glands in the forepaw [48] are also innervated from fibres originating in the SG, the latter are an exception to sympathetic physiology as they express choline acetyltransferase [37]. Temporary blockade of the SG is studied with regard to inflammatory processes in acute lung injury [49] or for treatment of hot flushes and sleep dysfunction [50], therefore the protocols at hand might offer a repertoire for mechanistic questions in these fields. […]

**It would be useful** to include additional information about the size and shape of the SG. Can the experimenter expect the SG to vary in size and shape between animals of the same sex and age, and between right and left SG?

We thank the reviewer for this question and fully agree that this will be useful information for the experimenter. Therefore, in consequence for this comment, we have now included new data about the size and shape of the SG.

*Figure 1D, E*

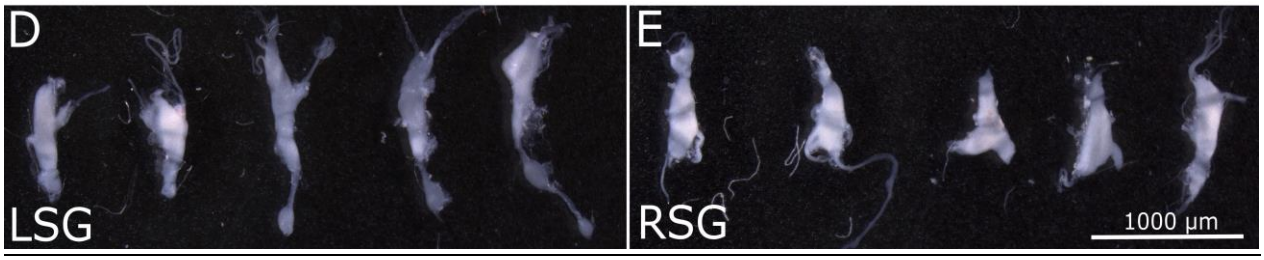


**Figure 1: Location, dissection and morphology of the murine stellate ganglia.** […] (D) LSG and (E) RSG from wild type, male C57Bl6 mice (16 weeks) were dissected and photographed to show the variations in morphology and size. Scale bar represents 1000 µm.

*Page 10, line 387 ff., Representative Results*

Morphology of the stellate ganglion differs between individuals. It often consists of a fusion of the inferior cervical and the first to the third thoracic ganglia [24]. Some variety that the experimenter can expect in murine SG is depicted in **Figure 1D and E**, where left and right SG of 5 male C57Bl6 wild type mice are photographed.

The materials and equipment table contains unnecessary German text (e.g. "vergällt mit" and "graduiert"). This table also contains irregular fonts.

We thank the reviewer for this thorough evaluation and excuse for the negligence. In consequence of this comment, we have carefully revised the materials and equipment table.

Previously published protocols are not cited properly for:

- "The location and dissection of murine stellate ganglia." There are some previously published descriptions of this method, albeit less detailed.

We thank the reviewer for pointing this out. In consequence to this comment we have now included citations of previously published protocols. In case we have accidentally omitted an important citation, we are grateful to learn about and include it in our revised manuscript.

*Page 3, line 112 ff., Protocol Step 1*

Note: Even though descriptions and drawings are mostly available in bigger species, some publications have previously described location of the SG in rats [24] and mice [25] using anatomical methods and fluorescent reporter lines, respectively.

- "Whole mount immunohistochemistry protocol." Please include a sentence at the start of section 2, with a reference to the protocol(s) this is based on.

We thank the reviewer for finding these flaws. We now include proper citations of the protocols this is based on.

*Page 4, line 163., Protocol*

[…] This protocol is adapted from cardiac whole mount stainings [4, 29].[…]

84: "With a IIb recommendation". This clinical jargon may be unfamiliar to the target audience.

We thank the reviewer for this remark. Thanks to this and to the question by reviewer 2, we now see that guideline-specific language for clinical recommendations is confusing for the target audience. In consequence, we have now excluded this jargon from the revised version of the manuscript.

*Page 2, line 84 ff., Introduction*

[…] Cardiac sympathetic denervation presents an option for patients with therapy-refractory VA with promising results [14, 16, 17]. […]

92: "reduction of sympathetic nerve activity, via ..., but in-depth characterization". Part of sentence missing.

We thank the reviewer for finding this editing mistake and have changed it accordingly.

*Page 3, line 90 ff., Introduction*

[…] Experimental studies present novel approaches to treat VA, for example the reduction of sympathetic nerve activity via optogenetics [22], but in-depth characterization of the SG is still lacking in many cardiac pathologies that go in hand with VA. […]

110: "Euthanize mouse". At what age range can this dissection be performed? Can you suggest any variations to the protocol for dissection of very young/old mice?

We thank the reviewer for this question. In consequence to this question and the remark by reviewer 1, we have now included age of the mice used in this study.

While we do not have experiences with mice younger than this age range, we have performed SG dissection in mice up to 60 weeks with this protocol. This is now included as a remark in the protocol.

*Page 3, line 104 ff., Protocol*

[…] Studies were performed using male and female (aged 10-24 weeks) C57BL/6 mice (stock number 000664, Jackson Laboratories) and mice homozygous (db/db) or heterozygous (db/het; control) for the diabetes spontaneous mutation (Leprdb; BKS.Cg-Dock7m+/+ Leprdb /J, stock number 000642, Jackson Laboratories). The authors have used the protocols at hand without variations for mice aged up to 60 weeks. […]

112: "in ripping off". Informal language.

Thank you, we removed informal language from the revised protocol.

*Page 3, line 122 ff., Protocol*

[…] Incorrect cervical dislocation can result in (1) breakage of the spine and damage of thoracic vessels leading to bleeding which hinders preparation or (2) in severing the sympathetic chain, so that SG are not in their correct position. […]

135: "Turn the forceps around by 180 °C" - remove C.

Thank you, we removed "C" from the revised protocol.

365: Fig1A/B. The absence of color in this image makes it much harder to identify different tissues. Please include a color image of your dissection.

We appreciate the feedback on the image in Figure 1A/B and agree that it might be hard to identify different tissues. In consequence to the suggestion by this reviewer, we have now revised Figure 1 and included new images of our dissection in color. Additionally, we have included a schematic drawing according to the suggestion of reviewer 1.

*Figure 1*

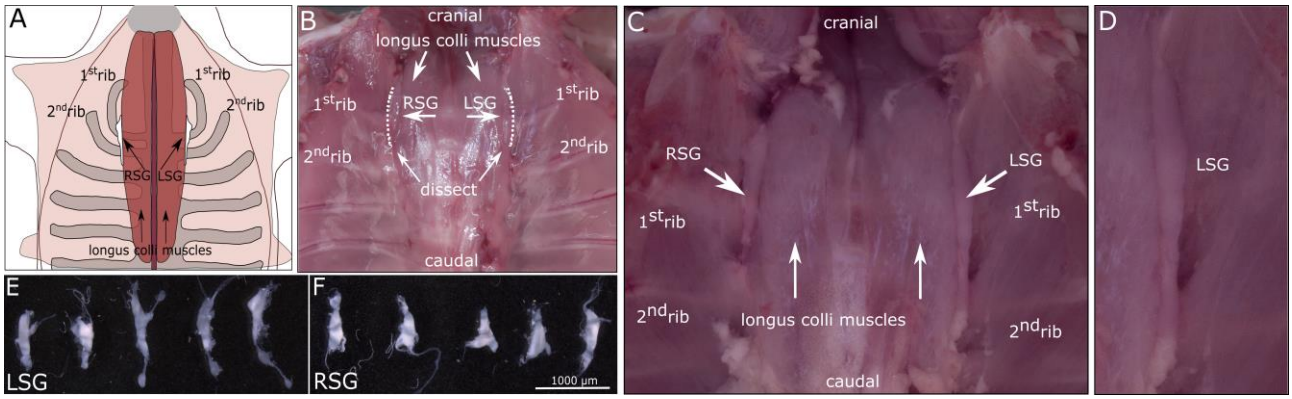


**Figure 1: Location, dissection and morphology of the murine stellate ganglia.** (A) Schematic drawing of the location of the stellate ganglia (SG). (B) View into the thorax after removal of the heart-lung package. It is important to note that the stellate ganglia are not immediately visible most of the time. The longus colli muscles are located lateral from the spine. The SG are located lateral from the muscles at the junction with the first rib. Carefully dissect lateral to the muscles (area marked by dotted line) to uncover the ganglia. After dissection, ganglia (left and right, LSG and RSG respectively) and the sympathetic chain can be made out as white, long structures. (C) An exemplary dissection showing the ganglia and anatomical landmarks. (D) Magnification of the LSG. (E) LSG and (F) RSG from wild type, male C57Bl6 mice (16 weeks) were dissected and photographed to show the variations in morphology and size. Scale bar represents 1000 µm.

*Page 10, line 381, Representative Results*

**Figure 1** visualizes how to identify and dissect the SG. **Figure 1A** shows a schematic drawing of the location, while **Figure 1B** presents the view into the thorax after removal of the heart-lung-package. The left and right longus colli muscles medial from the SG and the rib cage are important landmarks for orientation. Dissection is performed along the dotted lines between muscles and first rib. The SG and the sympathetic chain be-

come visible as white structures (Figure 1C). Figure 1D shows a magnification of the region between the left longus colli muscle and the first rib, where the left SG is located. […]

Fig 3: It is unclear how intracardiac neurons were obtained. Please include methods. It is unclear why SG neurons are compared to intracardiac neurons. Please clarify, or use a comparison to other ganglion neurons instead, such as from the SCG or parasympathetic ganglia.

We thank the reviewer for this feedback. The revised version of the manuscript now clarifies how to obtain SCG or parasympathetic ganglia as controls. As detailed methods for dissection of other ganglia was beyond the scope of this protocol, we have included recommendations and important key references.

*Page 9, line 335, Protocol*

Note: Include controls depending on your experimental design. This could be SGs with different genotypes and disease background and/or other autonomic ganglia, such as the sympathetic superior cervical ganglion (located in the neck area, see detailed description in Ziegler et al. [35]) or parasympathetic ganglia (such as intracardiac ganglia, see Jungen et al.[4]).

Fig legend of 3B should include statistical test used.

Thank you for finding this flaw. We have now included Mann-Whitney test as the statistical test used in Figure 3B.

*Page 11-12, line 460 ff., Figure legend*

[…] (B) This was performed in a mouse model of diabetes (100 cells per SG, n = 2 SG per genotype, data were compared using Mann-Whitney test). […]

Fig 3C is a comparison between fibres in the SG and cell bodies of ICN, therefore this is not an informative comparison. The SG does contain a very small percentage of cholinergic neuron cell bodies (<5%), which innervate the sweat glands in the forepaws, but most of the ChAT found in the SG is contained within the presynaptic sympathetic fibres. Please provide a rationale for this comparison, or compare SG to a different ganglion instead.

We thank the reviewer for this point. We agree that this comparison is not useful. Therefore, in consequence of this comment, we now state that the SG contains cholinergic neuronal cell bodies innervating the sweat glands in the forepaws, that cholinergic fibers in the SG are presynaptic sympathetic fibers and excluded the comparison with intracardiac neurons and replaced it with a panel of genes expressed in stellate ganglia.

*Page 13, line 505 ff., Discussion*

[…] It is important to note that the heart is not the only target of the SG. Amongst others, lungs [47] and sweat glands in the forepaw [48] are also innervated from fibres originating in the SG, the latter are an exception to sympathetic physiology as they express choline acetyltransferase [37]. […]

*Page 10, line 395 ff., Representative Results*

[…] TH-expressing neuronal somata are surrounded by nerve fibers staining positive for choline acetyltransferase (ChAT). These are most likely presynaptic fibers [37, 38]. […]

*Page 11, line 450 ff., Figure 2 Subtitle*

[…] (B) The magnification shows sympathetic, strongly TH-positive cell bodies and the presence of presynaptic, ChAT-positive nerve fibers surrounding neuronal somata. […]

*Figure 3C*

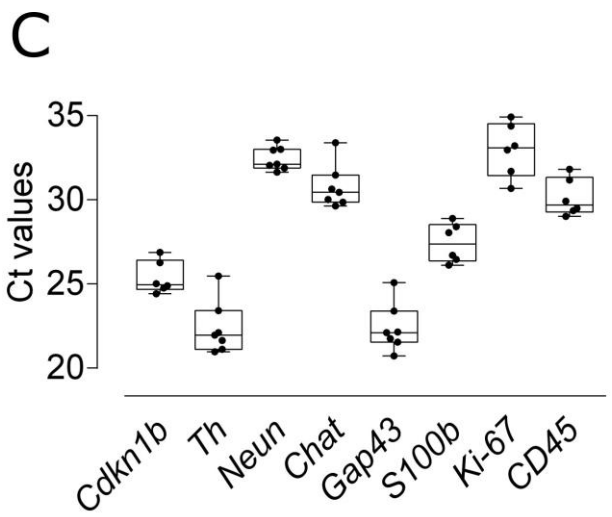


**Figure 3: Potential quantitative analyses and pitfalls**. […] (C) Exemplary genes expressed in the SG that might be useful for characterizing molecular processes. *Cdkn1b* as well as the neuronal marker *Neun/Rbfox3* (to account for the presence of other cell types) can be used for normalization. Tyrosine hydroxylase (*Th*) serves as a sympathetic marker, choline acetyltransferase (*Chat*) for cholinergic transdifferentiation, *Gap43* for neuronal sprouting. Genes expressed in non-neuronal cell types include *S100b* (glial cells), *Ki-67* (proliferating cells) and *Cd45* (immune cells).

*Page 10, line 413 ff., Representative results*

Figure 3C shows the expression of genes from different cell types of the SG (n = 6-7). Pooling of both SG from one animal allows gene expression measurements of approximately 24 assays (12 genes in duplicates). We typically normalize samples for *Cdkn1b* (detected at Ct values of 25.4 ± 0.97) as well as the neuronal marker *Neun/Rbfox3* (32.5 ± 0.7) if it is necessary to account for other cell types and neuronal purity of the dissection. Genes that we found useful for characterizing molecular processes in the SG include the sympathetic gene *Th* (22.4 ± 1.6), *Chat*, which could indicate cholinergic transdifferentiation (expressed at Ct values of 30.8 ± 1.3) and *Gap43*, a marker for neuronal sprouting (detectable at Ct values of 22.4 ± 1.4). Genes expressed in non-neuronal cell type include *S100b* (for glial cells, 27.3 ± 1.2), *Ki-67* (for proliferating cells, 33.0 ± 1.6) and *Cd45* (for immune cells, 30.2 ± 1.1).

Fig. 3F is unclear. What is the dark blue stain? Which side of the dotted line shows failed staining? Which characteristic can be used to identify this as failed staining? How does this image show that fat and connective tissue prevent proper staining here?

We thank the reviewer for his opinion on Figure 3F. In consequence to this comment, we have now included a different staining of the same specimen and clearly state that the dark blue stain is DAPI. We clarify anti-

bodies do only show a clear signal in some parts of the staining, even though DAPI shows that cells are present.

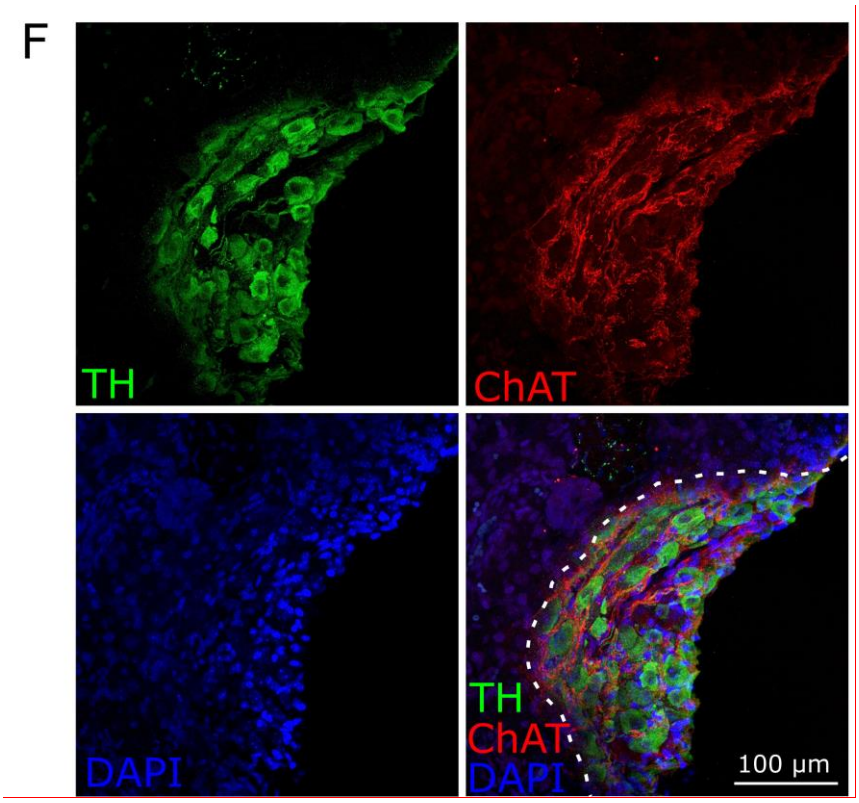


**Figure 3: Potential quantitative analyses and pitfalls.** […] (F) At some occasions we observed failure in antibody-based staining, most likely due to incomplete removal of the capsule. While ChAT (red) and TH (green) staining are only detectable in some parts of the SG, nuclei counterstained with DAPI (dark blue) are detectable throughout. The dotted line in the merged image separates successful staining (right of the line) from un-successful staining (left of the line).

*Page 11, line 423 ff., Representative results*

The SG is surrounded by a capsule of connective tissue [26], visualized via hematoxylin and eosin staining in **Figure 3D and E**. Occasionally, we observed inconsistencies in antibody-based staining as demonstrated in **Figure 3F**. While ChAT and TH staining are only detectable in some parts of the SG, nuclei counterstained with DAPI are detectable throughout. The dotted line in the merged image separates successful staining (right of the line) from unsuccessful staining (left of the line).

383: please clarify that the model of diabetes referred to here is db/db mice.

Please excuse for being unclear. We now clarify in the revised results section that the mouse model used here is the common type II diabetes model db/db.

*Page 3, line 104 ff., Protocol*

[…] Studies were performed using male and female (aged 10-24 weeks) C57BL/6 mice (stock number 000664, Jackson Laboratories) and mice homozygous (db/db) or heterozygous (db/het; control) for the diabetes spontaneous mutation (Leprdb; BKS.Cg-Dock7m+/+ Leprdb /J, stock number 000642, Jackson Laboratories). […]

410: "Due to its small size, manipulation of the murine SG in vivo is challenging." Should include mention of the location of the SG compared to the SCG as well. The SCG is much more accessible in vivo in mice.

We thank the reviewer for this suggestion. We have now mentioned that the SG is located within the thoracic cavity, while the SCG is location more assessable in the neck.

*Page 12, line 491 ff., Discussion*

Due to its small size and its location within the thoracic cavity [24], manipulation of the murine SG in vivo is challenging, although it has been performed successfully [23]. Still, for this reason, some studies focus on the superior cervical ganglia, which are located more accessible in the neck, upstream of the SG in the sympathetic chain behind the carotid bifurcation into internal and external carotid arteries [24, 35].

423: The statement "Chemical dissolving and permeabilization of connective tissue did not work successfully in our hands." is too vague. Need to specify methods used and explain what "did not work successfully" means.

We thank the reviewer for raising this point. We agree with the reviewer that this description is vague as it is rather anecdotal from establishment of the protocol. Therefore, in consequence of this remark, we have removed vague parts, and added relevant literature on this topic.

*Page 13, line 521 ff., Discussion*

[…] Some pitfalls should be kept in mind with the presented methods: we observed inconsistencies in antibody-based staining at some occasions and hypothesized that incomplete removal of the connective tissue capsule ensheathing the SG might be at fault, as they have been described to vary in permeability amongst different types of ganglia.[26] Mechanical removal of the capsule using fine forceps has been described in the superior cervical ganglion of rats up to postnatal day 10 [28] and desheathing is mentioned in literature for adult rat SG [54, 55] and mice [56]. Removal of the SG capsule might vary between age [28] and – due to size differences – species. In our experience, fresh dissection, removal of as much connective tissue as possible using fine forceps and thorough permeabilization as described in the protocol at hand, are important factors for successful staining. […]