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Nitroglycerin challenge induces lateralized headache in nasociliary nerve-ligated rats: implications for chronic migraine

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Background/aim: Chronic migraine is a common debilitating disease with limited treatment options. We aimed to develop a novel model for chronic migraine by ligating the nasociliary nerve (NCL) and administering nitroglycerin (NTG) to exacerbate acute headache attacks.

Materials and methods: Exacerbation of the headache was induced by NTG (10 mg/kg, subcutaneously) administered to male Wistar rats (n = 36) 14 days following unilateral NCL. Cutaneous and cold allodynia was tested using Von Frey (VF) filaments and acetone, respectively. Elevated plus maze (EPM) results and c-fos immunoreactivity of TNC were investigated.

Results: NTG administration significantly decreased VF threshold values only in the nasociliary nerve (NCN) territory and the ipsilateral forepaw (P = 0.0001, P = 0.02). Cold allodynia developed in bilateral NCN territories (P = 0.013). The number/rate of entrance to open arms in the EPM was significantly decreased in NCN-ligated rats (P = 0.042, P = 0.035). Immunohistochemistry disclosed significantly increased c-fos-positive neurons in ipsilateral brainstem TNC compared to the contralateral side (brain stem LI ipsilateral 25.4 ± 4.7, contralateral 11.8 ± 1.9, P < 0.05) in chronic NCN-ligated rats exposed to acute NTG.

Conclusion: The presented model provides a valid chronic migraine model relevant to humans, as NTG challenge in chronic NCL rats generated lateralized headache with cephalic and extracephalic allodynia, altered cold sensitivity, anxiety, and neuronal activation in the nociceptive laminae of brainstem trigeminal pain nuclei.

Key words: Rat, nitroglycerin, chronic migraine, headache, nasociliary nerve ligation, c-fos

1. Introduction

Chronic migraine is a major disabling condition causing loss of work and its treatment is challenging. Development of sensitization in both the peripheral and central parts of the trigeminovascular system plays a significant role in the transformation process from episodic to chronic migraine. Central sensitization can be recognized by cutaneous allodynia and muscle tenderness that develop as a result of reduced thresholds to both innocuous (touch) and noxious (pain, heat) stimuli and enlarged receptive fields (1). The existence of central sensitization is one of the characteristic features of chronic migraine as cutaneous allodynia has been reported in 40% of chronic daily headache patients (2). Allodynia affecting the periorbital region and spreading to extracephalic areas such as the upper extremities has been shown during migraine attack (3,4). Neuropathic pain features are therefore one of the characteristic aspects of chronic migraine headaches (5–7).

There are experimental acute migraine models in the literature, but no chronic migraine model mimicking migraine in humans is available. The nasociliary nerve (NCN), a rat correlate of the ophthalmic branch of the trigeminal nerve, transmits sensory information from the dura, arachnoid mater, pia mater, and intracranial structures (8–10). In addition, transection of the NCN inhibits trigeminal nerve-mediated vascular and neuronal effects and plasma protein extravasation in rats (10), while electrical stimulation of the NCN leads to increased cerebral blood flow (11). Nerve ligation is therefore a common approach to generate a neuropathic pain model (12,13) and we developed such a model by double-ligating the NCN. NCN ligation (NCL) in rats induced allodynia and hyperalgesia in the NCN territory between the ear and the eye on the ipsilateral forehead of the rats beginning around the postoperative 11th and 12th days (14).

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Chronic migraine patients experience headaches at least 15 days a month and severe attacks are induced by common triggers. We therefore aimed to simulate this condition by adding nitroglycerin (NTG) as a trigger. NTG is one of the well-known and commonly used challenges to induce a migraine attack in both humans and experimental animals. NTG administration generates a typical migraine attack within 4–6 h in up to two-thirds of migraine patients. Sumatriptan has been shown to cease NTG-induced headache in both human and animal studies (3,15).

We used the NCN ligation model to develop chronic headache and evaluated rat behavior following acute NTG administration in this study. We aimed to investigate headache by measuring mechanical pain thresholds, cold allodynia, anxiety/fear behavior, and activation of second-order trigeminal pain nuclei along with the cerebral pain matrix.

2. Material and methods

2.1. Animals

The study protocol was approved by the Gazi University Animal Studies Ethics Committee of Animal Experiments (Permit Number: GU ET-10.008). Experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All surgeries were performed under anesthesia and all efforts were made to minimize suffering.

Male Wistar rats weighing 250–300 g were housed in a controlled environment with a room temperature of 22 ± 2 °C and a dark/light cycle of 12 h of daylight and 12 h of darkness. The rats received food and water ad libitum.

2.2. Surgical procedure

Body temperature was kept constant at 37 ± 0.5 °C with a heating pad during all surgical procedures. Rats were anesthetized with an IP ketamine (Ketalar, 50 mg/kg) and xylazine (Alfazyne, 5 mg/kg) mixture. The depth of anesthesia was adjusted according to the hind leg pinch reaction. The surgical area was prepped and rats were placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). An incision was made medial to the right orbital rim and subcutaneous tissue was dissected using blunt dissection with small scissors. The ophthalmic nerve was found on the right side and followed into the orbita to localize the NCN, which was constricted twice 1 mm apart with 5.0 surgical sutures. The NCN was identified on the right side but not constricted in sham-operated rats (SHAM). The left side was not operated on in any rat and was used as a control. Surgery was terminated and a local anesthetic mixture (EMLA) was applied on the incision.

2.3. Behavior

All tests were performed between 0800 and 1600 hours. Behavioral tests of the head were performed on platforms 40 cm high. Rats were acclimatized to the platforms for 10 min before the experiments. Behavior tests were started 20 min following NTG administration.

On the postoperative 14th day, the area between the eye and ear denoting the nasociliary nerve territory was tested by Von Frey (VF) filaments using Chaplan's up and down method (16). Avoidance and grooming following VF testing was accepted as a positive response. VF threshold values were translated to grams using Dixon's nonparametric method (17). Rats with a VF threshold lower than 4 g after NCL were accepted as neuropathic and others were excluded.

On the 16th postoperative day (n = 6), acute headache was triggered with the subcutaneous administration of 10 mg/kg NTG (Adeka) in the NTG group or saline was administered. Behavioral tests were performed 20 min following NTG or saline administration. Mechanical allodynia was tested in the nasociliary nerve territory plus plantar regions using VF filaments. In addition, electronic VF (EVF) testing was employed for comparison with manual VF filaments. Cold allodynia was evaluated with acetone in the bilateral nasociliary nerve territory. Grooming and avoidance within 1 min was evaluated as allodynia. The test was repeated 5 times with 2-min intervals, starting from the contralateral side, and positive response was expressed by percentage.

Anxiety of the rats was evaluated by the elevated plus maze (EPM), a gold-standard test for anxiety. The time spent in the closed arms versus open arms within the first 5 min was recorded. Longer time spent in the closed arms and higher number of entrances to closed arms were in favor of anxiety (18–20).

2.4. Immunohistochemistry

The rats were sacrificed with a lethal dose of thiopental sodium 4–6 h after NTG administration. c-fos immunoreactivity was used to confirm neuronal activity. Rats were perfused transcardially with heparinized saline and afterwards with 4% 0.1 M paraformaldehyde. The brain and brainstem were postfixed in paraformaldehyde solution and then dipped into sucrose solution. Free floating brain sections were stained for c-fos-like immunoreactivity with the avidin–biotin–peroxidase method. Sections were washed in phosphate-buffered saline and placed in 0.3% hydrogen peroxide for 30 min. Endogenous proteins were blocked in 10% normal goat serum for 2 h followed by several washes. Primary antibody (rabbit anti-c-fos polyclonal antiserum; Calbiochem, San Diego, CA, USA) was used for incubation at a dilution of 1:5000 with 0.3% Triton X and 2% goat serum for 48 h at 4 °C. Sections were treated with biotinylated secondary antibody (goat

antirabbit IgG) at a dilution of 1:600 with 0.3% Triton X for 2 h. Afterwards, the avidin–biotin–peroxidase complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) was applied and diaminobenzidine was used as the chromogen. The slides were then dehydrated with ethanol, air-dried, and cover-slipped with Vectamount (Vector Laboratories) (10,21). The primary c-fos antibody was omitted in one well for a negative control. Tissue sections obtained from every 150 μm were examined for c-fos immunoreactivity under bright-field microscopy and c-fos counting of different brain regions according to the rat atlas (22,23) was performed by a blinded observer.

c-fos immunoreactivity in the brain and brainstem pain-related structures was studied. TNC laminae I–II and III–IV in the brainstem, the cingulate cortex, the claustrum, the insula in the cortex, and the hippocampus, putamen, amygdala, thalamus, and thalamic nuclei in the subcortical region were studied for c-fos-positive neurons as previously described (24).

2.5. Data analysis

Data were analyzed using SPSS 11.5. Variables were compared among groups for statistical significance by the one-way ANOVA and post hoc Tukey tests. Results were presented as mean ± standard error of the mean (SEM). $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Mechanical allodynia

VF thresholds of the nasociliary nerve territory were compared between the NCL rats and sham-operated rats. VF threshold values of the NCN territory were significantly decreased on the ipsilateral side in the NCN-ligated NTG-administered group (NCL NTG) compared to the sham-operated NTG-administered group (SHAM NTG) ($P < 0.05$, $P = 0.0001$, $P = 0.02$, Figure 1). There was no significant difference in EVF threshold values in the nasociliary nerve territory between the groups. The EVF duration on the ipsilateral NCN territory was significantly decreased in the NCL NTG group compared to the NCL saline-administered group ($P < 0.05$).

NTG administration significantly decreased VF threshold values in the ipsilateral and contralateral forepaw in the NCN-ligated NTG-administered group (NCL NTG) compared to the SHAM NTG group ($P = 0.0001$, Figure 2). EVF threshold values and the EVF duration in bilateral fore- and hind paws were similar among the groups.

3.2. Cold allodynia

Cold allodynia was tested by acetone administration. In the NCL NTG group, the percent positive response to acetone in bilateral NCN territories in the forehead was higher compared to the SHAM NTG group ($P = 0.013$, Figure 3).

3.3. EPM

In the NCL NTG group, the count and ratio of entrance to open arms in the EPM were significantly decreased compared to the SHAM NTG group ($P = 0.042$, $P = 0.035$, Figure 4). The time spent in the open arms of the EPM was similar between the groups. Rearing count in the closed arm of the EPM was significantly decreased in the NCL and NTG group compared to the SHAM NTG group ($P < 0.05$). Therefore, NCL increased anxiety in NTG-administered rats.

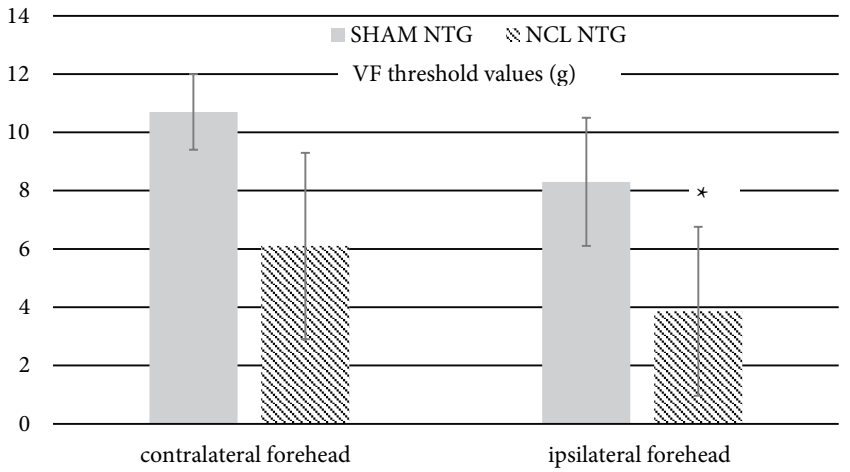

Figure 1. VF threshold values (g) in foreheads of NCL NTG group were significantly decreased compared to the SHAM NTG group. Only after NCL, allodynia reached neuropathic levels (mean ± SEM). *$P < 0.05$.
SHAM NTG: Sham-operated, IP NTG administered; NCL NTG: NCN ligated, IP NTG administered.

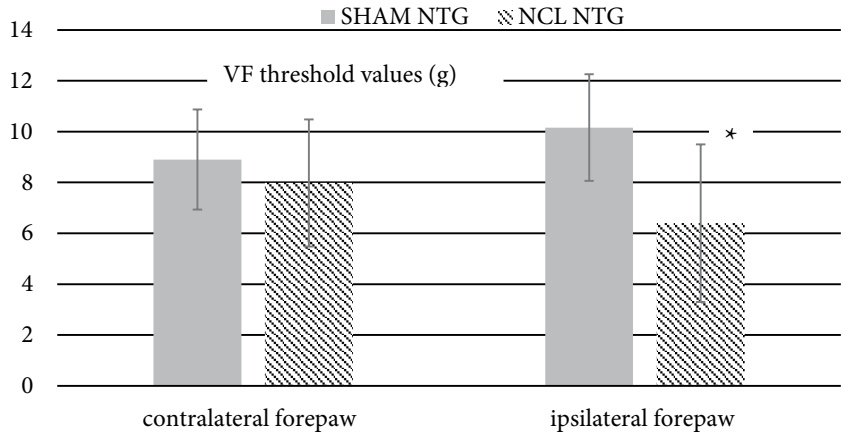

Figure 2. VF threshold values (g) in ipsilateral forepaws of NCL NTG were significantly decreased compared to SHAM NTG (mean ± SEM). *P < 0.05.
SHAM NTG: Sham-operated, IP NTG administered; NCL NTG: NCN ligated, IP NTG administered.

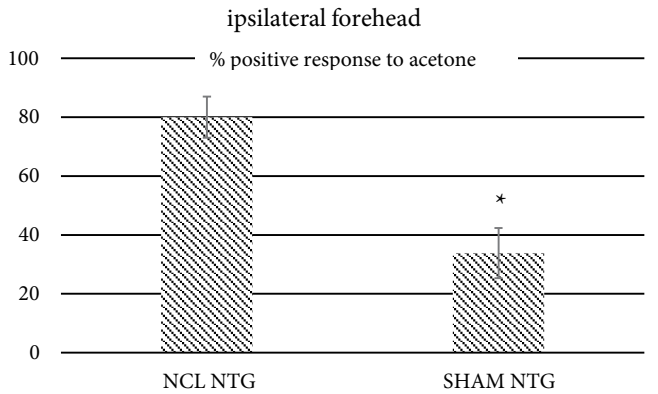

Figure 3. Cold allodynia was detected in NCN territory in ipsilateral foreheads in NCL NTG group (% positive response). *P < 0.05.
SHAM NTG: Sham-operated, IP NTG administered; NCL NTG: NCN ligated, IP NTG administered.

3.4. Immunohistochemistry

We counted the c-fos-positive neurons in the brainstem TNC laminae I–II and laminae III–IV under the light microscope. In the NCL group after NTG administration, the brainstem laminae I–II c-fos count was significantly increased compared to the contralateral side (25.4 ± 4.7, 11.8 ± 1.9, P < 0.05, Figure 5). In the NCL group after NTG administration, ipsilateral c-fos-positive neurons in TNC laminae I–II were significantly increased compared to the SHAM NTG group. In the NCL NTG group, total c-fos-positive neurons in lamina I–IV were significantly higher than in the SHAM NTG group (P < 0.05).

We evaluated the cingulate cortex, claustrum, insula, hippocampus, putamen, amygdala, and thalamic nuclei c-fos distribution in the brain. The NCL NTG group had more c-fos-positive neurons compared to the SHAM NTG group in the ipsilateral cingulate cortex, which indicates that NCL and NTG increase the cingulate cortex c-fos count (Figure 6).

The NCL group had a higher c-fos-positive neuron count in the amygdala compared with the SHAM group (P < 0.05). No significant difference in c-fos count was noted in the hippocampus, insula, and thalamic nuclei after NTG administration to NCL rats (Figure 6).

4. Discussion

We have developed a novel chronic migraine model where chronic headache was mimicked by unilateral NCL and acute headache exacerbation was triggered by systemic administration of NTG. NTG administration in NCL rats exhibited mechanical allodynia, cold allodynia in the NCN territory, increased anxiety, and c-fos activation in

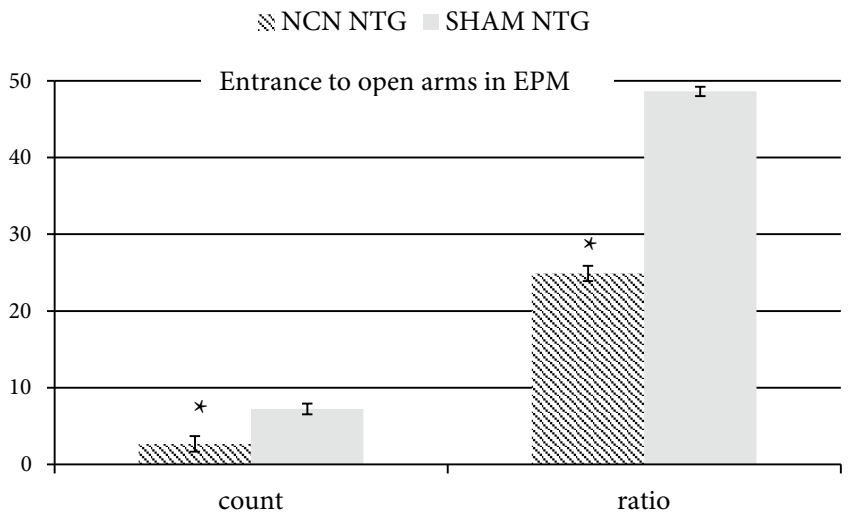

Figure 4. Count and ratio of entrances to open arms in the EPM were significantly decreased in NCL NTG compared to SHAM NTG group (mean ± SEM). *P < 0.05.
SHAM NTG: Sham-operated, IP NTG administered; NCL NTG: NCN ligated, IP NTG administered.

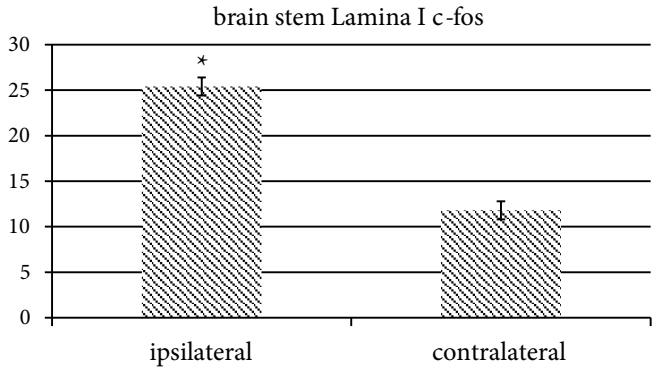

Figure 5. Ipsilateral c-fos-positive cells in laminae I–II of brain stem pain nuclei (trigeminal nucleus caudalis) were significantly increased compared to the contralateral side in the NCL NTG group (mean ± SEM). *P < 0.05.
NCL NTG: NCN ligated, IP NTG administered.

the pain matrix and brainstem trigeminal nuclei. These findings were all compatible with lateralized headache and central sensitization.

Mechanical sensitivity in the head and body is commonly reported during migraine. Patients who experience sensitivity in the head complain of throbbing pain and increased headache during activities increasing the intracranial pressure, such as bending and coughing. Sensitivity in the body causes pain while combing their hair, shaving, and wearing eyeglasses or earrings. Wearing tight clothes or being wrapped in a heavy blanket are also very disturbing for some patients during a migraine attack, due to cutaneous allodynia in extracranial regions. The threshold for touch, cold, heat, and mechanical stimuli has been reported to be significantly reduced during an attack in 79% of migraineurs. Cutaneous allodynia spreads to the contralateral side of the head and body indicating central sensitization in 67% of the patients (2,4,5,15). Migraine patients with allodynia are more susceptible to triggering factors compared to nonallodynic patients.

Recognizing the fact that chronic migraine and chronic headache are not definitely the same, a chronic migraine model should reflect the characteristic clinical features, triggers, drug responses, and chronicity of the disorder in accordance with the headache anatomy. The ophthalmic branch of the trigeminal nerve innervates the meningeal

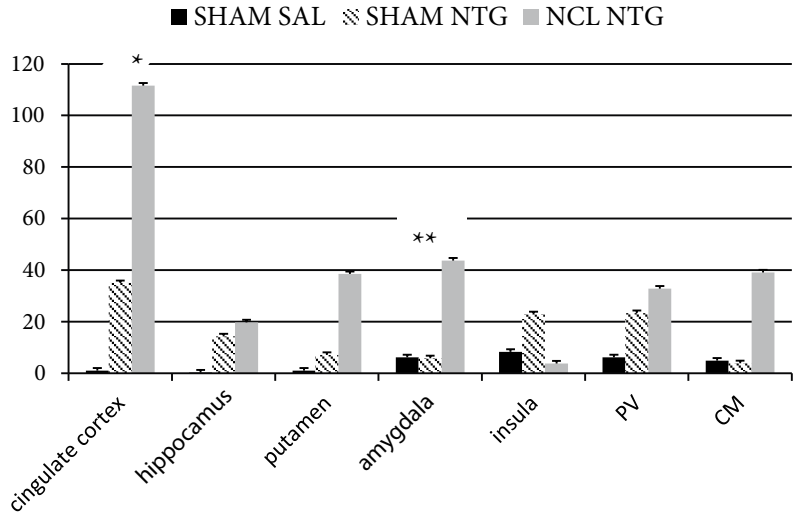

Figure 6. NTG challenge significantly induced c-fos-positive neurons in pain-related structures such as the cingulate cortex and amygdala (mean ± SEM). *Significance between SHAM SAL and NCL NTG groups at $P < 0.05$. **Significance between SHAM SAL and NCL NTG groups at $P < 0.05$.
SHAM SAL: Sham, IP saline administered; SHAM NTG: sham, IP NTG administered; NCL NTG: NCN ligated, IP NTG administered.

vessels, particularly the dura mater, and activation of perivascular trigeminal afferents plays a crucial role in migraine. The demonstration of trigeminovascular activation is therefore essential for a valid model for chronic migraine and there is no available model yet that can fulfill all the human migraine criteria. Infusion of the nitric oxide donor NTG is a well-known, reproducible, and approved way of inducing a delayed migraine headache in human migraineurs. NTG is also effectively used to induce acute headache in rodent models. Ligation of the nasociliary nerve, which is the equivalent of the ophthalmic nerve in humans, produces chronic headache.

We combined the 2 models above and produced a lateralized chronic migraine model with acute exacerbations. Our model was valid and relevant to human migraine as cutaneous allodynia and altered thresholds of heat were demonstrated in the periorbital area. In the literature, NTG-induced allodynia and hyperalgesia in rodents is reversed by sumatriptan (25–29).

The spinal trigeminal nucleus receives converging input from the meninges, scalp, and face and cephalic allodynia develops when these neurons are sensitized. Extracephalic allodynia reflects sensitization of the third-order trigeminovascular neurons in the posterior thalamic nuclei as they receive converging input from the meninges, face, and body (24). In NTG-administered NCL rats, increased sensitivity to mechanical and cold stimuli in the ipsilateral forepaws and bilateral head regions therefore indicated central sensitization. It is notable that NCL alone leads to allodynia limited to cephalic structures (14). The presented study demonstrates that NTG challenge upon NCL is a key factor in the sensitization and expansion of allodynia to extracephalic structures such as the forepaw in rats. The latter finding is relevant to human chronic migraine.

VF testing is the gold-standard method to evaluate allodynia in pain studies (30,31). VF and EVF tests both verify peripheral and central sensitization. In VF testing, repetitive measurements are performed with variable caliber filaments. EVF testing is advantageous due to its ease of use with a single measurement evaluating the reaction to increased force. Although EVF testing is not detailed as VF, it still reveals allodynia in the head and forepaw (30,31). In human studies, VF testing is considered unreliable for repetition while EVF seems more reliable (31). VF and EVF results were similar in our study but VF testing was more sensitive to a decreased threshold.

As heat allodynia is a clinically rare condition, cold stimulus is preferred as the thermal stimulus for neuropathic pain models (32–34). Acetone induces a pain-free cold sensation by evaporation of liquid from the normal scalp. Cold allodynia is defined as when acetone administration causes a nociceptive reaction as detected by grooming and/or avoidance behavior in our study. The presence of cold allodynia associated with mechanical allodynia in the same cutaneous territory confirms pain behavior induced by our chronic migraine model.

Repeated chemical stimulation of the dura mater has produced chronic trigeminal hypersensitivity and potentiated the response to NTG in a model while repeated

nociception caused a decrease in periorbital pressure thresholds (5,35,36). Stimulation of the dura mater also leads to allodynia and hyperalgesia in the trigeminal nerve's ophthalmic division along with increased neuronal responses in the brainstem trigeminal nuclei (37,38). Chronic nerve constriction injury has been shown to cause long-lasting (at least 2 months) hyperalgesia, allodynia, and spontaneous pain (39).

Pain has definitive and affective motivational aspects. Reflex withdrawal from tactile or thermal stimuli is a spinal reflex. However, animal experiences cannot be relied on and painful tests are therefore used for confirmation (40). Validation of pain in animals is controversial. Withdrawal from painful stimuli is the strict confirmation of pain. EVF testing is difficult to perform in the head region compared to the extremities and probably causes a more nociceptive stimulus. In NCL rats, c-fos-positive neurons were increased in the TNC parallel to the cingulate cortex, which is an important association region of pain, after NTG administration.

The EPM is used to detect anxiety and defensive response associated with nociception (41). In our study, the count and ratio of entrance to open arms and rearing in closed arms in the EPM were decreased, indicating anxiety/fear associated with our chronic headache model. In concordance with EPM results, c-fos expression in the amygdala was significantly increased in the NCL group compared to the sham group following NTG administration.

There are some limiting factors in this study. NTG, an established headache trigger in humans and rodents, was administered in higher doses than used in humans in our study. In addition, the study was conducted on male rats although migraine is more common in women. Further studies are needed to test whether a chronic migraine model in female rats would be more relevant.

In conclusion, the combination of NTG challenge following chronic NCN ligation is a novel model for mimicking chronic migraine in rats. The existence of cephalic and extracephalic allodynia, anxiety, and activation of lateralized trigeminal pain along with cortical structures constituting the pain matrix verified the validity of the model for chronic migraine. The presented model provides an opportunity to screen the therapeutic options for chronic migraine headache.

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