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Occipital afferent activation of second order neurons in the trigeminocervical complex in rat

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Abstract

Stimulation of the greater occipital nerve produces excitation of second order neurons in the trigeminocervical complex. Given that neck pain is very common in primary headache disorders, this convergent excitation may play a role in pain referral from cervical structures. While previous studies have demonstrated a physiological model for this convergence, this study sought an anatomical approach to examine the distribution of second order neurons in the trigeminocervical complex receiving greater occipital nerve input. In addition, the role of glutamatergic NMDA receptor activation within the trigeminocervical complex in response to cervical afferents was studied. Noxious stimulation of the occipital muscle in rat using mustard oil and mineral oil produced significantly altered Fos expression in the trigeminocervical complex compared with the surgical control ($H_4 = 31.3$, $P < 0.001$, Kruskal–Wallis). Baseline expression was 11 (median, range 4, 17) fos positive cells in the trigeminocervical complex, occipital muscle treated with mustard oil produced 23 (17, 33) and mineral oil a smaller effect of 19 (15, 25) fos positive cells, respectively ($P = 0.046$). The effects of both mustard and mineral oil were reversed by the NMDA-receptor antagonist MK801.This study introduces a model for examining trigeminocervical complex activity after occipital afferent stimulation in the rat that has good anatomical resolution and demonstrates involvement of glutamatergic NMDA receptors at this important synapse.

Keywords: Fos; Trigeminocervical complex; Trigeminal nucleus caudalis; Greater occipital nerve; Cervicogenic headache

Patients with headache often report discomfort in the neck in addition to pain in the frontal region [17]. The back of the head and upper neck are innervated by branches of the C_2 root, most particularly the greater occipital nerve (GON). It seems likely that crucial aspects of the pattern of pain in primary headache are related to referral of nociceptive afferent signals from the frontal region to the neck, or vice versa. Thus understanding convergence of afferent signals from the GON and the trigeminal nerve onto trigeminocervical complex neurons is fundamental to building a picture of the pathophysiology of primary headache.

It seems clear from experimental studies that there is extensive convergence of afferents from the face, teeth, neck and oral mucosa onto neurons on the trigeminal nucleus caudalis [25]. It also seems a general principle that afferents from pain-producing intracranial structures, such as the dura mater and large intracranial vessels, project into the trigeminal nucleus caudalis and dorsal horns of C_1 and C_2, taken together as a functional unit, the trigeminocervical complex [TCC [11,13,16]]. Moreover, activation of afferents in the greater occipital nerve, a branch of the C_2 root, increases metabolic activity in neurons with the same anatomical distribution, i.e. the TCC [12]. This convergent excitation may play a role in pain referral from cervical structures, such as the occipital muscles, to the head. Certainly greater occipital nerve (GON) activation leads to frontal head pain in humans [22], and GON injection has been used therapeutically in a range of primary headaches [1].

Whilst a physiological model for this phenomenon has been developed [2,8], an anatomical model to demonstrate convergence from the GON onto the TCC is needed. This study aimed to set-up a rat model to examine the activity of the second order neurons in the TCC that receive GON input activated by noxious stimulation and to explore the role of the glutamate-NMDA receptor in activation of TCC neurons. The work was presented in preliminary form at XIIth International Headache Congress, Kyoto, Japan [18].

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All experiments were conducted under the UK Home Office Animals (Scientific Procedures) Act (1986). Twenty-nine male Sprague–Dawley rats (300–325 g) were housed under diurnal lighting and allowed food and water ad libitum. Animals were anesthetised with intraperitoneal (i.p.) pentobarbital (60 mg/kg) and the femoral artery and vein cannulated to allow blood pressure monitoring and administration of supplemental anesthetic and test compound, respectively. A tracheal cannula was inserted for artificial ventilation. The rats were secured in a stereotaxic frame and blood pressure (BP), carbon dioxide (CO_2) and rectal temperature were monitored and remained within normal physiological limits throughout. The rats were maintained with 10 mg/kg pentobarbital i.v., as needed to maintain adequate anaesthesia tested by the withdrawal response to noxious hindpaw stimulation.

A midline cranial incision was made, extending from the posterior skull at the level of the ear to the dorsum of the neck as far as C_5. The occipital muscle was exposed on one side of the head by gentle blunt dissection of the skin away from the muscle. To minimise non-specific fos expression in the trigeminocervical complex following the surgery the animals were rested in the prone position in the stereotaxic frame for two hours as previously described [21].

Following the surgical preparation the rats were assigned to one of the following six groups:

1. Group 1 (surgical control) ($n = 4$) had the occipital muscle exposed and had saline administered at 2 h.
2. In the second group (mustard oil) ($n = 5$) saline (0.3 ml, i.v.) was administered at 2 h then 30 μl mustard oil (20% allyisothiocyanate in mineral oil, Sigma-Aldrich, UK) was injected unilaterally into the occipital muscle using a 26-gauge needle over a 5–10 s period at 2 h 30 min.
3. In the third group (vehicle control) ($n = 5$) saline (0.3 ml i.v.) was administered at 2 h then an equal volume of the vehicle control (30 μl mineral oil) was injected into the occipital muscle over a 5–10 s period at 2 h 30 min.
4. In order to assess the systemic effect of NMDA receptor antagonism on fos protein expression an intravenous injection of MK801 dissolved in saline (3 mg/kg) was administered to groups 4, 5 and 6 at 2 h. Group 4 (MK801) ($n = 5$) received no further intervention.
5. Group 5 (MK801 plus mustard oil) ($n = 5$) received a 30 μl mustard oil injection into the occipital muscle over a 5–10 s period at 2 h 30 min.
6. Group 6 (MK801 plus mineral oil) ($n = 5$) received 30 μl mineral oil injected into the occipital muscle over a 5–10 s period at 2 h 30 min [24].

Animals were then left in the prone position for a further 2 h [7]. Following the experiment, animals were deeply anesthetised with pentobarbital (80 mg/kg, i.v.), then perfused via the ascending aorta with 0.9% saline (200 ml), followed by 4% paraformaldehyde (500 ml) in 0.1 M phosphate buffer (PB; pH 7.4). The brain and spinal cord were removed and stored overnight in the same fixative and then placed in a cryoprotectant (20% sucrose, 30% ethylene glycol in 0.1 M PB) for 48 h before sectioning. The brainstems and upper cervical spinal cords were sectioned serially on a freezing cryostat. Sections were taken from the trigeminal nucleus caudalis to C_3 with every third 50 μm section saved and processed for immunohistochemistry.

Tissue sections were processed as free-floating sections with the avidin–biotin complex (ABC) procedure using commercially available kits. Sections were incubated in 0.03% hydrogen peroxide (VWR International Ltd, Lutterworth, UK) in 0.1 M phosphate-buffered saline (PBS; pH 7.0), in blocking solution (3% normal goat serum, Vector Laboratories Ltd, Peterborough, UK, in 0.1 M PBS with 0.25% Triton X-100; Sigma-Aldrich Company Ltd, Poole, UK) and then in the primary *fos* antibody overnight (Santa Cruz Biotechnology Inc, California, USA, obtained from Autogen Bioclear UK Ltd, Calne, UK) in a 1:10000 dilution in PBS (with 0.25% Triton X-100 and 0.01% sodium azide; VWR International Ltd, Lutterworth, UK). The secondary antibody (1/2000 in blocking solution) was biotinylated goat anti-rabbit serum (Vector Laboratories Ltd, Peterborough, UK). After washes in PBS, sections were placed in ABC-peroxidase complex (Vector Laboratories Ltd, Peterborough, UK) and then in a 40% solution of DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma-Aldrich Company Ltd, Poole, UK) and 0.003% hydrogen peroxide. After the DAB reaction, sections were mounted on slides, air-dried, dehydrated, cleared in xylene and coverslipped with DPX mountant (VWR International Ltd, Lutterworth, UK).

The methods for counting Fos protein stained nuclei have been described recently [3]. Briefly, for a fos cell to be positive, it must be visible under a light microscope at magnifications of 5×, 10× and 20× objective plus the 10× eyepiece. Laminae I, II, V and X were examined for Fos positive nuclei extending from the rostral spinal trigeminal nucleus to C_3. Previous studies have shown that Fos expression in the trigeminal nucleus caudalis is maximal at the caudal TCC and the mid-TCC transition zone following masseter muscle or temporomandibular joint stimulation [24]. Nuclear and laminae boundaries were defined according to the atlas of Paxinos and Watson [10]. In this study the slides were coded to enable blind counting of random slides at each of the above levels. Fos expression was, therefore, counted by a blinded observer and depicted on graphical representations of each section.

Data are reported as median with interquartile ranges for the groups as the Fos method cannot yield interval scale measurements [27]. Comparisons of fos expression between treatment groups were made using the Kruskal–Wallis one-way analysis of variance (ANOVA). Differences were taken to be significant at the $P < 0.05$ level.

The quality of Fos staining was consistent across all sections. Tables 1 and 2 show the level of expression at each lamina and level studied.

Fos expression was minimal in Group 1 (Tables 1 and 2).

Fos expression in the TCC was significantly altered by mustard oil and mineral oil when compared to control ($H_4 = 31.3$, $P < 0.001$, Kruskal–Wallis). Mustard oil produced large numbers of fos positive cells with a median of 23 (interquartile range 17–33). Counts were highest in laminae I and II, maximal at the level of trigeminal nucleus caudalis/C_1 but visible