CENTRAL NEURAL PATHWAYS INVOLVED IN CRANIOFACIAL NOCICEPTIVE REFLEX RESPONSES EVOKED IN JAW MUSCLES BY MUSTARD OIL INJECTION INTO THE TEMPOROMANDIBULAR JOINT REGION

BY

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A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Department of Oral Physiology
Faculty of Dentistry
University of Toronto

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ABSTRACT

Since the trigeminal subnucleus caudalis (Vc) has been viewed as the essential relay site of nociceptive information from craniofacial tissues to higher levels of the central nervous system, a series of studies were carried out in 135 anaesthetized rats to determine if Vc neurones are involved in craniofacial nociceptive reflex electromyographic (EMG) responses evoked in jaw muscles by the small-fibre excitant and inflammatory irritant mustard oil. Its injection into the temporomandibular joint (TMJ) region of intact rats evoked a bilateral increase in EMG activity of digastric (DIG) and masseter (MASS) muscles; surgical transection of Vc at the obex level significantly reduced this increased EMG activity of all four muscles whereas mustard oil injection into the contralateral TMJ region in these same rats or into the TMJ region of other groups of rats receiving sections medial or caudal to Vc still evoked an increase in EMG activity. Electrical micro-stimulation of Vc in another group of rats evoked EMG activity in only the ipsilateral DIG and MASS. The sites associated with the lowest thresholds and the shortest latencies were located in the caudal Vc. Neurones, as opposed to fibres of passage, particularly in the caudal Vc appear to be important in these excitatory effects because micro-injection of the cell excitant and excitatory amino acid glutamate
into the caudal Vc of another group of rats evoked a significant increase in EMG activity of the ipsilateral DIG and MASS; glutamate micro-injection into surrounding brainstem loci of other groups of rats did not evoke such EMG changes. The importance of neurones in the caudal Vc in the craniofacial nociceptive reflex pathways was further confirmed in other rats by the effectiveness of micro-injection into the caudal Vc of the neurotoxic chemical ibotenic acid in significantly reducing the mustard oil-evoked EMG activity and by findings that neurones in the caudal Vc responded to noxious stimulation, including mustard oil application, of the TMJ. Findings from this thesis thus suggest that Vc may be a critical element in the neural pathways underlying the reflex responses evoked bilaterally in DIG and MASS muscles by noxious stimulation within the TMJ region.
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<th>Description</th>
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<tbody>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>EMG</td>
<td>electromyographic</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory post-synaptic potential</td>
</tr>
<tr>
<td>DIG</td>
<td>digastric muscle</td>
</tr>
<tr>
<td>DNIC</td>
<td>diffuse noxious inhibitory controls</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory post-synaptic potential</td>
</tr>
<tr>
<td>LTM</td>
<td>low threshold mechanoreceptor</td>
</tr>
<tr>
<td>MASS</td>
<td>masseter muscle</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NS</td>
<td>nociceptive specific</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TENS</td>
<td>transcutaneous electrical nerve stimulation</td>
</tr>
<tr>
<td>TMJ</td>
<td>temporomandibular joint</td>
</tr>
<tr>
<td>V</td>
<td>trigeminal</td>
</tr>
<tr>
<td>Vc</td>
<td>trigeminal subnucleus caudalis</td>
</tr>
<tr>
<td>Vi</td>
<td>trigeminal subnucleus interpolaris</td>
</tr>
<tr>
<td>Vmo</td>
<td>trigeminal motor nucleus</td>
</tr>
<tr>
<td>Vo</td>
<td>trigeminal subnucleus oralis</td>
</tr>
<tr>
<td>Vp</td>
<td>trigeminal nucleus principalis</td>
</tr>
<tr>
<td>Vtr</td>
<td>trigeminal spinal tract</td>
</tr>
<tr>
<td>WDR</td>
<td>wide dynamic range</td>
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</tbody>
</table>
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CHAPTER 1. REVIEW OF LITERATURE

A. TEMPOROMANDIBULAR JOINT

A1. Anatomy and Histology

The bones associated with the TMJ are the convex condylar head of the mandible and the concave glenoid fossa on the undersurface of the squamous part of the temporal bone. As in other synovial joints, the TMJ is surrounded by a fibrous articular capsule which extends from the margins of the glenoid fossa, including the articular eminence, to envelop the head of the condyle before fusing inferiorly with the periosteum of the condylar process. The capsule encloses a joint cavity which is divided, by an articular disc interposed between the two bony components, into the lower joint space around the condyle and the upper joint space between the disc and the temporal bone (Griffin et al. 1975; Lindblom 1960; Sicher 1975). The anatomy of the TMJ varies considerably among mammals, depending upon masticatory requirements. In the carnivore, the TMJ is approximately level with the occlusal plane, which allows maximal gape with minimal distal displacement of the angular process so as not to interfere with the airway. Because the major function of the TMJ in the carnivore is to maintain stability once the prey has been caught, the condyle tends to be cylindrical and elongated transversely and the well-developed anterior and posterior bony flanges of the temporal bone clasp the condyle of the mandible. In the herbivore, the mandible has a high condylar process so that the TMJ is well above the occlusal plane. To allow a wide range of lateral and/or antero-posterior jaw movements in the herbivore, the glenoid fossa is shallow, the condyle is flat, and the TMJ ligaments are relatively lax but strong. In humans, the length of the jaw is moderate and the TMJ is unspecialized and able to move in a variety of directions; the articulation is flat and the TMJ ligaments are
lax but strong, providing both strength and a wide range of jaw movements (Miller 1988; Ten Cate 1994).

The bony surfaces of the condyle and the articular part of the temporal bone in adult mammals are covered by dense fibrous connective tissues which can be divided into four zones, i.e., from inside out, the calcified cartilage, the fibrocartilaginous zone, the proliferative zone, and the articular zone (see Ten Cate 1994). The articular disc consists of dense fibrous tissue and its shape conforms to the articular surfaces to which it is opposed. The most part of the peripheral border of the articular disc is attached to the TMJ capsule; however, it has not been firmly established whether there is a direct attachment between the anterior margin of the disc and the superior head of the lateral pterygoid muscle in humans (Marguelles-Bonnet et al. 1989). The internal surface of the capsule and the anterior and posterior limits of the TMJ are lined by a synovial membrane which secretes synovial fluid into the joint spaces. The synovial fluid lubricates the articular surfaces during joint movements and may also fulfill the metabolic needs of the avascular fibrous tissues associated with the joint (Ten Cate 1994). The TMJ is strengthened laterally by the temporomandibular ligament which is a fan-shaped reinforcement of the lateral wall of the capsule running obliquely backwards and downwards from the lateral aspect of the articular eminence to the posterior aspect of the condylar neck.

A2. Vascular and Nerve Supplies

The vessels and nerves supplying the TMJ of humans, monkeys and rats occur primarily in the joint capsule, the peripheral borders of the articular disc, the synovial membrane, the loose and wavy connective tissue behind the disc, the temporomandibular ligament and the periosteum (Bernick 1962; Boyer et al. 1964;
Choukas and Sicher 1960; Ichikawa et al. 1989, 1990; Johansson et al. 1986; Keller and Moffett 1968; Kido et al. 1991, 1993, 1995; Milam 1995; Thilander 1964; also see Mohl 1988; Ten Cate 1994); the central area of the disc is devoid of nerve tissues and blood vessels (Boyer et al. 1964; Choukas and Sicher 1960; Kido et al. 1991, 1993, 1995; Thilander 1961). The nerve fibres appear to be predominantly small-diameter afferents, i.e. thinly myelinated (Aδ) or unmyelinated (C) fibres with free nerve endings (Dreessen et al. 1990; Ichikawa et al. 1989, 1990; Kido et al. 1991, 1995; Milam 1995; Thilander 1961), although low-threshold non-nociceptive afferent fibres innervating the TMJ tissues have been found (Kawamura and Abe 1974; Klineberg 1971; Lund and Matthews 1981). Some of these small-diameter nerve fibres may contain substance P or calcitonin gene-related peptide (CGRP; Ichikawa et al. 1989, 1990; Johansson et al. 1986; Kido et al. 1993; Milam 1995) and may be associated with nociceptive stimuli; many of them project to Vc (Capra 1987) and thereby conduct nociceptive information from the TMJ to Vc neurones (Amano et al. 1986; Broton et al. 1988; Kojima 1990).

B. PRIMARY AFFERENT FIBRES OF THE TRIGEMINAL NERVE

The peripheral branches of V nerve (i.e. ophthalmic nerves, maxillary, mandibular) provide the predominant innervation of peripheral craniofacial tissues, but other cranial nerves as well as upper cervical spinal nerves may also contribute to the innervation of intraoral, periauricular, posterior mandibular or occipital regions (Dubner et al. 1978; Kerr 1979; Nazruddin et al. 1989; Pfaller and Arvidsson 1988). Like spinal nerve fibres, most V nerve fibres have their neurones located outside the CNS in the V (also called Gasserian or semilunar) ganglion (Shigenaga et al. 1986, 1988b, 1989; Takemura et al. 1991), except for those innervating muscle spindles in the jaw-closing muscles or some mechanoreceptors in the periodontium whose neurones are located in the V.
mesencephalic nucleus (Jerge, 1963; Mizuno et al. 1983; Nomura and Mizuno 1985; Shigenaga et al. 1988a, c, d, 1989, 1990). These primary afferent nerve fibres can be divided into three groups: large myelinated (Aβ; conduction velocity, c.v. > 50 m/s), small myelinated (Aδ; c.v. 2–50 m/s) and small unmyelinated (C; c.v. < 2 m/s) fibres (for review, see Darian-Smith 1966; Dubner et al. 1978). They may be associated with different peripheral receptors (e.g. specialized endings or nonspecialized free nerve endings) which may be sensitive to different types of stimulus energy (e.g. mechanical, thermal or chemical stimulation).

Many free nerve endings act as nociceptors which provide the peripheral structural and functional basis for craniofacial nociception in virtually all craniofacial tissues. They provide sensory-discriminative information about the quality, intensity, location and duration of the noxious stimulus and are associated with slow-conducting Aδ or C fibres that convey the nociceptive information into the V brainstem sensory nuclear complex (see Dubner et al. 1978; Sessle 1992). There are presently three known classes of V nociceptive primary afferent fibres supplying cutaneous and intraoral mucosal tissues (see Cooper and Sessle 1992; Dubner et al. 1978; Dubner and Bennett 1983; Hu and Sessle 1988). The first class are the high-threshold mechanoreceptive afferent fibres which are Aδ fibres and which can be activated only by intense mechanical stimulation; activation of this class usually signals some types of mechanical tissue injuries which are well localized. The second class are mechanothermal nociceptive fibres which are Aδ and which can be activated by noxious mechanical as well as noxious thermal stimuli (>45 °C, Bessou and Perl 1969; Dubner et al. 1974); this class of nociceptive fibres mediate the heat-pricking pain related to a heat-induced injury. The third class are polymodal nociceptive fibres which are C fibres and which can
be activated by intense mechanical, thermal and chemical stimuli (Beitel and Dubner 1976; Bessou and Perl 1969; Iggo 1960; Iggo and Ogawa 1971; Szolcsanyi 1987).

One chemical stimulus of particular interest to our laboratories (Bakke et al. 1996; Haas et al. 1992; Hu et al. 1992, 1993; Tsai et al. 1994a, b, 1996; Yu et al. 1993, 1994, 1995, 1996) is mustard oil (allyl iso-thiocyanate). This is an algesic chemical that can provoke pain or a burning sensation in humans (Handwerker et al. 1991; Jancso and Janka 1981). Mustard oil application has been regarded as a noxious stimulus and has been widely used in studies of mechanisms underlying nociception and sensitization of nociceptive neurones in somatosensory pathways (e.g. Cleland et al. 1994; Hartwig et al. 1996; Hu et al. 1992, 1993; Jancso et al. 1967, 1977; Lee and Beitz 1996; Woolf and Wall 1986; Yu et al. 1993). Although it is known to activate selectively small-diameter afferent fibres and produce inflammation at the site of application (Handwerker and Reeh 1991; Haas et al. 1992; Jancso et al. 1967; Jancso and Janka 1981; Woolf and Wall 1986), it is presently unclear whether mustard oil directly binds to specific receptors on nerve endings or releases endogenous algesic and/or inflammatory substances which subsequently activate small fibres.

When a single brief noxious stimulus is applied to the hand or foot of a human, the subject usually experiences first a pricking pain which is followed 1.0-1.5 s later (depending on conduction distance) by a second poorly localized, burning, throbbing or aching pain (Barrell and Price 1975; Campbell and LaMotte 1983; Dubner 1985; Price 1972; Price et al. 1976, 1977b); the former is mediated by activation of Aδ fibres and the latter by C fibres (Price et al. 1978, 1992b). Synchronous stimulation of Aδ and C afferent fibres reliably evokes a short-latency, high-frequency impulse discharge (which is attributable to A fibre inputs) followed by a long-latency impulse discharge (which is attributable to C fibre inputs) in the same second-order dorsal horn nociceptive
neurones (Price et al. 1978, 1992b; for review, see Price 1988; Willis 1985). Although the conduction distance of sensory nerves innervating craniofacial structures is too short to demonstrate clearly the first and second pain phenomena, the A and C fibre-evoked nociceptive neuronal impulse discharges can also be demonstrated in Vc (Hu 1990; Hu et al. 1992; Sessle and Hu 1991; Sessle et al. 1981, 1986). In addition, both the second pain (Barrell and Price 1975; Price 1972; Price et al. 1977b, 1989) and the long-latency C fibre-evoked impulse discharge of dorsal horn neurones (Mendell 1966; Davies and Lodge 1987; Dickenson and Sullivan 1987; Wagman and Price 1969; Price et al. 1971, 1978) progressively increase in magnitude (which reflects temporal summation of the primary afferent inputs) if the noxious stimulus is repeated at a frequency higher than 0.3 Hz: no such temporal summation occurs in case of the first pain or the short-latency A fibre-evoked impulse discharge. The temporal summation phenomenon of the long-latency impulse discharge of dorsal horn neurones (also known as neuronal “wind-up”) and the second pain are both evoked by C fibre inputs; the neural mechanisms underlying the C fibre-evoked temporal summation have been suggested to occur at the first synapse in nociceptive afferent pathways (Price 1988; Willis 1985). Temporal summation of C fibre-evoked dorsal horn neuronal responses has been thought to reflect some of the mechanisms underlying centrally mediated hyperalgesia that occurs after nerve injury or inflammation of damaged tissues (Dubner et al. 1987; Dubner 1991; Mao et al 1995; Price et al. 1992a; Ren et al. 1992; Sessle 1995; Sessle and Hu 1991; Woolf and Thompson 1991).

Noxious stimuli applied to somatic tissues may result in tissue damage which is accompanied by pain and hypersensitivity that outlast the stimuli. The persistent pain and hypersensitivity, which is mediated by sensitization of primary afferent nociceptors (LaMotte et al. 1982, 1983; Meyer and Campbell 1981), may reflect ongoing tissue
damage or lingering chemical irritants released by the original noxious stimuli and may also result from lasting changes in the peripheral nociceptors. Sensitization is defined as a leftward shift of the stimulus-response function that relates magnitude of the neural response to stimulus intensity (Meyer et al. 1989). Sensitization is characterized by a decrease in threshold, an augmented response to suprathreshold stimuli, and ongoing spontaneous activity (Beck et al. 1974; Beitel and Dubner 1976; Bessou and Perl 1969). Recent studies, however, have shown that there may be a central component to sensitization, i.e. "central sensitization". The sensitization of central sensory neurones is expressed as an increase of spontaneous or evoked neuronal activity, expansion of receptive fields, and reduction of thresholds (Dubner and Ruda 1992; Hu et al. 1992; Neugebauer and Schaible 1990; Simone et al. 1989; Woolf 1992; Woolf and King 1990; Yu et al. 1993) following application of noxious stimuli or induction of inflammation to the peripheral tissues (also see below).

C. TRIGEMINAL BRAINSTEM SENSORY NUCLEAR COMPLEX

The V nerve fibres enter the CNS at the pons level; they may ascend or descend in the V spinal tract (Vtr) before entering the V brainstem sensory nuclear complex where they synapse with second-order sensory neurones. The V sensory nuclear complex comprises the V nucleus principalis (Vp, also known as V main sensory nucleus) and the V spinal tract nucleus; the latter can be further subdivided into three subnuclei: oralis (Vo), interpolaris (Vi) and Vc. Neurones in each subdivision of the complex have axons that may project directly to the thalamus (Burton and Craig 1979; Burton et al. 1979; Funakoshi and Kerr 1979; Hayashi et al. 1984; Hockfield and Gobel 1978; Hu et al. 1981; Price et al. 1977a; Sessle and Greenwood 1976; Shigenaga et al. 1983; Stewart and King 1963). Many neurones, however, may project to neurones in adjacent
brainstem structures such as the reticular formation (Roberts and Matzke 1971; Stewart and King 1963). Some neurones may also project to the spinal cord, or to other subnuclei of the V sensory nuclear complex via intratrigeminal deep bundles (Dunn and Matzke 1968; Falls 1984; Hockfield and Gobel 1982; Hu et al. 1981; Ikeda et al. 1982; Jacquin et al. 1990b; Nasution and Shigenaga 1987; Roberts and Matzke 1971; Stewart and King 1963) and produce modulatory effects on neurones in these brainstem/spinal cord structures (Davis and Dostrovsky 1988; Greenwood and Sessle 1976; Hallas and Jacquin 1990; Sessle and Greenwood 1974). Furthermore, by virtue of their connections with the cranial nerve motor nuclei associated with muscle contraction or brainstem nuclei related to autonomic function (Burton et al. 1979; Dom et al. 1973; Erzurumlu and Killackey 1979; Robert and Matzke 1971; Travers and Norgren 1983; Stewart and King 1963), many V neurones may also serve as reflex interneurones in the numerous muscle and autonomic reflex responses that may be evoked by craniofacial stimuli (see Dubner et al. 1978; Hannam and Sessle 1994; Lund 1991; Olsson and Westberg 1989; Sessle 1990, 1992; Sessle and Hu 1991; Yoshida et al. 1995).

The most caudal component of the V spinal tract nucleus, Vc, begins rostrally at about the obex level and extends caudally into upper cervical spinal cord where it merges with the spinal dorsal horn (Hockfield and Gobel 1978; Pfalter and Arvidsson 1988; Shigenaga et al. 1988b). Because Vc has many morphological similarities with the spinal dorsal horn (Hockfield and Gobel 1978; Gobel et al. 1988) and Vc neurones generally have functional properties similar to those demonstrated for spinal dorsal horn neurones (Hoffman et al. 1981; Hu 1990; Hu et al. 1981; Price et al. 1976), it has been designated as the medullary dorsal horn. Two types of nociceptive Vc neurones, on the basis of their cutaneous receptive field properties, have been documented: (i) wide dynamic range (WDR) neurones that receive excitatory input from both large-diameter
(Aβ) mechanoreceptive afferents and small-diameter (Aδ and C) nociceptive afferents and respond to both innocuous and noxious stimulation to their peripheral receptive fields in a graded fashion as stimulation intensities increase into the noxious range; most of them also fire when the temperature of stimuli applied to the receptive field extends into the noxious range (45-52 °C; Craig and Dostrovsky 1991; Dubner 1985; Hu 1990; Hu et al. 1981; Price et al. 1976). Within the V brainstem nuclear complex, they exist in high concentrations in the deep portion of Vc but a few of them can also be found in other laminae of Vc and in the adjacent reticular formation, and in rostral components of the V brainstem complex, i.e. VP, Vo, and Vi (Amano et al. 1986; Broton et al 1988; Bushnell et al. 1984; Craig and Dostrovsky 1991; Dubner et al. 1976; Hayashi et al. 1984; Hu 1990; Hu et al. 1981, 1992; Price et al. 1976, 1977a; Raboisson et al. 1991, 1995; Villanueva et al. 1988). (ii) Nociceptive specific (NS) neurones that receive excitatory inputs exclusively from slowly conducting small-diameter nociceptive afferent (Aδ and C) fibres respond only to high-intensity stimuli (Amano et al 1986; Broton et al 1988; Bushnell et al. 1984; Dubner et al. 1976; Hu 1990; Hu et al. 1981, 1992; Price et al. 1976, 1977a; Villanueva et al. 1988); some of these neurones may also respond to noxious thermal stimuli. These neurones are usually found in the marginal and deep portion (laminae I, II and V) of Vc as well as in the rostral V brainstem complex, and each has a small receptive field (<2 cm²) that is usually smaller in size than those of WDR neurones. A third type of V sensory neurones, the low-threshold mechanoreceptive (LTM) neurones, respond to hair movement or to light tactile stimulation. They can code tactile stimulus intensity but show no increase in discharge with more intense stimulation into the noxious range. They can be subdivided into slowly adapting neurones, which have a maintained firing to a constant tactile stimulus, and rapidly adapting neurones, which discharge transiently as the stimulus is being
applied but cease firing within 2-3 s of a maintained stimulus (Hu et al. 1981, 1990; Price et al. 1976). They predominate in laminae III/IV of Vc as well as at other levels of the V brainstem complex.

Small-diameter primary afferent fibres innervating a variety of superficial and deep craniofacial tissues, including the TMJ, project to Vc (Arvidsson and Gobel 1981; Capra 1987; Hathaway et al. 1995; Jacquin et al. 1986; Lu and Bereiter 1995; Nishimori et al. 1986; Shigenaga et al. 1986, 1988a; Sugimoto et al. 1994; Strassman and Vos 1993; Strassman et al. 1994; Takemura et al. 1991). Moreover, nociceptive Vc neurones can be excited by noxious stimuli applied to these various craniofacial tissues (Amano et al. 1986; Broton et al. 1988; Hu et al. 1981; Price et al. 1976). They can code the spatial localization and intensity of noxious stimuli, and many have been shown to project directly to the thalamus (Craig and Dostrovsky 1991; Dubner et al. 1976; Hockfield and Gobel 1978; Hu et al. 1981; Kus et al. 1995; Price et al. 1976, 1977a; Williams et al. 1994; Yoshida et al. 1991; also see Dubner 1985; Hannam and Sessle 1994; Sessle 1987; Yokota 1985). Some Vc neurones have also been shown to project to cranial nerve motor nuclei (Burton et al. 1979; Dom et al. 1973; Erzurumlu and Killackey 1979; Robert and Matzke 1971; Stewart and King 1963) and thereby Vc has also been implicated as a site of interneurones serving in craniofacial muscle reflex pathways, autonomic reflexes and more complex integrative motor functions (Bereiter and Gann 1986, 1988; Bereiter et al. 1994; Dubner 1978; Panneton 1991; Panneton and Yavari 1995; Sessle 1995).

These findings also are consistent with earlier observations that V tractotomy near the obex in humans or analogous lesions in experimental animals produces a profound craniofacial analgesia and thermanesthesia, with much less complete loss of tactile sensibility and intraoral/perioral nociception such as tooth pain (Broton and Rosenfeld 1985; Kerr 1979; Young 1982; Young et al. 1981). Accumulating evidence seems to
suggest that the role of Vc in pain is primarily related to processing of nociceptive information from facial skin and deep craniofacial tissues, whereas the more rostral components such as Vp and Vo may be more involved in intraoral and perioral pain mechanisms (e.g. Broton and Rosenfeld 1986; Dallel et al. 1988, 1990; Raboisson et al. 1995; Sessle 1995).

D. POSSIBLE NEUROTRANSMITTERS INVOLVED IN NOCICEPTION AND SENSITIZATION

A variety of chemical substances have been found to accumulate near peripheral terminals of nociceptive afferent fibres following tissue injury. Potassium, histamine, and adenosine triphosphate leak out of damaged cells following tissue injury; they excite C fibres (Foreman et al. 1979; Kanaka et al. 1985; Kumazawa and Mizumura 1977; Mense 1977) and produce pain when injected into human skin (Lim and Guzman, 1967; Vyklicky 1984). Bradykinin, prostaglandins and leukotrienes are synthesized in the region of tissue damage or enter the injured area due to plasma extravasation; they are associated with inflammation and may excite or sensitize peripheral nociceptors (Beck and Handwerker 1974; Bisgaard and Kristensen 1985; Crossman and Fuller 1988; Kanaka et al. 1985; Levine et al. 1984). Nociceptors themselves also release substances into the extracellular space when they are activated (Chahl and Ladd 1976; Chapman et al. 1961; also see Couture and Cuello 1984; Handwerker and Reeh 1991; Kumazawa 1990). Substance P and CGRP for example have been found at the peripheral terminals of unmyelinated primary afferent nerve fibres (Del Fiacco and Cuello 1980; Furness et al. 1982; Hokfelt et al. 1975; Ichikawa et al. 1989; Johansson et al. 1986; Kido et al. 1993; Norregaard and Moskowitz 1985) and both can be released when the peripheral nerves are electrically stimulated at C-fibre intensity (Akagi et al. 1980; Pohl et al. 1992; Rosell
et al. 1981; Yaksh et al. 1980). In the periphery, substance P has been shown to cause vasodilation and increase vascular permeability (Furness et al. 1982; Gazelius et al. 1987; Lembeck and Holzer 1979; Norregaard and Moskowitz 1985) and to cause the release of histamine or serotonin from mast cells (LaMotte et al. 1983) which leads to more inflammation and release of more algesic chemicals; it may also activate or sensitize C fibres (Chahl and Ladd 1976; Chapman et al. 1961; Nakamura-Craig and Gill 1991; Woolf and Wiesend-Hallin 1986). It has also been shown that glutamate application to peripheral tissues, activating particularly kainate receptors, may also activate C-fibre primary afferents (Agrawal and Evans 1986; Ault and Hildebrand 1993; Evans et al. 1987).

There is evidence that the C fibre-evoked responses of dorsal horn nociceptive neurones are mediated by the co-release of glutamate/aspartate and substance P and their respective activation of N-methyl-D-aspartate (NMDA) and neurokinin-1 receptors, leading to prolonged depolarization of the nociceptive neurones (Dougherty and Willis 1991a, b, 1992; Ren et al. 1992; for review, see Mao et al. 1995). The release of glutamate and substance P in the spinal and medullary dorsal horn (Vc) is increased following noxious stimulation of the peripheral tissues (Angelucci 1956; Bereiter et al. 1996; Kawagoe et al. 1986; Kangrge and Randic 1990; Randic and Miletic 1977). Microidontophoresis of glutamate or substance P in the spinal or medullary dorsal horn (Anderson et al. 1978; Henry 1976; Henry et al. 1980; Hosli et al. 1981; Murase et al. 1982; Salt et al. 1982; Urban and Randic 1984; Wilcox 1991; Woolf and Thompson 1991; Wright and Roberts 1980) results in excitation of nociceptive dorsal horn neurones in particular (Henry 1976; Henry et al. 1980; Randic and Miletic 1977; Wright and Roberts 1980), and intraspinal injection of NMDA produces a behavioural hyperalgesia (Aanonsen and Wilcox 1987). NMDA receptor antagonists have been
shown to block the C fibre-evoked depolarization (Woolf and Thompson 1991), mechanoreceptive field expansion (Ren et al. 1992), and "wind-up" responses (Davies and Lodge 1987; Dickenson and Sullivan 1987; Dickenson et al. 1991) of dorsal horn neurones, the C fibre-evoked temporal summation related to second pain (Price et al. 1994), and noxious stimulation-evoked behavioural hyperalgesia (Cahusac et al. 1984; Ren et al. 1992; Woolf and Wall 1986) and reflex responses (Yu et al. 1996). Substance P antagonists have also been shown to attenuate dorsal horn neuronal responses (Kelstein et al. 1990) and peripheral vasodilatation (Rosell et al. 1981) evoked by C fibre stimulation and to increase behavioural nociceptive thresholds (Akerman et al. 1982; Lembeck et al. 1981; Rodriguez et al. 1983); there are however considerable numbers of substance P terminals in the dorsal horn which are not primary afferent in origin but are derived from spinal interneurones (Del Fiacco and Cuello 1980; Hunt et al. 1981) or descending fibres (Gilbert et al. 1982).

E. MODULATION OF NOCICEPTIVE TRANSMISSION

Although modulation of nociceptive transmission may occur at different stages of the nociceptive neural pathways, it appears to occur largely at earlier stages of nociceptive transmission, i.e. the V brainstem sensory complex and spinal dorsal horn (Fields 1987; Fields and Basbaum 1978, 1994; Sessle 1987, 1992, 1995). A complex interaction of various neural pathways and neurochemical mechanisms may be responsible for the modulation.

E1. Modulation by Peripheral Stimulation

The intensity of pain can be reduced by peripheral stimulation procedures such as acupuncture (e.g. Chen et al. 1996; Yanehara et al. 1992; Zhang et al. 1986; also see
Melzack 1984), transcutaneous electrical nerve stimulation (TENS; e.g. Johnson et al. 1989, 1991; Wang et al. 1992; also see Woolf 1984), and diffuse noxious inhibitory controls (DNIC; Bouhassira et al. 1988; Dallel et al. 1990; Dickenson and Le Bars 1983; Dickenson et al. 1980; Falinower et al. 1994; Hu 1990; Le Bars et al. 1979; Villanueva et al. 1984). The V neuronal or reflex responses evoked by craniofacial stimuli can also be modulated by other craniofacial stimuli or by stimulation applied to a remote site of the body (Bouhassira et al. 1988; Cadden 1985; Dallel et al. 1990; Dickenson and Le Bars 1983; Dickenson et al. 1980; Hu 1990; Sessle and Greenwood 1976; Sessle et al. 1981; Yu and King 1974). It is unclear whether the effectiveness of modulation by peripheral stimulation is mediated by segmental mechanisms or by recruitment of descending influences from higher brain region. Endogenous opioid-related mechanisms have been suggested to be involved, at least in part, in acupuncture or TENS-induced analgesic effects (Melzack 1984; Woolf 1984). Other endogenous chemicals, including serotonin, noradrenalin, and γ-aminobutyric acid (GABA) have also been considered as possible neurotransmitters that may be involved in the peripheral stimulation-induced modulation (Basbaum 1985; Chan and Yip 1979; Crisp et al. 1991; Lovick and Wolstencroft 1983; Salt and Hill 1983; Yeomans et al. 1992).

As pointed out above, trauma and inflammation may result in sensitization of peripheral nerve endings and enhancement of responses and neuroplastic changes of nociceptive neurones in V brainstem sensory complex or spinal dorsal horn (e.g. Hoheisel and Mense 1989; Hu et al. 1992; Hylden et al. 1989; Yu et al. 1993; for review, see Dubner and Ruda 1992; Sessle 1992; Woolf 1989). Deafferentation, a partial or total loss of a sensory nerve supply to a particular body region, may also lead to an increase in incidence of neurones with an extensive mechanoreceptive field and spontaneous activity (Devor 1987; Hu et al. 1986a; Kaas et al. 1983; Kwan et al. 1993; Lombard and
Larabi 1983; Mendell 1984; Tasker and Dostrovsky 1989; Wall 1983, 1984). Deafferentation is also thought to be involved in processes underlying the development of chronic pains such as causalgia, sensory neuropathies, postherpetic pain, phantom limb pain, and perhaps atypical facial pain, and several possible mechanisms have been suggested, viz. sprouting of collaterals of non-affected afferent fibres in the periphery or in the CNS, loss of segmental inhibition, disinhibition of existing but ineffective synapses, ectopic impulse generation in primary afferents, activation or facilitation of primary afferent fibres by sympathetic nerves (for review, see Dubner and Basbaum 1994; Fields 1987; Sessle 1992).

**E2. Modulation by Central Stimulation**

Pain can also be modulated by stimulation of various sites in the CNS (e.g. descending inhibition), including the somatosensory cortex, hypothalamus, periaqueductal gray (PAG), rostral ventromedial medulla (RVM), anterior pretectal nucleus, and parabrachial area of the pons (Baskin et al. 1986; Mayer and Liebeskind 1974; Reynolds 1969; Richardson and Akil 1977; also see Fields and Basbaum 1978; Mayer and Price 1976; Sessle 1987; 1995). Nociceptive neurones in the V brainstem sensory complex or the spinal dorsal horn as well as reflex responses to noxious stimuli can be inhibited by stimulation of these loci (Chiang et al. 1989, 1990, 1991, 1995; Dostrovsky et al. 1982, 1983; Hongo and Jankowska 1967; Hu et al. 1986b; Rees and Roberts 1987; Roberts and Rees 1986; Sessle et al. 1981, 1992; Yezierski et al. 1983).

The PAG receives inputs from hypothalamus (Beitz 1982a; Reichling and Basbaum 1990), frontal cortex (Hardy and Leichnetz 1981), amygdala (Gray and Magnuson 1992) and the adjacent pontomedullary reticular formation (Herbert and Saper 1992); it also receives a major projection from lamina I nociceptive neurones in the
spinal dorsal horn (Hylden et al. 1986; Menetrey et al. 1982). The PAG is the major source of projection to RVM (Aimone and Gebhart 1986; Behbehani et al. 1979; Gebhart et al. 1983). The RVM neurones send descending projections to the superficial layers and lamina V of the spinal dorsal horn via the dorsolateral funiculus (Fields et al. 1977; Willis et al. 1977). Although the PAG contains a large number of enkephalin, GABA and substance P-containing neurones (Hokfelt et al. 1977a, b; Moss et al. 1983), these neurones appear not to project to the RVM (Reichling and Basbaum 1990). Glutamate and/or aspartate may be the neurotransmitter in the PAG-to-RVM connection (Aimone and Gebhart 1986; Aimone et al. 1987), and RVM neurones may also receive serotonergic or noradrenergic inputs from other brain loci (Beitz 1982b; Clark and Proudfoot 1991). Serotonin and norepinephrine have been suggested to be the neurotransmitters involved in brainstem-produced analgesia (Crisp et al. 1991; Hylden and Wilcox 1983; Jordan et al. 1979; Randic and Yu 1976; Solomon and Gebhart 1992).

Endogenous opioids are also important in CNS stimulation-induced pain modulation. Stimulation-produced analgesia, some forms of stress-induced analgesia, and placebo analgesia of postoperative pain have all been reported to be reduced by the opioid antagonist naloxone (Fields 1988; Mogil et al. 1996; Sandler et al. 1994; van Bastelaere et al. 1995; Watkins and Mayer 1982). Intravenous, intraventricular, or intrathecal (i.e. to Vc) application of naloxone also "rekindles" the EMG activity of jaw muscles after the increased EMG response evoked by mustard oil injection into the TMJ region (see below) has returned to baseline levels (e.g. Yu et al. 1994). Cell bodies or nerve terminals containing opioids can be found in PAG, RVM, the spinal dorsal horn, and Vc (Bowker et al. 1988; Cruz and Basbaum 1985; Glazer and Basbaum 1981; Harlan et al. 1987; Hokfeld et al. 1979; Millan et al. 1986, 1987; Nishimori et al. 1988; Ruda et al. 1981, 1984; Standaert et al. 1986). Intrathecal naloxone reduces the antinociceptive
effects of electrical stimulation of RVM (Aimone et al. 1987; Zorman et al. 1982). Although there are direct projections to Vc and the spinal dorsal horn from opioid-containing RVM neurones (Beitz et al. 1987; Hokfeld et al. 1979), a substantial number of opioid terminals in Vc or the spinal dorsal horn are derived from local interneurones (Cruz and Basbaum 1985; Glazer and Basbaum 1981; Harlan et al. 1987; Nishimori et al. 1988; Standaert et al. 1986). Neurones in Vc or the spinal dorsal horn also contain high concentrations of opioid receptors (Arvidsson et al. 1995; Atweh and Kuhar 1977; Lamotte et al. 1976; Mansour et al. 1994; Pert et al. 1975). Spinal application of opioids produces analgesia and iontophoresis of opioids inhibits nociceptive neurones in Vc and spinal dorsal horn (Dickenson et al. 1990, 1991; Duggan and North 1984; Fleetwood-Walker et al. 1988; Yonehara et al. 1986). Opioids have also been found in central terminals of primary afferent fibres (Basbaum et al. 1986; Weihe et al. 1986) and local injection of opioids also produces antinociceptive effects (Haley et al. 1990; Stein et al. 1989). These findings thus indicate that opioids may produce analgesic effects via actions in both peripheral and central nervous systems.

F. TRIGEMINAL PREMOTONEURONES AND MOTONEURONES

Neurones directly projecting to the Vmo, the site of motoneurones supplying jaw-opening and jaw-closing muscles, have been found ipsilaterally in the V mesencephalic nucleus (Chandler et al. 1990; Mizuno et al. 1983; Nomura and Mizuno 1985) and bilaterally in the parvocellular reticular formation, supra- and inter-trigeminal nuclei, Vp, Vo, Vi, Vc and upper cervical (C1-C3) spinal dorsal horns (e.g. Chandler et al. 1990; Donga et al. 1990; Donga and Lund 1991; Landgren et al. 1986; Mizuno et al. 1978, 1983; Nomura and Mizuno 1985; Shigenaga et al. 1988b; Travers and Norgren 1983; Yoshida et al. 1994). Among these brainstem structures, the parvocellular reticular
formation, Vo, and supra- and inter-trigeminal nuclei have received most attention and have been studied more thoroughly than other nuclei due to their involvement in rhythmic jaw movements and orofacial reflexes. In the parvocellular reticular formation, three types of premotoneurones have been found: (i) inhibitory premotoneurones which produce inhibitory post-synaptic potentials (IPSPs) in masseter α motoneurones, (ii) excitatory premotoneurones which produce excitatory post-synaptic potentials (EPSPs) in α masseter motoneurones, and (iii) excitatory premotoneurones which produce EPSPs in digastric motoneurones (Nozaki et al. 1993). These premotoneurones receive direct inputs from neurones in the medial bulbar reticular formation, where the central pattern generator for rhythmic jaw movements is located (Chandler and Tal 1986; Chandler et al. 1990; Katoh et al. 1982; Nozaki et al. 1986), and show rhythmic firing patterns induced by repetitive stimulation of the cortical masticatory area (Nozaki et al. 1993). Excitatory premotoneurones of the digastric motoneurones have also been found in the ipsilateral Vo and Vi (Olsson and Westberg 1991; Shigenaga et al. 1988b; Sumino 1971; Yoshida et al. 1994, 1995), and inhibitory premotoneurones of masseter motoneurones have been found in Vo and the supratrigeminal nucleus (Kidokoro et al. 1968b; Nakamura et al. 1978; Yoshida et al. 1995); however, no inhibitory premotoneurones of the digastric motoneurones have been reported so far.

The V motoneurones can be topographically divided into two groups, i.e. jaw-opening and jaw-closing motoneurones, respectively. The former are located in the ventromedial portion of the Vmo and innervate the jaw-opening muscles (viz. anterior DIGs) which depress the mandible; the latter are located in the dorsolateral portion and innervate the jaw-closing muscles (viz. masseter, temporalis and the pterygoid muscles) that elevate the mandible (DeSantis et al. 1978; Lynch 1985; Matsuda et al. 1978; Mizuno et al. 1975; Nomura and Mizuno 1983; Rokx and van Willigen 1988; Sessle
The V motoneurones innervating the jaw-closing muscles can be further divided into α or γ motoneurones which innervate extrafusal and intrafusal fibres of the jaw-closing muscles, respectively, and control jaw muscle activity and jaw movements (Kubota 1976; Lund 1976; Matthews 1975; Rokx et al. 1987). The V γ motoneurones can be physiologically differentiated from V α motoneurones by their longer antidromic latency (and thus slower conduction velocity), higher threshold for antidromic activation, and a characteristic tonic discharge even with the jaw closed (Sessle 1977a).

G. CRANIOFACIAL JAW REFLEXES

G1. Jaw-Closing Reflex

The jaw-closing reflex, like the stretch reflex in the spinal system, is a monosynaptic reflex; its reflex arc consists of Ia primary afferent fibres which innervate muscle spindles in the jaw-closing muscles (Mizuno et al. 1983; Nomura and Mizuno 1985; Shigenaga et al. 1988a, c, 1990) and synapse centrally with jaw-closing α motoneurones (Hannam et al. 1970; Jerge 1963; Nakamura et al. 1967; Smith et al. 1967). The cell bodies of these muscle-spindle afferent fibres are located in the CNS, i.e. V mesencephalic nucleus (Jerge 1963; Mizuno et al. 1983; Nomura and Mizuno 1985; Shigenaga et al. 1988a, c, 1989, 1990; Smith et al. 1968; Szentagothai 1948).

Mechanical stimulation of periodontal tissues, e.g. tapping or pressing on the upper incisors, has also been shown to evoke excitatory EMG responses in the jaw-closing muscles (Funakoshi and Amano 1974; Goldberg 1971, 1972a; Sessle 1977b), which can be reduced by injection of lidocaine into the periodontal tissues or by section of the maxillary nerve. In awake humans, these periodontium-evoked reflexes can only be evoked when subjects maintain an isometric contraction of the jaw-closing muscles and are expressed as a short-latency excitatory response followed by inhibition of EMG
activity (Goldberg 1971). However, in anaesthetized rats, these reflexes are expressed in two phases of excitatory EMG activities in the jaw-closing muscles. The first phase is mediated by primary afferent fibres whose cell bodies are located in the ipsilateral V mesencephalic nucleus whereas the relay interneurones for the second phase of the response seem to be located in Vp; the V spinal tract nucleus seems not to be involved (Funakoshi and Amano 1974). Electrical stimulation of the TMJ evokes a short-latency reflex EMG response in the jaw-closing muscles, although the responses in the jaw-opening muscles are more prominent (Broton and Sessle 1988). We have also noticed that pressing the TMJ with a blunt glass rod also evokes increases in EMG activity of jaw-closing as well as jaw-opening muscles (Tsai et al. unpublished observations).

G2. Jaw-Opening Reflex

The jaw-opening reflex is a di- or multi-synaptic reflex evoked by low- and high-intensity orofacial stimulation, e.g. mechanical stimulation of periodontal or perioral/intraoral tissues or electrical stimulation of the tooth pulp or inferior alveolar nerve (Hannam and Matthews 1969; Sotgiu and Bellinzona 1991; Sumino 1971; Widmer 1987; Yamada et al. 1985); the efferent arm of the jaw-opening reflex involves motoneurones innervating the digastic muscle (Chiang et al. 1989, 1990, 1991; Clarke and Matthews 1985; Dessem et al. 1988; Tanaka and Toda 1982), although the lateral pterygoid, geniohyoid or mylohyoid muscles may also be involved in the reflex responses (Broton and Sessle 1988; Widmer 1987). In association with the jaw-opening reflex evoked by orofacial stimulation, there may be a corresponding reflex inhibition of the antagonistic jaw-closing muscles (Goldberg 1972b; Kidokoro et al. 1968a; Sherrington 1917; Sumino 1971). This inhibitory effect on the jaw-closing motoneurones may result from a powerful inhibition which masks the facilitatory effects of the same
peripheral stimulus, because intravenous application of the glycine receptor antagonist strychnine depresses the inhibitory effect and results in an enhancement of the jaw-closing motoneuronal activity (Sessle 1977b). The excitatory interneurones of the jaw-opening reflex are located in the ipsilateral Vo/Vi (Olsson and Westberg 1991; Sumino 1971; Yoshida et al. 1994, 1995) and the inhibitory interneurones of the jaw-closing reflex are located in the ipsilateral Vo and supratrigeminal nucleus (Kidokoro et al. 1968b; Nakamura et al. 1978; Yoshida et al. 1995).

The jaw-opening reflex has been suggested to provide protection to the masticatory apparatus from mechanical damage during biting and thus has been regarded as analogous to the spinal flexion reflex; it has been used as a behavioural model to study mechanisms underlying orofacial nociception and its regulation (see Dubner et al. 1978; Mason et al. 1985; Sessle 1979). However, since the jaw-opening reflex can be activated by low-intensity as well as high-intensity orofacial stimuli (e.g. Dallel et al. 1989; Kidokoro et al. 1968a; Sherrington 1917; Sessle 1977b; Sotgiu and Bellinzona 1991; Sumino 1971; Yamada et al. 1985; Young et al. 1981), the nociceptive nature of the jaw-opening reflex has been questioned (see Kidokoro et al. 1968a; Mason et al. 1985; Sessle 1979, 1987). Thus, the reflex may not be related to only a protective function but also may play a role in the integration of complex craniofacial neuromuscular activities such as mastication (Dubner et al. 1978; Lund 1991; Hannam and Sessle 1994; Lund and Sessle 1994).

G3. Craniofacial Nociceptive Reflexes

Previous studies in our laboratories have shown that application of algesic chemicals (KCl, 7% NaCl, histamine and mustard oil) into the TMJ or tooth pulp can evoke an increase in EMG activity of jaw-closing and jaw-opening muscles (Broton and
Sessle 1988; Sunakawa et al. 1993a, b; Tsai et al. 1994a, b; Yu et al. 1994, 1995). These algesic chemical-evoked EMG responses have been attributed to a centrally mediated reflex response because (i) the increased EMG activity occurs in muscles remote from and contralateral to the injection site (e.g., the ipsilateral DIG and genioglossus, and the contralateral DIG and MASS), (ii) the increased EMG activity can be blocked in rats receiving pre-administration of lidocaine into the TMJ region (Yu et al. 1995) or extirpation of the tooth pulp (Sunakawa et al. 1993a, b), (iii) the time course of the increased EMG activity is consistent with that of the mustard oil-evoked neuroplastic changes (e.g. expansion of mechanoreceptive fields) in nociceptive neurones in Vc (Hu et al. 1992; Sessle et al. 1995; Yu et al. 1993), and (iv) neonatal application of the C fibre neurotoxin capsaicin to afferent nerves prevents the central trigeminal neuronal excitatory effects of mustard oil (Sessle et al. 1995) and the mustard oil-evoked EMG responses (Tsai et al. unpublished data). Since application into the TMJ region of the vehicles (i.e. saline or mineral oil) of these algesic chemicals cannot evoke any significant change in EMG activity of the jaw muscles, these algesic chemical-evoked reflex responses have been regarded as craniofacial nociceptive reflexes.

The observations that noxious stimuli applied to the TMJ region co-activate both jaw-opening and jaw-closing muscles appear inconsistent with previous findings that orofacial stimulation can reflexly activate jaw-opening muscles and inhibit jaw-closing muscles (see above). However, the latter reflex effects are typically evoked by intraoral/perioral noxious or non-noxious stimuli whereas the conjoint activation of jaw-opening and jaw-closing muscles occurs specifically with noxious stimulation of the TMJ region. Moreover, our findings are consistent with findings that Vc is critically involved in craniofacial nociception and the rostral V brainstem sensory nuclei, i.e. Vp, Vo and Vi, are primarily related to intraoral and perioral pain mechanisms (see above).
These findings also agree with findings that the V tractotomy increases the threshold of neck and head reflexes/behaviours evoked by intraoral/perioral stimulation but cannot completely eliminate these responses (Dalle et al. 1989; Young et al. 1981) and that Vc is also involved in autonomic reflexes evoked by various craniofacial noxious stimuli (Bereiter and Gann 1986, 1988; Bereiter et al. 1996; Panneton 1991; Panneton and Yavari 1995).

H. STATEMENT OF THE PROBLEM AND AIMS OF THE STUDIES

The central neural pathways underlying the craniofacial nociceptive reflexes are presently unknown. Although the rostral components of the V spinal tract nucleus, i.e. Vp, Vo and Vi, have been implicated in orofacial nociceptive mechanisms related especially to intraoral/perioral pain (e.g. Broton and Rosenfeld 1986; Dalle et al. 1988, 1990; Hayashi et al. 1984; Luccarini et al. 1995; Raboisson et al. 1995; Sessle and Greenwood 1976; Yu and King 1974) and in reflexes evoked by low-intensity orofacial stimulation (Funakoshi and Amano 1974; Kidokoro et al. 1968a; Olsson and Westberg 1991; Shigenaga et al. 1988c; Sumino 1971; Yoshida et al. 1994, 1995), the caudal component of the V spinal nucleus, Vc, has traditionally been viewed as the essential relay site of nociceptive information from superficial and deep craniofacial tissues to higher levels of the CNS (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985). Indeed, in the case of relay mechanisms related to nociceptive inputs from deep craniofacial tissues, previous studies have shown that Vc receives projections from small-diameter primary afferent fibres, i.e., Aδ and C fibres, innervating deep tissues including the TMJ (Capra 1987; Nishimori et al. 1986; Shigenaga et al. 1988a), and that injection of mustard oil into the TMJ region of rats induces c-fos-like immunoreactivity in Vc (Hathaway et al. 1995). Our previous studies
have also shown that many Vc neurones, classified according to their cutaneous receptive field properties as WDR or NS neurones, also receive convergent primary afferent inputs from deep craniofacial tissues including the TMJ (Amano et al. 1986; Broton et al. 1988; Hu 1990; Hu et al. 1992; Kojima 1990; Sessle et al. 1986; Yu et al. 1993). Furthermore, Vc neurones have been shown to project to the Vmo (Dunn and Matzke 1968; Li et al. 1993; 1995; Mizuno et al. 1983; Roberts and Matzke 1971; Stewart and King 1963). These findings thus indicate that Vc may play an important role not only in superficial craniofacial pain but also in deep pain and possibly in reflexes evoked by noxious stimulation of deep craniofacial tissues. However, it is still unclear what are the central pathways for the nociceptive reflexes evoked in jaw-closing and jaw-opening muscles by noxious stimulation of the TMJ region. Therefore, the overriding objective of this thesis was to determine if Vc is involved in craniofacial nociceptive reflexes evoked by mustard oil injection into the TMJ region.

One approach to address this issue was to apply techniques that excite neurones in Vc to determine if such stimulation evokes EMG responses in both jaw-opening and jaw-closing muscles. In addition to electrical micro-stimulation, this approach utilized micro-injection into Vc of glutamate since it is an excitatory amino acid and cell excitant (see above). Glutamate, a cell excitant and excitatory amino acid, has been regarded as one of the most important excitatory neurotransmitters in the CNS (Curtis and Johnson 1974; Johnson 1978; Watkins and Evans 1981; also see Salt and Hil 1983). Glutamate receptors, both NMDA and non-NMDA subtypes, have been found to exist not only in the CNS but also in the peripheral nervous system (Berger et al. 1995; Shigemoto et al. 1992; for review, see Collingridge and Lester 1989; Erdo 1991; Monaghan et al. 1989; Nakanishi 1992). Functionally, non-NMDA receptor mechanisms are implicated in generating fast EPSPs in the CNS, whereas NMDA receptor mechanisms mediate more
prolonged EPSPs (for review, see Collingridge and Lester 1989; Monaghan et al. 1989; Nakanishi 1992) and are considered of primary importance in nociceptive transmission in ascending somatosensory pathways, particularly in "central sensitization" states that can be evoked by inflammation and other conditions associated with increased small-fibre nociceptive afferent inputs into the CNS (Aanonsen and Wilcox 1987; Dickenson and Sullivan 1987; Yaksh 1989; Woolf and Thompson 1991; for review, see Dubner and Ruda 1992; Coderre 1993; Meller and Gebhart 1993; Urban et al. 1994). Glutamate applied to the CNS may bind with NMDA and non-NMDA receptors at the site of injection and produce an excitatory effect on the neurones. It helps determine the importance of neurones, as opposed to fibres of passage, in the central neural pathways involved in a specific function.

Another approach was to disrupt Vc and determine if this interferes with the mustard oil/TMJ-evoked EMG responses in both jaw-opening and jaw-closing muscles. For this approach, surgical sections of certain caudal brainstem and spinal cord regions were used, as well as injections of the neurotoxic chemical ibotenic acid. Ibotenic acid, an excitatory amino acid, is an extract of the mushroom Amanita muscaria. Like other members in the excitatory amino acid family, e.g. glutamate, kainic acid, and aspartate, ibotenic acid possesses a neurotoxic effect specifically on neurones in the CNS, i.e. when injected into brain tissue it produces neuronal death but may not damage fibres of passage and nerve terminals of extrinsic origin (Aimone et al. 1988; Chiang et al. 1990; Schwarz et al. 1979). At the same concentrations, ibotenic acid produces a more limited and spherical lesion at the site of injection compared with injection of the same amount of kainic acid, which tends to produce more extensive and irregular-shaped lesions (Schwarz et al. 1979; Wuerthele et al. 1978). Because of its discriminative effect on
neurones versus fibres of passage and the limited lesion sizes it produces. Ibotenic acid has been used as a means of selective lesioning in the CNS.

The **first aim** of this thesis was to use surgical sections and extensive injection of the neurotoxic agent ibotenic acid to test whether lesions of Vc are effective in reducing the increased jaw-muscle EMG activities evoked by injection of mustard oil into the TMJ region (see Chapter 2). The **second aim** was to apply systematically electrical micro-stimulation in Vc to test whether stimulation of Vc produces any excitatory responses in jaw muscles and which portion of Vc is most critically involved in producing these excitatory responses (see Chapter 3). The **third aim** was to use micro-injection of the excitatory amino acid and cell excitant glutamate to test if neurones in the critical area of Vc are responsible for the excitatory responses in the jaw muscles (see Chapter 3). The **fourth aim** was to use more limited micro-injection of ibotenic acid to determine if indeed neurones in the crucial area of Vc are of critical importance in the craniofacial nociceptive reflexes evoked by injection of mustard oil into the TMJ region (see Chapter 4). The **fifth aim** was to test whether neurones in Vc respond to noxious stimuli, including mustard oil injection, of the TMJ region (see Chapter 5). The final chapter of the thesis provides a General Discussion linking the implications of these various studies.
CHAPTER 2. INVOLVEMENT OF TRIGEMINAL SUBNUCLEUS CAUDALIS (Vc) IN CRANIOFACIAL NOCICEPTIVE REFLEX ACTIVITY

INTRODUCTION

Mustard oil (allyl iso-thiocyanate) is an algesic chemical that can provoke pain in humans (Handwerker et al. 1991). Because it selectively activates small-diameter afferent fibres and produces inflammation at the site of application (Handwerker and Reeh 1991; Haas et al. 1992; Jancso et al. 1967; Woolf and Wall 1986), it has been widely used in studies of mechanisms underlying nociception and "central sensitization" of nociceptive neurones in somatosensory pathways (Hu et al. 1992, 1993; Jancso et al. 1967, 1977; Woolf and Wall 1986; Yu et al. 1993, 1994, 1995). Our previous studies have also shown that injection of mustard oil into the TMJ region evokes an increase in EMG activity in DIG and MASS muscles (Yu et al. 1994, 1995; Tsai et al. 1994a,b). This mustard oil-evoked EMG activity has been attributed to a centrally mediated reflex response because (i) the increased EMG activity occurs in muscles remote from and contralateral to the injection site (e.g., the ipsilateral DIG, and the contralateral DIG and MASS), (ii) the increased EMG activity can be blocked in rats receiving pre-administration of local anaesthetic into the TMJ region (Yu et al. 1995), (iii) the time course of the increased EMG activity is consistent with that of the mustard oil-evoked responses in nociceptive neurones in the V spinal tract nucleus (Sessle et al. 1995), and (iv) neonatal application of the C fibre neurotoxin capsaicin to afferent nerves prevents the central V neuronal excitatory effects of mustard oil (Sessle et al. 1995).
The central neural pathways underlying the mustard oil-evoked reflex EMG response in jaw muscles are presently unknown. Although the rostral components of the V spinal tract nucleus, i.e. Vo and Vi, have been implicated in orofacial nociceptive mechanisms related especially to intraoral/perioral pain (e.g. Dalle et al. 1988, 1990; Hayashi et al. 1984; Luccarini et al. 1995; Raboisson et al. 1995; Sessle and Greenwood 1976), the caudal component of the V spinal nucleus, Vc, has traditionally been viewed as the essential relay site of nociceptive information from superficial and deep craniofacial tissues to higher levels of the CNS (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985). Indeed, in the case of relay mechanisms related to nociceptive inputs from deep craniofacial tissues, previous studies have shown that Vc receives small-diameter primary afferent fibres, i.e., Aδ and C fibres, innervating deep tissues including the TMJ (Capra 1987; Nishimori et al. 1986; Shigenaga et al. 1988). Injection of mustard oil into the TMJ region of rats induces in Vc c-fos-like immunoreactivity (Hathaway et al. 1995). Since the c-fos is an immediate-early gene which encodes and expresses a nuclear phosphoprotein in neuronal nuclei following induction of inflammation or activation of nociceptive primary afferent fibres (Bullitt 1990; Hathaway et al. 1995; Hunt et al. 1987; Menetrey et al. 1989; Noguchi et al. 1991), the TMJ-induced expression of c-fos-like immunoreactivity in Vc thus suggests a projection to Vc neurones of nociceptive primary afferent fibres which innervate the TMJ region. Our previous studies have also shown that many Vc neurones, classified according to their cutaneous receptive field properties as WDR or NS neurones, also receive convergent primary afferent inputs from deep craniofacial tissues including the TMJ (Amano et al. 1986; Broton et al. 1988; Hu 1990; Hu et al. 1992; Sessle et al. 1986; Yu et al. 1993), and some can be excited by injection of mustard oil into the TMJ region in rats (Tsai et al. unpublished data). Furthermore, degenerative axons have been
observed in Vmo in monkeys, sheep and cats after Vc lesions (Dunn and Matzke 1968; Roberts and Matzke 1971; Stewart and King 1963), and some Vc neurones can be retrogradely labelled after horseradish peroxidase injection into Vmo (Li et al. 1993; Mizuno et al. 1983). These findings thus indicate that Vc may play an important role not only in superficial craniofacial pain but also in deep pain, including possibly reflexes evoked by noxious stimulation of deep craniofacial tissues. Therefore, the aim of this study was to use surgical and chemical lesions to test whether Vc is involved in the craniofacial nociceptive reflexes evoked by injection of mustard oil into the TMJ region. Some of these data have been previously presented in abstract form (Tsai et al. 1994b).

METHODS

This study was performed on 45 adult male Sprague-Dawley rats (250-350 g). Most of the methods have been described in detail previously (Yu et al. 1994, 1995; Hu et al. 1993) and so the following will focus on those methodological aspects not previously described. Under general anaesthesia (1/3 O₂, 2/3 N₂O and 1.5% halothane), a tracheotomy was performed and a tracheal cannula was inserted for artificial ventilation (3-4 ml/stroke, 75-80 strokes/min). The right femoral artery and vein were cannulated for blood pressure monitoring and fluid infusion, respectively. The rat was then placed in a stereotaxic apparatus with ear and incisor bars. Two screws were inserted into the exposed dorsal surface of the skull and an aluminum bar mounted on the stereotaxic apparatus was fixed to the screws with dental ligature wire and acrylic resin to suspend the head of the rat. A pair of bipolar EMG electrodes were inserted into each of the left DIG, the left MASS, the right MASS and the right DIG muscles. Electrode locations were confirmed by the increased EMG activity evoked in all four muscles by pressing the TMJ with a blunt glass rod and by dissection at the end of the experiment. A short 26-
gauge needle connected to a 25-μl Hamilton syringe via a polyethylene tubing (PE-50) was implanted into the TMJ region for the injection of mustard oil (20% in mineral oil).

The rats were divided into seven groups. Intact rats (INTACT, n=8) were used as a control group. Except for the INTACT group, a laminectomy of the first and second cervical vertebrae was produced and the dura was opened and retracted in the other six groups of rats to expose the caudal brainstem and the spinal cord.

For the TRAN rats (n=8), a 2 mm deep transection was produced in the left brainstem with a surgical blade (Bard-Parker, #11) which was fixed on a micromanipulator and moved according to the calibration on the stereotaxic apparatus. The blade was inserted into the brainstem at 2 mm lateral to the midline and moved to the lateral margin of the rostral end of the left Vc at the level 0.8-1.0 mm caudal to the obex.

In the IBO rats (n=5), ibotenic acid (0.03 M in phosphate-buffered saline; 0.5 μl; 0.02 μl/min) was injected into the left brainstem by a 26-gauge needle attached to a 1-μl Hamilton syringe. The syringe was mounted on the micromanipulator with an angle of 90° to the dorsal surface of the brainstem. The injections were made at two sites: (i) 2 mm lateral to the midline at a depth of 2 mm at the level 2 mm caudal to the obex, and (ii) 1 mm lateral to the midline at a depth of 1.5 mm at the level 5 mm caudal to the obex. To help prevent leakage of ibotenic acid from the needle track, the needle was kept in position for 5 minutes after completion of the ibotenic acid injection.

For the LAM rats (n=6), the surgical wound was sutured and no lesion was produced in the brainstem.

In the SAG rats (n=8), a 2 mm deep and 2 mm long sagittal section was produced medial to the left Vc from 1 mm caudal to the obex and 0.5 mm lateral to the midline.
For the C2-SECT rats (n=5), a 1.5 mm deep transection was produced at the caudal end of left Vc from 0.5 mm lateral to the midline to the lateral margin of left spinal cord at the entry of the second cervical spinal nerve.

In the PBS rats (n=5), phosphate-buffered saline (0.5 μl; 0.02 μl/min) was injected into the left brainstem at two sites comparable to those described in the IBO rats.

Following these procedures, the neck muscles and the overlying skin were sutured and the ear and incisor bars were removed from all of the rats. Halothane concentration was reduced and maintained at 0.8-1.0% until the end of the experiment. Heart rate, expired percent CO₂, blood pressure and core temperature of the rat were continuously monitored and maintained at normal physiological levels of 330-430 beats/min, 3.0-5.0%, 80-130 mmHg and 36-38°C, respectively. The flexion reflex of the hindlimb induced by noxious pressure applied with serrated forceps to the toes was used as an indication of recovery of the physiological conditions from deep anaesthesia and from any spinal shock that might have been produced by the lesions. If there was any sign of failure of recovery of the physiological conditions after the surgical preparation or brainstem lesion, the rat was sacrificed and any data obtained from the rat were discarded.

**EMG Recording**

The EMG activity of each muscle was amplified (2,000-5,000X; 30-3,000 Hz), displayed on oscilloscopes, and also recorded online with a data acquisition system (consisting of an IBM AT 486 computer, CED 1401 Plus hardware and the software "SPIKE2"; CED, Cambridge). Signal sampling rate was 2,000 Hz for each muscle. The first 20 min of recording in each muscle monitored resting EMG activity and was used to derive the baseline level of EMG activity for that muscle in each rat (see below). In the INTACT, LAM, SAG, C2-SECT and PBS rats, mustard oil was injected into the left TMJ
region at the beginning of the twenty-first min and the EMG activity was continuously recorded thereafter for another 40 min. The total recording time for rats in these five groups was 60 min.

In the TRAN and IBO rats, injections of mustard oil were made into the TMJ region bilaterally. In four TRAN rats and three IBO rats, after the first 20-min EMG recording period, the first mustard oil injection was made into the left TMJ region at the beginning of the twenty-first min; the EMG activity was then continuously recorded for another 40 min, after which time (the sixty-first min) the second mustard oil injection was made into the right TMJ region and the EMG activity was again continuously recorded for the other 40 min. In the remaining four TRAN rats and two IBO rats, the procedure was reversed, i.e. mustard oil was first injected into the right TMJ region and later the left TMJ region was injected. The total recording time for the TRAN and IBO rats was 100 min.

The EMG activity was subsequently rectified to transform negative EMG waves into positive waves and then integrated into EMG area by multiplying the mean EMG levels by the time interval of every 60 s. The EMG activity of each muscle was expressed with data points representing the integrated area for every 60-s segment. Data points of the EMG area for each muscle recorded during the first 20 min in each animal were pooled to produce a mean value which represented the baseline level of the EMG activity for that muscle. Relative changes in EMG area with respect to the baseline level were used to address the effects of mustard oil injection into the TMJ region. All data points were normalized relative to the mean value and expressed as a percentage value of the mean baseline level of the EMG activity. Changes in EMG area after the mustard oil injection were regarded as significantly increased if one or more EMG data points rose 2 standard deviations above the mean baseline level. The time period between the
initiation of the increased EMG activity and its recovery to the baseline EMG level was designated as the duration of the increased EMG activity evoked by injection of mustard oil into the TMJ region.

**Histological Examination**

At the end of each experiment, Evans blue dye (10 mg/kg) was injected intravenously into the rat. Ten min after Evans blue injection, the rat was sacrificed with an overdose of sodium pentobarbital (100 mg/kg). The rat was perfused with 200 ml warm saline followed by 150 ml 10% buffered formalin solution. The location of the mustard oil injection indicated by plasma extravasation of Evans blue was confirmed by postmortem dissection of the TMJ region. If the plasma extravasation did not include the TMJ capsule, data recorded from the rat were discarded. The brainstem was removed after perfusion and post-fixed in 10% buffered formalin solution for one week. The brainstem tissue was cut in 50-μm serial sections with a Vibratome and stained with cresyl violet. The lesion sites were examined under a light microscope and reconstructed with the aid of a camera lucida.

**Statistical Analysis**

Fisher's Exact Probability test was used for statistical comparison of the incidence of the mustard oil-evoked increases of EMG activity between the INTACT rats and the other groups. The Wilcoxon test was used for statistical comparisons between the mean value representing the baseline level of EMG activity (see above) and any data point of the post-injection periods. According to previous findings (Hu et al. 1993; Tsai et al. 1994a,b; Yu et al. 1994, 1995), the mustard oil-evoked EMG activity peaks at the first min after mustard oil injection. Therefore, the EMG area for the first min post-injection was selected to reflect evoked EMG activity in statistical comparisons. The Mann-Whitney test was used for statistical comparisons of the increased EMG activity.
between different groups of rats. The paired t-test was used for statistical comparisons of the increased EMG activity evoked by the two injections of mustard oil in the TRAN or IBO rats. A probability level of less than 0.05 (2-tailed) was regarded as significant. In this paper, all the values are given as mean±SD.

RESULTS

In agreement with our earlier findings (Haas et al. 1992; Yu et al. 1995) of the extent of plasma extravasation of Evans blue dye after mustard oil injection into the TMJ region, plasma extravasation could be seen in all animals in the TMJ capsule and periauricular tissues lateral to the TMJ injected with mustard oil. No such plasma extravasation could be found in tissues of the TMJ region that received no mustard oil injection; this finding is also consistent with our earlier findings (Haas et al. 1992; Yu et al. 1995).

For all animals in the seven groups, heart rate, expired percent CO₂, blood pressure and core temperature during EMG recording were maintained at normal physiological ranges as described above. There was no significant variation in EMG activity of any jaw muscle during the initial 20 min-period before injection of mustard oil into the TMJ region. This finding agrees with our earlier data (Yu et al. 1994, 1995) and indicates that a stable level of baseline EMG activity was achieved.

INTACT Rats

Mustard oil injection into the left TMJ region of the INTACT rats evoked an increase in EMG activity in all four muscles of all eight INTACT rats (see Table 2-1). The mustard oil-evoked EMG activity had a latency of 1-2 s, peaked within 1 min, lasted for 10-40 min, and was reflected in one or two phases of increased activity (Fig. 2-1A). This increased EMG activity was significantly greater than the baseline EMG level in all four
TABLE 2-1.
INCIDENCE OF INCREASED EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION
Values in round brackets, ( ), represent number of rats in each group; values in square brackets, [ ], indicate number of rats having a significant increase of EMG activity (EMG area that rose 2 SD above the mean baseline level) in each muscle. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region).

<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT (n=8)</td>
<td>100% [8]</td>
<td>100% [8]</td>
<td>100% [8]</td>
<td>100% [8]</td>
</tr>
<tr>
<td>TRAN-L (n=8)</td>
<td>50% [4]*</td>
<td>37.5% [3]*</td>
<td>50% [4]*</td>
<td>12.5% [1]**</td>
</tr>
<tr>
<td>TRAN-R</td>
<td>100% [8]#</td>
<td>87.5% [7]</td>
<td>62.5% [5]</td>
<td>75% [6]#</td>
</tr>
<tr>
<td>IBO-L (n=5)</td>
<td>0% [0]**</td>
<td>0% [0]**</td>
<td>40% [2]*</td>
<td>20% [1]**</td>
</tr>
<tr>
<td>IBO-R</td>
<td>100% [5]#</td>
<td>100% [5]#</td>
<td>80% [4]</td>
<td>60% [3]</td>
</tr>
</tbody>
</table>

*p<0.05 and **p<0.01 (Fisher's test) indicate significantly different compared with INTACT rats.
#p<0.05 (Fisher's test) indicates significantly different compared to incidence in the contralateral muscle evoked by mustard oil injection into the left TMJ region of the same group of rats.
Fig. 2-1 An example of EMG changes evoked by injection of mustard oil into the left TMJ region. A: the EMG traces are expressed in terms of the integrated EMG area for every 60 sec. B: dotted area shows the location of extravasated dye. The actual EMG traces recorded from the left digastric (L DIG), the left masseter (L MASS), the right masseter (R MASS) and the right digastric (R DIG) are shown in C, at 10 min, during mustard oil injection, and at 30 and 50 min. ↓, start mustard oil injection; ↑, end injection.
muscles (Fig. 2-2). The mean EMG activity (mean EMG area for the first min post-injection) and the mean duration of the mustard oil-evoked EMG activity of the INTACT rats are shown in Tables 2-II and 2-III.

TRAN Rats

The lesion in the left Vc of the TRAN rats interrupted the continuity of the V spinal nucleus at the Vi-Vc junction. The transection involved the Vtr, laminae I-IV, and most of laminae V and VI of the left Vc (Fig. 2-3C,D) whereas no signs of destruction were observed in the right Vc. EMG activity recorded from the four TRAN rats receiving injections of mustard oil into the left TMJ region and then the right TMJ region was not significantly different from that recorded from the other four TRAN rats receiving bilateral mustard oil injections in the reverse sequence. Data from these eight TRAN rats were pooled together for further analysis.

Mustard oil injection into the left TMJ region of the TRAN rats (TRAN-L rats) evoked a small increase in EMG activity of the ipsilateral DIG in four rats, of the ipsilateral MASS in three rats, of the contralateral DIG in four rats, and of the contralateral MASS in one of the eight TRAN rats. The incidence of the increased EMG activity in all four muscles of the TRAN-L rats was significantly lower than that of the INTACT rats (Table 2-I). The evoked EMG activity in all four muscles of the TRAN-L rats was not significantly greater than the baseline EMG level (Fig 2-3A), and was significantly smaller and significantly shorter in duration than in the INTACT rats (Tables 2-II and 2-III).

In contrast, mustard oil injection into the right TMJ region of the TRAN rats (TRAN-R rats) evoked an increase in EMG activity of the ipsilateral DIG in all eight rats, of the ipsilateral MASS in seven rats, of the contralateral MASS in six rats, and of the contralateral DIG in five of the eight TRAN rats. The incidence of the increased EMG
Fig. 2-2 Mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region of INTACT rats. Each data point represents the mean±SD of the normalized values relative to the baseline EMG activity in each rat (in some cases, symbols cover the SD bars), and the horizontal dotted line in each graph indicates the mean baseline EMG activity. "*" indicates that the amplitude of the EMG activity of that data point was significantly higher than the baseline EMG activity (Wilcoxon test. p<0.05).
EMG CHANGES OF INTACT RATS

Mustard oil
L TMJ

Time (min)

Relative changes in EMG activity (x 100%)

L DIG

L MASS

R DIG

R MASS

0 10 20 30 40 50 60 70

0 2 4 6 8

12

9

6

3

0
**TABLE 2-II.**
MEAN FIRST MINUTE (PEAK) EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION
Values representing EMG activity (EMG area for the first min post-injection) are expressed as mean±SD of the normalized value relative to the mean value of the pre-injection EMG activity (=100%, baseline activity) in each group of rats. Values in brackets indicate number of rats in each group. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region).

<table>
<thead>
<tr>
<th>Group</th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT (n=8)</td>
<td>570±276%</td>
<td>471±248%</td>
<td>731±479%</td>
<td>217±101%</td>
</tr>
<tr>
<td>TRAN-L (n=8)</td>
<td>138±65%***</td>
<td>113±39%***</td>
<td>143±66%**</td>
<td>102±19%**</td>
</tr>
<tr>
<td>TRAN-R</td>
<td>520±310%#</td>
<td>410±310%#</td>
<td>128±23%**</td>
<td>132±33%</td>
</tr>
<tr>
<td>IBO-L (n=5)</td>
<td>100±1%***</td>
<td>91±14%***</td>
<td>97±2%**</td>
<td>98±1%**</td>
</tr>
<tr>
<td>IBO-R</td>
<td>1270±783%#</td>
<td>434±237%#</td>
<td>146±30%**,#</td>
<td>132±21%#</td>
</tr>
<tr>
<td>LAM (n=6)</td>
<td>526±194%</td>
<td>371±178%</td>
<td>433±319%</td>
<td>176±87%</td>
</tr>
<tr>
<td>SAG (n=8)</td>
<td>454±236%</td>
<td>613±597%</td>
<td>476±512%</td>
<td>153±104%</td>
</tr>
<tr>
<td>C2-SECT (n=5)</td>
<td>737±296%</td>
<td>666±387%</td>
<td>327±185%</td>
<td>157±77%</td>
</tr>
<tr>
<td>PBS (n=5)</td>
<td>447±118%</td>
<td>473±435%</td>
<td>594±547%</td>
<td>125±50%*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 and ***p<0.001 (Mann-Whitney test) indicate significantly different compared with INTACT rats.
#p<0.05 (Paired t-test) indicates significantly different compared with the EMG activity evoked by mustard oil injection into the left TMJ of the same group of rats.
TABLE 2-111.
MEAN DURATION OF THE INCREASED EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION

Values representing duration of the increased EMG activity are expressed as mean±SD. Values in brackets indicate number of rats in each group. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region).

<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT (n=8)</td>
<td>25.9±14.9</td>
<td>15.8±5.3</td>
<td>17.9±11.4%</td>
<td>19.4±16.6</td>
</tr>
<tr>
<td>TRAN-L (n=8)</td>
<td>3.0±4.2%***</td>
<td>2.6±4.7***</td>
<td>2.4±3.6**</td>
<td>4.7±0.6***</td>
</tr>
<tr>
<td>TRAN-R</td>
<td>13.1±8.4#</td>
<td>14.0±10.1#</td>
<td>6.1±7.0*,#</td>
<td>5.0±8.1#</td>
</tr>
<tr>
<td>IBO-L (n=5)</td>
<td>0***</td>
<td>0***</td>
<td>0.4±0.5***</td>
<td>0.2±0.4***</td>
</tr>
<tr>
<td>IBO-R</td>
<td>10.4±6.1#</td>
<td>17.6±11.7#</td>
<td>2.2±2.3**,#</td>
<td>3.6±3.9*,#</td>
</tr>
<tr>
<td>LAM (n=6)</td>
<td>12.5±8.1</td>
<td>16.5±6.3</td>
<td>7.3±7.9</td>
<td>3.8±6.7</td>
</tr>
<tr>
<td>SAG (n=8)</td>
<td>20.8±7.7</td>
<td>18.4±14.7</td>
<td>16.4±12.8</td>
<td>9.0±14.0</td>
</tr>
<tr>
<td>C2-SECT (n=5)</td>
<td>18.8±4.4</td>
<td>15.2±5.2</td>
<td>16.0±14.7</td>
<td>5.0±4.8</td>
</tr>
<tr>
<td>PBS (n=5)</td>
<td>18.8±4.9</td>
<td>21.4±11.4</td>
<td>12.2±8.0</td>
<td>2.4±4.8*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 and ***p<0.001 (Mann-Whitney test) indicate significantly different compared with INTACT rats.

#p<0.05 (Pared t-test) indicates significantly different compared with the EMG activity evoked by mustard oil injection into the left TMJ of the same group of rats.
Fig. 2-3 The EMG activity of TRAN rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into left, and then right, TMJ region. B, example of transection of Vc at the obex level indicated as a dotted line on the dorsal surface of the brainstem. C, photograph of a transection in the brainstem. D, histological reconstruction of the transection in C. Value at the upper-right corner of the graph indicates the distance of the section from obex and the vertical and horizontal bars at the lower-right corner indicate 1 mm. Cu, cuneate nucleus; Gr, gracile nucleus; LR, lateral reticular nucleus; PT, pyramidal tract; RF, reticular formation; Vc, subnucleus caudalis; XII, hypoglossal nucleus.
activity in all four muscles of the TRAN-R rats was not significantly different from that of the INTACT rats (Table 2-I). This increased EMG activity in all four muscles was significantly greater than the baseline EMG level (Fig. 2-3A). Statistical comparisons showed that the evoked EMG activity in the ipsilateral DIG, ipsilateral MASS and contralateral MASS of the TRAN-R rats was not significantly different from that of the INTACT rats, but in the contralateral DIG it was significantly smaller and significantly shorter in duration than in the INTACT rats (Tables 2-II and 2-III). The comparisons within the TRAN rats (TRAN-L rats vs. TRAN-R rats) showed that the increased EMG activity was significantly smaller in the ipsilateral DIG and ipsilateral MASS and was significantly shorter in duration in all four muscles of the TRAN-L rats than in the TRAN-R rats (Tables 2-II and 2-III).

**IBO Rats**

The lesion in the left Vc of the IBO rats produced signs of neuronal death and microglial infiltration at the sites of ibotenic acid injection. The lesion was composed of two irregular spheres with diameters of about 1.5 mm that involved the medial portion of laminae I-IV and most of laminae V, VI, VII and VIII of the left Vc (Fig. 2-4C,D) whereas no signs of destruction were observed in the right Vc. The EMG activity recorded from the three IBO rats receiving injections of mustard oil into the left TMJ region and then the right TMJ region was not significantly different from that recorded from the other two IBO rats receiving bilateral mustard oil injections in the reverse sequence. Data from all of the five IBO rats were pooled together for further analysis.

Mustard oil injection into the left TMJ region evoked no increase in EMG activity of the ipsilateral DIG and ipsilateral MASS of any of the IBO rats (IBO-L rats); increased EMG activity of the contralateral DIG and contralateral MASS occurred in two and one of these rats, respectively. The incidence of the increased EMG activity in all four
Fig. 2-4 The EMG activity of IBO rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into left, and then right, TMJ region. B, example of ibotenic acid injections indicated by two dots on the dorsal surface of the brainstem. C, photograph of a ibotenic acid injection site. D, histological reconstruction of the injection track (black region) and the area with visible neuronal death and accumulation of microglia cells (hatched region) in C.
EMG CHANGES OF IBO RATS

A

Mustard oil
L TMJ

Mustard oil
R TMJ

Relative changes in EMG activity (x 100%)

Time (min)

B

C

D

-2.0
muscles of IBO-L rats was significantly lower than that of the INTACT rats (Table 2-I). The evoked EMG activity of the IBO-L rats was not significantly greater than the baseline EMG level in any of the four muscles (Fig. 2-4A), and was significantly smaller and significantly shorter in duration than in the INTACT rats (Tables 2-II and 2-III).

In contrast, mustard oil injection into the right TMJ region evoked an increase in EMG activity of the ipsilateral DIG and ipsilateral MASS in all five IBO rats (IBO-R rats) as well as of the contralateral DIG and contralateral MASS in four and three of these rats, respectively. The incidence of the increased EMG activity in all four muscles of IBO-R rats was not significantly different from that of the INTACT rats (Table 2-I). This increased EMG activity in the ipsilateral DIG, ipsilateral MASS and contralateral DIG (but not the contralateral MASS) was significantly greater than the baseline EMG level (Fig. 2-4A). Statistical comparisons showed that the evoked EMG activity in the ipsilateral DIG, ipsilateral MASS and contralateral MASS of the IBO-R rats was not significantly different from that of the INTACT rats, but in the contralateral DIG it was significantly smaller and significantly shorter in duration than in the INTACT rats (Tables 2-II and 2-III). The comparisons within the IBO rats (IBO-L rats vs. IBO-R rats) showed that the increased EMG activity was significantly smaller and significantly shorter in duration in all four muscles of the IBO-L rats than in the IBO-R rats (Tables 2-II and 2-III).

Other Groups of Rats

Mustard oil injection into the left TMJ region of the LAM, SAG, C2-SECT and PBS rats evoked an increase in EMG activity of bilateral DIG and MASS (Figs. 2-5, 2-6, 2-7, 2-8). The incidence of the increased EMG activity in all four muscles of these groups of rats was not significantly different from that of the INTACT rats (Table 2-I). The evoked EMG activity was significantly greater in the ipsilateral DIG, ipsilateral
Fig 2-5. Mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region of LAM rats.
EMG CHANGES OF LAM RATS

Mustard oil
L TMJ

L DIG

L MASS

R DIG

R MASS

Relative changes in EMG activity (x 100%)

Time (min)

0 10 20 30 40 50 60 70
Fig. 2-6 The EMG activity of SAG rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, example of sagittal section indicated as a dotted line on the dorsal surface of the brainstem. C, histological reconstruction of the sagittal section from the same rat shown in B.
EMG CHANGES OF SAG RATS

A

Mustard oil
L TMJ

L DIG

L MASS

R DIG

R MASS

Relative changes in EMG activity (x 100%)

Time (min)

0 10 20 30 40 50 60 70

B

C2

OBEX

C

-1.0

-1.5

-3.0

-2.0

-2.5
Fig. 2-7 The EMG activity of C2-SECT rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, example of transection indicated as a dotted line on the dorsal surface of the spinal cord. C, histological reconstruction of the transection from the same rat shown in B.
Fig. 2-8 The EMG activity of PBS rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, example of phosphate-buffered saline injections indicated by two dots on the dorsal surface of the brainstem. C, histological reconstruction of the injection from the same rat shown in B.
MASS and contralateral DIG (but not the contralateral MASS) of all of these groups of rats than the baseline EMG level (Figs. 2-5, 2-6, 2-7, 2-8), and was not significantly different from that of the INTACT rats in all four muscles, except that in the contralateral MASS of the PBS rats which was significantly smaller and significantly shorter in duration than in the INTACT rats (Tables 2-II and 2-III).

The incidence of the increased EMG activity in the bilateral DIG of the SAG rats and in the ipsilateral MASS of the C2-SECT rats was significantly higher (p<0.05) than that of the TRAN-L rats. The evoked EMG activity in all four muscles of the SAG and C2-SECT rats was significantly greater and significantly longer in duration (p<0.05, Mann-Whitney test) than in the TRAN-L rats. The incidence of the increased EMG activity in the ipsilateral DIG and ipsilateral MASS of the PBS rats was significantly higher (p<0.05) than that of the IBO-L rats. The evoked EMG activity in the ipsilateral DIG, ipsilateral MASS and contralateral DIG (but not the contralateral MASS) of the PBS rats was significantly greater and significantly longer in duration (p<0.05) than in the IBO-L rats.

DISCUSSION

The present study has provided the first documentation that Vc plays an important role in craniofacial nociceptive reflexes evoked by injection of the small-fiber excitant and inflammatory irritant mustard oil into the TMJ region. The magnitude and duration of the increased EMG activity evoked by mustard oil injection into the TMJ region are consistent with those of our previous studies showing the reflex basis and features of the increased EMG activity (Yu et al. 1994, 1995). The reduction of the mustard oil-evoked EMG activity as a result of transection of the brainstem at the obex level or ibotenic acid-induced lesions of the Vc is unlikely to reflect deterioration of the
physiological conditions of the rats because (i) heart rate, expired percent CO\textsubscript{2}, blood pressure and core temperature were maintained within normal physiological ranges, (ii) noxious pressure applied to the toes still induced a flexion reflex of the hindlimb after brainstem lesions, (iii) mustard oil injection into the TMJ region contralateral to the lesions still evoked an increase in EMG activity of the jaw muscles, and (iv) mustard oil injection into the ipsilateral TMJ region of rats with a sagittal section medial to the left Vc (SAG rats), with a transection of the left spinal cord at the C2 segment (C2-SECT rats) or with phosphate-buffered saline injections in the left Vc (PBS rats), evoked an increase in EMG activity of the jaw muscles. Discussion of the limitations and technical features of the study is provided in Chapter 6.

The V primary afferent fibres, including those innervating TMJ, enter the central nervous system at the pons level and may descend to Vc via Vtr (Capra 1987; Jacquin et al. 1990a; Shigenaga et al. 1988b; Takemura et al. 1991). Some Vc neurones project to rostral V sensory nuclei including Vmo (Dunn and Matzke 1968; Jacquin et al. 1990b; Li et al. 1993; Mizuno et al. 1983; Nasution et al. 1987; Roberts and Matzke 1971; Stewart and King 1963) via deep bundles in the V spinal tract nucleus (Falls 1984; Gobel and Purvis 1972; Jacquin et al. 1990b; Ikeda et al. 1982). Therefore, transection of the left caudal brainstem at the obex level, the approximate location of Vi/Vc junction (Strassman and Vos 1993; Takemura et al. 1991), could have interrupted inputs to and outputs from the left Vc and thus reduced the increased EMG activity in the jaw muscles reflexly evoked by injection of mustard oil into the left TMJ region. In contrast, sagittal section medial to the left Vc and transection of the left spinal cord at the C2 segment failed to reduce the mustard oil evoked EMG activity, indicating that neural pathways involved in these reflex responses were not interrupted by these two lesions. Since the transection of the caudal brainstem at the obex level, the sagittal section medial to Vc
and the transection at the C2 segment demarcate the Vc region of the caudal brainstem. these findings indicate that Vc is essential for the increased EMG activity reflexly evoked by injection of mustard oil into the TMJ region.

This conclusion is further supported by our observations of the effects of ibotenic acid which is a specific neurotoxic agent that produces neuronal death but may not damage fibres of passage and nerve terminals of extrinsic origin (Aimone et al. 1988; Chiang et al. 1990; Schwarcz et al. 1979). In the present study, histological examination of the loci of ibotenic acid injections in the brainstem indeed showed marked neuronal death at the injection sites. The lesion-induced reduction in the increased EMG activity evoked by mustard oil injection into the left TMJ region of the IBO rats and the ineffectiveness of the vehicle injection in the PBS rats strongly point to the importance of Vc neurones in craniofacial nociceptive reflex pathways. This conclusion is further supported by our recent observations that electrical micro-stimulation of Vc excites the ipsilateral DIG and ipsilateral MASS, with the lowest threshold sites being the medial portion of the caudal Vc (Tsai et al. 1995; also see Chapter 3) and that injection of an excitatory amino acid and neurone excitant glutamate into the caudal Vc (but not the C2 spinal segment or the adjacent reticular formation) evokes an increase in EMG activity of the ipsilateral, but not the contralateral, DIG and MASS (Chapter 3).

Reflex activation of jaw-opening muscles evoked by orofacial stimulation has been regarded as analogous to the limb flexion reflex and used as a behavioural model to study mechanisms underlying orofacial nociception and its regulation (see Dubner et al. 1978; Mason et al. 1985; Sessle 1979). However, since the jaw-opening muscles can be reflexly activated by low-intensity as well as high-intensity stimuli applied to orofacial tissues (e.g. Dallel et al. 1989; Sherrington 1917; Sotgiu and Bellinzona 1991; Sumino 1971), the nociceptive nature of the jaw-opening reflex has been questioned.
(see Kidokoro et al. 1968a; Mason et al. 1985; Sessle 1979, 1987). In association with the reflex activation of the jaw-opening muscles evoked by low-intensity orofacial stimulation, there may be a corresponding reflex inhibition of the antagonistic jaw-closing muscles (Kidokoro et al. 1968a; Sherrington 1917; Sumino 1971). The excitatory interneurones of the jaw-opening reflex are located in the ipsilateral Vo/Vi (Dalle et al. 1989; Olsson and Westberg 1991; Sumino 1971; Yoshida et al. 1994, 1995) and the inhibitory interneurones of the jaw-closing reflex are located in the ipsilateral Vo and supratrigeminal nucleus, respectively (Kidokoro et al. 1968b; Yoshida et al. 1995). The distinction between our findings (co-activation of jaw-opening and jaw-closing muscles with noxious stimuli) and earlier observations (activation of jaw-opening muscles and inhibition of jaw-closing muscles with low-intensity stimuli) suggests that craniofacial reflexes may involve different V nuclei depending on the nociceptive or non-nociceptive character of the stimulus and inputs, i.e. the former principally involving Vc and the latter mainly the more rostrally located V nuclei (Kidokoro et al. 1968b; Olsson and Westberg 1991; Sumino 1971; Yoshida et al. 1994,1995). We have used mustard oil for injection into the TMJ region (Yu et al, 1994,1995) since it is a C fibre excitant and inflammatory irritant (Handwerker and Reeh 1991; Haas et al. 1992; Jancso et al. 1967; Woolf and Wall 1986). Our finding that mustard oil injection into the TMJ region co-activates jaw-closing and jaw-opening muscles agrees with recent findings in the rat (Yu et al. 1994, 1995) and is consistent with the co-activation of jaw-closing and jaw-opening muscles that has been documented by injection of other algesic chemicals into the cat's TMJ region (Broton and Sessle 1988).

The present study raises several additional points. Firstly, since the lesions produced by transection and by ibotenic acid injection involved considerable portions of the Vc, the portion of Vc that is most critically involved in the TMJ-induced reflex is
unclear. Injection of mustard oil into the rat TMJ region induces a two-peak expression of c-fos-like immunoreactivity along the rostro-caudal extent in the caudal brainstem: one around the Vi/Vc junction and the other at the caudal Vc (Hathaway et al. 1995). Although there is evidence for TMJ afferent projections to the superficial laminae of Vc (Capra 1987; Hathaway et al 1995), most Vc neurones that respond to TMJ stimulation are found in laminae V and VI (Broton et al. 1988). Whether superficial or deep Vc neurones or both contribute to the increases of EMG activity evoked by injection of mustard oil into the rat TMJ region is not known. However, micro-stimulation of the rat Vc evoked EMG activity in the ipsilateral DIG and MASS, with the lowest threshold sites being superficial loci of the caudal Vc; the threshold and latency of the evoked EMG activity both increased when micro-stimulation was delivered more rostral or caudal to the caudal Vc (Tsai et al. 1995; also see Chapter 3). These preliminary results indicate that Vc may produce excitatory effects on jaw muscles, with the most effective loci located in the caudal Vc. More localized lesions of different parts of the caudal brainstem will help delineate the most important portion of Vc in this craniofacial nociceptive reflex pathway.

The pathway between Vc neurones and V motoneurones is also still unclear. Although Vc neurones can be retrogradely labelled by injection of horseradish peroxidase into the Vmo (Li et al. 1993, 1995; Mizuno et al. 1983) and degenerated axons can be seen in Vmo after lesions of Vc (Dunn and Matzke 1968; Roberts and Matzke 1971; Stewart and King 1963), the possibility that there exist multi-synaptic connections between Vc and Vmotoneurones cannot be discounted, e.g. Vc neurones project to Vp, Vo and Vi (Dunn and Matzke 1968; Hockfield and Gobel 1978; Hu et al. 1981; Jacquin et al. 1990b; Nasution and Shigenaga 1987; Roberts and Matzke 1971; Stewart and King 1963) and neurones in Vp, Vo and Vi project to Vmo (Fort et al. 1990;
Mizuno et al., 1983; Yoshida et al., 1994). Also, the latencies of the EMG activity evoked by micro-stimulation in Vc (Tsai et al., 1995; also see Chapter 3) suggest that these excitatory effects of Vc on Vmotoneurones may involve multi-synaptic or slowly conducting pathways from Vc to DIG and MASS motoneurones. Another possibility is that the Vc projection to Vp, Vo or Vi involves a facilitatory influence on reflex pathways that are mediated through more rostral brainstem structures. This possibility is supported by findings that the excitability of V primary afferent terminals in Vp and Vo (Dostrovsky et al., 1981; Young and King, 1972) and the mechanoreceptive field and response properties of Vp, Vo and Vi neurones (Greenwood and Sessle, 1976; Hallas and Jacquin, 1990) can be modulated by transection or cold block of Vc.

Our findings that Vc lesions markedly diminish the increased EMG activity in the right DIG and right MASS of the TRAN-L and IBO-L rats point to the importance of Vc neurones in activation of contralateral jaw muscles. These findings appear to rule out two possible pathways for TMJ-induced activation of the contralateral muscles: one involving a relay via TMJ primary afferent projections to ipsilateral rostral brainstem nuclei and then to contralateral V motoneurones, and another involving TMJ primary afferent projections to contralateral Vc and then to contralateral V motoneurones. The findings from the TRAN-L and IBO rats, plus the effectiveness of Vc lesions on reducing (but not abolishing) the increased EMG activity in the left DIG and left MASS of the TRAN-R and IBO-R rats, suggest that the TMJ nociceptive inputs may be relayed via TMJ primary afferent projections to ipsilateral Vc neurones, and then via contralateral Vc neurones to the contralateral V motoneurones. Thus, both left Vc and right Vc seem to be essential for the activation of contralateral jaw muscles. This possibility is supported by findings of interneuronal connections between left and right Vc (Hockfield and Gobel, 1981; Jacquin et al., 1990 a, b) and by c-fos-like immunoreactivity in the contralateral Vc.
as a result of mustard oil injection into the TMJ region (Hathaway et al. 1995). Other possible contralateral neural pathways that need to be tested include one involving Vc neurones projecting directly to contralateral V motoneurones (Mizuno et al. 1983) or to rostral brainstem sensory neurones (Dunn and Matzke 1968; Hockfield and Gobel 1978; Hu et al. 1981; Ikeda et al. 1982; Jacquin et al. 1990b; Nasution et al. 1987; Roberts and Matzke 1971; Stewart and King 1963) that project to contralateral V motoneurones (Mizuno et al. 1983; Rokx et al. 1986; Ter Horst et al. 1990).

Small-diameter primary afferent fibres innervating a variety of craniofacial tissues as well as the TMJ project to Vc (Arvidsson and Gobel 1981; Capra 1987; Hathaway et al. 1995; Jacquin et al. 1986; Lu and Bereiter 1995; Nishimori et al. 1986; Shigenaga et al. 1986, 1988a; Sugimoto et al. 1994; Strassman and Vos 1993; Strassman et al. 1994; Takemura et al. 1991). Moreover, Vc neurones can be excited by noxious stimulation applied to these tissues (Amano et al. 1986; Broton et al. 1988; Chiang et al. 1994; Hu et al. 1992; Pozo and Cervero 1993; Sessle et al. 1986; Yu et al. 1993). Electrical micro-stimulation or micro-injection of the excitatory amino acid glutamate into Vc produces excitatory EMG responses in the jaw muscles (Chapter 3) as well as autonomic responses, e.g. changes in plasma concentration of adrenocorticotropic, arterial blood pressure and heart and respiration rates (Bereiter and Gann 1986, 1988; Panneton and Yavari 1995). In addition, injection of various algesic chemicals into the TMJ region or tooth pulp evokes increases of EMG activity in both jaw-opening and jaw-closing muscles (Broton and Sessle 1988; Hu et al. 1995; Sunakawa et al. 1993a,b; also see Hu et al. 1994). These findings, plus observations that craniofacial stimulation also produces changes of autonomic responses which can be blocked by injection of lidocaine into Vc (Bereiter et al. 1994; Panneton 1991; Panneton and Yavari 1995), point to the likelihood
that Vc may be involved in craniofacial muscle and autonomic reflexes evoked by noxious stimuli applied to various craniofacial tissues.
CHAPTER 3. EXCITATORY EFFECTS ON JAW MUSCLE ACTIVITY OF ELECTRICAL MICRO-STIMULATION AND GLUTAMATE MICRO-INJECTION IN THE TRIGEMINAL SUBNUCLEUS CAUDALIS (Vc)

INTRODUCTION

Injection of the small-fibre excitant and inflammatory irritant mustard oil into the rat's TMJ region evokes a bilateral increase in EMG activity of DIG and MASS muscles (Yu et al. 1994, 1995; also see Chapters 2 and 4). This mustard oil-evoked EMG activity has been attributed to a centrally mediated nociceptive reflex response because (i) the increased EMG activity occurs in muscles remote from and contralateral to the injection site (e.g., the ipsilateral DIG, and the contralateral DIG and MASS), (ii) the increased EMG activity can be blocked in rats receiving pre-administration of local anaesthetic into the TMJ region (Yu et al. 1995), (iii) lesions of Vc, an important brainstem relay of craniofacial nociceptive information (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985), significantly reduce the mustard oil-evoked EMG activity (Tsai et al. 1994b; in press), (iv) i.v., i.c.p., or local (into the TMJ region) pre-administration of MK-801, a glutamate subtype (NMDA) receptor antagonist, also reduces the mustard oil-evoked EMG activity (Yu et al. 1996 in press), (v) the time course of the increased EMG activity is consistent with that of the mustard oil-induced neuroplastic changes in nociceptive neurones in Vc (Hu et al. 1992; Sessle et al. 1995; Yu et al. 1993) and (vi) neonatal application of the C fibre neurotoxin capsaicin blocks these neuroplastic changes in Vc nociceptive neurones (Sessle et al. 1995) and abolishes the mustard oil-evoked jaw muscle EMG activity (Tsai et al. unpublished data).
Although some neurones in the rostral V brainstem sensory nuclear complex or adjacent nuclei, e.g. Vp, Vo and Vi, or supratrigeminal nucleus may act as interneurones of the low-threshold orofacial reflexes (Funakoshi and amano 1974; Kidokoro 1968a, b; Shigenaga et al. 1988c; Sumino 1971; Yoshida et al. 1994, 1995), transection of the rostral Vc at the Vi/Vc junction significantly reduces the increased bilateral EMG activity in DIG and MASS muscles reflexly evoked by mustard oil injection into the TMJ region (Tsai et al. 1994b; also see Chapter 2). This mustard oil-evoked EMG responses can also be reduced by injection into Vc of the excitatory neurotoxic agent ibotenic acid (Chapters 2 and 4), which kills neurones but spares fibres of passage at the injection site (Aimone et al. 1988; Chiang et al. 1990; Schwarcz et al. 1979), indicating the importance of Vc neurones in craniofacial nociceptive reflex pathways. However, the surgical and chemical lesions in our previous study involved considerable portions of the Vc, and it is unclear which portion of Vc is most critically involved in these pathways. In addition, we have shown that the contralateral DIG and MASS may also be activated by mustard oil injection into the TMJ region (Tsai et al. 1994a,b; also see Chapters 2 and 4). Therefore, the aims of this study were to use electrical micro-stimulation and micro-injection of the excitatory amino acid and cell excitant glutamate (Curtis and Johnson 1974; Johnson 1978; Watkins and Evans 1981) in localized regions of Vc in order to test (i) whether stimulation of Vc produces excitatory EMG responses in the ipsilateral and contralateral jaw muscles, (ii) which portion of Vc is most critically involved in producing any such excitatory responses, and (iii) whether neurones or fibres of passage in Vc are responsible for these excitatory responses. Some of these data have been previously presented in abstract form (Tsai et al. 1995, 1996).
METHODS

This study was performed on 33 O₂/N₂O/halothane-anaesthetized adult male Sprague-Dawley rats (250-350 g). Most of the methods have been described in detail previously (Hu et al. 1993; Yu et al. 1994, 1995; also see Chapter 2) and so the following will focus on those methodological aspects not previously described. The trachea and right femoral artery and vein were cannulated for artificial ventilation, blood pressure monitoring and fluid infusion, respectively. All rats were fixed in a stereotaxic apparatus with ear and incisor bars. For rats used in the glutamate micro-injection study, their heads were suspended as previously described (Hu et al. 1993; Yu et al. 1994, 1995; also see Chapter 2). The EMG activity was recorded bilaterally from DIG and MASS with a pair of bipolar needle EMG electrodes in each muscle; electrode locations were confirmed by the increased EMG activity evoked in all four muscles by pressing the TMJ with a blunt glass rod and by dissection at the end of each experiment.

A C1-C2 laminectomy was carried out to expose the caudal brainstem and the spinal cord. Following these surgical procedures, halothane concentration was reduced and maintained at 0.8-1.0% until the end of the experiment. Heart rate, blood pressure and core temperature of the rats were continuously monitored and maintained at normal physiological levels to ensure that any changes in EMG activity of the jaw muscles were not produced by deterioration of the rat's physiological condition. The flexion reflex of the hindlimb induced by noxious pressure applied with serrated forceps to the toes was used as an indication of recovery of the physiological condition from deep anaesthesia.

Electrical Micro-stimulation

Eight rats were used for determination of thresholds and latencies of EMG activities evoked by electrical micro-stimulation at different brainstem loci. A varnish-coated tungsten micro-electrode (0.5-3 MΩ), which was mounted on a micro-
manipulator, was stereotaxically inserted perpendicularly into the left caudal brainstem for delivery of electrical current. Micro-electrode penetrations were made in eight rostro-caudal planes (between 1 and 11.5 mm caudal to the obex, inter-plane distance 1.5 mm), with three medio-lateral penetrations (in lateral Vc, medial Vc, or the reticular formation) in each plane. For each penetration three depths (0.25, 1 and 1.5 mm below the surface, referred to as superficial, intermediate and deep, respectively) were chosen for micro-stimulation. A total of 72 micro-stimulation sites in the left brainstem were studied in each rat. Micro-electrode penetrations were started from the most caudal planes and moved rostrally, from medial to lateral at the same plane, and from superficial layers to deeper layers in the same penetration. EMG activities were displayed on an oscilloscope for determination of the electrical thresholds and latencies of the evoked EMG activities.

Electrical micro-stimulation (single pulse, 200 μs, ≤ 1 mA) was made every 3 s by a pulse stimulator (301-T, WPI) and stimulus isolator (A360, WPI) with increasing intensity until an EMG response was evoked. Threshold was defined as the smallest electrical current producing an EMG response in a muscle in 50 % of the trials. After determination of the EMG threshold, five supra-threshold (2 xT) pulses were given and the shortest latency of the evoked EMG activity was determined; selected EMG traces were photographed from the oscilloscope.

To avoid damaging the neural pathways between Vc and Vmo, no electrolytic lesion was made in the above-mentioned eight rats to mark specific micro-stimulation sites. Instead, micro-stimulation was delivered in a ninth rat at four representative loci selected according to the results from these eight rats and thresholds and latencies of evoked EMG responses determined for each locus; an electrolytic lesion was then produced by passing DC current (8 μA, 15 s) through the micro-electrode to mark each of the four loci.
**Glutamate micro-injection**

In 24 rats, sodium glutamate (0.5 M in saline) was injected (Bereiter 1993) via a micro-pipette (outer diameter: 0.35 mm; inner diameter: 0.12 mm) into the rostral Vc (Vc at the levels 1-2.5 mm caudal to the obex, ROST-Vc rats, n=6), caudal Vc (Vc at the levels 4-5.5 mm caudal to the obex, CAUD-Vc rats, n=6), C2 segment (C2 rats, n=6), or the reticular formation at the obex level (RF rats, n=6). The EMG activity was also recorded from each of the left DIG, left MASS, right DIG and right MASS, as previously described (Hu et al. 1993; Yu et al. 1994, 1995; Tsai et al. in press). The first 20 min of recording in each muscle monitored resting EMG activity and was used to derive the baseline level of EMG activity for that muscle in each rat (see below). Glutamate was injected into the left brainstem or C2 segment at the end of 20 min and the EMG activity was continuously recorded thereafter for another 40 min. The total recording time was 60 min.

The EMG activity was subsequently rectified and integrated into EMG area (bin width: 60 s). Data points of the EMG area for each muscle recorded during the first 20 min in each animal were pooled to produce a mean value which represented the baseline level of the EMG activity for that muscle. Relative changes in EMG area with respect to the baseline level were used to address the effects of glutamate injection into different brainstem loci. All data points were normalized relative to the mean value and expressed as a percentage value of the mean baseline level of the EMG activity.

**Histological Examination**

All rats were sacrificed with an overdose of sodium pentobarbital (100 mg/kg) at the end of each experiment. Those rats receiving electrolytic lesions or glutamate micro-injection were perfused with 200 ml warm saline followed by 150 ml 10% buffered formalin solution. The brainstem was removed after perfusion and post-fixed in 10% buffered formalin solution for one week. The brainstem tissue was cut in 10-µm serial
sections and one of every five sections was selected and stained with cresyl violet. Since glutamate produces neurotoxic effects on central neurones (Choi 1985; Rothman et al. 1987; Sloviter and Dempster 1985), the sites of glutamate micro-injection as well as electrolytic lesions could be determined under a light microscope and reconstructed with the aid of a camera lucida.

**Statistical Analysis**

Repeated Measures ANOVA test was used for ranking the EMG thresholds of the 72 micro-stimulation loci for each muscle and for statistical comparisons (for each muscle, and between muscles) of the thresholds and latencies of EMG responses evoked at selected loci. Fisher's Exact Probability test was used for statistical comparisons of the incidence of the glutamate-evoked increases of EMG activity between the four groups of glutamate-injected rats. The Wilcoxon test was used for statistical comparisons between the mean value representing the baseline level of EMG activity (see above) and any data point of the post-injection periods of each group of rats. The time period of the post-injection data points that were 2 standard deviations above the mean baseline level was designated as the duration of the increased EMG activity evoked by glutamate micro-injection. The Mann-Whitney test was used for statistical comparisons of the mean EMG area for the first-min post-injection and the mean duration of the increased EMG activity between the four groups of glutamate-injected rats. A probability level of less than 0.05 (2-tailed) was regarded as significant. All values are given as mean±SD.
RESULTS

Electrical micro-stimulation

Electrical micro-stimulation at several sites in Vc evoked EMG responses in the ipsilateral DIG and MASS (Fig. 3-1A,B), but no EMG activity could be evoked in the contralateral DIG and MASS even when the stimulation intensity was increased to 4 xT (Fig. 3-1C). Therefore, the following focusses only upon the ipsilateral DIG and MASS data.

The sites associated with the lowest thresholds (114.4±58.0 μA for DIG and 192.5±110.5 μA for MASS) of the evoked EMG responses among the 72 loci were located in the medial portion of the superficial layers in the caudal Vc at the plane 4 or 5.5 mm caudal to the obex; the thresholds increased when micro-stimulation was delivered more rostral or caudal to these two planes (Figs. 3-2). Data recorded from these two planes were used for statistical comparisons of the EMG thresholds of each muscle in different layers of Vc. The thresholds of the EMG responses evoked from the superficial layers in both DIG and MASS were significantly lower than those evoked from the intermediate and deep layers, and DIG had significantly lower thresholds than MASS (Fig. 3-3). The shortest EMG latencies (4.5±1.6 ms for DIG and 5.3±1.6 ms for MASS; evoked at 2 xT) also were associated with responses evoked from loci in the caudal Vc at the plane 4 mm caudal to the obex; the latencies also increased when the micro-stimulation was delivered more rostral or caudal to this plane (Fig. 3-4).
Fig. 3-1 An example of EMG responses evoked by electrical micro-stimulation of the caudal Vc. A and B, EMG responses in the ipsilateral (IPSI) DIG and MASS evoked by 1 and 2 xT intensities, respectively. C, at 4 xT intensity, the amplitudes of the EMG responses were increased and the latencies were reduced in the ipsilateral DIG and MASS; however, no EMG activity was noticed in the contralateral (CONTRA) muscles. D, histological reconstruction of the micro-stimulation site. E, photograph of the micro-stimulation site in D. The arrow indicates the location of the tip of the micro-electrode. Bars below D and E indicate 1 mm.
EXCITATORY EMG RESPONSES EVOKED BY ELECTRICAL MICRO-STIMULATION OF Vc

A
IPSI DIG $T = 260 \, \mu A$

IPSI MASS $T = 280 \, \mu A$

100 $\mu V$
5 ms

B
IPSI DIG $2 \times T$

IPSI MASS

100 $\mu V$
5 ms

C
IPSI DIG $4 \times T$

IPSI MASS

CONTRA DIG

CONTRA MASS

100 $\mu V$
5 ms

D

E
OBEX-6.5
Fig. 3-2 Ranking of the 72 micro-stimulation loci according to their thresholds to evoke EMG responses in the ipsilateral DIG and MASS. The locus with the lowest threshold in each muscle, pointed by the arrow, was ranked as #1 and numbers of the other loci increased as their thresholds increased. Data from those loci in the box in each muscle were pooled for comparisons of the micro-stimulation thresholds in different layers of Vc.
RANKING OF THE 72 MICRO-STIMULATION LOCI

DIG

- OBEX-1
- OBEX-2.5
- OBEX-4
- OBEX-5.5
- OBEX-7
- C2 dorsal root entry zone
- OBEX-8.5
- OBEX-10
- OBEX-11.5

1 mm

MASS

- OBEX-1
- OBEX-2.5
- OBEX-4
- OBEX-5.5
- OBEX-7
- C2 dorsal root entry zone
- OBEX-8.5
- OBEX-10
- OBEX-11.5

1 mm
Fig. 3-3 Statistical comparisons of the mean (±SD) micro-stimulation thresholds in different layers of Vc. *: p<0.05, **: p<0.01. ***: p<0.001. Repeated Measures ANOVA on Ranks test. ###: p<0.001, paired t-test.
STATISTICAL COMPARISONS OF THE EMG THRESHOLDS IN DIGASTRIC AND MASSETER MUSCLES AT DIFFERENT DEPTHS IN Vc

- 0.25 mm below brainstem surface
- 1.0 mm below brainstem surface
- 1.5 mm below brainstem surface

Diagram shows threshold values for DIG and MASS muscles at different depths.
Fig. 3-4 Statistical comparisons of the mean (±SD) shortest latencies of the EMG activities evoked by micro-stimulation at each of the seven rostro-caudal planes in the caudal brainstem. Data from plane OBEX-11.5 were not compared due to missing data. *: p<0.05. **: p<0.01. Repeated Measures ANOVA on Ranks test. ###: p<0.001. paired t-test.
STATISTICAL COMPARISONS OF THE LATENCIES OF THE EVOKED EMG ACTIVITY IN DIGASTRIC AND MASSETER MUSCLES AT DIFFERENT ROSTRO-CAUDAL PLANES

LATENCY (msec)
Glutamate Micro-injection

For all 24 rats receiving glutamate micro-injection into the brainstem or spinal cord, there was no significant variation in EMG activity of any jaw muscle during the initial 20 min-period before glutamate micro-injection, indicating that a stable level of baseline EMG activity was achieved. Glutamate micro-injection into the left caudal Vc (CAUD-Vc rats) evoked a profound increase (compared with baseline EMG level, see above) in EMG activity (post-injection EMG area) of the ipsilateral DIG and MASS muscles in all six rats of this group, a small increase of the contralateral DIG in five rats, and no increase of the contralateral MASS in any of these rats (see Table 3-1). The glutamate-evoked EMG activity in the ipsilateral DIG and MASS of the CAUD-Vc rats had a latency of 4.2±2.8 s, peaked within 1 min, and was reflected in one prolonged phase of increased activity that lasted for 5-20 min (Fig. 3-5). The mean increased EMG activity (first min post-injection EMG area, 556±167% of baseline for ipsilateral DIG; 531±163% for ipsilateral MASS) was significantly greater than the baseline EMG level; the mean changes of EMG activity in the contralateral DIG (172±24%) and MASS (100±1%), however, were not significantly increased (Fig. 3-6).

In contrast, glutamate injection into the rostral Vc (ROST-Vc rats), the C2 segment (C2 rats) or the reticular formation at the obex level (RF rats) evoked a small increased EMG activity in the four muscles of various number of rats in each of these three groups (Table 3-1); the mean change in EMG area, however, was not significant in any muscle of these groups compared with the baseline EMG level, except for the ipsilateral DIG of the C2 rats (Fig. 3-7). The incidence of the increased EMG activity in the ipsilateral DIG, ipsilateral MASS and contralateral DIG of the ROST-Vc rats and in the contralateral DIG of RF rats was significantly lower than that of the CAUD-Vc rats (Table 3-1). The amplitude of the first min post-injection EMG area was significantly smaller and the
<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to glutamate injection</th>
<th>MASS ipsilateral to glutamate injection</th>
<th>DIG contralateral to glutamate injection</th>
<th>MASS contralateral to glutamate injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROST-Vc (n=6)</td>
<td>17% [1]***</td>
<td>17% [1]***</td>
<td>0% [0]***</td>
<td>0% [0]***</td>
</tr>
<tr>
<td>CAUD-Vc (n=6)</td>
<td>100% [6]</td>
<td>100% [6]</td>
<td>83% [5]</td>
<td>0% [0]</td>
</tr>
<tr>
<td>RF (n=6)</td>
<td>83% [5]</td>
<td>67% [4]</td>
<td>17% [1]***</td>
<td>0% [0]</td>
</tr>
</tbody>
</table>

***p<0.001 (Fisher's test) indicates significantly different compared with CAUD-Vc rats.
Fig. 3-5 An example of EMG changes evoked by injection of glutamate into the left caudal Vc. A, EMG traces are expressed in terms of the integrated EMG area for every 60 s. B, the EMG traces recorded from the muscles at 10 min, during glutamate injection (20 min), and at 25, 30 and 50 min. ↓: start glutamate injection; ↑: end injection. C, photograph of the glutamate micro-injection site in the rat from which the EMG traces in A and B were recorded.
Fig. 3-6 A, mean changes in EMG area in the jaw muscles evoked by injection of glutamate into the left caudal Vc. Each data point represents the mean±SE of the normalized values relative to the baseline EMG activity in each rat (in some cases, symbols cover the SE bars), and the horizontal dotted line in each graph indicates the mean baseline EMG activity. "*" indicates that the amplitude of the EMG activity of that data point was significantly higher than the baseline activity (Repeated Measures ANOVA on Ranks test, p<0.05). B, histological reconstruction of the glutamate micro-injection sites of the six rats in this group.
EMG CHANGES OF RATS WITH GLUTAMATE INJECTION INTO THE CAUDAL Vc

Glutamate Injection

Relative changes in EMG activity (x 100%)

Time (min)

IPSI DIG

IPSI MASS

CONTRA DIG

CONTRA MASS

Glu01

Glu03

Glu04

Glu06

Glu08

Glu07
Fig. 3-7 A, mean changes in EMG area in the jaw muscles evoked by injection of glutamate into the left rostral Vc (ROST-Vc), C2 segment (C2) or the reticular formation (RF). B, C and D, histological reconstructions of the glutamate micro-injection sites of rats in these three groups, respectively.
EMG CHANGES OF RATS WITH GLUTAMATE INJECTION INTO THE ROSTRAL Vc, C2 SEGMENT OR RETICULAR FORMATION

A

Glutamate Injection
- C2
- ROST-Vc
- RF
- Baseline EMG level

IPSI DIG

Relative changes in EMG activity

IPSI MASS

CONTRA DIG

CONTRA MASS

Time (min)

-10 0 10 20 30 40 50 60 70
duration of the increased EMG activity was significantly shorter in the ipsilateral DIG and MASS of the ROST-Vc, C2 and RF rats than in the CAUD-Vc rats (Tables 3-II and 3-III), except for the duration of the increased EMG activity in the ipsilateral DIG of the C2 rats which was not significantly different from that of the CAUD-Vc rats (Tables 3-III). The amplitude and duration of the evoked EMG activity in the contralateral DIG and MASS were not significantly different between these three groups.

Histological examination of the glutamate micro-injection sites showed profound neuronal death within a spherical area of 0.5-1.0 mm in diameter. Lesions of Vc in the CAUD-Vc rats were located in deep laminae; neurones in the superficial laminae or in the contralateral Vc seemed unaffected (Figs. 3-5 and 3-6).

**DISCUSSION**

The present study has provided the first documentation that electrical or chemical stimulation of Vc produces excitatory responses in the ipsilateral DIG and MASS. These findings, plus our previous findings that surgical or chemical lesions of Vc reduce the increased EMG activity of the ipsilateral DIG and MASS evoked by injection of the C-fibre excitant and inflammatory irritant mustard oil into the rat TMJ region (Tsai et al. 1994b, 1996; also see Chapters 2 and 4), indicate that Vc may be of critical importance in the neural pathways underlying the reflex excitation of the ipsilateral jaw muscles evoked by noxious TMJ stimulation. The co-activation of DIG and MASS evoked by micro-stimulation of Vc is also consistent with previous findings that noxious TMJ stimulation evokes co-activation of the jaw-opening and jaw-closing muscles (Broton and Sessle 1988; Yu et al. 1994, 1995; Tsai et al. 1994a, b, 1996; also see Chapters 2 and
TABLE 3-II.
MEAN FIRST MINUTE (PEAK) EMG ACTIVITY EVOKED BY GLUTAMATE INJECTION INTO DIFFERENT LOCI IN THE CAUDAL BRAINSTEM

Values representing EMG activity (EMG area for the first min post-injection) are expressed as mean±SD of the normalized value relative to the mean value of the pre-injection EMG activity (=100%, baseline activity) in each group of rats. Values in brackets indicate number of rats in each group.

<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to glutamate injection</th>
<th>MASS ipsilateral to glutamate injection</th>
<th>DIG contralateral to glutamate injection</th>
<th>MASS contralateral to glutamate injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROST-Vc (n=6)</td>
<td>103±2%*</td>
<td>109±10%*</td>
<td>102±1%</td>
<td>100±1%</td>
</tr>
<tr>
<td>CAUD-Vc (n=6)</td>
<td>556±167%</td>
<td>531±163%</td>
<td>172±24%</td>
<td>100±1%</td>
</tr>
<tr>
<td>C2 (n=6)</td>
<td>182±25%*</td>
<td>140±15%*</td>
<td>193±62%</td>
<td>99±2%</td>
</tr>
<tr>
<td>RF (n=6)</td>
<td>122±5%*</td>
<td>114±9%*</td>
<td>103±2%</td>
<td>100±1%</td>
</tr>
</tbody>
</table>

*p<0.05 (Mann-Whitney test) indicates significantly different compared with CAUD-Vc rats.
TABLE 3-III.
MEAN DURATION OF THE INCREASED EMG ACTIVITY EVOKED BY GLUTAMATE INJECTION INTO DIFFERENT LOCI IN THE CAUDAL BRAINSTEM
Values representing duration of the increased EMG activity are expressed as mean±SD. Values in brackets indicate number of rats in each group.

<table>
<thead>
<tr>
<th>Loci</th>
<th>DIG ipsilateral to glutamate injection</th>
<th>MASS ipsilateral to glutamate injection</th>
<th>DIG contralateral to glutamate injection</th>
<th>MASS contralateral to glutamate injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROST-Vc (n=6)</td>
<td>0.8±0.7*</td>
<td>0.7±0.7*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CAUD-Vc (n=6)</td>
<td>9.7±3.0</td>
<td>9.2±2.1</td>
<td>2.3±0.8</td>
<td>0</td>
</tr>
<tr>
<td>C2 (n=6)</td>
<td>6.3±2.4</td>
<td>3.3±1.2*</td>
<td>3.7±6.0</td>
<td>0</td>
</tr>
<tr>
<td>RF (n=6)</td>
<td>0.8±1.1*</td>
<td>2.8±1.4*</td>
<td>0.2±0.4</td>
<td>0</td>
</tr>
</tbody>
</table>

*p<0.05 (Mann-Whitney test) indicates significantly different compared CAUD-Vc rats.
4). Discussion of the limitations and technical features of the study is provided in Chapter 6.

In contrast to our findings with noxious stimulation of the TMJ region, intraoral/perioral stimulation has been shown to evoke reflex activation of the jaw-opening muscles and inhibition of the jaw-closing muscles; the interneuronal pathways involved in these orofacial reflexes are located in rostral brainstem nuclei, e.g. Vp, Vo, Vi, and supratrigeminal nucleus (Funakoshi and Amano 1974; Dallel et al. 1989; Kidokoro et al. 1968a, b; Olsson and Westberg 1991; Sumino 1971; Yoshida et al. 1994, 1995). The distinction between our findings and these earlier observations suggests that craniofacial reflexes may involve different V nuclei depending on the site of stimulation and the nociceptive or non-nociceptive character of the stimuli and inputs, i.e. the orofacial reflexes (especially those evoked by low-intensity intraoral/perioral stimuli) principally involving the more rostrally located V nuclei and the craniofacial nociceptive reflexes (e.g. those evoked by noxious stimuli applied to the TMJ) mainly involving Vc. This possibility is supported by findings that the V tractotomy produces a profound craniofacial analgesia and thermesthesia, with much less complete loss of tactile sensibility and intraoral/perioral nociception such as tooth pain (Broton and Rosenfeld 1985; Kerr 1979; Young 1982; Young et al. 1981), and significantly increases the threshold of the neck and head reflexes/behaviours evoked in animals by intraoral/perioral stimulation but cannot completely eliminate these responses (Dallel et al. 1989; Sumino and Nozaki 1977; Young et al. 1981). These findings also are consistent with documentations that Vc is critically involved in craniofacial nociception (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985) and autonomic reflexes evoked by craniofacial noxious stimulation (Bereiter et al. 1994; Bereiter and Gann 1986, 1988; Panneton 1991; Panneton and Yavari 1995), and
that the rostral V brainstem sensory nuclei are primarily related to intraoral and perioral pain mechanisms (Dalle et al. 1988, 1990; Hayashi et al. 1984; Luccarini et al. 1995; Kerr 1979; Raboisson et al. 1995; Sessle and Greenwood 1976).

The Vc at the levels 4-5.5 mm caudal to the obex, i.e. the caudal Vc, appears to be most critically involved in the excitatory effects because micro-stimulation at these levels could consistently evoke EMG responses in the ipsilateral DIG and MASS with low thresholds and short latencies. This finding is supported by findings that micro-injection of glutamate into the caudal Vc also evoked an increase in EMG activity of the ipsilateral DIG and MASS while glutamate micro-injection into the rostral Vc, the C2 spinal segment or the adjacent reticular formation produced no significant changes in EMG activity of any of the jaw muscles studied (except for the ipsilateral DIG of the C2 rats). Also relevant is earlier documentation that some V primary afferent fibres that project to Vc may contain glutamate (Clements et al. 1991; Clements and Beitz 1991) and glutamate receptors have been identified in some Vc neurones (Dohrn and Beitz 1994; Kondo et al. 1995; Magnusson et al. 1987). In addition, mustard oil injection into the TMJ region induces glutamate release in Vc (Bereiter and Benetti in press 1996) and pre-administration (i.v, i.c.p. or local) of MK-801, a non-competitive glutamate antagonist of the NMDA receptor subtype, significantly reduces the increased EMG activity of the jaw muscles evoked by mustard oil injection into the TMJ region (Yu et al. in press). Furthermore, glutamate iontophoresis in Vc excites Vc nociceptive neurones (Henry et al. 1980; McMahon et al. 1993; Wilcox 1990). These previous observations, plus present findings of excitatory effects of glutamate in Vc, suggest that glutamate receptors, especially of the NMDA subtype, may be involved in craniofacial nociceptive reflex pathways involving Vc.
The effectiveness of glutamate micro-injection into the caudal Vc in evoking EMG activity in the jaw muscles also point to the importance of Vc neurones in the reflex pathways, since glutamate is an excitatory amino acid and neurone excitant (Curtis and Johnson 1974; Johnson 1978; Watkins and Evans 1981). This finding is in agreement with our previous observations that injection into Vc of the neurotoxic agent ibotenic acid, which produces marked neuronal death but spares fibres of passage at the injection site (Aimone et al. 1988; Chiang et al. 1990; Schwarcz et al. 1979), abolishes the increased jaw muscle activity reflexly evoked by mustard oil injection into the TMJ region (Tsai et al. 1994b, 1996; also see Chapters 2 and 4). Nociceptive Vc neurones in particular may be responsible for these excitatory effects on the jaw muscles, because (i) nociceptive Vc neurones but not low-threshold mechanoreceptive Vc neurones respond to injection into the TMJ region of algesic chemicals, including mustard oil (Broton et al. 1988; Hu et al. 1992; also see Chapter 5), and (ii) injection into the TMJ region of the C-fibre excitant mustard oil (but not its vehicle mineral oil) or other algesic chemicals (7% NaCl, KCl and histamine) evokes an increase in EMG activity of the jaw muscles (Broton and Sessle 1988; Yu et al. 1994, 1995; also see Chapters 2 and 4) and also results in the expression of c-fos-like immunoreactivity in Vc (Hathaway et al. 1995). This c-fos-like immunoreactivity is expressed at two loci along the rostro-caudal extent in the ipsilateral caudal brainstem: one at the rostral Vc and the other at the caudal Vc (Hathaway et al. 1995). However, our present study has shown that electrical micro-stimulation or glutamate micro-injection in the latter region produces a marked excitatory EMG response in the ipsilateral DIG and MASS while comparable stimulation of the former region produces no EMG response in the same muscles. Recent evidence has shown that autonomic reflexes evoked by various craniofacial stimuli may be mediated via the rostral Vc (Bereiter and Gann 1986; Bereiter et al. 1994; Panneton and
Yavari 1995) and that micro-injection of glutamate or substance P into the rostral Vc evokes autonomic responses (Bereiter and Gann 1988, 1989). These findings thus suggest that neurones in the rostral Vc may be more involved in autonomic reflexes while neurones in the caudal Vc are more important for jaw muscle reflexes.

Our previous studies have shown that mustard oil injection into the TMJ region evokes an increase in EMG activity of the contralateral jaw muscles (Tsai et al. 1994a, b; Yu et al. 1994, 1995; also see Chapters 2 and 4). This increased EMG activity can be significantly reduced by Vc lesions ipsilateral or contralateral to the mustard oil injection (Chapters 2 and 4), indicating that intact left and right Vc appear to be essential for the reflex activation of the contralateral DIG and MASS evoked by noxious TMJ stimulation. Our present findings have however shown that glutamate or electrical micro-stimulation of Vc does not evoke any significant increase in EMG activity of the contralateral DIG and MASS. There are several possible explanations for the difference between these two sets of observations. Firstly, electrical micro-stimulation or glutamate micro-injection may not have excited a sufficient number of Vc neurones to evoke contralateral muscle activity compared to the number of Vc neurones activated by mustard oil injection into the TMJ region: this possibility is supported by the extensive area of c-fos-like immunoreactivity in Vc induced by mustard oil injection into the TMJ region (Hathaway et al. 1995) and by the small size of the glutamate lesions in the present study. Secondly, the electrical or glutamate micro-stimulation may have activated a different population of Vc neurones (e.g. receiving convergent afferent inputs from non-TMJ as well as TMJ tissues) resulting in excitation of ipsilateral V motoneurones and inhibition of contralateral V motoneurones whereas the TMJ application of mustard oil may have activated a different population of Vc neurones producing predominantly bilateral excitatory effects. Thirdly, at least in terms of the
difference between glutamate and mustard oil-evoked effects, the mustard oil injection may conceivably have resulted in release in Vc of several other neurotransmitters/neuromodulators as well as glutamate (e.g. Dougherty and Willis 1991; Henry et al. 1977, 1980) and so have more extensive effects than glutamate alone.

The latency values of the EMG activity in ipsilateral DIG and MASS evoked by micro-stimulation in the caudal Vc suggest that multi-synaptic or slowly-conducting mono-synaptic pathways may be involved in excitation of V motoneurones by Vc stimulation. The possibility of directly projecting neural pathways from Vc neurones to V motoneurones is supported by findings that Vc neurones can be retrogradely labelled by injection of horseradish peroxidase into the Vmo (Li et al. 1993, 1995; Mizuno et al. 1983) and degenerated axons can be seen in Vmo after lesions of Vc (Dunn and Matzke 1968; Roberts and Matzke 1971; Stewart and King 1963). On the other hand, the possibility that there exist multi-synaptic connections between Vc neurones and V motoneurones is supported by findings that Vc neurones project to Vp, Vo and Vi (Dunn and Matzke 1968; Hockfield and Gobel 1978; Hu et al. 1981; Jacquin et al. 1990b; Nasution and Shigenaga 1987; Roberts and Matzke 1971; Stewart and King 1963) and neurones in Vp, Vo and Vi project to Vmo (Fort et al. 1990; Mizuno et al. 1983; Yoshida et al. 1994). Our present study did not rule out the possibility that the Vc projection to Vp, Vo and Vi involves a facilitatory influence on the nociceptive reflex pathways that are mediated through more rostral brainstem structures. This possibility is supported by findings that the excitability of V primary afferent terminals in Vp and Vo (Dostrovsky et al. 1981; Young and King 1972) and the mechanoreceptive field and response properties of Vp, Vo and Vi neurones (Greenwood and Sessle 1976; Hallas and Jacquin 1990) can be modulated by transection or cold block of Vc.
CHAPTER 4. INVOLVEMENT OF NEURONES IN THE CAUDAL SUBNUCLEUS CAUDALIS (Vc) IN CRANIOFACIAL NOCICEPTIVE REFLEX ACTIVITY

INTRODUCTION

Interneuronal pathways involved in low-threshold jaw reflexes evoked by low-intensity stimulation of orofacial tissues are located in the rostral V brainstem sensory nuclear complex or adjacent nuclei, e.g. Vp, Vo or Vi, or supratrigeminal nucleus (Funakoshi and Amano 1974; Kidokoro et al. 1968a, b; Olsson and Westberg 1991; Shigenaga et al. 1988b; Sumino 1971; Yoshida et al. 1994, 1995). We have, however, recently shown that injection into the rat’s TMJ region of the small-fibre excitant and inflammatory irritant mustard oil (Handwerker and Reeh 1991; Haas et al. 1992; Jancso et al. 1967; Woolf and Wall 1986) reflexly evokes an increase in EMG activity of both jaw-opening (e.g. DIG) and jaw-closing (e.g. MASS) muscles (Tsai et al. 1994 a, b; Yu et al. 1994, 1995; also see Chapter 2) and that this nociceptive reflex can be significantly reduced by interruption of Vc (Chapter 2). The evoked EMG activity can, for example, be reduced by transection of the brainstem at the junction of Vi and Vc which may interrupt inputs to and outputs from Vc, and by extensive injections into Vc of the excitatory neurotoxic agent ibotenic acid which destroys neurones but spares fibres of passage at the injection site (Aimone et al. 1988; Chiang et al. 1990; Schwarcz et al. 1979). The caudal part of Vc may be especially involved since electrical micro-stimulation or micro-injection of the excitatory amino acid and neurone excitant glutamate into the caudal Vc evokes EMG activity in the ipsilateral, but not contralateral, DIG and MASS (Chapter 3). To test further the possible involvement of the caudal Vc in
the nociceptive reflex pathways, the present study was initiated in order to determine the effects of micro-injection of ibotenic acid specifically into the caudal Vc on the EMG activity in DIG and MASS evoked by injection of mustard oil into the TMJ region. Some of these data have been previously presented in abstract form (Tsai et al. 1996).

**METHODS**

This study was performed on 34 O<sub>2</sub>/N<sub>2</sub>O/halothane-anaesthetized adult male Sprague-Dawley rats (250-350 g). Most of the methods have been previously described in detail (Chapter 2 and 3) and so the following will focus on those methodological aspects not previously described. The trachea and right femoral artery and vein were cannulated. All rats were fixed in a stereotaxic apparatus with ear and incisor bars; a C1-C2 laminectomy was carried out to expose the caudal brainstem and the spinal cord.

Ibotenic acid micro-injection (0.03 M in phosphate-buffered saline, 20 nl, 1 nl/10 s) was made, via a micro-pipette (outer diameter: 0.35 mm; inner diameter: 0.12 mm) attached to a 1-μl Hamilton syringe, into the left caudal Vc of 14 rats (CAUD-Vc rats) at either one of two sites (1.5 mm lateral to the midline and a depth of 1 mm at a level 4 mm caudal to the obex, i.e. obex-4, n=3; or 1 mm lateral to the midline and a depth of 1 mm, at obex-5.5 mm, n=3) or at both sites (n=8). Ibotenic acid was also micro-injected into the left rostral Vc of six rats (ROST-Vc rats) at two sites (2.5 mm lateral to the midline and a depth of 1.5 mm, at obex-1 mm; and 2 mm lateral to the midline and a depth 1.5 mm, at obex-2.5 mm), into the left C2 segment of seven rats (C2 rats) at two sites (0.8 mm lateral to the midline and a depth of 1 mm, at obex-8.5 mm; and 0.5 mm lateral to the midline and a depth of 1 mm, at obex-10.5 mm), and into the reticular formation in the left caudal brainstem of seven rats (RF rats) at two sites (1 mm lateral to the midline and a depth of
1.5 mm, at obex-1 mm; and 1 mm lateral to the midline and a depth of 1.5 mm, at obex-2.5 mm).

Following these procedures, the surgical wound was sutured, the ear and incisor bars were removed, and the rat's head was suspended as previously described (Hu et al. 1993; Yu et al. 1994, 1995; also see chapter 2 and 3). A needle was implanted into the TMJ region for injection of mustard oil (20% in mineral oil; 20 µL). Halothane concentration was reduced and maintained at 0.8-1.0% until the end of the experiment. The flexion reflex of the hindlimb induced by noxious pressure, heart rate, blood pressure and core temperature were used to monitor the physiological condition of the rat. If the physiological condition failed to return to the physiological level (heart rate, 330-430 beats/min; blood pressure, 80-130 mmHg; core temperature, 36.3-38°C) after the surgical preparation and brainstem lesion, the rat was sacrificed and any data obtained from the rat were discarded.

**EMG Recording**

The EMG activity was recorded bilaterally from DIG and MASS with a pair of bipolar needle EMG electrodes in each muscle; electrode locations were confirmed by the increased EMG activity evoked by application of a blunt glass rod to the TMJ and by dissection at the end of the experiment. The EMG activity of each muscle was amplified and recorded as previously described (Hu et al. 1993; Yu et al. 1994, 1995; also see chapter 2 and 3). The first 20 min of recording in each muscle monitored resting EMG activity and was used to derive the baseline level of EMG activity for that muscle in each rat (see below). In the ROST-Vc, RF and C2 rats, mustard oil was injected into the left TMJ region at the beginning of the twenty-first min and the EMG activity was continuously recorded thereafter for another 40 min; the total recording time was 60 min. In the CAUD-Vc rats, after an initial 20-min EMG recording period, mustard oil
injection was first made into the left TMJ region and EMG activity was then continuously recorded for another 40 min, after which time (at the beginning of the sixty-first min) a second mustard oil injection was made into the right TMJ region and the EMG activity was again continuously recorded for the other 40 min; the total recording time was 100 min.

The EMG activity was subsequently rectified and integrated into EMG area (bin width: 1 min). The mean value of the first 20-min data points for each muscle in each animal represented the baseline level of the EMG activity for that muscle. Relative changes with respect to the baseline level were used to address the effects of mustard oil injection into the TMJ region; all data points were normalized and expressed as a percentage value relative to the mean baseline EMG level (100%).

**Histological Examination**

Evans blue dye (10 mg/kg) was injected intravenously into the rat ten min before the rat was sacrificed. The rat was then perfused with saline followed by 150 ml 10% buffered formalin solution. The brainstem was post-fixed in 10% buffered formalin solution for one week and then cut in 10-μm serial sections; one of every five sections was selected and stained with cresyl violet. The lesion sites were examined under a light microscope and reconstructed with the aid of a camera lucida. The location of the mustard oil injection indicated by plasma extravasation of Evans blue was confirmed by postmortem dissection of the TMJ region. If the plasma extravasation did not include the TMJ capsule, data recorded from the rat were discarded.

**Statistical Analysis**

Data of normal (INTACT) rats from our previous study (Chapter 2) are cited and used in this paper as control data. Fisher's Exact Probability test was used for statistical comparisons of the incidence of the mustard oil-evoked increases of EMG activity
between the INTACT rats and the other groups. The Wilcoxon test was used for statistical comparisons between the mean value representing the baseline level of EMG activity (see above) and any data point of the post-injection periods of each group of rats. The time period of the post-injection data points that were 2 SD above the mean baseline level was designated as the duration of the increased EMG activity evoked by injection of mustard oil into the TMJ region. The Mann-Whitney test was used for statistical comparisons of the mean EMG area for the first min post-injection and the mean duration of the increased EMG activity between different groups of rats; the paired t-test was used for statistical comparisons of the increased EMG activity evoked by the two mustard oil injections within the CAUD-Vc rats. A probability level of less than 0.05 (2-tailed) was regarded as significant; all values are given as mean±SD.

RESULTS

Ibotenic acid produced signs of neuronal death and microglial infiltration in a spherical area (0.5-1.0 mm diameter) at the sites of micro-injection; no signs of cellular destruction were observed in the non-injected side of the brainstem (Fig. 4-1). In agreement with our earlier findings (Haas et al. 1992; Yu et al. 1995; also see Chapter 2), plasma extravasation of Evans blue dye could be seen in all animals in the TMJ capsule and periauricular tissues lateral to the TMJ injected with mustard oil; no such plasma extravasation could be found in tissues surrounding the TMJ that received no mustard oil injection. There was no significant variation in EMG activity of any jaw muscle during the initial 20 min-period before injection of mustard oil into the TMJ region, indicating that a stable level of baseline EMG activity was achieved.
Fig. 4-1  An example of EMG changes evoked by injection of mustard oil into the TMJ region of a CAUD-Vc rat. A: EMG traces are expressed in terms of the integrated EMG area for every 60 sec. B: dotted area shows the location of extravasated dye. The actual EMG traces recorded from the left digastric (L DIG), the left masseter (L MASS), the right masseter (R MASS) and the right digastric (R DIG) are shown in C, at 10 min, at 20 min when mustard oil was injected into the left TMJ region, at 60 min when mustard oil was injected into the right TMJ region, and at 70 min. ↓, start mustard oil injection; ↑, end injection.
CAUD-Vc Rats

The EMG activity of the three CAUD-Vc rats receiving one ibotenic micro-injection in the caudal Vc at the obex-4 mm level was not significantly different from that of the other three CAUD-Vc rats receiving one ibotenic acid micro-injection at the obex-5.5 mm level; the EMG activity of these six CAUD-Vc rats was not significantly different from that of the eight CAUD-Vc rats receiving two ibotenic acid micro-injections at obex-4 and obex-5.5 mm levels. Therefore, data of these 14 rats were pooled for further statistical analysis.

Mustard oil injection into the left TMJ region (CAUD-Vc-L rats) evoked a small increase in EMG activity of the ipsilateral DIG and ipsilateral MASS in six of the 14 rats, of the contralateral DIG in three rats, and of the contralateral MASS in two rats. The incidence of the increased EMG activity in all four muscles of the CAUD-Vc-L rats was significantly lower than that of the INTACT rats (Table 4-I); the EMG activity was also significantly smaller in amplitude and significantly shorter in duration than in the INTACT rats (Tables 4-II and 4-III).

In contrast, mustard oil injection into the right TMJ region (CAUD-Vc-R rats) evoked an increase in EMG activity of the ipsilateral DIG, ipsilateral MASS and contralateral DIG in all 14 rats as well as of the contralateral MASS in seven of these rats (Fig. 4-2). The incidence of this increased EMG activity was not significantly different from that of the INTACT rats but was significantly higher than that of the CAUD-Vc-L rats, except that in the contralateral MASS which was significantly lower than that of the INTACT rats and was not significantly different from that of the CAUD-Vc-L rats (Table 4-I). The evoked EMG activity in the ipsilateral DIG and MASS of the CAUD-Vc-R rats was not significantly different from that of the INTACT rats, but it was significantly lower in amplitude and significantly shorter in duration in the contralateral
TABLE 4-1.
INCIDENCE OF INCREASED EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION

Values in round brackets, ( ), represent number of rats in each group; values in square brackets, [ ], indicate number of rats having a significant increase of EMG activity (EMG area that rose 2 SD above the mean baseline level) in each muscle. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region); dashed line separates data of mustard oil injection into the left or right TMJ region in the same group of rats.

<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUD-Vc-L (n=14)</td>
<td>43% [6]††</td>
<td>43% [6]††</td>
<td>21% [3]†††</td>
<td>14% [2]†††</td>
</tr>
<tr>
<td>CAUD-Vc-R</td>
<td>100% [14]###</td>
<td>100% [14]###</td>
<td>100% [14]###</td>
<td>50% [7]†</td>
</tr>
<tr>
<td>ROST-Vc (n=6)</td>
<td>100% [6]§</td>
<td>100% [6]§</td>
<td>100% [6]‡</td>
<td>100% [6]***</td>
</tr>
<tr>
<td>C2 (n=7)</td>
<td>100% [7]§</td>
<td>100% [7]§</td>
<td>71% [5]§</td>
<td>57% [4]</td>
</tr>
<tr>
<td>RF (n=4)</td>
<td>100% [4]†</td>
<td>100% [4]†</td>
<td>75% [3]†</td>
<td>25% [1]†</td>
</tr>
<tr>
<td>INTACT (n=8) ‡</td>
<td>100% [8]</td>
<td>100% [8]</td>
<td>100% [8]</td>
<td>100% [8]</td>
</tr>
</tbody>
</table>

*p<0.05 and **p<0.01 (Fisher's test) indicate significantly different compared with CAUD-Vc-L rats. # p<0.05 and ## p<0.01 (Fisher's test) indicate significantly different compared with the incidence of increased EMG activity evoked by mustard oil injection into the left TMJ region of the same group of rats. †p<0.05, ††p<0.01 and †††p<0.001 (Fisher's test) indicate significantly different compared with INTACT rats.
‡ Referred to data reported in Chapter 2.
TABLE 4-11.
MEAN FIRST MINUTE (PEAK) EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION

Values representing EMG activity (EMG area for the first min post-injection) are expressed as mean±SD of the normalized value relative to the mean value of the pre-injection EMG activity (=100%, baseline activity) in each group of rats. Values in brackets indicate number of rats in each group. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region).

<table>
<thead>
<tr>
<th>Group</th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUD-Vc-L (n=14)</td>
<td>163±104%††††</td>
<td>124±60%††††</td>
<td>134±80%†††</td>
<td>106±23%††††</td>
</tr>
<tr>
<td>CAUD-Vc-R</td>
<td>779±610%###</td>
<td>352±211%###</td>
<td>187±102%†††</td>
<td>126±39%††††</td>
</tr>
<tr>
<td>ROST-Vc (n=5)</td>
<td>616±340%**</td>
<td>634±331%***</td>
<td>297±199%**,†</td>
<td>139±52%**,†</td>
</tr>
<tr>
<td>C2 (n=7)</td>
<td>529±278%***</td>
<td>365±227%**</td>
<td>687±819%**</td>
<td>172±133%*</td>
</tr>
<tr>
<td>RF (n=4)</td>
<td>676±266%**</td>
<td>514±207%**</td>
<td>765±590%</td>
<td>102±4%</td>
</tr>
<tr>
<td>INTACT (n=8)‡</td>
<td>570±104%†</td>
<td>471±94%</td>
<td>731±181%</td>
<td>217±38%‡</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 and ***p<0.001 (Mann-Whitney test) indicate significantly different compared with CAUD-Vc-L rats. †p<0.05, ††p<0.01 and †††p<0.001 (Mann-Whitney test) indicate significantly different compared with INTACT rats.

#p<0.05 (Paired t-test) indicates significantly different compared with the EMG activity evoked by mustard oil injection into the left TMJ of the same group of rats.

‡ Referred to data reported in Chapter 2.
### TABLE 4-III.
**MEAN DURATION OF THE INCREASED EMG ACTIVITY EVOKED BY MUSTARD OIL INJECTION INTO THE TMJ REGION**

Values representing duration of the increased EMG activity are expressed as mean±SD. Values in brackets indicate number of rats in each group. Unless indicated otherwise, mustard oil was injected into the left (L) TMJ region (R: right TMJ region).

<table>
<thead>
<tr>
<th></th>
<th>DIG ipsilateral to mustard oil injection</th>
<th>MASS ipsilateral to mustard oil injection</th>
<th>DIG contralateral to mustard oil injection</th>
<th>MASS contralateral to mustard oil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAUD-Vc-L (n=14)</td>
<td>3.1±4.6††</td>
<td>1.6±3.7†††</td>
<td>2.4±6.5†††</td>
<td>0.5±1.3†††</td>
</tr>
<tr>
<td>CAUD-Vc-R</td>
<td>13.1±11.3#</td>
<td>11.2±9.7##</td>
<td>5.4±5.4##, ††</td>
<td>2.4±2.9##, ††</td>
</tr>
<tr>
<td>ROST-Vc (n=6)</td>
<td>11.5±5.9*</td>
<td>15.0±13.2**</td>
<td>11.2±11.8**</td>
<td>10.0±14.9**</td>
</tr>
<tr>
<td>C2 (n=7)</td>
<td>19.0±9.7**</td>
<td>13.6±9.0**</td>
<td>9.4±12.5</td>
<td>4.7±6.8†</td>
</tr>
<tr>
<td>RF (n=7)</td>
<td>23.3±15.4*</td>
<td>23.0±15.6**</td>
<td>15.8±16.0</td>
<td>0.3±0.5††</td>
</tr>
<tr>
<td>INTACT (n=8) ‡</td>
<td>25.9±14.9</td>
<td>15.8±5.3</td>
<td>17.9±11.4</td>
<td>19.4±16.6</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 and *** p<0.001 (Mann-Whitney test) indicate significantly different compared with CAUD-Vc-L rats. † p<0.05, †† p<0.01 and ††† p<0.001 (Mann-Whitney test) indicate significantly different compared with INTACT rats.

# p<0.05 (Paired t-test) indicates significantly different compared with the EMG activity evoked by mustard oil injection into the left TMJ of the same group of rats.

‡ Referred to data reported in Chapter 2.
Fig. 4-2 The EMG activity of CAUD-Vc rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left and then right TMJ region CAUD-Vc rats. Each data point represents the mean±SD of the normalized values relative to the baseline EMG activity in each rat (in some cases, symbols cover the SD bars), and the horizontal dotted line in each graph indicates the mean baseline EMG activity. "*" indicates that the amplitude of the EMG activity of that data point was significantly higher than the baseline EMG activity (Wilcoxon test, p<0.05). Histological reconstructions of the lesions in each of the 14 CAUD-Vc rats are shown in B.
EMG CHANGES OF RATS WITH IBOTENIC ACID INJECTION INTO THE LEFT CAUDAL VC

Mustard oil (L TMJ)  Mustard oil (R TMJ)

Relative Changes in EMG activity (%)

Time (min)

L DIG

L MASS

R DIG

R MASS

SAMI 03  SAMI 04  SAMI 05  SAMI 06
SAMI 14  SAMI 15  SAMI 16  SAMI 18
SAMI 35  SAMI 36  SAMI 40
SAMI 38  SAMI 39  SAMI 41
DIG and MASS; it was also significantly greater in the ipsilateral DIG, ipsilateral MASS and contralateral DIG and significantly longer in duration in all four muscles than in the CAUD-Vc-L rats (Tables 4-II and 4-III).

**ROST-Vc, C2 and RF Rats**

Mustard oil injection into the left TMJ region evoked a significant increase in EMG activity of all four muscles in the ROST-Vc and C2 rats (Figs. 4-3A, 4-4A). The incidence of the increased EMG activity in all four muscles of ROST-Vc and C2 rats was not significantly different from that of the INTACT rats and was significantly higher than that of the CAUD-Vc-L rats, except for the contralateral MASS of the C2 rats (Table 4-I). The amplitude and duration of the evoked EMG activity in the ipsilateral DIG and MASS of these two groups were not significantly different from those of the INTACT rats but were significantly greater than in the CAUD-Vc-L rats (Tables 4-II and 4-III). In the contralateral DIG and MASS the amplitude and duration were not significantly different from those in the INTACT rats, except for both muscles of the ROST-Vc rats the amplitude was significantly smaller and for the contralateral MASS of the C2 rats the duration was significantly shorter; they were significantly greater and significantly longer than those in the CAUD-Vc-L rats, except for the duration in both muscles of the C2 rats (Table 4-II and 4-III).

Of the seven RF rats, three showed deterioration of their physiological condition and no sign of recovery within 30 min after the ibotenic acid micro-injection; therefore, their EMG data were discarded. Mustard oil injection into the left TMJ region of the remaining four RF rats evoked a significant increase in EMG activity of the ipsilateral (but not the contralateral) DIG and MASS (Fig. 4-5A). The incidence of the increased EMG activity in all four muscles of the RF rats was not significantly different from that
Fig. 4-3 The EMG activity of ROST-Vc rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, histological reconstructions of the lesions in each of the six ROST-Vc rats.
EMG CHANGES OF RATS WITH IBOTENIC ACID INJECTION INTO THE LEFT ROSTRAL Vc

Mustard oil (L TMJ)

Relative changes in EMG activity (x 100%)

Time (min)

L DIG

L MASS

R DIG

R MASS

IAML02

IAML09

IAML10

IAML17

IAML18

IAML24
Fig. 4-4 The EMG activity of C2 rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, histological reconstructions of the lesions in each of the seven C2 rats.
EMG CHANGES OF RATS WITH IBOTENIC ACID INJECTION INTO THE LEFT C2 SEGMENT

<table>
<thead>
<tr>
<th></th>
<th>L DIG</th>
<th>L MASS</th>
<th>R DIG</th>
<th>R MASS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Relative change in EMG activity (%)</td>
<td>x 100%</td>
<td>x 100%</td>
<td>x 100%</td>
<td>x 100%</td>
</tr>
</tbody>
</table>

Mustard oil (L ml/L)

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
Fig. 4-5. The EMG activity of RF rats. A, mean changes in EMG area in the jaw muscles evoked by injection of mustard oil into the left TMJ region. B, histological reconstructions of the lesions in each of the four survived RF rats.
EMG CHANGES OF RATS WITH IBOTENIC ACID INJECTION INTO THE LEFT RETICULAR FORMATION

L DIG
L MASS
R DIG
R MASS

Relative changes in EMG activity (x 100%)
of the INTACT and CAUD-Vc-L rats, except for the contralateral MASS which was significantly lower than that of INTACT rats (Table 4-I). The amplitude and duration of the evoked EMG activity in all four muscles of the RF rats was not significantly different from that in the INTACT rats, except for the contralateral MASS the duration was significantly shorter; they were significantly greater in the ipsilateral (but not the contralateral) DIG and MASS of the RF rats than in the CAUD-Vc-L rats (Tables 4-II and 4-III).

The incidence, amplitude and duration of the increased EMG activity in all four muscles of the ROST-Vc,C2 and RF rats were not significantly different from that of the CAUD-Vc-R rats and were not significantly different between these three groups.

DISCUSSION

The present findings are consistent with our earlier observations that extensive surgical or ibotenic acid lesions of Vc significantly reduce the bilateral DIG and MASS activity reflexly evoked by injection into the TMJ region of the small-fibre excitant and inflammatory irritant mustard oil (Chapter 2). The findings have further extended these earlier observations by showing that more localized ibotenic acid lesions, specifically of the caudal component of Vc, are effective in significantly reducing the mustard oil-evoked EMG activity of both ipsilateral and contralateral DIG and MASS while ibotenic acid lesions of the rostral Vc produce a significant reduction in EMG activity of only the contralateral (but not ipsilateral) DIG and MASS; ibotenic acid lesions of the C2 segment or reticular formation at the obex level do not produce any significant change of this bilateral EMG response. The reduction of the mustard oil-evoked EMG activity as a result of ibotenic acid-induced lesions of the caudal Vc is unlikely to reflect deterioration of the physiological conditions of the rats because (i) mustard oil injection into the TMJ
region of the CAUD-Vc-R, ROST-Vc or C2 rats still readily evoked EMG activity in the jaw muscles, (ii) heart rate, expired percent CO₂, blood pressure and core temperature were maintained within normal physiological ranges, and (iii) noxious pressure applied to the toes still induced a flexion reflex of the hindlimb after brainstem lesions. The present findings have also indicated that neurones (as opposed to fibres of passage), particularly in the caudal Vc, may be critical elements for the reflex EMG activity evoked in the jaw muscles. This conclusion is supported by our recent findings that neurones in the caudal Vc can be activated by mustard oil injection into the rat TMJ region (Chapter 5) and that glutamate micro-injection in the caudal Vc (but not the rostral Vc, the C2 segment or the adjacent reticular formation) produces profound excitatory EMG responses in the ipsilateral DIG and MASS (Chapter 3). Discussion of the limitations and technical features of the study is provided in Chapter 6.

In the present study, one ibotenic acid micro-injection in the caudal Vc at the obex-4 or obex-5.5 mm level produced a significant reduction of the mustard oil-evoked EMG activity in the jaw muscles; the effect of a single micro-injection was as effective as (and not significantly different from) that produced by two ibotenic acid micro-injections at both loci. This finding indicates that partial damage to the caudal Vc may produce a profound interruption to the craniofacial nociceptive reflex pathways. Rats receiving ibotenic acid lesions in the left caudal Vc showed a highly significant (p<0.001) reduction in the amplitude and duration of the EMG activity of the right DIG and MASS evoked by mustard oil injection into the left TMJ region (CAUD-Vc-L rats); a small (but still significantly increased) EMG activity was evoked in the left DIG and MASS of the same rats by mustard oil injection into the right TMJ region (CAUD-Vc-R rats) which was also significantly smaller (p<0.01) in amplitude and significantly shorter (p<0.01) in duration compared with that of normal rats. Rats receiving ibotenic acid
lesions in the rostral Vc also showed a less significant (p<0.05) reduction in amplitude (but not duration) of the evoked EMG activity of the contralateral jaw muscles. These findings thus indicate that the rostral and especially the caudal components of Vc may be important for the relay of nociceptive TMJ inputs to contralateral V motoneurones and that these TMJ inputs may be relayed via ipsilateral Vc neurones to contralateral Vc neurones (Hockfield and Gobel 1982; Jacquin et al. 1990 a, b) and then to contralateral V motoneurones. In view of previous observations that mustard oil injection into the the TMJ region of normal rats induces a bilateral expression of c-fos-like immunoreactivity in rostral and caudal Vc (Hathaway et al. 1995) and evokes a bilateral increase in EMG activity of DIG and MASS (Yu et al. 1994, 1995; also see Chapter 2), these findings suggest that the mustard oil injection may produce excitation of a substantial number of Vc neurones which results in the reflex activation of the jaw muscles. Together with our earlier observations that glutamate or electrical micro-stimulation of the rostral or caudal Vc could not produce any excitatory EMG response in the contralateral DIG and MASS, the findings also suggest that reflex activation of contralateral V motoneurones may depend upon a summation of inputs from many neurones in the rostral and especially the caudal components of Vc.

Intraoral/perioral stimulation has been shown to evoke reflex activation of the jaw-opening muscles and inhibition of the jaw-closing muscles; interneurones involved in these orofacial reflexes are located in rostral brainstem nuclei, e.g. Vp, Vo, Vi, and supratrigeminal nucleus (Funakoshi and Amano 1974; Dallel et al. 1989; Kidokoro et al. 1968a, b; Olsson and Westberg 1991; Shigenaga et al. 1988b; Sumino 1971; Yoshida et al 1994, 1995). Although the V tractotomy produces a profound craniofacial analgesia and thermanalgesia and significantly increases the threshold of the neck and head reflexes/behaviours evoked in animals by intraoral/perioral stimulation but cannot
completely eliminate these responses (Dallel et al. 1989; Sumino and Nozaki 1977; Young et al. 1981), this procedure results in much less complete loss of tactile sensibility and intraoral/perioral nociception such as tooth pain (Broton and Rosenfeld 1985; Kerr 1979; Young 1982; Young et al. 1981) and increases the threshold of the neck and head reflexes/behaviours evoked by intraoral/perioral stimulation but cannot completely eliminate these responses (Dallel et al. 1989; Sumino and Nozaki 1977; Young et al. 1981). However, Vc has traditionally been viewed as the essential relay site of nociceptive information from superficial and deep craniofacial tissues to higher levels of the CNS (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985). Indeed, Vc receives projections of primary afferent fibres innervating deep tissues including the TMJ (Capra 1987; Nishimori et al. 1986; Shigenaga et al. 1988a) and injection of mustard oil into the TMJ region of rats induces c-fos-like immunoreactivity in Vc (Hathaway et al. 1995). Many Vc nociceptive neurones, classified according to their cutaneous mechanoreceptive field properties as WDR or NS neurones, also can be activated by convergent primary afferent inputs from deep craniofacial tissues including the TMJ (Amano et al. 1986; Broton et al. 1988; Hu 1990; Hu et al. 1981, 1992; Kojima 1990; Sessle et al. 1986; Yu et al. 1993; also see Chapter 5); some Vc neurones may project to Vmo (Dunn and Matzke 1968; Li et al. 1993, 1995; Mizuno et al. 1983; Roberts and Matzke 1971; Stewart and King 1963). The present series of investigations (Chapters 2, 3 and 5) have added further evidence to support the involvement of Vc, especially its caudal component, in the central neural pathways of craniofacial nociceptive reflexes. We can now suggest some possible central pathways that may be involved in nociceptive reflexes evoked by noxious stimulation of the TMJ (Figs. 4-6 and 4-7).
Fig. 4-6 Some possible neural pathways that may be involved in reflex activation of the ipsilateral DIG and MASS evoked by injection of mustard oil into the TMJ region. RF, reticular formation; Vc, trigeminal (V) subnucleus caudalis; Vi, V subnucleus interpolaris; Vmo, V motor nucleus; Vo, V subnucleus oralis; Vp, V nucleus principalis. "X" indicates that involvement of the particular pathway in TMJ-evoked nociceptive reflexes may be ruled out by our studies.
POSSIBLE CENTRAL NEURAL PATHWAYS FOR REFLEX ACTIVATION OF THE IPSILATERAL JAW MUSCLES

(i)

(ii)

(iii)
Fig. 4-7 Some possible neural pathways that may be involved in reflex activation of the contralateral DIG and MASS evoked by injection of mustard oil into the TMJ region. "X" indicates that involvement of the particular pathway in TMJ-evoked nociceptive reflexes may be ruled out by our studies.
POSSIBLE CENTRAL NEURAL PATHWAYS FOR REFLEX ACTIVATION OF THE CONTRALATERAL JAW MUSCLES

(a)  
(b)  
(c)  
(d)  
(e)
Based on the current literature and our recent findings, TMJ-induced reflex activation of the ipsilateral jaw muscles may conceivably involve nociceptive TMJ afferent inputs relayed (i) via ipsilateral caudal Vc neurones which project directly to ipsilateral V motoneurones (Dunn and Matzke 1968; Li et al. 1993, 1995; Mizuno et al. 1983; Roberts and Matzke 1971; Stewart and King 1963), (ii) via ipsilateral caudal Vc neurones through ipsilateral rostral brainstem interneurones (Hockfield and Gobel 1978; Hu et al. 1981; Ikeda et al. 1982, 1984; Jacquin et al. 1990a; Nasution et al. 1987) and then to ipsilateral V motoneurones, or (iii) via ipsilateral rostral brainstem interneurones which project to ipsilateral V motoneurones (Fig. 4-6). For activation of the contralateral jaw muscles, TMJ afferent inputs may be relayed (a) via ipsilateral rostral/caudal Vc neurones through contralateral Vc neurones and then to contralateral V motoneurones. (b) via ipsilateral rostral/caudal Vc neurones which project directly to contralateral V motoneurones (Li et al. 1993; Mizuno et al. 1983), (c) via ipsilateral Vc neurones through ipsilateral rostral brainstem interneurones then to contralateral V motoneurones (Donga et al. 1990; Mizuno et al. 1983; Rokx et al. 1986; Ter Horst et al. 1990), (d) via direct primary afferent projections to contralateral Vc neurones (Clarke and Bowsher 1962; Gobel and Binck 1977; Jacquin et al. 1982; Marfurt 1981; Nord and Rolince 1980; Torvik 1956) and then to contralateral V motoneurones, or (e) via ipsilateral rostral brainstem interneurones which project to contralateral V motoneurones (Fig. 4-7). Since we have documented that ibotenic acid lesions of Vc neurones significantly reduce the bilaterally increased EMG activity in DIG and MASS, nociceptive inputs from the TMJ region must first reach ipsilateral Vc neurones before they are relayed to other brainstem interneurones or V motoneurones. Therefore, pathway iii for activation of the ipsilateral jaw muscles and pathways d and e for activation of the contralateral jaw muscles are unlikely to be involved in this craniofacial nociceptive reflex. However, our data cannot
rule out the involvement of the remaining pathways in the reflex or delineate the exact number of synapses between Vc neurones and V motoneurones.
CHAPTER 5. RESPONSES OF NEURONES IN TRIGEMINAL SUBNUCLEUS CAUDALIS (Vc) TO MUSTARD OIL INJECTION INTO THE TEMPOROMANDIBULAR JOINT REGION

INTRODUCTION

Although there is considerable evidence that the rostral components of the V brainstem sensory nuclear complex, i.e. Vp, Vo, and Vi, may be involved in orofacial pain mechanisms (Dalle et al. 1988, 1990; Hayashi et al. 1984; Luccarini et al. 1995; Raboisson et al. 1995; Sessle and Greenwood 1976) and in reflexes evoked by low-intensity orofacial stimulation (Funakoshi and Amano 1974; Kidokoro et al. 1968a, b; Olsson and Westberg 1991; Shigenaga et al. 1988b; Sumino 1971; Yoshida et al. 1994, 1995), its caudal component, Vc, has traditionally been viewed as the essential relay site of nociceptive information from superficial and deep craniofacial tissues to higher levels of the CNS (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985). Indeed, with respect to deep craniofacial tissues, Vc has been shown to receive projections of primary afferent fibres innervating deep tissues including the TMJ (Capra 1987; Nishimori et al. 1986; Shigenaga et al. 1988a), and injection of the small-fibre excitant and inflammatory irritant mustard oil into the TMJ region of rats induces c-fos-like immunoreactivity in Vc (Hathaway et al. 1995). Many Vc nociceptive neurones, classified according to their cutaneous mechanoreceptive field properties as WDR or NS neurones, also can be activated by convergent primary afferent inputs from deep craniofacial tissues including the TMJ (Amano et al. 1986; Broton et al. 1988; Hu 1990; Hu et al. 1981, 1992; Kojima 1990; Sessle et al. 1986; Yu et al. 1993), and some show neuroplastic changes reflected in hyperexcitability and enlargement of their mechanoreceptive field following application of mustard oil to deep as well as cutaneous craniofacial tissues (Chiang et al. in press; Hu et al. 1992; Yu et al. 1993).
Also noteworthy are our findings that chemical (ibotenic acid) micro-lesions of Vc, especially its caudal component, can significantly reduce the increased EMG activity evoked in the jaw muscles by mustard oil injection into the TMJ region, suggesting the involvement of the caudal Vc in the central neural pathways involved in craniofacial nociceptive reflexes (Chapter 4). These findings have been supported by observations that micro-injection of the excitatory amino acid and cell excitant glutamate particularly into the caudal Vc also evokes an increase of EMG activity in the jaw muscles (Chapter 3). Although mustard oil injection into the TMJ region does produce neuroplastic changes of the rostral Vc neurones, none of these neurones appeared to be excited by this mustard oil stimulation (Yu et al. unpublished data). In view of the evidence that the caudal Vc may be the essential relay site in the neural pathways for mustard oil/TMJ-evoked nociceptive reflex activity, the aim of this study was to determine if noxious stimulation, including mustard oil injection, of the TMJ region indeed produces excitatory effects on neurones in the caudal Vc.

METHODS

This study was carried out on 10 adult male Sprague-Dawley rats (250-350 g) anaesthetized intraperitoneally with a mixture of chloralose (50 mg/kg) and urethane (1 g/kg); each animal also received a subcutaneous injection of atropine (0.02 mg/kg) to reduce tracheal secretions. Adequacy of anaesthesia was determined periodically by noting the lack of spontaneous movements by the animal, the lack of a response to a pinch stimulus applied to the paw, and the presence of constricted pupils; a supplementary anaesthetic dose (5 mg/kg chloralose, 100 mg/kg urethane, i.v.) was administered if necessary. Heart rate and expired percentage CO₂ were continuously monitored, and rectal temperature was maintained at 37.5 °C. Tracheal and venous
cannulae were inserted, and the animals were allowed to breathe spontaneously or artificially ventilated with an air/O₂ mixture after they were paralyzed with gallamine triethiodide.

Recording procedures

Each animal was placed in a stereotaxic frame, and single unit activity was recorded extracellularly in the left caudal Vc (4-5.5 mm caudal to the obex) by means of a varnish-coated tungsten micro-electrode which was introduced by a microdrive into the brainstem. The activity was amplified and displayed on oscilloscopes and also discriminated with a window discriminator; the discriminator-generated transistor-transistor logic (TTL) signals were recorded online with a data acquisition system (consisting of an IBM-clone computer, CED 1401-Plus hardware and the software "SPIKE2"; CED, Cambridge) and displayed as peristimulus histograms of their spontaneous or evoked activity.

Stimulation procedures and characterization of neurones

All neurones were examined for responses to a wide range of mechanical stimuli of the skin, which was shaved within 1 mm of the surface with animal clippers to aid in mechanoreceptive field delineation. Mechanical stimuli consisted of brushing with a camel-hair brush, gentle pressure with a blunt glass rod, and a pinch stimulus applied by serrated forceps. Neurones responding to noxious mechanical stimulation were usually tested also for responses to noxious radiant heat. The heat stimulus used was a 8-V, 50-W focused projector bulb (Osram 58-8007), which when run at 4 V and held 3-5 cm from the skin produced a skin temperature of 51-53 °C at the site of projection. When similarly applied to the experimenter's skin, the heat generated could produce a sensation of pricking pain about 3 s after the onset of the heat stimulus. On the basis of the mechanoreceptive field characteristics, neurones were classified into two major
groups of cutaneous nociceptive or non-nociceptive neurones. The non-nociceptive group, viz., LTM neurones, responded to light touch or brushing and exhibited no increase in discharge with more intense stimuli. There were two classes of cutaneous nociceptive neurones that responded to noxious facial or mucosal stimuli. The WDR neurones responded to low-threshold mechanical stimuli but increased their firing rate with increased mechanical stimulation intensity into the noxious range. Neurones not responding to low-intensity mechanical stimuli but responding to heavy pressure and pinch or only pinch were classified as NS neurones.

A 26-gauge needle connected to a 25-μl Hamilton syringe via a polyethylene tubing (PE-50) was implanted into the left TMJ region for noxious mechanical stimulation of the TMJ and for the injection of mustard oil (20% in mineral oil). Since the main purpose of this study was to determine if noxious stimuli, especially mustard oil injection, of the TMJ region produces excitatory effects on neurones in the caudal Vc, only neurones which responded to pricking of the TMJ were tested with mustard oil injection into the TMJ region. For recording of the mustard oil-evoked effects, the first 2 min of recording monitored resting activity of the neurone under study in order to derive the baseline level of neuronal activity (see below). Mustard oil was injected into the left TMJ region at the beginning of the third min and the neuronal activity was continuously recorded thereafter for another 58 min. The total recording time was 60 min. The neuronal activity was expressed with data points representing the number of spikes for every 5-s segment. Data points of the neuronal activity recorded during the first 2 min (24 data points) were pooled to produce a mean value which represented the baseline level of the neuronal activity. Changes in neuronal activity after the mustard oil injection were regarded as significantly increased if one or more data points rose 2 standard deviations (SD) above the mean baseline level; the time period between the initiation of
the increased neuronal activity and its recovery to the baseline level was designated as the duration of the increased neuronal activity. In two neurones which showed a transient increase of neuronal activity following mustard oil injection (see Results), the mechanoreceptive field was remapped 10 min after mustard oil injection so that any changes of the mechanoreceptive field size could be documented.

At the end of each experiment, an electrolytic lesion was produced by passing DC current (8 μA, 10 s) through the micro-electrode to mark the recording site of the neurone tested with mustard oil injection into the TMJ region (one neurone in each rat) and Evans blue dye (10 mg/kg) was injected intravenously into the rat. Ten min after Evans blue injection, the rat was sacrificed and perfused with 200 ml warm saline followed by 150 ml 10% buffered formalin solution. The location of the mustard oil injection indicated by plasma extravasation of Evans blue was confirmed by postmortem dissection of the TMJ region. The brainstem was removed after perfusion and post-fixed in 10% buffered formalin solution for one week. The brainstem tissue was cut in 10-μm serial sections and one of every five sections was selected and stained with cresyl violet. Verification of loci of the sites of electrolytic lesion was made under a light microscope and reconstructed with the aid of a camera lucida and microdrive readings of micro-electrode depth below the surface of the brainstem (Hu 1990; Hu et al. 1992; Yu et al. 1993).

RESULTS

A total of 52 neurones in the caudal Vc were functionally identified (22 LTM, 13 NS, and 17 WDR). Forty-two of these neurones (18 LTM, 11 NS, and 13 WDR) were tested with noxious mechanical stimulation (pricking with the implanted needle) of the ipsilateral TMJ; no LTM, eight (73%) NS, and eight (61%) WDR neurones responded to
the TMJ stimulation; note that the incidence of NS and WDR neurones responding to pricking of the TMJ was significantly higher than that for LTM neurones (Table 5-I).

Nine neurones (four NS and five WDR, all excited by pricking of the ipsilateral TMJ) identified in nine different rats were tested with mustard oil injection into the ipsilateral TMJ region; seven (two NS and five WDR) of the nine neurones tested showed an increase of neuronal activity following the mustard oil injection. These seven mustard oil-sensitive neurones were all located in the deep laminae (laminae IV-VI) of the caudal Vc. All seven neurones had a cutaneous mechanoreceptive field that covered part of or the whole ipsilateral ear, four of them also had a mechanoreceptive field that included skin overlying the lateral face and the TMJ, and one neurone had a mechanoreceptive field on neck skin; none of the seven neurones had an intraoral and perioral cutaneous mechanoreceptive field. The mustard oil-evoked neuronal activity was reflected in one (in one NS neurone) or two phases (in one NS and five WDR neurones) of increased neuronal activity (Figs. 5-1 and 5-2). The first phase had a latency of 0.5-5 s, peaked within the first 30 s, and lasted for 0.5 min up to 5 min; the second phase usually occurred 5-20 min after the initiation of the first phase, lasted for 0.5 min up to 30 min, and had various firing patterns (Figs. 5-1 and 5-2). These patterns included a long-lasting (30 min) continuous firing in one WDR neurone (see Fig. 1), a short burst of activity in one NS neurone and one WDR neurone (e.g. Fig. 2D), and multiple bursts of firing with variable interburst intervals and intraburst spikes that were seen in three WDR neurones (e.g. Figs. 2B, 2C). In one WDR neurone (Fig. 2A), a continuous low-amplitude first phase of activity was evoked by mustard oil injection, and 15 min following the injection there appeared a burst of high-amplitude activity which continued throughout the recording time.
TABLE 5-I. INCIDENCE OF NEURONAL RESPONSES TO MECHANICAL STIMULATION AND MUSTARD OIL INJECTION IN THE TMJ REGION

<table>
<thead>
<tr>
<th></th>
<th>Mechanical stimulation of TMJ</th>
<th>Mustard oil injection into the TMJ region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neurones tested</td>
<td>Neurones activated</td>
</tr>
<tr>
<td>LTM (n=22)</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>NS (n=13)</td>
<td>11</td>
<td>8***</td>
</tr>
<tr>
<td>WDR (n=17)</td>
<td>13</td>
<td>8***</td>
</tr>
</tbody>
</table>

***p<0.001 (Fisher's test) indicates significantly different compared with LTM neurones.
Fig. 5-1 Response properties of a WDR neurone and effects of 20% mustard oil injected into the TMJ region. The histologically confirmed recording locus of the neurone was located in the deep laminae of Vc, A. The mechanoreceptive field of the neurone is shown on the face figurine in B; note that the mechanoreceptive field was limited within the trigeminal region. C, the histogram (5 s bins) shows the neurone’s responses to touch, press, pinch, and noxious heat stimuli applied to its cutaneous mechanoreceptive field. D shows its response (five overlapped traces) to electrical stimulation (single pulses, 2 ms, 5 mA) of its mechanoreceptive field; the neurone received both A and C fibre inputs. E shows its response to mustard oil injection into ipsilateral TMJ region. The histogram shows the baseline (0-120 s) and the increased neuronal activity evoked by mustard oil injection (120-2400 s); note the biphasic and long-duration response of the neurone to the mustard oil injection. The horizontal dotted line indicates mean+2SD of the baseline activity.
A

B

C

D

E

Mustard oil injection

Time (s)

# of spikes

brush pinch

# of spikes

pinch heat

press

brush

1 mV

20 ms

10 s
Fig. 5-2 Different firing patterns of Vc neuronal activity evoked by mustard oil injection into the TMJ region. A, B, C, and D show the cutaneous mechanoreceptive fields and histograms of responses of four Vc neurones whose histologically confirmed recording sites were located in a, b, c, and d, respectively, in the drawing of brainstem. Neuronal activity is expressed in terms of the number of spikes for every 5 s. Horizontal dotted line on each graph represents mean+2SD of the baseline activity of each neurone. A, B, and C were WDR neurones while D was a NS neurone.
Two NS neurones, which fired transiently following the mustard oil injection, were carefully studied for changes in the size of their mechanoreceptive field. Compared with their pre-injection mechanoreceptive field size, they showed an expansion of the mechanoreceptive field 10 min after the mustard oil injection (Fig. 5-3). We were not able to follow any subsequent changes of the mechanoreceptive field size of these two neurones because they were “lost” during the experiment. The expansion of the mechanoreceptive field, however, seemed to be reversible because no mechanoreceptive field expansion was noticed in the other five neurones at 60 min after the mustard oil injection (by which time all mustard oil-evoked activity had long disappeared).

**DISCUSSION**

The present study has provided the first documentation that injection of the small-fibre excitant and inflammatory irritant mustard oil into the TMJ region produces excitatory effects on neurones in the caudal Vc. The latency value (0.5-5 s), mono- or bi-phasic responses, and duration (0.5-35 min for the first plus the second phases) of the mustard oil-evoked neuronal activity in the caudal Vc are comparable with those of the mustard oil-evoked EMG activity in the jaw muscles. These findings support our lesion and micro-stimulation data that neurones in the caudal Vc are indeed critical elements in the neural pathways underlying the reflex responses evoked in DIG and MASS muscles by noxious TMJ stimulation.

Only nociceptive neurones (and no LTM neurones) responded to pricking of the TMJ with the implanted needle, and a high percentage of these nociceptive neurones could be excited by mustard oil injection into the TMJ region, suggesting that nociceptive neurones in the caudal Vc may be particularly important in the reflex pathways. These findings are consistent with previous observations that (i) primary
Fig. 5-3 Two examples of NS neurones, A and B, exhibiting mechanoreceptive field expansion following injection of mustard oil into the TMJ region; each neurone could be activated by pinch of the cutaneous mechanoreceptive field. In each of A and B, the two face figurines in the top row show the pre-injection cutaneous mechanoreceptive fields (hatched areas) of the two neurones and those in the bottom row show the cutaneous mechanoreceptive fields 10 min following mustard oil injection. The two graphs in the middle row show the baseline (0-120 s) and the increased neuronal activity evoked by mustard oil injection; the latter lasted for about 1 min in the neurone represented in A and for about 30 s in the neurone represented in B. The bottom drawing shows the location (a and b) within Vc of the two neurones represented in A and B.
EXPANSION OF RECEPTIVE FIELDS OF Vc NEURONES INDUCED BY MUSTARD OIL INJECTION INTO THE TMJ REGION
afferent fibres innervating the TMJ region appear to be predominantly small-diameter fibres with unmyelinated nerve endings (Dreessen et al. 1990; Ichikawa et al. 1989, 1990; Kido et al. 1991, 1995; Thilander 1961). (ii) some of these afferent fibres, which may contain substance P or CGRP (Ichikawa et al. 1989, 1990; Johansson et al. 1986; Kido et al. 1993), may project to Vc (Capra 1987). (iii) injection into the TMJ region of algesic chemicals (7% NaCl, KCl, histamine) also excites only nociceptive Vc neurones (Broton et al. 1988), (iv) mustard oil selectively activates small-diameter afferent fibres and produces pain and burning sensations in humans (Handwerker et al. 1991; Jancso and Janka 1981; Jancso et al. 1967; Woolf and Wall 1986), and (v) neonatal application of the C fibre neurotoxin capsaicin blocks the mustard oil-evoked neuroplastic changes of Vc nociceptive neurones (Sessle et al. 1995) as well as the mustard oil-evoked increase of the jaw muscle EMG activity (Tsai et al. unpublished data). However, it is not known whether LTM neurones or some nociceptive neurones which cannot be activated by mechanical stimulation of the TMJ may also play a role in the reflex pathways. Future studies need to address this question.

In the present study, all of the mustard oil-sensitive neurones were located in the deep laminae of the caudal Vc. Although mustard oil injection into the TMJ region induces expression of c-fos-like immunoreactivity predominantly in the superficial laminae of Vc (Hathaway et al. 1995) and EMG activity evoked by electrical micro-stimulation of Vc had the lowest threshold when micro-stimulation was delivered in the superficial laminae (Chapter 3), neurones in the deeper laminae (but not in the superficial laminae) of Vc have been retrogradely labeled following injection of neuronal tracers into the Vmo (Li et al. 1993, 1995). Furthermore the ibotenic acid micro-lesions in the caudal Vc, which significantly reduced the mustard oil/TMJ-evoked EMG activity, were mostly located in the deep laminae of Vc (Chapter 4) and glutamate micro-injection into
the analogous loci also evoked a significant increase in EMG activity (Chapter 3). The roles of neurones in the superficial and deep laminae of Vc in the reflex pathways thus remain unclear and represent a direction for future studies.

Our findings of an enlarged cutaneous mechanoreceptive field of Vc nociceptive neurones induced by mustard oil injection into the TMJ region are consistent with our previous findings that mustard oil application to various craniofacial tissues induces mechanoreceptive field expansion of neurones in Vc (Chiang et al in press; Hu et al. 1992; Yu et al. 1993). A previous study (Yu et al. 1993) has argued that the mechanoreceptive field changes are unlikely to reflect nonspecific effects or fluctuations in mechanoreceptive field size unrelated to the mustard oil application because application to the gastrocnemius-soleus muscle does not produce such changes and mineral oil injection produces only an occasional small and insignificant mechanoreceptive field expansion.

The precise central neural mechanisms underlying the neuroplastic changes that we have documented are still unclear. Several possible mechanisms, including an increase in neuronal excitability, and increased peripheral inputs, and a decreased descending or segmental inhibition, have been suggested to account for spinal neuronal mechanoreceptive field expansion during inflammatory conditions (Cervero et al. 1992; Hoheisel et al. 1993; Ren et al. 1992, 1994). The NMDA receptor in particular, which has been suggested as a critical candidate for wind-up (Davies and Lodge 1987; Dickenson and Sullivan 1987; Mendell 1966; Price and Wagman 1970; Price et al. 1971; Ren 1994) and central sensitization (Cervero et al. 1993; Neugebauer and Schaible 1990; Willis 1993; Woolf 1992) of neurones in the central nervous system, may be an important factor in maintaining mechanoreceptive field size (Ren et al. 1992, 1994). Indeed, Yu et al. (1996) have shown that the NMDA antagonist MK-801 blocks the mustard oil/TMJ-
evoked EMG activity in jaw muscles. Our recent preliminary results have also shown that neonatal application of capsaicin blocks the increased jaw muscle EMG activity evoked by glutamate micro-injection into the caudal Vc (Tsai et al. unpublished data), indicating that both the central as well as the peripheral components of the neural pathway involved in craniofacial nociceptive reflexes may be altered by neonatal capsaicin and that excitatory amino acids are likely involved in this pathway. The neurochemical mechanisms involved in the different elements of the central nociceptive reflex pathway would appear to represent a fruitful avenue for future research.
CHAPTER 6. GENERAL DISCUSSION

This thesis has provided the first documentation that Vc, especially its caudal component, is an important relay in the central neural pathways involved in the reflex activation of both the jaw-opening (DIG) and jaw-closing (MASS) muscles. This reflex activation evoked by injection into the rat TMJ region of the small-fibre excitant and inflammatory irritant mustard oil is considered to be a craniofacial nociceptive reflex, as Yu et al. (1994, 1995) have explained. The central neural pathways involved in the craniofacial nociceptive reflexes appear to be different from those involved in orofacial reflexes evoked by intraoral/perioral stimuli because the craniofacial nociceptive reflex pathways may involve interneurones in the caudal part of the V brainstem sensory nucleus complex, Vc, whereas the orofacial reflexes involve interneurones mainly in the rostral brainstem nuclei, viz. Vp, Vo, Vi and the supratrigeminal nucleus (Dallel et al. 1989; Kidokoro et al. 1968 a, b; Olsson and Westberg 1991; Shigenaga et al. 1988b; Sumino et al. 1971; Yoshida et al. 1994, 1995). Furthermore, the craniofacial nociceptive reflex pathways produce excitatory effects on both the jaw-opening and jaw-closing muscles (Yu et al. 1994, 1995; also see Chapters 2 and 4) whereas the orofacial reflex pathways primarily produce an excitatory effect on the jaw-opening muscles and an inhibitory effect on the jaw-closing muscles (Goldberg 1972b; Kidokoro et al. 1968b; Sherrington 1917; Sumino 1971). Our findings also are consistent with previous documentations that Vc is the essential relay site of nociceptive information from craniofacial tissues to other levels of the central nervous system (for review, see Dubner et al. 1976, 1978; Sessle 1987; Sessle and Hu 1991; Yokota 1985) and that the rostral V brainstem sensory nuclei are primarily related to intraoral and perioral pain mechanisms (Dallel et al. 1988, 1990; Hayashi et al. 1984; Luccarini et al. 1995; Kerr 1979; Raboisson 143
Together with the possible involvement of Vc in head and neck reflexes/behaviours (Dallel et al. 1989; Sumino and Nozaki 1977; Young et al. 1981) and in changes of plasma concentration of adrenocorticotropin, arterial blood pressure, heart rate and respiration rate (Bereiter and Gann 1986, 1988; Bereiter et al. 1996; Panneton 1991; Panneton and Yavari 1995) evoked by various noxious craniofacial stimuli, these findings suggest that Vc is an important relay site in a variety of reflex responses to craniofacial noxious stimuli.

TECHNICAL CONSIDERATIONS

Halothane Anaesthesia

A mixture of 1/3 O₂, 2/3 N₂O and halothane has been used to anaesthetize our animals in the EMG studies. The advantages of halothane anaesthesia include fast induction, easy adjustment of the concentration, stable anaesthetic levels and a wide safe concentration range. At a 1.5-2% concentration, animals can be maintained at a deep and stable anaesthetic level (as indicated by constricted pupils, slow heart rate, low blood pressure, and absence of reflex responses to noxious stimulation) which is suitable for surgical preparation (Dallel et al. 1990; Hu et al. 1992, 1993; Le Bars et al. 1980; Raboisson et al. 1991, 1995; Yu et al. 1994, 1995). After completion of surgery, halothane concentration has been maintained at 0.8-1.0% during the EMG recording. At this halothane concentration, the rat's physiological condition can be maintained at normal physiological levels (heart rate, 330-430 beats/min; expired percent CO₂, 3-5%; blood pressure, 80-130 mmHg); rats also show a flexion reflex of their hindlimb following noxious mechanical stimulation applied to the toes but otherwise no spontaneous activity. These findings are consistent with observations that halothane concentration higher than 2% blocks the hindlimb flexion reflex evoked by noxious
electrical stimulation (15 mA, 2ms, 0.16 Hz) whereas 0.8% halothane allows the reflex to be reproduced regularly and steadily for a long period of time (Falinower et al. 1994).

Mustard Oil Injection into the TMJ Region

The TMJ is innervated by free nerve endings (Dreessen et al. 1990; Ichikawa et al. 1989, 1990; Kido et al. 1991, 1995; Milam 1995; Thilander 1961) which may contain substance P or CGRP (Ichikawa et al. 1989, 1990; Johansson et al. 1986; Kido et al. 1993; Milam 1995) and may be associated with small-diameter primary afferent fibres (Dreessen et al. 1990; Kido et al. 1995; Milam 1995; Thilander 1961). The nerve endings supplying the TMJ are mainly located in the joint capsule, the synovial membrane, the loose and wavy connective tissue behind the disc, and the periosteum; no nerve endings have been found in the articular disc (Bernick 1962; Boyer et al. 1964; Choukas and Sicher 1960; Ichikawa et al. 1989, 1990; Johansson et al. 1986; Keller and Moffett 1968; Kido et al. 1991, 1993, 1995; Milam 1995; Thilander 1964; also see Mohl 1988; Ten Cate 1994).

In previous studies in our laboratories, algesic chemicals have been directly applied into the joint space of the exposed TMJ in the cat (Broton and Sessle 1988; Broton et al. 1988). However, in order not to damage the overlying masseter muscle and the closely adjacent sensory and motor nerves which innervate the TMJ and the jaw muscles, respectively, mustard oil was injected into the rat TMJ region via a pre-implanted needle. Plasma extravasation of Evans blue dye, which was injected intravenously 10 min before sacrificing the rat, was confirmed by postmortem dissection of the TMJ region. Plasma extravasation involving the TMJ capsule and periauricular tissues lateral to the injected TMJ was seen in all animals tested (Chapers 2 and 4), indicating that mustard oil injection may have activated free nerve endings in
surrounding muscles and ligaments as well as the TMJ tissues. Mustard oil has previously been shown to activate selectively small-diameter afferent fibres (Handwerker and Reeh 1991; Jancso et al. 1967; Woolf and Wall 1986), produce inflammation at the site of application (Haas et al. 1992; Jancso and Janka 1981), and provoke pain or burning sensations in humans (Handwerker et al. 1991; Jansco and Janka 1981). Our laboratories have also shown that the central (Sessle et al. 1995) and reflex (Tsai et al. unpublished data) effects of mustard oil application to craniofacial tissues can be blocked by neonatal application of the C fibre neurotoxic agent capsaicin. It is currently unknown whether mustard oil activates small-diameter afferent fibres by binding to specific receptors on nerve endings or by releasing endogenous algesic chemicals and/or inflammatory substances, which subsequently activate small-diameter fibres. It is possible that other types of TMJ afferent fibres which are not sensitive to mustard oil may also play a role in neural pathways involved in craniofacial reflexes evoked by other types of TMJ stimulation, and this may be a factor explaining why the TMJ application of mustard oil evoked bilateral reflex effects (Chapters 2 and 4) whereas micro-stimulation in Vc evoked ipsilateral effects (Chapter 3); other possible explanations are outlined in Chapter 3.

**Current spread of electrical micro-stimulation in the brainstem**

Electrical current required to excite a nerve cell can be predicted by the Inverse Square Law Equation (Bagshaw and Evans 1976): \( I = a + kd^2 \), where \( I \) refers to the current required, \( a \) represents the minimum current required for activation of the nerve cell when the stimulating electrode is in direct contact with the cell, \( k \) is a constant that varies with individual electrodes, and \( d \) reflects the distance between the electrode and the cell. Given \( I_0 = a = 250 \ \mu A \) when \( d = 0 \ \text{mm} \) and \( I_{0.5} = 500 \ \mu A \) when \( d = 0.5 \ \text{mm} \).
would be 1,000 µA/mm². Therefore, in the case of the maximal stimulation intensity (1 mA), the effective current spread would be about 0.85 mm. The estimation of current spread seems reasonable and is consistent with the findings that a current of 1 mA delivered in the left Vc did not evoke EMG activity in the right DIG and MASS (see Chapter 3), since more than 4 mA (according to the equation) would be necessary to excite neurones in the contralateral Vc neurones which were more than 2 mm away from the stimulation locus.

**Glutamate and Ibotenic Acid Micro-Injections into the Brainstem**

Both glutamate and ibotenic acid are members of the excitatory amino acid family (Aimone et al. 1988; Chiang et al. 1990; Curtis and Johnson 1974; Johnson 1978; Schwarcz et al. 1979; Watkins and Evans 1981). Glutamate has been regarded as one of the most important excitatory neurotransmitters in the CNS (Curtis and Johnson 1974; Johnson 1978; Watkins and Evans 1981) and was micro-injected in Vc in order to determine the excitatory effects of Vc neurones on V motoneurones (Chapter 3). Ibotenic acid, which destroys specifically neurones but spares fibres of passage in the CNS (Aimone et al. 1988; Chiang et al. 1990; Schwarcz et al. 1979), was injected in Vc to determine the involvement of Vc neurones in craniofacial nociceptive reflex pathways (Chapters 2 and 4). Both glutamate and ibotenic acid possess neurotoxic effects (Schwarcz et al. 1979; Wuerthele et al. 1978); in the present studies, they produced signs of neuronal death in Vc and the area of neuronal death was used as an indication of the area involved by glutamate or ibotenic acid injection (Chapters 2, 3 and 4). It is unclear, however, whether the area of neuronal death reflected exactly the spread of these two chemicals in Vc. Nevertheless, glutamate micro-injection into the left caudal Vc did not evoke EMG activity in the right DIG and MASS (Chapter 3) and ibotenic acid micro-
injection into the left Vc did not significantly reduce the increased EMG activity in the right DIG and MASS evoked by mustard oil injection into the right TMJ region (Chapter 4). These findings suggest that the micro-injected glutamate and ibotenic acid did not spread to the contralateral Vc or more rostral nuclei including the Vmo. A systematic search in the brainstem of the effects of the chemicals on neuronal activity evoked by peripheral stimulation or the use of anatomical or histochemical markers of cell death and degeneration would be possible means to quantify the spread of the chemicals.

It has been reported that ibotenic acid may produce differential effects on different types (GABA-, dopamine-, or enkephalin-containing) of neurones with different dose-response relationships (Schwarcz et al. 1979). It would be important in the future to determine the differential and dose-response effects of glutamate and ibotenic acid on Vc neurones and on the associated EMG responses.

In previous studies (Aimone et al. 1988; Chiang et al. 1990; Curtis and Johnson 1974; Johnson 1978; Schwarcz et al. 1979; Watkins and Evans 1981), animals receiving ibotenic acid injections typically were sacrificed days or months after the injection for histological reconstruction of the ibotenic acid-induced lesions. In this thesis, EMG recording was started four hours following ibotenic acid injection into Vc, and EMG activity was significantly reduced by this acute treatment; the lesions in Vc were also histologically confirmed and produced signs of neuronal death and micro-glial infiltration in a spherical area (Chapters 2 and 4). These findings suggest that ibotenic acid is an effective neurotoxic agent and can be used for acute as well as chronic lesion studies.
EMG Recordings

The EMG activity is the electrical record of the activity of a muscle. It has been, and still is, a powerful tool for investigation and understanding the function of jaw muscles. Three general types of EMG electrodes have been used, i.e. surface, needle, and fine-wire electrodes. For recording especially from DIG and MASS muscles, fine-wire and needle electrodes help prevent signal contamination from the overlying facial muscles which may be a potential problem for recording with surface electrodes. The ease of insertion and implantation are the advantage of fine-wire electrodes over needle electrodes; the latter usually need special fixation to keep them in position. Due to these advantages, intramuscular fine-wire electrodes were selected for EMG recording in this thesis. However, since the studied muscles were not exposed, an independent confirmation of proper placement (e.g. by dissection at the end of experiments) was necessary.

The vagaries of amplitude measurement are another potential problem. The amplitude of the recorded signal depends on many factors, including the number of motor units recorded, the frequency at which they fire, the distance from the motor units to the electrodes, and the inter-electrode distance; these factors are often difficult to control. In this thesis, the EMG activity of the jaw muscles was continuously recorded with the same set of intramuscular electrodes before (for 20 min) and after injection of mustard oil (for 40 or 80 min, depending upon whether one or both TMJs were injected) and then rectified and integrated into values of EMG area for every 60 s period. The advantage of this data-processing method is that the amplitudes and firing frequencies of the action potentials of several motor units within the 60 s period have been taken into account.
Centrally Mediated Reflex Responses

Several lines of evidence suggest that the mustard oil-evoked EMG activity documented in this thesis reflects a centrally mediated reflex response. First, increased EMG activity can also be observed in muscles remote from or contralateral to the mustard oil injection site (Yu et al. 1994, 1995; also see Chapter 2 and 4), which is consistent with observations of injection of other algesic chemicals into the TMJ or injection of mustard oil into deep neck tissues (Broton and Sessle 1988; Hu et al. 1993). Second, the mustard oil-evoked EMG activity can be blocked by interruption at various sites along the neural pathways, e.g. pre-administration of local anaesthetic into the TMJ region (Yu et al. 1995), transection of the Vtr and V spinal sensory nucleus at the obex level (Chapter 2), or chemical lesions of interneurones in Vc (Chapters 2 and 4). Third, an increase in jaw muscle EMG activity can be evoked by electrical micro-stimulation or micro-injection of the cell excitant and excitatory amino acid glutamate in Vc (Chapter 3). Fourth, mustard oil injection into the TMJ region excites neurones in Vc and produces neuroplastic changes of Vc neurones (Chapter 5). Fifth, neonatal application of the C fibre neurotoxic agent capsaicin to afferent nerves blocks the excitatory effects of mustard oil on the central V neurones (Sessle et al. 1995) and the mustard oil-evoked EMG activity (Tsai et al. unpublished data); the locomotion of these rats, however, is not affected (Tsai et al. unpublished observations).

Experimental Design

In this thesis, mustard oil was first injected into the TMJ region of a group of intact rats (n=8); increased EMG activity (which had latencies of 1-2 s, peaked within 1 min, lasted for 10-40 min, and was reflected in one or two phases of increased activity) in each of the bilateral DIG and MASS muscles was recorded following mustard oil injection into the TMJ region. These findings are consistent with our previous
observations (Yu et al. 1994, 1995) and suggest further that this is a good animal model for studies of craniofacial nociceptive reflexes.

Since noxious stimuli applied to remote somatic tissues may produce DNIC on responses of V or spinal nociceptive neurones (Bouhassira et al. 1988; Dallel et al. 1990; Dickenson and Le Bars 1983; Dickenson et al. 1980; Hu 1990; Le Bars et al. 1979; Villanueva et al. 1984) and hindlimb flexion reflex responses (Falinower et al. 1994) evoked by noxious stimulation applied to the toes, it was therefore necessary to test whether the surgical preparation of the animals used in this thesis project produced any adverse effects on the mustard oil-evoked EMG activity. Mustard oil was injected into the TMJ region of another group of rats (n=6) receiving only laminectomy of C1 and C2. An increase in EMG activity of the jaw muscles could still readily be evoked in these rats; the amplitude and duration of the increased EMG activity of these rats were not significantly different from those of the intact rats. These findings indicate that the effects of the surgical procedures on the mustard oil-evoked jaw muscle EMG activity are minimal.

In order to infer that a given nuclear structure participates in a reflex pathway, three lines of evidence are necessary. First, removal of this structure should result in elimination of the reflex. Second, selective stimulation of this structure should produce a similar reflex response. Third, the structure should have anatomical and physiological connections with the afferent and efferent arms of the reflex. In this thesis, various strategies have been adopted to provide these three lines of evidence.

Surgical lesions were used to interrupt mechanically the neural pathways. Transection of Vc at its rostral end (n=8) significantly blocked the mustard oil-evoked jaw muscle EMG activity whereas sagittal section medial to Vc (n=8) or transection of Vc at its caudal end (n=5) did not produce any significant changes to the EMG activity
These three lesions thus defined an area in the caudal brainstem, Vc, which is critically involved in the mustard oil-evoked jaw muscle EMG activity. However, results from these surgical lesion studies did not allow us to differentiate the importance of neurones or fibres of passage in Vc in the reflex pathways. In order to answer this issue, the excitatory neurotoxic agent ibotenic acid which destroys neurones but spares fibres of passage was injected extensively into Vc (n=5); it significantly reduced the mustard oil-evoked EMG activity (Chapter 2). Extensive injection of phosphate-buffered saline (vehicle of ibotenic acid) into Vc (n=5) did not produce such neuronal death or reduction in EMG activity. These findings thus provide the first line of evidence of the involvement of Vc in craniofacial nociceptive reflexes.

If Vc is indeed involved in the neural pathways of the craniofacial nociceptive reflexes, stimulation of Vc should produce an analogous excitatory EMG response in the jaw muscles; electrical micro-stimulation of Vc and adjacent brainstem structures (n=8) was therefore used to test this possibility. Since Vc is a columnar structure which extends from the obex to the upper cervical spinal segments and merges with the spinal dorsal horn in rats (Arvidsson and Gobel 1981; Hockfield and Gobel 1978; Pfaller and Arvidsson 1988; Shigenaga et al. 1988b), and the surgical or chemical lesions that we created in Vc involved most of Vc as well as adjacent brainstem structures, a systematic mapping stimulation was applied to the caudal brainstem and upper cervical segments, including Vc (see Chapter 3). Electrical micro-stimulation did produce excitatory EMG activity in the ipsilateral DIG and MASS. The Vc at the level 4-5.5 mm caudal to the obex, i.e. the caudal Vc, appeared to be most critically involved in the excitatory effects because micro-stimulation at these levels could consistently evoke EMG responses in the ipsilateral DIG and MASS with low thresholds and short latencies. The thresholds and latencies of the evoked EMG activity increased when the micro-stimulation was
delivered to more rostral or caudal levels, suggesting that the caudal Vc is of critical importance in producing excitatory effects on jaw muscle EMG activity.

However, electrical micro-stimulation may activate fibres of passage as well as neurones in Vc. The excitatory amino acid and cell excitant glutamate was therefore used to differentiate the importance of Vc neurones or fibres of passage in the evoked EMG response. Glutamate micro-injection into the caudal Vc (n=6) evoked a significant increase in EMG activity of the ipsilateral DIG and MASS; glutamate micro-injection into the rostral Vc (n=6), the C2 segment (n=6) or the reticular formation (n=6) at the obex level, however, did not produce any significant change in jaw muscle EMG activity, except for the those rats receiving glutamate micro-injection into the C2 segment which showed a small but significant increase in EMG activity of the ipsilateral DIG. These findings thus confirm that neurones in Vc, especially its caudal component, produce excitatory effects on jaw muscles (Chapter 3) and provide the second line of evidence of the involvement of Vc in craniofacial nociceptive reflexes.

Mustard oil injection into the TMJ region evokes a bilateral increase in EMG activity of DIG and MASS (Yu et al. 1994, 1995; also see Chapter 2 and 4); electrical or glutamate micro-stimulation of Vc, on the other hand, produces excitatory EMG activity only in the ipsilateral DIG and MASS (Chapter 3). Primary afferent fibres innervating the rat TMJ project to both the rostral and caudal Vc (Capra 1987; Hathaway et al 1995); glutamate micro-injection of the caudal Vc (but not the rostral Vc) produces excitatory jaw muscle EMG responses. Ibotenic acid micro-injection into the caudal brainstem structures, including the caudal Vc (n=14), the rostral Vc (n=6), the C2 segment (n=7), or the reticular formation at the obex level (n=7), was therefore adopted for further clarification of the role of Vc in the reflex pathways. Ibotenic acid micro-injection into the left caudal Vc produced a highly significant reduction (compared with intact rats) in
EMG activity of the left and right DIG and MASS evoked by mustard oil injection into the left TMJ region; EMG activity of the left DIG and MASS evoked by mustard oil injection into the right TMJ region of these same rats was also significantly reduced and that of the right DIG and MASS was, however, unaffected (Chapter 4). Ibotenic acid micro-injection into the rostral Vc produced a small but also significant reduction only on the contralateral (but not ipsilateral) DIG and MASS; ibotenic acid micro-injection into the C2 segment or the reticular formation did not produce any significant changes to the mustard oil-evoked EMG activity. Considering all the evidence from the EMG studies in this thesis, we therefore conclude that (i) mustard oil injection into the TMJ region may produce excitation of a substantial number of Vc neurones which results in the reflex activation of the jaw muscles and (ii) reflex activation of the contralateral jaw muscles may depend upon a summation of inputs from many neurones in the rostral and especially the caudal components of Vc.

FUTURE DIRECTIONS

Central Neural Pathways Involved in Reflex Activation of Jaw-Opening and Jaw-Closing Muscles

Primary afferent fibres innervating TMJ tissues project to Vc (Capra 1987), mustard oil injection into the TMJ region induces expression of c-fos-like immunoreactivity in Vc (Hathaway et al. 1995) and noxious TMJ stimuli, including mustard oil injection, excite neurones in Vc (Broton et al. 1988; also see Chapter 5). These findings suggest that neurones in Vc may receive nociceptive afferent inputs from TMJ. Degenerative terminal fibres have been found in Vmo following lesions of Vc (Dunn and Matzke 1968; Roberts and Matzke 1971; Stewart and King 1963) and Vc neurones have been retrogradely labeled following injection of neural tracer into Vmo (Li
et al. 1993, 1995; Mizuno et al. 1983). These findings suggest that there may be direct connections between Vc neurones and V motoneurones. The shortest latency values (4.5±1.6 ms for DIG and 5.3±1.6 ms for MASS) of the EMG activity evoked by electrical micro-stimulation however raise the possibility that multi-synaptic pathways may exist between Vc neurones and V motoneurones. The latency values of DIG was also significantly shorter than those of MASS (Chapter 3), and intraventricular preadministration of the non-competitive NMDA antagonist MK-801 (for review, see Collingridge and Lester 1989; Wong and Kemp 1991) significantly reduces the magnitudes of the mustard oil-evoked EMG activity of MASS but has no significant effects on that of DIG (Yu et al. 1996). These findings, however do not rule out the possible involvement in craniofacial nociceptive reflexes of neurones in more rostral nuclei which also receive TMJ inputs (Capra 1987; Kawamura et al. 1967; Klineberg 1971; Sessle and Greenwood 1976). In addition, the TMJ is also innervated by non-nociceptive primary afferent fibres (Lund and Matthews 1981) and conceivably other nociceptive primary afferents which may not be sensitive to mustard oil, and nociceptive primary afferent fibres innervating various other craniofacial and intraoral/perioral tissues also project to Vc (Arvidsson and Gobel 1981; Capra 1987; Hathaway et al. 1995; Jacquin et al. 1986; Lu and Bereiter 1995; Nishimori et al. 1986; Shigenaga et al. 1986, 1988a; Sugimoto et al. 1994; Strassman and Vos 1993; Strassman et al. 1994; Takemura et al. 1991). Indeed, application into the cat's TMJ capsule of histamine, KCl, or 7% NaCl also evokes increased EMG activity (Broton and Sessle 1988); whether those TMJ afferents are also sensitive to mustard oil is not known. Mustard oil application to the rat's tooth pulp also reflexly evokes increased jaw muscle activity (Sunakawa et al. 1993a, b). It is thus unclear whether activation of mustard oil-insensitive primary afferent fibres, or application of mustard oil to other craniofacial tissues in the rats also evokes...
the nociceptive reflex responses and/or whether the reflex responses are also mediated via Vc. Further studies should aim to clarify the reflex effects of noxious stimulation of other craniofacial tissues and the possible involvement of different central neural pathways, particularly those involving more rostral brainstem nuclei, in nociceptive muscle reflexes, and also specifically test for different neural circuitries underlying the reflex activation of jaw-opening versus jaw-closing muscles.

**Dual Effects of Mustard Oil**

Mustard oil application to peripheral tissues excites peripheral and central nociceptive neurones and produces inflammatory responses at the site of application (Handwerker and Reeh 1991; Haas et al. 1992; Jancso et al. 1967; Woolf and Wall 1986). The time courses of the excitatory and inflammatory effects, however, do not coincide. The excitatory effects have latencies within seconds, peak within the first minute, and last for less than an hour (Handwerker and Reeh 1991; Harris and Ryall 1988; Woolf and Wall 1986; Yu et al. 1993, 1994, 1995; also see Chapters 2-5). The inflammatory effects, on the other hand, have a much slower onset, peak within an hour or hours, and may last for more than several hours (Haas et al. 1992; Yu et al. unpublished data). One factor that may be related to the difference in time courses could be the mechanisms by which mustard oil induces inflammation and afferent nerve excitation. It is noteworthy that mustard oil injection into the TMJ region of rats neonatally treated with capsaicin still readily induces an inflammatory reaction but does not evoke any significant increase in EMG activity of the jaw muscles (Tsai et al. unpublished data), indicating that the mustard oil-induced inflammation may not be completely neurogenic or that the inflammatory reaction may involve capsaicin-insensitive afferents whereas the central effects involve capsaicin-sensitive afferents.
Although some inflammatory mediators do excite and/or sensitize nociceptive primary afferent fibres (for review, see Levine et al. 1993; Levine and Taiwo 1994; Schaible and Grubb 1993), it is presently unclear whether mustard oil activates nociceptive primary afferent fibres by binding directly to specific receptors on the peripheral nerve endings or by releasing from tissues at the site of application inflammatory mediators which in turn excite nociceptive primary afferent fibres. Another factor in explaining the different time courses is that the mustard oil-evoked EMG activity can be “rekindled” by i.v. and i.t. application of the opioid antagonist naloxone (Hu et al. 1994; Yu et al. 1994), indicating that mustard oil application may also activate central opioid inhibitory systems that modulate or “override” the central excitatory effects of mustard oil. Further studies are needed to address the effects of mustard oil on the neural and immune systems and the relationship and interactions between these two systems.

**Neurochemical Mechanisms Involved in the Reflex Responses**

Glutamate receptors, both NMDA and non-NMDA subtypes, have been found to exist not only in the CNS but also in the peripheral nervous system (e.g. Berger et al. 1995; Shigemoto et al. 1992; for review, see Collingridge and Lester 1989; Erdo 1991; Monaghan et al. 1989; Nakanishi 1992). Functionally, non-NMDA receptor mechanisms have been implicated (for review, see Collingridge and Lester 1989; Monaghan et al. 1989; Nakanishi 1992) in generating fast EPSPs in the CNS whereas NMDA receptor mechanisms may be involved in mediating more prolonged EPSPs, in nociceptive transmission in ascending somatosensory pathways, and particularly in “central sensitization” states that can be evoked by inflammation and other conditions associated with increased small-fibre nociceptive afferent inputs into the CNS (Aanonsen and Wilcox 1987; Dickenson and Sullivan 1987; Yaksh 1989; Woolf and
Thompson 1991; for review, see Dubner and Ruda 1992; Coderre 1993; Meller and Gebhart 1993; Urban et al. 1994). In this thesis, micro-injection of glutamate, which might have activated various glutamate receptor subtypes (NMDA and non-NMDA) at the site of injection, into the caudal Vc evoked a significant increase in EMG activity of DIG and MASS (Chapter 3). Our previous studies have shown that i.v., i.c.p., and local (into the TMJ region) preadministration of the non-competitive NMDA receptor antagonist MK-801 significantly reduces the mustard oil/TMJ-evoked EMG response. It is unclear, however, whether non-NMDA receptors are also involved in the reflex activation of the jaw muscles. In addition, we have shown that neurokinin A and endogenous opioids may also be involved in the activation and modulation, respectively, of the mustard oil-evoked EMG responses (Bakke et al. 1996; Hu et al. 1994; Yu et al. 1994). More studies are therefore necessary to address the central neural pathways and neurochemical mechanisms involved in the activation and modulation of craniofacial nociceptive reflexes and to test whether different pathways and mechanisms are involved in the DIG and MASS activity evoked by noxious TMJ stimulation.

Our preliminary data have also shown that neonatal application of the C fibre neurotoxin capsaicin to afferent nerves blocks the increased EMG activity evoked both by mustard oil injection into the TMJ region and by glutamate micro-injection into the caudal Vc. These data suggest that neonatal capsaicin treatment not only produces a depletion of peripheral C fibres (Holzer 1991; Fitzgerald 1983) but may also produce changes in the central nervous system. However, more studies are necessary to clarify the mechanisms underlying these effects of neonatal capsaicin treatment on Vc neuronal activity and related craniofacial nociceptive reflexes.
Involvement of Superficial Laminae and Deep Laminae of Vc in the Reflex Responses

Ibotenic acid micro-lesions in the caudal Vc, which significantly blocked the mustard oil/TMJ-evoked EMG activity, were mostly located in the deep laminae of Vc (Chapter 4); glutamate micro-injection into the analogous loci also evoked a significant increase in EMG activity (Chapter 3). Neurones in the deep laminae (but not in the superficial laminae) of Vc can be retrogradely labeled following injection of neural tracers into Vmo (Li et al. 1993; Mizuno et al. 1983). On the other hand, mustard oil injection into the TMJ region results in expression of c-fos-like immunoreactivity most prominently in the superficial laminae of Vc (Hathaway et al. 1995); EMG activity evoked by electrical micro-stimulation of Vc also had the lowest threshold when micro-stimulation was delivered in the superficial laminae (Chapter 3). The relationship between neurones in the superficial or the deep laminae of Vc with respect to TMJ afferent inputs is thus unclear, and due to the small size of the rat brainstem and the thin cap-shaped superficial laminae of Vc, we were not able to lesion or excite selectively neurones in the superficial laminae. One possible explanation of these apparently conflicting findings is that neurones in the superficial laminae receive direct inputs from TMJ primary afferent fibres (LaMotte 1977; Gobel and Binck 1977; Light and Perl 1979; Light et al. 1979) and relay the nociceptive information to deeper neurones (Brown et al. 1976; Proschansky and Egger 1977) which then project to Vmo and excite V motoneurones. A test of antidromic activation of these two groups of neurones by electrical stimulation of Vmo, or selective micro-injection of ibotenic acid into the superficial or deep laminae of Vc of larger animals, should help address this matter.
CLINICAL IMPLICATIONS

Injection of algesic chemicals into the TMJ region produces a profound inflammatory response at the site of injection (Haas et al. 1992; Yu et al. 1994, 1995; also see previous chapters) and evokes a reflex increase in EMG activity of the jaw muscles (Broton and Sessle 1988; Yu et al. 1994, 1995; also see Chapters 2 and 4). Although recent reports have disputed whether chronic craniofacial pain may necessarily cause "hyperactivity" of the jaw muscles in humans (Lund et al. 1991, 1993; Lund and Sessle 1994), the present findings and earlier data from our laboratories suggest that an acute inflammation and pain in the TMJ region may reflexly and reversibly evoke an increase in EMG activity of the jaw muscles in rats and cats (Broton and Sessle 1988; Yu et al. 1994, 1995; also Chapters 2 and 4). The reflex co-activation of both jaw-opening and jaw-closing muscles following the induction of TMJ inflammation by mustard oil also suggests a "splinting" of the mandible. If such a phenomenon were to occur in humans, it might be expressed in some patients with a painful temporomandibular disorder (TMD) as a limitation of jaw movements and serve to prevent further damage to the already inflamed joint (see Bell 1986; Dubner et al. 1978; Laskin et al. 1983; Storey 1979); the neuroplastic changes induced by injection of mustard oil into deep craniofacial tissues (Hu et al. 1992; Yu et al. 1993; also see Chapter 5) have also provided explanations for the referral of pain, the lower pain threshold, and the altered sensation in the craniofacial region which may be observed in some TMD patients (see Sessle 1995).

There is an extensive overlap of the central projections of V and upper cervical sensory nerves (Pfaller and Arvidsson 1988; Ruggiero et al. 1981). Our findings that Vc, particularly its caudal component, is critically involved in the reflex activation of the jaw muscles, plus findings that mustard oil injection into the deep neck tissues (which are innervated by upper cervical nerves) also evokes an EMG increase in jaw muscles as
well as neck muscles (Hu et al. 1993), suggest that nociceptive inputs from craniofacial and deep neck tissues may converge at the brainstem/spinal cord junction and may be relayed to V motoneurones. These findings are supported by findings that small-diameter primary afferent fibres innervating a variety of craniofacial tissues project to Vc and upper cervical spinal segments (Arvidsson and Gobel 1981; Capra 1987; Hathaway et al. 1995; Jacquin et al. 1986; Kaube et al. 1993; Lu and Bereiter 1995; Nishimori et al. 1986; Pfaller and Arvidsson 1988; Ruggiero et al. 1981; Shigenaga et al. 1986; Strassman and Vos 1993; Takemura et al. 1991) and neurones in Vc and upper cervical spinal dorsal horn can be excited by noxious stimulation applied to these tissues (e.g. Dostrovsky et al. 1991; Sessle et al. 1986; Sessle and Hu 1991; Sunakawa et al. 1996; also see Chapter 5). Inputs from the V afferents also may be relayed to cervical spinal motoneurones (Abrahams and Richmond 1977; Burton and Loewy 1977; Hayashi et al. 1984; Sumino et al. 1981). These findings point to an overlap of central neural pathways underlying deep craniofacial and neck pain. Together with the neuroplastic changes (e.g. expansion of receptive fields and increase of activity) of central sensory neurones and increased excitability of motoneurones that can be induced by application of mustard oil to cutaneous and particularly deep tissues (Clarke et al. 1992; Hu et al. 1992; Woolf and Wall 1986; Yu et al. 1993), these findings suggest a possible substrate for the neural mechanisms underlying inflammation-induced referral of pain and reflex activation of several muscles in the craniofacial/neck region.

Excitatory amino acid receptors, especially the NMDA subtype, are involved in nociception and nociceptive reflexes (Henry et al. 1980; McMahon et al. 1993; Wilcox 1990; Yu et al. in press; also see Chapter 3). Glutamate-containing terminals and glutamate receptors have been found in the peripheral and central nervous system, including Vc (Azerad et al. 1992; Berger et al. 1995; Clements and Beitz 1991; Clements
et al. 1991; Magnusson et al. 1986; Shigemoto et al. 1992; Tallaksen-Greene et al. 1992; for review, see Collingridge and Lester 1989; Erdo 1991; Monaghan et al. 1989; Nakanishi 1992), and they have been suggested to be involved in mechanisms underlying neuronal "wind-up", thermal hyperalgesia, central sensitization and second pain (Davies and Lodge 1987; Dickenson and Sullivan 1987; Price et al. 1994; Ren et al. 1992, 1994). Mustard oil injection into the TMJ region induces a significant increase in glutamate release in Vc (Bereiter and Benetti 1996 in press) and EMG activity in the jaw muscles (Yu et al. 1994, 1995; also see Chapters 2 and 4) and micro-injection of glutamate into the caudal Vc also evokes an increase in EMG activity of the jaw muscles (see Chapter 3). Our previous study has also shown that i.v., i.c.p. or local (into the TMJ region) pre-administration of the non-competitive NMDA antagonist MK-801 (for review, see Collingridge and Lester 1989; Wong and Kemp 1991) blocks the increased jaw muscle EMG activity evoked by mustard oil injection into the TMJ region (Yu et al. 1996). These findings thus point to both peripheral and central NMDA receptor mechanisms in craniofacial nociception and nociceptive reflexes. They also suggest possible directions for clinical research into the management of TMD, viz. in according with the suggestion of Denucci et al. (1996) for the pharmacological control of pain associated with TMD, they suggest the possible clinical efficacy in such conditions of NMDA antagonist drugs so as to reduce the peripheral inflammation and the activation of glutamate/NMDA receptors in the peripheral and central nervous system.
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IMAGE EVALUATION
TEST TARGET (QA-3)

1.0
1.1
1.25
1.4
1.6

2.5
2.2
2.0
1.8

150mm

6"

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