

# Expression of NMDA and Oestrogen Receptors by Trigeminal Ganglion Neurons that Innervate the Rat Temporalis Muscle

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**Objective:** To assess whether N-methyl-D-aspartate (NMDA) receptor (NR) or oestrogen receptor (OR) expression plays a role in the differences that temporalis muscle afferent fibres are less sensitive to peripheral receptor activation than masseter muscle afferent fibres and do not exhibit sex-related differences in NMDA-evoked discharge.

**Methods:** Immunohistochemical techniques were used to examine the expression of NR1, 2A, and 2B subunits of the NMDA receptor in male and female rats and the co-expression of NR2B subunits with ORs in female rats by trigeminal ganglion neurons that innervate the temporalis muscle. In vivo electrophysiological recording methods were employed to assess the response of afferent fibres to injection of NMDA into the temporalis muscle in female rats.

**Results:** Approximately 20% of temporalis ganglion neurons expressed NR1, NR2A and NR2B subunits, respectively, and there was no sex-related difference in the expression of these subunits. In female rats, both OR $\alpha$  and OR $\beta$  receptors were identified in the trigeminal ganglion by Western blot. ORs were found on the majority (~80%) of temporalis ganglion neurons that expressed NR2B subunits. A significant positive correlation between blood oestrogen concentration and NMDA-evoked afferent discharge was identified.

**Conclusion:** The absence of sex-related differences in NMDA receptor expression may account for the lack of sex-related differences in NMDA-evoked temporalis afferent discharge. The association of elevated oestrogen concentration with increased afferent response to NMDA and the co-expression of NRs and ORs in temporalis ganglion neurons suggest that sensory input from the temporalis muscle may be modulated by oestrogenic tone.

**Key words:** nociception, estrogen, NMDA, temporomandibular disorders, sex differences

Temporomandibular disorders (TMDs) are a group of conditions that have pain in the masticatory muscles as one of their principal symptoms, which in many sufferers occurs in the absence of demonstrable tissue pathology<sup>1,2</sup>. There is a female predominance in TMD patients, with 2 times more women than men reporting

this pain condition in the community and up to 5 times more women seeking treatment for clinical symptoms of TMD<sup>2,3</sup>. Although the pathogenesis of pain in TMDs remains enigmatic, it was recently reported that the concentration of the excitatory amino acid glutamate in the masseter muscle of myofascial TMD sufferers was significantly elevated compared to healthy controls<sup>4</sup>. In healthy controls, injection of glutamate into the masticatory muscles causes short lasting pain and a longer lasting period of mechanical sensitivity reminiscent of the symptoms of TMD<sup>5,6</sup>.

Glutamate is the principal excitatory neurotransmitter involved in nociceptive transmission from trigeminal primary afferent fibres to brain stem trigeminal sensory neurons and acts, in part, through N-methyl-D-aspartate (NMDA) receptors (NRs)<sup>7-9</sup>. NRs are ligand-gated

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ion channels that bind glutamate and are expressed at excitatory synapses in the mammalian central nervous system, as well as peripherally on sensory afferent fibres that innervate skin, muscle and viscera<sup>10-12</sup>. Functional NRs are heteromeric complexes composed of both NR1 and one or more subtypes of the NR2 subunit, of which 4 have been identified (NR2A-D)<sup>13</sup>. Functional NRs contain at least one NR1 subunit and commonly contain either NR2A or NR2B subunits<sup>14</sup>. The NR1 subunit contains the glycine-binding site and the NR2 subunit contains the glutamate-binding site<sup>14</sup>. There is evidence that phosphorylation of the NR2B subunit increases NR currents and that this process is modulated by oestrogen<sup>15,16</sup>.

In previous studies, it was found that injection of glutamate into the rat masseter muscle evoked afferent discharge through activation of peripheral NRs and that this afferent discharge was greater in female than in male rats<sup>11,17,18</sup>. Subsequent investigation has determined that the sex-related difference in masseter afferent discharge in response to peripheral NR activation is oestrogen-dependent and due, in part, to a greater expression of NR2B subunits by masseter afferent fibres in female than in male rats<sup>11</sup>. In contrast, it has been found that temporalis muscle afferent fibres are less sensitive to peripheral NR activation and do not exhibit sex-related differences in afferent discharge<sup>19</sup>. This difference has also been found in healthy humans, where greater pain intensity in healthy women than men after injection of glutamate into the masseter muscle but not the temporalis muscle, has been reported<sup>20</sup>. It is not known why temporalis muscle afferent fibres respond differently than masseter muscle afferent fibres to peripheral NR activation.

The present study was conducted to determine the level of expression of NR1, 2A, and 2B subunits by temporalis muscle ganglion neurons and whether there are sex-related differences in the expression of one or more of these subunits. It was hypothesised that there would be no sex-related difference in the expression of NR subunits by temporalis ganglion neurons. Since oestrogen appears to modulate masseter afferent fibre excitability through NR2B expression, the expression of oestrogen receptor (OR)  $\alpha$  and  $\beta$  and their co-expression with the NR2B subunit on female rat temporalis muscle ganglion neurons were also examined to determine the extent to which NR expression could potentially be modulated by oestrogen in these neurons. Finally, *in vivo* electrophysiology was conducted to examine the relationship between oestrogen concentration and afferent response to NMDA in female rats.

## Materials and methods

### *Animal housing*

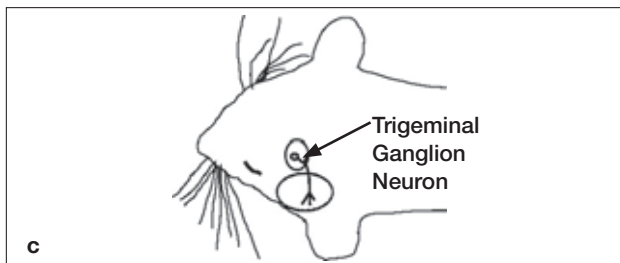
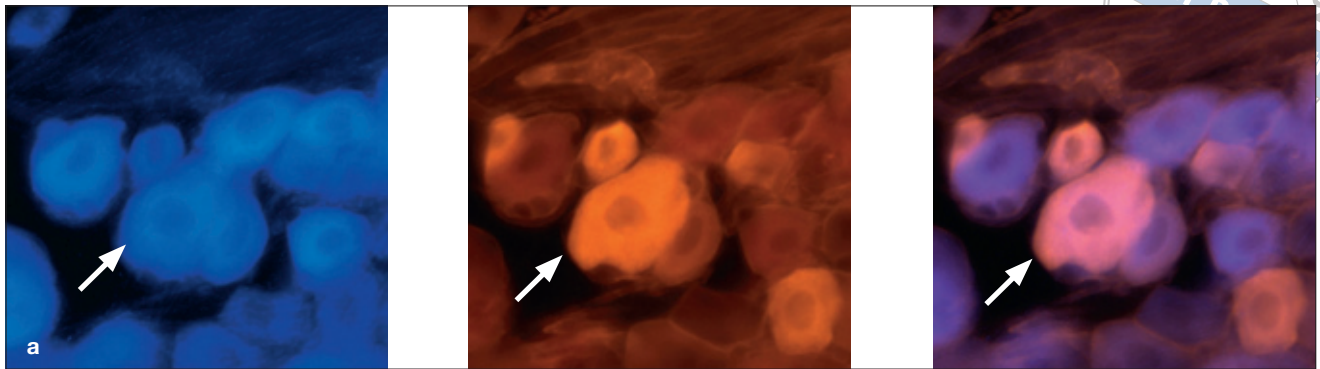
Adult Sprague-Dawley rats (6 males, 37 females) weighing 225 to 450g (Charles River) were housed two per cage (same-sex) on a 12:12 hr light:dark cycle with ad libitum access to food and water. All procedures were performed in adherence with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

### *Immunohistochemistry*

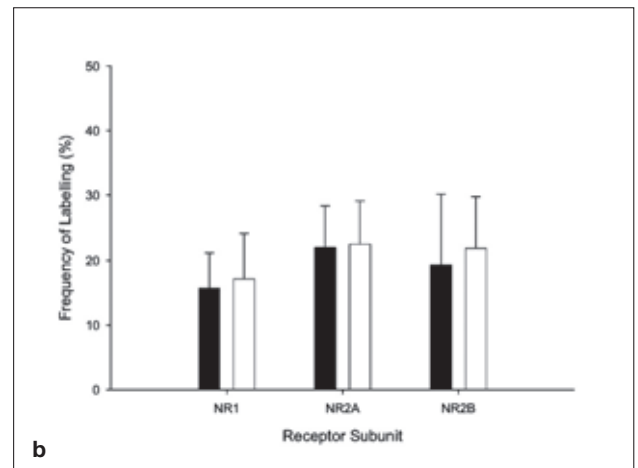
Rats (6 males, 10 females) were briefly anaesthetised with isoflurane to allow bilateral intramuscular injection of the fluorescent dye Fast Blue (2%, 10  $\mu$ l). Fast Blue dye was injected into the temporalis muscle to identify trigeminal ganglion neurons that innervate this muscle, i.e. temporalis muscle ganglion neurons (Fig 1a). Seven days after injection, rats were euthanised with an overdose of pentobarbital (100 mg/kg) and perfused with 120 ml cold saline followed by 120 ml of paraformaldehyde (4%). The right and left trigeminal ganglia were removed and cut into sections (40  $\mu$ m) with a vibratome. The sections were treated with 5% normal goat serum (NGS) in phosphate-buffered saline for 1 h for blocking.

Sections were incubated with primary antibodies against NR1 (mouse monoclonal, 1:500, BP Pharmingen, 556308), NR2A or NR2B subunits (rabbit polyclonal, Chemicon International) in 1% NGS overnight at 4°C. To determine the co-expression of the NR2B subunit and OR $\alpha$  or OR $\beta$  receptors, trigeminal ganglion neurons were incubated with NR2B mouse monoclonal antibody (Chemicon International, MAB5216), and either OR $\alpha$  polyclonal rabbit antibody (Abcam, AB21232) or OR $\beta$  polyclonal rabbit antibody (Chemicon International, AB1410) in 1% NGS overnight at 4°C.

The next day, the appropriate secondary antibodies were applied for incubation at room temperature for one hour. Cyanine (CY3)-conjugated goat anti-mouse (NR1) or goat anti-rabbit antibodies (NR2A and B; Jackson ImmunoResearch Laboratories) were used as the secondary antibodies. For co-expression of NR2B subunits with ORs, CY3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories) was used to visualise the expression of the NR2B subunit, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) was used to visualize OR $\alpha$  or OR $\beta$ . Sections were washed



**Fig 1** a) A Fast Blue labeled temporalis ganglion neuron with positive membrane labeling for the NR2B subunit is shown (see arrows); b) The bar graph illustrates the mean expression frequency of NRs in temporalis muscle ganglion neurons (6 male (M), 6 female (F)). There were no significant differences in NR expression by temporalis muscle ganglion neurons when male and female rats were compared.



with phosphate-buffered saline and mounted on slides with coverslips. A Leica DM fluorescent microscope was used to view labelling. The specificity of antibody labelling was evaluated by omission of the primary antibody.

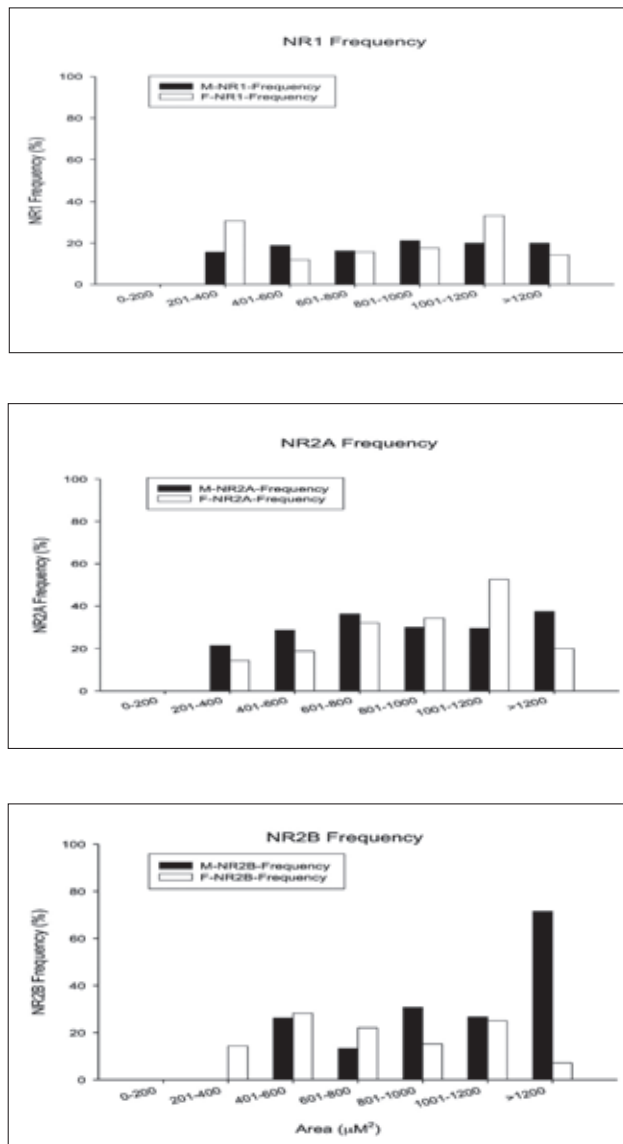
#### Western blot

Trigeminal ganglia obtained from 4 adult female Sprague–Dawley rats were homogenised on ice in lysis buffer (Sigma-Aldrich), centrifuged and transferred to a fresh Eppendorf tube. The total protein content was determined by the Bradford method<sup>21</sup>. Protein samples (20  $\mu$ g each) were run for 90 min on 7% SDS electrophoresis polyacrylamide gel. Proteins were then transferred onto 0.2  $\mu$ m nitrocellulose membrane (GE Healthcare), the membrane was blocked (5% non-fat dried milk) at room temperature for one hour and then incubated at 4°C overnight with either OR $\alpha$  polyclonal rabbit antibody (Abcam, AB21232) or OR $\beta$  polyclonal

rabbit antibody (Chemicon International, AB1410). The next day, the membrane was washed and then incubated with secondary antibodies. Receptor specific bands were visualised with an ECL Western blotting detection kit (Amersham). Images were captured with an Alpha Innotech FluorChem 8800 gel-box imager (Alpha Innotech).

#### Electrophysiology experiments

Adult female Sprague-Dawley rats (n = 23) were anaesthetised with isoflurane and prepared for acute *in vivo* recording of trigeminal afferent activity, as has been previously described in detail<sup>7</sup>. Single trigeminal afferent unit activity within the trigeminal ganglion was recorded by a parylene-coated tungsten microelectrode (A-M Systems). A blunt probe was applied to the temporalis muscle to identify trigeminal afferent fibres that had mechanoreceptive fields in the temporalis muscle. To determine whether the afferent fibre projected to the caudal brainstem, constant-current electrical stimuli (100  $\mu$ s



**Fig 2** The histograms of A, B and C illustrate NR1, NR2A and NR2B expression, respectively, in temporalis muscle ganglion neurons of different sizes in 6 males (M) and 6 females (F). There was a uniform but low-level expression of all 3 subunits among temporalis muscle ganglion neurons of different sizes, with no difference in this expression between male and female rats.

biphasic pulse, range 10–90  $\mu$ A, 0.5 Hz) were applied to a stimulating electrode that had been lowered into the ipsilateral caudal brainstem to evoke antidromic action potentials. Antidromic action potentials were collided with orthodromic action potentials evoked by mechanical stimulation of the temporalis muscle to confirm the projection of the afferent fiber to the caudal brainstem. After this characterisation procedure was completed,

baseline primary afferent fibre activity was recorded for 10 min. Then, a single 10  $\mu$ l intramuscular injection of 500 mM NMDA (Sigma; pH 7.4) was made into the temporalis muscle and the resulting afferent discharge monitored for 10 min post injection. At the end of the experiment, the distance between the stimulating and recording electrodes was measured, and divided by the latency of the antidromic action potential to give an estimation of the conduction velocity (CV) of the recorded afferent fibre<sup>7</sup>.

#### Measurement of serum oestrogen

To assess the association between circulating oestrogen levels and NMDA-evoked temporalis afferent discharge, blood samples were taken and plasma concentration of the unconjugated form of 17 $\beta$ -oestradiol was measured with a commercially available radioimmunoassay kit (ICN Biomedical).

#### Statistical analysis

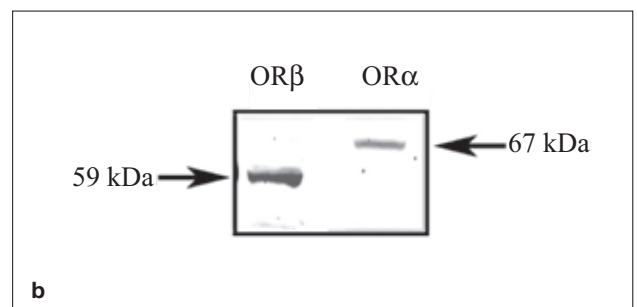
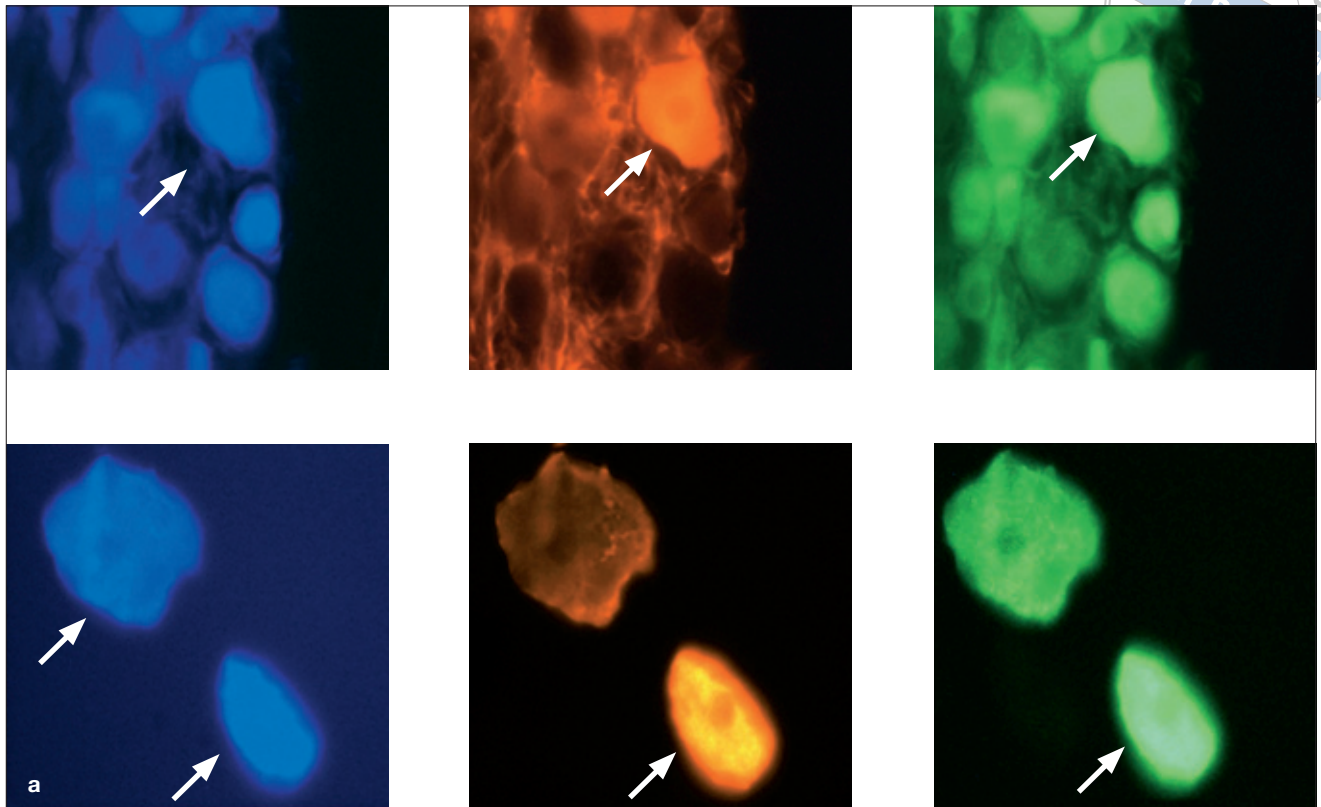
The WCIF Image J software program (NIH Image) was used to measure the area of Fast Blue positive cells. The percentage of receptor labelled temporalis muscle ganglion neurons was calculated by dividing the number of receptor positive neurons by the total number of Fast Blue labelled neurons. Coexpression was calculated by dividing the number of NR2B positive neurons by the number of NR2B positive neurons that were also OR positive. Two-way analysis of variance was used to assess differences in NR subunit expression between males and females. The Chi Square test was employed to assess differences in distribution of receptor expression amongst ganglion neurons of different sizes in male and female rats.

Cumulative afferent discharge was calculated by subtracting the summed action potential discharge for 10 min after injection from the baseline (spontaneous) afferent discharge 10 min prior to injection. Pearson product moment correlation was used to determine if there was a significant correlation between serum oestrogen concentration and NMDA-evoked cumulative discharge.

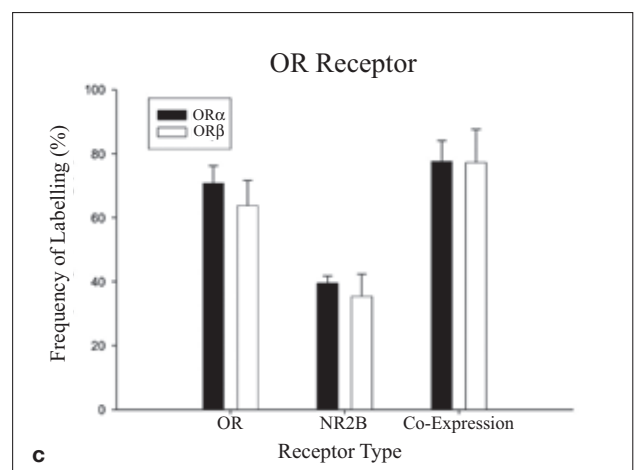
Population data are reported as the mean and standard error of the mean.

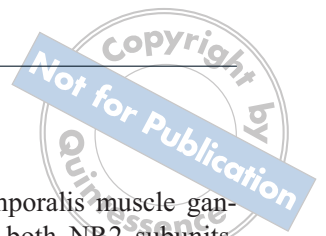
#### Results

Expression of NRs and ORs in temporalis muscle ganglion temporalis muscle ganglion neurons were examined for expression of NRs in 6 male and 6 female rats.



**Fig 3** a) The pictures show examples of Fast Blue-labelled (left) temporalis ganglion neurons that expressed the NR2B and OR $\alpha$  or OR $\beta$  receptors in a female rat. OR receptor labelling was obvious in the cytoplasm of these cells; b) Western blots for OR $\alpha$ , and OR $\beta$  receptors in the rat trigeminal ganglion neuron ( $n = 4$ ) are shown. The position of the molecular weight standard is indicated in kilodaltons (kDa). The asterisks denote the single bands that were labelled by the receptor specific antibodies (OR $\alpha$ : 67 kDa, OR $\beta$ : 59 kDa); c) The bar graph illustrates the frequency of expression of oestrogen receptors (OR $\alpha$  and OR $\beta$ ) in temporalis muscle ganglion neurons of female rats ( $n = 4$ ), and their co-expression with NR2B receptors. There was a high frequency of expression of both ORs by temporalis muscle ganglion neurons (70% and 62% respectively). There was a high co-expression (> 75%) of either OR in temporalis muscle ganglion neurons that also expressed NR2B subunits. The high co-expression of NR2B and ORs by temporalis muscle ganglion neurons indicates that the anatomical conditions necessary for receptor interaction are present.





There were no differences in the average expression of the three subunits in temporalis muscle ganglion neurons (Fig 1b). There were also no sex-related differences in the mean expression of the NR1, NR2A or NR2B, or interactions between the sex and receptor subtype expression. There was a uniformly low expression of NR subunits in temporalis muscle ganglion neurons of various sizes in both male and female rats (Fig 2). There were no sex-related differences in the frequency of expression ( $P > 0.05$ , Chi Square test).

We have previously found that the expression of NR2B subunits in trigeminal ganglion neurons that project to the masseter muscle is regulated by oestrogen levels in female rats<sup>11</sup>. To determine whether the lack of a sex-related difference in NR expression in temporalis muscle ganglion neurons might be a result of low OR expression in temporalis muscle ganglion neurons that express NRs, we assessed co-expression of ORs and NRs. A total of 8 ganglia from 4 female rats were examined for OR expression. Co-expression of both OR and OR was readily observed in individual temporalis muscle ganglion neurons (Fig 3a). Since a previous study reported finding little OR $\beta$  receptor labelling in the trigeminal ganglion, the Western blot technique was undertaken to confirm that the antibodies used were identifying proteins of different molecular weights<sup>22</sup>. Western blots confirmed that the OR $\alpha$  and OR $\beta$  antibodies labelled single bands at 67 and 59 kDa, respectively (Fig 3b). These bands corresponded to the expected molecular weights for these two receptors<sup>21,23</sup>.

Overall, OR $\alpha$  expression was found to be  $71 \pm 6\%$  and OR $\beta$  expression was found to be  $64 \pm 8\%$  in temporalis muscle ganglion neurons (Fig 3c). Co-expression of OR $\alpha$  and OR $\beta$  with NR2B subunits in the temporalis muscle ganglion neurons was found to be  $78 \pm 7\%$  and  $77 \pm 10\%$ , respectively (Fig 3c). Both OR receptors were evenly distributed among temporalis muscle ganglion neurons of various sizes (Fig 4). These findings indicate that there is in fact a high co-expression of ORs and NR2B subunits in temporalis muscle ganglion neurons.

#### *Serum oestrogen correlated with NMDA-evoked temporalis afferent fibre discharge*

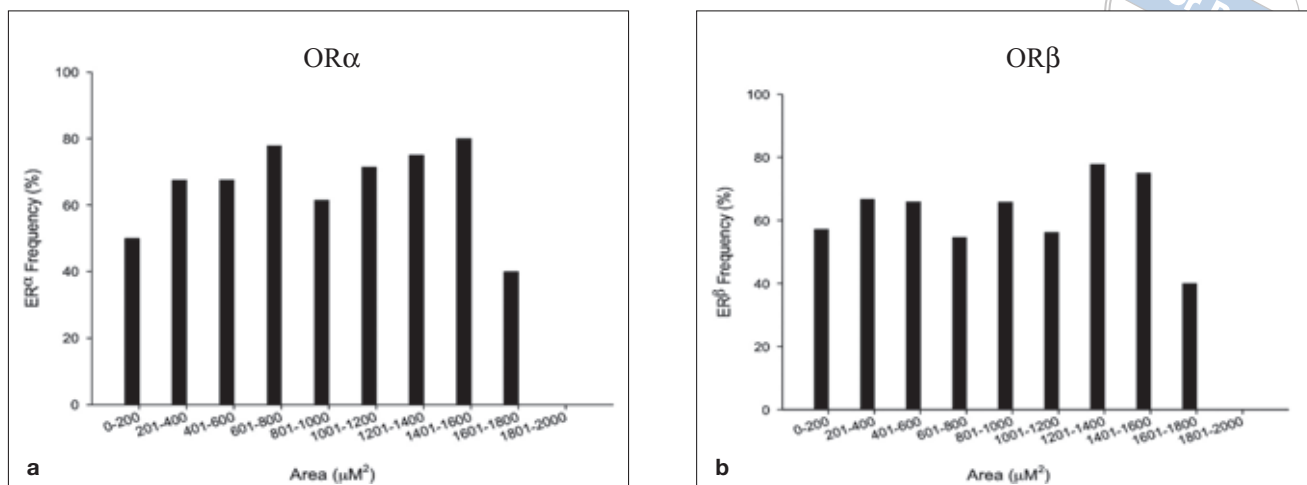
The relationship between serum oestrogen concentration and NMDA-evoked temporalis afferent fibre discharge was examined for 23 afferent fibres. The mean conduction velocity of these fibres was  $6.6 \pm 0.9$  m/s (22 A and 1 C fibre). There was a significant positive correlation ( $r = 0.435$ ) between NMDA-evoked afferent discharge and serum oestrogen concentration in these fibres (Fig 5).

## Discussion

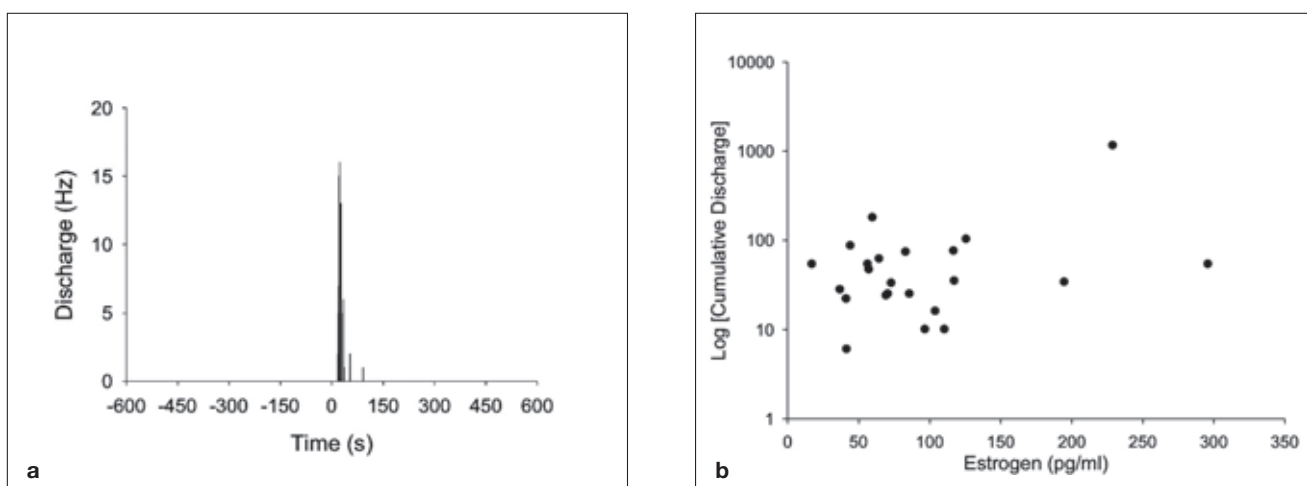
The present study found that temporalis muscle ganglion neurons express NR1 and both NR2 subunits, which is consistent with previous studies that labelled NRs in trigeminal and dorsal root ganglia (DRG)<sup>24–26</sup>. There was no sex-related difference in NR expression in temporalis muscle ganglion neurons when male and female rats were compared. This result may explain our previous finding of a lack of a sex-related difference in NMDA-evoked temporalis muscle afferent discharge<sup>19</sup>. The sex-related difference in masseter muscle afferent fibre discharge in response to peripheral NR activation is oestrogen-dependent and due, in part, to a greater expression of NR2B subunits by masseter muscle afferent fibres in female than in male rats<sup>11</sup>. We speculated that since oestrogen appears to modulate masseter muscle afferent fibre excitability through NR2B expression, the expression of OR and OR and their co-expression with the NR2B subunits on female rat temporalis muscle ganglion neurons might be very modest in temporalis muscle ganglion neurons. However, our results indicate that the expression of both OR subtypes is quite high in temporalis muscle ganglion neurons and that both ORs are highly co-expressed with NR2B subunits. Further, we found a significant positive correlation between NMDA-evoked temporalis afferent discharge and serum oestrogen levels, suggesting that oestrogenic tone may modulate peripheral NMDA receptor sensitivity in temporalis afferent fibres in female rats.

We have previously found that the expression of NR2A and NR2B subunits by masseter muscle ganglion neurons is 12% and 46% in females versus 20% and 20% in males, respectively<sup>5</sup>. The expression of NR2A and NR2B subunits by masseter and temporalis muscle ganglion neurons in males appears similar (masseter vs temporalis: NR2A: 20% vs 22%; NR2B: 20% vs 19%). In female rats, temporalis ganglion neurons express more NR2A and less NR2B subunit containing NRs than do masseter ganglion neurons (masseter vs temporalis: NR2A: 12% vs 23%; NR2B: 46% vs 22%). The increased expression of NR2B subunit containing NRs by trigeminal ganglion neurons that innervate the masseter muscle is consistent with findings of increased NMDA-evoked afferent discharge in female rats, since oestrogen modulates both the activity and expression of NR2B subunit containing NRs in females<sup>11,15,16</sup>.

Since functional NRs are heterodimers composed of NR1 and NR2 subunits, it would be expected that the NR2 subunit expression would be comparable to NR1 subunit expression. Indeed, NR2 subunit expression by temporalis muscle ganglion neurons more or



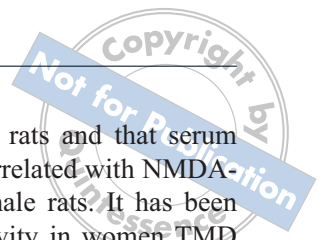
**Fig 4** The histograms of a) and b) illustrate OR $\alpha$  and OR $\beta$  expression, respectively, in temporalis muscle ganglion neurons of different sizes in female rats ( $n = 4$ ). There was a uniform expression of both ORs among temporalis muscle ganglion neurons of different sizes.



**Fig 5** a) The peri-stimulus histogram illustrates the response of a temporalis afferent fibre (conduction velocity = 5.9 m/s) to NMDA in a female rat (serum oestrogen: 126 pg/ml). The injection of 10  $\mu\text{l}$  500 mM NMDA into the temporalis muscle was made at time zero. The cumulative discharge for this afferent fibre was 103. b) The semi-log scatter plot shows the relationship between serum oestrogen level and NMDA-evoked temporalis afferent discharge. A significant positive correlation ( $r = 0.435$ ) was found between oestrogen concentration and NMDA-evoked afferent fibre cumulative discharge.

less equates with the NR1 subunit expression in these neurons. In the DRG, the NR2B subunit is expressed at a much higher level than the NR2A subunit, and it has been speculated that most NMDA receptors in this ganglion are composed of NR1 and NR2B subunits, since these subunits were found to be colocalized on  $\sim 90\%$  of DRG neurons<sup>24</sup>. Unlike in the DRG, it has been suggested that the NR2A subunit is predominant in the trigeminal ganglion in adult rats<sup>25</sup>. Our results suggest that expression of NR2 subunits by trigeminal ganglion neurons may depend, in part, on the tissue they innervate and the sex of the animal.

Oestrogens are steroid hormones that are involved with development of neurons, modulation of synaptic plasticity, neuronal excitability and neurogenesis, in addition to a possible role in sex-related differences observed in cognitive function as well as pain perception<sup>22,23</sup>. The most abundant oestrogen in humans is 17 $\beta$ -oestradiol. The transcriptional activity of oestrogen is mediated by OR $\alpha$  and OR $\beta$ . ORs are expressed by DRG neurons and both OR isoforms appear to be expressed at similar levels<sup>27-29</sup>. In contrast, an earlier study suggested that between 8 and 12% of trigeminal ganglion neurons in female rats expressed OR $\alpha$  but very



few trigeminal ganglion neurons expressed OR $\beta$ <sup>22</sup>. In the present study, we found the expression of both ORs to be relatively similar at around 60 to 70% in temporalis muscle ganglion neurons. Subsequent Western blot experiments indicated that the commercially acquired antibodies employed labelled two different proteins of the correct molecular weight to be OR $\alpha$  and OR $\beta$  receptors, respectively, and confirmed that both proteins were expressed in the trigeminal ganglion. These findings suggest that masticatory muscle afferent fibres have a much higher expression of ORs than nerve fibres that innervate other craniofacial tissues.

Oestrogen has been shown to modulate hippocampal NRs in the cortex and striatum of ovariectomised rats by increasing the expression of NRs and by activating Src tyrosine kinases to phosphorylate tyrosine residues on the NR2 subunit<sup>30</sup>. Changes in oestrogen levels during the oestrus cycle in female rats were also found to be associated with changes in activated and phosphorylated levels of extracellular signal-regulated kinase 2 (ERK2) that are further associated with tyrosine phosphorylation of the NR2 subunit of NMDA receptors<sup>10</sup>. These findings seem to suggest that in addition to regulation of genomic events through binding to ER receptors, oestrogen also can exert rapid effects on cells and tissues by activating intracellular cascades such as the ERK/MAP kinase pathway to phosphorylate tyrosine residues on NMDA receptors. Our results suggest that expression of NR2B by temporalis muscle ganglion neurons in female rats is less likely to be influenced by oestrogen modulation than in masseter muscle ganglion neurons<sup>11</sup>. Despite the high co-expression of the oestrogen receptors with NR2B subunits (78% and 77%), the expression of NR2B by temporalis muscle ganglion neurons was similar in males and females. Nevertheless, the high expression of ORs by temporalis muscle ganglion neurons in female rats suggested that their activity could be modulated by oestrogen levels. Indeed, there was a significant positive correlation between NMDA-evoked temporalis afferent discharge and serum oestrogen levels, which provides evidence that oestrogen concentration may modulate the sensitivity of afferent fibres that innervate this muscle through a mechanism that does not involve increased NR receptor expression.

The reasons for sex-related differences in the occurrence of masticatory muscle pain in conditions such as myofascial TMD are not well understood, however, it is likely that both biological and psychosocial factors contribute to these differences. The results of the present study indicate that there is a robust expression of oestrogen receptors by the nerve fibres that innervate

the temporalis muscle of female rats and that serum oestrogen levels are positively correlated with NMDA-evoked afferent discharge in female rats. It has been reported that muscle pain sensitivity in women TMD sufferers increases during large changes in oestrogen concentrations, such as those which occur at ovulation and menstruation<sup>31</sup>. OR expression by ganglion neurons that innervate the temporalis muscle suggests that oestrogen may be able to act directly on masticatory muscle nociceptors to modulate muscle pain sensitivity in females.

### Acknowledgements

Brian E. Cairns is the recipient of a Canada Research Chair. The authors declare no conflicts of interest. This work was supported by Canadian Institutes of Health Research Grants MOP 77538 and CGR-86827.

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