Neural circuits engaged in mastication and orofacial nociception

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Omistettu miehelleni Dimitrille ja tyttärellemme Zoelle

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ABSTRACT

A deeper understanding of both movement control and the effects of nociceptor inputs on our motor systems is critical for proper clinical diagnosis of musculoskeletal dysfunctions and for development of novel rehabilitation schemes. In the jaw system, masticatory movements are produced by a central pattern generator (CPG) located in the brainstem. Considerable efforts have been made in deciphering this neuronal network. The present thesis contributes towards an increasingly detailed understanding of its essential elements, and presents a hypothesis of how deep somatic pain (i.e. muscle pain) may be evoked and interferes with the masticatory CPG circuitry.

In Paper I, the expression of c-Fos-like protein was used as a molecular marker to visualize brainstem neurons that were active during induced fictive mastication in the anesthetized and paralyzed rabbit. Our findings provide a previously lacking detailed record of the neuronal populations that form the masticatory motor pattern. Certain cells were located in brainstem areas previously suggested to be involved in the masticatory CPG. However, it was a new finding that neurons in the dorsal part of the trigeminal main sensory nucleus (NVsnpr-d) may belong to this circuitry. Paper II focused on the discovered neurons in NVsnpr in an *in vitro* slice preparation from young rats. Intracellular recordings allowed us to define two cell types based on their response to depolarizing current. Microstimulation applied to the trigeminal motor nucleus, its reticular border, the parvocellular reticular formation and the nucleus reticularis pontis caudalis, elicited postsynaptic potentials in 81% of the neurons tested. Responses obtained were predominately excitatory and sensitive to glutamatergic antagonists DNQX or/and APV. Some inhibitory and biphasic responses were also evoked. Bicuculline methiodide or strychnine blocked the IPSPs indicating that they were mediated by $GABA_A$ or glycinergic receptors. About one third of the stimulations activated both types of neurons antidromically. Neurons in NVsnpr-d seem to gather all the conditions that can theoretically account for a role in masticatory rhythm generation.

In Paper III, the masticatory model system was used to investigate the possible role of muscle spindle primary afferents in development of persistent musculoskeletal pain. Following intramuscular acidic (pH 4.0) saline injections of rat masseter muscles, *in vitro* whole cell recordings were done from jaw closing muscle spindle somata located in the trigeminal mesencephalic nucleus (NVmes). Compared to control neurons, the somata of afferents exposed to acid had more hyperpolarized membrane potentials, more hyperpolarized thresholds for firing, high frequency membrane oscillations and ectopic bursting of action potentials. These changes in membrane properties lasted for up to 35 days. Within the same time frame experimental animals showed hypersensitivity to touch on the skin covering the injected muscle. Similar saline injections also resulted in a significant increase of activity dependent c-Fos expression in NVmes neurons compared to controls. Immunofluorescence and lectin binding studies indicated that small-caliber muscle afferents containing known nociceptor markers (CGRP, SP, $P2X_3$, TRPV1 and IB4) and expressing glutamate receptors are found close to the annulo-spiral endings of the NVmes afferents. Combined, our new observations support the hypothesis that excessive release of glutamate, within muscle spindles due to ectopically evoked antidromic action potentials, could lead to development of persistent musculoskeletal pain by activation and/ or sensitization of adjacent muscle afferent nociceptors.

Key words: rhythmical jaw movements; central pattern generator; trigeminal system; rabbit; Rat; c-Fos, nociceptors; muscle pain; muscle spindle afferents; glutamate

ORIGINAL PAPERS

This thesis is based on the following papers that will be referred to in the text by their Roman numerals:

I. **Athanassiadis, T.**, Olsson, K.Å., Kolta, A. & Westberg, K-G. (2005) Identification of c-Fos immunoreactive brainstem neurons activated during fictive mastication in the rabbit. *Experimental Brain Research*, **165**, 478-489.

II. **Athanassiadis, T.**, Westberg, K.G., Olsson, K.Å. & Kolta, A. (2005) Physiological characterization, localization and synaptic inputs of bursting and non-bursting neurons in the trigeminal principal sensory nucleus of the rat. *European Journal of Neuroscience,* **22**, 3099-3110.

III. Lund, J.P., Sadeghi, S., **Athanassiadis, T.**, Caram-Salas, N., Auclair, F., Thiverge, B., Arsenault, I., Rompré, P., Westberg, K-G. & Kolta, A. (2009) Evidence that muscle spindle mechanoreceptor afferents play a role in chronic muscle pain. *Submitted manuscript*.

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ABBREVIATIONS

c-Fos:	*c-Fos-like immunoreactivity*
mPeriV:	*Medial part of the reticular borderzone of the trigeminal motor nucleus (Regio h)*
NVmes:	*Trigeminal mesencephalic nucleus*
NVmt:	*Trigeminal motor nucleus*
NVmt-dig:	*Digastric motoneuron subnucleus of the trigeminal motor nucleus*
NVmt-mass:	Masseteric *motoneuron subnucleus of the trigeminal motor nucleus*
NVsnpr:	*Main sensory trigeminal nucleus*
NVsnpr-d:	*Dorsomedial part of* the m*ain sensory trigeminal nucleus*
NVspo-β:	*Subnucleus-β of the oral nucleus of the spinal trigeminal tract*
NVspo-γ:	*Subnucleus-γ of the oral nucleus of the spinal trigeminal tract*
NintV:	*Intertrigeminal nucleus*
NsV:	*Supratrigeminal nucleus*
NVII:	*Facial motor nucleus*
NXII:	*Hypoglossal motor nucleus*
Regio h (Paper I):	*Reticular borderzone of the trigeminal motor nucleus comprising NsV, NintV and mPeriV*
PeriV (Paper II):	*The reticular borderzone of the trigeminal motor nucleus comprising NsV, NintV and mPeriV*
Rgc:	*Nucleus reticularis gigantocellularis*
Rgc-α:	*Pars α of nucleus reticularis gigantocellularis*
Rpc:	*Nucleus reticularis parvocellularis*
Rpc-α (Paper I):	*Pars α of nuclei reticularis parvocellularis*
PCRt (Paper II):	*Pars α of nuclei reticularis parvocellularis*
RPc (Paper I):	*Nucleus reticularis pontis caudalis*
RPc-α (Paper I):	*Pars α of nucleus reticularis pontis caudalis*
NPontc (Paper II):	*Nucleus reticularis pontis caudalis and its pars α*

Additional abbreviations are given in the text

INTRODUCTION

Fundamental studies on motor behaviors, that allow us to eat, talk, drink, and to express our feelings, are of great interest to oral biology and the medical professions. When eating, mastication is the first step in the feeding process in which food is prepared for further digestion and absorption in the gastrointestinal tract. The eating behavior is also recognized to have an important social function, because it contributes significantly to the general well-being of humans. Therefore, a healthy masticatory function will have favorable influences both on an individual's nutritional status and on life satisfaction (see: Brennan *et al*., 2008).

On casual inspection, chewing can appear repetitive and stereotyped (i.e. jaw opening and jaw closing). However, it is recognized that mastication involves a sequence of precisely coordinated movements of the mandible from the ingestion of a food morsel to its final swallow (Luschei & Goodwin, 1974; Orchardson & Cadden, 1998; Woda *et al*., 2006). When analyzed in detail, a masticatory sequence can be divided into several distinct components that comprise a number of characteristic jaw movement cycles (Schwartz *et al*., 1989). In the first component, called the "preparatory" or "manipulative" phase, food is placed within the anterior parts of the mouth and split into smaller pieces. During the following component, the "reduction phase", food is moved from the anterior to the posterior teeth by movement of the cheeks and tongue to be split up by grinding between the molar teeth. During the third component, the "pre-swallowing" phase, coordinated activities in mylohyoid, anterior digastric, medial pterygoid and tongue muscles transport the bolus of pulverized materials, mixed with saliva, towards the pharynx where it is prepared for swallowing (see: Lund, 1991; Jean, 2001).

The repertoire of movements required during normal mastication is generated by the combined actions of more than 20 orofacial muscles or muscle portions (compartments), which are able to control movement trajectories of the mandible as well as directions and magnitudes of force vectors acting on the teeth (see: Korfage *et al*., 2005a, b). These muscles are controlled by nervous circuits that generate the motor programs needed to activate individual muscles or muscle compartments according to task requirements. For instance, during a biting task, when a piece of food is split between the front teeth, the vertical force can readily reach more than 100 N. In humans this force is generated by activation of certain compartments both within the temporal and masseter muscles (Blanksma & van Eijden, 1995). In contrast, during the reduction phase, there is a combination of vertical and horizontal

forces that fragments the food particles between posterior teeth (Lund, 1991). In simplified terms, the vertical force vector is mainly generated by actions of the deep anterior portion of the masseter muscle and the anterior part of the temporal muscle, while activities in the lateral and medial pterygoid muscles produce horizontal force components (see: Hannam & McMillan, 1994). It is clear that the control of mandibular movements and of force acting on the occlusal surfaces of teeth is based on a dynamic context dependent selection of motor units. How the setting of different parameters to control jaw muscle activity during mastication are governed by the peripheral and central components of the nervous system is an interesting question for analysis (see: Kawamura, 1972, Landgren & Olsson, 1977, Olsson, 1979; Westberg, 1990; Trulsson, 1993). Moreover, any interference with these salient and often not fully appreciated orofacial functions, may sometimes, under certain circumstances, have an often unexpected and most disturbing significance to the patient. Pain, particularly persistent musculoskeletal pain in the orofacial and cranial region is an example of a condition that may severely compromise these normal functions.

Mastication

A central pattern generator evokes the masticatory rhythm

In the first part of the last century, Sherrington (1917) proposed that a chain of jaw opening and closing reflexes formed the basic units of masticatory movements. When stimulating the oral mucosa bordering the teeth or hard palate of decerebrated cats, he observed a brisk opening of the jaw, which was immediately followed by a short latency jaw closing reflex elicited by the stretch evoked input from activated muscle spindles in the jaw closing muscles. According to Sherrington, this "*would bring into operation once again the jaw-opening stimulus. And so, after being started by a first bite, a rhythmic masticatory reflex tends to keep itself going so long as there is something biteable between the jaws*". When Sherrington made his observations it was already known that "masticatory-like" movements could be induced from the cerebral cortex of different animal species (Ferrier, 1886). Later on, it was also shown by several authors that repetitive electrical stimulation of areas at the most lateral cortical precentral gyrus and the adjacent post central gyrus, both in man and primates, not only elicit one type of jaw movement but a variety of masticatory related orofacial movements including rhythmical jaw movements accompanied by coordinated

rhythmical tongue movements as well as secretion of saliva (see: Kawamura, 1972; Luschei & Goldberg, 1981; Lund, 1991; Nakamura & Katakura, 1995).

In early work, Bremer (1923) divided the rabbit's cortical masticatory area (CMA) into three compartments according to the different forms of naturally resembling masticatory movements produced by cortical surface stimulation. Further detailed intracortical microstimulation of anterior sites within the rabbit CMA was shown to elicit vertical movements resembling those seen during the "preparatory" phase of mastication, while stimulation of posterior sites evoked grinding like movements characterized by a vertical movement of the mandible followed by a laterally directed swing during jaw closure as seen during the "reduction" phase of mastication (Lund *et al*., 1984). This indicated that masticatory like movements were represented in a kinesiotopic like manner at the cortical level and that these subareas may play a role in adapting certain aspects of the masticatory motor outputs to the needs of the animal (see: Lund *et al.*, 1998; see also: Hatanaka *et al*., 2005). Since the cortically induced rhythmic jaw opening and closing movements indeed resemble natural masticatory movements, this strongly suggested that the neuronal elements involved also take part in the generation of natural mastication. Because of these arguments, the CMA induced rhythmical jaw movement, has been used as a model to study the basis of the natural masticatory behavior.

To explain the fact that rhythmical mastication could be evoked by stimulation of the cortical masticatory area, Rioch (1934) proposed a modified version of Sherrington´s reflex-chain theory. According to her hypothesis, mastication is initiated when tonic activation of descending paths "*relaxes the jaw closers and excites the jaw openers*". The resulting jaw opening movement then evokes a stretch reflex induced jaw closing. However, as the jaw closes, the stretch dependent muscle spindle input gradually ceases and relaxation of the jaw closers again occurs. Alternate reflex based cycles of closing and opening movements are then repeated as long as tonic inputs from the cerebral cortex inhibit jaw closing motoneurons and excite jaw opening motoneurons.

The first evidence that masticatory movements are not dependent on reflex actions but are generated by a central neural pattern generating circuit, referred to as the masticatory central pattern generator (CPG), came from experiments conducted by Lund & Dellow (1971). They showed, that repetitive electrical stimulation of descending corticobulbar pathways at the pontine level in paralyzed decerebrate rabbit evoked alternating masticatory like bursts in jaw opening and closing motor nerves (i.e. fictive mastication). Thus, once initiated rhythmic mastication does not require phasic cycle by

cycle feedback from peripheral afferents as postulated in the earlier models. During normal function, however, a fully balanced behavioral expression depends on a dynamic dialogue between both central and peripheral nervous control mechanisms (see: Olsson & Westberg, 1989; Lund, 1991; Nakamura & Katakura, 1995; Trulsson & Johansson, 1996). For instance, sensory information from the mouth has to interact with the centrally generated rhythm to produce the variations in cyclic oral behavior that occur with food texture and consistency changes during the triturating process (see: Woda *et al.,* 2006). This is illustrated by the fact that intra- and perioral deafferentation influence on the normal spatiotemporal sequence of muscle activetions and thereby reducing masticatory efficiency (see: Lund, 1991). The motor effects of sensory inputs from jaw muscle spindles and periodontal mechanoreceptors, which both have movement phase modulated signaling during mastication, have been particularly well studied (Olsson *et al.*, 1986b; Masuda *et al.*, 1997). During ongoing mastication these inputs boost firing frequency and burst duration in jaw closing motoneurons. They can thereby induce appropriate motor adjustments during the closing phase to overcome the increased resistance that occurs when teeth contact the food morsel (Lavigne *et al.*, 1987; Morimoto *et al.*, 1989). These "positive feedback" effects have been considered to be mediated via short latency reflex paths. However, motoneuron firing can not be expected to be properly adjusted in a simple (online) sensory feedback manner because masticatory movements are fast and reflex loops are slow, due to nerve conduction and synaptic delays. It is therefore likely, that they are regulated in a predictive (feedforward) manner where the characteristics of the upcoming movement cycle are preprogrammed by support of information from orofacial sensory organs gathered during previous cycles (see: van der Bilt *et al.*, 2006; see also: Wolpert & Flanagan, 2001; Johansson & Flanagan, 2009). However, there are also some evidence that parts of the movement generated sensory inflow can be used for reflex mediated (feedback) corrections when unexpected perturbations (i.e. "prediction errors" of food properties) occur, for instance at the moment of contact between the bolus and the teeth (Komuro *et al.*, 2001; see also: Lund & Olsson, 1983).

Proposed localization and organization of the masticatory CPG

Conceptual CPG models for coordinated rhythmic movements such as locomotion and respiration generally postulate the involvement of two essential components; one for *rhythm generation*, which produces the oscillatory onset and offset of the involved motoneurons during each movement cycle and another for *intra-cycle motor pattern generation*, which coordinates and shapes the spatiotemporal pattern of motoneuron outflow during a given

movement task (see: Grillner, 2006; McCrea & Rybak, 2008). At the network architectural level, rhythm generation and pattern formation have been viewed as either subserved by the same set of neurons or by separate groups of neurons. In the trigeminal motor system, some evidence have indicated the latter to be the case, because blockade of glycinergic synapses with strychnine "*do not substantially alter*" the frequency of cortically evoked rhythmical EPSPs in trigeminal motoneurons, but produce significant effects on their burst durations (see: Goldberg & Chandler, 1990). Thus, the absence of chloride mediated postsynaptic inhibition does not seem to influence on the operation of the cycle-timing network, but does affect the network responsible for intra-cycle pattern generation (Goldberg & Chandler, 1990).

In the 1980s, Japanese and American research groups were the first to localize neuronal populations in the brainstem that could constitute the rhythm generating circuitry of the masticatory CPG (Nozaki *et al*., 1986a, b; Chandler & Tal, 1986). By doing transection experiments in an anaesthetized and paralyzed guinea pig model, they were able to demonstrate that a circumscribed block of neural tissue in the medial bulbar reticular formation between the obex and the facial nucleus (NVII), seemed to contain the minimal circuitry needed to generate alternate masticatory like activity in the jaw opening and closing motor nerves (see Fig 1A, B).

In subsequent electrophysiological experiments, Nozaki *et al*., (1986b) further showed that the rhythm was generated in a two stage process, when evoked by stimulation of the CMA. In the first stage, crossed monosynaptic corticobulbar inputs, which travel in the pyramidal tract, induced tonic firing of neurons located in the contralateral paragigantocellular nucleus (Rgc-α; Fig 1B). These were connected to a nearby group of neurons in the giganto-cellular nucleus (Rgc; Fig 1B) and, when excited, the Rgc neurons started to fire rhythmic bursts of action potentials with the same rhythm as the fictive masticatory motoneuron output recorded from the digastric (jaw opening) motor nerve. These latter neurons were therefore suggested to represent the output stage of a rhythm generating network based on two interacting neuronal elements. This type of network was found on both sides of the brainstem. Although each was capable of generating independent rhythms to left and right side trigeminal motoneurons, they were suggested to work together during natural conditions because of interconnecting commissural axons that cross the brainstem (see: Nakamura & Katakura, 1995).

However, additional transection studies have shown that more rostral brainstem nuclei also seem to hold neurons capable of generating trigeminal rhythmogenesis (Kogo *et al*., 1996; Tanaka *et al*., 1999; Enomoto *et al*., 2002). By using an *in vitro* isolated brainstem preparation from the neonatal

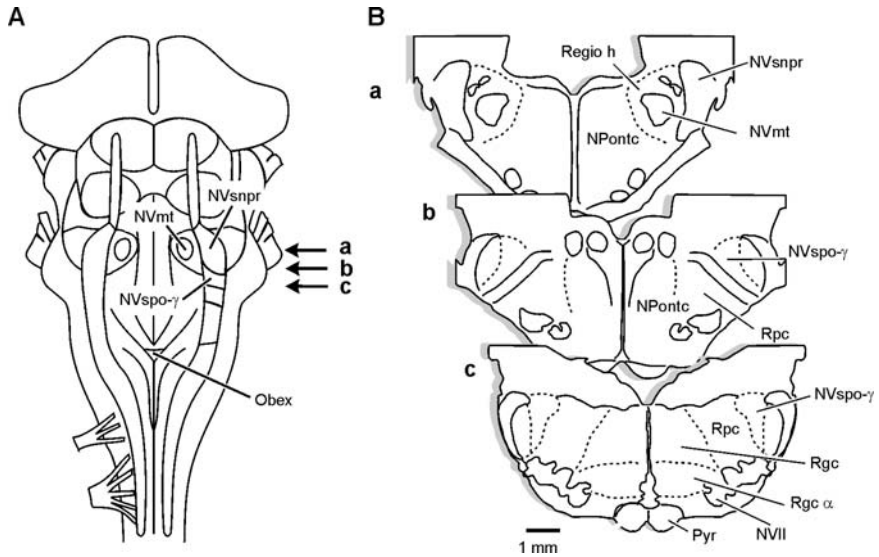

Figure 1. Diagram showing location of trigeminal and brainstem nuclei suggested to be involved in the generation of rhythmical masticatory jaw movements. **A**. Drawing of the brainstem seen from a dorsal view where outlines of the left and right trigeminal nuclei are given. Borders between the subnuclei are indicated to the right (From: Landgren & Olsson, 1977). Arrows mark level of sections shown in B. **B.** Illustration of coronal histological sections to schematically denote the location of certain nuclei within the brainstem.

NVsnpr: main sensory trigeminal nucleus; *NVmt:* trigeminal motor nucleus; *NVspo-*γ : subnucleus-γ of the oral nucleus of the spinal trigeminal tract; *Rgc-*α*:* pars α of nucleus reticularis gigantocellularis; *Rgc:* nucleus reticularis gigantocellularis; *NPontc:* nucleus reticularis pontis caudalis; *Rpc:* nucleus reticularis parvocellularis; *Regio h:* reticular borderzone of the NVmt; *Pyr:* pyramid; *NVII:* facial motor nucleus.

rat, these authors showed that masticatory rhythm could still be recorded from the motor branch of the trigeminal nerve, when brainstem regions caudal to the facial nucleus were disconnected by a coronal transection. In addition, when further transections rostral to the facial nucleus were made, the induced rhythm "*progressively deteriorated until the trigeminal motor nucleus was reached, at which all activity ceased*" (Tanaka *et al*., 1999). In line with this observation is the finding that mastication is severely compromised in *krox-20* knockout mice, in which the third and fifth rhombomeres fail to develop normally (Jacquin *et al*., 1996). This part of the brainstem comprises the main sensory trigeminal nucleus (NVsnpr); the reticular borderzone (Regio h) surrounding the trigeminal motor nucleus (NVmt); the parvocellular reticular formation (Rpc); the rostral division of the oral

subnucleus of the spinal trigeminal tract (NVspo-γ) and the nucleus reticularis pontis caudalis (NPontc). These areas have all been implicated to form part of the CPG because they contain neurons that receive convergent descending and oro-facial primary afferent inputs and that fire rhythmically in phase with the jaw motoneurons during induced fictive mastication (Olsson *et al*., 1986a, b; Donga *et al*., 1990; Donga & Lund, 1991 Westberg & Olsson, 1991; Inoue *et al*., 1992, 1994; Westberg *et al*., 1995, 1998; Tsuboi *et al*., 2003).

It is well known that the output of a particular CPG (i.e. its motor pattern) depends on multiple mechanisms including the synaptic connectivity between the involved neurons and their intrinsic properties (see: Marder & Bucher, 2001; Grillner, 2006). The former decides possible avenues of information transfer between cells, while the latter determine how these synaptic signals are processed before being conveyed to other cells in the network. Despite noteworthy advances on the organization of the masticatory CPG, discussed above, much still remains unknown. Therefore, to gain a better understanding of this particular network, further studies are needed to identify neurons that comprise the circuitry, to delineate their synaptic interactions and to investigate their intrinsic membrane properties (i.e. ion channel subtypes).

Due to certain restrictions inherent to electrophysiological studies in acute experiments on anesthetized animals the possibilities to localize neurons forming the core of the masticatory CPG are limited. Nevertheless, modern neurobiological techniques offer new alternative means to identify and differentiate neurons belonging to the masticatory CPG microcircuitry at a cellular level. For example, several immediate-early-genes are useful in order to detect transsynaptically activated neurons (see: Morgan & Curran, 1991; Herdegen & Leah, 1998). The rapid and transient expression of these gene products has allowed them to be used as “functional activity markers” (see: Sagar *et al*., 1988; Herrera & Robertson, 1996; Kovács, 1998; Harris, 1998). The most frequently studied of these genes is the *c-fos*, which protein product (c-Fos) can be visualized by immunohistochemistry (see: Dragunow & Faull, 1989). This technique has been used to map neuronal groups in the mesencephalic locomotor region, caudal brainstem and spinal cord activated during natural and fictive limb movements such as scratching and locomotion (see: Barajon *et al*., 1992; Noga *et al*., 2009). The development of single cell recordings in trigeminal slice preparations and studies of circuit properties in isolated brainstem preparations of young rats have also opened up new possibilities to expand our knowledge on details concerning the connectivity and function of the masticatory CPG microcircuit (see: Lund *et al*., 2009). These methods have been applied in this thesis.

Orofacial musculoskeletal pain

Pain and jaw motor function

Normally, jaw motor function, whether during mastication or during speech articulation, permits the execution of precise and highly coordinated movements of the mandible. However, in individuals suffering acute or persistent pain from the orofacial, neck and cranial regions, the output of the masticatory CPG is influenced by the nociceptive inputs, in a manner different from what is the case during physiological conditions. The most typical motor disability being reduced amplitude and velocity as well as irregular jaw movements. When in pain, patients are reported to complain that their ability to bite and chew not only hurts but are more difficult to perform. They also have difficulties in opening their mouth and when closing they get the feeling that their bite seems to have changed (for review see: Stohler, 1999; Svensson 2007; Lund, 2008). At the beginning of the 1980s, pain and dysfunctional symptoms or signs, such as limitations of jaw opening, asymmetric movements and temporomandibular joint sounds, were given the all inclusive label temporomandibular disorders (TMD), after having had a terminology that changed over the years alluding to the different suggestions of the origin, ethiology and pathogenesis.

The diagnosis and treatment of orofacial pain, especially with regard to management of temporomandibular disorders, has been most problematic for many decades (see: Greene & Laskin, 2001; Laskin, 2008). However, the field of orofacial pain is undergoing a radical change, primarily because of pain physiology and pharmacology becoming an extremely active area of research (see: Dubner & Ren, 2006; Costigan *et al*., 2009). The increased knowledge, has led to the view that the TMD disorders presently are defined as a collective term that embraces distinct clinical problems that engage the (1) masticatory muscles (myogenous TMD), (2) the temporomandibular joint (arthrogenous TMD) and (3) the associated structures (deLeeuw, 2008). This improved concept of TMDs, under further consideration, indicates that the different disturbances are part of the constellation of musculoskeletal disorders requiring a broad biomedical perspective to understand and manage within a proposed bio-psycho-social framework (see: McNeill *et al*., 2008; Maixner, 2008).

Primary afferents and persistent pain

Pain is defined as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" (Loeser & Treede, 2008). As pain is a conscious experience, it is an interpretation influenced by memories, emotional, pathological, genetic and cognitive factors. Pain is, therefore, a highly subjective experience, as illustrated by the definition.

The sensation of acute superficial (cutaneous) pain or nociceptive pain is evoked by activation of thinly myelinated (Aδ) and unmyelinated (C) fibers that respond to noxious stimuli with a first rapid, well localized, sharp pain, and a second delayed more diffuse, dull pain. Acute pain stops rather quickly after the noxious stimuli have been removed and is seldom referred. Superficial somatic pain caused by injury and the subsequent inflammatory response to aid healing and repair leads to increased neuronal excitability due to peripheral sensitization of the nociceptors (inflammatory pain). The prolonged neuronal discharges cause central sensitization of neurons in the central nervous system, manifesting as increased pain response in and around the injured skin area to a noxious stimulus (secondary *hyperalgesia*) and painful response to a stimulus that is normally not painful (*allodynia*). Typically, inflammation pain disappears after resolution of the initial tissue injury. However, if it becomes persistent and maladaptive ("chronic"), the pain may last for years and be considered a disease. The mechanisms underlying the chain of processes leading to persistent pain, which are not yet fully understood, may include, among others, peripheral sensitization.

Muscle pain, differs from cutaneous pain in many ways. Thus, the sensory manifestation of deep somatic pain in a muscle, is described as a poorly localized, tearing, cramp-like diffuse aching. It is often referred to other deep somatic tissues and is difficult to tolerate. This pain is evoked by activation of muscle nociceptors, which are the free nerve endings of group III (Aδ-fiber) and group IV (C-fiber) muscle afferents (Mense, 1993). The endings contain substance P (SP), calcitonin gene-related peptide (CGRP), somatostatin and vasoactive intestinal peptide. The muscle nociceptors, like cutaneous nociceptors, are equipped with a multitude of membrane receptors for noxious mechanical, chemical and thermal stimuli including, among others, inflammatory substances, protons, ATP, growth factors and glutamate (see: Julius & Basbaum, 2001; Mense, 2009). Transition to persistent musculoskeletal pain syndromes may be induced following either inflammatory or non-inflammatory states. In noninflammatory conditions, minimal or no peripheral tissue damage is seen. Peripheral and central

sensitization act together and play important roles in maintaining these pain conditions, but the initiators of the changes are only at the beginning of being unraveled (see: Mense, 2008).

In the orofacial region, most of the sensory neurons that innervate low threshold mechanoreceptors and nociceptors, have their cell bodies in the trigeminal ganglion, which is the equivalent to the dorsal root ganglia in the spinal cord. However, the primary muscle spindle Ia afferents of jaw closing muscles and some of the afferents, which innervate periodontal mechanoreceptors, have their cell bodies located in the trigeminal mesencephalic nucleus (NVmes) of the brainstem (Jerge, 1963). The NVmes neurons have intrinsic electrical properties, resembling those of dorsal root ganglion neurons, which allow them to generate membrane bistability and fast oscillations for prolonged periods of time (Pedroarena *et al*., 1999, Verdier *et al*., 2004, Wu *et al*., 2001). They also have the unique characteristic to receive synaptically transmitted inputs, which may play a role in central modulation of peripherally generated signals (Lazarov, 2002).

The possible importance of fast conducting large diameter myelinated afferent fibers in persistent pain is presently under serious examination. It has been observed that this group of spinal primary afferents may generate inappropriate action potentials following injury (Amir *et al*., 1999; Hutcheon & Yarom, 2000). This impulse generation has been suggested to arise at the site of injury ("ectopic electrogenesis"). However, in early work Wall & Devor (1983) reported that such firing also occurs at the cell soma in the dorsal root ganglion. Action potentials fired at the soma of primary afferents is considered to be ectopic, since it is generally assumed that normal firing of these neurons is generated in peripheral sensory receptors. This type of firing has been suggested to contribute to the initiation and development of neuropathic pain since it constitutes a direct signal that can trigger and maintain central sensitization (see: Devor, 2005).

There is the possibility that similar changes could also play a role in the development of non-neuropathic (dysfunctional) pain states, such as myofacial pain (myogenous TMD) and fibromyalgia. Therefore, a more detailed understanding of the role of large caliber primary muscle afferents in the development of persistent muscle pain is of great interest to explore.

AIMS OF THE THESIS

Paper I

The first aim of this thesis was to identify neuronal groups in the brainstem that could be part of the masticatory CPG. This was done by using the c-Fos technique to localize populations of brainstem neurons that were activated during the evoked masticatory-like motor behavior in anesthetized and paralyzed rabbits.

Paper II

The second aim was to investigate if neurons in the main sensory trigeminal nucleus (NVsnpr), and one of the identified neuronal populations in Paper I, had the appropriate functional properties that would allow them to form part of the masticatory CPG. This was done by using combinations of sharp intracellular recordings, microstimulations and pharmacological techniques, in an *in vitro* brainstem slice preparation taken from young rats.

Paper III

The third aim was to test the hypothesis that a change in electrical membrane properties of trigeminal muscle spindle afferents could contribute to the development of persistent musculoskeletal pain. This was done in experiments on rats with a combination of behavioral, electrophysiological and immunohistochemical techniques.

MATERIALS AND METHODS

A brief summary of the experimental set-ups is given below. Detailed descriptions of the methods and data analysis are presented in the respective papers. All procedures had been approved by the institutional animal care committee in Umeå and complied with the European Communities Council (86/609/ECC) directive and the Canadian animal committee for laboratory animal care and use.

Paper I

Fictive mastication and expression of c-Fos in brainstem neurons

All experiments were carried out on urethane anesthetized and paralyzed New Zealand White rabbits.

These experiments were performed on ten animals that were separated into an Experimental group (n=5) and a Control group (n=5). Surgery was followed by a recovery period of at least one hour. After this, bouts of masticatory like jaw movements were evoked by electrical stimulation of the CMA on the left sensory-motor cortex in the Experimental group. A recording electrode was positioned within the digastric motoneuron pool of the trigeminal motor nucleus on the right side of the brainstem to monitor rhythmic motoneuron activity under muscle paralysis. The bouts of masticatory-like motor activity usually lasted 15-120 seconds and were induced at regular intervals separated by rest periods of about 2-5 minutes. The total accumulated evoked movement time varied between 60-130 minutes during a 3.5-5.5 h period. The cortical microstimulation was placed so that rhythmical jaw movements, evoked in the nonparalyzed state, resembled the motor output seen during the “reduction” stage of natural mastication i.e. a lateral swing of the mandible during the closing phase of the movement cycle. These types of jaw movements were chosen because they correspond to the motor activity observed during the main part of the masticatory behavior (see: Lund *et al.*, 1984; Schwartz *et al.*, 1989; Westberg *et al.*, 1998). The animals of the Control group were treated in the same way as the Experimental animals with regard to surgery, paralysis and artificial ventilation. However, no fictive jaw movements were induced.

To detect the c-Fos-like protein, the animals were perfused and their brainstems were cryosectioned and processed immunocytochemically. The c-Fos neurons were recognized by their dark brown precipitate of the DAB reaction. Every second section, from the level of the rostral limit of the trigeminal motor nucleus to obex, was examined and profiles of positively stained neuronal cell nuclei were counted. Because there was consistency in nuclear size and since the nuclear diameters were small in comparison with the section thickness used (see: Coggeshall & Lekan, 1996; Ma *et al.*, 2001), the total number of nuclear profiles was not corrected for split nuclei. All experiments were analyzed in random order before the statistical analysis, in order to avoid counting bias. Some brainstem nuclei were also re-examined at a different occasion and the two counts of stained neurons were then compared to evaluate the intraexaminer bias, which turned out to be small.

Paper II

In vitro recorded NVsnpr neurons

All experiments were carried out on Sprague-Dawley rats. Intramuscular injections were carried out under cryoanesthetized conditions.

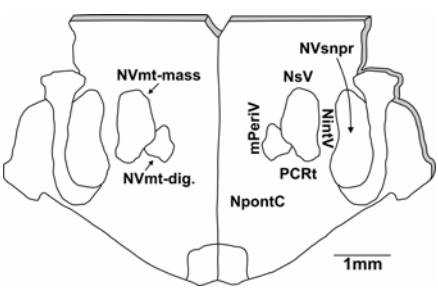

Figure 2. Illustration of a brainstem section, which shows the areas where microstimulation was applied. *NsV*: supratrigeminal nucleus; NintV: intertrigeminal nucleus; *PCRt*: nuclei reticularis parvocellularis pars α; *mPeriV*: medial Regio h; *NPontc*: nucleus reticularis pontis caudalis; *NVmt-mass* and *NVmt-dig*: masseterric and digastric subnuclei of NVmt, respectively. The recording electrode was positioned within the trigeminal main sensory nucleus (*NVsnpr*).

In these experiments masseteric and digastric motoneurons were labelled by muscular injections of $DiIC_{18}$ and cholera toxin conjugated to fluorescein of rats (0-2 days old). Nine to twenty-five days later the animals were quickly decapitated. Their brainstems were rapidly removed and immersed in oxygenated cutting solution. After sectioning the slices, containing the NVsnpr and NVmt, were transferred into an interface-type chamber and recording commenced after 1 h incubation (Kolta, 1997). Intracellular recordings were carried out with conventional sharp electrodes and intrinsic membrane properties were electrophysio-

logically characterized. Neurons were included in the study if they had stable resting membrane potentials, more negative than -50 mV, and overshooting action potentials. In order to evoke synaptic responses from defined brainstem nuclei, extracellular bipolar nichrome electrodes (25 μm diam) were used. The stimulus intensity and duration (200 ± 33 μA and 0.15-0.25 ms) was adjusted subthreshold for action potential initiation.

Figure 2 shows the areas of investigation in the brainstem slice. Retrogradely stained motoneurons were visualized with an epifluorescence microscope and were used as guidance for positioning the electrodes in brainstem areas known from our *in vivo* rabbit model to contain masticatory CPG-related interneurons. Excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) were identified on the basis of their voltage dependency and their responses to bath applied antagonists of glutamate-ergic, glycinergic or $GABA_A$ receptors. Some of the recorded cells were injected with neurobiotin for morphological examination.

Paper III

Role of muscle spindle mechanoreceptors in persistant muscle pain

All experiments were conducted on Sprague-Dawley rats. Intramuscular injections were carried out under Isoflurane anesthesia.

Analysis of nocifencive behavior

Twelve rats were used, of which 6 were given an injection of 0.9% NaCl (pH 7.2) into each masseter muscle (Control group) and the remaining 6 received bilateral injections of acidic saline (pH 4; Experimental group). The injections were repeated three days later, following the protocol established by Sluka *et al.* (2001). Sensory testing was done with a series of von Frey filaments, which were applied in order of increasing load (maximum loads of 10, 15, 26 and 60 g). Testing was repeated 10 times for each muscle. The filaments were applied at the center of the skin covering the muscle for 1s with 2s between applications and the number of responses was noted. Testing was performed on 3 Control and 3 Experimental rats on each of three days prior to the injections. All animals were then tested on days 5, 7, 10, 17, 24, 31, 38 and 45 after the first injection. In the other 6 animals (3

Experimental, 3 Control), additional testing was done on the day after each of the injections, to look for early changes in nocifencive responsiveness.

Analysis of c-Fos expression

Thirty-three rats (13-16 days old) were used, of which 5 animals did not receive any injections and served as unoperated Controls. Animals in the Experimental group (n=14) were injected with 20 µl of 0,9% NaCl (pH 4) into the ipsi- and contralateral masseter muscle. The Control animals (n= 14) were injected in the masseter muscles with the same volume of NaCl with pH 7.2. Four hours (n=8), 24 h (n=10) or 96 h (n=10) after the injections, the animals were perfused. To detect the c-Fos-like protein, the brainstems were cryosectioned and processed immunocytochemically. Profiles expressing c-Fos labeling on both sides were counted on every second section obtained from Control and Experimental animals by an examiner who was unaware of the group's experimental condition. NVmes neurons, immunoreactive to c-Fos, were counted bilaterally from the rostral level of NVmt to the level of the rostral pool of the oculomotor nucleus.

Analysis of masseter muscle spindle afferent electrical properties

In these experiments the somata of masseter muscle spindle afferents were retrogradely labelled by injections into the masseter muscle of Cholera toxin β subunit conjugated to Alexa Fluor 488. Seventy-one rats were divided into three groups; (1) bilateral injections of 0.9% NaCl (pH 4) into the masseter muscles; (2) injections of 0.9% NaCl, pH 7.2, into one masseter, and with pH 4 on the opposite side; (3) bilateral injections of 0.9% NaCl (pH 4). A second injection was given two days later. Electrophysiological experiments were carried out at 7 different times after the second injection: T1: 2-8 hrs; T2: 1 day; T3: 2-5 days; T4: 6-8 days; T5: 9-11 days; T6; 12-14 days; T 7: 32-35 days.

At least 4 days after tracer injections, animals were decapitated, the brain was quickly removed and coronal brainstem slices were cut and transferred to a submerged type chamber for cell recordings. Labelled cells were identified using epifluorescence and the recording electrode was positioned under infrared microscopy. Whole cell patch-clamp recordings in current-clamp mode were made. Incrementing and decrementing depolarizing and hyperpolarizing currents steps and ramps were injected in every cell to measure basic electrophysiological properties. Only cells with a resting membrane potential (RMP) below -50 mV were used in the experiments. The membrane potentials, which had an abrupt change in the slope of the I-V

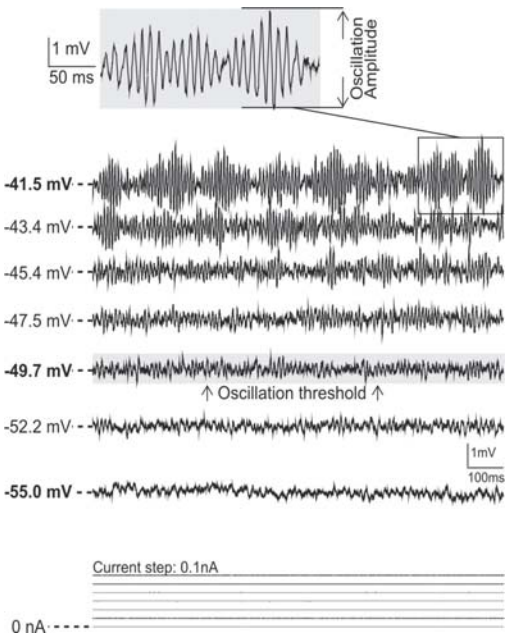

Figure 3. Illustration of recordings from a neuron in the NVmes. Incrementing depolarizing current steps were injected to measure maximum oscillation amplitude (inset), and threshold for high frequency oscillation and for burst firing.

relationship, were taken as the thresholds of inward or outward rectification. In order to examine the basic firing characteristics of the cells, single spikes were evoked by brief intracellular pulses imposed (1 ms) on the holding potential. The following measurements were made: firing threshold, peak amplitude of the action potential from RMP, action potential half-width, amplitude of the after-hyperpolarization and its duration. Incrementing 1s current steps were also used to measure the threshold of bursting when present and of the high-frequency membrane oscillations. Maximum amplitude and frequency of the oscillations (Fig 3) were measured at the membrane potential at which the amplitude appeared to be maximal (see: inset Fig 3).

Means were calculated from the three cycles that had the greatest amplitude. Patterns of spontaneous firing and firing during maintained depolarization were described.

Analysis of the anatomical relationship between muscle spindle mechanoreceptor and nociceptor afferents

Sixty-two rats (24–29 days old) were deeply anesthetized and perfused. The masseter muscles and trigeminal ganglia (used for positive control) were cryosectioned along their long axes and processed for immunofluoroscence and lectin binding (PGP9.5, VGLUT1, CGRP, SP, TRPV1, $P2X_3$, GluR1, NMDAR2B, mGluR5, TH and IB4).

RESULTS AND DISCUSSION

Paper I

Localization of c-Fos immunoreative brainstem neurons following fictive mastication

Following periods of robust jaw motoneuron activity, the Experimental group showed significant bilateral increases in the number of c-Fos immunoreactive cells in several nuclei between the level of the trigeminal motor nucleus and obex. In the rostral part of the brainstem significantly increased numbers of stained neurons were seen in the dorsomedial part of the trigeminal main sensory nucleus (NVsnpr-d) and the dorsal and lateral parts of Regio h (Fig 4A-B and Fig 5A). Caudally to NVmt increased numbers

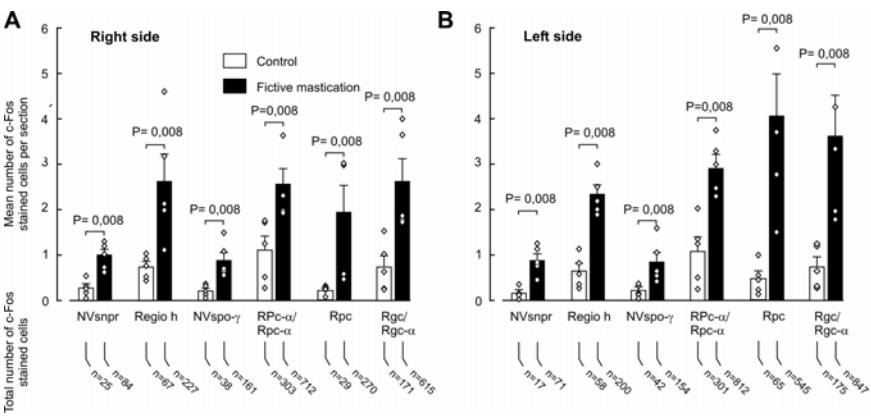

Figure 4. Histograms, which show quantitative distributions (mean ± SE) of labelled nuclear profiles (neurons) per section in the examined nuclei on the right (**A**) and left (**B**) sides of the brainstem. White and filled bars illustrate data from Control and Experimental animals, respectively. Diamonds give mean values from individual animals. Statistical differences were evaluated by using Mann-Whitney U-test. For abbreviations, see abbreviation list.

occurred mainly in subnucleus-γ of the oral nucleus of the spinal trigeminal tract (NVspo-γ; see: Fig 4A-B and Fig 5B). Masticatory related stained cells were also seen in two areas located more medially; (1) in the pontine and parvocellular (RPc-α/ Rpc-α) parts of the reticular formation, (2) the nucleus reticularis gigantocellularis (Rgc) and its pars α (Rgc-α; Fig 4A-B and Fig 5B-D). At the level of the facial nucleus, stained neurons appeared

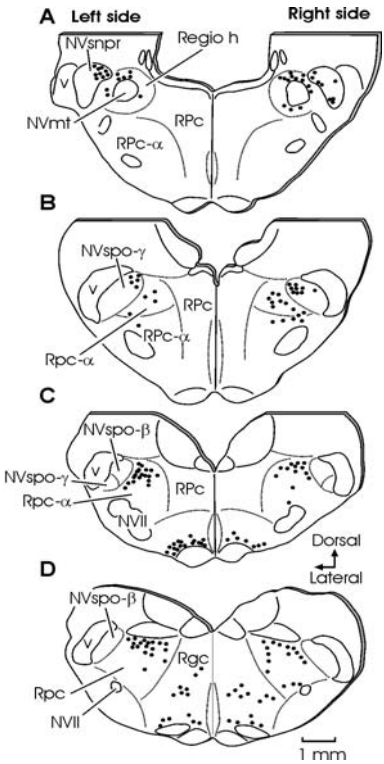

Figure 5. Tracings of stained sections to illustrate the location of c-Fos stained neurons in the brainstem. The sections were taken from: **A** the middle level of NVmt; **B** approximately 700 µm rostral to the facial motor nucleus (NVII); **C** mid level NVII; and **D** about 600 µm caudal to NVII. Distributions of neurons in nuclei with significantly increased labelling are indicated by dots. The illustrated data (A-D) were plotted from four consecutive sections taken from four animals in the experimental group. For abbreviations, see abbreviation list.

within the ventral portion of the Rgc/ Rgc-α area. This group of neurons formed two separate clusters further caudally as illustrated in Fig 5D.

In the region caudal to the facial nucleus a significant bilateral increase in the number of c-Fos positive neurons was also observed within the nucleus reticularis parvocellularis (Fig 5 D; Rpc).

As mentioned in the introduction, Nozaki and colleagues (Nozaki *et al.*, 1986a, b) were the first to propose a model of the CPG based on activity of a neuronal network located within the Rgc and adjacent Rgc-α. They suggested that the initial step in masticatory pattern generation was tonic excitation of a group of neurons in Rgc-α. These then induced rhythmic firing in a dorsal group located in Rgc, which distributed rhythmical commands to the trigeminal motoneurons. The enhanced numbers of c-Fos stained neurons in Rgc and Rgc-α, which were observed in this paper, confirm that these neuronal groups are active during the motor task. However, as mentioned in the introduction, their role as "unique" rhythm generators has been challenged on the basis of reports suggesting that additional elements may be found rostral to the facial nucleus (Kogo *et al.*, 1996; Tanaka *et al.,* 1999; Jacquin *et al.*, 1996).

The groups of c-Fos activated neurons that we observed in the pontine and parvocellular parts of the reticular formation (RPc-α; Rpc/Rpc–α) may represent such additional CPG elements. When neuronal activity in these

parts of the rabbit brainstem is blocked by focal lidocaine injections, the pattern of mastication is modified in a way that is dependent on the site of injection. When neurons in the ventral half are silenced, digastric EMG burst duration and burst area are increased, while similar blocks applied to the dorsal part gives more variable effects, although burst duration and area are often decreased (see: Westberg *et al*., 2001; Scott *et al*., 2003). This data suggests that neurons within the pontine and parvocellular reticular formations are primarily related to the regulation of jaw motoneuron burst patterns rather than generating the oscillatory masticatory rhythm. These neurons do not project directly to the trigeminal motor nucleus. Instead, they have axons that project to nuclei in the lateral brainstem (i.e. Regio h, NVspo-γ; see Fig 5). The fact that neurons in these lateral brainstem areas integrate short latency oral- and perioral primary afferent inputs with rhythmic drive signals during ongoing mastication also support their involvement in motor pattern formation (Hiraba *et al*., 1988; Donga *et al*., 1990; Donga & Lund, 1991; Inoue *et al*., 1992, 1994; Tsuboi *et al*., 2003; Westberg *et al*., 1998; Kolta *et al*., 2000, Dal Bo *et al*., 2005). Furthermore, many of these neurons have bilateral projections making them ideal to coordinate muscle activity on both sides of the mandible (Mizuno *et al*., 1970; Landgren *et al*., 1986; Donga *et al*., 1990; Li *et al*., 1993, Kamogawa *et al*., 1988).

Neurons in the parvocellular reticular formation have in addition been implicated as masticatory CPG elements on the basis of their behavior when recorded in brainstem slices of newborn rats (Min *et al*., 2003). This behavior, including rhythmic firing, has been suggested to result from synaptic interactions, because the majority does not possess intrinsic rhythm generating abilities when studied *in vitro* (Bourque & Kolta, 2001). In contrast, such an intrinsic bursting capacity seems to be present in some neurons that are located in the dorsal part of Regio h (Hsiao *et al*., 2007). In this respect, it is interesting to note that most of our c-Fos-stained neurons in Regio h were confined to its dorsal half (i.e. NsV; Fig 5A).

Our observation of c-Fos stained cells in the dorsomedial part of the main sensory trigeminal nucleus (NVsnpr-d) is a particularly interesting finding. As a whole, this nucleus has traditionally been viewed as a relay in the trigemino-thalamic pathway to the primary somato-sensory cortex. However, some of its neurons seem to possess characteristics, which now indicate that they instead could be part of a masticatory CPG. First, neuroanatomical studies have shown that the NVsnpr does not only contain projecting neurons, but also local-circuit neurons (see: Torvik, 1957; Darian-Smith, 1965; Yoshida *et al*., 1998). Some of the latter are labelled when retrograde tracers are injected into orofacial motor nuclei, which suggest

they may function as premotoneurons (Landgren *et al*., 1986; Yoshida *et al*., 1998; Pinganaud *et al*., 1999; Kolta *et al*., 2000). Second, recordings made during fictive mastication in the anaesthetized and paralyzed rabbit show, that the dorso-medial part of the NVsnpr contains rhythmically active neurons (Tsuboi *et al*., 2003). Since movement related afferent inputs are lacking during paralysis, the bursting in these neurons must be based on central mechanisms. Thus, besides relay cells, other types of neurons may exist in the main sensory trigeminal nucleus. For instance, neurons that may serve in a masticatory CPG function. However, in order to support such a speculative proposal, further electrophysiological documentation was undertaken as reported in paper II.

Paper II

Intracellular recordings were made *in vitro* from 106 NVsnpr neurons and two main categories of cells were defined on the basis of their firing patterns: non-bursting (63%, *n*=67) and bursting (37%, *n*=39).

Electrophysiological characteristics of NVsnpr neurons

In all bursting cells a depolarizing current pulse induced an initial burst consisting of 2-3 spikes (intra-burst frequency: 140-454 Hz; duration: ≤ 21.8 ms, measured at 0.2 nA), which was followed by single spikes or recurrent bursts. The non-bursting cells fired single action potentials in response to membrane depolarization as well as at resting membrane potential. Our categorization of neurons, based on their firing pattern in response to depolarization, agrees with an earlier report assigning approximately half of the NVsnpr neurons intrinsic properties for plateau potential and burst generation. The remaining half was reported as non-bursting (Sandler *et al*., 1998). These authors did not relate the different firing patterns to location within the nucleus. Interestingly, we found a remarkable distributional difference, with bursting neurons concentrated in the dorsomedial NVsnpr while the non-bursting neurons were scattered throughout the nucleus (Fig 6B). It is known that the dorsal cap of the nucleus contain neurons that fire rhythmically in phase with trigeminal motoneurons during cortically evoked fictive mastication. Some of these neurons receive inputs from muscle spindles and periodontal receptors (Tsuboi *et al*., 2003) and project to jaw closing motoneurons (Kolta *et al*., 2000). This is in accordance with the results of our *in vivo* study (Paper I) where the number of c-Fos immunoreactive neurons in NVsnpr-d increased significantly following the cortically induced fictive mastication.

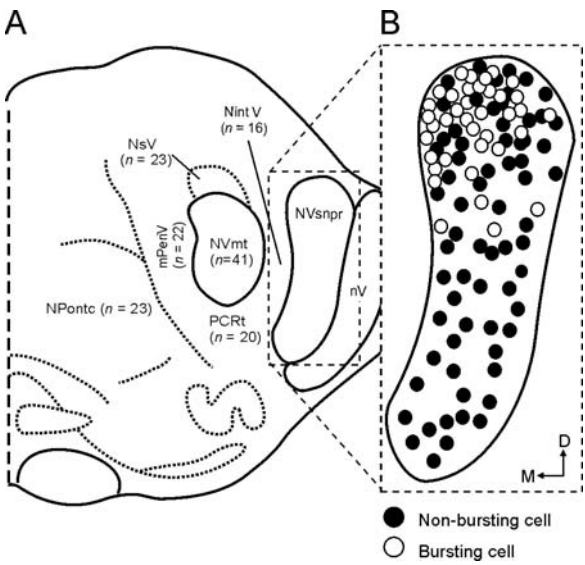

Figure 6. Illustration showing the areas of investigation and distribution of recorded neurons in the main sensory trigeminal nucleus (*NVsnpr*). **A**. Drawing of a transverse section at the level of the trigeminal motor nucleus to show the nuclei where microstimulation was delivered. The number of stimulation trials in each area is given within the parenthesis. The outlines of the nuclei are indicated: NsV and NintV: supratrigeminal and intertrigeminal nuclei, respectively; nV: trigeminal tract; PCRt: nucleus reticularis parvocellularis; *mPeriV:* medial Regio h and; NPontc: nucleus reticularis pontis caudalis. **B**. Enlargement of the framed area in (A) shows the distribution of different neuronal subtypes within the NVsnpr according to their firing properties.

Synaptic inputs on NVsnpr neurons from brainstem nuclei

In the majority of cases (77%) microstimulation in the reticular borderzone around NVmt (see: Fig 6A) evoked postsynaptic potentials. Applied stimulation in its four subareas resulted mainly in DNQX and APV sensitive excitatory responses. Different proportions of IPSPs and biphasic responses sensitive to glutamatergic, GABAergic and glycinergic receptor antagonists were also recorded (Fig 7A-C). These observations are all in line with immunocytochemical investigations reporting glutamatergic, GABAergic and glycinergic immunoreactive neurons in the borderzone of the trigeminal motor nucleus (Li *et al*., 1996; Turman & Chandler, 1994a, b; Yang *et al*., 1997; Kolta *et al*., 2000). Axonal projections from nucleus reticularis parvocellularis (PCRt) and the supratrigeminal nucleus (NsV) to NVsnpr has

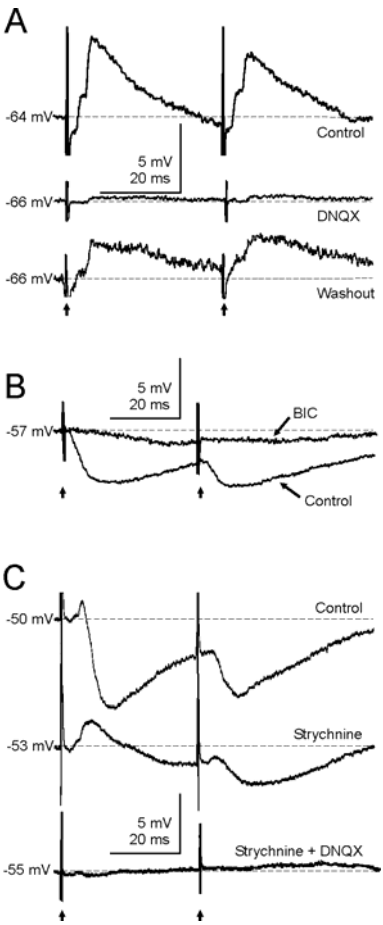

Figure 7. Records showing postsynaptic potentials. **A**. Recording of an excitatory postsynaptic potential elicited following stimulation of the mPeriV area. Addition of 6, 7-dinitroquinoxaline (DNQX) to the bath abolished the EPSP response (*middle trace*). The *bottom trace* shows recovery of the EPSP after partial wash out of the antagonist. Arrows indicate the onset of the stimulation pulse. **B.** Recording of an IPSP evoked by stimulation of NVmt and that was abolished by administration of Bicuculline (BIC). **C.** Stimulation of PCRt evoked a biphasic PSP consisting of a small amplitude EPSP followed by a large IPSP. Strychnine abolished the IPSP and revealed an EPSP with larger amplitude than observed in the control. The excitatory response was subsequently blocked by DNQX.

previously been demonstrated by extracellular injection of biocytin (Bourque & Kolta, 2001). In agreement with this we acquired monosynaptic responses from PCRt and NsV.

Axonal projections from the lateral (NintV) and medial borderzone of NVmt (mPeriV) to NVsnpr have not been described in the literature. However, we observed some postsynaptic responses (PSP) from these two areas that had latencies in the monosynaptic range (≤ 1.7 ms). In 70% of the cases, when stimulations were applied to NVmt, the recorded postsynaptic potentials in NVsnpr fell in the monosynaptic range. Because trigeminal motoneurons lack recurrent axon collaterals, it is likely that these responses were caused by stimulation of interneurons (see Sessle, 1977; Ter Horst *et al.*, 1990; Shigenaga *et al.*, 1988; Olsson & Landgren, 1990 McDavid *et al.*, 2008). Most of the evoked responses obtained in NVsnpr neurons were DNQX sensitive EPSPs, but we also obtained some IPSPs mediated by $GABA_A$ or glycine receptors, and a few mixed responses as well. These data corroborate with immunocytechemical studies conducted on the rabbit and rat indicating the presence of small neurons, presumably interneurons with-in NVmt, that are immunoreactive to

glycine, glutamic acid decarboxylase and GABA (Li *et al*., 1996; Kolta *et al*., 2000; McDavid *et al*., 2006).

Half of the responses evoked by stimulation of dorsal nucleus pontis caudalis (NPontc) occurred within a monosynaptic latency (1.1 - 2.0 ms), which suggests that there also exist direct projections from this nucleus to NVsnpr. Mostly excitatory input was elicited, but a mix of glutamate and GABA immunoreactive neurons in the NPontc has been reported (Kolta *et al*., 2000). Taking into account that stimulation of NPontc evokes synaptic responses frequently in NVsnpr, but never in NVmt as reported by Bourque & Kolta (2001) and Dal Bo *et al*. (2005), strongly argues against the likelihood of current spread being responsible for the recorded responses since NVmt is much closer to the stimulation site than NVsnpr. However, it has been shown that there are frequently occurring projections from NPontc neurons to the border zone surrounding the NVmt (see: Kolta *et al*., 2000) and these connections could be responsible for some of the longer latency responses.

As the two neuronal types (i.e. bursting vs. non-bursting) received post-synaptic potentials in similar proportions from all areas, a distinction has not been made between the two categories of cells based only on the distribution of their inputs. However, it is interesting to note that there appear to be a difference in characteristics of the excitatory inputs. Our data suggest that inputs to non-bursting neurons are a result of non-NMDA receptor activation, while bursting neurons receive input mediated by AMPA/kainate and/or NMDA receptors. The role of NMDA receptor activation in jaw movement production has been pointed out by Kogo *et al*., (1996). They reported that rhythmical burst discharges, obtained from the trigeminal nerve in an *in vitro* brainstem preparation, were blocked by NMDA antagonists.

Projections of NVsnpr neurons

Our NVsnpr neurons were antidromically activated in 23% of the stimulation trials, which suggests that they may contact trigeminal motoneurons and/or different types of brainstem interneurons. The majority of the antidromic responses were evoked from the masseteric pool of NVmt (35%) and the dorsal NPontc (29%). It is likely that the antidromic potentials recorded by us is a result of direct activation of local axon collaterals terminating in the lower brainstem area. The reason why stimulus spread is unlikely is that the ascending axons projecting to thalamus from dorsally located NVsnpr

neurons, travel principally in the ipsilateral reticular formation, ventral to the trigeminal mesencephalic nucleus (Torvik, 1957; Mizuno, 1970). By using anterograde tracer techniques, it has also been shown that the dorsomedial part of NVsnpr sends projections to NPontc and PCRt (see: Ter Horst *et al*., 1991). The reciprocal connectivity between neurons in the dorsal part of the NVsnpr, with brainstem areas containing putative masticatory CPG neurons, further strengthens the hypothesis that this particular part of the main sensory trigeminal nucleus may be involved in the masticatory generating circuitry.

Paper III

Acidic saline injections and nocifencive behavior

Prior to masseter muscle injection, mechanical thresholds for evoking nocifencive behaviors were recorded by testing with von Frey filaments. The filament producing a load of 26g, caused 0-3 responses per side, while a filament giving a load of 15g caused even fewer. Following injection of 0.9% NaCl with a pH 7.2 (Control), the frequency of responses rose slightly for tests with the stiffer filament only. However, in the Experimental group responses to both filaments increased to a great extent and remained significantly higher than baseline levels up to 38 days after the first injection.

In accordance with observations presented by Sluka *et al*. (2003), we found significant differences between Experimental and Control groups with filaments that had bending thresholds of 15 and 26 grams. On the other hand, our observations differ from a study by Ambalavanar *et al*. (2007) who reported that repeated masseter injections of saline of pH as low as 3.0 had no effect on the mechanosensitivity. Opposite to us, these authors defined a withdrawal threshold as the mean force of five pressures applied with a rigid filament connected to a force transducer, while we compared the frequency of head withdrawal in response to defined von Frey hair loading, similar to those used by Sluka *et al*. (2001) on the limbs. Thus, the lack of effect reported by Ambalavanar *et al*. (2007) may be related to the lower sensitivity of their testing method (see: Ren, 1999)

c-Fos expression in NVmes neurons following acidic saline injections.

The NVmes neurons, immunoreactive to c-Fos, were counted from the rostral level of NVmt to the level of the rostral pool of the oculomotor nuclei.

The number of immunoreactive neurons was significantly higher 4 h after intramuscular injections in both the normal saline Control (0.9% NaCl, pH 7.2) and acidic saline Experimental group (0.9% NaCl, pH 4) in comparison with the unoperated Controls. However, the cell counts of the normal saline Control group did no longer differ from those of the unoperated Controls at 24 h and 96 h post-injection, whereas those of the Experimental group remained significantly higher. The counts in the Experimental group were also significantly higher than those of the normal saline Control group at 24 hrs and 96 hrs (see Fig 8).

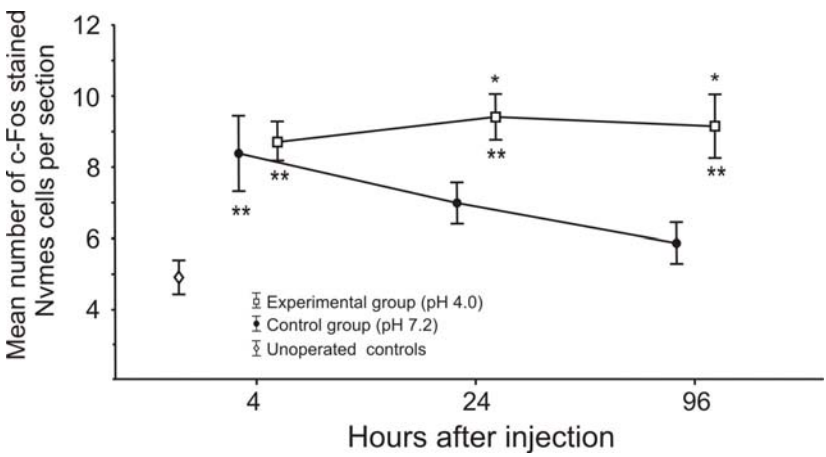

Figure 8. Graph showing the mean number ± S.E. of c-Fos stained NVmes cells in unoperated controls (open diamond), and experimental (open squares) and sham-control group (filled circles) at 4, 24 and 96 hours after injection. Asterisk above points: significance compared to Control group. Asterisk below points: significance to unoperated controls. *, $p<0.05$; **, $p<0.01$

In the saline Controls the increase and return in numbers of marked NVmes neurons resemble observations of c-Fos expression seen in the dorsal horn that also returns to base level 8-24 hours after acute nociceptor stimulation (Draisci & Iadarola, 1989). However, the number of c-Fos neurons remained high for 96 hrs in our Experimental group. This finding suggests that acidic saline causes prolonged increase in expression as a consequence of increased neuronal activity. This proposal is in line with previous studies by Fields *et al.* (1997), who reported a similar strong link as observed in an *in vitro* study on dissociated dorsal root ganglion neurons.

Change of membrane and firing properties of NVmes neurons following acidic saline injections

Intracellular recordings from 308 NVmes neurons were analyzed. It turned out that the treatment protocol of the different groups of animals had a significant effect on: (1) resting membrane potential, (2) firing threshold, (3 & 4), threshold of inward and outward rectifications, (5 & 6) oscillation threshold and amplitude, and (7) burst threshold. Within the first 8 hours after the second injection (days <1) distinct discrepancies between the Control and Experimental groups were present, and were generally maintained throughout the experiment. The Experimental neurons had a lower resting membrane potential and lower threshold for firing, inward and outward rectification. The amplitude of oscillations was higher and bursting occurred more frequently (Fig 9A).

About 10% (17/197) of the cells from the Experimental group were spontaneously active at resting membrane potential, whereas none of the Control cells were active. This difference was significant ($p<0.0001$). Following injection of a 1s suprathreshold depolarizing current, four types of firing patterns could be classified: (1) Adapting, (2) Train, (3) Train and Bursting, and (4) Bursting (Fig 9B). There was a significant between-group difference in the distribution of firing patterns ($p= 0.019$). Most Control neurons were of the Adapting type, and were thus unable to sustain their firing.

The changes in membrane properties that Experimental neurons displayed after acidic injections made them function at more negative membrane potentials and become more excitable and more likely to fire spontaneously. We hypothesize that the observed neuronal changes are a result of exposure of their terminals to a local increase in $[H^+]$. Interestingly, NVmes neurons do not express the acidic sensitive channel $ASIC_3$ (Molliver *et al.*, 2005), but isolated NVmes neurons still do respond to low pH, although in a different way than trigeminal ganglion neurons (Connor *et al.*, 2005). Thus, small changes in pH between 7.4 and 6.0 cause large changes in inward current in ganglion neurons but only small changes in NVmes neurons. However, additional reductions in pH produce small currents in ganglion neurons, but large currents in NVmes, suggesting that the latter are especially sensitive to low pH.

The NVmes neurons show certain distinctive electrical properties. Several reports point to the fact that their strong inward and outward rectifications make it difficult to uphold their firing during depolarization, or to keep them maintained in a hyperpolarized state (Del Negro & Chandler, 1997; Khakh &

Henderson, 1998; Tanaka *et al*., 2003; Verdier *et al*., 2004). The outward rectification probably explains why most Control NVmes neurons ceased firing during maintained depolarization (Adapting neurons). However, neurons belonging to the Experimental groups were more likely to discharge continuously than Control neurons and to fire spontaneously, despite having a lower threshold for outward rectification and a more hyperpolarized resting membrane potential. This difference in firing pattern may result from changes in membrane spontaneous oscillations. Oscillations in primary afferents were initially described by Puil & Spiegelman (1988) in trigeminal ganglion neurons and later by Amir *et al*. (1997), who found that 35% of large diameter dorsal root ganglion neurons showed oscillations in the 88-195 Hz range when depolarized.

There are now a number of observations of a similar behavior of NVmes nerve cells. Unlike dorsal root ganglion cells, the somas of these primary afferents are, as mentioned earlier, equipped with a variety of transmitter receptors and receive synaptic inputs (Lazarov, 2002, Verdier *et al*., 2004). Their oscillations, however, are not dependent on synaptic inputs because they persist in calcium-free medium (Wu *et al*., 2001; Verdier *et al*., 2004). When firing occurs, the spikes appear during the depolarizing crests of the oscillations (Verdier *et al*., 2004).

The ion channel antagonist TTX abolishes oscillations in both dorsal root ganglion and NVmes neurons because it blocks a voltage-sensitive persistent inward sodium current (I_{NaP}) responsible for the depolarizing phase (Wu *et al*., 2001, 2005). Although the repolarizing phase requires an outward potassium current (Amir *et al*., 2002; Puil *et al*., 1989), Amir *et al.* (2002) showed that intracellular infusions of Cs^+, which blocks a wide spectrum of K^+ conductances, and TEA and 4-AP that block voltage-dependant K^+ currents, all increase the amplitude of the oscillations and repetitive firing pattern in dorsal root ganglion neurons. They therefore, suggested that a passive voltage-independent K^+ leak is responsible for the repolarizing phase. We also observed that the changes in membrane properties were accompanied by some spontaneous firing as well as significant increase in the occurrence of sustained firing during depolarization. Such an increase in spontaneous ectopic firing is seen in the cell bodies of injured A fibres, including muscle afferents and intact C fibres in rodent neuropathic pain models (Boucher *et al*., 2000; Liu *et al*., 2000). Amir & Devor (1997) reported that dorsal root ganglion neurons, which failed to show oscillations even during deep depolarization, were unable to generate continuous discharges, signifying the importance of the oscillatory process for repetitive firing.

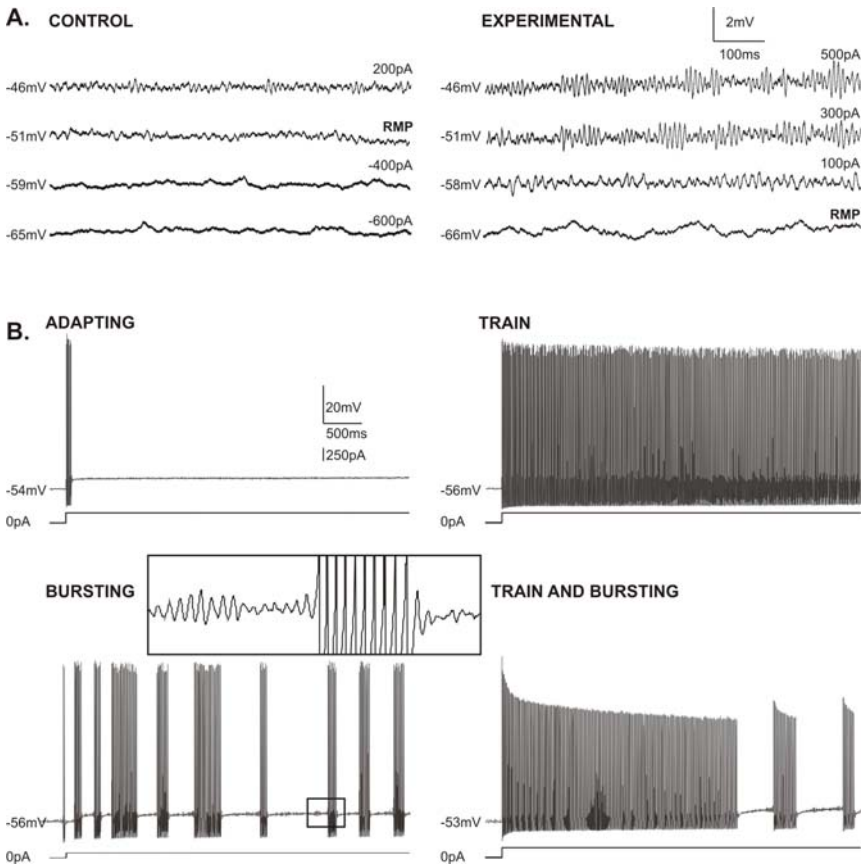

Figure 9. Diagram illustrating high frequency membrane oscillations and firing patterns of NVmes neurons. **A**. Example of recordings made 14 days after the second injection in Control (left) and Experimental neurons (right) showing the difference in oscillation amplitude at similar membrane potentials. The amount of current injected is given on the right hand side of each trace. Calibration bars apply to both panels. **B:** Examples of the four types of firing patterns induced by current injections. The inset illustrates a section of the "bursting" trace at higher magnification to show that the bursts coincided with a period of high amplitude oscillations. Calibration bars in top left panel apply to all panels.

Long-term changes in the dorsal horn nociceptive pathways caused by ectopic firing in the spinal cord primary afferents have previously been reported (Woolf, 1983; Devor, 2005). Furthermore, there are reports indicating that Aβ fibre terminals may sprout into dorsal horn laminae normally occupied by nociceptive afferent terminals after nerve injury (Woolf *et al*., 1995; Kohama *et al*., 2000). The possibility for similar changes in the brainstem exists, because masseter spindle afferents were reported to send collaterals into regions of the spinal nucleus of the trigeminal tract that also receives nociceptive inputs (Luo *et al*., 1995; Westberg *et al*., 1995). An additional possibility is that action potentials arising from central axons or somata of the muscle spindle afferents, can also travel antidromically (see: Gossard *et al*., 1999; Amir *et al*., 2005).

Expression of nociceptor markers on masseter muscle spindle afferents

Immunofluoroscence staining showed that the annulo-spiral endings and the myelinated axons of the masseter Ia primary afferents were found to be intensely labelled with PGP9.5 (see Fig 10A, C, D), the general marker of peripheral neuronal processes (Winarakwong *et al*., 2004). This was true also for VGLUT1 (Fig 10B), the marker of glutamate containing fibres and terminals (Bellocchio *et al*., 2000; Takamori *et al*., 2000). Spindle annulo-spiral endings were always negative for CGRP, SP, $P2X_3$, TRPV1 or IB4, markers known to be linked to nociceptors. Interestingly PGP9.5 also labelled some thin fibers within muscle spindles (Fig 10A', C', D'). These axons turned out to be immunoreactive for CGRP (Fig 10A', B'), SP (Fig 10 C, C') and TRPV1 (Fig 10D, D'). CGRP or $P2X_3$ (Fig 11A, B) were present in many spindles, in close apposition to the intrafusal muscle fibres and intersected, in a number of locations, with the surrounding annulo-spiral endings. Intrafusal thin fibers immunoreactive for SP (Fig 10C) or TRPV1 (Figs. 10D, 11C) were seen much less frequently. Furthermore, labelling of annulo-spiral endings were always intensely mGluR5-positive, and many small-calibre fibres also appeared immunoractive for the glutamate receptor subunit mGluR5 (Fig 11A'-C').

Single axons or bundles of small unmyelinated fibres have been shown before inside the capsules of cat tenuissimus muscle spindles (Santini & Ibata, 1971). Our new findings show that many of the unmyelinated fibers within the masseter muscle contain peptides and receptors usually found in nociceptors but that have also been described occasionally in other types of primary afferents (Kruger *et al*., 1989; McCarthy & Lawson, 1990; Hoheisel *et al*., 1994; Lawson *et al*., 2002). The purinergic $P2X_3$ receptor has been

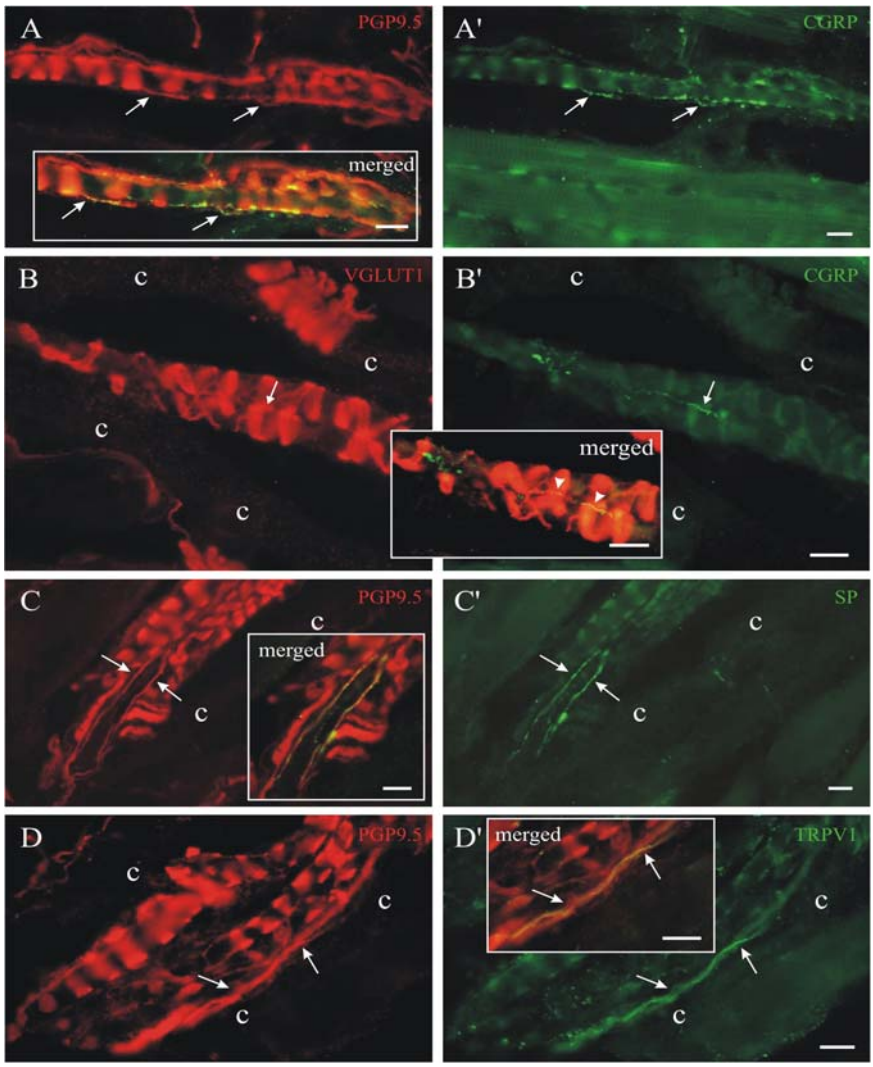

Figure 10. Examples of small-calibre axons within muscle spindles labelled with nociceptor markers . Left and right photomicrographs have identical frames with different sets of fluorescence filters. In each case, portions of the photomicrographs were digitally merged in boxed areas. **A**: Fibres immunoreactive for PGP9.5 and containing CGRP (A'). **B**: A small green CGRP-positive fibre (B', thin arrow) runs across three VGLUT1-positive loops of an annulo-spiral ending. None of the VGLUT1-positive fibres in B corresponded to the CGRP-positive fibres in B'. In the merged image, the fibre to the right appears yellow for most of its length (arrowheads) because it passes over red VGLUT1-positive fibres. **C**: Fibres immunoreactive for both PGP9.5 and SP. **D**: Fibres immunoreactive for PGP9.5 and the capsaicin receptor, TRPV1. c: spindle capsule wall. All scale bars = 10 µm.

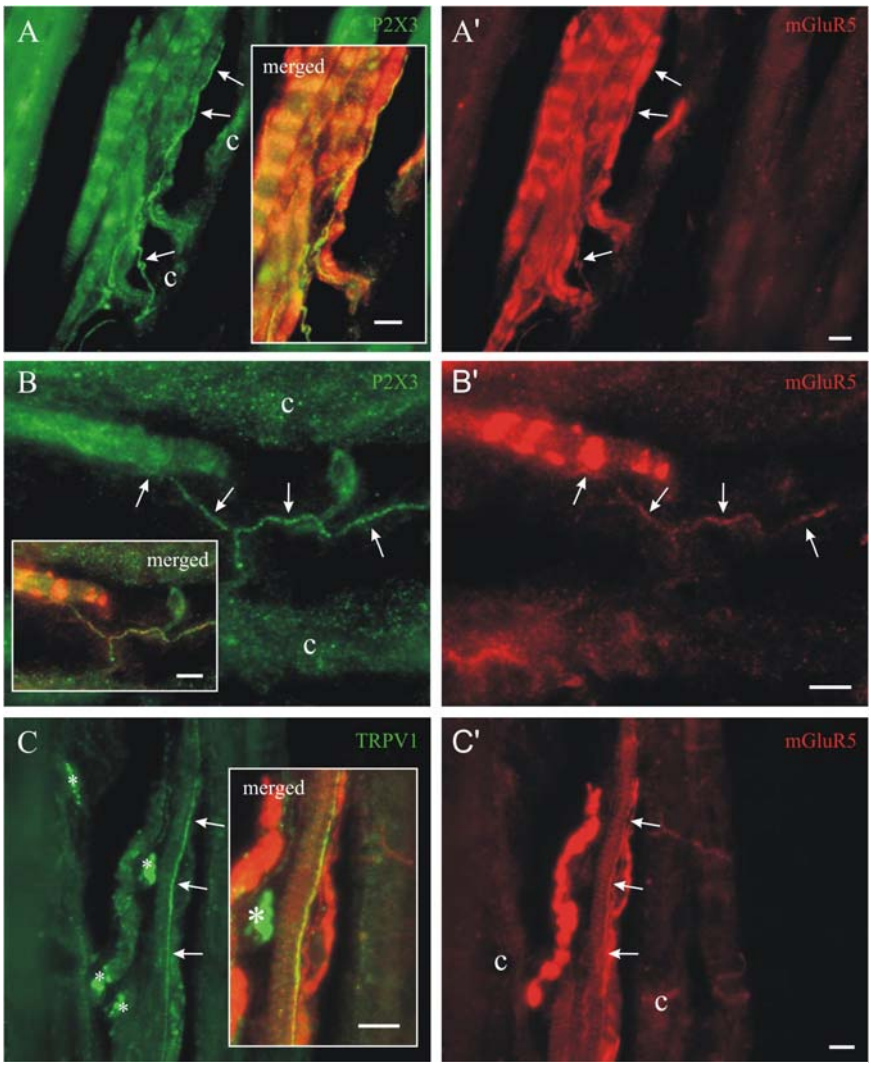

Figure 11. Examples of small-calibre fibres double-labelled for nociceptor markers, and metabotropic mGluR5 glutamate receptors. In all cases, the photomicrographs from the left and right columns have the exact same frames but with different set of fluorescence filters. Merged portions of these photographs were placed in boxed areas to show the yellow-appearing double-labelled fibres. **A** and **B**. Photomicrographs of nerve fibres immunoreactive for P2X3 and mGluR5 (A',B'). C: Photomicrographs of a nerve fibre immunoreactive for both TRPV1 and mGluR5 adjacent to an intrafusal muscle fibre. *: fluorescent artefact. c: spindle capsule wall. All scale bars = 10 µm.

suggested to be exclusively expressed by nociceptors (see: Connor *et al.*, 2005). We found that many masseter spindles contained CGRP-positive small-caliber fibres, but few contained SP. We also observed $P2X_3$-positive small-calibre fibres in masseter spindles. This is in line with the report by (Ambalavanar *et al.* (2005) that 22% of trigeminal ganglion masseter afferent neurons contain CGRP and 22% of the neurons express $P2X_3$, but only 5% contain SP. It is known that NVmes neurons express mGluR5 receptors from birth (Munoz *et al.*, 1999, Turman *et al.*, 2001) and our findings now show that annulo-spiral endings bind mGluR5 antibodies.

Pain is evoked in humans when glutamate or its agonists are injected into muscles, including the masseter (Svensson *et al.*, 2003, Castrillion *et al.*, 2008). Moreover, in experimental models, glutamate excites small-diameter muscle afferent fibres (Dong *et al.*, 2006). In addition, glutamate concentrations have been shown to be increased in patients with trapezius myalgia *(Rosendal et al.*, 2004) as well as in human experiments on delayed onset muscle soreness (Tegeder *et al.*, 2002).

Taken together, in this study we have found that intramuscular injections of acidic saline cause nocifensive behavior that mimics persistent muscle pain (see: Sluka, 2001). We have also observed that small-calibre terminals, which are likely to be nociceptors, are located close to masseter muscle spindle mechanoreceptor endings. Furthermore, our analysis has revealed that acidic muscle injections cause phenotypic changes in the cell soma of muscle spindle afferent NVmes neurons leading to pathophysiological membrane oscillations and ectopic firing. Such an ectopic firing could cause release of glutamate stored in the muscle spindle peripheral ending and activate the identified glutamate receptors on nociceptors close nearby. This transformation of NVmes neuronal excitability could be of importance for the development of myogenous (dysfunctional) pain.

GENERAL DISCUSSION

The masticatory CPG comprise a distributed network of interneurons and motoneurons, which upon appropriate stimulation generates an organized motor rhythm that replicates the patterns of motor activity seen during repetitive jaw movements. Trying to understand how the masticatory CPG is organized requires identification of its components. Our results have shown that the c-Fos method is a useful research tool in elucidating neuronal components that may form this neuronal network. However, the introduction of genetic tools in an attempt to explore the molecular and cellular mechanisms underlying the organization of central pattern generators, at the level of the spinal cord, has opened up additional avenues. In such experiments it is possible to molecularly identify and genetically manipulate specific neuronal cell types (see: Grillner et al., 2005; Goulding, 2009). If used in the masticatory system, integrated with physiological and computational analysis, this additional way of classifying and investigating functions of individual circuit components should further deepen our understanding of the masticatory CPG.

One of our identified nuclei containing "activity marked" cell populations, i.e. NVsnpr-d, is of particular interest, as it seems to gather all the conditions to form part of a CPG. Although some NVsnpr neurons possess intrinsic properties for bursting, new evidence show that burst firing modes can also carry stimulus-related information in sensory systems that are qualitatively different from information encoded by tonic firing (see: Krahe & Gabbiani, 2004). In thalamo-cortical sensory pathways, bursting is thought to enhance the transmission of sensory information by being a more reliable way of transmitting spikes across "unreliable" synapses. A number of evidence also supports the hypothesis that sensory information transmitted under the bursting mode improves the signal-to-noise ratio (see: Krahe & Gabbiani, 2004). However, this would not explain the increased c-Fos labelling we found, nor the bursting observed *in vivo* during fictive mastication where sensory afferents are silent (see: Tsuboi *et al.*, 2003). In addition, if bursting of NVsnpr-d neurons had a sensory function; one would expect this function to be distributed across NVsnpr and not to be limited to the dorsal part that was found to have neurons with antidromically documented projection to the jaw closing motoneuron pool of the trigeminal motor nucleus.

We rather propose that the dorsal cap of NVsnpr should be included as a part of the core, of the masticatory CPG because: (1) under appropriate conditions most of its neurons have the intrinsic ability to generate recurring bursts (Brocard *et al.*, 2006; Kolta *et al.*, 2007). (2) it receives direct inputs

from corticobulbar fibers and intraoral afferents (Eisenman *et al.*, 1963; Tsuru *et al.*, 1989; Zhang & Sasamoto, 1990), both known to trigger mastication. (3) it is reciprocally connected with areas supposed to play an important role in the circuitry responsible for masticatory motor output, i.e. NVmt, PeriV and NPontc.

Brocard *et al.* (2006) has shown that the repetitive bursting in dorsal NVsnpr neurons, which develops after birth in parallel with the behavioral switch from suckling to mastication, is mediated by a persistent sodium current (I_{NaP}).This current, which can produce endogenous bursting, is proposed to control membrane excitability in the voltage region just sub-threshold to spike production (see: Ramirez *et al.,* 2004). Furthermore, there is also evidence that this particular current can be activated by a reduction of extracellular [Ca^{2+}] and cause rhythmical firing in NVsnpr neurons (see: Brocard *et al.*, 2006, Kolta *et al.*, 2007). In this context, it is interesting to note that bursting in many brain areas is accompanied by drops of extracellular $[Ca^{2+}]_E$(Cohen & Fields, 2004). Likewise, a similar fall in $[Ca^{2+}]_E$ causing bursting in NVsnpr-d neurons may be involved in the initiation of mastication (see: Kolta *et al.*, 2007). The proposed mechanism is founded on the fact that when mastication is evoked by stimulation of the masticatory cortex or intraoral afferents, NVsnpr-d neurons are likely to receive intense synaptic activation. This would lead to local depletion of extracellular calcium and activation of the I_{NaP} (Kolta *et al.,* 2007). The intriguing chain of cellular events may explain the relatively slow onset of mastication observed when experimentally induced by stimulation of descending or afferent pathways. In fact, when electrically stimulating the masticatory area, a train of pulses lasting several hundred milliseconds is normally required to induce the first movement cycle. Similarly, prolonged activity in sensory afferents by mechanical stimulation of the buccal mucosa in the retromolar region is necessary to trigger mastication (Dellow & Lund, 1971). In this scenario, the pacemaker activity kindled by sensory or descending inputs may provide sufficient excitation to activate other components of the masticatory CPG network. Thus, we hypothesize that embedded pacemaker neurons, like those described by us in NVsnpr-d, may be an important mechanism for the masticatory network to overcome a quiet activity state and turn into a condition that generates the rhythmic masticatory motoneuron activity.

In this context, it should also be pointed out that mastication not only involves jaw muscles, but also tongue and facial muscles. During mastication all these are coordinated but they can also function independently in other movement contexts, such as respiration and licking movements. This has raised the question of whether trigeminal, hypoglossal and facial moto-

neurons are controlled by one or several CPG´s. The latter seem to be the case, because studies on isolated brainstem preparations from the newborn rat and mouse have indicated that separate "unit" rhythm generators may be located segmentally close to the respective orofacial motor nuclei (see: Nakamura *et al.,* 2004; see also: Grillner et al., 2008). However, future studies are needed to fully understand the organization of these networks and how they interact to generate the multifaceted motor repertoires expressed during normal orofacial behaviors.

Pain that persists represents a major health problem. Increased understanding of the underlying pathophysiological processes is needed in order to reach the goal of mechanism-based treatments for the many different troublesome clinical problems, especially musculoskeletal pain syndromes. Studies of the behavior of patients with acute or persistent pain in the oral and craniofacial regions show that they also suffer disturbed motor functions, which in turn may be unfortunate. In an attempt to explain the possible relationship between pain and motor function, a hypothesis called "the pain adaptation model" has been set up. The model proposes, with respect to masticatory movements, a facilitation of inhibitory pathways to jaw closing motorneurons in the agonist movement phase (jaw closing) and a facilitation of excitatory pathways in the antagonist (jaw opening) phase (Lund *et al*., 1991). This motor strategy, generated by the masticatory CPG, is considered adaptive and functionally meaningful. It limits movements in order to minimize further damage and promotes healing of injured tissue (see: Lund, 2008).

Under certain circumstances, such as myogenous TMD, pain may be experienced with no damage or any detectable inflammation. What causes the manifestation or persistence of this pain, called "dysfunctional pain", is unclear (see: Dubner & Ren, 2006; Costigan *et al*., 2009). However, both peripheral and central mechanisms contribute to the development of this maladaptive type of pain. Thus, both central descending endogenous brainstem modulatory systems and peripheral nociceptor inputs can induce plasticity and hyperexcitability of neurons within the CNS involving also neuroinflammation (see: Dubner & Ren, 2006; see also: Mense, 2008; Tillu *et al*., 2008). The descending brainstem pathways provide the neural mechanisms by which the attentional, motivational and cognitive variables modulate the ascending sensory information. The fact that not all muscle pain becomes persistent implies that the transition requires not only the mentioned mechanisms but also other ones e.g. genetic predisposition (Maixner, 2008; Mense, 2008).

Our new data now show that large caliber muscle afferents may play a role in the development of the condition of “dysfunctional pain”. Thus, our present work shows that annulo-spiral endings in the masseter muscle express high levels of the glutamate transporter VGLUT1, which is responsible for the transport of glutamate into synaptic vesicles before exocytotic release (Wu *et al*., 2004; Pang *et al*., 2006). The presence of synaptically stored glutamate in the peripheral endings might have transmitter-like functions, and it has been suggested to be coupled to activity dependent autogenic regulation of spindle excitability (Bewick *et al*., 2005). Interestingly, our observations of a close apposition between annulo-spiral endings and nociceptive afferents equipped with glutamate mGluR5 receptors allow the hypothesis that excessive release of glutamate from muscle spindles due to ectopically evoked antidromic action potentials, may also under critical circumstances, lead to persistent musculoskeletal pain by activation and/ or sensitization of adjacent nociceptors. Taken together, it seems reasonable to hypothesize that this new mechanism could be involved in the development of long lasting “dysfunctional” musculoskeletal pain (e.g myogenous MPD).

CONCLUSIONS

Mastication is a physiological function promoting a healthy life. When orofacial persistent pain occurs, comprising normal function, its diagnosis and treatment is a challenge to health care providers. Within oral physiology, studies of motor control and of pain mechanisms have been major topics in basic research since the beginning of the 1970s. In this thesis, attempts have been made to elucidate aspects related to both of these areas.

The experiments on mastication have expanded our understanding of the organization of the brainstem masticatory CPG microcircuit.

The experiments on pain have revealed the basis for a new hypothesis of how “dysfunctional” musculoskeletal pain (e.g. myogenous MPD), known to interfere with orofacial motor functions, may develop and become persistent in some individuals.

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REFERENCES

Ambalavanar, R., Yallampalli, C., Yallampalli, U. & Dessem, D. (2007) Injection of adjuvant but not acidic saline into craniofacial muscle evokes nociceptive behaviors and neuropeptide expression. *Neuroscience*, **149**, 650-659.

Amir, R. & Devor, M. (1997) Spike-evoked suppression and burst patterning in dorsal root ganglion neurons of the rat. *J. Physiol. (Lond.),* **501**, 183-196.

Amir, R., Michaelis, M. & Devor, M. (1999) Membrane potential oscillations in dorsal root ganglion neurons: role in normal electrogenesis and neuropathic pain. *J. Neurosci.*, **19**, 8589-8596.

Amir, R., Michaelis, M. & Devor M. (2002) Burst discharge in primary sensory neurons: triggered by subthreshold oscillations, maintained by depolarizing afterpotentials. *J. Neurosci.*, **22**, 1187-1198.

Amir, R., Kocsis, J.D. & Devor, M. (2005) Multiple interacting sites of ectopic spike electrogenesis in primary sensory neurons. *J. Neurosci.*, **25**, 2576-2585.

Barajon, I., Gossard, J.P. & Hultborn, H. (1992) Induction of fos expression by activity in the spinal rhythm generator for scratching. *Brain Res.*, **588**, 168-172.

Bellocchio, E.E., Reimer, R.J., Fremeau, R.T. Jr. & Edwards, R.H. (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science, **289**, 957-960.

Bewick, G.S., Reid, B., Richardson, C. & Banks, R.W. (2005) Autogenic modulation of mechanoreceptor excitability by glutamate release from synaptic-like vesicles: evidence from the rat muscle spindle primary sensory ending. *J. Physiol.*, **562**, 381-394.

Blanksma, N.G. & van Eiden, T.M.G.J. (1995) Electromyographic heterogeneity in the human temporalis and masseter muscles during static biting, open/close excursions and chewing. *J. Dent. Res.,* **6**, 1318-1327.

Boucher, T.J., Okuse. K., Bennett, D.L., Munson, J.B., Wood, J.N. & McMahon, S.B. (2000) Potent analgesic effects of GDNF in neuropathic pain states. *Science,* **290**, 124-127.

Bourque, M.J. & Kolta, A. (2001) Properties and interconnections of trigeminal interneurons of the lateral pontine reticular formation in the rat. *J. Neurophysiol.*, **86**, 2583-2596.

Bremer, F. (1923) Physiologie nervuse de la mastication chez le chat et le lapin. Réflexes de mastication. Réponses masticatrices corticales et centre cortical du gout. *Arch. Int. Physiol.*, **21**, 308-352.

Brennan, D., Spencer, J.A. & Roberts-Thomson, K. (2008) Tooth loss, chewing ability and quality of life. *Qual. Life Res.*, **17**, 227-235.

Brocard, F., Verdier, D., Arsenault, I., Lund, J.P. & Kolta, A. (2006) Emergence of intrinsic bursting in trigeminal sensory neurons parallels the acquisition of mastication in weanling rats. *J. Neurophysiol.*, **96**, 2410-24.

Castrillon, E.E., Cairns, B., Ernberg, M., Wang, K., Sessle, B., Arendt-Nielsen, L. & Svensson, P. (2008) Glutamate-evoked jaw muscle pain as a model of persistent myofacial TMD pain? *Arch. Oral Biol.*, **53**, 666-676.

Chandler, S.H. & Tal, M. (1986) The effects of brain stem transections on the neuronal networks responsible for rhythmical jaw muscle activity in the guinea pig. *J. Neurosci.*, **6**, 1831-1842.

Cohen, J.E. & Fields, R.D. (2004) Extracellular calcium depletion in synaptic transmission. *Neuroscientist*, **10**, 12-7.

Coggeshall, R. E. & Lekan, H. A. (1996) Methods for determining numbers of cells and synapses: A case for more uniform standards of review. *J. Comp. Neurol.*, **364**, 6-15.

Connor, M., Naves, L.A. & McCleskey, E.W. (2005) Contrasting phenotypes of putative proprioceptive and nociceptive trigeminal neurons innervating jaw muscle in rat. *Mol. Pain*, **1**, 31.

Costigan, M., Scholz, J. & Woolf, C.J. (2009) Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu. Rev. Neurosci.*, **32**, 1-32.

Dal Bo, G., Lund, J.P., Verdier, D. & Kolta, A. (2005) Inputs to nucleus pontis caudalis from adjacent trigeminal areas. *Eur. J. Neurosci.*, **22**, 1987-1996.

Darian-Smith, I. (1965) Tactile sensory pathways from the face. *Proc. Aust. Assoc. Neuro.*, **3**, 27-39.

deLeeuw, R. (2008) Temporomandibular disorders. *Orofacial Pain Guidelines for Assessment, Diagnosis and Management*. Quintessence Publ. Co, Inc, Chicago, pp 131.

Dellow, P.G. & Lund, J. P. (1971) Evidence for central timing of rhythmical mastication. *J. Physiol.*, **215**, 1-13.

Del Negro, C.A. & Chandler, S.H. (1997) Physiological and theoretical analysis of K+ currents controlling discharge in neonatal rat mesencephalic trigeminal neurons. *J. Neurophysiol.*, **77**, 537-553.

Devor, M. (2005) Responses of nerves to injury in relation to neuropathic pain. The pathophysiology of damaged peripheral nerve. In: McMahon, S.L. & Koltzenberg, M. (eds.), *Textbook of Pain*, Churchill-Livingston, London, pp 905-927.

Dong, X.D., Mann, M.K., Sessle, B.J., Arendt-Nielsen, L., Svensson, P. & Cairns, B.E. (2006) Sensitivity of rat temporalis muscle afferent fibers to peripheral N-methyl-D-aspartate receptor activation. *Neuroscience*, **141**, 939-945.

Donga, R., Lund, J.P. & Veilleux, D. (1990) An electrophysiological study of trigeminal commissural interneurons in the anaesthetized rabbit. *Brain Res.*, **515**, 351-354.

Donga, R. & Lund, J.P. (1991) Discharge patterns of trigeminal commissural last-order interneurons during fictive mastication in the rabbit. *J. Neurophysiol.*, **66**, 1564-1578.

Dragunow, M. & Faull, R. (1989) The use of c-fos as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Methods*, **29**, 261-265.

Draisci, G. & Iadarola, M.J. (1989) Temporal analysis of increases in c-fos, preprodynorphin and preproenkephalin mRNAs in rat spinal cord. *Brain Res. Mol. Brain Res.*, **6,** 31-37.

Dubner, R. & Ren, K. (2006) Persistent orofacial pain. In: Laskin, D.M., Greene, C.S. & Hylander, W.L. (eds.), *Temporomandibular Disorders*. Quintessence Publ, Co, Ins, Chicago, pp 89-96.

Eisenman, J., Landgren, S. & Novin, D. (1963) Functional organization in the main sensory trigeminal nucleus and in the rostral subdivision of the nucleus of the spinal trigeminal tract in the cat. *Acta Physiol. Scand*., **59** (suppl 214), 1-44.

Enomoto, A., Kogo, M., Koizumi, H., Ishihama, K. & Yamanishi, T. (2002) Localization of premotoneurons for an NMDA-induced repetitive rhythmical activity to TMNs. *Neuroreport,* **13**, 2303-2307.

Ferrier, D. (1886) *The Functions of the Brain*. Smith, London.

Fields, R.D., Eshete, F., Stevens, B. & Itoh, K (1997) Action potential-dependent regulation of gene expression: temporal specificity in Ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. *J. Neurosci*., **17**, 7252-7266.

Goldberg, L.J. & Chandler, S.H. (1990) Central mechanisms of rhythmic trigeminal activity. In: Taylor, A. (ed.), *Neurophysiology of the Jaws and Teeth*., Macmillan, London, pp 268- 293.

Gossard, J.P., Bouyer, L. & Rossignol, S. (1999) The effects of antidromic discharges on orthodromic firing of primary afferents in the cat. *Brain Res*., **825**, 132-145.

Goulding, M. (2009) Circuits controlling vertebrate locomotion: moving in a new direction. *Nat. Rev. Neurosci*., **10**, 507-18.

Greene, C. S. & Laskin, D.M. (2001) Temporomandibular disorders; moving from a dentally based to a medically based model. *J. Dent. Res*., **79**, 1736-1739.

Grillner, S., Kozlov, A. & Hellgren Kotaleski, J. (2005) Integrative neuroscience: linking levels of analyses. *Curr. Opinion Neurobiol*., **14**, 614-621.

Grillner, S. (2006) Biological pattern generation: the cellular and computational logic of networks in motion. *Neuron*, **52**, 751-766.

Grillner, S. Wallén, P. Saitoh, K., Kozlov, A. & Robertson, B. (2008) Neural bases of goal-directed locomotion in vertebrates – An overview. *Brain Res. Rev*., **57**, 2-12.

Hannam, A.G. & McMillan, A.S. (1994) Internal organization in the human jaw muscles. *Crit. Rev. Oral Biol. Med*., **5**, 55-89.

Harris, J.A. (1998) Using c-fos as a neural marker of pain. *Brain Res. Bull*., **45**, 1-8.

Hatanaka, N., Tokuno, H., Nambum, A., Inoue, T. & Takada, M. (2005) Input-output organization of jaw movement-related areas in monkey frontal cortex. *J. Comp. Neurol*., **492**, 401-425.

Herdegen, T. & Leah, J.D. (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res. Rev*., **28**, 370-490.

Herrera, D.G. & Robertson, H.A. (1996) Activation of c-fos in the brain. *Prog. Neurobiol*., **50**, 83-107.

Hiraba, K., Taira, M., Sahara, Y. & Nakamura, Y. (1988) Single-unit activity in bulbar reticular formation during food ingestion in chronic cats. *J. Neurophysiol.*, **60**, 1333-1349.

Hoheisel, U., Mense, S. & Scherotzke R. (1994) Calcitonin gene-related peptide-immunoreactivity in functionally identified primary afferent neurones in the rat. *Anat. Embryol.,* **189**, 41-49.

Hsiao, C.F., Gougar, K., Asai, J. & Chandler, S.H. (2007) Intrinsic membrane properties and morphological characteristics of interneurons in the rat supratrigeminal region. *J. Neurosci. Res.*, **16**, 3673-3686.

Hutcheon, B. & Yarom, Y. (2000) Resonance, oscillation and the intrinsic frequency preferences of neurons. *Trends Neurosci.*, **23**, 216-222.

Inoue, T., Masuda, Y., Nagashima, T., Yoshikawa, K. & Morimoto, T. (1992) Properties of rhythmically active reticular neurons around the trigeminal motor nucleus during fictive mastication in the rat. *Neurosci. Res.*, **14**, 275-294.

Inoue, T., Chandler, S.H. & Goldberg, L.J. (1994) Neuropharmacological mechanisms underlying rhythmical discharge in trigeminal interneurons during fictive mastication. *J. Neurophysiol.*, **71**, 2061-2073.

Jacquin, T.D., Borday, V., Schneider-Maunoury, S., Topilko, P., Ghilini, G., Kato, F., Charnay, P. & Champagnat, J. (1996) Reorganization of pontine rhythmogenic neuronal networks in Krox-20 knockout mice. *Neuron*, **17**, 747-758.

Jean, A. (2001). Brain stem control of swallowing: Neuronal network and cellular mechanisms. *Physiol. Rev.*, **81**, 929-960.

Jerge, C.R. (1963) Organization and function of the trigeminal mesencephalic nucleus, *J. Neurophysiol.*, **26**, 379-392.

Johansson, R.S. & Flanagan, J.R. (2009) Coding and use of tactile signals from the fingertips in object manipulation tasks. *Nat. Rev. Neurosci.,* **10**, 345-359.

Julius, D. & Basbaum, A. (2001) Molecular mechanisms of nociception. *Nature*, **43**, 203-210.

Kamogawa, H., Hanashima, N., Naito, K. & Kagaya, K. (1988) Candidate interneurons mediating peripherally evoked disynaptic inhibition of masseter motoneurons of both sides. *Neurosci. Lett.*, **95**, 149-154.

Kawamura, Y. (1972) Advances in the physiology of mastication. In: Emmelin, N. & Zotterman, Y. (eds.), *Oral Physiology*. Wenner-Gren Center International Symposium series, vol 20, pp 163-204.

Khakh, B.S. & Henderson, G. (1998) Hyperpolarization-activated cationic currents (Ih) in neurons of the trigeminal mesencephalic nucleus of the rat. *J. Physiol.*, **510**, 695-704.

Kogo, M., Funk, G.D. & Chandler, S.H. (1996) Rhythmical oral motor activity recorded in an in vitro brainstem preparation. *Somatosens. Motor Res.*, **13**, 39-48.

Kohama, I., Ishikawa, K. & Kocsis, J.D. (2000) Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by A-beta afferents. *J. Neurosci.*, **20**, 1538-1549.

Kolta A. (1997) In vitro investigation of synaptic relations between interneurons surrounding the trigeminal motor nucleus and masseteric motoneurons. *J. Neurophysiol.*, **78**, 1720-1725.

Kolta, A., Westberg, K-G. & Lund, J.P. (2000) Identification of brainstem interneurons projecting to the trigeminal motor nucleus and adjacent structures in the rabbit. *J. Chem. Neuroanat.*, **19**, 175-195.

Kolta, A., Brocard, F., Verdier, D. & Lund, J.P. (2007) A review of burst generation by trigeminal main sensory neurons. *Arch. Oral Biol.*, **52**, 325-328.

Komuro, A., Morimoto, T., Iwata, K. , Inoue, T., Masuda, Y., Kato, T. & Hidaka, O. (2001) Putative feed-forward control of jaw-closing muscle activity during rhythmic jaw movements in the anesthetized rabbit. *J. Neurophysiol.,* **86**, 2834-2844.

Korfage, J.A.M., Koolstra, J.H., Langenbach, G.E.J. & van Eijden, T.M.G.J. (2005a) Fiber-type composition of the human jaw muscles—(Part 1) Origin and functional significance of fiber-type diversity. *J. Dent. Res.*, **84**, 774-783.

Korfage, J.A.M., Koolstra, J.H., Langenbach, G.E.J. & van Eijden, T.M.G.J. (2005b) Fiber-type composition of the human jaw muscles—(Part 2) Role of hybrid fibers and factors responsible for inter-individual variation. *J. Dent. Res.*, **84**, 784-793.

Kovács, K.J. (1998) c-Fos as a transcriptional factor: a stressful (re) view from a functional map. *Neurochem. Int.*, **33**, 287-297.

Krahe, R. & Gabbiani, F. (2004) Burst firing in sensory systems. *Nat. Rev. Neurosci.*, **5**, 13-23.

Kruger, L., Silverman, J.D., Mantyh, P.W., Sternini, C. & Brecha, N.C. (1989). Peripheral patterns of calcitonin-gene-related peptide general somatic sensory innervation: cutaneous and deep terminations. *J. Comp. Neurol.*, **280**, 291-302.

Landgren, S., Olsson, K.Å. & Westberg, K-G. (1986) Bulbar neurones with axonal projections to the trigeminal motor nucleus in the cat. *Exp. Brain Res.*, **65**, 98-111.

Landgren, S. & Olsson, K.Å. (1977) *Tuggapparatens Nervösa Styrmekanismer*. Studentlitteratur, Lund.

Laskin, D.M. (2008) Temporo-mandibular disorders; a term past its time? *J. Am. Dent. Assoc.*, **139**, 124-128.

Lavigne, G., Kim, J.S., Valiquette, C. & Lund, J.P. (1987) Evidence that periodontal presso-receptors provide positive feedback to jaw closing muscles during mastication. *J. Neurophysiol.*, **58**, 342-358.

Lawson, S.N. (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. *Exp. Physiol.*, **87**, 239-244.

Lazarov, N.E. (2002) Comparative analysis of the chemical neuroanatomy of the mammalian trigeminal ganglion and mesencephalic trigeminal nucleus. *Prog. Neurobiol.*, **66**, 19-59.

Li, Y.Q., Takada, M. & Mizuno, N. (1993) Identification of premotor interneurons which project bilaterally to the trigeminal motor, facial or hypoglossal nuclei: a fluorescent retrograde double-labeling study in the rat. *Brain Res.*, **611**, 160-164.

Li, Y.Q., Takada, M., Kaneko, T. & Mizuno, N. (1996) GABAergic and glycinergic neurons projecting to the trigeminal motor nucleus: a double labeling study in the rat. *J. Comp. Neurol.*, **373**, 498-510.

Liu, C.N., Wall, P.D., Ben Dor, E., Michaelis, M., Amir, R. & Devor, M. (2000) Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. *Pain*, **85**, 503-521.

Loeser, J.D. & Treede, R-D. (2008) The Kyoto protocol of IASP basic pain terminoloy. *Pain*, **137**, 473-477.

Luo, P., Wong, R. & Dessem, D. (1995) Projection of jaw-muscle spindle afferents to the caudal brainstem in rats demonstrated using intracellular biotinamide. *J. Comp. Neurol.*, **358**, 63-78.

Lund, J.P. & Dellow, P.G. (1971) The influence of interactive stimuli on rhythmical masticatory movements in rabbits. *Arch. Oral Biol.*, **16**, 215-223.

Lund, J.P. & Olsson, K.Å. (1983) The importance of reflexes and their control during jaw movement. *Trends in Neurosci.*, **6**, 458-463.

Lund, J.P., Sasamoto, K., Murakami, T. & Olsson, K.Å. (1984). Analysis of rhythmical jaw movements produces by electrical stimulation of motor sensory cortex of rabbits. *J. Neurophysiol.*, **52**, 1014-1029.

Lund, J.P. (1991) Mastication and its control by the brain stem. *Crit. Rev. Oral Biol. Med.*, **2**, 33-64.

Lund, J.P., Donga, R., Widmer, C.G. & Stohler, C.S. (1991) The pain-adaptation model: a discussion of the relationship between chronic musculoskeletal pain and motor activity. Can. *J. Physiol. Pharmacol.*, **69**, 683-94.

Lund, J.P., Kolta, A., Westberg, K-G. & Scott, G. (1998) Brainstem mechanisms underlying feeding behaviors. *Curr. Opin. Neurobiol.*, **8**, 718-724.

Lund, J.P. (2008) Persistent pain and motor dysfunction. In: Sessle, B.J., Lavigne, G.J., Lund, J.P. & Dubner, R. (eds.), *Orofacial Pain.* Quintessence Publ. Co, Inc, Chicago, pp 117-124.

Lund, J.P., Kolta, A. & Sessle, B. (2009) Trigeminal system. In: Squire, L.R. (ed.), New *Encyclopedia of Neuroscience.* Elsevier Ltd, pp. 1167 -1171.

Luschei, E.S. & Goodwin, G.M. (1974) Patterns of mandibular movement and jaw muscle activity during mastication in the monkey. *J. Neurophysiol.*, **37**, 954-966.

Luschei, E.S. & Goldberg, L.J. (1981) Neural mechanisms of mandibular control: mastication and voluntary biting. In: Brooks, V.B. (ed.), *Handbook of Physiology. Motor Control, Vol II.* American Physiological Society, Washington, pp 1237-1274.

Ma, J.J., Novikov, L.N., Wiberg, M. & Kellerth, J.O. (2001) Delayed loss of spinal motoneurons after peripheral nerve injury in adult rats: a quantitative morphological study. *Exp. Brain Res.*, **139**, 216-223.

Maixner,W. (2008) Biopsychological and genetic risk factors for temoporomandibular joint disorders and related conditions. In: Graven-Nielsen, T., Arendt-Nielsen,L. & Mense, S. (eds.), *Fundamentals of Musculoskeletal Pain.* IASP Press, Seattle, pp 263-279.

Marder, E. & Bucher, D. (2001) Central pattern generators and the control of rhythmic movements. *Curr. Biol.*, **11**, 986-996.

Masuda, Y., Morimoto, T., Hidaka, O., Kato, T., Matsuo, R., Inoue, T., Kobayashi, M. & Taylor, A. (1997) Modulation of jaw muscle spindle discharge during mastication in the rabbit. *J Neurophysiol.*, **77**, 2227-2231.

McCarthy, P.W. & Lawson, S.N. (1990) Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. *Neuroscience*, **34**, 623-632.

McCrea, D.A & Rybak, I.A. (2008) Organization of mammalian locomotor rhythm and pattern generation. *Brain Res. Rev.*, **57**, 134-146.

McDavid, S., Verdier,D., Lund, J.P. & Kolta, A. (2008) Electrical properties of interneurons found in the trigeminal motor nucleus. *Eur J. Neurosci.*, **28**, 1136-1145.

McDavid, S., Lund, J.P., Auclair, F. & Kolta, A. (2006) Morphological and immuno-histochemical characterization of interneurons within the trigeminal motor nucleus. *Neuroscience*, **139**, 1049-1059.

McNiell, C., Dubner, R. & Woda, A. (2008) What is pain and how do we classify orofacial pain. In: Sessle, B.J., Lavigne, G.J., Lund, J.P. & Dubner,R. (eds.), *Orofacial Pain*, Quintessense publ. Co, Inc., Chicago, pp 3-11.

Mense, S. (1993) Nociception from skeletal muscle in relation to clinical pain. *Pain*, **54,** 241-289.

Mense, S. (2008) Peripheral and central mechanisms of musculoskeletal pain. In: Castro-Lopes, J., Raja, S. & Schmelz, M. (eds.), *Pain 2008- An updated review*. IASP Press, Seattle, pp 55-62.

Mense, S. (2009). Algesic agents exciting muscle nociceptors. *Exp.Brain Res.*, **196**, 89-100.

Min, M.Y., Hsu, P.C. & Yang, H.W. (2003) The physiological and morphological characteristics of interneurons caudal to the trigeminal motor nucleus in rats. *Eur. J. Neurosci.*, **18**, 2981-2998.

Mizuno, N. (1970) Projection fibers from the main sensory trigeminal nucleus and the supratrigeminal region. *J. Comp. Neurol.*, **139**, 457-471.

Molliver, D.C., Immke, D.C., Fierro, L., Paré, M., Rice, F.L. & McCleskey, E.W. (2005) ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. *Mol. Pain*, **1**, 35.

Morgan, J.I. & Curran, T. (1991) Proto-oncogene transcription factors and epilepsy. *Trends Pharmacol. Sci.*, **12**, 343-349.

Morimoto, T., Inoue, T., Masuda, Y. & Nagashima, T. (1989) Sensory components facilitating jaw-closing muscle activities in the rabbit. *Exp. Brain Res.*, **76**, 424-440.

Muñoz, A., Liu, X.B. & Jones, E.G. (1999) Development of metabotropic glutamate receptors from trigeminal nuclei to barrel cortex in postnatal mouse. *J. Comp. Neurol.*, **409**, 549-566.

Nakamaura, Y. & Katakura, N. (1995) Generation of masticatory rhythm in the brainstem. *Neurosci. Res.*, **23**, 1-19.

Nakamura, Y., Katakura, N., Nakajima,M. & Liu, J. (2004) Rhythm generation for food-ingestive movements. *Prog. Brain Res.*, **143**, 97-103.

Noga, B.R., Johnson, D.M.G., Riesgo, M.I. & Pinzon, A. (2009) Locomotor-activated neurons of the cat. I. Serotonergic innervation and co-localization of 5-HT7, 5-HT2A and 5-HT1A receptors in the thoraco-lumbar spinal cord J Neurophysiol., *In press*, doi: 10.1152/jn.91179.

Nozaki, S., Iriki, A. & Nakamura, Y. (1986a) Localization of central rhythm generator involved in cortically induced rhythmical masticatory jaw-opening movement in the Guinea Pig. *J. Neurophysiol.*, **55**, 806-825.

Nozaki, S., Iriki, A. & Nakamura, Y. (1986b) Role of corticobulbar projection neurons in cortically induced rhythmical masticatory jaw-opening movement in the guinea pig. *J. Neurophysiol.*, **55**, 826-845.

Olsson, K.Å. (1979) Hypothalamic and cortical control of jaw reflexes. Thesis, Umeå University, Med. Diss. New Ser., No 52.

Olsson, K.Å., Sasamoto, K. & Lund, J.P. (1986a) Modulation of transmission in rostral trigeminal sensory nuclei during chewing. *J. Neurophysiol.*, **55**, 56-75.

Olsson, K.Å., Landgren, S. & Westberg, K-G. (1986b) Location of, and peripheral convergence on, the interneuron in the disynaptic path from the coronal gyrus of the cerebral cortex to the trigeminal motorneurons in the cat. *Exp. Brain Res.*, **65**, 83-97.

Olsson, K.Å. & Westberg, K-G. (1989) Interneurones in the trigeminal motor system. In: van Steenberghe, D., De Laat, A. (eds.), *EMG of Jaw Reflexes in Man*. Leuven University Press, Leuven, pp 19-50.

Olsson, K.Å. & Landgren, S. (1990) Primary afferent and descending cortical convergence on the interneurons in the border zone of the trigeminal motor nucleus: A comparison between trigeminal and spinal interneurons. In: Taylor, A. (ed.), *Neurophysiology of the Jaws and Teeth*. MacMillan Press LTD, London, pp 162-191.

Orchardson, R. & Cadden, S.W (1998) Mastication. In: Linden, R.W.A. (ed.), *Frontiers of Oral Biology*. Karger, Basel, pp 76-121.

Pang, Y.W., Li, J.L., Nakamura, K., Wu, S., Kaneko, T. & Mizuno, N. (2006) Expression of vesicular glutamate transporter 1 immunoreactivity in peripheral and central endings of trigeminal mesencephalic nucleus neurons in the rat. *J. Comp. Neurol.*, **498**, 129-141.

Pedroarena, C.M., Pose, I.E., Yamuy, J., Chase, M.H. & Morales, F.R. (1999) Oscillatory membrane potential activity in the soma of a primary afferent neuron. *J. Neurophysiol.* **82**, 1465-1476.

Pinganaud, G., Bernat, I., Buisseret, P. & Buisseret-Delmas, C. (1999) Trigeminal projections to hypoglossal and facial motor nuclei in the rat. *J. Comp. Neurol.*, **415**, 91-104.

Puil, E. & Spiegelman, I. (1988) Electrophysiological responses of trigeminal root ganglion neurons in vitro. *Neuroscience*, **24**, 635-646.

Puil, E., Miura, R. & Spiegelman, I. (1989) Consequences of 4-aminopyridine applications to trigeminal root ganglion neurons. *J. Neurophysiol.*, **62**, 810- 820.

Ramirez, J-M., Tryba, A.K. & Pena, F. (2004) Pacemaker neurons and neuronal networks: an integrative view. *Curr Opinion Neurobiol.*, **14**, 1-10.

Ren, K. (1999) An improved method for assessing mechanical allodynia in the rat. *Physiol. Behav.*, **67**, 711-716.

Rioch, J.M. (1934) The neural mechanism of mastication. *Am. J. Physiol.*, **108,** 168-176.

Rosendal, L., Larsson, B., Kristiansen, J., Peolsson, M., Sogaard, K., Kjaer, M., Sorensen, J. & Gerdle, B. (2004) Increase in muscle nociceptive substances and anaerobic metabolism in patients with trapezius myalgia: microdialysis in rest and during exercise. *Pain*, **112**, 324-334.

Sagar, S.M., Sharp, F.R. & Curran, T. (1988) Expression of c-fos protein in the brain: Metabolic mapping at the cellular level. *Science*, **240**, 1328-1331.

Sandler, V.M., Puil, E. & Schwarz, D.W. (1998) Intrinsic properties of bursting neurons in the nucleus principal trigemini of the gerbil. *Neuroscience*, **83**, 891-904.

Santini, M. & Ibata, Y. (1971) The fine structure of thin unmyelinated axons within muscle spindles. *Brain Res.*, **33**, 289-302.

Schwartz, G., Enomoto, S., Valiquette, C. & Lund, J.P. (1989) Mastication in the rabbit: a description of movement and muscle activity. *J. Neurophysiol.*, **62**, 273-287.

Scott, G., Westberg, K-G., Vrentzos, N. & Lund, J.P. (2003) Effect of lidocaine and NMDA injections into the medial pontobulbar reticular formation on mastication evoked by cortical stimulation in anaesthetized rabbits. *Eur. J. Neurosci.*, **17**, 2156-2162.

Sessle, B.J. (1977) Modulation of alpha and gamma trigeminal motoneurons by various peripheral stimuli. *Exp. Neurol.*, **54**, 323-339.

Sherrington, C. S. (1917) Reflexes elicitable in the cat from pinna, vibrissae and jaws. *J. Physiol.*, **51**, 404-431.

Shigenaga, Y., Yoshida, A., Tsuru, K., Mitsuhiro, Y., Otani, K. & Cao, C.Q. (1988) Physiological and morphological characteristics of cat masticatory motoneurons - intracellular injection of HRP. *Brain Res.*, **461**, 238-256.

Sluka, K.A., Kalra, A. & Moore, S.A. (2001) Unilateral intramuscular injections of acidic saline produce a bilateral, long lasting hyperalgesia. *Muscle & Nerve*, **24**, 37-46.

Sluka, K.A., Price, M.P., Breese, N.M., Stucky, C.L., Wemmie, J.A. & Welsh M.J. (2003) Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. *Pain*, **106**, 229-239.

Stohler, C.S. (1999) Craniofacial pain and motor function: pathogenesis, clinical correlates, and implications. *Crit. Rev. Oral Biol. Med.*, **10**, 504-518.

Svensson, P., Cairns, B.E., Wang, K., Hu, J.W., Graven-Nielsen, T., Arendt-Nielsen, L. & Sessle, B.J. (2003) Glutamate-evoked pain and mechanical allodynia in the human masseter muscle. *Pain*, **101**, 221-227.

Svensson, P. (2007) What can human experimental pain models teach us about clinical TMD? *Arch. Oral. Biol.*, **52**, 391-394.

Takamori, S., Rhee, J.S., Rosenmund, C. & Jahn R. (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature*, **407**, 189-194.

Tanaka, S., Kogo, M., Chandler, S.C. & Matsuya, T. (1999) Localization of oral-motor rhythmogenic circuits in the isolated rat brainstem preparation. *Brain Res.*, **821**, 190-199.

Tanaka, S., Wu, N., Hsaio, C.F., Turman, J. & Chandler, S.H. (2003) Development of inward rectification and control of membrane excitability in mesencephalic V neurons, *J. Neurophysiol.*, **89**, 1288-1298.

Tegeder, L., Zimmermann, J., Meller, S.T. & Geisslinger, G. (2002) Release of algesic substances in human experimental muscle pain. *Inflamm. Res.*, **51**, 393-402.

Ter Horst, G.J., Copray, J.C., Van Willigen, J.D. & Liem, R.S. (1990) Contralateral projections of cells in the motor trigeminal nucleus of the rat. *Neurosci. Lett.*, **113**, 260-266.

Ter Horst, G.J., Copray, J.C., Liem, R.S. & Van Willigen, J.D. (1991) Projections from the rostral parvocellular reticular formation to pontine and medullary nuclei in the rat: involvement in autonomic regulation and orofacial motor control. *Neuroscience*, **40**, 735-758.

Tillu, D.V., Gebhart, G.F. & Sluka, K.A. (2008) Descending facilitatory pathways from the RVM initiate and maintain bilateral hyperalgesia after muscle insult. *Pain*, **136**, 331-339.

Torvik, A. (1957) The ascending fibers from the main trigeminal sensory nucleus; an experimental study in the cat. *Am. J. Anat.*, **100**, 1-15.

Trulsson, M. (1993) Orofacial Mechanoreception in Man. Thesis, Umeå University, Odont. Diss. Abstract No 44.

Trulsson, M. & Johansson, R. S. (1996) Encoding of tooth loads by human periodontal afferents and their role in jaw motor control. *Prog. Neurobiol.*, **49**, 267-284.

Tsuboi, A., Kolta, A., Chen, C.C. & Lund, J.P. (2003) Neurons of the trigeminal main sensory nucleus participate in the generation of rhythmic motor patterns. *Eur. J. Neurosci.*, **17**, 229-238.

Tsuru, K., Otani, K., Kajiyama, K., Suemune, S. & Shigenaga, Y. (1989) Central terminations of periodontal mechanoreceptive and tooth pulp afferents in the trigeminal principal and oral nuclei of the cat. *Brain Res.*, **485**, 29-61.

Turman, J.E. & Chandler, S.H. (1994a) Immunohistochemical evidence for GABA and glycine-containing trigeminal premotoneurons in the guinea pig. *Synapse*, **18**, 7-20.

Turman, J.E. & Chandler, S.H. (1994b) Immunohistochemical localization of glutamate and glutaminase in guinea pig trigeminal premotoneurons. *Brain Res.*, **634**, 49-61.

Turman, J.E., Hiyama, L., Castillo, M. & Chandler, S.H. (2001) Expression of group I and II metabotropic glutamate receptors in trigeminal neurons during postnatal development. *Dev. Neurosci.*, **23**, 41-54.

van der Bilt, A., Engelen, L., Pereira, L.J., van der Glas, H.W, & Abbink, J.H. (2006). Oral physiology and mastication. *Physiol. Behav*., **89**, 22-27.

Verdier, D., Lund, J.P. & Kolta, A. (2004) Synaptic inputs to trigeminal primary afferent neurons cause firing and modulate intrinsic oscillatory activity. *J. Neurophysiol*., **92**, 2444-2455.

Wall, P.D. & Devor, M. (1983) Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. *Pain*, **17**, 321-339.

Westberg, K-G. (1990) Interneurones in the trigeminal motor system. An experimental neurobiological study in the cat. Thesis, Umeå University, Odont. Diss. Abstract No 37.

Westberg, K-G. & Olsson, K.Å. (1991) Integration in trigeminal premotor interneurones in the cat. 1. Functional characteristics of neurones in the subnucleus-γ of the oral nucleus of the spinal trigeminal tract. *Exp. Brain Res*., **84**, 102-114.

Westberg, K-G., Sandström, G. & Olsson, K.Å. (1995) Integration in trigeminal premotor interneurons in the cat. 3. Input characteristics and synaptic actions of neurons in subnucleus-γ of the oral nucleus of the spinal trigeminal tract with a projection to the masseteric motorneurone subnucleus. *Exp. Brain Res*., **104**, 449-461.

Westberg, K-G., Clavelou, P., Sandström, G. & Lund, J.P. (1998) Evidence that trigeminal brainstem interneurons form subpopulations to produce different forms of mastication in the rabbit. *J. Neurosci*., **18**, 6466-6479.

Westberg, K-G., Scott, G., Olsson, K.Å. & Lund, J.P. (2001) Discharge patterns of neurons in the medial pontobulbar reticular formation during fictive mastication in the rabbit. *Eur. J. Neurosci*., **14**, 1709-1718.

Winarakwong, L., Muramoto, T., Soma, K. & Takano, Y. (2004) Age-related changes and the possible adaptability of rat jaw muscle spindles: immunohistochemical and fine structural studies. *Arch. Histol. Cytol*., **67**, 227-240.

Woda, A., Mishellany, A., Peyron, M.A. (2006) The regulation of masticatory function and food bolus formation. *J. Oral Rehab*., **33**, 840-849.

Wolpert, D.M. & Flanagan, J.R. (2001) Motor prediction. *Curr. Biol*., **11**, 729-732.

Woolf, C.J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature*, **306**, 686-688.

Woolf, C.J., Shortland, P., Reynolds, M., Ridings, J., Doubell, T. & Coggeshall, R.E. (1995) Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J. Comp. Neurol*., **360**, 121-134.

Wu, N., Hsiao, C.F. & Chandler, S.H. (2001) Membrane resonance and subthreshold membrane oscillations in mesencephalic V neurons: participants in burst generation. *J Neurosci*., **21**, 3729-3739.

Wu, S.X., Koshimizu, Y., Feng, Y.P., Okamoto, K., Fujiyama, F., Hioki, H., Li, Y.Q., Kaneko, T. & Mizuno, N. (2004). Vesicular glutamate transporter immunoreactivity in the central and peripheral endings of muscle-spindle afferents. *Brain Res*., **1011**, 247–251.

Wu, N., Enomoto, A., Tanaka, S., Hsiao, C-F., Nykamp, D.Q., Izhikevich, E. & Chandler, S.H. (2005) Persistent Sodium Currents in mesencephalic V neurons participate in burst generation and control of membrane excitability. *J. Neurophysiol.*, **93**, 2710-2722.

Yang, H.W., Min, M.Y., Appenteng, K. & Batten T.F. (1997) Glycine-immunoreactive terminals in the rat trigeminal motor nucleus: light- and electron-microscopic analysis of their relationships with motoneurones and with GABA-immunoreactive terminals. *Brain Res.*, **749**, 301-319.

Yoshida, A., Hiraga, T., Moritani, M., Chen, K., Takatsuki, Y., Hirose, Y., Bae, Y.C. & Shigenaga, Y. (1998) Morphologic characteristics of physiologically defined neurons in the cat trigeminal nucleus principalis. *J. Comp. Neurol.*, **401**, 308-328.

Zhang, G. & Sasamoto, K. (1990) Projections of two separate cortical areas for rhythmical jaw movements in the rat. *Brain Res. Bull.*, **24**, 221-230.