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The effect of 5-HT on the sustained afferent excitability induced by epidural direct current stimulation

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Serotonins effekt på långvarigt ökad excitabilitet i afferenter efter epidural likströmsstimulering

Degree Project in Medicine

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Contents

Abstract

The effect of 5-HT on the sustained afferent excitability induced by epidural direct current spinal stimulation.

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Background – Epidural spinal stimulation (ESS) is a therapeutic method used clinically in patients with spinal cord injury. Epidural cathodal direct current stimulation (DCS) has recently been found to result in an up to sevenfold increase in primary afferent excitability, lasting over an hour. As patients with spinal cord injury often are concomitantly treated with drugs affecting serotonergic systems – and serotonin (5-HT) receptors being known to modulate excitability in spinal pathways – the outcome of DCS might depend on the presence or absence of serotonergic drugs.

Objective – To investigate whether 5-HT, ionophoretically administered in the dorsal column, affects the sustained excitability increase induced in peripheral afferents by epidural cathodal direct current stimulation.

Methods – Acute in vivo experiments were carried out on adult deeply anesthetized rats. Epidural cathodal stimulation (1.0 µA) was applied for 1 minute in lumbar segments at the level of the peroneal and tibial motor nuclei. Antidromic responses were recorded in afferents stimulated just above threshold intensity. 5-HT was administered locally in the dorsal column by ionophoresis (30-60 nA) during 20-30 minutes of the post polarization period.

Results – During DCS, afferent responses increased with on average 407% (95% CI 228%-586%) ($p<0.0001$, n=16, 3 rats), compared to baseline. The effect remained at least 95% (5%-186%) increased ($p=0.0371$, n=16, 3 rats) throughout the post polarization period, without being significantly affected by 5-HT administration ($p=0.9999$).

Conclusions and implications – The results indicate that the sustained effect following ESS is not dependent on serotonergic modulation. Thus, concurrent treatment with serotonergic drugs most likely has no, or very little, interaction with ESS.

Key words: epidural spinal stimulation, direct current, serotonin

Abbreviations

5-HT 5-hydroxytryptamine; serotonin

CPG central pattern generator

DC direct current

DCS direct current stimulation

ESS epidural spinal stimulation

GABA γ-aminobutyric acid

L lumbar

SCI spinal cord injury

SCS spinal cord stimulation

Th thoracic

Background

When a direct current (DC) is passed through a tissue, the tissue becomes either negatively or positively electrically polarized depending on the direction of the current. I.e. differences in electrical charges are formed throughout the tissue. This is also true for neurons, which are either hyper- or depolarized depending on the current flow direction in relation to the spatial orientation of the neuron[1]. This ability to modify signaling probability of action potentials and subsequently the information flow within the nervous system has been used as part of the clinical therapeutic arsenal for over 50 years, and for a vast array of indications (e.g. as recently described in a review by Hamani)[2]. Some of the more well-known indications are chronic pain, cardiac arrythmias and depression, but the method is also utilized for modulation of motor functions. In 2012 it was estimated that more than 40,000 spinal cord stimulation systems were implanted annually worldwide[2], with a presumably rapidly increasing incidence. Hence, further research on any potentially antagonistic or potentiating effects of concomitant drug treatments is of uttermost relevance in order to minimize side effects and maximize desired treatment outcome.

DC can be applied transcranially, transspinally, or epidurally, and in animal experiments even intraspinally. DCS administered epidurally, also called epidural spinal stimulation (ESS), is a type of spinal cord stimulation (SCS) used in humans by implanting an array of electrodes in the epidural space, attached to a subcutaneous stimulator. SCS has been used to treat various pain conditions for over five decades[3], and is currently a common therapeutic method in chronic pain treatment and motor rehabilitation[4,5] after spinal cord injury (SCI). The method has shown great potential in facilitating regain in motor control even in previously paralyzed animals of several species, including cat[6,7], rat[8], mouse[4], and fish[9]. Moreover, recent studies even imply facilitation of rehabilitation and regain of voluntary motor function in humans[10-12] As summarized in a recent review by Laliberte[13], these modulatory effects of ESS on both nociception and motor function arguably depend on stimulation of sensory afferents in the dorsal column[13]. However, many details of the physiological mechanisms through which the afferent excitability is altered are not yet fully understood. This is particularly true regarding

motoafferents, despite several studies reporting a subsequent recruitment of proprioceptive interneurons and motoneurons[13].

The direct benefits of altering afferents' excitability are perhaps more intuitively obvious concerning pain syndromes and nociception, but motoafferents (primary sensory afferents from muscle spindles and tendon organs together with cutaneous afferents) likewise play a key role in motor function. Complex rhythmic locomotor patterns can be produced in the spinal cord independent of input from afferents or supraspinal centers, but primary afferent input is necessary to adapt and correct motor output to outer disturbances (see e.g. Grillner and Zangger[14] for review). Multiple studies have indicated the central role of proprioception in walking [9,15,16], especially when the parameters of the surroundings change [8,17,18]. Proprioceptive input seems to play an even greater role for humans than for other animals, as upright bipedal locomotion requires more complex motor output than quadrupedal movement[19]. Input from motoafferents is not only forwarded to supraspinal centers (e.g. to cerebellum in aiding accommodation and fine-tuning of motor activation and output), but additionally modulates locally generated locomotor pattern output from spinal central pattern generators (CPG:s)(for recent reviews on spinal cord motor circuits see e.g. Arber (2012)[20] and Kiehn (2016)[21]). As recently summarized in a review by Kiehn, motoafferents promote transition between opposite locomotor phases (i.e. flexion/extension and left/right alteration) via direct synapses on both α-motoneurons and interneurons[21]. The relevance of afferent stimulation in motor function modulation is further supported by recent findings indicating that activation of proprioceptive feedback during locomotor training could be essential for establishment of detour circuits and locomotor recovery after SCI [4].

The duration of ESS effects depends on intensity, duration and frequency of the stimulation (see Truong (2018)[22] for a recent review). Generally surmised a stimulation of at least a few minutes is required to achieve any persistent effect, which builds up during about ten minutes after termination of the stimulation. This has been recorded in studies on humans as well as animals, using transspinal, transcranial and intraspinal stimulation[23]. However, Jankowska et al. recently reported a hitherto seemingly overlooked phenomenon; ESS with DC induced an immediate excitability change in primary afferents, lasting over an hour[24]. The effects

required mere seconds of total ESS duration at a 0.3 µA intensity, leveling off at an ESS intensity near 0.8 µA. Cathodal and anodal DC evoked excitability increases and decreases respectively, with cathodal DC yielding up to a 700% immediate increase in afferent excitability followingly remaining above 200%. The phenomenon was not reproduced by transspinal, intraspinal, or peripheral stimulation of primary afferent axons, leading to the hypothesis that the observed plasticity is induced presynaptically, directly in the primary axons. The phenomenon has also shown to be activity independent[24,25]. Thereby likely excluding Hebbian plasticity, the question remains whether this presumably extra- or presynaptic modulation of primary afferent excitability is secondary to actions of modulatory interneurons, or in fact an intrinsic mechanism inherent to afferent axons.

Serotonin (5-HT) is one of the main neurotransmitters mediating supraspinal modulation of spinal sensory and motor pathways/signals. Serotoninergic projections in the central nervous system arise in the raphe nuclei in the brain stem[26]. Four of these nuclei, *nucleus Raphe Magnus*, *n. Pallidus*, *n. Obscurus* and *n. Pontis*, give rise to descending tracts that terminate within the spinal cord[27]. They constitute the main source of spinal 5-HT, as practically no intrinsic 5-HT neurons can be found in the mammalian spinal cord[28]. The contribution of serotonin to the above-mentioned phenomenon – both in itself as well as a mechanism in clinical therapies – is of further relevance, not only because serotonin is highly involved in neuroregeneration after SCI[29], but also as ESS treatment often is concomitant with treatment with drugs affecting serotonergic systems.

Within motor systems, the physiological mechanisms enabling the serotoninergic modulatory effect on spinal circuitry has been found to operate as both excitatory and inhibitory, pre- and postsynaptically, on synapses relaying signals to and from afferents, and via both synaptic and volumetric transmission (see e.g. Rossignol (2006)[30], Fuxe et al. (2010)[31], Perrier (2015)[32] and Grillner (2003)[33] for reviews). Over 15 5-HT receptor subtypes are currently identified, some inducing hyperpolarization and some depolarization, the majority of receptor subtypes being G-protein coupled and only a handful direct ion channels (See e.g. Perrier (2015)[32] for review). Several studies indicate that serotonin exerts an overall inhibitory effect on both nociceptive and motor afferents in the dorsal horn[32]. Adding to that, preliminary data indicate

that serotonin administered ionophoretically in the dorsal horn inhibits the development of sustained changes in excitability induced by intraspinal DC in the dorsal horn, both when serotonin was administered before as well as after DC[34]. It is highly relevant to study whether serotonin would exert similar effects regarding epidural DC. The equivalent would be administering serotonin in the dorsal column, i.e. around and between afferent axons, as this is effectively the part of the afferents stimulated by epidural DC.

Specific objectives

The primary aim of this thesis is to investigate if and to what extent local administration of 5-HT in the dorsal column affects the sustained increased excitability in primary afferents induced by a brief episode of epidural cathodal DC polarization over correlating motor nuclei.

Ethics

All experiments were approved by the regional Ethics Committee for Animal Research (Göteborgs Djurförsöksetiska Nämnd) and complied with European Union and NIH guidelines for animal care. The animals were housed under veterinary supervision at the Laboratory for Experimental Biomedicine (Sahlgrenska Academy, University of Gothenburg), where all experiments were also carried out. The animals were kept on a 12-hour light/dark cycle, with access to food and water ad libitum. Everyone handling the animals had first taken and passed an Animal Handling Course (provided by the Laboratory for Experimental Biomedicine, Core Facilities, University of Gothenburg).

The number of animals used was kept at minimum, e.g. by simultaneously recording from two nerves, recording several series from the same animal when possible, and stimulating at both threshold and suprathreshold intensities. The animals were carefully and continuously monitored during the procedures to minimize pain and stress.

Methods

Surgical procedures

Acute experiments were performed on 16 adult rats (Sprague Dawley, all female, 2–6 months old, 200–450 g).

Animal anesthesia was induced by isoflurane (4% in air (Baxter Medical, Kista, Sweden)) delivered by a pressure vaporizer, followed by intraperitoneal injection of sodium pentobarbital and α-chloralose (both 30 mg/kg)(Distansapoteket Hässleholm, Sweden, and Acros Organics, Geel, Belgium, respectively). Anesthesia was maintained throughout the experiment by administration of additional doses of α-chloralose intraperitoneally, as needed (3 mg/kg, up to 60 mg/kg). Ringer acetate buffer (10-20 ml) was administered subcutaneously during the initial surgical procedures to compensate for fluid losses during the experiment. Branches of the sciatic nerve (*n. peroneus* and *n. tibialis*) were dissected and cut distally.

The animal was thereafter mounted in a ventral recumbent position to a custom-made metal frame (S. Berg, Gothenburg University), to which the head, spinal column, tail and hindlegs were fixed. A laminectomy was performed on spinal column level L2-L5, after which the incision was covered in a pool of mineral oil created by skin-flaps. The dissected nerves were mounted on dual silver electrodes in a pool of mineral oil created using skin-flaps. All surgical procedures and drug preparations are described in further detail in Appendix I.

Following these procedures the animal was, via insertion of two subcutaneous electrodes, connected to a two-channel real time oscilloscope (TDS 210, Tektronix, Inc.,), by which the electrocardiogram was monitored, and depth of anesthesia assessed, regularly throughout the experiment. Muscle relaxation was initiated by intravenous administration of around 20 mg gallamine triethiodide (20 mg/ml, Sigma-Aldrich, G8134), and maintained by administration of additional doses of on average 4 mg when needed. At cessation of spontaneous respiration, artificial ventilation was administered via a respiratory pump (SAR-830/P, CWE, Inc., Ardmore, PA, USA) connected to the tracheostomy. End expiratory CO_2 was continuously monitored (End-Tidal CO_2 analyzer CapStar-100 CWE Inc., U.S.A.), and maintained between 3.5-4.2% by respiratory rate adjustments (rate range 50-70 breaths per minute, volume about

2.5 ml, pressure controlled at around 2.40 cmH_2O). Experiments were only continued for as long as the end tidal CO_2 and the heart rate remained within physiological ranges. Body temperature was continuously monitored via a rectal thermometer and maintained at 38.0±0.5 °C by servo-controlled heating lamps.

Experiments were terminated by a supralethal dose of pentobarbital, followed by excision of the heart after confirmed cardiac arrest.

Electrodes

Tungsten electrode

For the epidural polarization and antidromic activation of afferents, a tungsten electrode was used (50-320kΩ) (Microneurography active needle, UNA35FNM, FHC, Bowdoin, ME, U.S.A.), insulated except for a tip of 20-30 µm.

To confirm that the tungsten electrode is perfectly straight, and thus also that stepwise manipulation of the holder will give accurate manipulation of the electrode, the electrode is mounted on a holder (*Figure 2C, page 13*). The electrode is viewed through the spotting scope, and the tip aligned to the cross marked on the scope (Figure 2*F*). Any skewness in the electrode will subsequently manifest as deviation from the alignment to the cross when the electrode holder is rotated.

Glass micropipette electrode

The glass micropipettes used for the microelectrode ionophoresis were drawn from capillary tubes (1,20 x 1,65 x 100 mm, Vitrex Medical A/S, Denmark), using a custom designed pipette puller (*Figure 2A*)(S. Berg, University of Gothenburg). The tip was manually broken by gentle collision with a blunt ended glass rod under a microscope (magnification 10x, Figure 2*D*). The tip diameter was measured under 50x magnification (Figure 2*E*), ensuring inner diameters of 2.5-3µm, impedance 7-20 MOhm were used. The tips were broken to appropriate diameter by hand rather than by a machine, granting the napped edges necessary for penetration of the adult spinal cord tissue (*Figure 2E*). Using a micropipette (GELoader, Eppendorf Quality™, 0.5 – 20 µl, 62 mm) the glass pipette was filled with approximately 8 µl 0.2 M 5-HT (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany)(dissolved in HCl, pH

4.5), pressed out into the tip by positive air pressure. The glass pipette microelectrode was checked under microscope (magnification 50x) to ensure absence of air bubbles in the solution, as these would interfere with passing of current and subsequent movement of drug ions.

Mounting and placement of electrodes

The tungsten electrode and the glass micropipette filled with 0.2M 5-HT were mounted in the remotely controlled[35] dual head micromanipulator[36] (illustrated in Figure 2, electrodes in holder X and Y, respectively)(dual head model designed by I. Engberg et al., and micromanipulator by E. Eide and Y. Källström, University of Gothenburg), allowing stepwise manipulation with 2 µm precision. When holder X is lowered both holders move, so that they remain positioned in the same place relative to each other. Holder Y can however be moved independently of holder X, minimizing the time of retaining current needed to be applied.

Once mounted in the holder, the electrode tips were aligned with respect to each other under microscopic control, to allow precise positioning of the electrodes with the glass micropipette electrode 0.5-1 mm rostral of the tungsten electrode (*Figure 2*).

The tungsten electrode tip is positioned just lateral of the central vein, ipsilaterally to the nerves recorded from, with the holder tilted 7° medially. The tungsten electrode is lowered enough to indent the dura mater, to minimize shunting of current in the cerebrospinal fluid, but without applying pressure to the spinal cord. Stimulus, alone or together with DC, was applied through the tungsten electrode against a silver reference electrode inserted in back muscles approximately one cm rostral of the tungsten electrode. See *Figure 3*, page 13, for a schematic illustration of the electrode setup.

The tip of the glass micropipette electrode containing 5-HT was kept in the paraffin pool but above the spinal cord until just before 5-HT administration, at which point a negative retaining current of 10 nA was passed and the tip of the electrode was advanced 200 µm into the spinal cord through a 0.5-1 mm^2 opening in the *dura mater* carefully made with fine tip surgical tweezers.

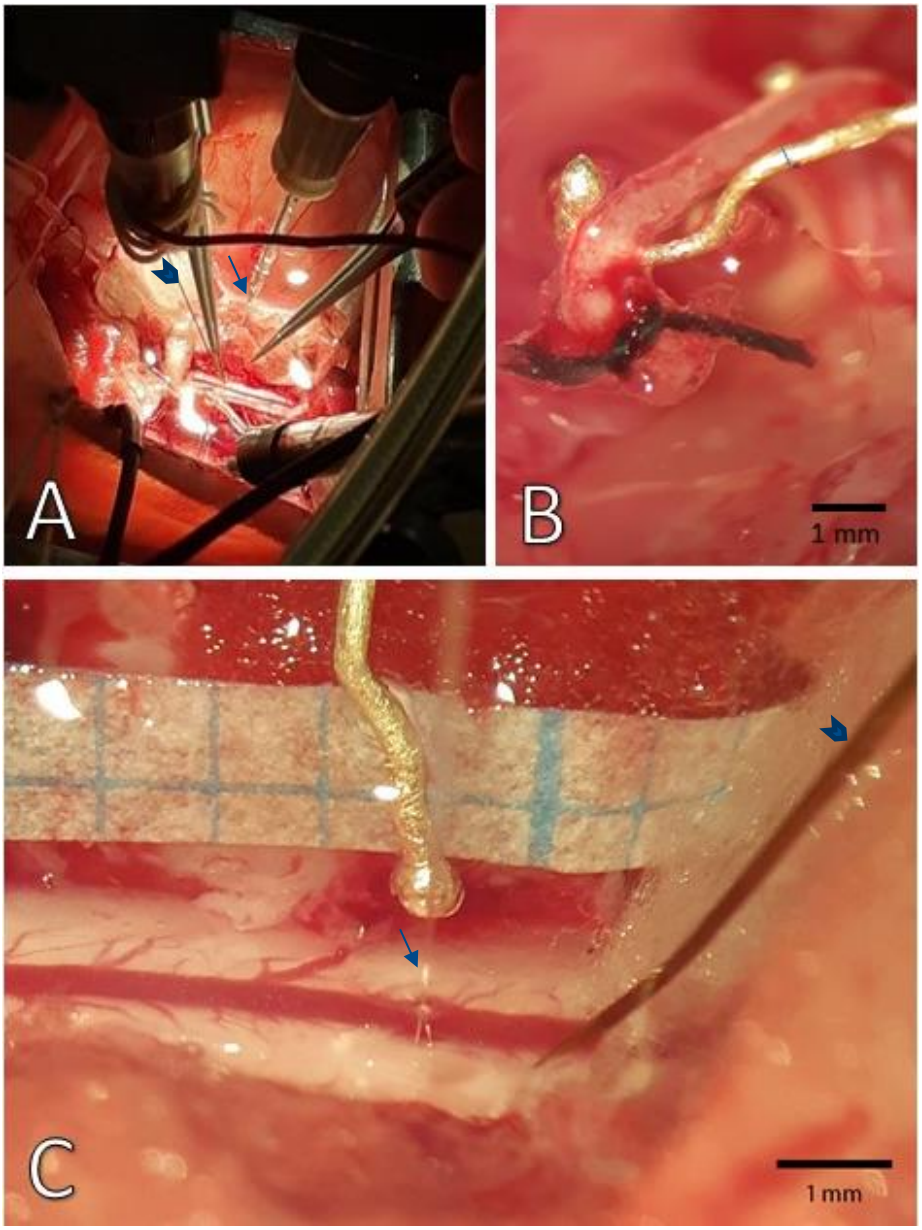

Figure 1: *Surgical preparations. Dura mater being opened with a pair of tweezers, in a pool of mineral oil (A). Peripheral nerve dissected free with intact epineurium and vascularization, mounted on a dual silver ball electrode in a pool of mineral oil (B). Tungsten electrode indenting dura mater and glass micropipette advanced 200 µm into the dorsal column of the spinal cord (C). Arrows: glass micropipette electrode, arrowheads: tungsten electrode.*

Stimulation

Stimulation was supplied via a stimulator unit (custom designed by E. Eide, University of Gothenburg) containing 3 independent stimulators[37]. Epidural stimulation was applied via the tungsten electrode previously described. Single stimuli of 200 µs constant current (CC) were applied epidurally continuously throughout the experiments at two intensities (between 10 and 20 µA); one just above threshold intensity and one at 1.5 times threshold intensity, at an intraintensity frequency of 1 Hz and interintensity frequency of 2 Hz. Stimulation of peripheral afferents was applied through the dual silver electrodes (one per nerve) in succession used for recording responses in the same positions.

Optimal epidural dorsal column stimulation sites were primarily mapped out by stimulating the peripheral nerves. A third dual silver electrode, with one silver ball epidurally on the spinal cord and one as a reference electrode placed in muscle tissue lateral to the spine, was used to monopolarly register responses along the surface of the dorsal column around the spinal segment levels where afferents from *n. peroneus* and *n. tibialis* enter the spinal cord. At the levels showing the strongest response, further and more precise positioning of the tungsten electrode was made using a mechanical step device by which the dual electrode holder was mounted to the metal frame base, allowing minimal steps of the spinal column electrodes of about 20 µm accuracy along both the rostrocaudal and the lateromedial axis[36]. The tungsten electrode was positioned so that it indented the dura mater without damaging it or pressing on the spinal cord. This served to minimize the volume of cerebrospinal fluid (CSF) between the electrode and the spinal cord, thereby increasing the control of the current flow, as CSF has a very high conductance compared to surrounding tissues (see e.g. Holsheimer et al. (2015)[38] for a review). A disposable paper scale denoting millimeter spans was placed just laterally along the spinal cord to facilitate rostrocaudal orientation throughout the duration of experiments.

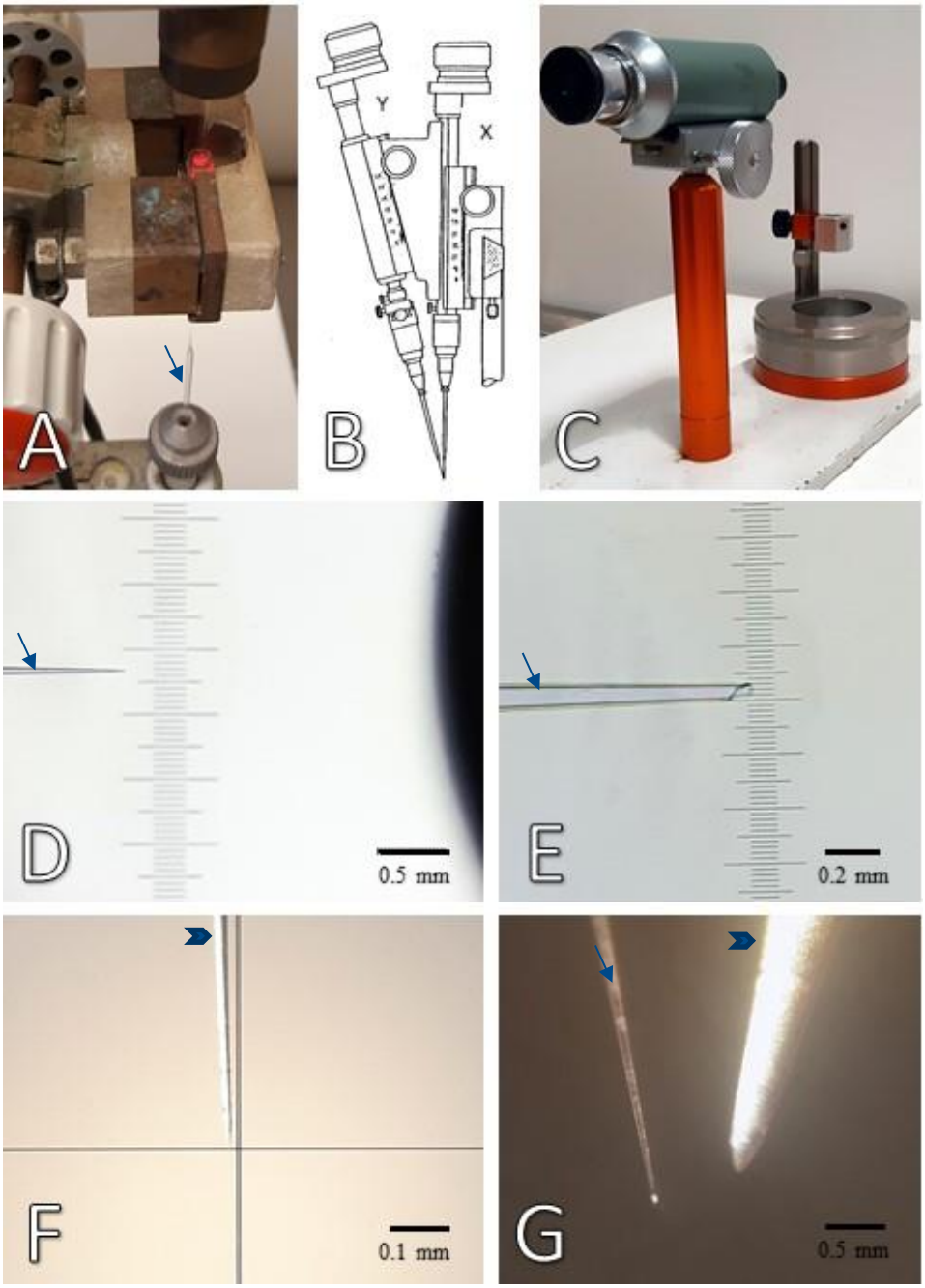

Figure 2: *Glass and tungsten electrode preparations. Glass micropipette electrode being manually drawn from a capillary tube (A). Dual head electrode manipulator for independent manipulation of two electrodes (B)*[35]*. Spotting scope (magnification 10x) and rotatable electrode holder for visual inspection of electrodes (C). Tip of micropipette under 20x magnification, before manual breaking of tip against glass rod (right) (D). Napped edges of glass micropipette achieved by manual breaking. Tip diameter 5.5-6µm, 50x magnification (E). Tungsten electrode viewed through spotting scope in C (F). Alignment of electrode tips under 20x magnification (G). Arrows: glass micropipette electrode, arrowheads: tungsten electrode.*

DC- evoked polarization

Epidural polarization by DC was supplied using a custom-made battery driven CC stimulator with ability to supply a range of 0-1.000 µA CC/DC (D. Magnusson, University of Gothenburg). In both groups, 1.0 µA DC was applied for 1 minute (60±10 s) via the tungsten electrode. Polarization and stimulation was supplied through the same electrode, as DC has shown to not affect stimuli under the current experimental conditions[39].

Recording

The epidurally evoked nerve volley and field potential responses were recorded diphasically via dual silver ball electrodes via TL-1 DMA interface (Axon Instruments, Foster City, CA) at a 33 kHz sampling frequency, with time resolution of 30 µs per address. A conventional high-impedance amplifier was used (low pass filters 15 or 1 Hz, high-pass filter 5 or 3 Hz), as well as cancellation of noise from mains hum (50 Hz) using a HumBug Noise Eliminator (Digitimer Ltd., UK). Single records and averages of 10 successive records (obtained online) were saved at a minimum every minute, for later offline analysis.

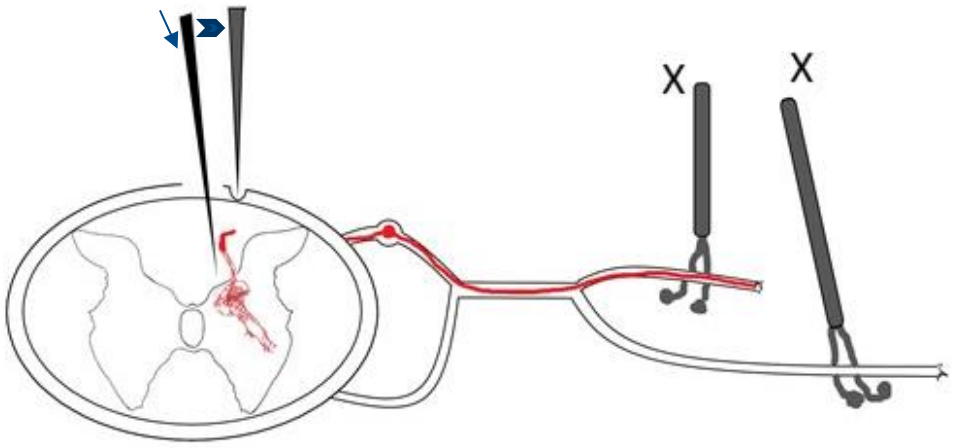

Figure 3: *Schematic illustration of the positioned electrodes in the experimental setup. Arrow: Glass electrode containing 0.2 M 5-HT. Arrowhead: tungsten electrode for epidural cathodal DC polarization and antidromic activation of primary afferents. Red: illustration of a primary afferent in a peripheral nerve, and primary afferent branching in a transection view the spinal cord, adapted from Hongo et al. (1987)[40]. X: Dural silver ball electrode for registration of peripheral nerve volleys.*

Experimental design

The data consisted was divided into one experimental group (5-HT group), and one control group. Data were collected before, during and after DC polarization with 1 minute of 1.0 µA cathodal epidural DC stimulation. DC was delivered after a minimum of four minutes of stable responses had been recorded (baseline activity). In the 5-HT group, 5-HT was administrated ionophoretically (30-60 nA, for 20-30 minutes), starting 10 minutes after polarization with DC. Changes in responses in relation to the series baseline were compared between the two experimental groups. Data obtained in a particular experimental variant – e.g. records from one nerve, stimulated at one site, in one animal – is referred to as a "series".

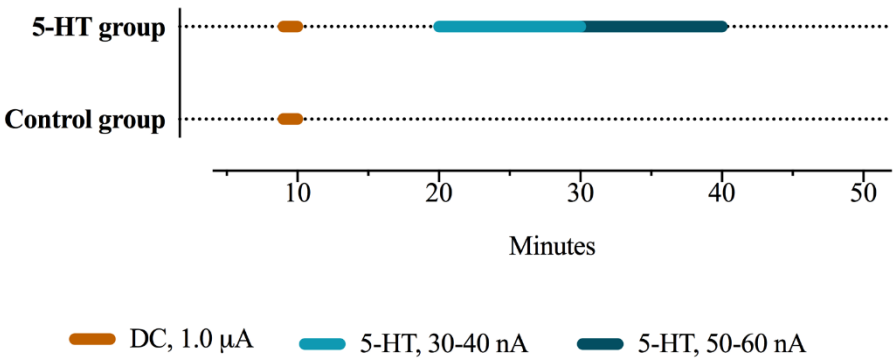

Figure 4: *Schematic illustration of the experimental design. 1 minute of epidural polarization with cathodal DC stimulation (both groups) was followed by ionophoretic administration of 5-HT in the spinal cord dorsal column (5-HT group). Antidromically evoked primary afferent responses were registered peripherally throughout the duration of the experiments. DC: direct current.*

Data collection

5-HT group

To compare the epidurally evoked nerve volleys, the areas of the earliest components of the averaged responses were measured using a system of analysis designed and created by E. Eide (VBDOS, at University of Gothenburg), which expresses areas of responses in arbitrary units. The analysis was restricted to the first 0.32 – 1.12 ms of the compound response

volleys. Data from each series was normalized with respect to the mean of areas of the baseline data from the same series, thus expressed as ratio of baseline response.

Data was plotted in a scatter plot graph, and extreme outliers identified. The outliers were compared to any timed comments in protocol notes, and if these comments showed alteration of conditions correlating to change in neural excitability (e.g. changes in ECG, simultaneous administration of drug, mechanical impact), the outlier was excluded from analysis. Four series were excluded completely, as the 5-HT micropipette electrode was blocked during these series, and the mass and timing of any 5-HT administered subsequently uncontrolled and most likely not present.

Control group

The data collected in the present series of experiments were compared with control data obtained from data collections by Jankowska et al., results published 2017[24]. These data were collected and normalized the same way as above described for the 5-HT group, but from *nervus peroneus* and *nervus suralis* rather than *nervus peroneus* and *nervus tibialis*.

Statistical methods

For analysis of the effect of DC, the normalized areas from all series were averaged for each testing period (i.e. baseline, during DC, between DC and 5-HT, during 5-HT and after 5-HT). Difference from baseline was calculated through a one-way ANOVA with repeated measures (rm ANOVA), correcting for multiple comparisons with Dunnett's test, and for non-sphericity using Geisser-Greenhouse correction.

Differences between averaged responses between the control group and the expression group, at each time point, were calculated through multiple unpaired t-tests. The Holm-Sidak method was used to adjust for multiple comparisons (37 separate t-tests), without assuming consistent standard deviations. Difference between the groups was further calculated on the testing period averages by performing a two-way rm ANOVA, with Geisser-Greenhouse and Sidak corrections for non-sphericity and multiple comparisons, respectively.

Calculations were made using Microsoft Excel and GraphPad Prism 8.

Results

Effect of DC stimulation

A total of 24 series were collected in 5 experiments. Of those were 16 series collected in 3 experiments included in the study. The control group data was collected from experiments previously performed by Jankowska et al. and consisted of 16 series collected in 4 experiments. In both groups, DC was delivered only after a stable baseline had been established. During DC stimulation, afferent response increased by over 400% (407±179%, n = 16, *Table 1*, Figure 5) compared to the baseline response, and remained increased by at least 95% (95±90%, *Table 1*) throughout at least 40 minutes post polarization. This increase in afferent response induced by DC was not statistically significantly different from the increase observed in the control group (292±98% during DC, increase remaining at least 181±44%, *n* = 16, (*Table 1, Figure 5*). Thus, a significant increase in excitability remained after termination of the polarization.

Table 1: *Average increase from baseline response of antidromically activated primary afferent responses at the five experimental time periods, per experimental group. Control group data acquired from data previously published in Jankowska et al. (2017)*[24].

	Control group		**5-HT group**	
Minutes after DC	Increase (95% CI)	p-value	Increase (95% CI)	p-value
-1 - 0 (during DC)	292% (194-390%)	<0.0001	407% (228-586%)	<0.0001
1 - 10	222% (149-295%)	<0.0001	195% (89-300%)	0.0005
11 - 17 (30-40nA 5-HT)	171% (112-231%)	<0.0001	133% (47-219%)	0.0024
18 - 32 (50-60nA 5-HT)	169% (113-226%)	<0.0001	121% (37-204%)	0.0045
33+	181% (137-224%)	<0.0001	95% (5-186%)	0.0371

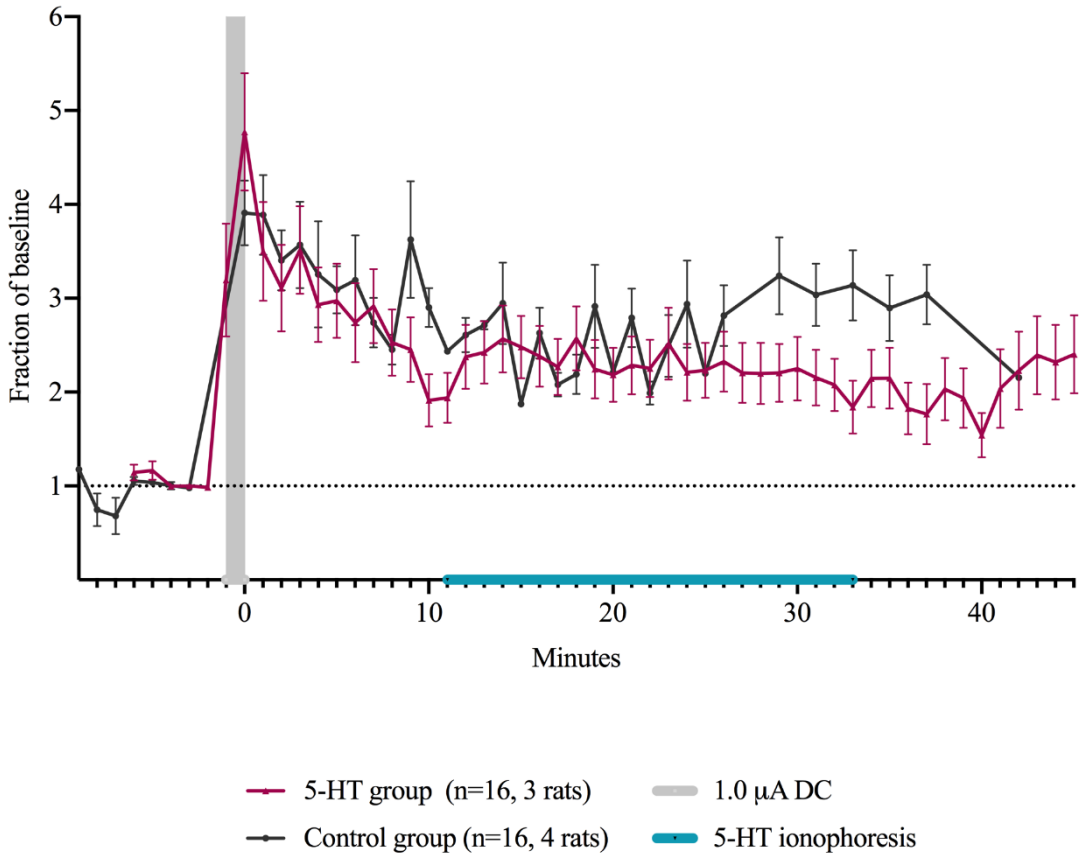

Figure 5: *Changes in averaged responses over time in 5-HT group and control group animals, before, during and after epidural polarization with cathodal DC stimulation (both groups) followed by ionophoretic administration of 5-HT in the spinal cord dorsal column during the post polarization period (5-HT group). Error bars depict 95% confidence interval. DC: direct current.*

Effect of 5-HT

5-HT was applied ionophoretically at 11-32 minutes after DC polarization, when the increased excitability of the primary afferents was deemed to have stabilized. The afferent responses remained increased by over 120% during 5-HT ionophoresis and by at least 95% after ionophoresis (*Table 1*). No statistically significant difference was found when comparing responses during and after DC – including ionophoresis period of the 5-HT group – between the two groups, neither when calculated using multiple t-tests ($p = 0.9999$, corrected for multiple comparisons), nor when calculated with two-way rm ANOVA ($p = 0.7555$). In other

words, no statistically significant effect of administration 5-HT during the post polarization period was found.

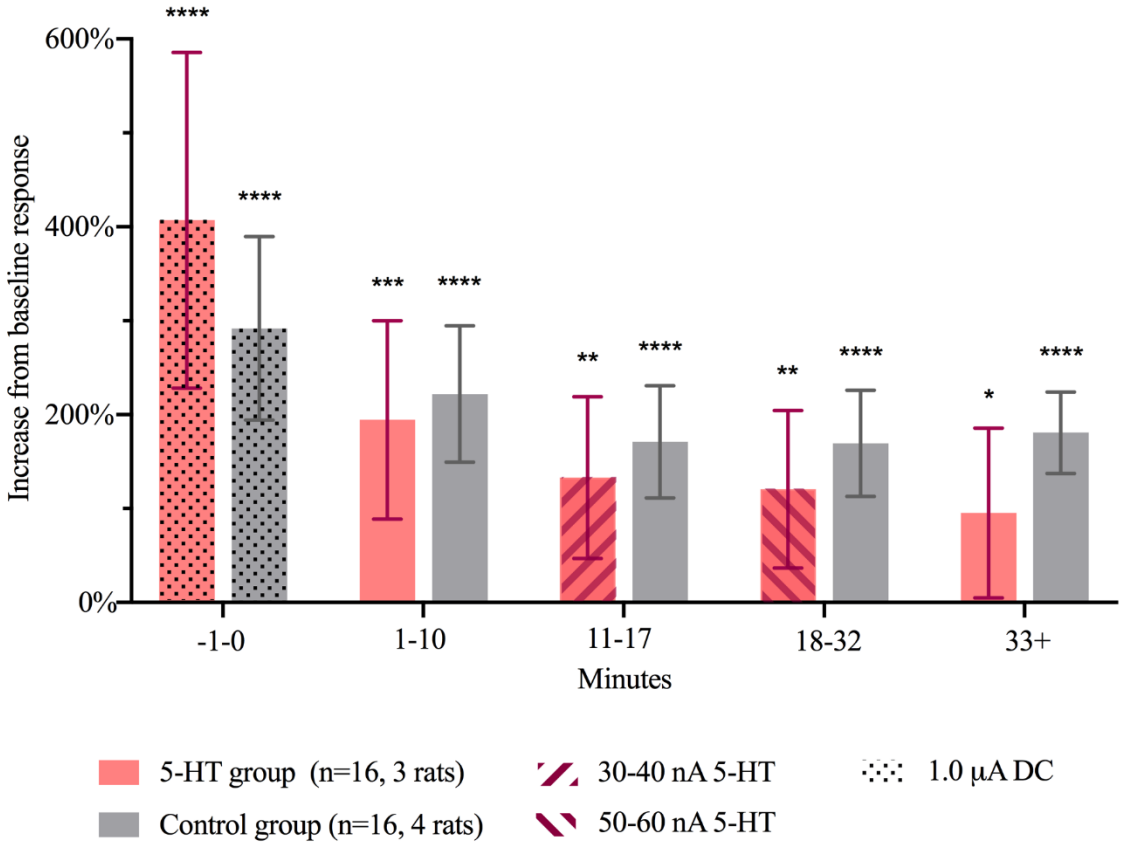

Figure 6: *Increase in primary afferent response during and after 1 minute of epidural cathodal DC polarization, with 5-HT administered in the spinal cord dorsal column by ionophoresis for 21 minutes during the post polarization period in the 5-HT group (one-way repeated measures ANOVA, Dunnett correction for multiple comparisons and Greenhouse-Geiger non-sphericity correction). DC: direct current, error bars: 95% CI, **** p<0.0001, *** p<0.001, **p<0.01, *p<0.05*.

A statistically significant interaction was found between the two variables time period and 5-HT ($p = 0.0002$), making up about 4% of the total variation in data. In comparison, the state of the series (during DC or time interval during post polarization period) accounted for 42% ($p = <0.0001$, *Table 2*) of the total variation, and interseries variation for 33% ($p = <0.0001$, *Table 2*). Sphericity could not be assumed ($\varepsilon=0.3352$) and was thus corrected for using the Greenhouse-Geiger method.

Table 2: *ANOVA-table of two-way ANOVA with repeated measures in one factor. P-values presented as corrected for with Sidak's multiple comparisons test and Greenhouse-Geiger method for non-sphericity.*

Source of variation	% of total variation	P-value	F (DFn, DFd)	SS
Time x 5-HT	3.87	0.0002	F (5, 150) = 5.321	19.2
Time	41.61	<0.0001	F (1.796, 53.89) = 57.24	206.1
5-HT	0.11	0.7555	F (1, 30) = 0.09873	0.5
Series	32.60	<0.0001	F (30, 150) = 7.473	161.4
Residual				108.0

Descriptive statistics

The series in the 5-HT group displayed a larger range of increase in afferent response during DC polarization, compared to the control group (*Figure 7*). Data was also collected more frequently and for on average 14 minutes longer after termination of DC polarization in the 5-HT group, compared to the control group (Table 3).

Table 3: *Total collected data volumes and durations of data collection after termination of epidural spinal cord polarization with 1.0 μA cathodal DC stimulation, per experimental group. ∑: sum, SD: standard deviation, DC: direct current.*

Group	Data points used for calculations ∑(mean ± SD)	Duration of data collection after DC mean (range)	Number of rats included/total	Number of series included/total
5-HT	73,100 (4,600 ± 600)	44 (38-39) min	3/4	16/24
Control	34,900 (1,900 ± 600)	30 (12-42) min	4/unknown	16/unknown

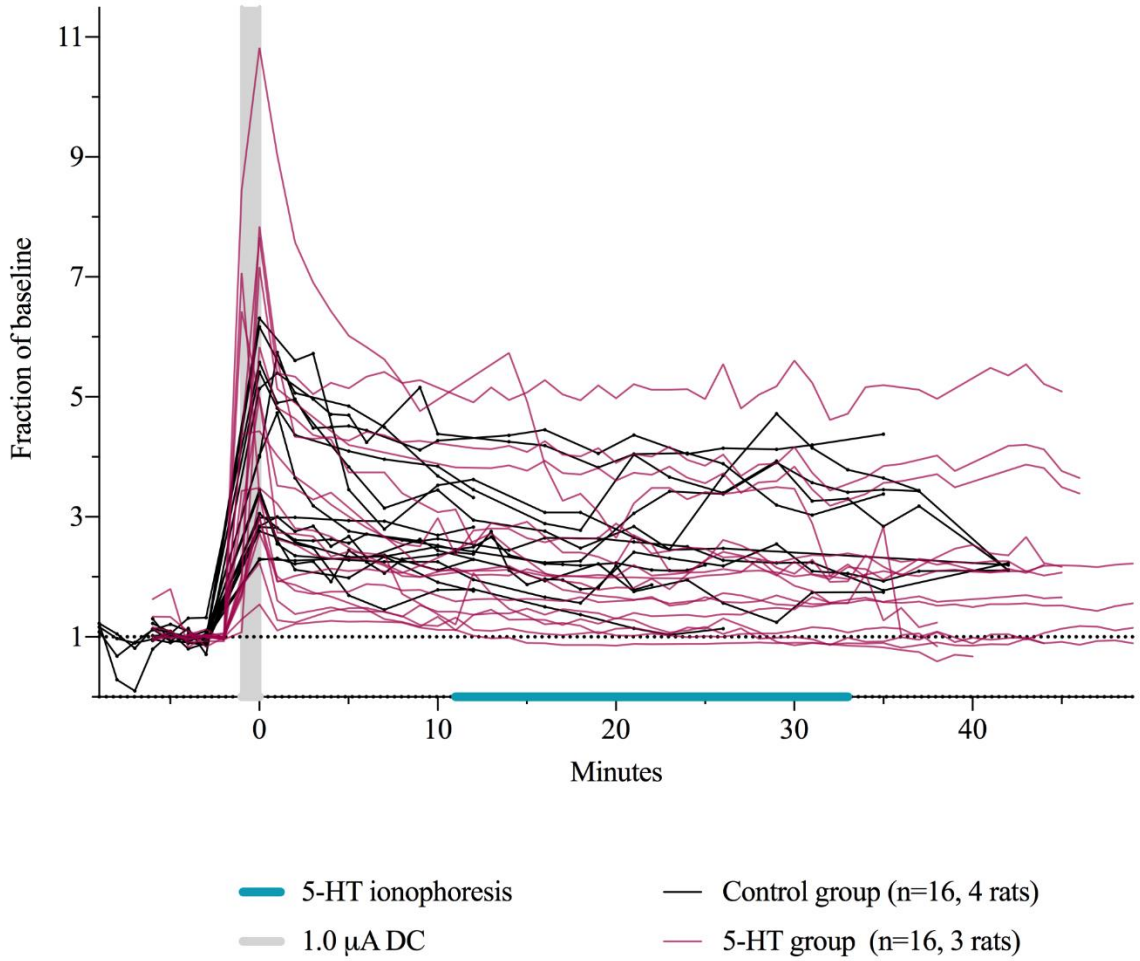

Figure 7: *Overview of all data collected. Primary afferent responses in all series, before, during and after epidural polarization with cathodal DC stimulation (both groups) followed by ionophoretic administration of 5-HT in the spinal cord dorsal column (5-HT group). DC: direct current.*

Table 4: *Descriptive time point statistics for the ionophoretic administration of 5-HT in the spinal cord dorsal column after termination of epidural spinal cord polarization with 1.0 µA cathodal DC stimulation (n=16, 3 rats). DC: direct current, SD: standard deviation.*

5-HT	**Start time, minutes after DC** mean (range, SD)	**End time, minutes after DC** mean (range, SD)	**Duration, minutes** mean (range, SD)
30-40 nA	11 (8-15, 3)	18 (12-24, 4)	7 (4-9, 2)
50-60 nA	17 (13-20, 3)	33 (27-40, 5)	16 (12-21, 3)

Discussion

The results of this study demonstrate that the DC-evoked long-lasting increase in the excitability of peripheral afferent fibers traversing the spinal cord dorsal columns is unlikely to be affected by a local increase in 5-HT. This suggests that the phenomenon of DC-induced axon excitability increase is not inhibited by 5-HT at an axonal level, and that intrathecal administration of serotonergic drugs are unlikely to significantly affect ESS.

Interpretation of results

The results did not show any statistically significant effect of ionophoretic administration of 5-HT in the dorsal column as measured by the area of peripheral afferent response volley after epidural cathodal DC polarization, compared to the control. It should however be noted that a statistically significant interaction was found between the variables "experimental time period" and "experimental group" (*Table 2*). In other words - over time, the two experimental groups differed in their response to DC. There was no statistically significant difference between average group responses at individual time points nor at pooled time interval means, but overall, the DC effect decreased faster in the 5-HT group. This discrepancy is likely due to variations in data sampling between the groups. As illustrated in *Figure 7*, page 21, the range of afferent response (presented in units of fraction of baseline response) during DC is much wider in the 5-HT group than in the control group. Additionally, series with only minor sustained increase after DC were included in the 5-HT group, whereas the series included in the control group all seem to demonstrate a high effect of DC polarization. This difference in DC-effect amplitude is theoretically very likely to affect the decline rate of the same and would explain the observed difference between the two experimental groups' response stability. The difference in group response stability was present before the administration of 5-HT and could therefore mask any minor changes in afferent excitability induced by 5-HT ionophoresis. Ultimately, despite being statistically significant this difference in DC effect only accounted for 4% of the total data variability (see *Table 2* on page 20) and becomes clinically irrelevant compared to interseries variations being the source of 33% of the total data variability. Studies by our research group indicate that the placement of the DC polarization electrode is a key factor to the amplitude and stability of the DC-induced

excitability increase[41]. More specifically, the effect of DC seems to be dependent on the location of the epidural stimulation in relation to the level of branching points of primary afferents, with stimulation over a higher number of afferent branching yielding a greater effect. On that basis, dividing data series according to the distance of epidural stimulation site from nearest primary afferent branching point would be a promising method to further decrease the interseries variation, subsequently allowing identification of smaller changes in afferent response excitability.

The results differ from previous preliminary findings indicating that 5-HT interacts with both development and maintenance of DC induced long term increase in afferent excitability[34]. Importantly, the long-term effects in that pilot study was however induced by intraspinal DC, where the terminals of afferent fibers were polarized, as opposed to the afferents traversing the dorsal column as in this study. The different effects of intraspinal and epidural DC is further supported by the recent results published by Bolzoni et al. (2019)[42], showing that the $GABA_A$ inhibitor Baclofen, administered either intravenously or ionophoretically in the dorsal column, decreases primary afferent responses before and during intraspinal DC, but does not interfere with epidural DC. This is of uttermost relevance for clinical studies in both the aspect that DCS in clinical use is currently delivered epidurally, and that preclinical studies are investigating intraincisional 5-HT administration as a possible method to increase recovery after SCI. However, as most serotonergic acting drugs used concurrently with ESS are administered systemically and systemic versus local administration of 5-HT modulating drugs have demonstrated adverse effects on gait patterns (see e.g. Perrier (2017)[32] for a recent review), it is still highly relevant to study whether systemic administration of serotonergic drugs affects the DC-induced long-term increase in primary afferent excitability.

Methodological considerations

Experimental design

A less significant limitation of this study is arguably the time resolution and duration of data points collected in the control group. The decision to use control data obtained in a previous study was primarily ethical, as it would be excessive to sacrifice animals for the sake of merely repeating the same experiment. As the study from which the control data was obtained

was designed to answer a different hypothesis the corresponding study design had requirements other than those regarding this study, and hence yielded a limited number of data points in the control group (See Table 3, page 20, for descriptive statistics).

The mechanical and electrophysiological effect of ionophoresis itself was not controlled for by adding a third experimental group with ionophoresis of a vehicle solution, as it has been shown in previous studies that passing a current of 20 nA positive current from a microelectrode glass pipette filled with a NaCl solution of pH 8 or 4 does not affect the neuron response in this set up[43].

Blinding was not possible in this type of preclinical electrophysiological in vivo-design, seeing as setting the measuring windows for the response volley amplitudes requires manual analysis of each individual sweep by someone able to differentiate between various forms of sporadic disturbances and changes in latency. This can therefore not be performed automatically with analysis set for a predetermined window.

Although not likely to have any significant impact on the results, it could be noted that the 5-HT group consisted of all female rats, whilst the control group contained animals of both sexes.

Optimal epidural stimulation site

As the afferents from *nervus peroneus* and *nervus tibialis* will not branch at the exact same positions, the place chosen for epidural polarization, afferent antidromic stimulation and 5-HT ionophoresis will inevitably have to be a tradeoff between the best signal from the two different nerves. The distance in this case is however in the range of no more than a millimeter or so, and other factors than wanting to record two nerves simultaneously – such as e.g. physical limitations by overlying blood vessels - will have more impact on the electrode positionings.

Drugs and anesthesia

The procedure of first sedating the animal with inhalation anesthesia prior to the intraperitoneally injected one can be debated. Both procedures contain elements of stress for

the animal, but the decision to combine the two methods was based on both a need to minimize stress for the animal and because i.p. injections are less reliable in their success than inhalation sedation. By combining the methods repeatability is ensured, as any potentially failed i.p. injection can be corrected for by additional 10-20% of the original dose[44], without causing additional stress to the animal.

α-chloralose is a frequently used anesthetic in labs as this drug has a less impact on autonomic and cardiovascular responses[44], and the drug exerts it's anesthetic effect by increasing the affinity of GABA to $GABA_A$ receptors fivefold, as well as increasing the efficacy of receptor activation[45]. Anesthetic dose rate in rat is recommended to be 55-65 mg/kg i.p., for 480-600 min light anesthesia[44]. It is also long acting, but effects are fully attained after about 15 min, why the drug is usually combined with a drug with more rapid induction, such as barbiturates[44]. Sodium pentobarbital is a barbiturate commonly used for animal anesthesia and euthanasia[46]. Additionally, the drug also inhibits glutamate, adding to the depressant effect[47]. Recommended dose is 40-50 mg/kg i.p. for 15-60 min light anesthesia, but when used in combination with α-chloralose, a lower mass is needed[44].
Both sodium pentobarbital and α-chloralose are hypnotic drugs with little analgesic effects. Both drugs alter GABAergic signaling, and sodium pentobarbital additionally also inhibits glutamate. Thus, the drugs will inevitably have some effect on the signaling in the spinal circuits, and to what extent this altered the results in the experiments for this thesis is unknown. However, the chosen drugs have less impact than other anesthetic drugs, and there are significant advantages in using in vivo methods rather than in vitro when investigating not yet fully mapped pathways and connections between supraspinal and spinal networks. We are, however, currently in addition working on developing an ex vivo model.

Further studies

To further study the effect of 5-HT on the DC-induced long-term excitability increase in primary afferents, it would be of interest to replicate the experiments with administration of specific 5-HT receptor agonists and antagonists rather than 5-HT, as different receptors have shown to exert a wide range of effects on their target neurons in the spinal cord[32]. An experimental method closer resembling the natural release of 5-HT in the spinal cord could

be electrically stimulating the raphe nuclei in the brainstem, and study whether this more natural intrasynaptic release of the neurotransmitter affects the long-term DC-induced increase in afferent excitability. If any such effect were to be seen, this could additionally be confirmed by studying the effect of spinalization. Additionally, bilateral registration in peripheral primary afferents would allow for comparisons between the effects of ipsilateral and contralateral DC polarization and 5-HT administration, which could be of interest seeing as 5-HT modulation of spinal CPG:s plays a key role in coordination of opposing muscle groups (right/left and flexor/extensor)[32]. Finally, development of an ex vitro method allowing intravenous administration of serotonergic drugs would allow studies on the effects of systemic drug administration on ESS-induced long-lasting increase in primary afferent excitability.

Conclusions and Implications

From the results it can be concluded that an increase of 5-HT in the ipsilateral dorsal column after DC does not seem to have any significant effect on sustained afferent excitability achieved through cathodal epidural DC. The conclusion yet implies that the expression of the sustained DC effect is not dependent on presynaptic serotonergic modulation in the ipsilateral dorsal column. Furthermore, a clinical implication of these results is that local administration of serotonergic drugs or agonists after SCS may have little or no significant impact on the effects of SCS therapy.

Populärvetenskaplig sammanfattning

Serotonins inverkan på nervers långvarigt ökade retbarhet efter elektrisk ryggmärgsstimulering

Om man skickar en svag ström genom en vävnad bildas det olika elektriska laddningar i vävnaden. Eftersom signaler inom nervceller fortleds med hjälp av elektriska strömmar, kan man öka eller minska sannolikheten för att en cell ska fortleda en signal genom att stimulera omkringliggande vävnad med ström. Vilken effekt stimuleringen har på nervcellen beror på strömmens riktning i relation till nervcellens rumsliga orientering. Denna elektriska egenskap hos nervceller utnyttjar man bland annat som behandling av smärttillstånd och rehabilitering av muskelfunktion efter ryggmärgsskador.

Serotonin är ett signalämne som nerver använder för att kommunicera med varandra. Ämnet kan både användas för att skicka vidare en signal från en cell till en annan, och för att öka eller minska sannolikheten för signalfortledning i andra nervceller. Det sistnämnda kallas för en neuromodulatorisk egenskap. Serotonin är ett av de främsta signalämnena för neuromodulering av ryggmärgssignaler för både sensorik och motorik, och ämnet frisätts i ryggmärgen från utskott från nervceller i hjärnstammen. Det är också vanligt att behandling med ryggmärgsstimulering sker samtidigt som behandling med läkemedel som på olika sätt påverkar serotoninsystemen.

För några år sedan såg man att stimulering precis utanför ryggmärgshinnorna ger en starkare effekt som håller i sig längre än stimulering av nervsystemet på andra ställen. Kunskap om hur detta fenomen uppstår skulle kunna innebära bättre effekt och mindre biverkningar av ryggmärgsstimulering för patienter med sådan behandling. På grund av den centrala roll serotonin spelar i neuromodulering, rörelsemönster och behandling av ryggmärgsskada har den här studien undersökt vilken roll serotonin spelar i den långvarigt ökade retbarheten i nervceller som vidarebefordrar information om musklerna till ryggmärgen. Detta gjordes genom experiment på djupt sövda råttor, som avlivades efter försöken. Resultaten visade att en minuts katodal likströmsstimulering på 1,0 µA gav upp till fem gånger så stor respons på en bestämd stimulering i nervceller som skickar information om musklerna, och responsen

fortsatte att vara dubblad så länge försöken pågick (som längst 49 minuter). Tillförsel av serotonin bland nervcellernas förgreningar i ryggmärgen gav inte någon signifikant påverkan på effekten av ryggmärgsstimuleringen. Man kan därför dra slutsatsen att en ökad mängd serotonin kring muskelafferenters axon i ryggmärgen troligen varken är nödvändigt för eller märkbart påverkar effekten av ryggmärgsstimulering. Vad gäller serotonin i andra delar av nervsystemet kan inga slutsatser dras kring signalämnets eventuella betydelse för det studerade fenomenet. Fenomenet behöver alltså kartläggas vidare genom ytterligare studier.

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Appendix I – Surgical procedures

Tracheotomy

Open surgical tracheotomy was initiated by a small skin incision ventrally on the neck. Connective tissues and strap muscles were subsequently parted by blunt dissection (using forceps and blunt scissors) until reaching the trachea. 3-4 preparatory ligatures threads were passed under the trachea. Further, a small horizontal incision is made between two adjacent tracheal rings, followed by a vertical incision through about two tracheal rings caudal of the horizontal incision. The tracheostomy tube was then inserted into the trachea and secured with the previously prepared ligatures. If necessary, excess skin incision was sutured up to minimize fluid loss from open wound, and the tracheostomy secured with extra cutaneous sutures. Spontaneous respiration was confirmed.

Intravenous tail catheter

One to two peripheral venous tail catheters were placed by first making an incision in the skin over a vein, proximal to the root of the tail. Blunt dissection was carefully done around the vein to free it from surrounding tissue, thus minimizing potential motor response when cannulating. A small catheter (Neocatheter, BD Neoflon TM, 24GA, Becton Dickinson infusion Therapy AB, Sweden), was subsequently inserted into the vein, and secured with several ligatures around the entire tail. Placement was confirmed by flushing the catheter with a small volume of sterile saline.

Laminectomy

Preparations for a laminectomy were made by – after incision and freeing of the skin – exposing the dorsal part of spinal column segments T10-L6 by blunt dissection. Laminectomy was performed on L2-L5. When needed, excessive bleeding was stopped by monothermical burning. Intraincisional analgesia (lidokain, 10 mg/ml, Distansapoteket Hässleholm) was administered in generous doses. Ringer acetate buffer (10-20 ml) was administered subcutaneously during the initial surgical procedures to compensate for fluid losses during the experiment.

Peripheral nerve dissection

To expose the peripheral nerves, the skin was first incised on the dorsal aspect of the calf and separated from adventitia and fascia by gentle blunt dissection. *Musculus biceps femoris* was dissected free, proximally ligated and subsequently removed, enabling visualization of *n. peroneus* and *n. tibialis*. These nerves were cut and ligated distally, and then dissected free from surrounding tissue, carefully as not to remove epineural tissue and vascularization (Figure 1, page 11). The nerve branches were cut distally and mounted on dual silver electrodes in a pool of mineral oil created using skin-flaps (Figure 1).

Mounting of animal to frame

The animal was mounted in a ventral recumbent position to a custom-made metal frame (Figure 8, page IV), and initially supported by a metal mesh under the chest and abdomen. The head was fixed by a clamp (placed between posteriorly of the superior incisors and above the nose), and one metal rod gently secured in each exterior auditory canal (Figure 8). The spinal column was thereupon fixed to the frame by a custom-made metal holder, bilaterally placed just under transverse spinal processes a few segments rostral of the aimed laminectomy. The hindlegs were secured to the frame with ligatures around the ankles, and the supporting mesh removed. A laminectomy was performed, exposing the second to fifth lumbar segments (L2-L5) without disrupting the *dura mater*. Skin flaps on the back and hindleg were tied to the frame, and the incisions filled with paraffine oil, thereby creating electrically isolated pools covering the exposed spinal cord and column respectively the peripheral nerves.

Appendix II – Additional figures

Figure 8: *Spotting scope for final alignment of electrode tips (A). Metal frame, carefully electrically grounded, to which the preparation is secured (B). Plush toy rat demonstrating fixation of the head (C), spinal column (D) and tail (E) to the metal frame.*

Appendix III – Drug procedures and protocols

5-HT

5-hydroxytryptamine hydrochloride, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. 0.2 mg 5-HT powder was diluted in 4.70 ml HCl of pH 4.5, giving a solution of 0.2 M 5-HT. The powder and diluted solution were stored refrigerated (+4-6°C) in UV-protected containers.

α-chloralose

α-chloralose, 97%, ACROS Organics, Geel, Belgium, stored UV-protected in room temperature.

60.0 mg α-chloralose powder was mixed with 20.0 ml distilled water, heated for about 30 sec in microwave and, thoroughly stirred in order for the solute to properly dissolve. The solution was then filtered through a coffee filter and stored at +4-6°C.

Pentobarbital

Pentobarbitalsodium, Distansapoteket Hässlehol, Sweden, of concentration 60 mg/ml, was diluted in homemade saline (0.09% NaCl in distilled water) to a concentration of 3 mg/ml. All concentrations of pentobarbital were stored at +4-6°C.

Gallamintriethiodid

Gallamintriethiodid, ≥98%, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. 100.0 mg gallamintriethiodid was diluted in 5.00 ml homemade saline (0.09% NaCl in distilled water), yielding a solution of 20 mg/ml. Diluted solutions were stored at +4-6°C.

CO_2 meter maintenance

The CO_2 meter is cleaned and calibrated according to manufacturer instructions. Cleaning is performed when needed, using Alconox detergent (Sigma-Aldrich, Germany), which is diluted 1:100 in warm distilled deionized water, and about 30 ml of it slowly flushed through the meter. Thereafter the meter is rinsed by slowly flushing it with 20 ml 99.5% methanol, followed by drying with low pressure air flow for several minutes.