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Confinia Cephalalgica 2024; 34:15730

Normative values of the nociceptive blink reflex habituation

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ABSTRACT

Introduction: Habituation is a physiological phenomenon, characterized by response reduction to repeated stimulus presentation. In headache disorders, habituation studies have involved different paradigms with several stimulation parameters and sensory modalities, and consistently showed impaired habituation in primary headaches in the interictal phase. The nociceptive blink reflex (nBR) and its related R_2 response, modulated by a polysynaptic network in the trigeminocervical complex, is one of the most studied in the field. The lack of nBR habituation normative data hampered the possibility to draw conclusions regarding the functional status of individual patients. The present study aims to define normative values for the nBR habituation process in healthy subjects without a personal diagnosis and family history of migraine, or other headache disorders.

Methods: We enrolled 40 healthy subjects (24 females, 32.7±11.6 years) for nBR recording and nBR habituation assessment. To assess the habituation of nBR, 26 consecutive stimuli were administered at three different and randomized stimulus frequencies (0.2, 0.3, 0.5 Hz). After excluding the first response, the remaining 25 area under the curve (AUC) were divided in 5 blocks, and the average values of the AUC was calculated for each block. The percentage reduction in the AUC of the fifth block, compared to the first, represents the habituation index (HI) value. We considered a one-tailed 10^{th} percentile threshold as the lower threshold of normative values for nBR HI.

Results: The habituation phenomenon was confirmed for all study frequencies. The absolute AUC of the R_2 component across the five blocks of stimulation was higher in female subjects when compared to male for 0.5 Hz (p=0.021) and 0.2 Hz (p=0.007). We found a frequency-dependent habituation pattern, being lower at the 0.2 Hz stimulation when compared to 0.5 Hz (p=0.001), and 0.3 Hz (p=0.008). The average HIs were 73.1±13.6 at 0.5 Hz, 69.2±15.0 at 0.3 Hz, and 61.1±21.4 at 0.2 Hz. HIs were comparable between male and female subjects, without correlations with age, intensity of stimulation, and latency of the R_2 component. The 10th percentile of the HIs was 43.5% for 0.5 Hz, 55.8% for 0.3 Hz, and 28.6% for 0.2 Hz.

Conclusions: We investigated the nBR habituation in a population of healthy subjects for normative data collection. We described a frequency-dependent degree of habituation, being more pronounced at higher frequencies of stimulation. Moreover, we described gender-related features of response behaviour, which is extremely important in the migraine field. Our study further characterized the physiological habituation phenomenon in healthy controls exposed to a nociceptive stimulation. The definition of a normative habituation value will open novel possibilities in the study of migraine, as well as other headache and pain disorders.

Key words: headache, migraine, trigeminal autonomic cephalalgias, electrophysiology, sensitization.

Introduction

Habituation, defined as the reduction in responsiveness with repeated no longer biologically relevant stimulus presentation, is a physiological phenomenon that occurs in different sensory modalities across neuronal circuits of varying complexity (1). Habituation and sensitization represent pivotal events in several primary headaches, including migraine, cluster headache and paroxysmal hemicrania and their study provided relevant information about their physiopathology, such as the modulation of the trigeminovascular-system across the migraine cycle and role of hypothalamic dysfunction in cluster headache (2-5). In particular, migraine patients are characterized by an alteration of habituation, which appears to fluctuate during the migraine cycle. When studied outside a migraine attack (interictal phase), electrophysiological studies consistently showed a lack of habituation for different sensory modalities, to suggest an endophenotypic marker of migraine (6). Similarly, a defective habituation to both non-nociceptive and nociceptive trigeminal stimulation has been detected in cluster headache regardless of the phase of the disease activity, but consistent with the affected side (5, 7).

Habituation studies have involved different paradigms, including – but not limited to – visual evoked potentials, somatosensory evoked potentials, P300 recording, laser evoked potentials, and the blink reflex (8-12).

The blink reflex, a non-invasive method to study transmission of the trigeminal and facial nerves in humans, involves stimulating the supraorbital nerve and recording from the orbicularis oculi muscle. It comprises three components: i) an early R_1 response ipsilateral to the stimulation side and mediated by Aβ fibers; ii) a bilateral R_2 component mainly mediated by Aδ fiber; and iii) a tardive, fast-adapting R_3 component considered part of a startle reaction (13). The R_2 component gained attention in the migraine field because it is primarily modulated by a polysynaptic neural network in the trigeminocervical complex (TCC).

Previous research identified the *nociceptive* blink reflex (nBR) triggered by a peculiar stimulating electrode (14). This electrode is designed to create high current planar density at low intensities, selectively activating superficial nociceptive Aδ fibers in the dermis. The nBR exclusively includes the R_2 response and has proved valuable for investigating trigeminal nociception, modulation of the TCC and related reflex responses (15).

Notably, patients with migraine with and without aura showed a reduced habituation of the nBR (7, 15).

During acute migraine attacks, habituation normalizes, independently from acute intake of lysine acetylsalicylate or zolmitriptan (15, 16). Moreover, in migraine, no differences were observed between nBR habituation following stimulation of the headache and non-headache side (7). In episodic migraine, the severity of the habituation deficit peaks a few days before an attack (*i.e.* preictal phase), subsequently normalizing during the attack (ictal phase) (4). In chronic migraine, the habituation deficit is less prominent or absent, leading to the idea that these patients are locked in a never-ending migraine attack (4). A pronounced lack of habituation of the nBR is observed on the symptomatic side in cluster headache patients both during and outside the bout (5, 17). By contrast, only a few signs of deficient habituation have been observed in subgroups of tension-type headache patients (18). Most studies showing nBR habituation deficit in migraine are cross-sectional studies, and the overall findings are the results of the average behaviour of study groups (5, 7). By contrast, the inter-individual variability, and the lack of normative data of the physiological habituation of the nBR has hampered the possibility to draw conclusions regarding the functional status of individual patients. For these reasons, the nBR habituation deficit did not qualify as a migraine biomarker and its clinical utility has been criticized.

The aim of present study is to define normative values for the nBR habituation process in healthy controls without a personal diagnosis or a family history of migraine, or diagnosis of other headache disorders.

Results

Study population. We enrolled 40 healthy controls (24 females, 32.7±11.6 years, range 20-61 years). The age was comparable between male (33.0±10.2) and female (32.6±12.6) subjects (p=0.913).

Baseline parameters of the nociceptive blink reflex. Regarding reflex threshold (RTh), sensory threshold (STh) was 1.0±0.5 mA, while RTh was 3.5±3.7. The average latency of the R_2 component was 35.9±4.8 msec, while the average area under the curve (AUC) was 1.8±0.9 μV × msec. None of the baseline parameters correlated with age and we did not detect differences. Latency of the R_2 component was shorter in female subjects (p=0.003) (**Table 1**).

Frequency-dependent habituation of the nociceptive blink reflex-area under the curve expressed as absolute values (μV × msec). The habituation phenomenon, a progressive decrease of the AUC across the five blocks of stimulation, was confirmed for all study frequencies (factor TIME: p=0.001 for all study frequencies).

The AUC of the first block of stimulation was higher in female when compared to male subjects (0.5 Hz: p=0.006, 0.3 Hz: p=0.013, and 0.2 Hz: p=0.001) (**Supplementary Tables 1-3**). In addition, we found a frequency-dependent behaviour of the AUC of the first block of stimulation (p=0.034) as the post-hoc analysis showed a lower amplitude with 0.5 Hz when compared to 0.2 Hz frequency (p=0.036) (**Figure 1A**). For all the study frequencies, the AUC of the first block did not correlate with age (0.5 Hz: p=0.454, 0.3 Hz: p=0.950, and 0.2 Hz: p=0.561), and intensity of stimulation (0.5 Hz: p=0.300, 0.3 Hz: p=0.752, and 0.2 Hz: p=0.855). A negative correlation was found between the AUC of the first block at 0.2 Hz and latency of R_2 component (Spearman -0.580, p=0.001).

The absolute AUC of the R_2 component across the five blocks of stimulation was different among the three study frequencies (factor Hz: p=0.001) (**Figure 1B**). The *post-hoc* analysis confirmed a frequency-dependent behaviour of AUC during nBR habituation recording, being lower with 0.5 Hz stimulation when compared to 0.3 Hz (p=0.007), and 0.2 Hz (p=0.001), and with 0.3 Hz when compared to 0.2 Hz (p=0.013). This implies a less pronounced response decrement (*i.e.* less habituation) for lower frequencies of stimulation.

The absolute AUC of the R_2 component across the five blocks of stimulation was higher in female subjects when compared to male for 0.5 Hz (factor SEX: p=0.021) and 0.2 Hz (factor SEX: p=0.007) (**Figure 2A** and **C**). In addition, a significant interaction was described for the 0.3 Hz (interaction TIME × SEX: p=0.033).

Frequency-dependent habituation of the nociceptive blink reflex with area under the curve expressed as percentage variation from the first block of stimulation (normalized to 100%). When the modification of the AUC was expressed as percentage variation from the first block of stimulation, the habituation phenomenon was confirmed for all study frequencies across the five blocks of stimulation (factor TIME: p=0.001 for all study frequencies).

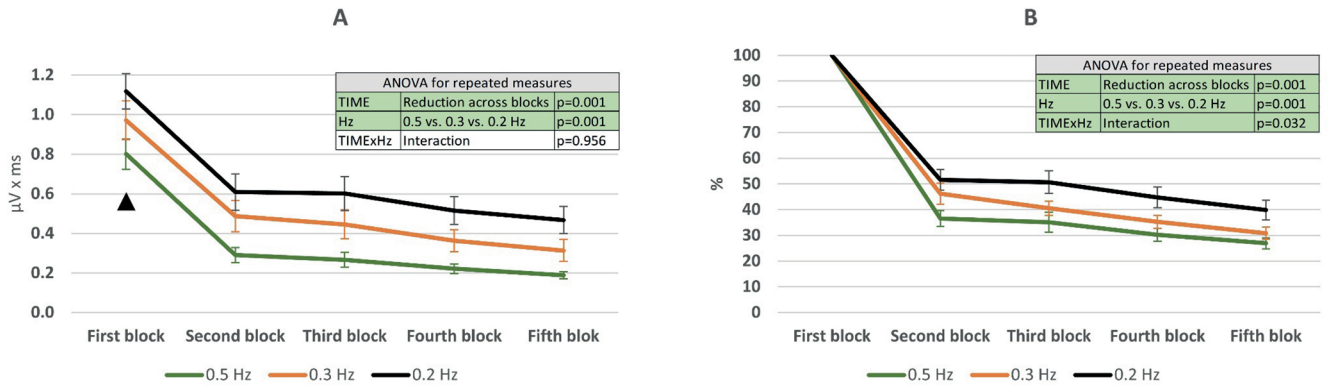
The percentage variations of AUC of the R_2 component across the five blocks of stimulation was different among the three study frequencies (factor Hz: p=0.001) (**Figure 1B**). The *post-hoc* analysis confirmed a frequency-dependent behaviour of AUC during nBR habituation recording, being higher at the 0.2 Hz stimulation when compared to 0.5 Hz (p=0.001), and 0.3 Hz (p=0.008); no differences were found between 0.3 Hz and 0.5 Hz frequencies (p=0.154). In addition, a significant TIME × Hz was described (p=0.032). This implies a less pronounced response decrement (*i.e.* less habituation) for lower frequencies of stimulation.

The percentage variations of AUC of the R_2 component across the five blocks of stimulation did not differ between male and female subjects for all study frequencies (**Figure 1D-F**).

Table 1. Neurophysiological parameters of the nociceptive blink reflex.

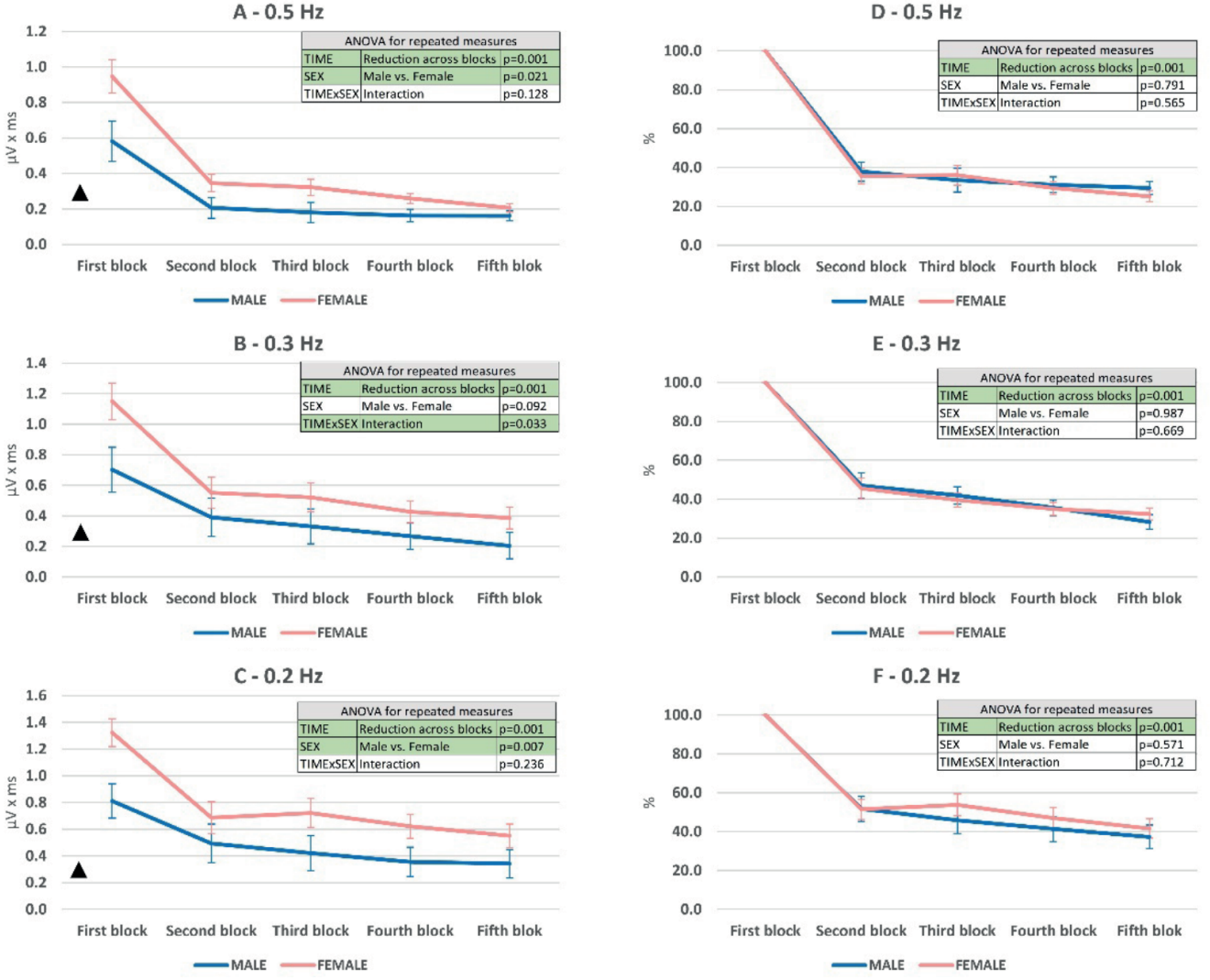
	Total	Male	Female	p
N. subjects	40	16	24	-
STh, mA	1.0±0.5	0.8±0.6	1.1±0.4	0.150
RTh, mA	3.5±3.7	4.6±5.6	2.7±1.2	0.924
NRS RTh	4.8±1.8	5.0±2.0	4.7±1.7	0.594
RTh R_2 latency, msec	35.6±4.8	38.4±5.3	34.2±3.7	0.003
RTh R_2 AUC, μV X msec	1.8±0.9	1.6±0.5	2.0±1.3	0.672
HI 0.5, Hz (%)	73.1±13.6	70.5±14.5	74.8±13.1	0.404
HI 0.3, Hz (%)	69.2±15.0	71.7±9.3	67.6±17.9	0.713
HI 0.2, Hz (%)	61.1±21.5	62.7±20.7	60.0±22.3	0.754

STh, sensory threshold; RTh, reflex threshold; NRS, numeric rating scale; AUC, area under the curve; HI, habituation index.



Δ: p<0.050 among the three stimulation frequencies in the first block of stimulation; the *post-hoc* analysis showed a lower amplitude with 0.5 Hz when compared to 0.2 Hz frequency (p=0.036).

Figure 1. Habituation of the nociceptive blink reflex R_2 area. A) Absolute change in area under the curve when compared to first block of stimulation (normalized to 100%). B) Percentage change in area under the curve when compared to first block of stimulation (normalized to 100%).



Δ: p<0.050 between the male and female in the first block of stimulation.

Figure 2. Gender differences in the habituation of the nociceptive blink reflex R_2 area. A-C) Absolute change in area under the curve. D-F) Percentage change in area under the curve when compared to first block of stimulation (normalized to 100%).

Habituation indexes and proposed normative values. The average habituation indexes (HIs) were 73.1±13.6 (range 35.4-91.1) at 0.5 Hz, 69.2±15.0 (range 31.2-90.6) at 0.3 Hz, and 61.1±21.4 (range 6.9-89.7) at 0.2 Hz. At all study frequencies, the HIs were comparable between male and female subjects (**Table 1**), and no correlations with age, intensity of stimulation, and latency of the R_2 component were found. At 0.5 Hz, the HI positively correlated with the AUC of the first block of stimulation (Spearman's ρ 0.409, p=0.009); by contrast, this correlation was not found at the lower stimulation frequencies of 0.3 Hz (Spearman's ρ 0.020, p=0.903), and 0.2 Hz (Spearman's ρ 0.099, p=0.543).

The percentile distribution of the HIs for each stimulation frequencies are illustrated in **Table 2**. The 10th percentile of the HIs, namely the thresholds of the proposed normative values, was 43.5% for 0.5 Hz, 55.8% for 0.3 Hz, and 28.6% for 0.2 Hz.

Discussion

In the present study, we investigated the habituation of the nBR in a population of healthy subjects in order to provide normative values for this physiological phenomenon.

The main results of our study may be summarized as follows. The habituation phenomenon was recorded and confirmed across 26 consecutive nBR recordings. We described a frequency-dependent degree of habituation, being more pronounced when higher frequencies of stimulation were applied. Indeed, the 10th percentile of the habituation indexes differed according to the stimulation frequency, with a normative threshold set at 43.5% for 0.5 Hz, 55.8% for 0.3 Hz, and 28.6% for 0.2 Hz.

Based on our findings, we propose that values of HIs below the 10th percentile for each stimulus frequency identify a subset of subjects with a habituation deficit behaviour. We could also speculate that HI values above the 90th percentile may identify a subset of subjects with an extremely pronounced habituation phenomenon, but this probably represents an increased physiological response more than a pathological sensory processing. Compared to men, female subjects showed a larger amplitude of the R_2 component of the nBR and a more pronounced habituation phenomenon at intermediated stimulation frequencies. It is worth noting that this gender differences normalized when habituation was assessed as percentage variation of the second to the fifth blocks from the AUC of the first block. The gender-related habituation trend observed in our study is extremely important in the migraine field. Indeed, considering the higher prevalence of migraine in the female population, we underlie the importance to balance clinical and demographic features in future studies.

The nBR and related habituation evaluation represent a valid tool to explore the functional modulation of the trigemino-vascular system in migraine. The nBR is closely linked to the pain-related trigeminal processing, indeed the afferent trigeminal arch is stimulated with a nociceptive-specific electrode in the V1 area, and a set of descending fibres project to a bilateral polysynaptic network in the TCC before reaching the efferent branch to the pontine nucleus of the facial nerve (14, 19). Thus, the nBR evaluation may provide insights into two key pathways for headache disorders, namely the trigemino-cervical complex and, indirectly, the thalamo-cortical relay.

The pathophysiological substrate of the interictal migraine habituation deficit is not fully understood. A first hypothesis takes into account a reduced activity in brainstem monaminergic pathways, resulting in a low level of cortical pre-activation (20, 21). This may also explain why several neurophysiological responses were reduced in amplitude in migraine patients after the very first stimulations (22, 23). In addition, a disrupted intracortical short-range lateral inhibition as well as functional disconnection between the thalamus and the cortex (*i.e.* thalamocortical dysrhythmia) may account for the observed habituation deficit (23, 24). These mechanisms are not mutually exclusive and may coexist, with a different degree of involvement across the migraine spectrum. Indeed, a recent elegant study demonstrated how chronic migraine with medication overuse headache patients features a combination of increased thalamocortical drive and aberrant cortical inhibitory mechanisms (25). In addition, another intriguing pathophysiological hypothesis explaining the defective habituation in primary headaches rooted on the link between the dysfunction of the hypothalamic axis in mice and the lack of habituation of the startle responses (26). It has been suggested that this dysfunction produces a chronic stress-like condition leading to abnormal processing of relevant environmental stimuli (26).

nBR habituation was consistently found impaired in migraine and other headache disorders (4). However, different paradigms were adopted to assess nBR habituation, thus limiting the generalization and the possibility to directly compare results obtained by different researchers (**Table 3**) (6, 7, 27-34). Nonetheless, our findings are largely consistent with a broader set of habituation features, as revised by Rankin *et al.* (35). Indeed, some of the main habituation characteristics described, such as the progressive decrease in response parameters, the spontaneous recovery after stimulus withdrawal, and the more pronounced habituation to higher stimulus frequencies, are in line with our results. Rankin *et al.* described other features of the habituation phenomenon, for example the potentiation of habituation induced by a carry-over effect achieved by means of repeated series of habituation training and recovery. The evaluation of these habituation components required longer and ad-hoc stimulation protocols. In our study, we voluntary avoided the study of the potentiation of habituation by allowing a prolonged rest between the sessions and randomizing the frequencies of stimulation. Moreover, we avoided an excessively supra-threshold stimulus. These choices were made to not over- or under-estimate our normative parameters.

Parameters with possible effects on the resulting habituation are the number of stimulations per block, the overall number of stimulations, the frequency of stimulation, the interval between blocks of stimulation and stimulation intensity. The present

Table 2. Habituation indexes of the nociceptive blink reflex at different stimulation frequencies.

Percentile	0.5 Hz, %	0.3 Hz, %	0.2 Hz, %
5th	35.3	52.0	24.8
10th	43.5	55.8	28.6
25th	63.4	65.3	41.2
50th	74.2	73.4	70.8
75th	82.7	77.8	80.0
90th	85.0	82.7	85.0
95th	91.1	90.6	89.5

The 10th percentile represents the normative value for the habituation index. Values below the 10th percentile are indicative of a habituation deficit behaviour.

recording protocol is consistent with previous experiences by our group. We decided to narrow the range of frequencies, excluding those above 0.5 Hz and below 0.2 Hz. This is because no differences between migraine patients and controls were found within 0.05-0.1 Hz stimulation frequencies, and we aimed to reduce the duration of the nBR recording session to increase tolerability, feasibility and future applications. Several limitations should be acknowledged for our study. First of all, the sample size is limited, and involves a quite young population. Thus, our results cannot be generalized to the overall population, but they should be applied to a comparable sample. It is worth noting that the study of habituation does not have pure clinical implication, and it is restricted to research purposes. In addition, most of the studies on primary headaches involve subjects with demographic features comparable to our study population. As previously described, we adopted a specific nBR habituation protocol, and we cannot generalize the proposed His if the study is performed with clearly different parameters. A strength of our study is the adoption of a set of precise and multiple stimulation frequencies.

Conclusions

Our study further characterized the physiological habituation phenomenon in healthy controls exposed to a nociceptive stimulation. The definition of a normative habituation value will open novel possibilities in the application of nBR habituation in the study of migraine, as well as other headache and pain disorders. This can allow to determine the functional neurophysiological status of a single patient, to facilitate future tailored and individualized approaches. Additionally, we hope that further normative values will be published, exploring habituation of other sensory modalities and reflex responses.

Materials and Methods

We enrolled healthy subjects aged 18-60 years. The inclusion criteria were: i) subjects not affected by primary or secondary headache disorders according to the International Classification of Headache Disorders-3rd edition with the exception for *2.1 Infrequent episodic tension-type headache*; ii) negative first relatives family history of primary headaches. Exclusion criteria were: i) diagnosis of any neurological, psychiatric, or chronic pain conditions; ii) use of chronic medications interfering with central or peripheral nervous system function; iii) diagnosis of concomitant medical conditions likely to influence study results according to the investigator; iv) intake of analgesic or anti-migraine drugs in the 24 hours before the nBR recording. All subjects who fulfilled inclusion/exclusion criteria signed a written informed consent and underwent a single evaluation of the nBR and nBR habituation, according to a previously published method (16).

Table 3. Summary of the findings of pivotal papers on nociceptive blink reflex habituation in migraine.

First author	Study population	Habituation paradigm	Main findings
De Marinis *et al.* (27)	30 migraine without aura 30 HS	Stimulation intensity: 7 times detection threshold intensity Frequency: every 20, 10, 5, 4, 3, 2 and 1 seconds Number of stimuli: 10 responses for each time frequency Number of blocks: 7 Habituation definition: change in R_2 areas during the habituation test	nBR habituation was reduced in migraine patients who had a migraine attack in the following 72 hours after the electrophysiological recording
Katsarava *et al.* (15)	17 migraine patients in three conditions: i) interictal phase; ii) during spontaneous headache within 6 hours of onset; iii) after acute treatment 15 HS	Stimulation Intensity: 1.5 times the pain threshold Frequency: pseudorandomized inter-stimulus interval 15 to 17 seconds Number of stimuli: 6 per block Number of blocks: 2 Habituation definition: regression coefficient for each block	Lack of nBR habituation in migraine when compared to HS Normalization of habituation during the acute migraine attack or after acute drug treatment
Di Clemente *et al.* (28)	15 migraine without aura (interictal phase) 15 HS	Stimulation intensity: 1.5 times the pain threshold Frequency: pseudorandomized inter-stimulus interval of 15 to 17 seconds Number of stimuli: 6 stimuli per block (first excluded for startle) Number of blocks: 10 (2 minutes inter-block interval) Habituation definition: percentage change of the R_2 area	nBR habituation deficit in migraine when compared to HSnBR habituation positively correlated with attack frequency Positive correlation between habituation of pattern reversal visual evoked potentials and nBR
Di Clemente *et al.* (6)	16 migraine without aura (interictal phase) 15 HS without family history of migraine 14 HS with at least one first degree relative suffering from migraine (HV-F)	Stimulation intensity: 1.5 times the pain threshold Frequency: pseudorandomized inter-stimulus interval of 15 to 17 seconds Number of stimuli: 6 stimuli per block (first excluded for startle) Number of blocks: 10 (inter-block interval of 2 minutes) Habituation definition: percentage change of the R_2 area	nBR habituation deficit in migraine and HV-F when compared to HVnBR habituation positively correlated with attack frequency

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Table 3. Continued from previous page.

First author	Study population	Habituation paradigm	Main findings
Perrotta *et al.* (20)	22 migraine without aura (interictal phase) 27 CH patients during the active phase 20 HS	Stimulus intensity: 1.3 times the reflex threshold Frequency: randomized frequencies of 0.2, 0.3, 0.5, 0.7 and 1 Hz Number of stimuli: 16 responses (first excluded for startle) Number of blocks: 3 Habituation definition: percentage of the R_2 area	nBR habituation deficit in migraine without aura and cluster headache when compared to HSThe nBR habituation deficit was more pronounced in CH when compared to migraine at specific stimulation frequencies
Hansen *et al.* (29)	5 FHM-1 (R583Q or C1369Y mutations) 4 FHM-2 (R_2 02Q or R763C mutations) -7 HS	Stimulation intensity: 1.5 times the pain threshold Frequency: pseudorandomized inter-stimulus interval of 15-17 s Number of stimuli: 6 responses per block (first excluded for startle) Number of blocks: 5 (inter-block interval of 2 minutes) Habituation definition: percentage change R_2 area	FHM had a more pronounced nBR habituation when compared to HS
de Tommaso *et al.* (30)	33 migraine without aura patients randomly assigned to 3 months of treatment with: i) nBR biofeedback; ii) nBR biofeedback plus topiramate 50 mg (b.i.d.); iii) topiramate 50 mg (b.i.d.)8 HS	Stimulus intensity: 1.5 times the pain threshold Frequency: pseudorandomized inter-stimulus interval of 15 to 17 seconds Number of stimuli: 6 responses per block Number of blocks: 10 (inter-blocks interval of 2 minutes) Habituation definition: percentage change R_2 area	nBR biofeedback reduced the R_2 area, without improving R_2 habituation
Perrotta *et al.* (7)	29 migraine without area patients (interictal phase) 17 migraine with aura (interictal phase) 30 HS	Stimulation intensity: 1.5 times the reflex threshold Frequency: randomized frequencies of 0.05, 0.1, 0.2, 0.3, 0.5, and 1 Hz Number of stimuli: 6 per block (first excluded for startle) Number of blocks: 5 Habituation definition: percentage change R_2 area	nBR habituation deficit in migraine with and without aura when compared to HS

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Nociceptive blink reflex registration procedure. The nBR was elicited using a planar concentric electrode (Bionen, Florence, Italy) placed 10 mm above the emergence of the supraorbital nerve. For each subject, the right side was used for stimulation and recording. Every study session was conducted between 9.00 and 11.00 in the morning. Participants were asked to stay caffeine-free in the 12 hours before the session. Females were investigated during the follicular phase to avoid fluctuation related with the menstrual cycle.

The stimulation (single monopolar stimulation, duration 0.3 ms) was delivered by a constant current stimulator (electric stimulator DS7A, Digitimer, Hertfordshire, UK).

The surface electromyographic recording was carried out at the level of the orbicularis oculi muscle through a pair of surface electrodes, with the reference electrode on the side of the eye, and the recording electrode on the midline of the lower eyelid. The ground electrode was placed on the subject's forehead. The recording parameters were: filter bandpass between 3 Hz and 3 kHz, sampling rate of 2.5 kHz, analysis time was 200 ms, and sensitivity of 100 mV. All signals were amplified and full-wave rectified (CED Powerlab interface 1401, Cambridge Electronic Design, UK; electronic amplifier BM623, Biomedica Mangoni, Pisa, Italy).

During the recording, the subjects were comfortably seated in an armchair in a quiet room, relaxing with their eyes open. First, a single nBR was recorded. A progressive staircase increase in the stimulation intensity (0.2 mA at time, with a 3-minute pause in between) was used to evaluate the RTh, defined as a stable R_2 response in at least 3 consecutive stimulations (amplitude exceeding 50 μV for at least 20 msec). At RTh, subjects were asked to indicate the perceived painful stimulus on a numeric rating scale (NRS) from 0 (no perception) to 10 (worst possible pain). The R_2 latency (msec) and amplitude (μV × msec) were recorded. The stimulus intensity (mA) at which the subjects first perceived a non-painful sensory feeling was recorded (STh).

Paradigm to study the habituation phenomenon. To assess the habituation of nBR, 26 consecutive stimuli were administered at three different and randomized stimulus frequencies (0.2, 0.3, 0.5 Hz). The stimulation intensity of the habituation study was equal to 1.5 times the RTh. Of these stimulations, the first sweep was removed from the analysis to eliminate the startle response. The remaining 25 electromyographic sweeps were used to assess the habituation phenomenon. In offline analysis, for each electromyographic sweep the AUC of the R_2 compo-

Table 3. Continued from previous page.

First author	Study population	Habituation paradigm	Main findings
Di Lorenzo *et al.* (31)	18 migraine without aura patients before and after 1 month of ketogenic diet	Stimulation intensity: 1.2 times the pain threshold Frequency: pseudorandomized inter-stimulus interval between 30 and 35 seconds Number of stimuli: 6 per block (first excluded for startle) Number of blocks: 2 (inter-blocks interval of 2 minutes) Habituation definition: slope of the linear regression of R_2 area	nBR habituation deficit did not change after 1 month of ketogenic diet
Thiele *et al.* (32)	22 episodic migraine patients at baseline (T0) and three months (T3) after treatment with anti-CGRP mAbs22 HS	Stimulation intensity: 1.5 times the pain threshold Frequency: inter-stimuli interval of 15 to 17 seconds Number of stimuli: 6 per block Number of blocks: 10 (inter-blocks interval of 2 min) Habituation definition: slope of the linear regression of R_2 area	nBR habituation of the non-stimulated side was enhanced from T0 to T3 nBR habituation of the stimulated side was not modified from T0 to T3
Casillo *et al.* (33)	20 migraine patients (15 chronic migraine and 5 episodic migraine) at baseline (T0), and 28 days (T1), and 56 days (T2) after erenumab 70 mg	Stimulation intensity: 1.2 times the pain threshold Frequency: pseudorandomized inter-stimulus interval of 30 to 35 seconds Number of stimuli: 6 per block (first excluded for startle) Number of blocks: 2 (inter-block interval of 2 minutes) Habituation definition: slope of the linear regression of R_2 area	nBR habituation did not change after erenumab treatment nBR AUC was lower at T1 and T2 when compared to baseline
Sebastianelli *et al.* (34)	15 chronic migraine patients at baseline (T0), and after 1 and 3 months after BoNT-A treatment	Stimulation intensity: 1.5 times the pain threshold Frequency: inter-stimuli interval of 40 seconds Number of stimuli: 6 responses per block (first excluded for startle) Number of blocks: 3 (inter-block interval of 2 minutes) Habituation definition: slope of the linear regression of R_2 area	nBR habituation enhancement at 3 months after a single session of BoNT-A treatment

HS, healthy subjects; nBR, nociceptive blink reflex; CH, cluster headache; FHM, familial hemiplegic migraine; AUC, area under the curve.

nent was measured and expressed in μV × msec. For each electromyographic sweep, the onset and end of the nBR R_2 component were manually identified: i) the onset of the R_2 component was visually determined and confirmed if the offset exceeded the isoelectric line of 50 μV for at least 20 msec, and ii) the end of the R_2 component was visually determined and confirmed if the electromyographic signal returned to the isoelectric line for at least 200 msec. The area of the nBR R_2 component was calculated within this manually determined time window using the AUC function of the Signal software, Version 5.08, for Windows (Cambridge Electronic Design, UK). The obtained 25 AUC were divided in 5 blocks, and the average values of the AUC was calculated for each block. The habituation phenomenon was assessed with absolute AUCs values of the 5 blocks (μV × msec) as well as with AUC expressed as percentage variation from the first block of stimulation (normalized to 100%).

The percentage reduction in the AUC of the fifth block, compared to the first, represents the HI value. Thus, high His indicate a pronounced habituation, while low HI are indicative of a weak habituation phenomenon. Between the three different stimulus frequencies, participants waited at least 20 minutes to avoid a carry-over effect.

Statistical analysis. Statistical analysis was conducted with the SPSS software, ver. 21 (IBM Corp., Armonk, NY, USA) and with "R: A language and environment for statistical computing" (R Foundation for Statistical Computing, Vienna, Austria), Version 1.2.5033, for Windows. The Kolmogorov-Smirnov test proved a non-normal distribution of a subset of data (for example absolute AUC in different blocks across consecutive stimulation), thus non-parametric tests were used; of note, the distribution of the His for all the study frequencies were normally distributed. Categorical data are reported as absolute numbers and percentages, while continuous variables as mean ± standard deviation. For continuous variables, differences between groups were analysed using the Mann-Whitney U test, while for categorical variables, statistical analysis was performed with the chi-square test. Differences among the three study frequencies in the AUC of the first block of stimulation were assessed with the Friedman test. Correlation analysis was performed with Spearman's correlation test. To evaluate the modification of AUC (absolute values and percentage modification) across consecutive stimulations, we used two non-parametric models for repeated measures (36). The first model compared the habituation among different stimulation frequencies and included the following factors: factor TIME

(within subjects, 5 levels: first to fifth blocks), and factor Hz (within subjects, 3 levels: 0.5 Hz *vs*. 0.3 Hz *vs*. 0.2 Hz). The second model compared the habituation between male and female subjects and included the following factors: factor TIME (within subjects, 5 levels: first to fifth blocks), and factor SEX (between subjects, 2 levels: male *vs*. female).

As we aimed to assess a limit for the habituation deficit, we considered a one-tailed 10th percentile threshold as the lower threshold of normative values.

The level of significance was set at $\alpha<0.05$, corrected for multiple comparison with Bonferroni when necessary.

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Online supplementary material:

Supplementary Table 1. *Absolute values of the area under the curve of the R_2 component of the nociceptive blink reflex at 0.5 Hz.*

Supplementary Table 2. *Absolute values of the area under the curve of the R_2 component of the nociceptive blink reflex at 0.3 Hz.*

Supplementary Table 3. *Absolute values of the area under the curve of the R_2 component of the nociceptive blink reflex at 0.2 Hz.*

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Conflict of interest: the authors declare no potential conflict of interest.

Funding: this study was supported by a Research Grant from the Italian Ministry of Health to IRCCS Mondino Foundation (Ricerca Corrente 2022–2024).

Ethics approval: the study protocol was approved by the local Ethics Committee of Pavia during the 09/05/2018 session (P-20180025265).

Availability of data and material: raw data are available upon reasonable request at: www.zenodo.org (10.5281/zenodo.10605959).

Received: 14 February 2024. Accepted: 2 April 2024.

Confinia Cephalalgica 2024; 1:15730. doi:10.4081/cc.2024.15730