

Ca²⁺ changes in sympathetic varicosities and Schwann cells in rat mesenteric arteries - relation to noradrenaline release and contraction

Hansen, Thomas; Tarasova, Olga S.; Khammy, Makhala M.; Ferreira, Avelino; Kennard, James A.; Andresen, Jørgen; Staehr, C; Brain, Keith; Nilsson, Holger; Aalkjær, Christian

DOI:

[10.1111/apha.13279](https://doi.org/10.1111/apha.13279)

License:

Unspecified

Document Version

Early version, also known as pre-print

Citation for published version (Harvard):

Hansen, T, Tarasova, QS, Khammy, MM, Ferreira, A, Kennard, JA, Andresen, J, Staehr, C, Brain, K, Nilsson, H & Aalkjær, C 2019, 'Ca²⁺ changes in sympathetic varicosities and Schwann cells in rat mesenteric arteries - relation to noradrenaline release and contraction', *Acta Physiologica*, vol. 226, no. 4, e13279. <https://doi.org/10.1111/apha.13279>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

ACTA PHYSIOLOGICA

[Ca²⁺] changes in sympathetic varicosities and Schwann cells in rat mesenteric arteries – relation to noradrenaline release and contraction

| | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Journal: | <i>Acta Physiologica</i> |
| Manuscript ID | APH-2018-07-0319.R1 |
| Manuscript Type: | Regular Paper |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Hansen, Thomas; Aarhus University, Biomedicine Tarasova, Olga; Faculty of Biology, M.V. Lomonosov Moscow State University, Department of Human and Animal Physiology Khammy, Makhala; Aarhus University, Biomedicine Ferreira, Avelino; Aarhus University, Department of Biomedicine Kennard, James; College of Medical and Dental Sciences, University of Birmingham, Institute of Clinical Sciences, Andresen, Joergen; Aarhus University, Biomedicine Staeher, Christian; Aarhus University, Biomedicine Brain, Keith; University of Birmingham, Neuropharmacology and Neurobiology Nilsson, Holger; University of Gothenburg, Institute of Neuroscience and Physiology Aalkjær, Christian; Aarhus University, Biomedicine |
| Key Words: | amperometry, confocal imaging, neurotransmission, prejunctional modulation, small arteries, sympathetic |
| Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online. | |
| Suppl. video 1.avi | |

1
2
3
4 **[Ca²⁺] changes in sympathetic varicosities and Schwann cells in rat mesenteric arteries –**
5
6 **relation to noradrenaline release and contraction**
7
8
9

10
11 **Thomas Hansen¹, Olga S. Tarasova^{2,3}, Makhala M Khammy¹, Avelino Ferreira¹, James A**
12
13 **Kennard⁴, Jørgen Andresen¹, Staehr C¹, Keith L. Brain⁴, Holger Nilsson⁵, Christian Aalkjær¹**
14

15
16 *¹Department of Biomedicine, University of Aarhus Denmark*
17

18 *²Faculty of Biology, M.V. Lomonosov Moscow State University, Moscow, Russia*
19

20 *³State Research Center of the Russian Federation - Institute for Biomedical Problems, Moscow,*
21
22 *Russia.*
23

24
25 *⁴Institute of Clinical Sciences, College of Medical and Dental Sciences, University of Birmingham,*
26
27 *UK*
28

29 *⁵Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy at the*
30
31 *University of Gothenburg, Sweden*
32
33

34
35
36
37
38 **Short title:** Ca²⁺ in sympathetic nerves
39

40
41 **Author for correspondence:** Christian Aalkjær, Dept Biomedicine, Aarhus University
42

43 Ole Worms Alle 4, Aarhus C, Denmark; tel: +45 3045 4306; e-mail: ca@biomed.au.dk
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

Aim

This study aimed to assess intracellular Ca^{2+} dynamics in nerve cells and Schwann cells in isolated rat resistance arteries and determine how these dynamics modify noradrenaline release from the nerves and consequent force development.

Methods

Ca^{2+} in nerves was assessed with confocal imaging, noradrenaline release with amperometry, and artery tone with wire myography. Ca^{2+} in axons was assessed after loading with Oregon Green 488 BAPTA-1 dextran. In other experiments, arteries were incubated with Calcium Green-1-AM which loads both axons and Schwann cells.

Results

Schwann cells but not axons responded with a Ca^{2+} increase to ATP. Electrical field stimulation of nerves caused a frequency dependent increase of varicose $[\text{Ca}^{2+}]_v$ ($[\text{Ca}^{2+}]_v$). ω -conotoxin-GVIA (100 nM) reduced the $[\text{Ca}^{2+}]_v$ transient to 2 Hz and 16 Hz by 60% and 27%, respectively; in contrast ω -conotoxin GVIA inhibited more than 80% of the noradrenaline release and force development at 2 and 16 Hz. The K_v channel blocker, 4-aminopyridine (10 μM), increased $[\text{Ca}^{2+}]_v$, noradrenaline release, and force development both in the absence and presence of ω -conotoxin-GVIA. Yohimbine (1 μM) increased both $[\text{Ca}^{2+}]_v$ and noradrenaline release but reduced force development.

Acetylcholine (10 μM) caused atropine-sensitive inhibition of $[\text{Ca}^{2+}]_v$, noradrenaline release and force. In the presence of ω -conotoxin-GVIA, acetylcholine caused a further inhibition of all parameters.

Conclusion

Modification of $[\text{Ca}^{2+}]_v$ in arterial sympathetic axons and Schwann cells was assessed separately. $\text{K}_v3.1$ channels may be important regulators of $[\text{Ca}^{2+}]_v$, noradrenaline release, and force

1
2
3
4 development. Presynaptic adrenoceptor and muscarinic receptor activation modify transmitter
5
6 release through modification of $[Ca^{2+}]_v$.
7
8
9

10
11 **Keywords**
12

13 Amperometry, confocal imaging, neurotransmission, prejunctional modulation, small arteries,
14
15 sympathetic.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

INTRODUCTION

Sympathetic nerves are important regulators of resistance artery tone. Extensive information on the pharmacology of transmitter release and transmitter effect in the vascular wall has accumulated over many years. This pharmacological characterization combined with evidence from other tissues suggest that the concentration of Ca^{2+} in the varicosities ($[\text{Ca}^{2+}]_v$) is important for transmitter release. It is also well known that several presynaptic receptors modify transmitter release and that pharmacological modification of ion channel activity can modify the release. Remarkably, however, there is no direct information on $[\text{Ca}^{2+}]_v$ in the vascular sympathetic nerves and therefore no direct information as to whether, for example, presynaptic receptors or ion channel activity modify transmitter release through modification of $[\text{Ca}^{2+}]_v$. This is largely because the small size of the terminal branches of the sympathetic axons and the varicosities (which typically have diameters around $1 \mu\text{m}$ ¹) and the presence of Schwann cells¹ make it difficult to directly assess $[\text{Ca}^{2+}]_v$ and other second messengers in the varicosities.

In this study we have applied Ca^{2+} -sensitive dyes, amperometry and myography to assess the relationship between $[\text{Ca}^{2+}]_v$, intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in axons and Schwann cells, noradrenaline (NA) release, and force development in rat resistance arteries. Because we wanted to assess both $[\text{Ca}^{2+}]_v$ in axons and $[\text{Ca}^{2+}]_i$ in Schwann cells we used two calcium dye loading protocols. To ensure selective dye loading of axons, we used a protocol developed for selective loading of axons in vas deferens²⁻⁵. To load axons and Schwann cells simultaneously we used the traditional loading of a lipophilic Ca^{2+} sensitive dye.

We have characterized the effect of known modulators of NA release on $[\text{Ca}^{2+}]_v$ and related this to their effects on NA release and force development, and evaluated the differential effect of ATP on

1
2
3
4 $[Ca^{2+}]_v$ and Schwann cell $[Ca^{2+}]_i$. We compared our $[Ca^{2+}]_v$ data with $[Ca^{2+}]_v$ data obtained in the
5
6 sympathetic nerve terminals of vas deferens ²⁻⁵.
7
8
9

10 11 RESULTS

12 13 14 15 *$[Ca^{2+}]_i$ transients in nerve fibers loaded with Calcium Green-AM.*

16
17 Following loading of Calcium Green-AM, nerve fibers were easily seen (Fig. 1A) lying
18 immediately outside the smooth muscle cells. The pattern seen after loading with Calcium Green-
19 AM was similar to the pattern seen after staining with the catecholamine-specific stain glyoxylic
20 acid (Suppl. Fig 1) consistent with the structures stained with Calcium Green-AM are nerve fibers
21 (i.e. axons and Schwann cells). It was not possible to distinguish varicosities. With field stimulation
22 a frequency-dependent increase in $[Ca^{2+}]_i$ was seen (Fig. 1B), which was TTX sensitive (insert Fig
23 1B). At low frequencies the effect of individual stimuli could be discerned, and a cumulative
24 increase of $[Ca^{2+}]_i$ consequent to a slow net decrease of the $[Ca^{2+}]_i$ transient was apparent (Fig. 1B).
25 Where ≥ 3 nerve fibers crossed, the fluorescence was often bright (single arrow in Fig. 1A). In 20-
26 30% of these crossings, the nuclear stain Syto16 revealed the presence of nuclei in the nerve fiber
27 (Fig. 1C, 1D and Suppl. Video 1).
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42

43 The increase of $[Ca^{2+}]_i$ to 2 and 16 Hz stimulation is seen in nerve fibers (Fig. 1E) and in areas
44 where nerve fibers cross (Fig. 1F) though the increase in $[Ca^{2+}]_i$ at both 2 Hz and 16 Hz was small
45 in areas where nerve fibers cross. Prior incubation with 10 μ M ATP did not significantly increase
46 the response to field stimulation in either of these areas (Fig. 1E and 1F). In non-activated arteries
47 10 μ M ATP caused a substantial increase of $[Ca^{2+}]_i$ in the area where nerve fibers cross (Fig. 2,
48 column 2). In the nerve fibers outside the crossing areas the increase did not obtain statistical
49 significance (Fig. 2, column 1).
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7 The increase in $[Ca^{2+}]_i$ with field stimulation was partly inhibited by 100 nM of the N-type Ca^{2+}
8 channel blocker ω -conotoxin GVIA (Fig. 3). The L-type Ca^{2+} channel blocker nifedipine (0.3 μ M)
9 and the predominantly T-type Ca^{2+} channel blocker mibefradil (1 μ M) were without effect on
10 $[Ca^{2+}]_i$ (the fluorescence relative to the time control value were 1.02 ± 0.02 and 1.01 ± 0.01 for
11 nifedipine and mibefradil, respectively, $n=7$). However, both 0.3 μ M nifedipine and 1 μ M
12 mibefradil reduced the force response to stimulation with exogenous NA (with tension relative to
13 the time control tension of $5 \pm 1\%$ and $4 \pm 1\%$ for nifedipine and mibefradil, respectively, $n=7$),
14 indicating that they were used in relevant concentrations. The ω -conotoxin GVIA insensitive
15 component of the $[Ca^{2+}]_i$ response to field stimulation increased with increasing frequencies and
16 only about 40% of the $[Ca^{2+}]_i$ response to 16 Hz stimulation was inhibited by ω -conotoxin GVIA
17 (Fig. 3).
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33

34 *$[Ca^{2+}]_i$ transients in axons loaded with Oregon Green 488 BAPTA-1 dextran*

35
36
37
38

39 In arterial segments loaded through the suction pipette with Oregon Green 488 BAPTA-1 dextran,
40 varicosities and intervaricose segments were easily distinguished (Fig. 4A) while, as expected,
41 extracellular structures, smooth muscle cells or endothelial cells were not seen.
42
43
44
45
46
47

48 Field stimulation resulted in a frequency-dependent increase in $[Ca^{2+}]_v$ (Fig. 4B). With Oregon
49 Green 488 BAPTA dextran loading, the transient increase in $[Ca^{2+}]_v$ to a single electrical pulse had
50 a higher amplitude and a faster decline than the $[Ca^{2+}]_i$ transient seen after Calcium Green-AM
51 loading (Fig. 4C). The $[Ca^{2+}]_v$ transients were fully inhibited by 100 nM TTX at all stimulation
52 frequencies (data not shown).
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7 Addition of ATP caused a significant reduction of the $[Ca^{2+}]_v$ amplitude to 2 Hz and 16 Hz (Fig.
8
9 4D). In contrast to what was seen with Calcium Green AM loaded arteries, ATP did not increase
10
11 baseline $[Ca^{2+}]_v$ (Fig. 2, bar labeled Oregon Green).
12
13
14

15 16 *Effect of pharmacological interventions on $[Ca^{2+}]_v$, noradrenaline release, and force*

17
18 We assessed whether presynaptic pharmacological intervention modified NA release and force via
19
20 an effect on $[Ca^{2+}]_v$. Fig. 5 shows traces from an experiment where NA release and force were
21
22 assessed. Fig. 6 shows the mean values for the frequency-response curves for $[Ca^{2+}]_v$, NA release,
23
24 and force. Based on these data it was decided to concentrate on stimulation with 2 Hz and 16 Hz.
25
26 The drugs used in these experiments did not affect $[Ca^{2+}]_v$, NA, or force under baseline conditions
27
28 (i.e. before the stimulation).
29
30
31
32
33

34 *Ca^{2+} channel inhibition with ω -conotoxin GVIA.*

35
36 The effect of ω -conotoxin GVIA on $[Ca^{2+}]_v$ was frequency-dependent and a larger proportion of
37
38 the $[Ca^{2+}]_v$ response was inhibited at low frequencies compared to higher frequencies (Fig. 7A)
39
40 similarly to what we observed for $[Ca^{2+}]_i$ after Calcium Green AM loading. To assess whether 100
41
42 nM ω -conotoxin GVIA was a supramaximal concentration, inhibition of $[Ca^{2+}]_v$ transient with 100
43
44 nM and 300 nM ω -conotoxin GVIA was compared. No additional effect of 300 nM ω -conotoxin
45
46 GVIA was seen (the response remaining to 16 Hz stimulation was $74\pm 8\%$ and $71\pm 7\%$ in the
47
48 presence of 100 and 300 nM ω -conotoxin GVIA, respectively, $n=4$, n.s. paired Student t-test). In
49
50 contrast 100 nM ω -conotoxin GVIA inhibited more than 80% of the NA release and force
51
52 development (Fig. 7B and 7C) while having no effect on force development to exogenously applied
53
54 NA (Fig. 8).
55
56
57
58
59
60

K⁺ channel inhibition.

In the presence of 10 μM 4-AP, the field stimulation elicited $[\text{Ca}^{2+}]_v$ transient, NA release and force development all increased (Fig. 7), while 10 μM 4-AP had no effect on force development to exogenously applied NA (Fig. 8). In the presence of ω -conotoxin GVIA, 4-AP still increased the responses (Fig. 7). To assess whether 10 μM 4-AP was sufficient to cause full effect, NA release and force at 16 Hz was assessed with 10 μM , 100 μM , and 200 μM 4-AP. The potentiation elicited with 100 μM 4-AP was greater than elicited with 10 μM 4-AP; with 200 μM 4-AP no further potentiation of NA release and force was seen (Fig. 9). The effect of 4-AP on tension development to 16 Hz was similar to the effect on NA release. To further characterize the K^+ -channels affected by 4-AP, the effects of charybdotoxin, TEA, and dendrotoxin were assessed. Charybdotoxin (100 nM) had no effect on NA release (Fig. 9B) but potentiated the tension response to 16 Hz. TEA (0.5 mM in the presence of charybdotoxin) increased the release of NA to the same extent as 100 μM 4-AP. TEA at 1 and 2 mM caused a further increase in NA release. The effects of TEA on the tension response to 16 Hz was similar to the effect on NA release. Dendrotoxin (0.1 μM) had no effect on field stimulation-induced force generation (2 Hz: $0.31 \pm 0.07 \text{ Nm}^{-1}$ and $0.26 \pm 0.05 \text{ Nm}^{-1}$ and 16 Hz: $2.71 \pm 0.41 \text{ Nm}^{-1}$ and $2.19 \pm 0.36 \text{ Nm}^{-1}$, control and dendrotoxin, respectively ($n=8$ and 8)). **These experiments were made with 5.9 mM extracellular K^+ . We also determined the effect of 10 μM 4-AP and 0.1 μM dendrotoxin at 3 mM K^+ . Also at the lower K^+ concentration 4-AP increased the NA release to 16 Hz field stimulation ($293 \pm 49 \%$, $n=3$), while dendrotoxin was without effect ($126 \pm 28\%$, $n=3$).**

α -Adrenoceptor inhibition with yohimbine.

In the presence of yohimbine (1 μM) $[\text{Ca}^{2+}]_v$ and NA release increased (Fig. 7A and 7B) but force development was inhibited (Fig. 7C). In the presence of ω -conotoxin GVIA, yohimbine had no

1
2
3
4 effect on $[Ca^{2+}]_v$ transients and NA release but did inhibit the force response at 16 Hz (Fig. 7). In
5
6 the presence of 200 μ M 4-AP or 2 mM TEA, yohimbine had no effect on NA release (Fig. 9) or
7
8 tension response to 16 Hz. Yohimbine caused a rightward-shift of the concentration-force curve to
9
10 exogenously applied NA (Fig. 8).
11
12
13
14

15 *ACh receptor activation with ACh.*

16
17 ACh (20 μ M) caused inhibition of $[Ca^{2+}]_v$, NA release and tension at both 2 Hz and 16 Hz (Fig. 7).
18
19 In the presence of ω -conotoxin GVIA, ACh caused a further inhibition of $[Ca^{2+}]_v$, NA release and
20
21 tension (Fig. 7). ACh caused a rightward shift of the concentration-force curve to exogenously
22
23 applied NA (Fig. 8). The effects of ACh on NA release and force were prevented by application of
24
25 1 μ M atropine ($n=4$, data not shown).
26
27
28
29
30
31

32 **DISCUSSION**

33
34
35
36 Despite its critical importance for control of vascular tone no direct measurements have been made
37
38 of the intravascular signaling that leads to transmitter release or modifies transmitter release
39
40 following depolarization of vascular sympathetic nerves. In this study we assessed Ca^{2+} transients in
41
42 varicosities using a fluorescent dye technique that specifically loads axons of perivascular nerves.
43
44 Using a different protocol for dye loading we also assessed Ca^{2+} transients in nerve fibers which
45
46 include axons and Schwann cells.
47
48
49
50
51

52 In one approach, we applied Calcium Green-AM, which is expected to load both Schwann cells and
53
54 axons. In the second approach, we exposed the cut vessel ends to Oregon Green 488 BAPTA-1
55
56 dextran. The latter technique exploits an axonal transport system present in nerves to transport the
57
58
59
60

1
2
3
4 dye to downstream axons that have not been in direct contact with the dye. Images in the
5
6 downstream areas therefore reflect calcium in axons only. This was confirmed by several
7
8 observations. Calcium Green-AM loading led to images of relatively thick nerves with a fuzzy
9
10 appearance and with characteristic areas of high fluorescence intensity in areas where nerve fibers
11
12 crossed. This contrasts with the sharply defined, thin fibers with distinct varicosities following
13
14 Oregon Green 488 BAPTA-1 dextran loading. Staining with the nuclear dye Syto16 revealed that
15
16 the areas of high Calcium Green fluorescence intensity often were **contained** nuclei. As cell nuclei
17
18 present in nerve fibers are Schwann cell nuclei, our observations suggest that Schwann cell somas
19
20 often locate to areas where nerve fibers cross. It will be relevant in further ultrastructural and
21
22 stereological studies to test this conclusion. Additional evidence that the areas with high dye
23
24 intensity are Schwann cells comes from the finding that ATP causes a substantial increase in $[Ca^{2+}]_i$
25
26 in these areas while field stimulation causes only small increases in $[Ca^{2+}]_i$. The response to ATP
27
28 confirms that the limited response to field stimulation in these areas is not due to dye saturation.
29
30 Notably, ATP did not increase fluorescence when axons were loaded with Oregon Green 488
31
32 BAPTA-1 dextran. Schwann cells in the sympathetic nerves respond to ATP with an increase of
33
34 $[Ca^{2+}]_i$ ^{6,7}, so our data strongly suggests that Schwann cells take up Calcium Green-AM and that
35
36 $[Ca^{2+}]_i$ changes following intervention reflect Schwann cell function. We suggest that Schwann cell
37
38 pharmacology can be assessed by imaging these areas following Calcium Green-AM loading.
39
40
41
42
43
44
45
46
47

48 The Calcium Green-AM loaded nerves fibers outside the areas where nerve fibers cross responded
49
50 with an increase of $[Ca^{2+}]_i$ to field stimulation in a frequency-dependent manner. Several
51
52 observations suggest that these responses are dominated by the axons; 1) the responses were
53
54 reminiscent of the response in the Oregon Green 488 BAPTA-1 dextran loaded arteries with minor
55
56 quantitative differences (i.e. a slower transient), 2) the responses to electrical stimulation were small
57
58
59
60

1
2
3
4 in the areas of the putative Schwann cell body, 3) the N-type calcium channel blocker ω -conotoxin
5
6 GVIA had similar effects in Oregon Green 488 BAPTA-1 dextran and Calcium Green-AM loaded
7
8 arteries, 4) blockers of L-type and T-type calcium channels, which are expressed in Schwann cells⁸
9
10 were without effect in the Calcium Green-AM loaded arteries, and 5) the responses were TTX-
11
12 sensitive.
13
14

15
16
17
18 Following Oregon Green 488 BAPTA-1 dextran loading the axons responded with a frequency-
19
20 dependent increase of $[Ca^{2+}]_i$. Both $[Ca^{2+}]_i$ in the intervaricose segments and $[Ca^{2+}]_v$ increased to
21
22 field stimulation and the responses were fully inhibited with TTX, confirming their expected
23
24 dependence on TTX-sensitive voltage-gated Na^+ channels.
25
26

27
28
29 N-type Ca^{2+} channel inhibition only partly reduced the Ca^{2+} increase in the axons. Even at low
30
31 stimulation frequencies, when ω -conotoxin GVIA was most effective, a substantial part of the field
32
33 stimulation-induced $[Ca^{2+}]_v$ increase remained. We routinely used 100 nM ω -conotoxin GVIA.
34
35 Application of 300 nM did not cause any further inhibition of $[Ca^{2+}]_v$ indicating that the remaining
36
37 $[Ca^{2+}]_v$ increase is unlikely to be via incompletely blocked N-type Ca^{2+} channels. A similar inability
38
39 of ω -conotoxin GVIA to fully block the $[Ca^{2+}]_v$ increase to field stimulation has been reported for
40
41 vas deferens^{2,4}. We do not know which pathway is important for the remaining $[Ca^{2+}]_v$ increase.
42
43 Given that it was not blocked with nifedipine and mibefradil it is unlikely to be mediated by L-type
44
45 and T-type calcium channels. In the vas deferens, Brain and Bennett (1997) found that the $[Ca^{2+}]_v$
46
47 increase remaining after ω -conotoxin GVIA was also insensitive to 100 nM of the P/Q-type Ca^{2+}
48
49 channel blocker, ω -agatoxin TK. It will be important to determine the source of Ca^{2+} responsible
50
51 for the ω -conotoxin GVIA -insensitive increase of $[Ca^{2+}]_v$ and determine its physiological
52
53 importance. In contrast to the effect of 100 nM ω -conotoxin GVIA on Ca^{2+} , 100 nM ω -conotoxin
54
55
56
57
58
59
60

1
2
3
4 GVIA almost completely inhibited NA release and force development. This could suggest a steep
5
6 relationship between $[Ca^{2+}]_v$ and NA release. Against this possibility is the finding that in the
7
8 presence of 4-AP and ω -conotoxin GVIA $[Ca^{2+}]_v$ is very close to normal levels while NA release is
9
10 substantially reduced. The alternative possibility, that a substantial part of the Ca^{2+} increase in the
11
12 varicosities during field stimulation is irrelevant for NA release, is therefore more likely.

13
14
15 The voltage-gated K^+ -channel inhibitor 4-AP is reported to increase transmitter release in the
16
17 vascular wall ^{9,10}. 4-AP is expected to modify the action potential and consequently enhance the
18
19 $[Ca^{2+}]_v$ increase; this has been observed with 10 mM 4-AP in the vas deferens ⁴. We found in the
20
21 vascular wall that 10 μ M 4-AP led to a significant increase of $[Ca^{2+}]_v$ associated with an increase of
22
23 NA release and force development. 4-AP not only increased the ω -conotoxin GVIA-sensitive
24
25 component but also the ω -conotoxin GVIA-insensitive component, which suggests that the ω -
26
27 conotoxin GVIA-insensitive $[Ca^{2+}]_v$ increase is controlled by the membrane potential and at least in
28
29 part can contribute to NA release. At 10 μ M, 4-AP had, as expected, no effect on the force
30
31 development to exogenously applied NA, and the increased tension during field stimulation can be
32
33 explained by the effect on NA release. The effect of 10 μ M 4-AP on $[Ca^{2+}]_v$, NA release and force
34
35 development is consistent with the finding that 10 μ M 4-AP also increases ³H-noradrenaline
36
37 overflow from the rabbit tail artery ¹⁰ and that 20 μ M 4-AP blocks the endothelin-mediated
38
39 inhibition of NA release from gastric nerves ¹¹. Since 100 μ M 4-AP produced a maximal effect, the
40
41 $K_V3.1$ channel, which is the only channel with an IC_{50} for 4-AP below 100 μ M ^{12,13}, is the most
42
43 likely channel candidate. K_V1 channels are suggested to be important in varicosities in vas deferens
44
45 ⁴, but K_V1 channels have an IC_{50} for 4-AP of ≥ 160 μ M ¹³ and are therefore less likely candidates in
46
47 perivascular nerves. To further test the potential role of K_V1 we used 100 nM dendrotoxin, which
48
49 effectively blocks K_V1 channels ¹³. The lack of effect of dendrotoxin further supports $K_V3.1$
50
51 channels as the most likely candidate. The effect of 4-AP and lack of effect of dendrotoxin was seen
52
53
54
55
56
57
58
59
60

1
2
3
4 at 5.9 mM extracellular K^+ as well as the 3 mM K^+ which is a more physiological K^+ concentration.
5
6 $K_v3.1$ channels are sensitive to TEA at sub-mM concentrations ¹³. Our finding that 0.5 mM TEA
7
8 enhanced NA release and force development to the same extent as 200 μ M 4-AP further supports a
9
10 role for $K_v3.1$ channels. However, since 1 and 2 mM TEA had additional effects on NA release and
11
12 force development, other K^+ -channels are likely to be important in the vascular varicosity: these are
13
14 unlikely to be BK_{Ca} channels since charybdotoxin was without effect.
15
16
17
18
19

20 Several presynaptic receptors modify transmitter release, although their mechanisms of action are
21
22 not clear. We have assessed the signaling from cholinergic, adrenergic, and purinergic receptors.
23
24 ACh caused a modest reduction of $[Ca^{2+}]_v$, particularly at 16 Hz, but a strong inhibition of NA
25
26 release and force, mediated by muscarinic receptors. These effects were reminiscent of the effect of
27
28 ω -conotoxin GVIA, and it seems likely that ACh works through inhibition of ω -conotoxin GVIA-
29
30 sensitive Ca^{2+} influx. However, ACh added in the presence of ω -conotoxin GVIA caused a further
31
32 reduction of $[Ca^{2+}]_v$, suggesting that ACh may also partly decrease the ω -conotoxin GVIA-
33
34 insensitive increase of $[Ca^{2+}]_v$. The data reinforces the possibility that NA release can be modified
35
36 through mechanisms independent of ω -conotoxin GVIA-sensitive Ca^{2+} influx. As expected, ACh
37
38 also reduced force to exogenously applied NA, probably via an endothelium dependent mechanism.
39
40 Therefore, the effect of ACh on field stimulation induced force development is difficult to interpret.
41
42
43 Yohimbine, which is predominantly an α_2 -adrenoceptor antagonist, increased field stimulation-
44
45 induced $[Ca^{2+}]_v$ increase and NA release, suggesting that presynaptic α_2 -adrenoceptor-mediated
46
47 negative feedback of NA release signals through reduction of $[Ca^{2+}]_v$. Furthermore, since
48
49 yohimbine had no effect on NA release in the presence of 4-AP or TEA, the presynaptic α_2 -
50
51 adrenoceptors most likely inhibits NA release via activation of $K_v3.1$ channels. Despite the
52
53 yohimbine induced potentiation of field stimulation-induced increase of $[Ca^{2+}]_v$ and NA release,
54
55
56
57
58
59
60

1
2
3
4 yohimbine inhibited force development. Yohimbine also reduced the sensitivity to exogenously
5
6 applied NA. This is despite the fact that smooth muscle cells of rat mesenteric small arteries only
7
8 have α_1 -adrenoceptors ¹⁴, suggesting that 1 μ M yohimbine may also inhibits α_1 -adrenoceptors in
9
10 this preparation ¹⁵. We also found an inhibitory effect of ATP on the $[Ca^{2+}]_v$ increase to field
11
12 stimulation. It will be of interest to determine whether this is mediated via presynaptic P2Y
13
14 receptors, as suggested for the sympathetic terminals of the mouse vas deferens ⁵.
15
16
17
18
19

20 *Conclusion*

21
22 In this study we have made a pharmacological characterization of Ca^{2+} homeostasis in varicosities
23
24 and Schwann cells in the vascular wall and compared this to NA release and force development. We
25
26 confirmed that Schwann cells respond with a $[Ca^{2+}]_i$ increase to ATP. This contrast with
27
28 varicosities, where a decrease of the $[Ca^{2+}]_v$ transient to field stimulation is seen. Our data further
29
30 suggests that a large part of the electrical field induced increase of $[Ca^{2+}]_v$ is irrelevant for NA
31
32 release. The pharmacological analysis suggests that $K_v3.1$ is an important regulator of $[Ca^{2+}]_v$, NA
33
34 release and force and suggest that both presynaptic adrenoceptors and muscarinic receptors modify
35
36 transmitter release through effects on $[Ca^{2+}]_v$.
37
38
39
40
41
42

43 **MATERIALS AND METHODS**

44
45
46
47
48 *Animals.* Male Wistar rats (16-19 weeks) were used. The investigation conforms to the Guide for
49
50 the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH
51
52 Publication No. 85-23, revised 1996) as well as the guidelines from Directive 2010/63/EU of the
53
54 European Parliament and Danish national guidelines for animal research. Rats were anesthetized
55
56
57
58
59
60

1
2
3
4 with CO₂ inhalation and killed by decapitation. Branches (2nd and 3rd order) of the mesenteric artery
5
6 were dissected out; the arteries had inner diameters of 200-250 μm.
7
8
9

10
11 *Mounting and normalization of artery segments in myographs for isometric force recordings.* For
12
13 Ca²⁺ imaging 2-mm-long artery segments were mounted as rings in a myograph (360CW, Danish
14
15 Myo Technology A/S, Denmark) for simultaneous recording of isometric force and confocal
16
17 imaging as described previously ¹⁶. Force transducer readings were recorded at 100 Hz using
18
19 PowerLab and LabChart (ADInstruments, New Zealand).
20
21
22

23
24 A passive tension-length curve was obtained and the arteries set, based on the passive tension-
25
26 length curve, to a value where near-maximal active force is developed ¹⁷. After equilibration in
27
28 physiological salt solution (PSS; for composition see below) at 37°C for about 1 hour, the arteries
29
30 were activated twice with 10 μM NA in PSS. The relaxed artery was then stimulated after one of
31
32 several protocols. The basis for the protocols was a series of square-wave field stimulations from
33
34 platinum electrodes placed in the mounting heads of the myograph as described previously ¹⁸.
35
36 Stimulations were with electrical pulses with pulse width between 0.2 and 0.4 ms and a current
37
38 strength of 40 mA. The pulses had frequencies from 0.5 to 16 Hz and were run for 3 s at each
39
40 frequency. **These frequencies were chosen because 1-2 Hz is the physiologically most relevant**
41
42 **frequency¹⁹ and 16 Hz gives the maximal nerve-specific activation in this preparation²⁰.** An interval
43
44 of 3 minutes was used between the different frequencies. In some protocols the arteries were
45
46 stimulated only at 2 Hz and/or 16 Hz each for 3 s but otherwise using the same stimulation
47
48 parameters. The arteries were incubated with drugs for 15 minutes before the electrical field
49
50 stimulations were initiated. The drugs were used in the following concentrations: ω-conotoxin
51
52 GVIA (CTX) 100 nM and 300 nM, 4-aminopyridine (4-AP) 10 μM, 100 μM, and 200 μM;
53
54
55
56
57
58
59
60

1
2
3
4 tetraethylammonium (TEA) 0.5 mM, 1 mM, and 2 mM; tetrodotoxin (TTX) 0.1 μ M; dendrotoxin
5 0.1 μ M; charybdotoxin 0.1 μ M; yohimbine 1 μ M; ACh 20 μ M and cocaine 3 μ M. In some
6
7
8 experiments, exogenous NA and ATP effects were assessed.
9

10
11
12
13 *Loading of nerve fibers.* After mounting and normalization of an artery segment in a myograph the
14 artery was incubated with 5 μ M of the Ca^{2+} -sensitive dye Calcium Green-1-AM in PSS for two
15
16
17 times 1.5 hours at 37°C.
18
19

20
21
22
23 *Axon-specific loading.* A 3-4 mm long artery segment was dissected. The proximal end of the artery
24 was secured in a glass suction pipette filled with phosphate-free PSS (for composition see below)
25 containing 43 mM Oregon Green 488 BAPTA-1 10 kDa dextran. The distal part of the artery was
26
27
28 kept in PSS. The solution remained at room temperature and the segments were loaded for 5-6
29
30
31 hours. Thereafter the artery was released from the glass suction pipette and maintained in PSS for a
32
33
34 minimum of 3 hours at room temperature, after which the segment was mounted in a myograph as
35
36
37 described above.
38
39

40
41 *Imaging.* Imaging of Ca^{2+} in axons and nerve fibers was made with either a Noran Odyssey
42
43
44 confocal unit mounted on a Nikon Inverted Microscope Eclipse TE2000-U with a 60 \times , water
45
46
47 dipping objective or a Zeiss Axiovert 200M confocal microscope with a 40 \times water dipping
48
49
50 objective. The excitation was from the 488 nm line of an argon laser, a dichroic mirror of 505 nm
51
52
53 was used and on the emission side a bandpass filter of 515-555 nm was used. Time resolution was
54
55
56
57
58
59
60 at least 4 images per second.

1
2
3
4 During Ca^{2+} imaging the PSS contained either 3 μM wortmannin or 1 μM α,β -mATP and 0.2 μM
5
6 prazosin which reduced contractions to field stimulation to less than 10% and consequently reduced
7
8 movement artifacts. In these experiments 3 μM cocaine was present to inhibit NA uptake into the
9
10 varicosities.
11
12

13
14
15
16 *Loading and imaging cell nuclei with Syto16.* To label nuclei the arteries mounted in the myograph
17
18 were incubated with 1 μM Syto16 in PSS for 10 min at 37°C. After a wash in PSS, the arteries were
19
20 imaged with the Zeiss confocal microscope with the settings used for Ca^{2+} imaging.
21
22

23
24
25 *Measurement of NA release.* In artery segments mounted in myographs for isometric force
26
27 recording, NA release was measured using a MicroC™ Picoammeter/Potentiostat (WPI) with
28
29 nafion coated carbon fiber electrodes (diameter 30 μm ; length 1 mm), across which a potential of
30
31 0.4 V was applied. The fiber electrodes were activated before use in accordance with the
32
33 instructions from the company. After mounting the arteries in the myograph, the mounting heads
34
35 and the artery were rotated to place the artery in a vertical position. Using a micromanipulator, the
36
37 electrode was placed alongside the artery, with the electrode gently touching the artery as judged
38
39 through the microscope. An Ag/AgCl electrode was used as reference electrode. Cocaine (3 μM)
40
41 was present in all experiments. The signal was recorded simultaneously with force at 100 Hz. For
42
43 calibration of the signal, NA was applied to the myograph chamber in increasing concentrations
44
45 without an artery mounted in the myograph.
46
47
48
49
50

51
52
53 *Solutions.* PSS contained (in mmol L^{-1}): NaCl 119, KCl 4.7, KH_2PO_4 1.18, MgSO_4 1.17, NaHCO_3
54
55 25, CaCl_2 1.6, EDTA 0.026, glucose 5.5, was gassed with 5% CO_2 in air or 5% $\text{CO}_2/95\% \text{O}_2$
56
57 (during Oregon Green BAPTA-1 dextran loading) and had a pH of 7.4. In phosphate-free PSS
58
59
60

1
2
3
4 KH₂PO₄ was omitted. In low K⁺ solution KCl was reduced to 1.82 mM. ω-conotoxin GVIA was
5
6 obtained from Alomone Labs (Jerusalem, Israel); 4-AP, yohimbine, NA, ACh, ATP, α,β-methylene
7
8 ATP, TTX, prazosin, dendrotoxin, and charybdotoxin, and TEA were obtained from Sigma; cocaine
9
10 was from Aarhus University Hospital. The Ca²⁺-sensitive dyes and Syto16 were obtained from
11
12 Molecular Probes. Drug nomenclature is consistent with the Concise Guide to Pharmacology ²¹.
13
14 During experiments the PSS was kept at 37°C.
15
16
17
18
19

20 *Calculations and statistics.*

21
22 Fluorescence in regions of interest (ROIs) placed in nerve fibers was used for analysis in the
23
24 Calcium Green-1-AM loaded arteries (5 to 10 ROIs per preparation) (Fig. 1A). In Oregon Green
25
26 488 BAPTA-1 dextran-loaded arteries ROIs encompassed single varicosities or intervaricose
27
28 segments (5 to 10 ROIs per preparation).
29
30
31

32
33
34 Fluorescence intensity for ROIs was calculated as mean values of an interval of 3 s immediately
35
36 before activation and for the last 1 s of the activation. The mean intensity from a ROI was obtained
37
38 on all images of the stimulation series. Any movement of the ROI during the stimulation was
39
40 corrected for by an automated procedure, written in MatLab, which tracked the varicosity between
41
42 every frame based on the strongest local fluorescence signal compared with the background.
43
44 Background values were obtained from ROIs immediately outside the nerves and subtracted from
45
46 the values in the nerve specific ROIs.
47
48
49

50
51
52 For each ROI, values during activation were expressed as the percentage of the value before
53
54 activation, and the data for all ROIs in the image were averaged. No calibration of the fluorescence
55
56 was made. The data obtained in the presence of CTX, 4-AP, yohimbine or ACh are expressed as the
57
58
59
60

percentage of the control values (i.e. without the drug) for individual ROIs and then averaged for the preparation.

The analysis of NA concentration and force was performed in a similar manner, i.e. by obtaining a baseline value in a 3 s period before activation and the value during the last 1 s of the activation.

The effects of drugs are expressed as percent of the baseline values.

Statistical analyses were made with Student's paired or unpaired *t*-test, two-way ANOVA, or extra sum-of-squares *F*-test as indicated. Data were expressed as mean±SEM, *n* is the number of arteries (one artery per rat) and *p*<0.05 was considered significant.

ACKNOWLEDGEMENT

This work was supported by the Novo Nordic Foundation (# 11789 to C.A), The Danish Heart Foundation no (R97-A5232 to C.A.), Russian Foundation for Basic Research (#16-44-730649 to O.S.T.).

CONFLICT OF INTEREST: None declared.

REFERENCES

1. Luff SE. Ultrastructure of sympathetic axons and their structural relationship with vascular smooth muscle. *Anat Embryol (Berl)*. 1996;193(6):515-531.
2. Brain KL, Bennett MR. Calcium in sympathetic varicosities of mouse vas deferens during facilitation, augmentation and autoinhibition. *J Physiol*. 1997;502 (Pt 3):521-536.
3. Brain KL, Trout SJ, Jackson VM, Dass N, Cunnane TC. Nicotine induces calcium spikes in single nerve terminal varicosities: a role for intracellular calcium stores. *Neuroscience*. 2001;106(2):395-403.
4. Jackson VM, Trout SJ, Brain KL, Cunnane TC. Characterization of action potential-evoked calcium transients in mouse postganglionic sympathetic axon bundles. *J Physiol*. 2001;537(Pt 1):3-16.
5. O'Connor SC, Brain KL, Bennett MR. Individual sympathetic varicosities possess different sensitivities to alpha 2 and P2 receptor agonists and antagonists in mouse vas deferens. *Br J Pharmacol*. 1999;128(8):1739-1753.

- 1
- 2
- 3
- 4
- 5 6. Lin YQ, Bennett MR. Varicosity-Schwann cell interactions mediated by ATP in the mouse vas deferens. *J Neurophysiol.* 2005;93(5):2787-2796.
- 6 7. Lin YQ, Bennett MR. Schwann cells in rat vascular autonomic nerves activated via purinergic receptors. *Neuroreport.* 2006;17(5):531-535.
- 8 8. Verkhratsky A, Steinhauser C. Ion channels in glial cells. *Brain Res Brain Res Rev.* 2000;32(2-3):380-412.
- 9 9. Msgghina M, Gonon F, Stjärne L. Paired pulse analysis of ATP and noradrenaline release from sympathetic nerves of rat tail artery and mouse vas deferens: effects of K⁺ channel blockers. *British Journal of Pharmacology.* 1998;125(8):1669-1676.
- 10 10. Uhrenholt TR, Nedergaard OA. Calcium channels involved in noradrenaline release from sympathetic neurones in rabbit carotid artery. *Pharmacol Toxicol.* 2003;92(5):226-233.
- 11 11. Nakamura K, Shimizu T, Tanaka K, Taniuchi K, Yokotani K. Involvement of presynaptic voltage-dependent Kv3 channel in endothelin-1-induced inhibition of noradrenaline release from rat gastric sympathetic nerves. *European Journal of Pharmacology.* 2012;694(1-3):98-103.
- 12 12. Judge SIV, Bever Jr CT. Potassium channel blockers in multiple sclerosis: Neuronal Kv channels and effects of symptomatic treatment. *Pharmacology & Therapeutics.* 2006;111(1):224-259.
- 13 13. Coetzee WA, Amarillo Y, Chiu J, et al. Molecular diversity of K⁺ channels. *Annals of the New York Academy of Sciences.* 1999;868:233-285.
- 14 14. Nielsen H, Pilegaard HK, Hasenkam JM, Mortensen FV, Mulvany MJ. Heterogeneity of postjunctional alpha-adrenoceptors in isolated mesenteric resistance arteries from rats, rabbits, pigs, and humans. *J Cardiovasc Pharmacol.* 1991;18(1):4-10.
- 15 15. Artigues-Varin C, Richard V, Varin R, Mulder P, Thuillez C. Alpha2-adrenoceptor ligands inhibit alpha1-adrenoceptor-mediated contraction of isolated rat arteries. *Fundam Clin Pharmacol.* 2002;16(4):281-287.
- 16 16. Peng H, Matchkov V, Ivarsen A, Aalkjaer C, Nilsson H. Hypothesis for the initiation of vasomotion. *Circ Res.* 2001;88(8):810-815.
- 17 17. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res.* 1977;41(1):19-26.
- 18 18. Angus JA, Broughton A, Mulvany MJ. Role of alpha-adrenoceptors in constrictor responses of rat, guinea-pig and rabbit small arteries to neural activation. *J Physiol.* 1988;403:495-510.
- 19 19. Nilsson H, Ljung B, Sjoblom N, Wallin BG. The influence of the sympathetic impulse pattern on contractile responses of rat mesenteric arteries and veins. *Acta Physiol Scand.* 1985;123(3):303-309.
- 20 20. Nilsson H. Different nerve responses in consecutive sections of the arterial system. *Acta Physiol Scand.* 1984;121(4):353-361.
- 21 21. Alexander SP, Kelly E, Marrion N, et al. The Concise Guide to PHARMACOLOGY 2015/16: Overview. *Br J Pharmacol.* 2015;172(24):5729-5743.
- 46
- 47
- 48
- 49
- 50
- 51
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
- 60

LEGENDS TO FIGURES

Fig. 1. A) Loading of nerve fibers in rat mesenteric small arteries with Calcium Green-AM. The single arrow indicates an area of high dye intensity, where nerve fibers cross; the double arrow indicates area of lower dye intensity, which are nerve fibers. The red arrows indicate a smooth muscle cell, which is out of focus. Bar indicates 1 μm . B) Traces (typical of 5 experiments) showing the effect of field stimulation at increasing frequencies (in Hz: 0.5, blue; 1, red; 2, violet 4, green; 16, light blue) on $[\text{Ca}^{2+}]$ in nerve fibers (double arrow in fig. 1A). The insert shows the effect of 0.1 μM tetrodotoxin (TTX) on 8 Hz field stimulation (typical of 3 experiments). C) and D) show two images of the same nerve fibers loaded with Calcium Green-AM before (C) and after (D) staining with Syto16. An area of high Calcium Green intensity where several nerve fibers cross is seen. In 20-30% of these areas, Syto16 gave a strong fluorescence demonstrating the presence of a nucleus. Images are typical of 4 experiments. Bars indicate 1 μm . E) and F) Mean effect ($n = 5$) of field stimulation at 2 Hz and 16 Hz under control conditions (grey bars) and in the presence of ATP (hatched bars). In E nerve fibers (double arrow in fig. 1A), and in F areas where nerves cross (single arrow in fig. 1A) were imaged. There was no significant difference between control and ATP, two-way ANOVA) for both E and F.

Fig. 2. The effect of 10 μM ATP on nerve fibers loaded with Calcium Green-AM (column 1 and 2) or axons loaded with Oregon Green 488 BAPTA-1 dextran. The response of nerve fibers (column 1; double arrow in fig. 1A) or areas with high dye load (column 2; single arrow in fig. 1A) to bath application of ATP was measured. * $p < 0.05$; compared to fluorescence without ATP paired t -test; $n = 5$.

1
2
3
4
5
6
7 Fig. 3. Effect of 100 μM ω -conotoxin GVIA on $[\text{Ca}^{2+}]_v$ in nerves as function of stimulation
8 frequency in Calcium Green-AM loaded arteries. The responses to field stimulation are given as the
9 percentage of the response at respective frequency in the absence of ω -conotoxin GVIA, $n=6$.
10
11
12
13
14

15
16 Fig. 4. A) Image of axons in rat mesenteric small arteries loaded with Oregon Green 488 BAPTA-1
17 dextran; bar indicates 1 μm . B) Traces showing the effect of field stimulation at increasing
18 frequencies (in Hz: 0.5, blue; 1, red; 2, violet 4, green; 16, light blue) on $[\text{Ca}^{2+}]_v$ in Oregon Green
19 488 BAPTA-1 dextran loaded axons. C) Traces showing the effect of a single pulse on Calcium
20 Green-AM loaded nerve fibers (black trace) and Oregon Green 488 BAPTA-1 dextran loaded axons
21 (violet). D) The effect of 10 μM ATP on $[\text{Ca}^{2+}]_v$ transients to field stimulation at 2 and 16 Hz in
22 axons. * $p<0.05$; control vs. ATP two-way ANOVA, $n=5$.
23
24
25
26
27
28
29
30
31
32
33

34 Fig. 5. Traces of noradrenaline release (top) and force (bottom) of rat mesenteric artery to field
35 stimulation with frequencies of 2 Hz and 16 Hz under control conditions and in the presence of 10
36 μM 4-aminopyridine.
37
38
39
40
41
42

43 Fig. 6. The average effect of field stimulation on $[\text{Ca}^{2+}]_v$ (Oregon Green 488 BAPTA-1 dextran) (A,
44 $n=14$), noradrenaline (NA) release (B, $n=16$), and force (C, $n=16$). The data plotted in B and C were
45 recorded simultaneously from the same preparations. Data are given as the percentage of the
46 response at 16 Hz.
47
48
49
50
51
52
53

54 Fig. 7. Effects of 100 nM ω -conotoxin GVIA (CTX), 10 μM 4-aminopyridine (4-AP), 1 μM
55 yohimbine (Yoh), and 20 μM acetylcholine (ACh) on $[\text{Ca}^{2+}]_v$ (Oregon Green 488 BAPTA-1
56
57
58
59
60

1
2
3
4 dextran loaded axons) (A), noradrenaline release (B), and force (C) during 2 and 16 Hz stimulation
5
6 of rat mesenteric small arteries. The hatched bars are the responses in the presence of CTX.

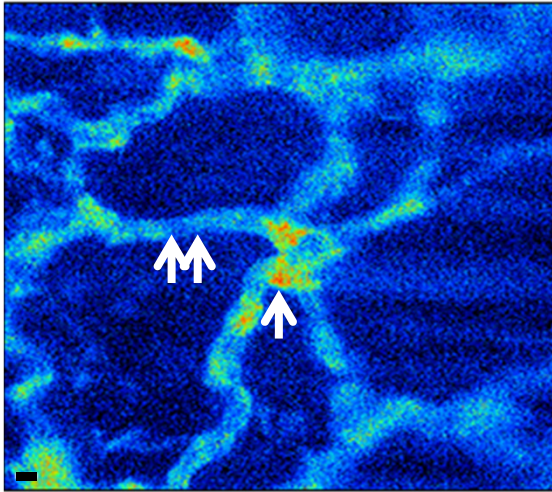
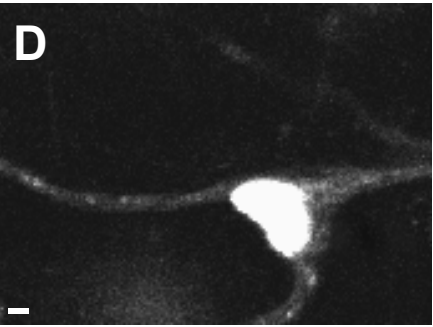
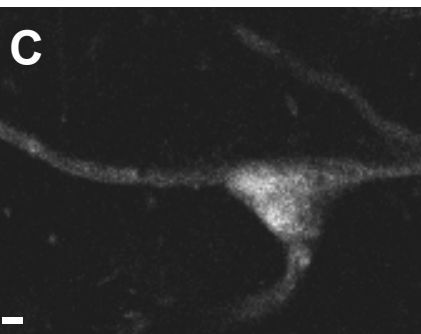
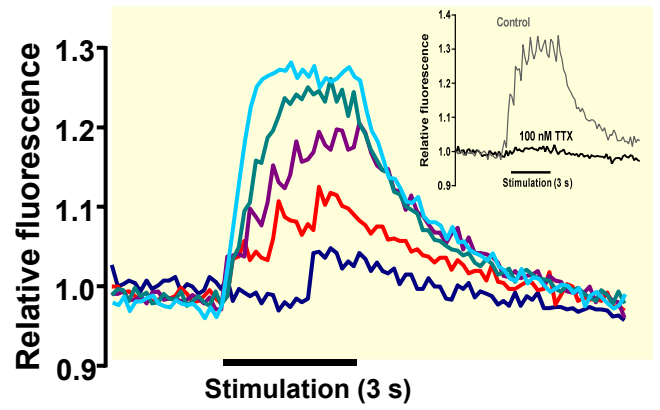
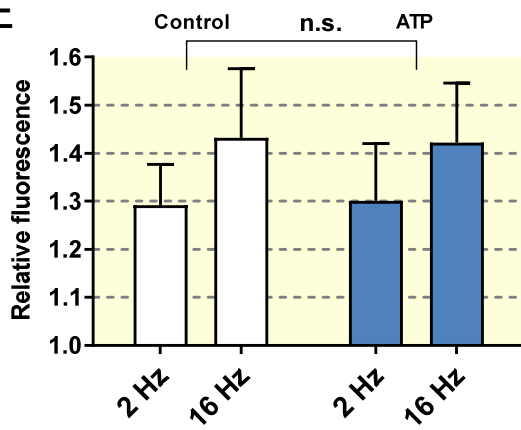
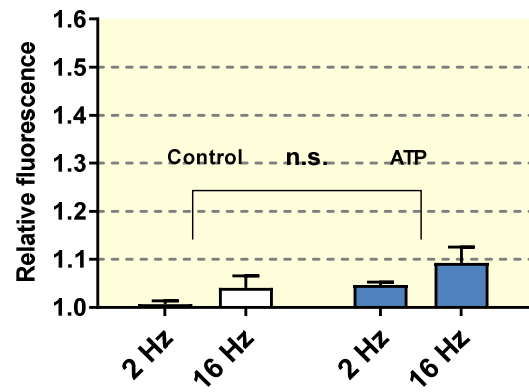
7
8 Responses are given as the percentage of the responses of the same preparations at respective
9
10 frequency in the absence of drugs. * $p < 0.05$; paired t -test; different from 100 % (i.e. significant
11
12 effect of the drug); @ $p < 0.05$; unpaired t -test; different from the response in the presence of CTX
13
14 alone (i.e. effect of the drug in the presence of CTX); $n = 3-8$.
15
16
17
18
19

20
21 Fig. 8. Effects of 100 nM ω -conotoxin GVIA (A, $n = 5$), 10 μ M 4-AP (B, $n = 5$), 1 μ M yohimbine (C,
22
23 $n = 5$) and 20 μ M ACh (D, $n = 5$) on force development to exogenously applied noradrenaline. E
24
25 shows time-control (two consecutive concentration-response relationships obtained from the same
26
27 preparation, $n = 4$). The responses were obtained in the presence of 3 μ M cocaine and given as the
28
29 percentage of the maximum response. * $p < 0.05$; extra sum-of-squares F -test.
30
31
32
33

34
35 Fig. 9. Effect of 4-aminopyridine (4-AP), charybdotoxin, tetraethylammonium (TEA), and
36
37 yohimbine on 16 Hz field stimulation-induced noradrenaline release and tension development. A)
38
39 $n = 10$; B) $n = 8$. The columns represent cumulative addition of the drugs. * $p < 0.05$; paired t -test.
40
41

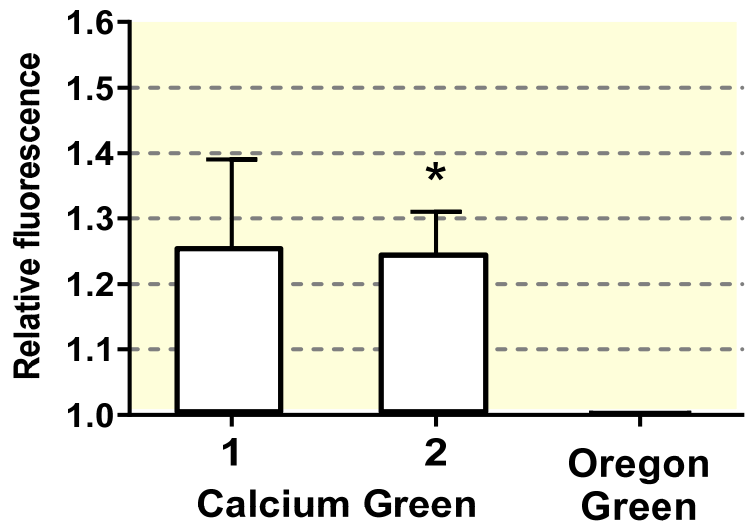
42
43 **Suppl. Fig. 1. Staining of nerve fibers with Calcium Green-AM (A) and glyoxylic acid (B) in rat**
44
45 **mesenteric small arteries. In A cell nuclei are stained with Syto16. The preparation in A is shown as**
46
47 **a 3D structure in Suppl. Video 1. In Suppl. video 1 note that the nucleus marked with a double**
48
49 **arrow in (A) is in the nerve fiber, whereas the nucleus marked with a single arrow in (A) is outside**
50
51 **the nerve fiber. Bar indicates 4 μ m.**
52
53

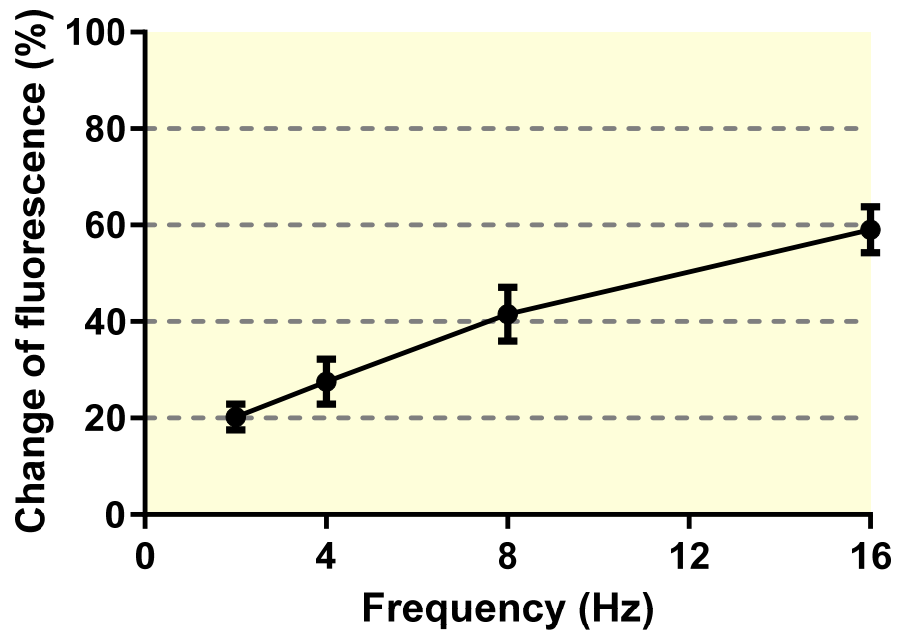
54
55
56 **Suppl. Video 1. A rat mesenteric small artery stained with Calcium Green-AM An image stack is**
57
58 **obtained with confocal microscopy and presented as a 3-D image.**
59
60

A**B****E****F**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

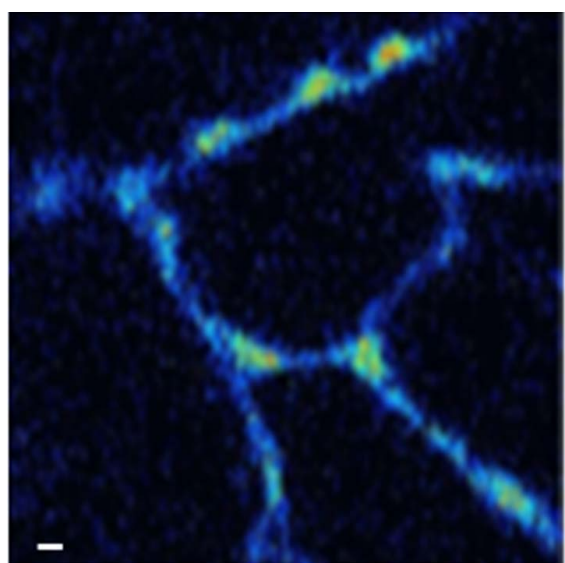




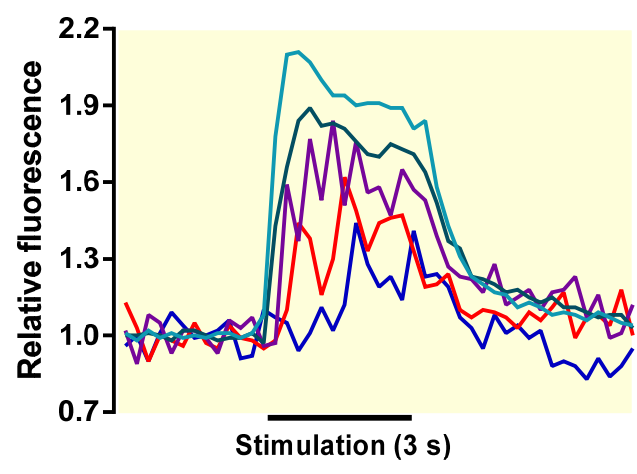
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

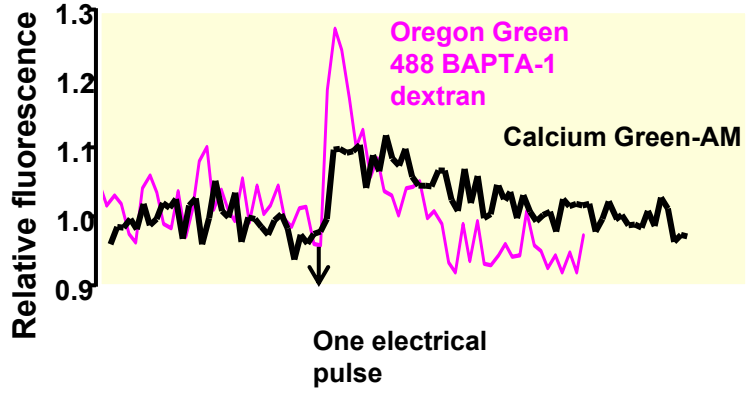
A



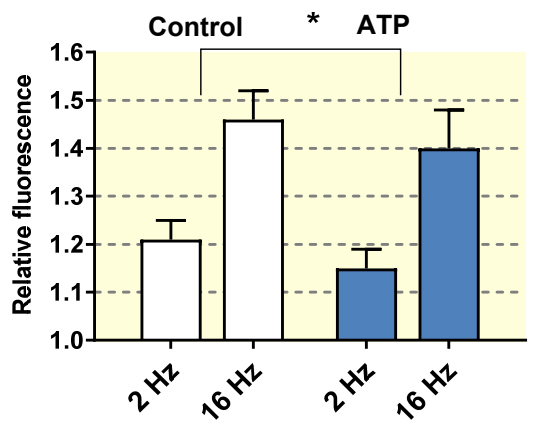
B

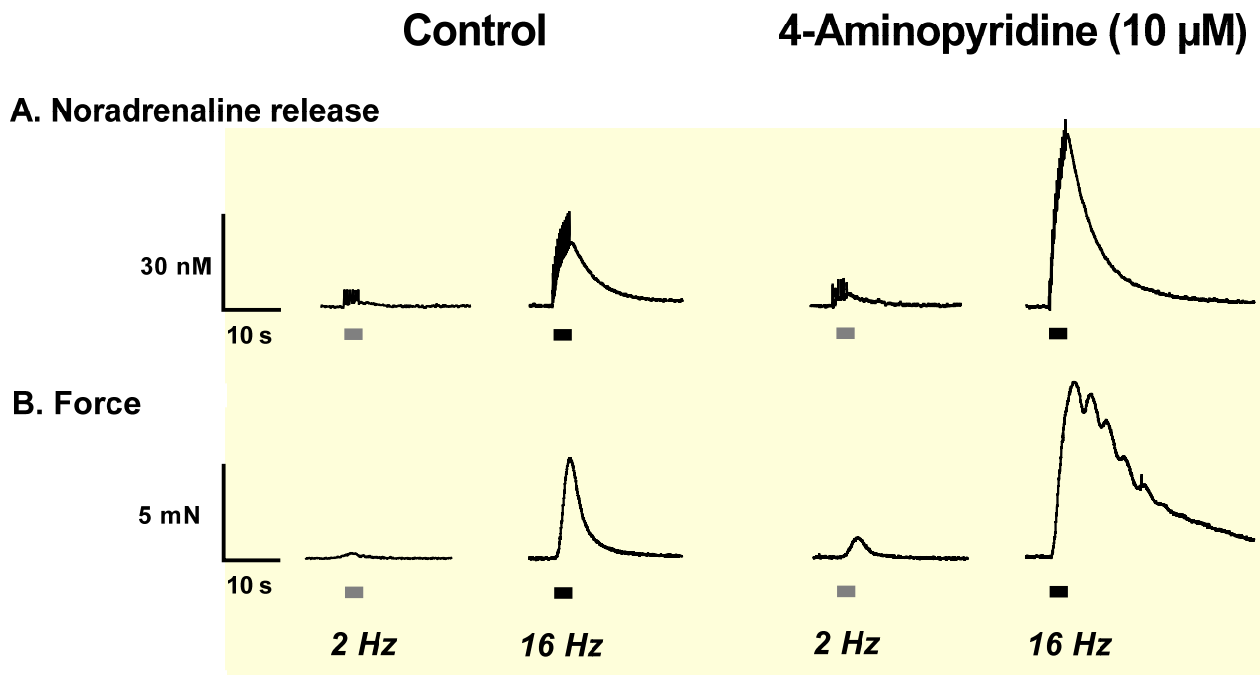


C

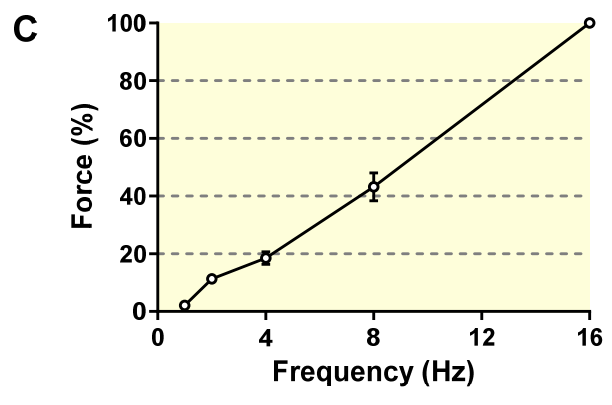
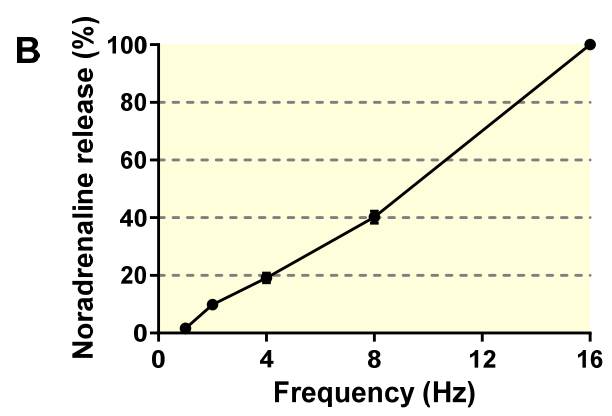
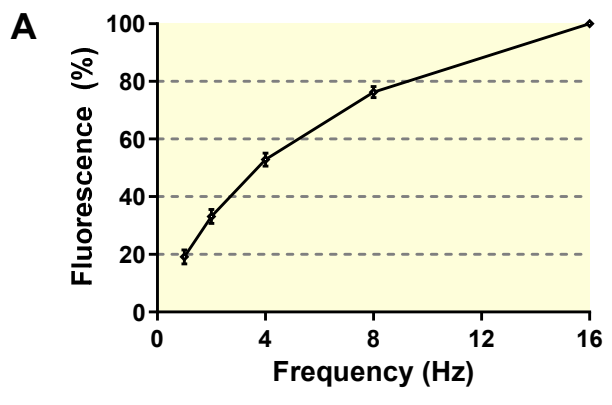


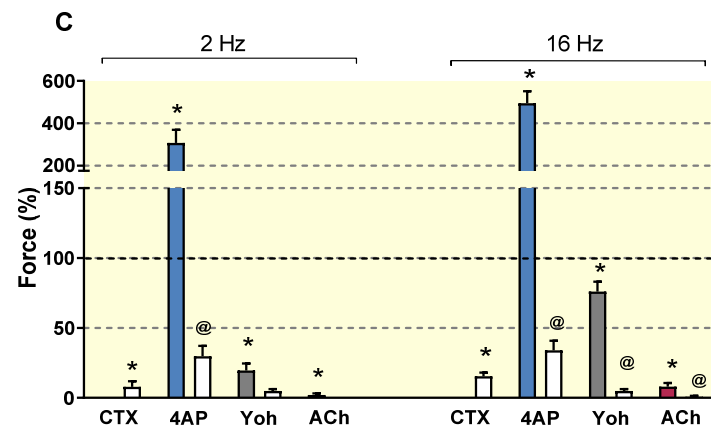
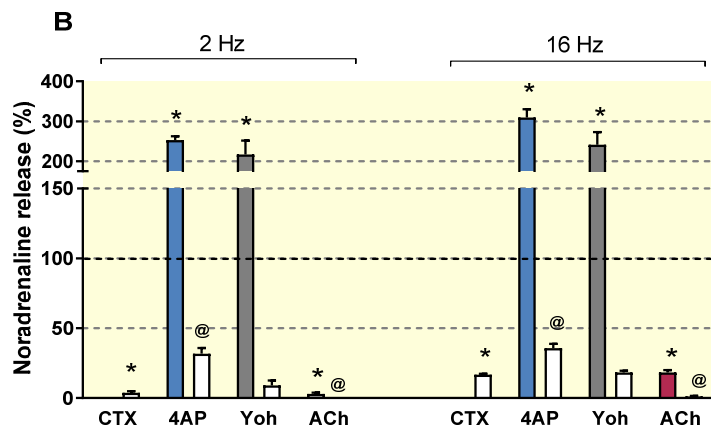
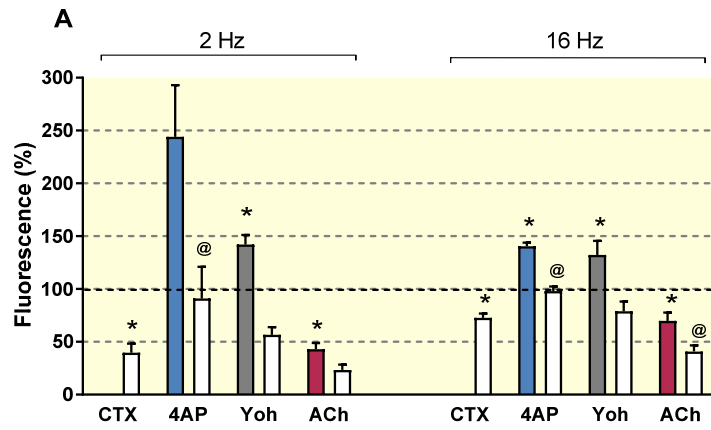
D



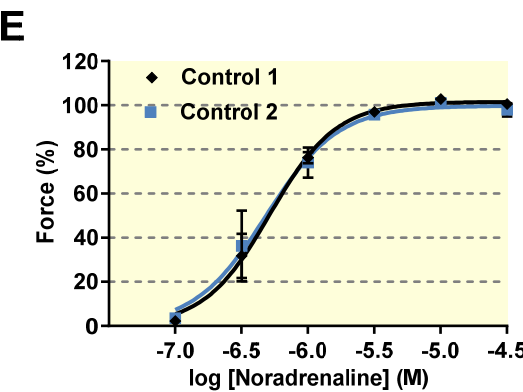
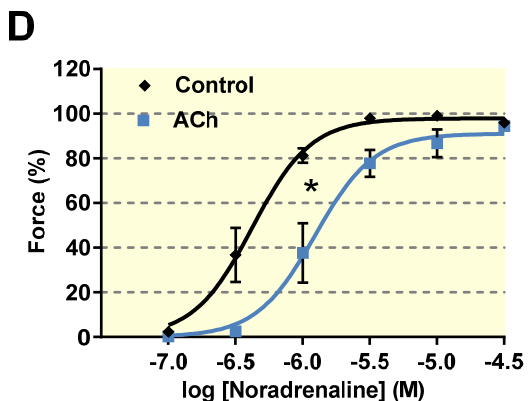
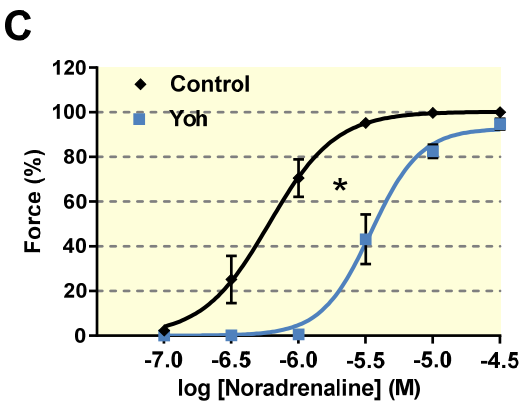
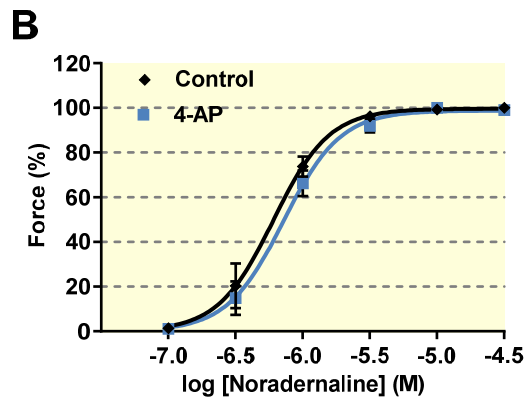
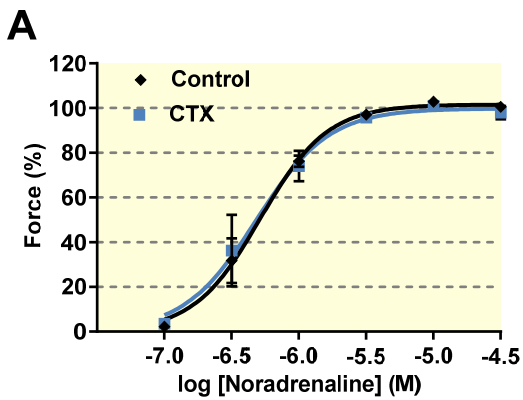


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



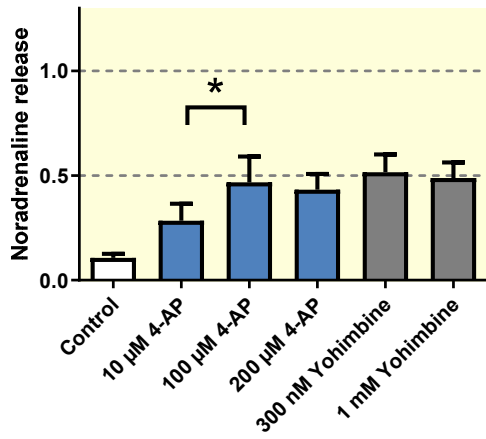


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

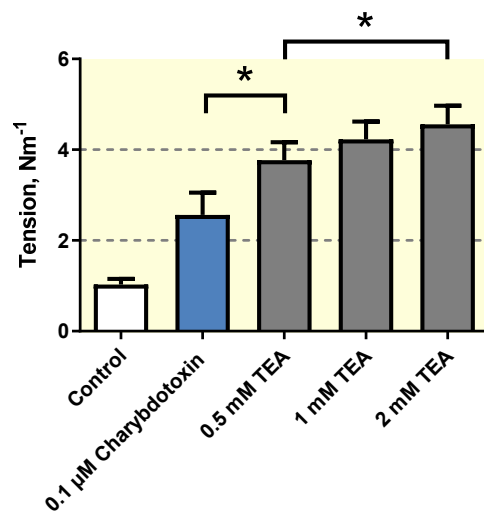
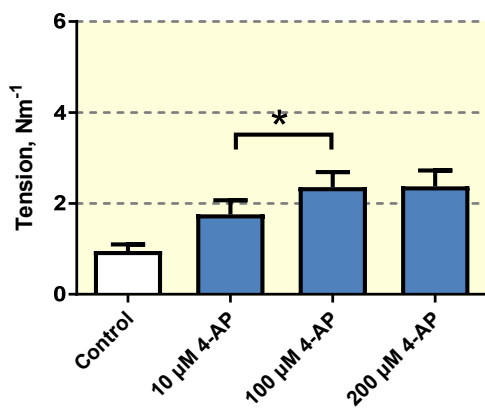
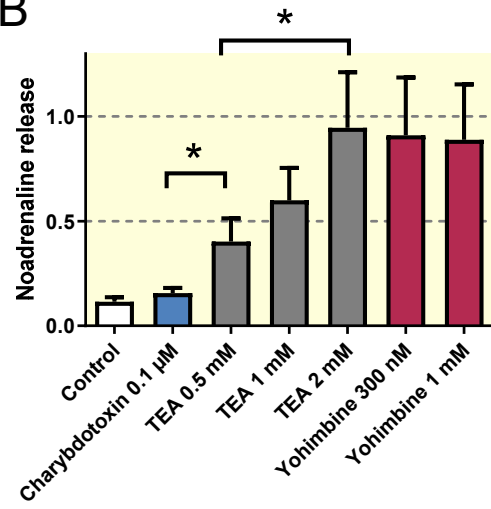


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

A

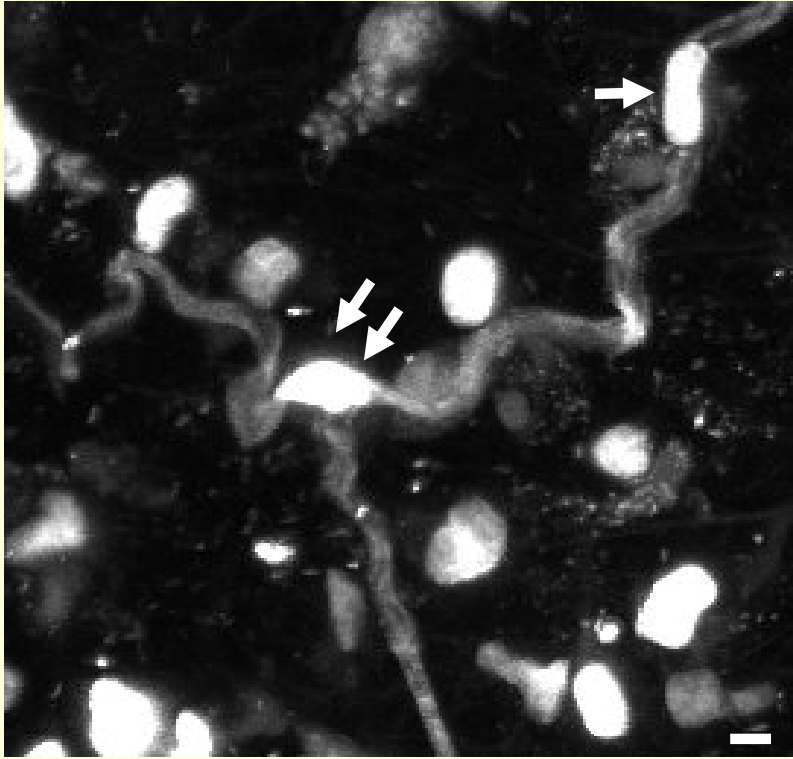


B

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

A



B

