ORIGINAL ARTICLE - NEUROSURGICAL ANATOMY



Usefulness of external anal sphincter EMG recording for intraoperative neuromonitoring of the sacral roots—a prospective study in dorsal rhizotomy

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Received: 3 July 2020 / Accepted: 5 October 2020 / Published online: 16 October 2020 \odot Springer-Verlag GmbH Austria, part of Springer Nature 2020

Abstract

Background In conus medullaris and cauda equina surgery, identification of the sacral nerve roots may be uncertain in spite of their anatomical/radiological landmarks. Mapping the sacral roots by recording the muscular responses to their stimulation may benefit from EMG recording of the External Anal sphincter (EAS) in addition to the main muscular groups of the lower limbs. **Method** In a consecutive series of 27 lumbosacral dorsal rhizotomy (DRh), authors carried out a prospective study on the reliability of the EMG recording of the EAS for identification of the S1 and S2 sacral roots.

Results An EAS-response was recorded in all the 27 (bilaterally) explored individuals, testifying good sensitivity and selectivity of the method. EAS-responses were obtained in 96.3% of the 54 stimulated sides of the S2 root versus in only 16.66% for the S1 root, so that an absence of response would indicate S1 rather than S2 level. Furthermore, comparison between myotomal distribution of the S1 and S2 roots showed a significant difference (p < 0.00001), so that myotomal profile may help to identify root level.

Conclusions EMG recording of the EAS can be recommended for current intraoperative neuromonitoring. This simple method also provides—indirectly by extrapolation—information on the sacral motor pathways of the external urethral sphincter (EUS), as the later has the same somatic innervation via the pudendal nerve and related S2, S3, and S4 roots. Method can be helpful not only for DRh, of all varieties, but also for spine surgery, correction of dysraphisms, lipomas and/or tethered cord, and tumor resection.

Keywords Sacral nerve roots \cdot External anal sphincter \cdot Intraoperative neuromonitoring \cdot Dorsal rhizotomy \cdot Selective dorsal rhizotomy \cdot Lumbo-sacral surgery \cdot Functional anatomy

Introduction

Recto-anal and micturition functions are at risk during surgeries in conus medullaris and lumbo-sacral spinal nerve root regions especially when performed for tumor resection or

This article is part of the Topical Collection on Neurosurgical Anatomy

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repair of spinal dysraphism pathologies. Furthermore, identification of the sacral root levels can be challenging in some situations, notably in dorsal rhizotomy for treating spastic diplegia. Therefore, intraoperative neuromonitoring (ION) of the sacral nerve root pathways can be beneficial in these indications.

A number of different methods have been developed to map and monitor the sacral nervous system and have been particularly explored and reported in detail by Deletis and coworkers [44]. The sensory pathways conveyed through the dorsal sacral roots can be traced through the potentials evoked by electrical stimulation of the dorsal penile or the clitoral nerves. Assessments can be made directly on the surgically exposed dorsal roots, by recording the pudendal dorsal root action potentials (DRAPs). Stationary waves of these pudendal somatosensory evoked potentials (SEPs) can also be recorded using subdurally placed electrodes on the spinal cord surface at the thoracic or the conus medullaris level. Also the P40 cortical wave corresponding to the travelling SEP can be recorded with scalp electrodes at the level of the cerebral cortex; these cortical potentials are very sensitive to anesthetics. All these above-mentioned procedures are for controlling the integrity of the corresponding sensory pathways during surgery. Same applies for the monitoring of the bulbocavernous reflex (BCR), which is recorded from the anal sphincter muscle under stimulation of the dorsal penile or the clitoral nerves; its reliability is far from being established for current practice. Over the recent years, the checking of the motor descending pathways has benefited from the development of the intraoperative motor evoked potential (MEP) technology; recordings come from the anal sphincter under transcranial electric stimulation of the motor cortex.

On the other hand, identification of the sacral myotomes by studying the muscle responses to ventral (=motor) root stimulation is an important part of ION during lumbo-sacral root surgery. Mapping of the sacral roots can be achieved by EMG recordings of the striated muscle sphincters, namely the external urethral sphincter (EUS) and the external anal sphincter (EAS). Although corresponding to different functions, according to basic anatomical knowledge, both of them obtain dominant somatic motor innervation from the S2, S3, and S4 ventral roots via the pudendal nerves [3, 5, 35]. Because considered easier to perform, EMG recordings are most frequently carried out on the sole EAS. Besides, they are estimated to provide reliable—although indirect—information about the EUS motor pathways, due to a similar somatic innervation [15, 44].

Only few among a large number of publications on dorsal rhizotomy (DRh) reported in detail on the mapping of the sacral roots using EMG recordings of the EAS; one publication even concluded on its lack of reliability [26]. Therefore, we have conducted a prospective study on this particular topic. In our protocol for DRh—of the keyhole interlaminar dorsal rhizotomy type [38]—that systematically explored the myotomal innervation of the (L2–S2) lumbo-sacral roots [12, 38], the muscle responses to electrical stimulation of the ventral (and also dorsal) roots were recorded not only from the main muscular groups of both lower limbs but also at level of the EAS to assess the motor sacral pathways.

The goal of the study was to evaluate the reliability of the EAS EMG recording, namely the "localizing value" of the EAS muscle responses to the stimulation of the S1 and S2 sacral root levels and therefore its usefulness for ION, especially for guidance of the DRh procedures.

Material and methods

The method used for this study was the one that we currently applied for DRh to treat spastic diplegia or quadriplegia in children with cerebral palsy (CP) [12, 38]. In brief, the protocol included (1) systematic mapping of the muscle responses to individual stimulation of the L2–S2 lumbar and sacral ventral (=motor) roots (VR), for identification of respective myotomes, and additionally (2) stimulation of their corresponding dorsal roots (DR), for estimation of the degree of hyperexcitability of their reflexive segmental circuitry in order to determine the amount of rootlets to be cut. The Institution's Medical Ethical Committee approved the protocol. An informed consent was received from all children's parents.

Patient selection for the study

Candidates for DRh were children whose disability was in relation to an excess of spasticity, resistant to physical therapy, and all conservative treatments including botulinum toxin injections. Timing for surgery was established on the regression on their functional development, calculated from the Gross Motor Function Measure (GMFM) score [31] and/or the deterioration of their locomotor apparatus (appearance of musculo-tendinous contractures, articular ankyloses, bone deformities....) [40].

Patients enrolled were those meeting the following criteria: spastic diplegia or quadriplegia amenable to L2–S2 lumbosacral DRh, who had complete intraoperative mapping including the S1 and S2 sacral roots, with EMG recording of the external anal sphincter (EAS).

Exploration of the muscle responses to radicular stimulation aimed to:

- · identify the root anatomical levels,
- define their corresponding myotomes, known to have important interindividual variations [11]
- confirm or modify the surgical plan that was preestablished by the multi-disciplinary team, based on the particular clinical features and objectives of each individual [12].

Surgical and monitoring protocol

Installation and setting All patients were operated under general anesthesia, without muscle relaxants to keep response to stimulation recordable. Installation was in prone position, with lower limbs accessible for visual observation and palpation by a trained physical therapist.

Bipolar needle-electrodes were inserted on each lower limb in the seven following main muscular groups (according to classical knowledge of their dominant motor root(s) of innervation): adductor longus (L2, L3), quadriceps rectus femoris (L3, L4), tibialis anterioris (L4), extensor hallucis (L5), hamstring biceps femoris (L5, S1, S2), triceps surae soleus (S1), and flexor digitorum (S2). In addition to them, the external anal sphincter (EAS) was recorded using two rigid bipolar needle-electrodes, 14 mm in length and 0.4 mm (27G) in diameter and uninsulated all over their length. Each needle electrode was inserted deep into the striated anal muscle: one at 3 o'clock, the other at 9 o'clock of the anal circumference (Fig. 1). Distance between the two tips of the bipolar needle-electrode was approximately of 5 to 7 mm. Proper insertion was checked, before antiseptic application and drapping of the lumbo-sacral operative region, by verifying impedance and eliciting grapho-elements on screen with repetitive finger tapping at the anal orifice.

Recording the external urethral sphincter (EUS) was not performed for the following reasons: first, the need for a special urodynamic bladder diagnostic unit with electrode-built urethral catheter [14], second, the fact that the EUS has according to classical knowledge—same motor somatic innervation as the EAS; thus, some extrapolation can be estimated possible [15, 16].

Surgical approach Approach was the keyhole interlaminar dorsal rhizotomy (KIDr) modality that we already presented [38]. Its principle is to preserve the spinous processes and interspinous ligaments to minimize destabilization of the lumbar spine, while allowing access to all of the L2 to S2 roots independently and successively (Fig. 2).

Approach consisted of three enlarged midline interlaminar (IL) "openings," currently at L1–L2 interspace to access L2 and L3 roots, at L3–L4 to access L3 and L4 roots, and at L5–S1 for accessing S1 and S2 roots. The height of each (enlarged) interlaminar space and corresponding (midline) dural opening was on average 2 cm. This permits access to two adjacent roots on the same side (one upper and one

lower). The S1 and S2 sacral roots (the only levels considered for this study) were reached through the L5–S1 interlaminar dural opening. Their ventral (and dorsal) components were individually targeted at the exit from (and entry to) their corresponding dural sheath, and separated on a length of 7 mm \pm 2 mm. Dissection and manipulation of roots were minimal to avoid altering their electric conduction.

Stimulation and recording Root stimulation was performed using a fine, flexible, bipolar probe, with an interpole distance of 4 ± 1 mm. The probe was kept in close contact with the root for a few seconds to obtain a steady state sufficient to interpret the muscular responses. Three consecutive stimulations provoking similar responses were required before interpreting responses (Fig. 3).

Stimulation of the ventral root (VR) consisted of a square biphasic wave of 0.1-ms duration, with 2 Hz of frequency, at low intensity (200 μ A). This intensity, slightly above threshold for triggering motor response (currently at 50 μ A), was chosen to minimize the risk of spreading the current to neighboring roots. It was also verified that, applied to dorsal root, this low intensity did not elicit reflex muscle contraction. Additional stimulation of the dorsal roots was systematically performed to evaluate the degree of their involvement in tone circuit excitability and to grade responses according to Fasano's classification [9, 10]. This was deemed important to help determine the amount of dorsal rootlets to be cut [12]. However, this will be briefly mentioned as it is not the subject of the present study.

The EMG responses were visualized on the screen of the recording machine (Nimbus, i-Care, Innopsys Medical Device, Parc d'Activités Activestre, 31390 Carbonne,

Fig. 1 Left: Patient installed in prone position with needleelectrodes inserted in the seven main muscular groups of both lower limbs, and in the external anal sphincter (EAS). Right: Insertion of the two pairs of needle electrodes on each side of the striated EAS



Lower limbs & External anal sphincter



(two) Bipolar needle electrodes in EAS



Fig. 2 Upper left: Schematic drawing of interlaminar (IL) vertebral levels where selected roots can be targeted for DRh: at L1–L2 for roots L2 and L3; at L3–L4 for roots L4 and L5; at L5–S1 for roots S1 and S2. The IL spaces to be opened are determined according to the preoperative plan for root sectioning (tailored operation) [38]. Fenestrations represent the IL vertebral levels where targeted roots are approached intradurally. The post-op X-ray of lumbar spine (antero-posterior view) shows the (enlarged) IL fenestrations with respect of lamina (approximately one-fourth of each) and spinous processes. Roots S1 and S2 are targeted at the L5–S1 IL space (arrow). Lower left: Operative view of the interlaminar (enlarged) openings at L1–L2, L3–L4, and L5–S1 levels, with preservation of the spinous processes and interspinous ligaments. At each fenestrated level, the inferior two-thirds of upper lamina and the superior three-

France) through a free-running recording. To accept a graphoelement as a compound motor action potential (CMAP), criteria were the following: (a) morphological aspect of depolarization and repolarization wave, (b) synchronization with the root stimulation, and (c) amplitude higher than 120 μ V. Information recorded from ION was used to instantaneously decide, i.e., confirm or modify the surgical pre-planning, regarding the levels and amount of the dorsal roots that needed to be cut [12].



Intra-dural access to (left) S1 & S2 roots at dural sheath

fourths of lower lamina are resected on midline and flavum ligament removed, so that dura and arachnoid can be opened (on midline) over 20–30 mm in height. The S1 and S2 roots will be accessed through the L5–S1 IL space (arrow). Right column: Operative views showing steps of S1 and S2 root exposure, from top to bottom: • Fenestration at L5–S1 (enlarged) IL space, seen from right side of the patient; • Incision of dura on midline and suspension with stitches to optimize intradural approach; • Cauda equina, covered by arachnoid, exposed; • Access to (left) S1 and S2 roots at their respective foraminal dural sheath; view is from right side through an oblique trajectory passing underneath the arch formed by the (respected) interspinous ligament (=keyhole principle). Ventral root (VR) and dorsal root (DR) are accessed at exit to (for VR) and entry from (for DR) at respective dural sheaths

Study design

Only the EMG recordings of the responses to the stimulation of the S1 and the S2 (ventral) roots were selected for the study. The recordings corresponding to the whole targeted roots (i.e., L2 to S2) have already been analyzed and published elsewhere [11].

As the exploration was bilateral in all 27 enrolled individuals, the total number of recordings was 54.



S1 (ventral) stimulation

Fig. 3 Examples of (free-running) recordings of compound motor action potentials (CMAPs) of the triceps surae (soleus), hamstrings, flexor digitorum and external anal sphincter (anal), as displayed on the "paused" monitoring screen. *X* axis represents the duration in milliseconds (from 0 to 600 ms) and *Y* axis represents the amplitude in microvolt (from -250

The muscular groups presumed to belong to the myotomes of S1 and of S2 roots were those manifesting an obvious contraction on clinical observation by the physical therapist and a typical CMAP on the EMG recording. For each considered muscular group, the total number of cases with response to stimulation and its percentage out of the 54 explored sides were calculated. Thus, an averaged "root profile" was established for S1 and for S2, independently.

Profiles from S1 and S2 sacral roots were then compared using chi-square test for statistical analysis. Indeed this would be of practical importance when respective identification of the S1 and S2 roots appears to be difficult during surgery. Interindividual variations between patients' sacral myotomes were watched for. Also symmetry/asymmetry in the myotomal innervation of the S2 (ventral) sacral root between left and right side was studied.

Results

Of the 27 enrolled individuals, 20 were males and 7 females. Age ranged between 5 and 17 years, with an average of 8 years. In the 27 individuals, exploration was bilateral, so that data from the 54 sides (cases) were studied. Analysis of the various muscle-responses in the 54 cases showed some

S2 (ventral) stimulation

to 250 μ V). Left view: CMAPs evoked by stimulation of the (ventral) root of S1, in soleus, hamstrings, flexor digitorum, but not in anal sphincter. Right view: CMAPs evoked by stimulation of the (ventral) root of S2, in hamstrings and flexor digitorum and anal sphincter (yellow arrow on CMAP)

degree of interindividual variability between patients and sides. Detailed data for the L2–S2 explored roots, as a whole, has been previously reported elsewhere [11].

EAS EMG responses to S2/S1 root stimulation

An EAS muscle response to stimulation of the (ventral) sacral roots was obtained in all 27 explored individuals, bilaterally in 25 of the 27 and unilaterally in 2. An EMG response to right side stimulation was observed in all of the 27 individuals, and in 25 to left side stimulation; difference was not significant.

The near consistency of the EAS to respond to (the S2) sacral root stimulation, as well as the constancy of the EMG recording to catch responses, testifies to the good sensitivity and reliability of this (simple) method for use in the current practice.

Comparison of the EAS muscle responses between the S2 and S1 (ventral) roots

• Stimulation of the S2 root provoked an EAS-response in 52 of the 54 sides, i.e., in 96.30% of the cases, bilaterally in 25 patients and unilaterally in 2.

• Stimulation of the S1 root elicited an EAS-response in only 9 of the 54 sides, i.e., in 16.66% of the cases, on both sides in 2 patients and on one side in 5.

Study shows that innervation of the EAS is comes predominantly by the S2 root and less by the S1 root (96.30% vs 16.66%), as illustrated in Fig. 4.

Myotomal profiles of the S2 and S1 (ventral) roots

Recordings of not only the EAS and of the main muscular groups of the lower limb allowed defining a myotomal profile of muscle-responses for each of the considered S2 and S1 sacral roots. Illustration of respective averaged profiles is given in Fig. 4. Importantly, those profiles are significantly different as shown by statistical analysis ($x^2 = 69.652$, p < 0.00001). Such difference should help to identify S1 and S2 respectively when anatomical surgical landmarks are practically uncertain.

Symmetry/asymmetry of myotomal innervation of the S2 sacral root

Profiles were considered asymmetrical when there was a difference at a muscular group's response between the two. Comparison of the myotomal profiles between the two sides plication (ulceration, hematoma, infection...) or side effect (pain, dysesthesias...). The CMAPs obtained from the EAS—although of lower

the anal canal did not produce any immediate or delayed com-

Insertion of a the double needle-electrode on each side of

The CMAPs obtained from the EAS—although of lower amplitude that those recorded from the main muscular groups



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of the S2 sacral roots showed an asymmetrical pattern in 7 of the 27 individuals, i.e., in 26%.

Discussion

Validity of the method

Bipolar needle-electrodes were chosen, similarly to the ones that we were currently using for recording the muscle responses of the lower limbs [11]. The intramuscular needletype was preferred to the surface-type because of better stability linked to its location inside the deep-seated striated sphincter, as pointed out from comparative studies published in literature [17–19, 30, 32, 45]. Furthermore, compared with surface-electrodes located in peri-anal position, the needleelectrodes did not record responses from the surrounding muscles, especially the levator ani and the gluteus muscles [44]. Hook-wire electrodes were not used in the study, although advocated to enhance accuracy [4, 5].



of the lower limbs—were sufficiently obvious to be recognized from the running-EMG baseline. CMAPs were interpretable in all the 27 patients of the series, so that *reliability* can be considered good. Because CMAPs were obtained using a rather low intensity of stimulation (200 μ A), *sensibility* of the method can be estimated as good. Selectivity was also good, as any particular diffusion to the EAS of muscular twitches from the neighboring muscles of the lower limbs was noticed. This was verified particularly with the gluteal muscles; on stimulation of the ventral root of L5 root, known to predominantly innervate the gluteal muscles, there were no CMAP responses on the EMG recordings of the EAS.

Interpretation of results

CMAPs were obtained in 96.3% of the cases that received S2 (ventral) root stimulation versus 16.66% of the cases under S1 (ventral) root stimulation (Fig. 4). This difference seems to be important enough to interpret an absence of response in the explored root as corresponding to the S1 level rather than the S2 level. Comparison to literature data cannot be established as to our knowledge there was no similar study with such systematic methodology, even in the reference publications by Phillips and Park (123 patients) and Schirmer et al. (129 patients) [29, 34].

When considering the muscle responses of the whole recorded muscular groups belonging to the lower limbs, study shows different profiles of myotomal distribution for the S1 and the S2 sacral roots (Fig. 4), with a significant difference (p < 0.00001). Such difference in profile may be helpful to differentiate the S2 from the S1 root when surgeon encounters a challenging operation. Some asymmetry in myotomal innervation of the S2 root was noticed in 26% of the individuals. We do not consider this asymmetry important enough as to compromise the validity of the concept of root profile. However, potential asymmetry pleads for bilateral exploration of the sacral roots in ION procedures. Of note, symmetry of innervation in the pelvic floor and corresponding organs has been occasionally noticed in the literature [6, 7].

Limitations of the study

An important limitation of our study was *the absence of additional systematic individual stimulation of the S3, S4, and S5 (ventral) sacral roots.* This limitation was imposed by the design of the surgical approach, which did not allow accessing these roots at their respective intrasacral dural sheath. For ethical reason, we did not extend approach just for scientific purpose. It is well known that S2 is not the sole root of the sacral S2–S4 sacral roots at the origin of the pudendal nerve. However, in a majority of our patients, we stimulated the hemicauda equina below S2, as a whole and, as expected, important CMAP responses were observed on the monitor screen in all of the tested cases. But these recordings were not included in the study because they were not part of the protocol.

Another important limitation was the absence of EMG recording of the external urethral sphincter (EUS). For such recording, special equipment would have been necessary [3, 14]. This was not foreseen when protocol was launched as it was deemed that this would increase the complexity of the surgery, taking into account the already relatively long duration of the procedure (5 h \pm 1 h on average). Another reason was that recording of the sole EAS, which according to classical anatomical knowledge, has the same somatic innervation as the EUS from the pudendal nerves and corresponding S2, S3, and S4 sacral roots would be sufficient for accuracy and safety of the surgery. This however can be argued, especially regarding protection of the micturition function.

Extrapolation of EAS neurophysiological exploration to the EUS and consequently the micturition function

Based on a same somatic innervation of the striated urethral (EUS) and the striated anal (EAS) sphincters, it could be postulated that recording of just the EAS—easier to perform might give information on the micturition function, by extrapolation [8, 15, 44]. Participation of the S2 root to innervation of the EAS was constantly found in our 27 patients' series. However, S2 is not the sole root at the origin of the pudendal nerve. It has been even more pointed out the predominance of the S3 and S4 roots in the innervation of either one of the two sphincters [2].

Participation of the S1 root to the EAS innervation has only been mentioned before in literature once by Vodusek and Deletis [44]. This might be due to the fact that this observation was somehow neglected. Involvement of S1 root could be via anastomoses of the sacral plexus with its upper level [21].

Reliability of extrapolation of EAS monitoring to EUS interpretation should be nuanced for several anatomical/ physiological reasons. Motricity of both sphincters is known to be physiologically influenced by contraction of the levator ani, which is predominantly innervated by the pudendal nerve. Furthermore, micturition entails strong coordinated relationship with detrusor; but motricity of detrusor is triggered by the same sacral roots and the pudendal nerve.

Even more important is the role-played by the autonomic nervous system, which includes the parasympathetic system (transmitted via the sacral roots) and the orthosympathetic system (arising from the thoracolumbar chain and travelling through the hypogastric nerves to innervate the visceral smooth musculature). Both autonomic systems innervate the EAS and the EUS, the anal internal sphincter the rectal wall, the vesical internal sphincter complex, and the detrusor wall [3, 44]. At present state of ION, clinical exploration of the autonomic nervous system is developed enough to be used in current intraoperative practice.

Usefulness of the EAS EMG recordings

Intraoperative mapping of the sacral roots using EMG recording of EAS can help when identification of the sacral roots is difficult during surgery. This applies particularly into the differentiation between S1 and S2 root levels when performing DRh. Whereas S1 root is generally a target, S2 targeting is always matter of dilemma, due to its involvement in micturition and sexual function [4, 13, 20]. Cutting S2 dorsal rootlets, not only bilaterally but also unilaterally although to a lesser extent, exposes to urinary retention [44]. Conversely, by avoiding cutting S2 rootlets might leave reflexive circuits that continue to drive spasticity in lower limb, principally in the hamstrings' muscular group. Decision is a matter of estimation of the degree of harmful spasticity that could be driven through the S2 root. Decreasing tonicogenic input in the S2 circuitry may be particularly useful in patients with severe hyperactive bladder and uncontrolled micturition [12]. Mapping by stimulation of the ventral component of the root, testing of the hyperexcitability of the reflexive circuitry by stimulation of its dorsal component is wise conduct [1, 12, 22–25, 27, 28, 43]. Targeting S2 root for neurophysiological assessment before decision to treat or not, and-if so-determine which amount of dorsal rootlets should be cut, is our current practice [12]. This ION protocol appeared useful in adjusting preoperatively the plan established by the multidisciplinary team caring the child.

Besides ION for DRh, similar mapping (and testing) methods can be applied to other lesioning procedures, namely the microsurgical lesioning in the dorsal root entry zone [41, 42]. Particular indication is for hyperspastic paraplegias in severely disabled adult patients, when they are not amenable to intrathecal baclofen therapy. The microsurgical DREZotomy (MDT) procedure can also be applied to patients affected with well-circumscribed chronic cancer pain or deafferentation neuropathic pain, notably in the perineum [36, 37, 39].

More generally, EAS EMG recording of the sacral roots can be useful in the surgery for lumbo-sacral dysraphism, lipomas, and tethered cord [17–19, 32, 33, 44]. Besides, EAS EMG recording is currently integrated to the monitoring of the MEPs under transcranial stimulation of the motor cortex, purpose being to control the integrity of the conus medullaris and cauda equina neural structures along surgery for spine and lumbo-sacral tumor resection [5, 17].

Conclusions

EMG recording of the EAS under sacral stimulation permits reliable identification of the sacral roots, particularly for differentiation between the S2 and the S1 roots, when anatomical landmarks prove insufficient. The rates of EAS-responses under respective stimulation of their VRs are very different (96.3% for the S2 root vs 16.66% for the S1 root), as well as their respective myotomal profiles (p < 0.00001).

The simplicity and reliability of the method—classical, but not quite routinely utilized—justify a more widely use in current neurosurgical practice.

The data that were harvested from the study may bring some more precise insights into the sacral root functional anatomy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (name of institute/committee) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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