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 R2 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 10th 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 R2 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 correlation with age, intensity of stimulation, and latency of the R2 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.
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. pre-ictal 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 R2 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 R2 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 the R2 component (Spearman -0.580, p=0.001).

The absolute AUC of the R2 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 R2 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 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 R2 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 R2 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.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. subjects</td>
<td>40</td>
<td>16</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>STh, mA</td>
<td>1.0±0.5</td>
<td>0.8±0.6</td>
<td>1.1±0.4</td>
<td>0.150</td>
</tr>
<tr>
<td>RTh, mA</td>
<td>3.5±3.7</td>
<td>4.6±5.6</td>
<td>2.7±1.2</td>
<td>0.924</td>
</tr>
<tr>
<td>NRS RTh</td>
<td>4.8±1.8</td>
<td>5.0±2.0</td>
<td>4.7±1.7</td>
<td>0.594</td>
</tr>
<tr>
<td>RTh R2 latency, msec</td>
<td>35.6±4.8</td>
<td>38.4±5.3</td>
<td>34.2±3.7</td>
<td>0.003</td>
</tr>
<tr>
<td>RTh R2 AUC, µV × msec</td>
<td>1.8±0.9</td>
<td>1.6±0.5</td>
<td>2.0±1.3</td>
<td>0.672</td>
</tr>
<tr>
<td>Hl 0.5, Hz (%)</td>
<td>73.1±13.6</td>
<td>70.5±14.5</td>
<td>74.8±13.1</td>
<td>0.404</td>
</tr>
<tr>
<td>Hl 0.3, Hz (%)</td>
<td>69.2±15.0</td>
<td>71.7±9.3</td>
<td>67.6±17.9</td>
<td>0.713</td>
</tr>
<tr>
<td>Hl 0.2, Hz (%)</td>
<td>61.1±21.5</td>
<td>62.7±20.7</td>
<td>60.0±22.3</td>
<td>0.754</td>
</tr>
</tbody>
</table>

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 R2 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 R2 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%).
Habitation indexes and proposed normative values. The average habitation 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 R2 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 frequency 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 habitation 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 habitation phenomenon was recorded and confirmed across 26 consecutive nBR recordings. We described a frequency-dependent degree of habitation, being more pronounced when higher frequencies of stimulation were applied. Indeed, the 10th percentile of the habitation 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 habitation deficit behaviour. We could also speculate that HI values above the 50th percentile may identify a subset of subjects with an extremely pronounced habitation 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 R2 component of the nBR and a more pronounced habitation phenomenon at intermediate stimulation frequencies. It is worth noting that this gender differences normalized when habitation was assessed as percentage variation of the second to the fifth blocks of the AUC of the first block. The gender-related habitation 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 habitation 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 habitation deficit is not fully understood. A first hypothesis takes into account a reduced activity in brainstem monaminergic pathways, resulting in a lower 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 intra-cortical short-range lateral inhibition as well as functional disconnection between the thalamus and the cortex (i.e. thalamo-cortical dysrhythmia) may account for the observed habitation 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 thalamo-cortical drive and aberrant cortical inhibitory mechanisms (25).

In addition, another intriguing pathophysiological hypothesis explaining the defective habitation in primary headaches rooted on the link between the dysfunction of the hypothalamic axis in mice and the lack of habitation 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 habitation was consistently found impaired in migraine and other headache disorders (4). However, different paradigms were adopted to assess nBR habitation, 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 habitation features, as revised by Rankin et al. (35). Indeed, some of the main habitation characteristics described, such as the progressive decrease in response parameters, the spontaneous recovery after stimulus withdrawal, and the more pronounced habitation to higher stimulus frequencies, are in line with our results. Rankin et al. described other features of the habitation phenomenon, for example the potentiation of habitation induced by a carry-over effect achieved by means of repeated series of habituation training and recovery. The evaluation of these habitation components required longer and ad-hoc stimulation protocols. In our study, we voluntarily avoided the study of the potentiation of habitation 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 habitation 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. Habitation indexes of the nociceptive blink reflex at different stimulation frequencies.

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

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

<table>
<thead>
<tr>
<th>First author</th>
<th>Study population</th>
<th>Habituation paradigm</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Marinis et al. (27)</td>
<td>30 migraine without aura</td>
<td>Stimulation intensity: 7 times detection threshold intensity</td>
<td>nBR habituation was reduced in migraine patients who had a migraine attack in the following 72 hours after the electrophysiological recording</td>
</tr>
<tr>
<td></td>
<td>30 HS</td>
<td>Frequency: every 20, 10, 5, 4, 3, 2 and 1 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of stimuli: 10 responses for each time frequency</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of blocks: 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Habituation definition: change in R2 areas during the habituation test</td>
<td></td>
</tr>
<tr>
<td>Katsarava et al. (15)</td>
<td>17 migraine patients in three conditions:</td>
<td>Stimulation Intensity: 1.5 times the pain threshold</td>
<td>Lack of nBR habituation in migraine when compared to HS</td>
</tr>
<tr>
<td></td>
<td>i) interictal phase; ii) during spontaneous headache</td>
<td>Frequency: pseudorandomized inter-stimulus interval 15 to 17 seconds</td>
<td>Normalization of habituation during the acute migraine attack or after acute drug treatment</td>
</tr>
<tr>
<td></td>
<td>within 6 hours of onset; iii) after acute treatment</td>
<td>Number of stimuli: 6 per block</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 HS</td>
<td>Number of blocks: 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Habituation definition: regression coefficient for each block</td>
<td></td>
</tr>
<tr>
<td>Di Clemente et al. (28)</td>
<td>15 migraine without aura (interictal phase)</td>
<td>Stimulation intensity: 1.5 times the pain threshold</td>
<td>nBR habituation deficit in migraine when compared to HSnBR habituation positively correlated with attack frequency</td>
</tr>
<tr>
<td></td>
<td>15 HS</td>
<td>Frequency: pseudorandomized inter-stimulus interval 15 to 17 seconds</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Number of stimuli: 6 stimuli per block (first excluded for startle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of blocks: 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 minutes inter-block interval)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Habituation definition: percentage change of the R2 area</td>
<td></td>
</tr>
<tr>
<td>Di Clemente et al. (6)</td>
<td>16 migraine without aura (interictal phase)</td>
<td>Stimulation intensity: 1.5 times the pain threshold</td>
<td>nBR habituation deficit in migraine and HV-F when compared to HVnBR habituation positively correlated with attack frequency</td>
</tr>
<tr>
<td></td>
<td>15 HS without family history of migraine (HV-F)</td>
<td>Frequency: pseudorandomized inter-stimulus interval 15 to 17 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of stimuli: 6 stimuli per block (first excluded for startle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of blocks: 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(inter-block interval of 2 minutes)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Habituation definition: percentage change of the R2 area</td>
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</tr>
</tbody>
</table>

To be continued on next page
Table 3. Continued from previous page.

<table>
<thead>
<tr>
<th>First author</th>
<th>Study population</th>
<th>Habitation paradigm</th>
<th>Main findings</th>
</tr>
</thead>
</table>
| 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  
Habitation definition: percentage of the R2 area  | nBR habituation deficit in migraine without aura and cluster headache when compared to HS  
The 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 (R02Q or R763C mutations)  
-7 HS  |  
Stimulus 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)  
Habitation definition: percentage change R2 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 reflex 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)  
Habitation definition: percentage change R2 area  | nBR biofeedback reduced the R2 area, without improving R2 habituation |
| Perrotta et al. (7)     | 29 migraine without area patients (interictal phase)  
17 migraine with aura (interictal phase)  
30 HS  |  
Stimulus 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  
Habitation definition: percentage change R2 area  | nBR habituation deficit in migraine without aura when compared to HS |

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 R2 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 R2 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 R2 compo-
nent was measured and expressed in µV × msec. For each electromyographic sweep, the onset and end of the nBR R2 component were manually identified: i) the onset of the R2 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 R2 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 R2 component was calculated within this manually determined time window using the electromyographic signal returned to the isoelectric line for at least 200 msec, and ii) the end of the R2 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 R2 component was calculated within this manually determined time window using the AUC function of the Signal software, Version 5.08, 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 stimulations), 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 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.

**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.
Corrado et al. | Confinia Cephalalgica 2024; 34:15730

(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 tiple comparison with Bonferroni when necessary.

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

References


