Early nociceptive evoked potentials (NEPs) recorded from the scalp

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Abstract

Objective

Neurophysiological investigation of nociceptive pathway has so far been limited to late cortical responses. We sought to detect early components of the cortical evoked potentials possibly reflecting primary sensory activity.

Methods

The 150 IDE micropatterned electrode was used to selectively activate Aδ intraepidermic fibres of the right hand dorsum in 25 healthy subjects and 3 patients suffering from trigeminal neuralgia. Neurographic recordings were performed to assess type of stimulated fibres and check selectivity. Cortical evoked potentials were recorded from C3'-Fz and Cz-Au1.

Results

Neurographic recordings confirmed selective activation of Aδ fibres. Early components were detected after repetitive stimulation (0.83/s rate and 250-500 averages); the first negative component occured at 40 ms (N40) on the contralateral scalp.

Conclusions

The provided data support the hypothesis that N40 could be the cortical primary response conducted by fast Aδ fibres.

Significance

This is the first report of early, possibly primary, cortical responses in humans by nociceptive peripheral stimulation. Although not perfected yet to allow widespread diagnostic use, this is probably the only method to allow fully objective evaluation of the nociceptive system, with important future implications in experimental and clinical neurophysiology.

Keywords

Neurophysiology

Nociception

Pain

Evoked Potentials

Primary response

Micropatterned

1 Introduction[1]

Objective investigations in clinical neurophysiology are still limited by a seemingly insuperable barrier: nociception. Whilst the whole lemniscal system carrying discriminative touch and proprioception can be studied in detail by means of somatosensory evoked potentials (Muzyka and Estephan, 2019) and peripheral nerve conduction studies (Tavee, 2019), the nociceptive afferents remain largely unexplored. The reasons for this situation are twofold. Firstly, the traditional method to elicit nerve depolarization with electric pulses recruits the large myelinated fibres first, so activity arising in nociceptive fibres is lost in the aftermath of the earlier and larger non nociceptive response. Secondly, the methods to overcome such pitfall, based on laser evoked potentials (LEPs), are far from satisfactory, because of their cost, potential skin damage, lack of specificity (Leandri et al., 2006; Mouraux and Iannetti, 2009) and uncertain synchronization due to the very nature of the stimulus. Nevertheless, in lack of a better method, LEPs are widely employed and the late components recorded from the scalp regarded as the "gold standard" to study nociceptive afferents (Cruccu et al., 2004). Although microneurographic recordings can detect even C fibre activity after laser stimulation (Jonas et al., 2018; Qiu et al., 2003), the afferent volley is far too small and desynchronized to be seen with standard near nerve electrodes, and the only chance to get some reflection of the stimulus occurs after substantial processing and amplification by the cerebral cortex. Matter of fact, on one hand the very latencies of LEPs are compatible with slow afferent fibres and subsequent cortical processing, on the other they seem far too long to be generated by the fastest nociceptive fibres, which should reach the cortex at latencies not later than two or three times the primary cortical response evoked by electric stimulation of the median nerve, that is approximately 20 ms. Fast peripheral A fibre nociceptive activity has now been demonstrated in humans (Nagi et al., 2019) after having been documented for some time in animals (Burgess and Perl, 1967; Lawson, 2002). Similarly, sensory primary cortical areas were found to be involved in nociception in monkeys and humans (Treede and Apkarian, 2008). The above evidence led us to believe that, provided an electric stimulus could

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Abbreviations

150 IDE 150 μm InterDigitated Electrode

1000 IDE 1000 μm InterDigitated Electrode

IASP International Association for the Study of Pain

LEP(s) Laser Evoked Potential(s)

PREP(s) Pain Related Evoked Potential(s)

SEP(s) Somatosensory Evoked Potential(s)

NEP(s) Nociceptive Evoked Potential(s)

VAS Visual Analog Scale

selectively activate fast nociceptive afferents, a much earlier activity than LEPs ought to be recorded from the scalp. Attempts at delivering selective electric stimuli have been performed since the turn of the century and several types of electrodes have been designed for the purpose (Inui et al., 2002; Kaube et al., 2000), but only late scalp responses similar to LEPs were recorded (Inui et al., 2002; Inui and Kakigi, 2012; Katsarava et al., 2006). No early response was described with this and subsequent modified versions (Lelic et al., 2012), while the selectivity of the design, at least for some of them, was challenged in later experiments (La Cesa et al., 2018; Perchet et al., 2012) and computer simulations (Leandri et al., 2018). The recent introduction of a new micropatterned electrode ought to make it possible to perform a selective electric stimulation of a relevant number of intraepidermal fibres without undesired activation of large diameter afferents, and, what is most important, allow a long sequence of stimuli to be delivered without fear of skin damage (Leandri et al., 2018). So the right conditions are now set to detect even small evoked responses from scalp derivations after electric stimuli with nociceptive character. The main aim of this paper is to describe the early, small scalp responses found after such stimulation, which we think should arise from the primary somatosensory cortex and could be considered equivalent to the N20 component usually obtained after stimulation of the median nerve.

2 Methods

2.1 General design

Our study was meant to be a proof of concept research whose results were expected to be the basis for further more extensive investigations in normal subjects and in patients, hence we limited our research to the minimum necessary number of subjects, preferring to perform different types of experiments that could be framed into four sets, as follows. First, we sought an experimental demonstration that the micropatterned interdigitated electrode with interrail gap of 150 μm (150 IDE) excited a comparatively large number of slow conducting fibres without coactivating fast bundles. This was done by near nerve recordings from the radial and infraorbital nerves, taking advantage of the new possibility of delivering a high number of stimuli at relatively fast rate. Second, we wanted to confirm that the slow random 150 IDE stimulation evoked similar responses as those traditionally obtained after laser pulses, to be compared with the repetitive stimuli that we used to detect the early components. The third set of experiments was aimed at investigating if it was possible to record cortical responses after the 150 IDE stimulation with latencies compatible with the conduction velocities detected at peripheral level. Such responses were to be compared with those obtained after the unselective stimulation by a similarly shaped but unselective electrode with interrail gap of 1000 μm (1000 IDE), where large Aβ fibres would be involved (Leandri et al., 2018). Fourth, we designed an ancillary study on the intensity needed to reach perception threshold as a function of the number of pulses constituting each stimulus, which would provide further evidence about

selectivity of the stimulation used and was expected to confirm that the 10 pulse stimulus only needed current intensities near the rheobase for the activated fibres, hence the lowest possible.

2.2 Subjects

The study involved 25 healthy subjects and 3 patients suffering from “essential” trigeminal neuralgia. Table 1 summarizes the experiments (columns) undergone by each subject (rows). The patients with trigeminal neuralgia (labelled as a, b and c) underwent a standard diagnostic procedure for trigeminal neuralgia which implies comparison of the recordings from the affected with the healthy side (Leandri and Favale, 1991). In our subjects the near nerve recordings from the infraorbital trunk were performed on the healthy side, to rule out possible (but very unlikely) alterations due to impairment of nerve conduction at its peripheral branches. The healthy subjects (labelled from 1 to 25) were volunteers recruited from the medical personnel of the Department of Neurology at the University of Genova and Sapienza University of Rome. Subjects from 1 to 10 underwent the experiments implying recordings from the scalp and in 3 of them also the near nerve study from the radial trunk was performed. Subjects from 11 to 25 were studied as to their perception threshold as a function of stimulus burst duration. In two of them the radial near nerve recording was carried out. Age in healthy subjects ranged from 25 to 45 years. All subjects were right handed. The study was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and had been approved by the local ethical committee. Informed consent was always obtained.

Subject n	Age/Sex	Radial Near nerve 150 IDE 1000 IDE	Trigem Near nerve 150 IDE 1000 IDE	C3'-Fz Early NEPs 150 IDE	C3'-Fz Early SEPs 1000 IDE	C4'-Fz Ipsilateral Early NEPs 150 IDE	Cz-Au1 Late NEPs 150 IDE	Perc 150 IDE
a	58/F		**R**					
b	65/M		**L**					
c	59/F		**L**					
1	27/F			●	●		●	
2	36/M	●		●	●		●	
3	28/M			●	●	●	●	
4	26/M			●	●		●	
5	29/F			●	●	●	●	
6	30/F			●	●	●	●	
7	45/M	●		●	●		●	
8	44/F	●		●	●		●	
9	40/F			●	●	●	●	
10	42/M			●	●	●	●	
11	39/M	●						●
12	25/F							●
13	25/F							●
14	26/M							●
15	30/F							●
16	28/F							●
17	27/M							●
18	25/M							●
19	27/F							●
20	31/M							●
21	34/F	●						●
22	26/M							●
23	26/F							●
24	32/F							●
25	30/M							●

Table 1. *General plan of experiments on 25 healthy subjects and 3 patients suffering from trigeminal neuralgia. The first two columns report identification, with letter (patients) or number (healthy subjects) , age and sex. The third column labelled "Radial" shows which subjects underwent near nerve recordings from the radial nerve after stimulation of the hand dorsum with the selective 150 mm and unselective 1000 mm interdigitated electrode (150 and 1000 IDE). The fourth, "Trigem" column, relates to patients suffering from essential trigeminal neuralgia, and the side of recording (right "R" or left "L"). The fifth column, marked "C3'-Fz, Early NEPs, 150 IDE" shows the subjects where the hand dorsum had been stimulated with the 150 IDE (0.83/s stimulation rate) to record contralateral early nociceptive evoked potentials (NEPs) from the C3'-Fz scalp derivation. Sixth column (labelled C3'-Fz, Early SEPs, 1000 IDE): the same subjects were also stimulated with the 1000 IDE to obtain non nociceptive contralateral somatosensory evoked potentials (SEPs). Seventh column (C4'-Fz, Ipsilateral, Early NEPs, 150 IDE): to show the 5 subjects where recordings were also performed ipsilaterally for comparison with the contralateral side. Eight column (Cz-Au1, Late NEPs, 150 IDE): subjects with recordings from Cz-Au1 scalp derivation after random stimulation with selective 150 IDE. Ninth column (Perc, 150 IDE): subjects where stimulation with 150 IDE had been tested at different burst durations to assess the perception threshold.*

2.3 Stimulation

This study was focused on the micropatterned interdigitated electrode (150 IDE) object of a previous publication (Leandri et al., 2018). The reader is referred to that paper for details of electrode design and analysis of electric characteristics. The electrode was meant to generate in the whole covered area a uniform and very shallow electric field, depolarizing a comparatively large number of intraepidermal nerve endings and fibres without involving subepidermal axons. It was considered to combine selectivity with efficiency in recruitment of small slow fibres (Leandri et al., 2018). In the current work, the electrode dimensions were 15x15mm (used on the hand) or 10x10 mm (on the lip) according to the experiment performed. The electrode is manufactured under license by Bionen S.A.S. (Florence, Italy). As an alternative method of stimulation a similarly interdigitated electrode but with a much wider interrail gap of 1000 mm (1000 IDE) was used to generate electric fields in deeper skin layers, so to activate large fibres. The same electrode had been proposed in the original paper (Leandri et al., 2018) to investigate the effect of a non selective activation of nerve fibres at the same sites where the 150 IDE was used. For each site, the dimensions of the 1000 IDE were identical to the 150 IDE. In order to activate the thin Aδ fibres with as little intensity as possible, a burst consisting of 10 short duration pulses (0.2 ms width and with interpulse period of 1 ms) was generally used. Only when short latency near nerve recordings were performed, just a single pulse stimulus was used (see sections 2.5 and 2.6). In experiments dealing with perception thresholds a whole series of different bursts (composed by 1, 2, 4, 6, 8, 10, 15 and 20 pulses) was used. Stimulation sites were the right hand dorsum, between the 1st and 2nd metacarpal bone in the healthy subjects and the upper lip of the unaffected side in patients with trigeminal neuralgia. Intensity of stimulation was always 1.5 times the perception threshold when the 10 pulse bursts were used (perception threshold ranged from 0.2 to 3.9 mA). In the case of 1 pulse stimulus, the intensity was kept at the perception threshold (ranging from 2.0 to 5.6 mA). Bursts were generated by an arbitrary waveform generator TG2511 (Thurlby Thandar Instruments Ltd, Huntingdon, UK) triggering a constant current stimulator (DS7A, Digitimer Ltd, UK). With 150 IDE two

different stimulations were used a) random stimulation every 5-10 seconds to obtain nociceptive scalp responses in the range of the intermediate/late event related responses, with 30-40 averages as in traditional LEPs or PREPs; b) continuous repetitive stimulation at the rate of 0.83/s to detect the small early responses, with 250-500 averages. When random stimulation was used in order to generate the late cortical responses, attention was monitored asking the subject to report after each stimulus the intensity of perception according to the Visual Analog Scale (VAS) from 0 (no sensation) to 10 (strongest possible painful sensation). During repetitive stimulation the subject was just asked to lay supine as relaxed as possible, with eyes open, and to report if there were any relevant variations in the perception of the stimulus.

2.4 Signal and data processing

Signals from near nerve and scalp electrodes were amplified between 50,000 and 100,000 times, with bandpass 0.1-2,000Hz, 2nd order Butterworth analogic filtering (LT amplifiers by Vertigo, Genova, Italy). They were then processed by an analog to digital converter (NI PCIe-6320, X Series Multifunction DAQ, 16 Bit, 250 KS/s sampling rate by National Instruments, Austin, Texas). A dedicated software, developed with the graphic language LabView 2014® (National Instruments, Austin, Texas), acquired 25,000 samples for 1000 ms after each stimulus, thus providing a high definition recording with a dwell time of 0.04 ms. Each single response was stored onto hard disc and kept for later averaging. At the offline analysis all records were visually inspected to discard those with biological artefacts or electromagnetic interference. Due to the very small amplitude of the scalp early responses, the high quality of the averaged signals was of paramount importance. As an adjunct to the recordings from the electrodes, per each single response we also monitored and stored onto disc the actual intensity and voltage of the delivered stimulus. This was done through a purpose built device set as an interface between stimulator and subject. Intensity was assessed at the output of the stimulator as voltage drop across a series mounted 200 Ohm resistor. Voltage was read as the potential difference between the terminals of a parallel mounted 200 KOhm resistor. The obtained values were processed by an active circuit and transmitted to the NI PCIe-6320 board via an optically isolated interface to maintain the floating characteristics of the subject's electrical circuit. The software converted the data into the desired units and also calculated the impedance of the electrode related to the actual pulse delivered, which was between 16 and 30 KOhm for the 150 IDE.

2.5 Technique for the radial near nerve recordings

Near nerve recordings were performed in 5 subjects from the radial nerve at its wrist notch on the radium. After having coarsely detected the nerve location with a surface stimulator, an active electrode 0.35 mm in diameter and 40 mm long, Teflon™ insulated but for 1 mm uncovered tip (Bionen S.A.S., Florence, Italy) was inserted at the same site. Its approach to the nerve was guided by delivering a 1 mA current pulse of

0.2 ms duration, with a rate of 3/s until the subject reported definite perception in the peripheral innervation area. The needle was then tentatively moved until a sensory threshold of at least 0.2 mA was reached, with perception irradiated on the area selected for stimulation, so the tip could be considered having penetrated the epineurium. The reference electrode was an uninsulated needle 0.30 mm diameter (Bionen S.A.S. Florence, Italy) inserted at distance of 10 mm from the active electrode perpendicularly to the nerve. Stimulation was performed on the hairy skin of the hand dorsum, between the I and II metacarpal bone. The 1000 IDE was used for non selective stimulation of the site, whilst the 150 IDE was used for selective stimulation of the nerve free endings at the same site, which had been marked with dermatographic ink. Stimulus rate was 0.83/s as for the scalp recordings, to avoid fatigue of the slow conducting fibres. Only one single stimulus could be used because of the short latencies involved. Duration of the stimulus was 500 μs, suitable also for small fibres. This way, the Aδ stimulation was not optimal, but it was considered that the situation was a conservative one: if a difference could be demonstrated between the two recordings in this unfavourable condition, all the more a difference would exist when a more selective type of stimulation for Aδ fibres would be used. Stimulus intensity was always set at the perception threshold. Fifty responses were averaged and each type of stimulation was repeated three times, to assess reproducibility of the peaks.

2.6 Technique for infraorbital near nerve recording

In three patients a near nerve recording was performed from the infraorbital nerve after stimulation of the upper lip. Two identical Teflon™ coated needle electrodes with 0.35 mm diameter and 40 mm long (Bionen S.A.S. Florence, Italy) were inserted into the infraorbital foramen for about 5mm. The electrodes were considered suitably positioned if the threshold current was at least as low as 0.15 mA. Stimulus rate was 0.83/s. At difference from the radial nerve recording, the two needles were inserted one near the other, according to the usual technique (Leandri et al., 1985). The upper lip was stimulated first with the unselective 1000 IDE and then with the 150 IDE selective electrode, always with a single 500 μs pulse at the rate of 0.83/s and intensity at the perception threshold.

2.7 Recordings from the scalp

Subdermal or surface electrodes (impedances always kept below 5000 Ohm) were used to detect signals from the scalp at the following sites according to the 10-20 EEG system: i) Cz referred to the ear contralateral to the stimulated side (Au1) and ii) the scalp sites overlying the hand projection on the somatosensory cortical homunculus (C3') referred to Fz. In five cases also the ipsilateral hand projection was explored with derivation C4'-Fz.

2.8 Statistical analysis

The two-tailed Student t-test for paired data was used for the following comparisons: 1) conduction velocity of the first component obtained after radial nerve stimulation: difference between 150 IDE and 1000 IDE; 2) conduction velocity of the first component obtained after infraorbital nerve stimulation: difference between 150 IDE and 1000 IDE; 3) early nociceptive evoked potentials (NEPs): N40 amplitude as measured on the ipsilateral versus contralateral recording; 4) latency of the early NEPs after stimulation using 150 IDE versus 1000 IDE; 5) NEP N40 latency using 150 IDE versus 1000 IDE. In the experiments on perception threshold, a repeated measures ANOVA (Analysis of Variance) with "number of pulses" as within-subjects factor was performed. The Shapiro Wilk test was run to confirm normal distribution of data. Significance level was set for $p<0.01$.

3 Results

3.1 Near nerve recordings from the radial nerve.

An example of the neurographic recording obtained in subject #2 is shown in Fig.1. Each set is composed of three traces obtained after averaging 50 responses in three consecutive sessions to check for reproducibility of components. The upper set s shows the activity evoked after non selective stimulation of the hairy skin at the hand dorsum with the 1000 IDE electrode. At perception threshold the stimulus was felt as a tap, but once it was increased to 1.5 times, a painful pin prick perception was added up. The first large negative peak pertains to the fastest group of fibres, conducting at 43.26 m/s, followed by several smaller peaks. The lower set of traces shows the response to selective stimulation through the 150 IDE electrode; this time the stimulus was felt as a pin prick since the perception threshold and the painful sensation increased when intensity was raised to 1.5 times. The first negative peak is related to fibres conducting at 25.28 m/s, about 13 m/s slower than the first peak evoked by non selective stimulation, followed by a smaller peak linked to fibres conducting at 31 m/s. The mean conduction velocities related to the two negative peaks in the five subject group were as follows: for the 1000 IDE stimulation 46.3 +/-2.1 m/s and for the 150 IDE 27.0+/-1.6 m/s, their difference being extremely significant ($p = 2.1 \times 10^{-5}$).

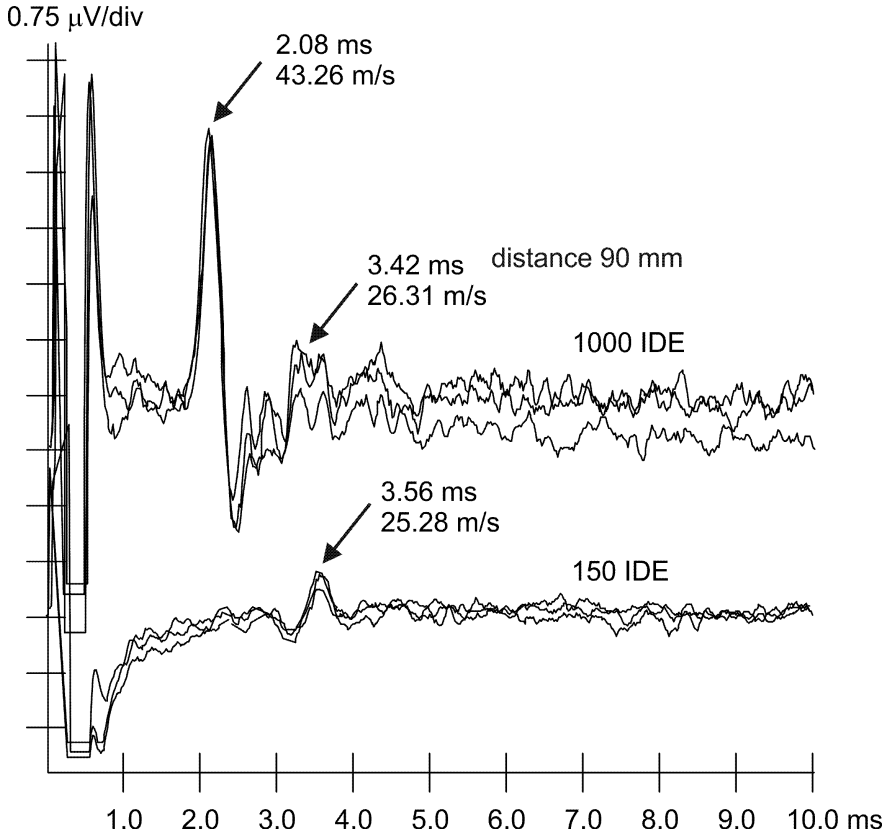

Fig. 1. *An example of near nerve recording from the radial nerve at the wrist in subject #2, after stimulation of the dorsal hand skin with the 1000 IDE (1000 μm interdigitated electrode)(upper set) and 150 IDE (150 μm interdigitated electrode) (lower set). Each set consists of three superimposed averages from as many consecutive sessions, as a check for reliability. Negative upwards. The unselective 1000IDE stimulation*

evokes a first negative peak at 2.08 ms (linked to an underlying velocity of 43.26 m/s) compatible to Aβ activity and a second negative peak at 3.42 ms (underlying velocity of 26.31 m/s) from Aδ activity. Stimulation through the 150IDE (lower set) only evokes the Aδ slower peak at 3.56 m/s (underlying velocity 25.28 m/s). This type of recording can be considered as evidence of selectivity of the 150IDE for Aδ fibres.

3.2 Near nerve recordings from the infraorbital nerve.

The example recordings of Fig. 2 are from subject b, each set showing three traces (averages of 50 responses) obtained in three consecutive sessions. Stimulation performed at 1.5 times the threshold current with the 1000 IDE electrode produced very similar results as those already reported (Leandri et al., 1987) and a first negative peak conducting at the mean velocity of 32.3+/-0.9m/s could be recorded. Stimulation with the 150 IDE evoked a low amplitude response with the first negative peak at the mean velocity of 20.7+/-1.3 m/s. The difference between the two peaks was extremely significant (p=0.0008) Perception was always a pin prick either at threshold level or at 1.5 times it.

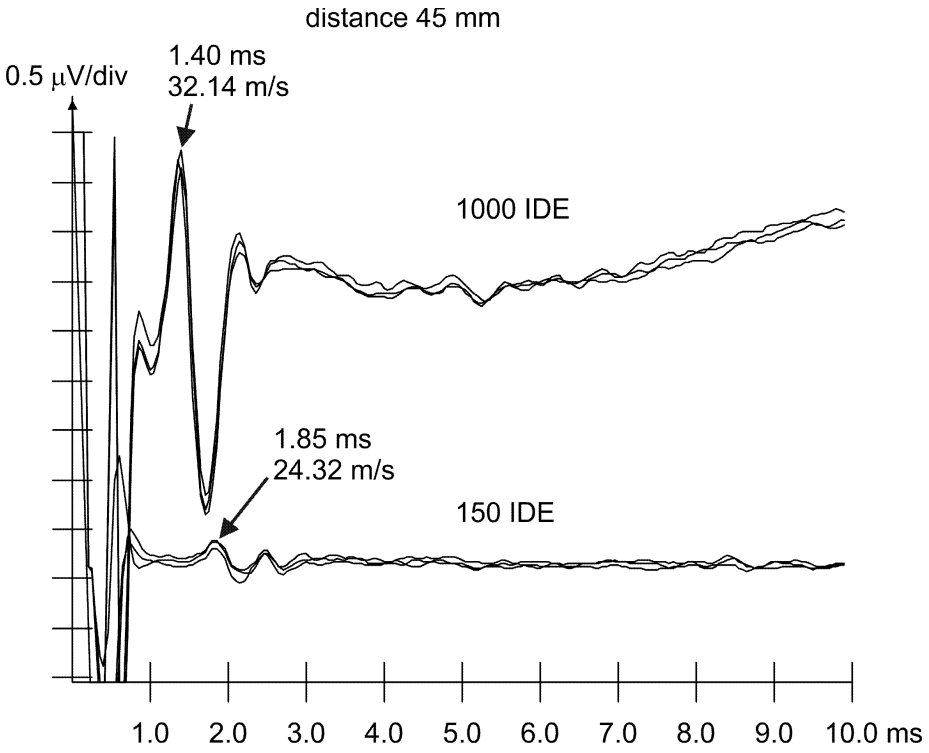

Fig. 2. *This is another demonstration of selectivity of the 150IDE (150 μm interdigitated electrode) for Aδ fibres in a different nerve than the radial. Here are sample recordings performed in patient b (suffering from essential trigeminal neuralgia) on the healthy side. Two needles have been positioned into the infraorbital foramen in contact with the homonymous nerve. Stimulation of the upper lip skin has been performed with the unselective 1000 IDE (1000 μm interdigitated electrode) and the selective 150IDE. Negative upwards. The 1000IDE evokes a first negative peak corresponding to a conduction velocity of 32.14 ms, hence in the Ab conduction range, whilst the 150IDE gives rise to a first negative peak corresponding to a 24.32 m/s velocity, in the Aδ range. No Aδ activity can be seen in the 1000IDE response as the travelled distance does not allow clear separation of the two components.*

3.3 Late nociceptive evoked potentials.

Random 10 pulse bursts were delivered through the 150 IDE electrode in 10 subjects. An example from subject # 8 is shown in Fig. 3. Three traces (30-40 averages each) are superimposed in the two sets. The upper set shows the recordings from Cz-Au1 derivation and the lower set those from C3'-Fz.. The usual N2-P2 component could be observed in Cz-Au1, with mean latencies of 130.4 +/- 12.3 ms (N2) and 235.8 +/- 33.0 ms (P2). The mean peak to peak amplitude of the N2-P2 complex was 36.9 +/- 9.4 μV. In derivation C3'-Fz (Fig. 3 lower set) component N1 was visible at the mean latency of 82.82+/-7.3 ms, with amplitude from the zero line of 11.23 +/- 7.2 μV. It is worth remarking that in this set two of the three superimposed traces, just before the start of the N1 ascending slope, two small negative peaks are visible at 40 and 50 ms approximately (marked with arrows and question marks) which could be related to the N40 and N60 described in section 3.4 (for a discussion about their doubtful reliability see section 4.2). All subjects reported a perception on the VAS scale graded between 2 and 3, from light to moderate pain.

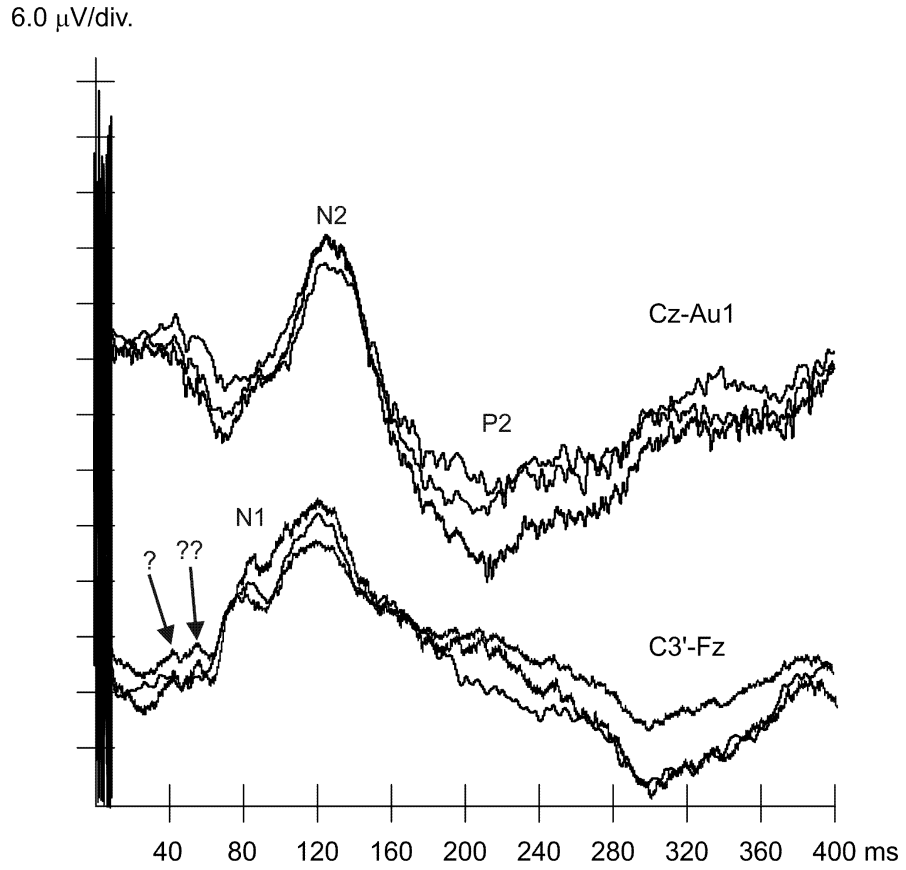

Fig. 3. *Late nociceptive evoked potentials were recorded after random stimulation with 150 IDE (150 μm interdigitated electrode) of the hand dorsum in subject #8. Attention was directed to the stimulus. Three traces are superimposed for reliability in each set. The waveforms are similar to those already published in the literature as PREPs (pain related evoked potentials) and LEPs (laser evoked potentials).The upper set shows the recordings from Cz-Au1 derivation, where the usual N2-P2 complex can be seen. The lower set shows the components from the C3'-Fz derivation aimed at recording the components from the contralateral somatosensory areas. The N1 component is clearly visible, but at its origin two small negative peaks can be noted, marked by arrows and question marks at 40 and 50 ms. Such peaks are not seen in the*

third trace, they are just shoulders in the uprising slope of the N1 and might be due to noise superimposing to the firs part of N1. However, they are in the same latency range of the N40 and N60 to be seen in Fig. 4 and could be enhanced by a more thorough averaging process, not allowed here by the few responses.

3.4 Early nociceptive evoked potentials.

In Fig. 4 are shown two examples of recordings obtained after rythmic repetitive stimulation at 0.83/s rate. The upper set of three traces is from subject # 2 and the lower set is from subject # 7. This type of stimulation consistently abated the event related late responses and enhanced an early group of four components. These could be recorded in all the ten investigated subjects from C3'-Fz. The sequence was the following: positive peak (P30) with mean latency of 31.2 +/- 2.6 ms (amplitude 1.0 +/-0.62 μV) ; negative (N40) at 41.18 +/- 2.18 ms (amplitude 1.65 +/-0.37 μV); positive peak (P50) at 51.5 +/- 4.0 ms (amplitude 1.50+/-0.92 μV); negative peak (N60) at 63.7 +/-5.1 ms (amplitude 1.19+/-0.40 μV) (Fig. 4, upper and lower sets of traces). After these first four peaks, two others could be observed in 6 out of the 10 subjects (Fig. 4, lower set): a positive peak (P80) at 83.49 +/- 7.51 ms(amplitude 1.0+/-0.98 μV) and negative peak (N100) at 102.43+/- 7.20 ms (amplitude 0.40+/-1.18 μV). The latter was sometimes followed by a positive peak (P150) averaging 156+/-15.8 ms (amplitude 0.20+/-1.42 μV) (Fig. 4, lower set of traces). The N100 peak was coincident with the event related N1, but, of course, of smaller amplitude and slightly longer latency than the N1 recorded after random stimulation.

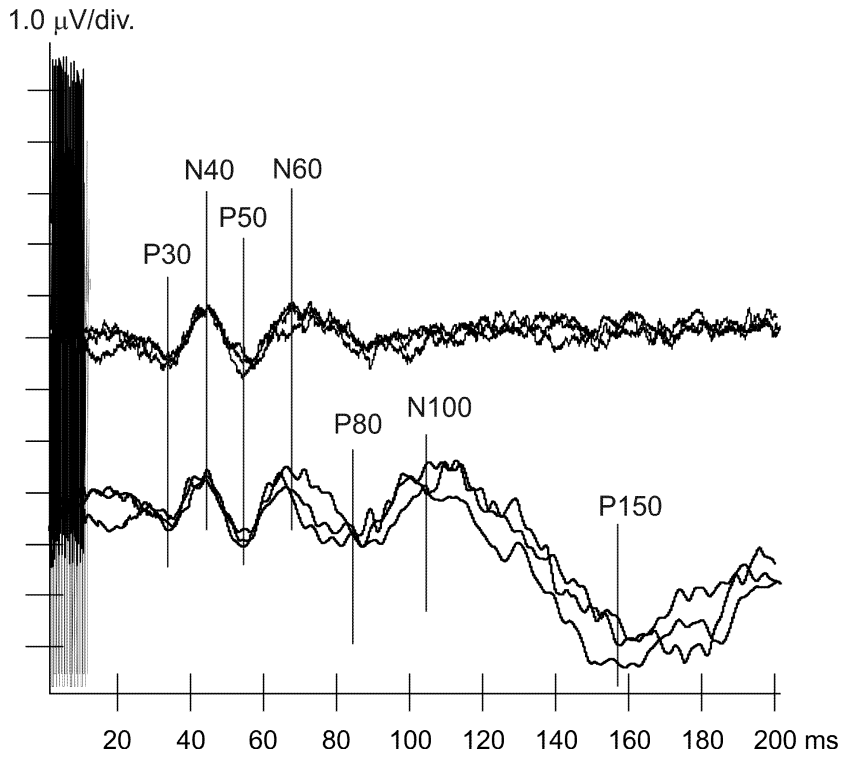

Fig. 4. *Two examples of early NEPs (nociceptive evoked potentials) from C3'-Fz after repetitive stimulation (0.83/s) through the 150IDE (150 μm interdigitated electrode), with 500 averages. The upper set is from subject # 2 and the second set from subject #7. Negative is upwards and there are three traces per each set from consecutive sessions. There is a striking difference from the lower set of Fig 4, the same derivation as*

here, where N1 was well visible, alongside further late waves of comparably large amplitude. The change in stimulus rhythm (from random in Fig. 3 and repetitive here) and number (500 here, and approximately 50 in fig. 3) rhythm made the N1 and further waves to completely disappear in subject #2 (upper set) and strongly abated them in subject #7 (lower set). Now higher amplification could be used and four early waves could be enhanced, marked as P30, N40, P50 and N60. The lower set is to show how in some subjects waves following N60 and of similar latency but much lesser amplitude as those of PREPs (pain related evoked potentials) or LEPs (laser evoked potentials) could be observed.

3.5 Early responses from C3'-Fz and C4-Fz

Fig. 5 shows an example of recordings from the contralateral (C3'-Fz) and ipsilateral (C4'-Fz) hemisphere in subject #3 after 150 IDE repetitive stimulation to check on amplitude lateralization of the early components. The procedure was carried out in 5 subjects. The mean amplitude of N40, referred to the baseline, was 1.43 +/- 0.39 μV contralaterally and 0.50 +/- 0.31 μV ipsilaterally. Their difference was highly significant with p = 0.003.

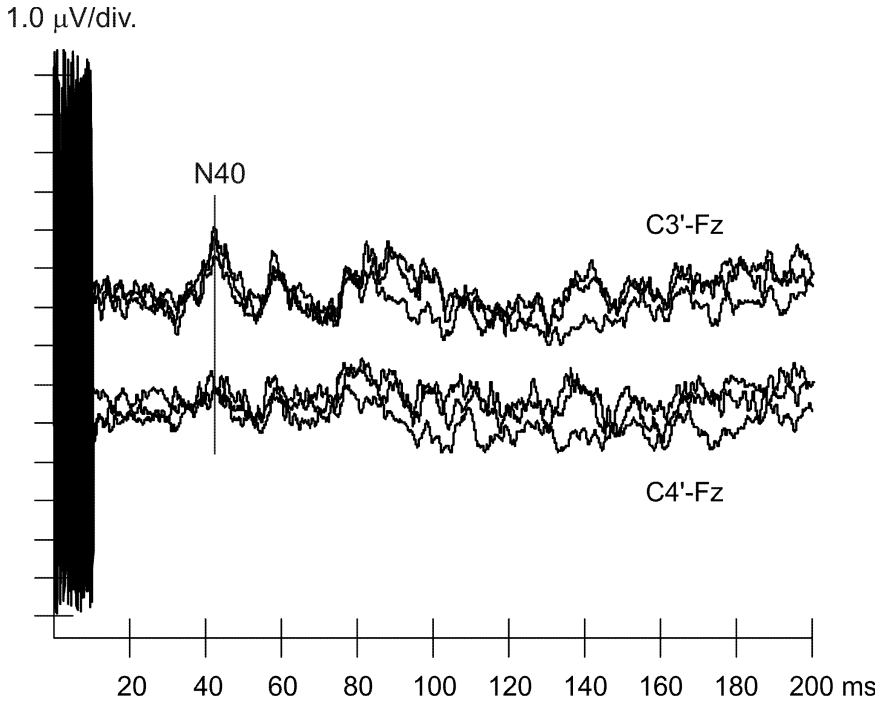

Fig 5. *Example (subject #3) of contralateral (C3'-Fz) and ipsilateral (C4'-Fz) recordings of early NEPs (nociceptive evoked potentials) components after repetitive stimulation (500 stimuli) at the rate of 0.83/s. On the ipsilateral scalp component N40 is significantly smaller than contralaterally.*

3.6 Early responses after stimulation with 150 IDE and 1000 IDE.

In the same session aimed at recording responses after repetitive stimulation with 150 IDE (Fig. 6 upper set) also the electrode 1000 IDE was used on the same spot (Fig. 6 lower set). To make a comparison as direct as possible, the 10 pulse burst was used even in the case of the 1000 IDE electrode. The experiment was carried out in 10 subjects. The first component had mean latency of 21.29+/-1.72 ms and mean amplitude (referred to the zero line) of 1.28+/-0.52 μV, followed by a positive peak at 28.51+/- 2.08 ms with amplitude of 1.79+/-1.06 μV and then by a negative peak at 40.23+/-4.97 ms and amplitude of 3.06+/-1.19 μV. Between the two negative events there was a mean latency difference of 9.9 ms which was obviously extremely significant ($p=3.39x10^{-7}$). However, there was no significant difference when the latency of the 150 IDE N40 was compared to the 1000 IDE N40.

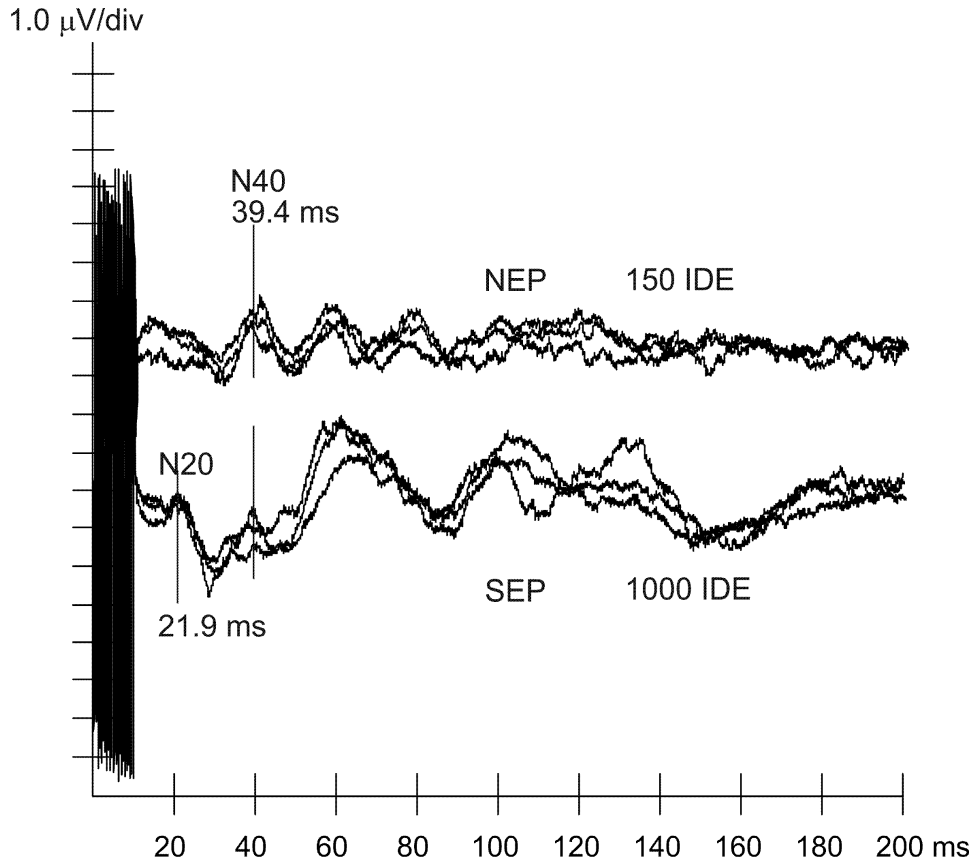

Fig 6. *Recordings from subject #1. Early NEPs (nociceptive evoked potentials) after selective nociceptive stimulation with 150 IDE (150 μm interdigitated electrode) obtained from C3'-Fz derivation are shown in the upper set of traces. In the lower set are shown SEPs (somatosensory evoked potentials) from the same derivation after non selective stimulation with the 1000 IDE (1000 μm interdigitated electrode). N 20 is only visible after non selective stimulation, whilst the firs negative peak after selective stimulation is N40. There is some morphological similarity between the SEP components after the occurrence of N20 and peaks and valleys in the NEP.*

3.7 Perception curve

Different effects of burst duration on perception threshold with the 150 IDE electrode are illustrated in the graph of Fig. 7. This experiment was aimed at assessing how the intensity of the stimulus (dependent variable) had to be adjusted in consequence of varying burst duration (independent variable) in order to keep perception at threshold level, so to obtain a sort of strength-duration curve. Fifteen subjects (from #11 to #25 in Table1) took part to the experiment. The 150 IDE electrode was placed on the hand dorsum between 1st and 2nd metacarpal bone and impedance was maintained between 16 and 30 KOhm. The duration of the burst was 1, 2, 4, 6, 8, 10, 15 and 20 ms containing as many pulses. In the given order, from 1 pulse up to the 20 pulse stimulus, the mean intensities in mA necessary to reach the perception threshold were 3.0 +/- 1.2, 2.5 +/- 1.0, 1.9 +/- 0.8, 1.7 +/-0.7, 1.5 +/- 0.7, 1.2 +/- 0.7, 1.1 +/- 0.6, 1.0 +/- 0.6. Perception threshold values were normally distributed for each number of pulse category. The values significantly decreased with increasing number of pulses $F[7,140]=44.3$, $p<0.0001$) and the calculated equation (Excel) of the best fitting curve was $y = -1.006 \ln(x) + 3.0707$ with $R^2 = 0.9936$, of the logarithmic type. Such results are summarized in the graph shown in Fig. 3, where the mean values of intensity necessary to reach perception threshold are plotted against number of pulses, or burst duration. Quality of perception at the threshold level was very definitely a light pin prick. No other types of perception, like touch or heat or cold, were reported but for three subjects (#14, #18 and #20) who besides the sharp pin prick perceived a sort of slight dull sensation lingering a few moments after the stimulus at durations of 15 and 20 ms.

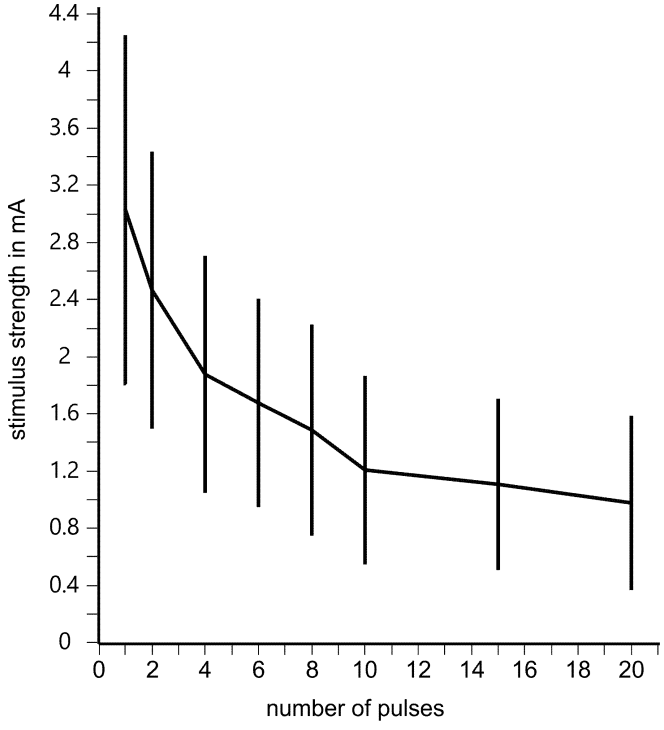

Fig. 7. Graph showing the mean values and standard deviations (as error bars) of the stimulus strength needed to reach the nociceptive perception threshold (pin prick) when a burst of n pulses is delivered through the 150 IDE (150 μm interdigitated electrode) on the hand dorsum.

4 Discussion

4.1 Selectivity of 150 IDE supported by neurographic recordings

Evidence about selectivity by the 150 IDE electrode has already been provided (Di Stefano et al., 2020; Leandri et al., 2018), but so far it has never been supported by direct recording of the neurogram from the stimulated area. Here we provided such evidence in two areas, the hand dorsum innervated by the radial nerve (Fig.1) and the upper lip innervated by the infraorbital nerve (Fig.2). In both cases we compared the recordings after stimulation using the unselective 1000 IDE with the selective 150 IDE. Also, we took the first negative peak as a reference for identifying the fastest group of fibres, since the first positive peak, although visible, was not always identified with sufficient reliability. The mean conduction velocity relative to the first negative peak after 1000 IDE unselective stimulation was estimated to be 46.3 m/s for the radial nerve and 32.3 m/s for the infraorbital nerve, both in the range of the Aβ large myelinated fibres. On the other hand, stimulation through the 150 IDE yielded responses with underlying mean conduction velocities of 27.0 and 20.7 m/s for the two nerves, both representative of the upper part of the Aδ range. So the difference in conduction velocities of the fastest fibre bundles was of approximately 18 ms for the radial nerve and of 8 ms for the infraorbital nerve. Such wide gap clearly demonstrates that at the stimulus intensities that we used, the 150 IDE stimulation did not activate fast myelinated fibres. It may be wondered why the conduction velocity, especially after the 1000 IDE stimulation was definitely faster in the case of the radial than infraorbital area. A possible explanation could be that this outcome could be related to the comparatively slow conduction velocity of the infraorbital nerve where the recordings had been performed from a much more peripheral part of the nerve trunk. Lastly, it is of some relevance that in the case of radial nerve, where the longer distance allowed a better separation of the neurogram components at the site of recording, the large first negative peak by 1000 IDE stimulation was followed by a smaller negative peak at approximately 3.5 ms (Fig. 1, upper set) which was coincident with the first negative peak observed after the 150 IDE stimulation (Fig. 1, lower set). It was evident that, at the used stimulus intensity and duration, the 1000 IDE activated not only the Aβ but also the slower Aδ fibres. The above evidence is a further strong support of the notion that the 150 IDE electrode, as used in our setting, never activated large fibres, and that it is a reliable instrument to obtain Aδ evoked cortical potentials. A limitation of this part of the study is the low number of subjects investigated: just 3 for the infraorbital nerve and 5 for the radial nerve. However, the reproducibility of the results was very high as suggested by the calculated standard deviations (see section 3.1 and 3.2), so the obtained measurements could well be considered representative enough.

4.2 The early components of nociceptive evoked potentials (early NEPs)

We recorded from derivation C3'-Fz a whole set of early cortical components named P30, N40, P50 and N60 taking place well before the already well known and lateralized N1 (see Fig. 4 for the early

components and Fig. 3 lower set for N1). All of these are very small in amplitude, which is something to be expected given the scanty amount of activated peripheral fibres. Nevertheless, after careful discarding of biological artefacts, these have been recorded in all our subjects. A word should be spent here to clarify the acronym NEPs that we propose instead of PREPs, as the latter is currently used when referring to cortical responses obtained through electrical stimulation applied by various contrivances. All PREPs components pertain to the late event or endogenous related responses, linked to the painful quality of perception. Either the old and the new definition of "pain" by IASP committees (International Association for the Study of Pain, 1994; Raja et al., 2020) are centred upon the term "experience", implying that cortical processes and consciousness are strongly involved. Such definition well suits the nature of the PREPs described so far. But the early components that we report here are much probably of a different nature, with little cortical processing and very likely independent from attention (they were obtained in conditions of low attention and with repetitive stimulation, as reported by the end of section 2.3). In that sense, they pertain more to the purely biological process of nociception, hence we propose to name them NEPs, nociceptive evoked potentials. It may look surprising that such responses had not been noticed before, but there are several reasons for such overlooking. The first one is related to the means of stimulation. Obviously laser stimuli can only be delivered in a very limited amount to avoid excessive skin damage, hence it is impossible to reach the number of necessary averages to detect such small waves. The second one is the tiny amount of receptors that the laser spot can activate, definitely smaller than the 150 IDE. The third is the poor synchronization of the laser pulse, as depolarization only occurs by heating the receptors or nerve fibres, with the infrared radiation taking a comparatively long and unequal time to heat up each single target. So a desynchronized volley is generated. The fourth and final reason, but by no means the least important is the fact that in the presence of the comparatively large components N1 and N2, the small early components are dwarfed by the upwards going slopes, and cannot be clearly detected. The same pitfalls have also taken place when electrical stimulation with concentric or pin electrodes has been used in previous reports (a review of such methods has been recently published (Lefaucheur, 2019)) . Such electrodes have been demonstrated to coactivate Aβ fibres as well (La Cesa et al., 2018; Perchet et al., 2012), so early somatosensory non nociceptive components should have been noticed in the scalp recordings. However these have never been described, to our knowledge, in such cases. It can be surmised that the attention of researchers has been directed to the slow and large components N1 and N2 recorded after laser stimulation and heavy filtering of the signal, with lowpass often set from 30 to 50 Hz to give a smoother aspect to the traces and get rid of the annoying mains ripple (Beydoun et al., 1993; Inui et al., 2002; Linde et al., 2020). When we recorded the traditional late components after random stimulation from Cz-Au1 and C3'-Fz we did not employ that sort of filtering, but set the lowpass filter to 2,000 Hz (see section 2.4), which is the customary setting to record early cortical or subcortical SEPs and other evoked potentials (Cruccu et al., 2008). In Fig. 3, the recording from C3'-Fz (lower set) shows a large N1, peaking at approximately 80 ms

but the slope preceding the peak starts at about 50-60 ms and could dwarf any components at that latency range. Matter of fact, in our example two negative peaks at 40 and 50 ms can be observed (marked by arrows with one and two question marks) , they are small in comparison to N1 and it is difficult to decide whether they are something reliable or not (hence the question marks). They are replicated in two traces but not in the third, so we cannot take them as for genuine activity related to the stimulus. The number of averages was low, as the aim of this type of recording is to enhance the late waves, but the reliability of such small waves can only be assessed with a much larger number of replications. As predicted in the original work introducing the 150 IDE (Leandri et al., 2018), we had now the chance to use a high stimulus repetition rate combined with reliable selectivity for Aδ fibres. The fast repetition rate of the stimulus not only allowed each session to be performed in a reasonable time, but also had the paramount advantage of naturally reducing the amplitude of late components, starting from N1. Such effect had already been brought to attention (Leandri et al., 2018) and can be observed in our recordings by comparing the traces in Figs. 3 and 4. The large N1 and N2 well visible in Fig. 3 after random and slow rate stimulation either disappear (Fig. 4, upper set) or are greatly reduced (Fig.4, lower set). Do note that Figs. 3 and 4 are differently scaled. So, we were able to highlight the early components just by using the appropriate rate of stimulation, without recurring to distorting hardware or software filters. Components P30, N40, P50 and N60 could be observed from derivation C3'-Fz in all our subjects and showed limited variability of their latency, suggesting that they were originated at sites after just few synapses had been passed through, as it may be expected in the case of fast Aδ fibres reaching the primary cortex. The later peaks: P80, N100 and P150 were seen in just a part of the subjects and demonstrated also increasing latency variability, suggesting their origin after further cortical processing. Derivation C4'-Fz, ipsilateral to the stimulus, was compared to C3'-Fz in 5 subjects to assess possible lateralized differences. As expected, the N40 component was definitely larger on the contralateral side with a difference of 0.92 μV which corresponded to a reduction of approximately 65% from the contralateral to the ipsilateral side. The other components showed lesser differences which were not statistically significant. The strong lateralization of N40 suggests that such component arises from the sensory cortex contralateral to the stimulus, and its latency makes an origin from the primary cortex as highly plausible. We chose to use the scalp location commonly used for SEPs after stimulation of the median and radial nerves (Treede and Kunde, 1995), as evidence in humans and animals had been provided in favour of a representation of the fast Ad afferents at that site (Treede and Apkarian, 2008). It was therefore logical to expect that the best chances to record early cortical activity by fast Aδ fibre after hand nociceptive stimulation would be the hand projection area of the sensory homunculus. Additional scalp recording sites would have been necessary to provide a definite evidence about the distribution of N40, but our study was not aimed at obtaining a detailed map of the early waves, which will be investigated in a further research. On the other hand, the very different latency ranges of the components and techniques of stimulus presentation do not allow comparisons with previous works on

scalp distribution and depth recordings (Frot et al., 2014; Hu et al., 2014; Peyron et al., 2002; Valeriani et al., 2004), as they were focused on slower nociceptive afferents and late cortical events.

4.3 Comparison between early NEPs and SEPs

Stimulation of the radial area with the unselective 1000 IDE evoked a cortical response (SEP) similar to the one obtained after stimulation of the nerve trunks, starting with the N20 component, albeit of smaller amplitude, confirming previous reports (Leandri et al., 2018). Such component was missing in the case of the 150 IDE stimulation, where the first negative component was N40. If, on the basis of its latency and lateralization, we suppose that N40 of the NEP is originated from the primary somatosensory hand area, then it could be compared to the non nociceptive N20. The transmission time would be approximately twofold, which would suit reasonably well with a slower pathway. When comparing the morphology of the responses, it is evident that there is an intriguing similarity between the two types of responses in the temporal epoch following the SEP N20. It may be wondered if the SEP middle and late components could reflect, at least in part, some activation of the slower Aδ fibres. The issue related to possible contribution by slow conducting afferents to middle and late SEP components had been raised since the very first reports and though the general consensus was against a role of slow conducting fibres, there has never been a definite clarification (Anderson et al., 1986; Halliday and Wakefield, 1963; Jabbari et al., 1990). The usual stimulation employed in SEPs is not painful, hence it is extremely unlikely that a significant contribution of Aδ fibres would take place. In our experiments, however, we employed a burst of 10 pulses even in the case of the 1000 IDE, which was considered an ideal stimulus for Aδ excitation. As a matter of fact, the 1000 IDE stimulation was perceived as a tap at the threshold level, but as a pin prick when intensity was raised by 1.5 times. The pin prick perception could be an indicator that besides Aβ activation, also Aδ fibres were involved. Such mixed activation occurred at comparatively low intensities, possibly because the structure of the 1000 IDE electrode implied narrow conductive rails with a high electric resistance at the skin interface. High interface resistance is a factor that contributes to keep a superficial and localized electric field, leading to activation of the intraepidermal fibres (Leandri et al., 2018). So, the 1000 IDE stimulation used for SEP recording cannot be considered as solely depolarizing Aβ fibres, but should be deemed as a suitable means of Aδ coactivation. Such feature may well be responsible for the similarities between the two types of recordings.

4.4 Perception threshold and strength duration curve

A relevant feature of the 150 IDE stimulation that can easily be experienced is the quality of perception, which is pin prick like since the very basic sensory threshold. This subjective experience is a further support of the selective property of the electrode, since any previous activation of Aβ cutaneous fibres would elicit a localized tapping sensation only turning to pin prick with increasing stimulus intensity. The burst stimulus

has been adopted in lieu of a long duration square pulse to bypass the high resistance barrier of the electrode to skin interface, exploiting capacitive transmission of electricity (Leandri et al., 2018), in that sense the duration of the burst can roughly be assimilated to a square pulse of the same duration. We then wondered if the pin prick quality of sensation would be maintained with bursts shorter than 10 ms, and what would happen with longer durations. The assessment of the necessary intensity to achieve perception threshold as function of burst duration showed that the pin prick sensation was always the only one to be evoked independently from duration, but with bursts shorter than 4 ms the necessary intensity increased steeply. The trend that we obtained, very similar to the strength duration curves obtained by direct stimulation of nerve fibres, suggests that the temporal summation effect of the burst pulses may occur at the peripheral fibre level rather than at central synapses, where a saturation effect would have taken place. The matter, anyway, needs further investigation in order to ascertain the role of pulse frequency on summation effect , also according to the fibre group (Aβ or Aδ). Based on the evidence provided in this experiment, it is possible to conclude that the 10 ms burst is the optimal one to achieve a nociceptive perception threshold with the minimum intensity and still leaving an artefact free epoch to allow accurate study of evoked potentials.

4.5 Are small fibres other than fast nociceptive Aδ group stimulated?

The intraepidermal free nerve endings and their fibres are the only excitable structures supposedly activated by electric stimuli delivered through the 150IDE (Leandri et al., 2018). Those free nerve endings pertain to small myelinated Ad fibres and unmyelinated C fibres that also carry thermal and tactile sensations (Rice and Albrecth, 2008; Treede, 2008; Treede et al., 1998; Vallbo et al., 1993). So it would be conceivable that membranes of such non nocieptive afferents could be depolarized by our stimulation. As remarked in the report of the experiment searching for the intensity needed to reach perception threshold as a function of the burst pulse number (sections 3.7, 4.4 and Fig. 7) seven subjects out of ten just felt a light pin prick for stimuli of any burst duration. But 3 subjects, besides the very short pin prick sensation, perceived a longer lasting dull pain when the burst duration was increased to 15 and 20ms. Whilst the pin prick sensation is linked to fast Ad activation, the dull long lasting pain should be linked to C fibre depolarization. It is just possible that with a stimulus of sufficient duration also the C fibres were involved. We can only rely our hypothesis on the reported perception, as obviously the early NEPs are not the correct instrument to investigate C fibre activity. Besides, even our near nerve recordings could not show slower components than those pertaining to fibres conducting at 25 m/s. No thermal or other sort of perception were ever reported from our subjects, and we can only speculate that perhaps the characteristics of our stimuli, like waveform, duration and rate of stimulation may not suit the needs for thermal afferents to be activated.

5 Conclusions

This research has brought novel evidence about selectivity of the 150 IDE micropatterned electrode as a safe and reliable means to stimulate intraepidermal nerve Aδ nerve endings. By exploiting its features, it has been possible to record nociceptive evoked responses (NEPs) from the scalp at latencies that are compatible with the first cortical activation (N40) analogous to the N20 primary response after median nerve stimulation. It is the first time that a primary cortical response after peripheral nociceptive stimulation has been detected. N40 and the other components of NEPs are of very small amplitude and careful recording techniques are necessary to detect them. Also, the stimulating procedure requires attention and care, as the 150 IDE electrode has some critical issues, mainly linked to skin moisture which may hamper its performance by shortening the conductive rails and compromising its selectivity. Such limitations make the early NEPs a difficult tool for extensive diagnostic application, nevertheless they are an invaluable means for experimental study of the Aδ nociceptive afferents, that now can be investigated in the same manner, though not as easily, as the lemniscal afferents.

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Giulia Di Stefano: investigation, data curation, review and editing.

Andrea Truini: investigation, data curation, review and editing

Lucio Marinelli: conceptualisation, methodology investigation, data curation, review and editing.