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# Réponses des neurones du noyau sensoriel principal du trijumeau à la stimulation de leurs afférences primaires

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**Résumé**

La mastication est une activité générée par un réseau de neurones communément nommé générateur de patron central ou GPC, qui se trouve dans le tronc cérébral et qui peut être activé par des influx corticaux ou sensoriels. De plus en plus d'évidences suggèrent que la partie dorsale du noyau sensoriel principal du trijumeau, (NVsnpr) pourrait former le cœur du GPC de la mastication. Le but de cette étude était de déterminer si l'activation tonique des inputs sensoriels à ce noyau génère une activité rythmique dans ses neurones. Pour tester cette hypothèse, nous avons effectué des enregistrements extracellulaires de neurones du NVsnpr dans une préparation de tranche *in vitro* et examiné les effets de la stimulation répétitive du tractus du trijumeau et de l'application locale de NMDA et d'APV en présence de différentes concentrations de calcium extracellulaire, ($[Ca^{2+}]_e$). Les effets de la stimulation répétitive sur les patrons de décharge des neurones du NVsnpr sont divers (excitateurs ou inhibiteurs) mais dans quelques cas la stimulation peut entraîner un changement du patron de décharge des cellules du NVsnpr (tonique à rythmique en bouffées). Dans ces cas, un index de rythmicité (RI) calculé démontre que la décharge devient rythmique après la stimulation ($RI \geq 0.01$), alors qu'elle ne l'était pas avant. L'effet rhythmogénique de la stimulation du tractus peut être mimé par l'application locale de NMDA et peut être bloqué par l'application locale d'APV. Dans plusieurs cas d'enregistrements multiples, les neurones qui changent leur patron de décharge deviennent synchrones. Ces résultats suggèrent que la stimulation répétitive des afférences sensorielles *in vitro* dans des $[Ca^{2+}]_e$ physiologiques peut initier dans les neurones du NVsnpr des activités rythmiques en bouffées qui ressemblent à la mastication et cet effet dépend de l'activation des récepteurs NMDA.

**Mots clés :** Génération de patron central, mastication, noyau sensoriel principal du trijumeau, inputs sensoriels, tractus du trijumeau, calcium, récepteurs NMDA.

**Abstract**

Mastication is an activity generated by a network of neurons that is called a central pattern generator or CPG. The masticatory CPG is located in the brainstem and can be activated either by cortical or sensory inputs. Increasing evidence suggests that neurons in dorsal part of the trigeminal main sensory nucleus (NVsnpr) have the intrinsic properties and synaptic connections that could allow it to form the core of the masticatory CPG. The purpose of this study was to determine whether the activation of peripheral inputs contributes to generation of rhythmic activity in NVsnpr. To test this hypothesis we recorded extracellularly from neurons in NVsnpr in a brainstem slice *in vitro* preparation and studied the effect of sustained stimulation of the trigeminal tract and local application of NMDA and APV under different extracellular concentrations of calcium, ($[Ca^{2+}]_e$). The effects of tonic stimulation on the firing pattern of NVsnpr neurons were diverse (either excitatory or inhibitory) but in some cases tonic stimulation switched the neurons firing pattern from tonic firing to bursting. A rhythm index (RI) calculated in these cases shows that cell firing becomes rhythmic after stimulation ($RI \geq 0.01$). The rhythmogenic effect of trigeminal tract stimulation could be reproduced by application of NMDA and blocked by local application of APV. In several cases of multiple unit recodings the neurons that change their firing pattern became synchronous. These results suggest that stimulation of sensory afferents *in vitro* and in physiological $[Ca^{2+}]_e$ can elicit masticatory-like rhythmic bursting activities in NVsnpr neurons and that this effect relies on the activation of NMDA receptors.

**Key words:** Central pattern generation, mastication, trigeminal main sensory nucleus, peripheral inputs, trigeminal tract, calcium, NMDA receptors.

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## LIST OF ABBREVIATIONS

ACSF: Artificial cerebrospinal fluid

ADP: After-depolarization

AHP: After-hyperpolarization

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid

APV: D,L-2-amino-5-phosphonovaleric acid,

BK: Big $Ca^{2+}$ activated $K^+$ conductances

$[Ca^{2+}]_e$ : Extracellular concentration of $Ca^{2+}$

CMA: Cortical Masticatory Area

CPG :Central Pattern Generator

DNQX: 6,7-dinitro-quinoxaline

dPGC :Paragigantocellular reticular formation

EMG :Electro-myogram

EPSP: Excitatory postsynaptic potential

GABA: γ-aminobutyric acid

GC :Gigantocellular reticular nucleus

GCo :Gigantocellular reticular nucleus pars oralis

HRP: Horse-radish peroxidase

INaP: Persistent sodium conductance

IPSP: Inhibitory postsynaptic potential

JC: Jaw-closing

JO: Jaw-opening

MRF : Medial bulbar reticular formation

NintV :Intertrigeminal region

NMDA: N-methyl-D-aspartic acid, NMDA

nPontC:Nucleus Pontis pars caudalis

nPontO : Nucleus Pontis pars oralis

NVd : dorsal portion of NVsnpr equivalent in the cat

NVmes: Trigeminal mesencephalic nucleus

NVmot: Trigeminal motor nucleus

NVsnpr: Trigeminal main sensory nucleus

NVsp: Trigeminal spinal nucleus

NVspc: Trigeminal spinal nucleus pars caudalis

NVspi: Trigeminal spinal nucleus pars interpolaris

NVspo: Trigeminal spinal nucleus pars oralis

NVv: ventral portion of NVsnpr equivalent in the cat

PCRt: Parvocellular reticular formation

PeriV: Peri-trigeminal reticular area

PKC: Protein Kinase C

PSPs: Postsynaptic potentials

Regio h : PeriV equivalent in the rabbit

SK: Small $Ca^{2+}$ activated $K^{+}$ conductances

SupV : Supratrigeminal region

TNSC: Trigeminal nuclear sensory complex

TTX: Tetrodotoxin

VPM: Ventral Posterio-medial Thalamus

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Alex Pastor Bernier.

# SECTION I

# INTRODUCTION

# INTRODUCTION

## 1. PREAMBLE

Many forms of animal behaviour are rhythmic, including different types of locomotion such as walking, swimming, crawling and flying, and vital behaviours such as breathing and chewing. The rhythmic movements involved in these behaviours are governed by central pattern generators (CPGs) (Lund & Dellow, 1969; Von Euler, 1983; Grillner *et al.*, 1985; Pearson *et al.*, 1985; Eisenhart *et al.*, 2000). A CPG is an assembly of neurons that can produce and maintain a rhythmic pattern of activity in the absence of sensory feedback or descending central commands because of intrinsic properties and/or connectivity (Rossignol & Dubuc, 1994). This study is part of a larger project that investigates the mechanisms by which rhythmogenesis is triggered in the trigeminal main sensory nucleus (NVsnpr), a brainstem nucleus that has both the synaptic connectivity and intrinsic properties that are required to constitute the core of the CPG for mastication (Kolta *et al.*, 2007 for a review). We know from our previous work that prolonged (>100ms) repetitive stimulation of cortical or sensory fibers is required to initiate mastication *in vivo* (Dellow & Lund, 1971; Lund *et al.*, 1984). Here we examined the effects of long lasting repetitive stimulation of sensory afferents (trigeminal tract) on the firing pattern of NVsnpr neurons. Special attention is payed to the capacity of sensory afferent stimulation to elicit rhythmic activities and the cellular mechanism underlying the process.

## 1.1 THE MASTICATION PROCESS.

### 1.1.1 Definition

Mastication is a natural process that is the first step of digestion in mammals. It is a complex act that requires the coordinated activity of the jaw, tongue and facial muscles that enables the positioning, reduction, and grinding of foods. A closer look to the process reveals that it consists of a number of different movement patterns depending on the size and texture of the bolus (Thexton *et al.*, 1980; Weijs & Dantuma, 1981). Sensory feedback is essential to enable modifications of the movement patterns depending on the type of food we encounter. In experimental animals, repetitive electrical stimulation of the cortical masticatory area (CMA) and subcortical areas including the amygdala, the internal capsule, putamen, globus pallidus, substantia nigra, lateral hypothalamus, thalamic reticular nucleus, the mesencephalic reticular formation and the pyramidal tract at the level of the Pons (Kawamura & Tsukamoto, 1960; Dellow & Lund, 1971; Nakamura & Kubo, 1978; Hashimoto *et al.*, 1989) can induce rhythmical jaw-opening and jaw-closing movements ressembling mastication.

The CMA partly overlaps the representation of the jaw, tongue and facial muscles. In primates, the CMA covers the inferiolateral end of the motor cortex and the adjacent postcentral gyrus (Lund & Lamarre, 1974). Destruction or anaesthesia of this region does not only disrupt mastication itself but also ingestion and swallowing (Sessle *et al.*, 2005). The stimulation needed to produce mastication consists of long-lasting medium-frequency trains (ranging from 10-100Hz). Single pulses or short trains applied to the same area will evoke twitch contractions of individual jaw, tongue and facial muscles rather than rhythmical jaw movements (Dellow & Lund, 1971; Lund *et al.*, 1984). The motor neuron bursts induced by cortical stimulation represent a true CNS pattern rather than a series of brain-stem reflexes (Sherrington, 1917), since they occur in absence of sensory feedback "fictive mastication" (Lund & Dellow, 1969) and of afferent signals from the vascular and respiratory systems (Lund & Dellow, 1971).

However, even if mastication can be produced in absence of sensory feedback, tonic sensory inputs from the periphery can drive the process. Fictive as well as real mastication can be elicited by innocuous mechanical stimulation of the oral mucosa or the teeth in decerebrated rabbits (Bremer, 1923), by placing an object like a balloon in the mouth (Lund & Dellow, 1971; Olsson *et al.*, 1986), in response to a tonic pressure on the hard palate (Van Willigen & Weijs-Boot, 1984; Juch *et al.*, 1985), or by electrical stimulation of the lips in young pup rats and rabbits (Thexton *et al.*, 1980; Thexton *et al.*, 1988; for a review see Lund, 1991; Nakamura & Katakura, 1995).

# 2. THE NEURAL CORRELATES OF MASTICATION

## 2.1 OROFACIAL INNERVATION

### 2.1.1 The trigeminal nerve

The trigeminal nerve is the largest of the cranial nerves. It is the fifth cranial nerve (nerve V). The name "*trigeminal*" comes from the fact that it has three major branches: ophthalmic (V1), maxillary (V2) and mandibular (V3). These three branches emerge from the trigeminal ganglion (also called ganglion of Gasser) which is the equivalent to the spinal cord ganglia. The trigeminal nerve is a mixed nerve that has both sensory and motor functions. The ophthalmic and maxillar branches are entirely sensory, while the mandibular branch contains both motor and sensory fibers. The mandibular branch leaves the skull through the foramen ovale (Fig. 1).

### 2.1.2 The motor branches of the trigeminal nerve

The motor fibers in the mandibular branch project to the following muscles: masseter, temporalis, medial and lateral pterygoides, tensor veli palatine, mylohyoides, the anterior belly of the digastric and tensor tympani. With the exception of tensor timpani, all these muscles are involved in mastication (Fig. 1).

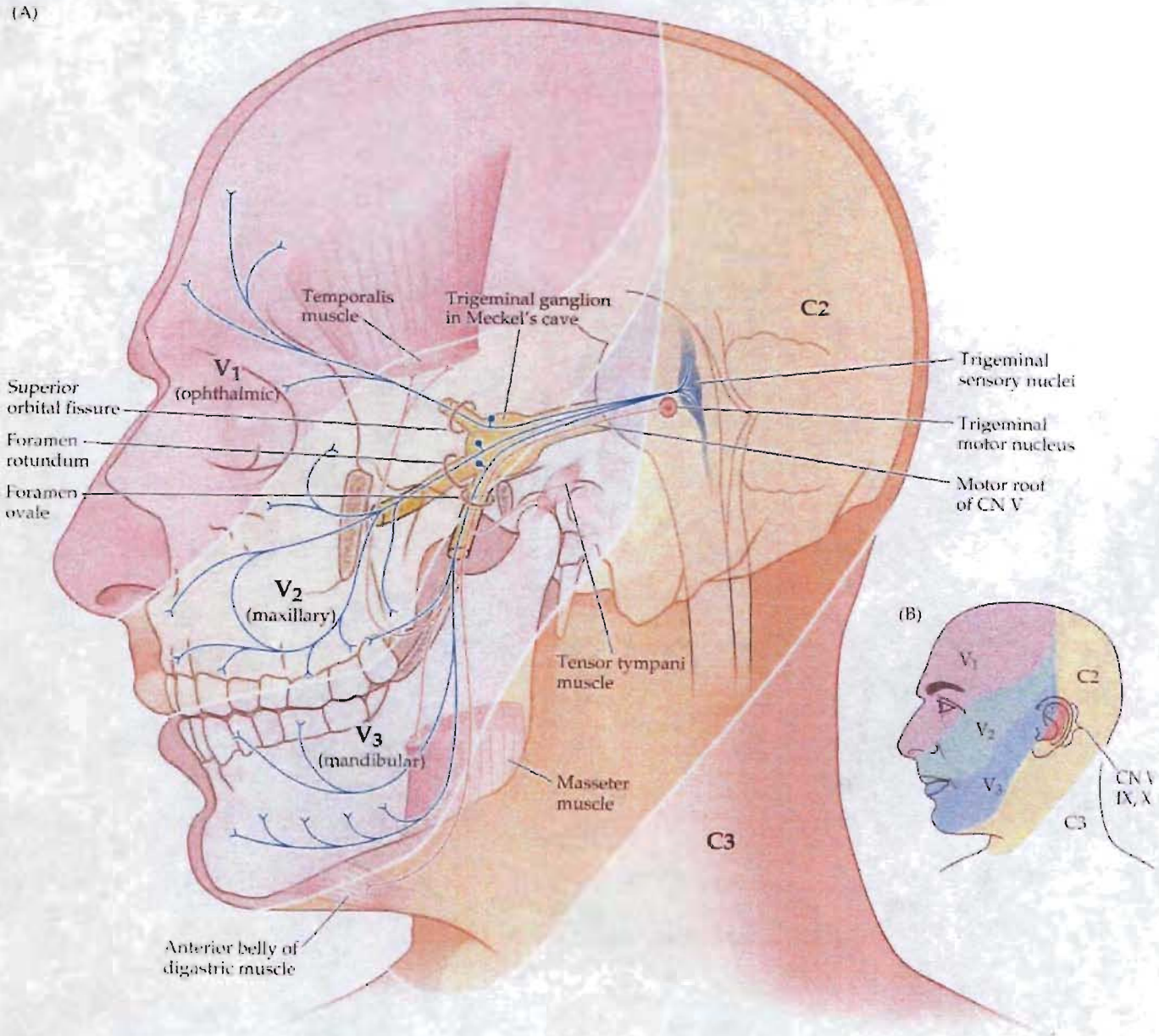


**Figure 1. A. Branches of the trigeminal nerve and their fields.** Motor branches are indicated in red and sensory branches in blue. The three main areas innervated by the trigeminal nerve V1, V2 and V3 are shown with different colours. The ophthalmic branch enters the skull through the orbital fissure and the maxillary branch through the foramen rotundum. Both branches converge on the trigeminal ganglion and join the mandibular branch that enters through the foramen ovale. **B. General somatic innervation of the face (dermatomes).** Adapted from Blumenfeld, (2002).

In mammals, the anterior digastric and mylohyoid muscles open the jaw, whereas the temporalis, masseter and pterygoides muscles close the jaw (Blumenfeld, 2002). The motoneuron cell bodies are found in the trigeminal motor nucleus in the brainstem (NVmot). Motoneurons of distinct muscles of the jaw are distributed in distinct locations within NVmot. The ventromedial motoneuron pool contains cells that innervate jaw-opening muscles (digastric and mylohyoideus) whereas the dorsolateral motoneuron pool innervates jaw-closing muscles (temporalis and masseter). The medial- and lateral pterygoides motoneuron pools are located between the other two (Weijs & Datuma, 1981; Jacquin *et al.*, 1983a; Mizuno *et al.*, 1983; Lynch, 1985).

### 2.1.3 The sensory branches of the trigeminal nerve

Trigeminal sensory fibers are found in all three branches and carry information from epithelial mechanoreceptors (in the skin, hair and mucosa), periodontal mechanoreceptors (in the periodontal ligaments), temporomandibular joint afferents (in the temporomandibular capsule), muscle afferents (primary and secondary muscle spindles, Golgi tendon organs and thermal and nociceptive afferents. Most of the cell bodies of these primary sensory fibers form the trigeminal ganglion (Fig. 1). However, all afferents that innervate the jaw-closing muscle spindles (primary and secondary) and a subpopulation of periodontal afferents have their cell bodies in the brainstem in the trigeminal mesencephalic nucleus (NVmes) (Gottlieb *et al.,* 1984) (Fig. 2).

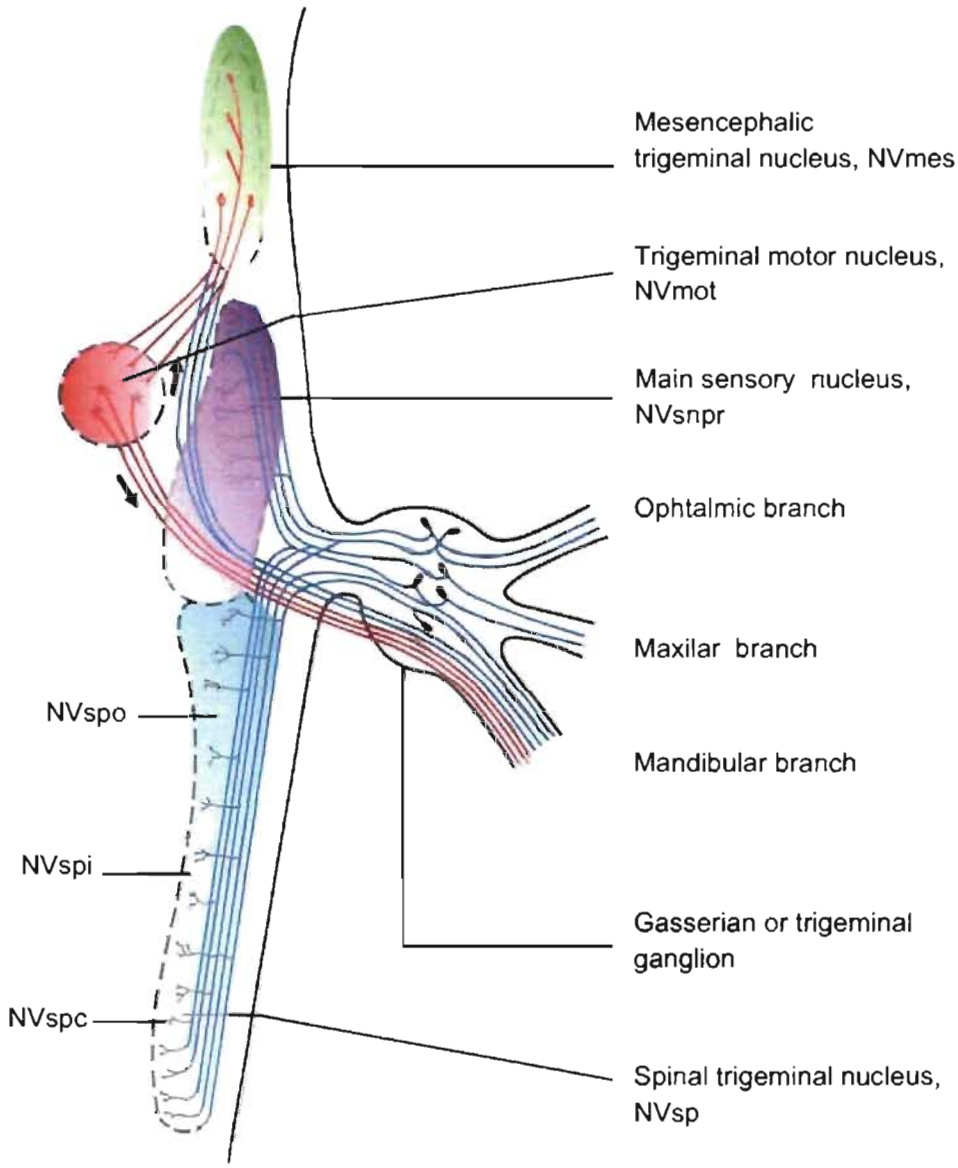


**Figure 2. Schematic figure depicting trigeminal sensory (blue) and motor (red) fibers and their associated nuclei (View from a sagital cut across the brainstem).** The main sensory nucleus (NVsnpr), the spinal nucleus (NVsp), subdivided in three regions (NVspo, NVspi, NVspc) and the mesencephalic nucleus, NVmes receive sensory inputs. NVmes sends efferents to the trigeminal motor nucleus NVmot, that contain motoneuron cell bodies for each group of muscles involved in mastication. Adapted from Cajal, S. Ry, (1909).

The central branches of neurons of the trigeminal ganglion enter the Pons ventrally via the trigeminal sensory root and then bifurcate in ascending and descending branches that are also called the trigeminal tract. Large-diameter (myelinated) primary sensory neurons mediating fine touch and dental pressure travel through the ascending branches of the trigeminal tract and project to the main sensory nucleus (NVsnpr) in the brainstem. Medium and small-diameter (unmyelinated) primary sensory fibers that convey crude touch, pain and temperature sensations enter the lateral Pons through the descending branch of the trigeminal tract (spinal tract) and project to the ipsilateral trigeminal spinal nucleus (NVsp) in the brainstem (Fig. 2).

## 2.2 THE TRIGEMINAL SYSTEM

The trigeminal sensory nuclear complex (TSNC) is formed by the NVmes, the trigeminal main sensory nucleus (NVsnpr) and the trigeminal spinal nucleus (NVsp) (Meessen & Olszewski, 1949; Olszewski, 1950) (Fig. 3). NVsnpr is the brainstem analog of the dorsal column nuclei, whereas NVsp is an extension of the dorsal horn of the spinal cord. NVsp is continuous with NVsnpr and runs caudally to the upper segments of the spinal cord (C2). NVsp is subdivided into nucleus oralis (NVspo) interpolaris (NVspi) and caudalis (NVspc), and NVspo is furtherly subdivided in two cytoarchitectonic distinct regions from rostral to caudal: NVspo $\gamma$ and NVspo $\beta$ (Eisenman *et al.*, 1963). Both nuclei, NVsnpr and NVsp are bordered laterally by the trigeminal tract and medially by the parvocellular reticular formation (PCRt) (Fig. 4A). NVmes is located dorsoanteriorly to NVmot and lies in the upper to middle regions of the Pons extending from the superior colliculus to the caudal edge of NVsnpr. NVmot is located medial to NVsnpr and lateral to nucleus Pontis Caudalis (nPontC) (Fig. 3). A shell of the parvocellular reticular formation (PeriV) surrounds NVmot and is subdivided into several regions: The supratrigeminal nucleus (SupV) dorsal to NVmot, the intertrigeminal region (NintV) between NVmot and NVsnpr, the medial PeriV (mPeriV) located medial to NVmot and PCRt ventral and caudal to NVmot (Paxinos & Watson, 1982) (Fig. 4A). nPontC lies in the medial reticular formation bordering PeriV (Fig. 4B).

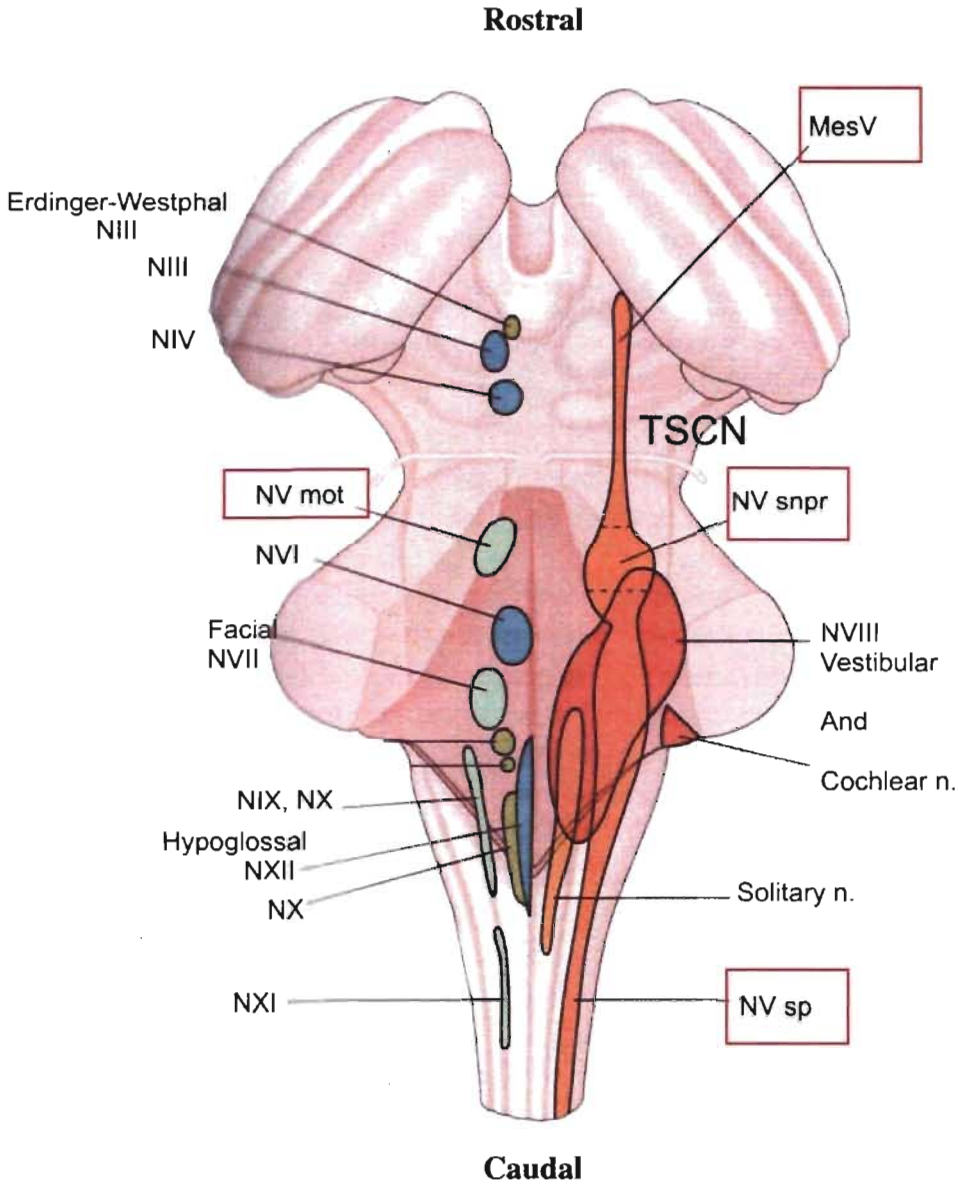


**Figure 3. The trigeminal nuclear complex.** Horizontal view of the brainstem seen from top of cerebellum (The cerebellum itself has been removed and we only see the peduncles and the brainstem structures beneath). We observe how the trigeminal sensory complex (orange) is organized in relation to other trigeminal nuclei (red, blue, green). Adapted from Kandel *et al.* (2000).

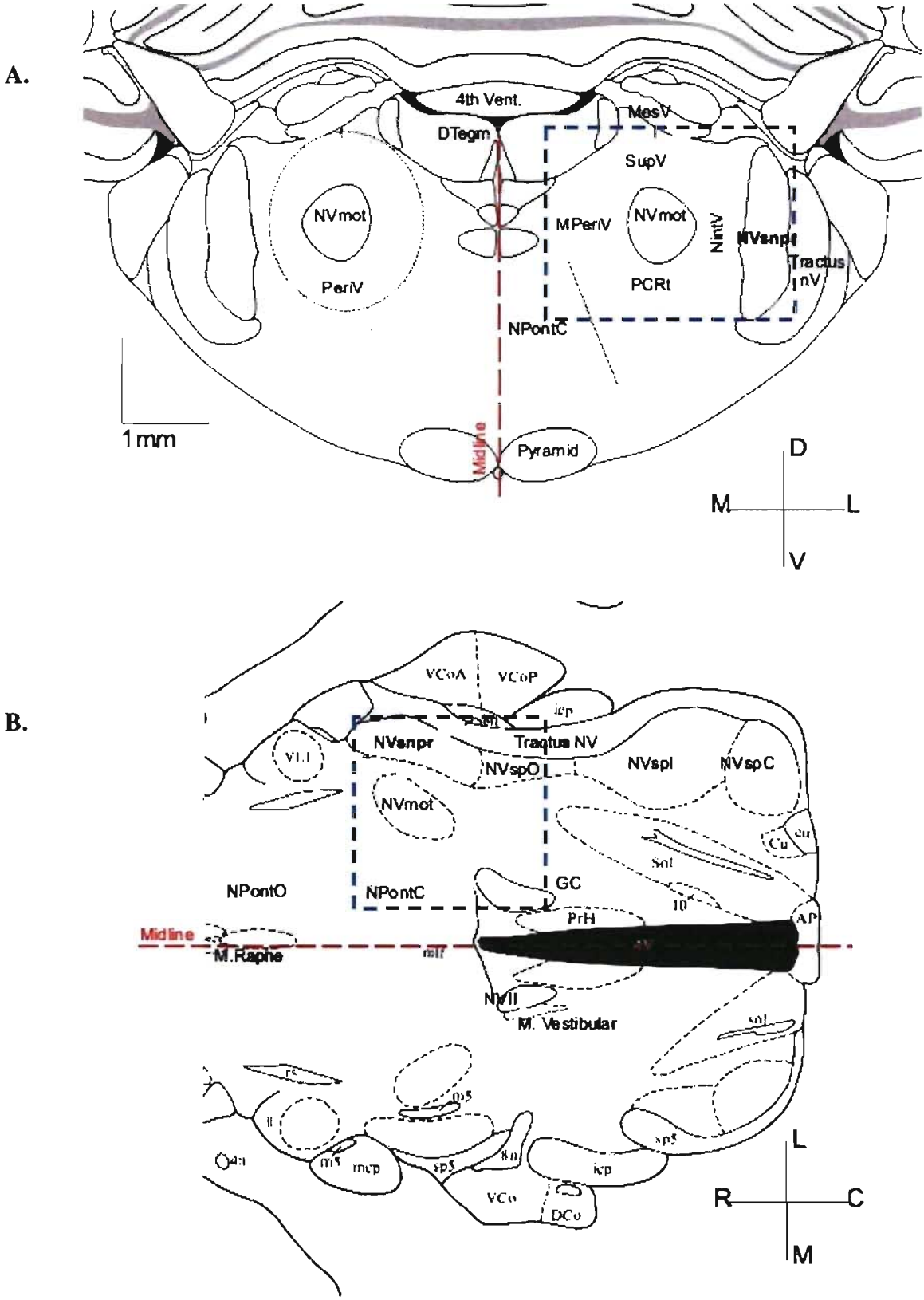


**Figure 4. NVsnpr and surrounding structures A.** transverse and **B.** horizontal sections of the rat brainstem. In blue, region described by Tanaka *et al.* (1999). Adapted from Paxinos & Watson (1982).

## 2.3 THE MASTICATORY CENTRAL PATTERN GENERATOR, CPG

### 2.3.1 Definition

The first attempts to describe the rhythmic process of mastication date from the experiments performed by Sherrington at the beginning of the 20th century. To Sherrington, rhythmic mastication could be explained by an alternate activation of two simple brainstem reflexes, a jaw opening reflex triggered by pressure on the teeth or tactile stimulation of perioral areas, and a jaw-closing reflex triggered by stretching of the jaw closing muscles during the opening. Both reflexes would follow each-other indefinitely until an external command interceded to stop the cycle (Sherrington, 1917) The idea of rhythmic orofacial-movements proper to mastication being patterned by central motor commands rather than as a consequence of pure reflex sensory arcs was put forward by Bremer, (1923). Later, Dellow & Lund (1971) demonstrated that the isolated brainstem can generate rhythmic coordinated activity ressembling mastication even in absence of afferent inputs and postulated the existence of a central pattern generator (CPG) for mastication. A CPG is an assembly of neurons that can produce and maintain a rhythmic pattern of activity in the absence of sensory feedback or descending central commands because of intrinsic properties and/or connectivity (Rossignol & Dubuc, 1994). CPGs are basic functional elements of essential processes such as respiration (von Euler, 1983), locomotion (Grillner, 1985) and mastication (Lund & Dellow, 1969).

Feldman & Ellenberger (1988) suggested that the respiratory CPG could be subdivided functionally into two different groups of neurons generating separate stages of the motor pattern. A first CPG component would produce the rhythm of respiration (the timing and length of the cycle); whereas a second component would shape the motoneuron output (duration and amplitude of EMG bursts). Other evidence suggested that this could also be the case for other complex mammalian motor rhythms such as locomotion (Rossignol *et al.*, 2006) and mastication (Lund, 1991 for a review). In the rabbit (Lund *et al.*, 1984) it was shown that the rhythm and the motoneuron output can be varied independently from each other. Increases in intensity of repetitive stimulation of different points of the cortical masticatory area in anaesthetized preparations could either increase the frequency of the rhythm without modifying the pattern of jaw movement or vice-versa. However, more recent evidence suggest that both functions may be accomplished by a single population of neurons forming the core of the CPG (Athanassiadis *et al.*, 2005a; Brocard *et al.*, 2006).

### 2.3.2 Localization of the masticatory CPG

It was postulated more than 30 years ago that the fundamental pattern of mastication, consisting of rhythmic opening and closing of the jaws, with associated repetitive movements of the tongue, cheeks and lips, could be generated by an assembly of neurons in the lower pons and the medulla (Lund & Dellow, 1969; 1971). Early experiments showed that mastication could be obtained in precollicular decerebrate animals (Bazett & Penfield, 1922), indicating that descending central commands were not essential for the initiation of the rhythmical motor output. Complementary sets of experiments using intraoral anesthetics in human subjects (Schaerer *et al.*, 1966), extensive orofacial denervation in rabbits (Inoue *et al.*, 1989) or selective lesions in NVmes (Goodwin & Luschei, 1975), showed that peripheral inputs were not essential for mastication (Lund, 1991 for a review). Dellow and Lund further demonstrated in 1971 that the isolated brainstem alone suffices to generate the typical rhythmic motor output of mastication. Stimulation of the corticobulbar tract in the brainstem of paralyzed and decerebrated animals could produce alternating bursts of activity in jaw-closing and jaw-opening motoneurons and in hypoglossal motoneurons. The rhythmic motor output of mastication did not dependent on the cardiac or respiratory rhythms.

### 2.3.3 Early models of the masticatory CPG

Nakamura's group was the first to propose a sequential model of the CPG for mastication that involved neurons within the medial bulbar reticular formation (MRF) between the facial motor nucleus (NVII) and hypoglossal nucleus (NXII) (Nozaki, *et al.* 1986a,b; Nakamura & Katakura, 1995). They suggested that the initial step in generation of a masticatory rhythm was the excitation of a group of neurons in the dorsal pole of the paragigantocellular reticular nucleus (dPGC) which receives direct projections from the contralateral cortical masticatory area in the guinea pig. They proposed that dPGC neurons project directly to the oral portion of the gigantocellular reticular nucleus, GCo, an area that contains neurons that burst rhythmically in phase with mastication (Fig. 5). After studying the latency of the local field potentials evoked in dPGC and GCo by cortical stimulation, Nakamura's group reported that the masticatory rhythm started with tonic excitation of neurons in dPGC. This area then drove the rhythmic firing of neurons in GCo (Nozaki *et al.*, 1986b). They added a third group of cells in the circuit, a group of interneurons within the caudal parvocellular reticular formation (cPCRt) located caudal to dPGC and GC, and next to NXII. This last group of cells projected to NVmot to control the trigeminal motor output (Nozaki *et al.*, 1993; Nakamura & Katakura, 1995; Nakamura *et al.*, 1999).

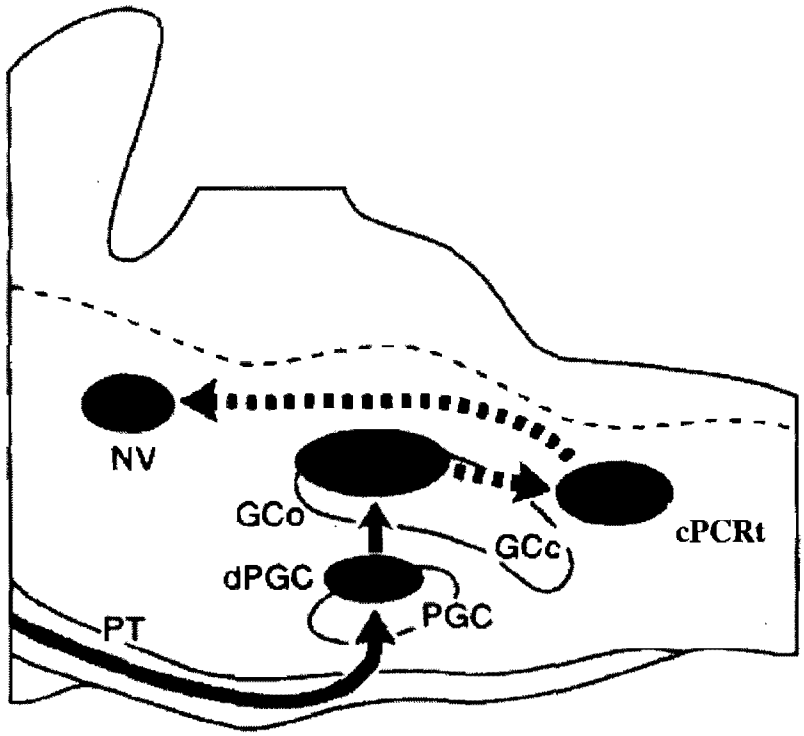


**Figure 5. Early CPG model.** In Nakamura's model, the descending central command travels through the corticobulbar path to the paragigantocellular nucleus and its dorsal portion, dPGC which then activate neurons in the gigantocellular nucleus, GCo and GCc, that project to the more caudal parvocellular reticular formation, cPCRt. The later acting as the last relay to the motor nucleus. Adapted from Nakamura & Katakura (1995).

Nozaki *et al.* (1986a) observed that the CPG for mastication was organized bilaterally in the guinea pig. When the two sides of the caudal pons and medulla were separated, each hemisection was still able to generate an unilateral pattern of mastication. Anatomical evidence showed that commissural axons connect the two half sides of the masticatory CPG (Nozaki *et al.,* 1991; Chandler & Tal, 1986; Landgren *et al.*, 1986; Bourque & Kolta, 2001). Chandler & Tal (1986) also demonstrated that a transection at the level of NXII disconnecting cPCRt from more rostral areas did not abolish mastication indicating that cPCRt was not essential for mastication. On the basis of these observations, Lund (1991) suggested a revision of Nakamura's model, and proposed that GCo projected to neurons of the rostral parvocellular reticular formation, PCRt pars α adjacent to NVspo γ. However, further evidence indicated that the areas that are essential for rhythm generation are located more rostraly in the brainstem (Lund, 1991 for a review). The elimination of caudal GC by transection does not disrupt mastication *in vitro* (Kogo *et al.*, 1996; Kogo *et al.*, 1998; Katakura, 1999). Using en bloc brainstem preparations *in vitro,* Tanaka *et al.* (1999) showed that the minimal portion of brainstem required to initiate rhythmic masticatory-like motor-output could be reduced to the region between the rostral border of NVmot and the rostral border of NVII. When considering medial to lateral dimensions, the preparation extends 400μm from the midline to the lateral border This region includes NVsnpr, NVspo-γ, NVmot, PeriV including PCRt and a portion of NPontC (Fig. 4).

The majority of the projections to trigeminal motoneurons arise from these areas, which all show increased c-Fos-like immunoreactivity after bouts of rhythmic mastication (Athanassiadis *et al.*, 2005b), and which all contain neurons with bilateral projections to NVmot indicating that they may participate to the bilateral coordination of the jaw (Mizuno *et al.*, 1983; Landgren *et al.*, 1986; Rokx *et al.*, 1986; Appenteng & Girdlestone, 1987; Appenteng *et al.*, 1990; Donga & Lund, 1991; Li *et al.*, 1993; Li *et al.*, 1995; Westberg *et al.*, 1995; Li *et al.*, 1996; Kolta *et al.*, 2000). In addition all of these areas contain neurons that fire rhythmically in phase with the trigeminal motoneurons during cortically induced mastication in the anaesthetized and paralysed rabbits, guinea-pigs and rats (Donga & Lund, 1990; Donga *et al.*, 1990; Inoue *et al.*, 1992; Westberg *et al.*, 1998; Tsuboi *et al.*, 2003).

However, it is unclear whether rhythmic firing in these neurons results from intrinsic properties or rhythmic synaptic inputs originating elsewhere. Neurons in PeriV and PCRt do not seem to possess intrinsic rhythm generating properties when studied *in vitro* (Bourque & Kolta, 2001), suggesting that these areas could be recruited by excitatory or inhibitory inputs rather than participate in the process of generating the masticatory rythm per se. In contrast, NVsnpr neurons have membrane conductances that produce plateau potentials. They have intrinsic rhythmic bursting properties as was shown in *in vitro* experiments in brainstem slice preparation of gerbils and rats (Sandler *et al.*, 1998; Brocard *et al.*, 2006). This feature is found in neurons forming endogenous oscillators observed in other rhythm generating circuits (Calabrese, 1995; Brocard *et al.*, 2006). Additionally, electrophysiological experiments conducted *in vitro* showed that NVsnpr neurons have synaptic connections with

ipsilateral NVmot, NPontC, PeriV and PCRt (Arsenault *et al.*, 2004; Athanassiadis *et al.*, 2005a) and with contralateral NVmot (Donga & Lund, 1991).

In addition, Tsuboi *et al.* (2003) showed that about one third of the neurons found in dorsal NVsnpr fire rhythmically in phase with trigeminal motoneurons (MNs) during fictive mastication. These evidence suggest that NVsnpr is an area that may contain the core of the masticatory CPG.

# 3. THE MAIN SENSORY NUCLEUS, NVsnpr

## 3. 1 NVsnpr morphology and localization

The NVsnpr is a rather compact nucleus with cells of uniform size and shape. It has a kidney-like shape in coronal section and is ovoid in the horizontal plane. It has a rostrocaudal extent of about 1.2mm and is found approximately 4.4 to 5.6mm rostral to the obex in rat. It is located 2.8 to 3.4mm from the midline (Paxinos & Watson, 2004) and measures 1.8mm from dorsal to ventral (Paxinos & Watson, 2004) (Fig. 4A, B). NVsnpr starts roughly at the same level that NVmot in the rostro-caudal axis although it extends more caudally (1400μm in the cat) (Eisenman *et al.*, 1963; Marfurt & Rajchert, 1991). NVsnpr ends where the fibres of NVII travel across the medulla and is followed caudally by NVspo γ. Classical morphological studies have reported cytoarchitectonic differences between the dorsal (NVd) and ventral (NVv) parts in the cat (Eisenman *et al.*, 1963; Shigenaga *et al.*, 1986a). According to Shigenaga *et al.*, (1986a) NVd has a higher cell density than NVv. However, this morphological distinction was not observed in the rat (Ide & Killackey, 1985). The average diameter of the cells within NVsnpr ranges from 10μm in the rat, and 11 to 13μm in platypus and echidna, two australian monotrema (Ashwell *et al.*, 2006) to 15 to 30μm in the cat (Eisenman *et al.*, 1963). NVsnpr can be distinguished from other nuclei of the trigeminal sensory complex by the high density of small to medium sized round or ovoid neurons. NVsp oralis has conversely large polygonal neurons (22 to 40μm diameter) and its neuropil is interrupted by distinctive rostrocaudal oriented bundles of myelinated axons (Ide & Killackey, 1985).

## 3.2 NVsnpr connectivity

### 3.2.1. Cortical inputs

Anatomical and electrophysiological studies conducted by Hernandez-Peon (1955) and Brodal *et al.* (1956) showed cortical inputs to NVsnpr in adult cats. These observations were supported by lesion studies of the sensory-motor cortex conducted by Woolsey (1955) and Gobel *et al.* (1971) who observed degenerating corticofugal endings in NVsnpr using electron microscopy. The corticofugal endings were characterized as small myelinated fibers (1μm diameter), containing few synaptic vesicles and establishing synapses mainly on dendrites. Few synapses were reported on axons and none on somas. These results confirmed the observations of Brodal (1956). In 1985, Yasui *et al.*, made HRP injections in the cat motor cortical masticatory area and observed labelling in the median NVsnpr as one of the projection targets of the descending pathway. Labelling was also observed in the dorsal and median portion of NVspo and median NVspi.

### 3.2.2. Peripheral inputs: Somatotopy of NVsnpr

Primary sensory fibers in the spinal trigeminal tract of nerve V are organized somatotopically in the cat (Marfurt, 1981) and in the rat (Gregg *et al.*, 1973; Jacquin *et al.*, 1983a, b; Shigenaga *et al.*, 1988). Sensory fibers from the ophthalmic division are found ventrolaterally while those from the mandibular division are found dorsomedially. Fibers from the maxillary division are found between the other two (Fig. 6A). Darian Smith *et al.* (1963a, b) reported that neurons in NVspo and NVsnpr in cat conserve the somatotopy of the sensory fibers and form a clear somatotopic map. This consists of an inverted representation of the face in the dorso-ventral aspect of the nucleus, with the mandible region represented dorsally, the ophthalmic region represented ventrally and the maxillary between both (Fig. 6B). Shigenaga *et al.* (1986a, b; 1988) studied exhaustively the anatomical and functional projections of the primary afferents in the cat using horse radish and *phaseolus vulgaris* leucoaglutinin injections in peripheral nerves innervating oral and facial structures. They confirmed the inverted representation of the face on the dorso-ventral aspect of the nucleus and showed that the dorsal part of NVsnpr is the principal target for intraoral afferents, whereas the ventral part receives both intraoral and facial afferents (mental, infraorbital and frontal nerve afferents). Infraorbital afferents end up in the ventral most part of the nucleus and lingual afferents are located in the dorsalmost part of NVsnpr.

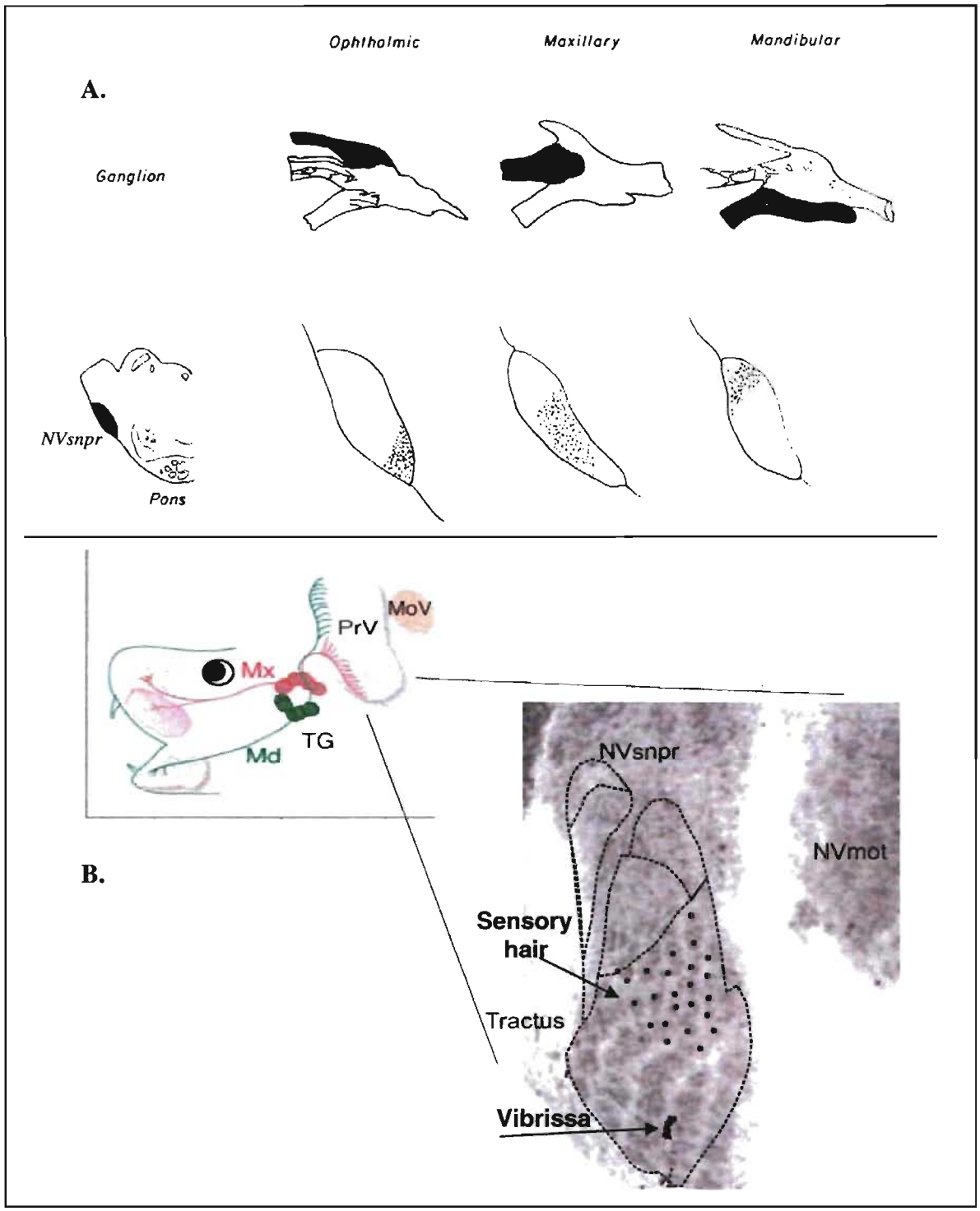


**Figure 6. Somatotopic organization of primary afferents within the trigeminal ganglion and NVsnpr**. **A.** HRP tracing studies reveal that the three trigeminal branches project to distinct areas of NVsnpr in the dorso-ventral axis. Adapted from Kerr *et al.* (1968) **B. Inverted-face representation**. Note that the afferents projecting from the mandible, Md are dorsal while the maxillary, Mx are in the middle of the nucleus. We can additionally see cytochrome oxidase staining of barrel-like spots in the ventral part of the nucleus due to the clustered projections from vibrissa and sensory hair (large black polygons and small black dots, respectively). Adapted from Erzurumlu *et al.* (2006) and Oury *et al.* (2006).

Arvidsson, (1982) also reported an inverted-face representation in NVsnpr and revealed in addition the localization of vibrissae columns organized in 'barrel-like' structures when observed in transverse sections of the brainstem. Although the dorso-ventral somatotopic representation of the face in NVsnpr has been generally accepted, there is some controversy about the organization of NVsnpr in the rostro-caudal axis. Eisenman *et al.* (1963) found that the majority of perioral touch cells in the rostral half of NVsnpr had their receptive fields on the lips, while the majority of the cells found in its caudal half had receptive fields located in the hairy skin surrounding the mouth and more remote areas. This group also reported that a larger number of cells with receptive field on whiskers were located on the rostral half of NVsnpr. Other early experiments demonstrated that the face and oral cavity were represented somatotopically but disproportionately in all rostrocaudal levels of the entire sensory trigeminal complex (Kruger & Witkovsky, 1961a; Kruger *et al.*, 1961b; Kruger & Michel, 1962a, b; Kerr *et al.*, 1968). Shigenaga, (1986a) reported that each area of the anterior face and oral cavity is indeed represented in rostrocaudally oriented columns that run parallel from NVsnpr to NVspc. These results were in agreement with the electrophysiological works of Kruger & Michel (1962a) and anatomical works by Arvidsson *et al.* (1982).

### 3.2.3. Neurochemical studies

A number of studies have tried to characterize the neurochemical content of fibers projecting to NVsnpr (Clements & Beitz, 1991; Bae *et al.*, 2000; Waite *et al.*, 2000). Bae *et al.* (2000) used HRP-conjugated cholera toxin B-subunit to label primary afferents in the trigeminal ganglion together with immunostaining against glutamate in the trigeminal main sensory complex. They suggested that large-caliber primary afferent neurons use glutamate as a neurotransmitter. These synapses were subject to pre-synaptic modulation by GABAergic fibers and the authors suggested that this would specially apply for NVsnpr where a higher degree of pre-synaptic control might be required for sharpening spatial somatosensory information. These observations were corroborated by the electron-microscopy studies of Clements & Beitz (1991). Waite *et al.* (2000) studied anatomical and functional development of the trigeminal sensory complex in rats from age E13 to P6. They found that responses to stimulation of the trigeminal ganglion in NVsnpr neurons were NMDA and AMPA dependent. Additionally GABA-A excitatory responses were also observed and blocked through bicuculline application. GABA-A responses were observed at all ages but were maximal from E20 to P1, which is consistent with the development of whisker-related patterns (Landers & Zeigler, 2006 for a review).

### 3.2.4 Inputs to NVsnpr from other nuclei in the brainstem

Extracellular injections of biocytin and intracellular recordings *in vitro* in conjunction with micro-stimulation were used to show that all areas of PeriV, PCRt and NPontC project to NVsnpr monosynaptically (Bourque & Kolta, 2001; Athanassiadis *et al.*, 2005a). The majority of responses detected in NVsnpr (80%) could be blocked by DNQX and APV (antagonists of AMPA and NMDA receptors respectively) indicating that these projections are glutamatergic. Fewer stimuli (20%) elicited IPSPs, and these were sensitive to GABAergic and glycinergic antagonists (Bourque & Kolta, 2001 ; Athanassiadis *et al.*, 2005a). This observation is consistent with immunohistochemical studies conducted in rat and rabbit (Li, *et al.*, 1996; Turman & Chandler, 1994a,b; Kolta *et al.*, 2000) which showed a mixed population of excitatory and inhibitory neurons in PeriV. In the study conducted by Athanassiadis *et al.* (2005a), NVmot stimulation also resulted in a mixture of excitatory and inhibitory evoked responses in NVsnpr. The latency of many NVmot evoked responses fell within the monosynaptic range. Since motoneurons in NVmot lack recurrent axon collaterals and are exclusively cholinergic (Lauterborn *et al.*, 1993; Ichikawa & Shimizu, 1998; Saad *et al.*, 1999) these responses were attributed to projections from interneurons within the nucleus (Sessle, 1977; Ter Horst *et al.*, 1990). More recently it has been shown that NVmot contains a mixed population of excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) interneurons (Li, *et al.* 1996; Kolta, *et al.* 2000; McDavid *et al.*, 2006).

NVsnpr also receive projections from NPontC, and one half of the responses evoked by stimulation of the dorsal NPontC occurred at monosynaptic range (Bourque & Kolta, 2001; Athanassiadis *et al.* 2005a). Projections from neurons in NPontC and PCRt of the rat have been also described using HRP and *phaseoulus vulgaris* leucoaglutinin tracers (Shammah-Lagnado *et al.*, 1987; Ter Horst *et al.*, 1991; Shammah-Lagnado *et al.*, 1992).

### 3.3. Outputs from NVsnpr

#### 3.3.1 Projections to higher centers: Thalamus

The NVsnpr has been classically described as a sensory relay to the thalamus. Later it has been reported that secondary neurons in the nucleus send collaterals to reticular areas such as PeriV (Jacquin *et al.*, 1982; Yoshida *et al.*, 1998; Zerari-Mailly *et al.*, 2001), NVmot (Yoshida *et al.*, 1994; Buisseret-Delmas *et al.*, 1997; Kolta *et al.*, 2000), NVmes or higher areas such as the cerebellum (Huerta *et al.*, 1983; Steindler, 1985). In fact, the most important projections from NVsnpr travel to the thalamus in the two reticulothalamic pathways (Blumenfeld, 2002). The first pathway contains axons from NVsnpr neurons that carry somesthetic information from the face. It constitutes a large projection arising in the ventral two thirds of the nucleus. The neurons give off ascending axons that ascend with the medial lemniscus, cross the midline to the contralateral side of the brainstem and then project to the ventral-posterior-median nucleus of the thalamus, VPM. This has been confirmed in retrograde studies in the cat (Smith, 1975) and in the dog and pig (Michail &

Karamanlidis, 1970; Brodal, 1981 for a review). Darian-Smith *et al.* (1963b) performed electrical stimulation of the contralateral arcuate nucleus of the thalamus in the cat and observed both antidromic and trans-synaptic responses in NVsnpr. Medial-lemniscal neurons were also identified through stimulation, and responses were observed throughout NVspo and the caudal part of NVsnpr. These neurons form an homogeneous group located in the dorsolateral part of each nuclei. This data was confirmed by further anatomical studies (Mizuno, 1970; Shigenaga *et al.*, 1979, 1983). The second pathway arises from neurons that convey proprioceptive information, touch and pressure sensation from the oral cavity, including the teeth. These axons arise from cells located in the dorsomedial third of NVsnpr and give off uncrossed axons that travel to ipsilateral VPM in the dorsal trigemino-thalamic tract (Walker, 1939; Carpenter, 1957). Torvik reported half a century ago that the dorsal NVsnpr contained ascending axons in the ipsilateral reticular formation that project to the thalamus (Torvik, 1957; Mizuno, 1970). Luo & Dessem (1995) performed anatomical studies in the rat using HRP neurotracer injections in VPM and labelled neurons contralaterally in SupV, NVsnpr, NVspo, NVspi and PCRt. Steindler (1985) obtained similar results in the mouse, and observed important projections to the cerebellar cortex and deep cerebellar nuclei from NVsnpr and NVspi. This was also reported in the rat (Huerta *et al.*, 1983).

Yoshida *et al.* (1998) conducted HRP tracing studies in the cat in order to determine cell morphology and local projections from cells whithin NVsnpr. They found three classes of projecting neurons based on their axonal and dendritic arborisation pattern. Class Ia neurons were sensory neurons having an ascending stem axon and no branching. This class correspond to the thalamus projecting neurons

reported by Torvik (1957) and Mizuno (1970). Class IIa contained neurons that had both ascending axons and collaterals to lower brainstem nuclei, especially projecting to dorsal NVmot. Yoshida et al. suggested that these neurons could be involved into sensory discrimination and the jaw closing reflex. Class IIb neurons were local circuit neurons that only send collaterals to neighbouring brainstem structures. Neurochemical studies on thalamus-projecting neurons from NVsnpr reported that the majority of them were glutamatergic (Magnusson *et al.*, 1987), although some local branching neurons were GABAergic (Ginestal & Matute, 1993; Avendano *et al.*, 2005).

### 3.3.2 Projections to the reticular formation and NVmot

As described above, NVsnpr neurons project to nearby reticular nuclei such as SupV, PCRt, NintV, mPeriV, and to NVmot (Jacquin *et al.*, 1982; Yoshida *et al.*, 1998; Zerari-Mailly *et al.*, 2001; Arsenault I., 2004; Athanassiadis *et al.*, 2005a). *In vitro* electrophysiological studies conducted by Athanassiadis in a rat brainstem slice preparation showed that antidromic activation in NVsnpr could be obtained through stimulation in the masseteric pool of NVmot and dorsal NPontc (Athanassiadis *et al.*, 2005a). This is in agreement with the unpublished observations of Arsenault *et al.* (2004). They found that stimulation of dorsal NVsnpr evokes PSPs in the masseteric pool of NVmot suggesting that dorsal NVsnpr send direct projections to jaw closing motoneurons. PSPs could be also obtained in the digastric pool of NVmot following stimulation of all parts of NVsnpr, but the latency of responses elicited by stimulation of the ventral part was much shorter, suggesting that ventral NVsnpr sends direct projections to jaw opening motoneurons. These observations support the findings of Li

*et al.* (1995) who reported that NVsnpr project to jaw-opening motoneurons. Anatomical studies using rhodamine dextran techniques combined with immunohistochemistry have shown that at least some of the NVsnpr neurons projecting to NVmot are GABAergic and/or glycinergic (Li *et al.*, 1996). Other groups have also reported the existence of glycinergic cells in NVsnpr (Ginestal & Matute, 1993; Turman & Chandler, 1994a; Rampon *et al.*, 1996; Avendano *et al.*, 2005) although they found more dense labellings in NVspc and NVspi. Employing similar techniques in the rabbit, Kolta *et al.* (2000) reported the existence of GABAergic and glutamatergic neurons among premotoneurons in dorsal NVsnpr projecting to NVmot. Similar findings were obtained by Turman and Chandler in the guinea-pig (Turman & Chandler, 1994a,b).

### 3.4 Evidence that NVsnpr may form the core for the CPG for mastication

As described above, some NVsnpr neurons project to NVmot and other structures of the lateral and medial reticular formation, and our group has raised the possibility that these cells could be involved in motor processing because they fulfill most of the requirements expected from CPG neurons (Tsuboi *et al.*, 2003; Athanassiadis *et al.*, 2005a; Brocard *et al.*, 2006). First, they receive massive inputs from the cortex and sensory afferents (Eisenman *et al.*, 1963; Dellow & Lund, 1971; Tsuru, *et al.* 1989; Zhang & Sasamoto, 1990; Yoshida, *et al.* 1998) and have outputs to premotorneurons and MNs of PeriV, NVmot and NPontC (Inoue *et al.*, 2002; Arsenault *et al.*, 2004; Athanassiadis *et al.*, 2005a). Second, *in vivo* studies have shown that about one third of the neurons in dorsal NVsnpr fire rhythmically in phase with trigeminal motoneurons when fictive mastication is elicited by stimulation of the CMA in paralyzed rabbits (Tsuboi *et al.*, 2003). Third, the expression of c-Fos-like protein, a functional marker of activity, increases in this area during fictive mastication (Athanassiadis *et al.*, 2005a). Fourth, in *in vitro* brainstem slice preparations, many NVsnpr neurons burst and have plateau properties in the gerbil (Sandler *et al.*, 1998) and in the rat (Brocard *et al.*, 2006). Our group has shown that appearance of these properties coincides with the emergence of mastication in new-born rats (Brocard *et al.*, 2006). The bursts and plateaux not only persist in $Ca^{2+}$ free ACSF (a condition that blocks synaptic transmission), but are enhanced in these conditions indicating that this property depends on intrinsic cell-membrane properties (Brocard *et al.*, 2006) (Fig. 7).

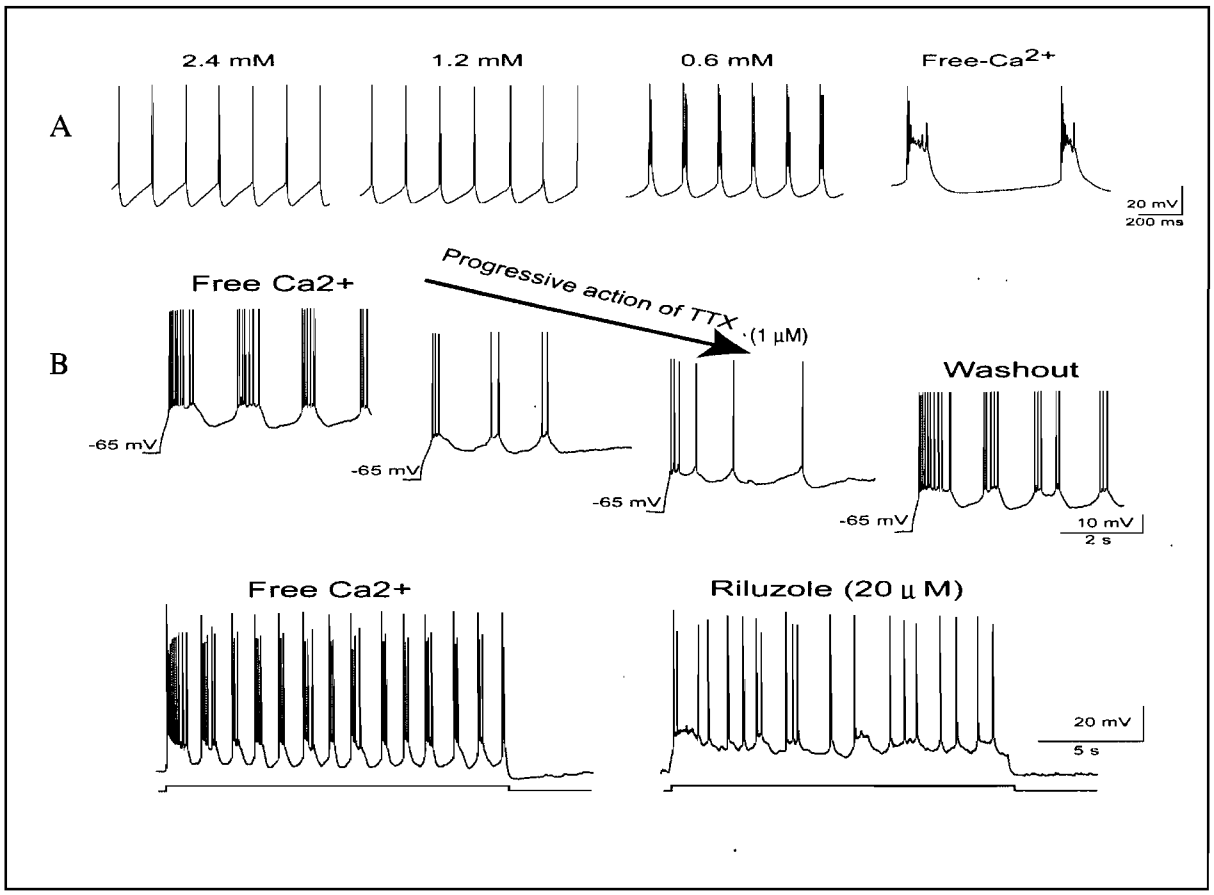


**Figure 7. Intrinsic bursting properties of NVsnpr neurons. A.** Whole-cell patch-recordings from NVsnpr neurons in an *in vitro* brainstem slice preparation showing bursting in $Ca^{2+}$ free conditions. **B.** Rhythmic bursting depend on a persistent sodic conductance, INaP, as it is abolished by the local application of TTX or the more specific antagonist Riluzole. Adapted from Brocard *et al.* (2006).

Removal of $Ca^{2+}$ from the perfusing solution results in conversion from tonic firing to repetitive bursting in 80% of neurons (Brocard *et al.*, 2006; Kolta *et al.*, 2007 for a review). Intracellular application of BAPTA, a $Ca^{2+}$ chelator, did not cause or prevent bursting, suggesting that the initiation of rhythmic bursting in the NVsnpr is related to changes in the extracellular space and not within the cell. Brocard *et al.* (2006) used a pharmacological approach to dissect the ionic mechanisms underlying recurrent bursting in NVsnpr neurons. The role of small (SK) and big (BK) $Ca^{2+}$-dependent $K^+$ conductances was assessed with bath application of Apamin and Charibdotoxin respectively. These drugs did not stop the spontaneous bursting of NVsnpr neurons in $Ca^{2+}$ free ACSF, indicating that neither one of these $K^+$ conductances was essential for bursting. Blocking $K^+$ conductances with TEA, or the Ih current with ZD7288 did not prevent bursting in $Ca^{2+}$ free ACSF, although the burst frequency was reduced and burst duration increased by TEA. Bath perfusion of tetrodotoxin, (TTX) in $Ca^{2+}$-free ACSF was used to test whether $Na^+$ conductances were involved in burst generation. TTX reversibly abolished bursting and plateau potentials at low doses before abolishing action potentials.

Taken altogether, this data suggested that both the plateaux and the bursts depended on sodium conductances. Riluzole, a specific antagonist of the persistent sodium current, INaP, blocked rhythmic bursting without affecting spikes, indicating that NVsnpr bursts and plateau properties were INaP dependent (Fig. 7). INaP-dependent rhythmic activities have been associated with rhythmic physiological processes such as respiration (Feldman & Smith, 1989; Del Negro *et al.*, 2002; Rybak *et al.*, 2003), locomotion (Tazerart *et al.*, 2007; Zhong *et al.*, 2007), whisking (Cramer *et al.*, 2007) and heartbeat pattern generation (Lu *et al.*, 1999). Brocard *et al.* (2006) showed that

the INaP-mediated rhythmic bursting in NVsnpr depends on the membrane potential Figure 9 shows a cell that does not burst close to the resting membrane potential (-65mV) but does burst when it is gradually depolarized. Cell firing becomes tonic at potential more depolarized than -51mV, indicating that rhythmic firing occurs only within a precise voltage range that correspond to activation and inactivation voltages of INaP (Azouz *et al.*, 1996; Su *et al.*, 2001; Del Negro *et al.*, 2002; Darbon *et al.*, 2004; Brocard *et al.*, 2006). More interestingly, Brocard *et al.* (2006) also showed that the amplitude and duration of the INaP-mediated plateau that underly bursting depend on $[Ca^{2+}]_e$. An example of this is shown in figure 8.

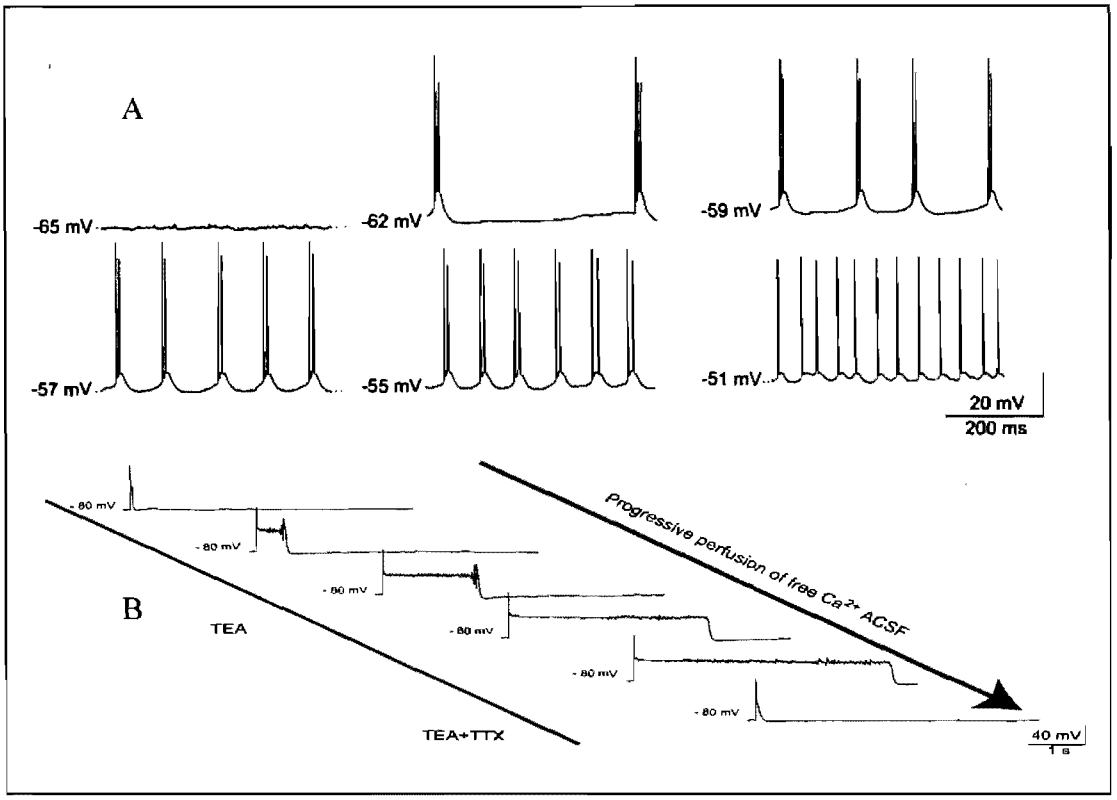


**Figure 8. Intrinsic properties of neurons in NVsnpr A.** Rhythmic bursting is linearly dependent with the membrane potential. The cell does not burst close to resting membrane potential (-60mV) but does it when we gradually depolarize the cell. At -51mV the cell becomes tonic indicating that the rhythmic firing occurs only within a precise voltage range that is consistent with the activation of INaP. **B.** The plateau potential has also been isolated pharmacologically and depends on INaP conductance. The plateau also depends strongly on $[Ca^{2+}]_e$. As we see the gradual perfusion of $Ca^{2+}$ free ACSF increases burst duration. Adapted from Brocard *et al.* (2006).

## 4. THE GENERAL HYPOTHESIS

There is evidence that changes in the intensity of neuronal activity causes important fluctuations in $[Ca^{2+}]_e$ in the spinal cord (Somjen, 1980; Murase & Randic, 1983); cortex (Nicholson *et al.*, 1978; Rusakov & Fine, 2003) and in hippocampal slices (Benninger *et al.*, 1980; Cohen & Fields, 2004) for a review. As pointed out in section 1.1, prolonged (>100ms) repetitive stimulation (10-100Hz) of cortical or sensory fibers is usually required to trigger mastication in experimental animals. Therefore, we postulate that the sustained activation of these inputs to NVsnpr causes a drop of $[Ca^{2+}]_e$ leading to activation of INaP and to recurrent bursting. A number of mechanisms could be responsible for the local changes in $[Ca^{2+}]_e$: ionotropic receptors such as NMDA, $Ca^{2+}$-ATPase pumps and $Na^+/Ca^{2+}$ exchangers. There is evidence that NMDA receptors can contribute to synaptically- evoked $[Ca^{2+}]_e$ depletion (Rusakov & Fine, 2003). Glial cells are also good candidates for $[Ca^{2+}]_e$ depletion, and are sensitive to a number of neurotransmitters, such as glutamate, GABA, acetylcholine, and ATP or ions such as $K^+$ released during neuronal firing (Verkhratsky *et al.*, 1998; Grosche *et al.*, 1999; Grosche *et al.*, 2002). Other projects being conducted in our laboratory are designed to dissect the contribution of glia and neurons to changes in $[Ca^{2+}]_e$. This study investigates whether long lasting repetitive stimulation of sensory afferents (trigeminal tract) elicits rhythmic bursting in NVsnpr neurons under physiological levels of $[Ca^{2+}]_e$ and evaluate the role of NMDA receptors in the process of rhythmogenesis.

## 5. HYPOTHESIS OF THIS STUDY

**a) Repetitive but not single activation of sensory inputs to NVsnpr in physiological $[Ca^{2+}]_e$ can elicit rhythmic bursting.**

**b) NMDA receptors do play a role in this process.**

## 6. THE OBJECTIVES OF THIS STUDY WERE:

**a) First to confirm that bursting in NVsnpr neurons depends on age and $[Ca^{2+}]_e$.**

**b) To test in a brainstem slice preparation, the effects of single and repetitive stimulation of the sensory tract on the firing pattern of NVsnpr neurons under $[Ca^{2+}]_e$ where only few neurons burst spontaneously.**

**c) Test the effects of NMDA receptors agonist and antagonist on the firing pattern of NVsnpr neurons.**

# SECTION II

# ARTICLE

**T. Freund**

# « Effect of the stimulation of sensory inputs on the firing of neurons of the trigeminal main sensory nucleus in the rat »

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**Keywords :** central pattern generation, main sensory nucleus, peripheral stimulation

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**Number of figures** 12

## ABSTRACT

Mastication may be triggered by repetitive stimulation of the cortex or of sensory inputs, but is patterned by a central pattern generator (CPG) in the brainstem. This CPG may lie in the dorsal part of the principal trigeminal sensory nucleus (NVsnpr) where neurons burst repetitively when the extracellular concentration of $Ca^{2+}$ ($[Ca^{2+}]_e$) drops (Brocard *et al.*, 2006). Here we examine the effects of repetitive stimulation of sensory afferents in the descending tract on activity of NVsnpr neurons recorded extracellularly *in vitro* under physiological $[Ca^{2+}]_e$ (1.6mM). Most spontaneously active cells had either a tonic or a bursting firing pattern. Stimulation of the tract altered burst duration and/or frequency in bursting cells and firing frequencies in most tonic cells. In 28% of the latter, the firing pattern switched to rhythmic bursting. This effect could be blocked by APV and mimicked by local application of NMDA. Rhythm indices (Sugihara *et al.*, 1995) calculated to assess rhythmicity were negative (non-rhythmic) in all cases before stimulation and significant ($\geq$ 0.01; rhythmic) after stimulation. The mean and median (±SE) bursting frequency were 8.32±0.72Hz and 6±0.5Hz respectively. In 6 cases where firing switched to rhythmic, two units were recorded and crosscorrelation analysis showed that, in all pairs, the units became synchronized after stimulation. Optimal stimulation parameters for eliciting rhythmic bursting consisted in 500ms trains of pulses delivered at 40 - 60Hz. Together, our results show that repetitive stimulation of sensory afferents *in vitro* can elicit masticatory-like rhythmic bursting in NVsnpr neurons at physiological $[Ca^{2+}]_e$.

## INTRODUCTION

The basic pattern of mastication is generated by a central pattern generator (CPG) located in the brainstem in response to tonic inputs from higher centers or from trigeminal sensory afferents (Dellow & Lund, 1971; Lund & Dellow, 1971). Neither input is essential because CPGs can produce repetitive movements in absence of inputs from either the superior centers or sensory afferents (Rossignol *et al.*, 2006 for a review). Tonic stimulation of either type of input can activate the masticatory CPG even in paralysed animals (fictive mastication) (Sumi, 1969; Dellow and Lund, 1971; Lund and Dellow, 1971), and this is not unique to mastication, since activation of sensory afferents and descending inputs elicit locomotion (Grillner *et al.*, 1981; Fleshman *et al.*, 1984; Rossignol, 2000). However, the cellular mechanisms by which sustained activation of these inputs is converted into a rhythmic output by the CPG are unknown. Our previous work suggests that the dorsal part of the trigeminal main sensory nucleus (NVsnpr) may form the core of the masticatory CPG (Tsuboi *et al.*, 2003; Athanassiadis *et al.*, 2005a; Brocard *et al.*, 2006; Kolta et *al.*, 2007 for a review). NVsnpr receives massive inputs from the masticatory area of the cortex and from trigeminal sensory afferents, and neurons of its dorsal part project directly to the trigeminal motor nucleus (Travers & Norgren, 1983; Li *et al.*, 1993; Yoshida *et al.*, 1998; Kolta *et al.*, 2000). The expression of c-Fos (a neuronal marker of activity) increases in neurons of dorsal NVsnpr after bouts of fictive mastication (Athanassiadis *et al.*, 2005b), and about a third of neurons recorded in there fire rhythmically in phase with trigeminal motoneurons during fictive mastication (Tsuboi *et al.*, 2003). These rhythmical neurons receive inputs from sensory receptors that provide important feedback during mastication: intraoral touch receptors, periodontal pressoreceptors &

mucles spindles (Tsuboi *et al.,* 2003). *In vitro* studies have shown that many dorsal NVsnpr neurons are intrinsic bursters, and our most recent work has shown that repetitive bursting in these cells depends on a persistent sodium conductance (INaP) that is voltage dependent and modulated by the extracellular concentration of $Ca^{2+}$ ($[Ca^{2+}]_e$). Under physiological $[Ca^{2+}]_e$, (1.6mM) only 18% of neurons could be made to burst by depolarizing current injections; however, reducing $[Ca^{2+}]_e$ caused a progressive increase in the percentage of neurons that burst spontaneously (Brocard *et al.*, 2006). Brocard *et al.* (2006) proposed that sustained activation of inputs to NVsnpr causes a fall in $[Ca^{2+}]_e$ and that, in turn, activates INaP and triggers rhythmic bursting. Decreases of $[Ca^{2+}]_e$ associated to sustained or intense neuronal activities have been described in the spinal cord (Somjen, 1980; Murase & Randic, 1983); cerebellum (Nicholson *et al.,* 1978); cortex (Somjen, 1980); and in hippocampal slices (Benninger *et al.,* 1980; Rusakov & Fine, 2003). The objective of this study was to test whether repetitive stimulation of sensory afferents to NVsnpr at physiological concentrations of $Ca^{2+}$ can elicit rhythmic bursting in these neurons.

## MATERIALS AND METHODS

*Brain slice preparation*

Sixty one Sprague-Dawley rats (13 to 34 days old) were used for this study. Retrograde labelling of trigeminal motoneurons was performed prior to slice preparation to help positioning the recording electrode in NVsnpr. This was done by injection of 5-10µl of Alexa fluor 488 conjugated cholera toxin (Subunit B) in the masseter muscle of cryoanaesthetized rat pups. One to two weeks later, the animals were decapitated, the brainstem rapidly removed and immersed in oxygenated ice-cold (4°C) sucrose-artificial cerebrospinal fluid, [(ACSF) composition in mM: Sucrose 252, KCl 3. $KH_2PO_4$ 1.25, $MgSO_4$ 4, $CaCl_2$ 0.2, $NaHCO_3$ 26, D-Glucose 25 bubbled with 95% $O_2$ and 5% $CO_2$, pH 7.4]. Transverse slices (400µm thick) containing NVsnpr and the trigeminal tract were prepared using a vibratome (Leica, VT1000 S, Germany). Slices were maintained at 28-32°C in an interface chamber saturated with a humidified mixture of 95% $O_2$ - 5% $CO_2$. The chamber was perfused with normal ACSF (composition in mM: NaCl 125, KCl 3, $KH_2PO_4$ 1.25, $MgSO_4$ 2.8, $CaCl_2$ 1.6, $NaHCO_3$ 26, D-Glucose 25) at a rate of 1ml/min. The slices were incubated at least 1h before the recordings. Four concentrations of $CaCl_2$ were used during recording of 280 neurons: physiological level (1.6mM, n: 174, Jones & Keep, 1988); low calcium (1.2mM, n: 69) or high calcium (2.4mM, n: 15). In some experiments (n: 22), we began with $Ca^{2+}$ free ACSF to facilitate the detection of bursting neurons then changed to physiological concentration in 17 cells. $Ca^{2+}$ free solutions were prepared by equimolar substitution of $CaCl_2$ with $MgCl_2$ to maintain the concentration of divalent ions constant. Once a bursting unit was detected, the $Ca^{2+}$ concentration was raised.

All procedures for dye injections and slice preparation conformed to national ethics committee guidelines and were approved by the institutional animal care committees at the Université de Montréal, Canada.

*Electrophysiological recording*

Extracellular recordings were made with conventional borosilicate electrodes (1.0mm OD, A-M systems, Inc USA) containing 2.5mM filtered NaCl and an A-M systems 1800 amplifier. The trigeminal tract was stimulated extracellularly using bipolar nichrome electrodes (25μm diam.) positioned close to the recording electrode in NVsnpr. The intensity (<1.5mA) was set to the minimum required to obtain responses for each cell. Pulse duration was 0.25ms. Repetitive stimulation was performed at 40Hz for all cases and assessed at different frequencies (20 to 100Hz) in some cases. The train duration varied from 100ms to 2s. The data was digitized at 10kHz and stored using pClamp 9 (Axon instruments, Molecular Devices Corporation, USA). The cells selected for analysis had a stable firing pattern under resting conditions during at least 10min of baseline. For each unit, the protocol of stimulation was repeated ten times for single pulses and five times for trains, with a minimum of one min between each stimulation protocol.

*Drug applications*

D,L-2-amino-5-phosphonovaleric acid, (APV; Sigma-Aldrich) was either bath-applied (75μM) or locally applied (50μM). N-methyl-D-aspartic acid, NMDA (50μM; Sigma-

Aldrich USA) was always locally applied using a Picospritzer II ejection system (General Valve) with a patch-like pipette (5μm tip diameter).

*Analysis*

Data was analyzed off-line using Spike2 (Cambridge Electronics Division, CED. UK). Spike detection and unit discrimination were performed with the template matching and wavemark processing tools. Burst detection was performed using the script Burst.s2s. Interval histograms, Poststimulus histograms, PSTH and Autocorrelograms were generated with Spike2 build-in analysis software. Autocorrelograms were performed for all neurons that appeared to change from tonic firing to bursting after trigeminal tract stimulation, and for 10 neurons that did not appear to burst. Interval histograms were used to set the time bin size of the autocorrelograms (5 to 10ms) as described in Lang *et al.* (1997 and 1999). The peaks and valleys in the autocorrelograms were identified if their amplitude exceeded ± 2SD of the baseline using the mathematical criteria described in Sugihara *et al.* (1995). The degree of rhythmicity was quantified as the rhythm index (RI), calculated from autocorrelograms using the method described by Sugihara *et al.* (1995). Firing patterns were considered to be rhythmic if RI≥0.01. When the autocorrelogram had no recognizable peaks and valleys, a value of zero was given to the RIs. The rhythm frequency was defined as the reciprocal of the interval between the start of the autocorrelogram and the first peak. Alternatively, Fast Fourier Transforms (FFTs) were performed to identify the predominant frequency. The RIs and FFTs were carried out with Matlab. All statistical calculations were run in SigmaStat 3.1 (Systat Soft, California US), and three dimensional plots (3D-spline surfaces) were made with Matlab (Mathworks, MA. US).

# RESULTS

## Age dependency of firing pattern

Recordings were obtained from 280 neurons spread throughout the entire dorso-ventral extent of the nucleus. Two main categories of spontaneous firing patterns were identified: tonic and bursting (Fig. 1A). Tonic units fired single spikes, whereas bursting units fired clusters of spikes separated by silent periods. Under physiological calcium concentration ($[Ca^{2+}]_e$: 1.6 mM, n: 191) spontaneous bursting was almost non existent in animals younger than 15 days old, and in agreement with our previous findings (Brocard *et al.*, 2006), the proportion of bursting neurons increased with age and reached nearly 40% from 17 days old (Fig. 1B).

## $[Ca^{2+}]_e$ dependency of firing pattern

The $[Ca^{2+}]_e$ had a marked effect on the firing pattern. Figure 2 shows the distribution of rhythmic and tonically firing neurons recorded under different $[Ca^{2+}]_e$. More than 90% of cells had a spontaneous bursting pattern in $Ca^{2+}$ free ACSF, while more than 90% fired tonically when $[Ca^{2+}]_e$ was 2.4mM. From these, 17 were recorded first in $Ca^{2+}$ free ACSF, and it was confirmed that they could burst spontaneously in this condition. Twelve of these (71%) became tonic after increasing $[Ca^{2+}]_e$ to 1.6mM.

**Effect of stimulation of the trigeminal tract on cells firing pattern**

All stimulations were carried out with $[Ca^{2+}]_e$: 1.6mM, and on neurons from rats at least 13 days old. Single shocks delivered to the trigeminal tract had little sustained effects on the spontaneous firing of NVsnpr neurons, whereas repetitive stimulation was effective in 77% of cases. In our sample, 124 neurons fired tonically before stimulation, 46 were bursting cells and 21 were silent. These silent cells were detected only after stimulation which produced tonic firing that lasted 5.3±1.3s (mean±SE) in all 21 cases (Fig 3A). Of the 124 tonically firing units, 52 (42%) increased their firing frequency (from 6.2±0.8Hz to 28.5±3.2Hz) for a similar period (4.4±0.6s) after stimulation (Fig 3B). Inhibition of firing lasted in average 2s±0.5 and were observed in 17 cells (14%) (Fig 3C). Twenty cells (16%) did not respond to stimulation. Finally, in 35 cells (28%), stimulation caused tonic firing to become rhythmic bursting in an average frequency of 8.3±0.7Hz for about 2.2±0.5s. The effects on burst firing units (n: 46) were also both excitatory and inhibitory (Fig. 4). In 8 cells (17%) stimulation caused an increase of burst frequency from 5.7±1.8Hz to 21.5±5.1Hz that lasted 1.5±0.56s (Fig. 4A). In 10 cases (22%) stimulation increased burst duration from 37±8.2ms to 180.4±43.6ms for about 3.7±1.5s (Fig. 4B). Bursting was suppressed in four neurons (9%) (Fig. 4C) for 1.7±0.36 and bursting was unchanged in 24 cases (52%).

**Distribution of neurons and effects of repetitive stimulation**

Different recording and stimulation positions were assessed. Figure 5 shows the distribution of the effects obtained according to their recording locations. Effects were more readily observed when cells were recorded from the dorsal half of the nucleus and the stimulation electrode was located "vis-à-vis" the recording electrode. Stimulation of the dorsal portion of the tract produced an effect in 87% of cases, whereas stimulation of the ventral portion of the tract had an effect in only 13% of cases. Excitatory effects (on tonic firing and bursting neurons) were mostly seen in neurons of the dorsal half whereas inhibitory effects were majoritarily observed in the middle part of the nucleus. Conversions from tonic to burst firing were exclusively observed in the dorsal part of the nucleus. Cases where stimulation had no effect were spread throughout the nucleus.

**Analysis of conversion from tonic to burst firing**

Analyses were carried out on the 35 units that were initially identified by visual inspection of data. The neuron shown in figure 6 was first identified as a bursting cell in $Ca^{2+}$ free ACSF (Panel A, 3$^{rd}$ trace). The two top traces show action potentials (Events) and bursts detected by the acquisition software (Spike 2). Even though bursting is somewhat unregular under this condition, a RI of 0.9 was calculated from the autocorrelogram (middle) which shows distinct peaks and a dominating frequency of 6.5Hz in the FFT analysis (bottom). Raising $[Ca^{2+}]_e$ to the physiological concentration of 1.6mM caused the cell to fire tonically (Panel B). The autocorrelogram was flat and the RI droped to -0.25. However, repetitive stimulation

of the trigeminal tract (40Hz for 1s) switched the firing pattern to rhythmic bursting (Panel C). Bursting was even more regular than in zero $Ca^{2+}$. The RI increased to 3.0, but the dominant frequency remained at 6.5Hz.

All neurons that converted from tonic firing to bursting (n: 35) had RIs that were negative prior to repetitive stimulation of the tract (Fig. 7A) and all RIs became significant after stimulation (Fig. 7B) (mean: 1.9±0.17). As a control, 10 neurons were selected from neurons that were tonically excited (5) or inhibited (5) by stimulation. In all cases, the RIs were negative before & after stimulation.

Post stimulus bursting frequencies calculated from the autocorrelograms and from FFTs gave similar results (mean of 8.75±0.78Hz calculated on autocorrelograms and 8.32±0.72Hz using FFT). The median was 6.25Hz using both methods. Figure 8 shows the distribution of the frequencies calculated with FFTs. These ranged from 1 to 20 Hz. Among the 35 neurons that began to fire rhythmically after stimulation, two units could be clearly detected in the raw data in 6 cases. In such cases, crosscorrelograms were also computed. Figure 9 shows an example where two units were recorded, one with a large action potential that fired tonically and one with a smaller action potential that fired in bursts. There was no clear temporal relationship between firing of both units before stimulation as shown in the flat crosscorrelogram (Fig. 9A, bottom). However, stimulation of the tract caused firing of the large unit to become rhythmic and bursts were synchronized in both units at 8.3Hz (Fig. 9B). In this case, the latency between firing in the two units was 10 ± 1.5ms as shown in the crosscorrelogram (arrowhead, bottom, panel B). Synchronization between two units was also observed in the other 5 cases and the average latency between units calculated in the crosscorrelograms was 9.17± 1.54ms.

**Optimal parameters of stimulation for the initiation of rhythmic activity**

Stimulation parameters were varied in frequency (20-100Hz) and duration (100-2000ms) to identify the optimal conditions for burst generation in 9 cases of conversion from tonic to bursting (Fig. 10). The Z axis in the figure represents the number of neurons that burst for each combination of frequency (X) and duration (Y). A rhythmic bursting case is represented only once in each different combination of parameters so the Z axis represents the number of cells that burst after tract stimulation in this combination of parameters. The peak in the figure was found for the combination 40 to 60Hz in the frequency domain and 500ms in duration. That was the combination used for most cases.

**Role of NMDA receptors in rhythm generation**

The contribution of NMDA receptors to changes from tonic to rhythmic bursting was assessed with local applications of NMDA and APV. Figure 11A shows tonic firing of a cell in control conditions ($[Ca^{2+}]_e$:1.6mM). About 8s after NMDA application, the cell began to burst (5Hz), (Fig. 11B) but firing returned to a tonic pattern again with the washout of NMDA (Fig. 11C). NMDA was tested in 22 of the 191 cases recorded in physiological $[Ca^{2+}]_e$. In half of these, an increase of tonic firing was observed, but rhythmic bursting was induced in 7 cases (33%). No effects were seen in 2 cases. In 2 of 22 cases (6%) rhythmic bursting could be obtained with NMDA application and also with stimulation of the trigeminal tract.

We also tested the ability of APV to block rhythmic bursting caused by stimulation of the tract in 15 neurons in physiological [$Ca^{2+}$]. In 9 of these cases (60%) APV completely blocked the effect of stimulation. Figure 12 shows an example of a tonic firing neuron (Fig. 12A) that burst rhythmically after repetitive stimulation of the trigeminal tract (Fig. 12B). Local application of APV (Fig. 12C) prevented the change in firing pattern, which recovered after washout (FIG. 12D).

## DISCUSSION

The goal of this study was to assess the effects of repetitive stimulation of peripheral inputs on the firing pattern of NVsnpr neurons *in vitro* under physiological $[Ca^{2+}]_e$, and especially to see if it is capable of eliciting rhythmic bursting within the frequency range of mastication, and of modifying spontaneous bursting patterns. Trigeminal primary afferents make monosynaptic glutamatergic connections with neurons in NVsnpr (Clements & Beitz, 1991; Bae *et al.*, 2000; Waite *et al.*, 2000), so we were not surprised that the majority of the short-latency and tonic effects observed were excitatory. The few inhibitory responses are probably due to activation of inhibitory neurons that have been found within NVsnpr and surrounding areas (Li *et al.*, 1996; Turman & Chandler, 1994). We have shown that stimulation of adjacent trigeminal and reticular areas elicit $GABA_A$ and glycine sensitive IPSPs in NVsnpr (Bourque & Kolta, 2001; Athanassiadis *et al.*, 2005a).

### Effects on bursting

Neurons that fired spontaneously in 1.6mM $[Ca^{2+}]_e$ were found throughout the nucleus, but only those in the dorsal $3/5^{th}$ were affected by the stimulation (Fig. 5). About $1/3^{rd}$ fired in bursts, and stimulation increased burst frequency or duration of about 40% of these. $2/3^{rds}$ fired tonically, and in about 30% of these, firing became rhythmic after stimulation. Many of these transformed neurons were found to be in the most dorsal portion of the nucleus. We showed that conversion from tonic firing to rhythmical bursting during stimulation was due to the release of glutamate, because it was blocked by APV and mimicked by NMDA application.

Stimulus-evoked rhythmical firing in NVsnpr has been reported previously *in vivo* by Tsuboi *et al.* (2003). They recorded extracellular neuronal activity during repetitive stimulation of the masticatory area of the cerebral cortex of decerebrate and paralyzed rabbits, and found a group of neurons that fired bursts in phase with fictive mastication. This group was located in the rostral and dorsal 1/3 of the nucleus, a region that projects to the trigeminal motor nucleus (Li *et al.*, 1993; Kolta *et al.*, 2000). Tsuboi *et al.* (2003) showed that these rhythmical masticatory neurons receive sensory inputs from muscle spindle, periodontal and mucosal afferents.

Three *in vitro* studies described intrinsic rhythmic fluctuations of membrane potential and associated bursting in NVsnpr of gerbils (Sandler *et al.*, 1998) and rats (Athanassiadis *et al.,* 2005a; Brocard *et al.*, 2006). Brocard *et al.* (2006) showed that bursting depended on a persistent sodium current (INaP). Recently, persistent sodium currents have also been described in spinal cord neurons linked to pattern generation in locomotion (Tazerart *et al.,* 2007; Zhong *et al.*, 2007).

**Link to mastication and whisking**

Brocard *et al.* (2006) found that both INaP and bursting were potentiated by reducing $[Ca^{2+}]_e$. They showed that the proportion of NVsnpr neurons that burst spontaneously in low $[Ca^{2+}]_e$ increased dramatically in the second postnatal week, in parallel with maturation of INaP, and with the appearance of mastication in rat pups (Westneat & Hall, 1992). Our results are consistent with this because bursting whether spontaneous or evoked by stimulation was very low in animals younger than 15 days.

Furthermore, stimulation was carried out with $[Ca^{2+}]_e$ at 1.6 mM, which is at the high end of the physiological range. Resting $[Ca^{2+}]_e$ in rat cerebrospinal fluid ranges from a

high of about 1.6 mM in the fetus to 1.2 mM in adults (Jones and Keep, 1988). Our finding that bursting activity can be modified or evoked *in vitro* by synaptic inputs from trigeminal sensory afferents provides further support to the hypothesis that NVsnpr rhythmic activity may be an essential component of the masticatory central pattern.

Westneat and Hall (1992) showed that the frequency of EMG bursts in the jaw muscles of freely behaving rats ranged from 5 to 11 Hz with a mean of 8.5Hz, which corresponds very well to the burst frequency of the great majority of our neurons.

The burst frequency of 6 of our neurons was above 12 Hz, suggesting that these could correspond to the "non-masticatory" rhythmic neurons of Tsuboi *et al.* (2003). These were found ventral to the masticatory neurons, and fired bursts at 2-3 times the masticatory rate that were not phase-linked to the masticatory motor pattern. They had receptive fields on the lips and face, suggesting that they could be part of a CPG for whisking, which has a frequency range of 5-15 Hz in rats (Berg and Kleinfeld, 2003). NVsnpr, particularly the ventral part, projects to facial motoneurons in NVII (Pinganaud *et al.,* 1999; Li *et al.,* 1997).

The masticatory CPG can be activated by tonic inputs from the motor cortex, and from the oral cavity (Sumi, 1969; Dellow and Lund, 1971; Lund and Dellow, 1971). Both project monosynaptically to NVsnpr, and both are glutamatergic. However, the brief trains of high frequency pulses that produce intense corticobulbar or corticospinal volleys and strong short-latency muscle twitches in jaw or limb muscles never trigger mastication. Instead, mastication is produced by trains of intermediate frequency (20-100Hz; Lund and Dellow, 1971; Huang *et al.,* 1989). The optimum stimulation frequency found to be most efficient in this study (40-60 Hz) was in the middle of this

range. *In vivo,* trains can last up to 10 sec and still evoke mastication, but in our preparation, the optimal train duration was about 500 ms. This may be due to the fact that the sensory fibers have been severed from their cell bodies, and so prolonged stimulation may deplete transmitter stores.

**Synchronization and phase-coupling**

Synchronization of neuronal firing and phase-coupling are fundamental features of CPGs (Grillner, 2006). We were able to examine these in 6 pairs of spontaneously-firing neurons that fired in bursts after stimulation. In all cases, the bursts were of the same frequency after stimulation, and they were phase-coupled. There was also a tendency for spikes of one to occur about 9ms after the other. The relatively long latency between the spikes of the two units argues against direct coupling through gap junctions and even direct excitatory projections from one neuron to the other. However, NVsnpr is strongly linked to interneurons in adjacent structures: the peritrigeminal area surrounding NVmot, NVmot itself, and nucleus pontis caudalis, and these are similarly interconnected by polysynaptic pathways which however, follow relatively high frequencies (e.g. 50-70 Hz; Bourque and Kolta, 2001; McDavid *et al.,* 2006; Athanassiadis, 2005a). We presume that these circuits participate in the pattern generating process, and indeed, many neurons in these nuclei fire rhythmically during fictive mastication. However, very few have intrinsic bursting properties, and neurons firing in the two phases (jaw opening, jaw closing) are intermingled. This is unlike NVsnpr, in which most of the neurons that fire during closing occupy the most dorsal and anterior part of the nucleus, while opener neurons are clustered more

posteriorly and ventrally (Tsuboi *et al.,* 2003). Our finding of burst phase coupling between adjacent neurons is consistent with this.

**Functional Implications**

Rhythmic bursting in NVsnpr neurons is initiated by activation of INaP , and can only occur when the membrane potential is between -59 and -41mV, which corresponds to activation range of this persistent sodium current (Azouz *et al.*, 1996; Su *et al.*, 2001; Del Negro *et al.*, 2002; Darbon *et al.*, 2004; Brocard *et al.*, 2006). We have proposed that sustained activity of depolarizing afferent inputs to the nucleus not only brings the level of depolarization into the INaP range, but also causes the $Ca^{2+}$ depletion that is necessary to activate it (Brocard *et al.,* 2006; Kolta *et al.,* 2007). Sustained neural activity does lead to large drops in $[Ca^{2+}]_e$ in other systems (e.g. Yue *et al.,* 2005). Within the INaP range, the frequency of bursting depends on the level of depolarization, providing a mechanism by which the frequency of masticatory movements can be adjusted by sensory feedback to the changing mechanical properties of the food as it is broken down by the teeth (Thexton *et al.*, 1980; Peyron *et al.*, 1997; Lund & Kolta, 2006).

## CONCLUSION

Neurons of dorsal NVsnpr have several properties that suggest a major role in masticatory pattern generation. They fire rhythmically during fictive mastication, project to trigeminal, facial and hypoglossal motor nuclei, and to other premotor regions. In addition, they have an intrinsic capacity to burst that depends in part on a persistent sodium current. This study provides evidence that tonic release of glutamate by terminals of trigeminal sensory afferents can trigger and modify rhythmic bursting, and suggest that it does so by a combination of depolarization and reduction of the extracellular concentration of calcium.

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## ABBREVIATIONS

ACSF, artificial cerebrospinal fluid; APV, D-(-)2 amino-5-phosphovaleric acid; $[Ca^{2+}]_e$, extracellular concentration of calcium; CPG, central pattern generator; FFT, fast Fourier transform; INaP, sodium persistant conductance; NMDA, N-methyl-D-aspartic acid; NVsnpr, trigeminal main sensory nucleus; RI, rhythm index.

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## FIGURE LEGENDS

**Figure 1:** Patterns of spontaneous activity. **A.** On top, trace of two bursting cells and amplification of a burst (inset). Below, a typical neuron that fires tonically a train of single action potentials as illustrated in the inset. **B.** Percentages of tonic and bursting neurons of all animals recorded in physiological $[Ca^{2+}]_e$:1.6mM (N: 191) at different ages (13 days and more).

**Figure 2:** Percentages of tonic and bursting neurons at different $[Ca^{2+}]_e$ (N: 280) of 13 days old and more. Numbers above the histograms indicate the number of neurons. 17 neurons were recorded in $Ca^{2+}$ free ACSF and in 1.6mM.

**Figure 3:** Effects of repetitive stimulation of the trigeminal tract on silent and tonically firing NVsnpr neurons. Excitatory effects included activation of silent units (**A.** n: 21, 11%) and long-lasting increases of tonic firing rate (**B.** n: 52, 42%). Inhibition was also of long duration (**C.** n: 17, 14%). In some cases stimulation caused tonic firing to become rhythmic for a short period (**D.** n: 35, 28%).

**Figure 4:** Effects of stimulation on spontaneously bursting neurons **A.** Increase of burst frequency (n: 8, 17%). **B.** Increase of burst duration (n: 10, 22%). **C.** Inhibiton of bursting (n: 4, 9%).

**Figure 5:** Localisation of the effects of repetitive stimulation of the trigeminal tract (dorsoventral aspect). The majority of the excitatory responses (black upright triangles and white circles) occurred in the dorsal half of the NVsnpr, as did conversion from tonic to burst firing (crosses). The inhibitory effects (black downward triangles and black circles) were more frequent in the middle of the nucleus and the cases were the stimulation had no effect were spread throughout the nucleus (white squares and white triangles).

**Figure 6:** Analysis of burst firing units. The third trace from the top shows the raw records, the traces above show spikes (Events) and bursts (Bursts) detected by the software. The middle part of each panel represents the autocorrelogram (bin size is 10ms), while the bottom graphs show the fast fourier transforms. **A.** $Ca^{2+}$ free ACSF. **B.** $[Ca^{2+}]_e$ (1.6mM) in the perfusing ACSF. **C.** Stimulation of the trigeminal tract switches the firing pattern back to rhythmic bursting.

**Figure 7:** Rhythm index distribution. In all 35 cases that changed firing pattern after stimulation, the rhythm indices were negative before stimulation (**A**) and above significancy level (0.01) after stimulation (**B**) ranging from 0.56 to 3.9. The average RI after stimulation was 1.90±0.17. The median is 2±0.4.

**Figure 8:** Frequency of the bursts elicited by repetitive stimulation calculated from the Fourier transform analysis (FFT).

**Figure 9:** Synchronization of two units after repetitive stimulation. **A.** In the control condition a large neuron (a) fires tonically 9Hz and a small neuron (b) bursts at 4Hz. The crosscorrelation shows that the two units fire asynchronously (bottom). **B.** 800ms after repetitive stimulation of the trigeminal tract, the two neurons fire in rhythmic bursts that are synchronous. The first peak in the crosscorrelogram at 10ms (arrowhead) indicates the latency between firing of the 2 units. Bursting frequency, calculated from the autocorrelograms was 8.3Hz in both cases.

**Figure 10:** Effects of stimulation parameters on probability of bursting. The arrow indicates the combination (40-60Hz, 500ms) that caused a maximal number of cells to burst (6/9).

**Figure 11:** Effect of NMDA application. Upper trace: Continuous recording of a cell before, during and after local application of NMDA (Black bar). Three periods are shown on an expanded time scale in the panels below together with the action potentials (Events) and bursts detected by the software. **A.** Before application of NMDA the cell fires tonically. **B.**Tonic firing (A) became rhythmic 8s after the application of NMDA. **C.** Nearly one min of constant perfusion with ACSF reversed the effect.

**Figure 12**: Rhythmic bursting involves NMDA receptors. A cell that fires tonically in physiological $[Ca^{2+}]_e$:1.6mM (**A**) changes its firing pattern to rhythmic bursting after repetitive stimulation of the trigeminal tract (**B**). Local application of APV prevents this change from occurring (**C**). The effect of stimulation is recovered 30s after washout of APV (**D**).

**Figure 1.**

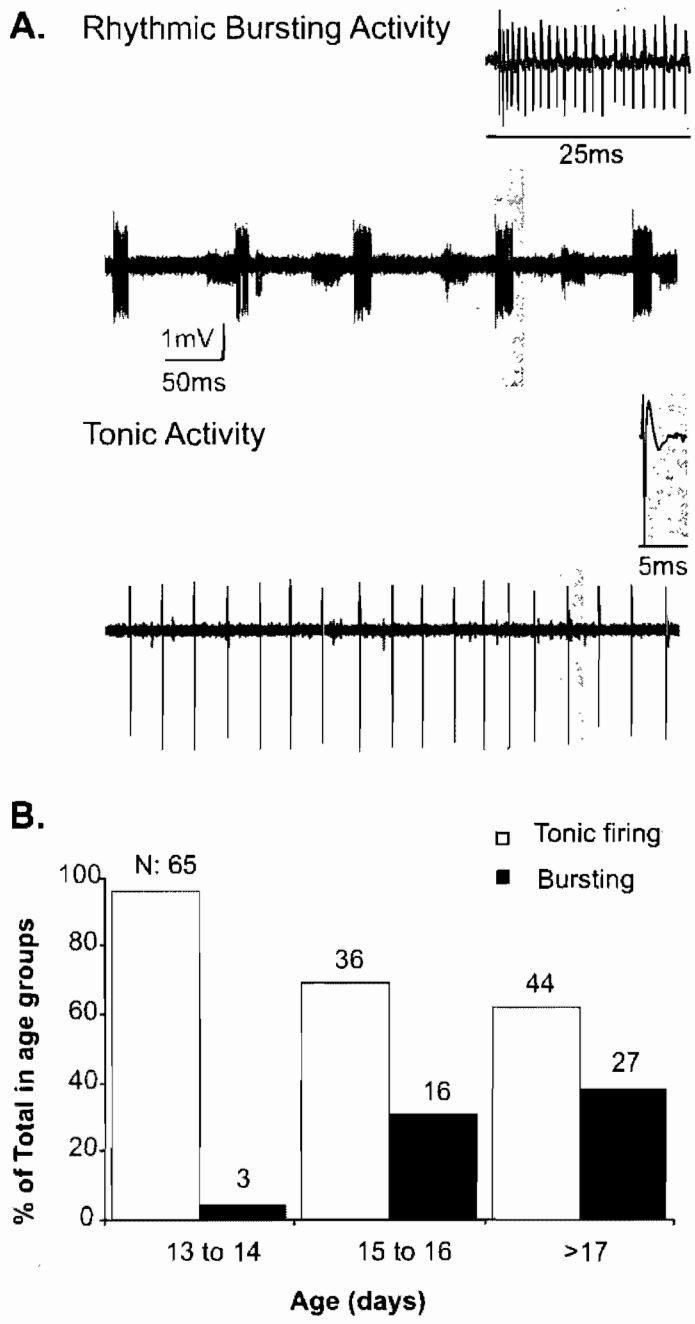

A.
Rhythmic Bursting Activity
25ms
1mV
50ms
Tonic Activity
5ms
B.
Tonic firing
Bursting
N: 65
100
80
60
40
20
0
% of Total in age groups
36
44
3
16
27
13 to 14
15 to 16
>17
Age (days)

**Figure 2.**

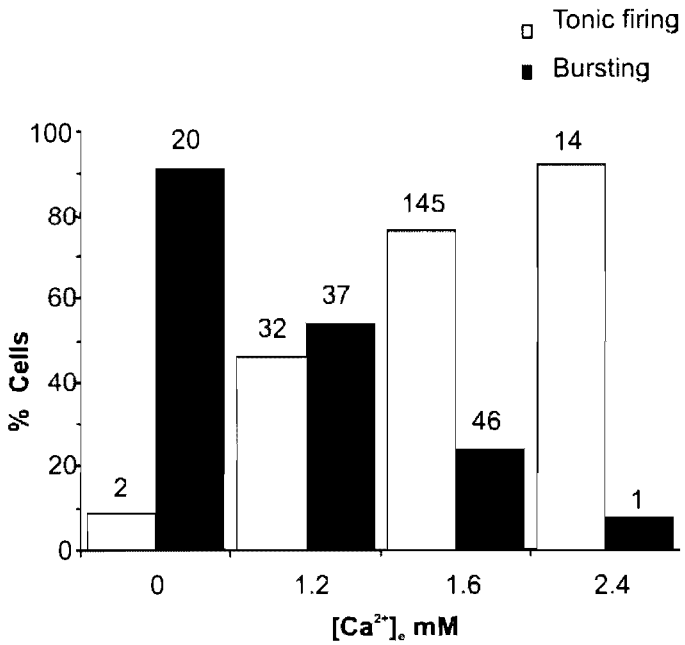

Tonic firing
Bursting
100
80
60
40
20
0
% Cells
20
14
145
37
32
46
2
1
0
1.2
1.6
2.4
[Ca2+]o mM

**Figure 3.**

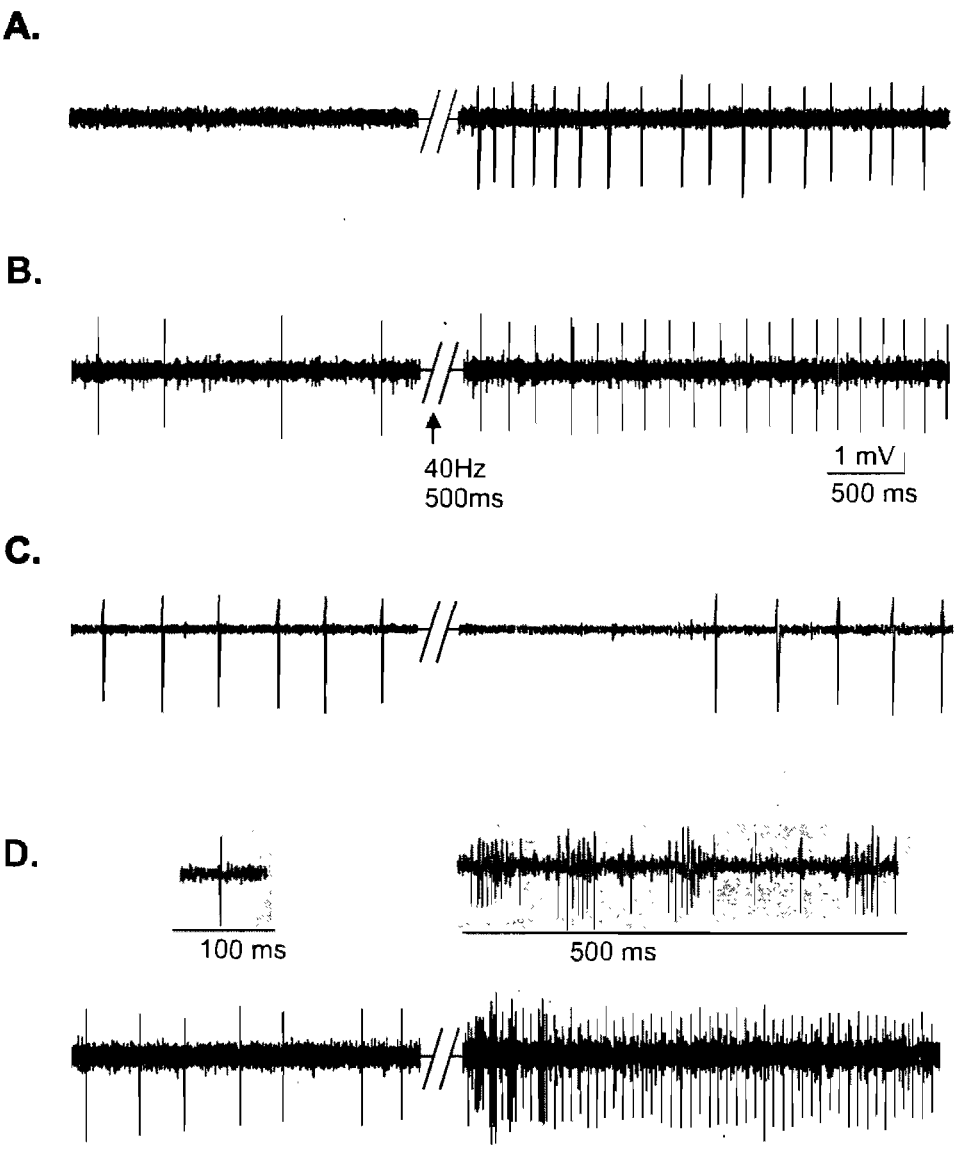


**Figure 4.**

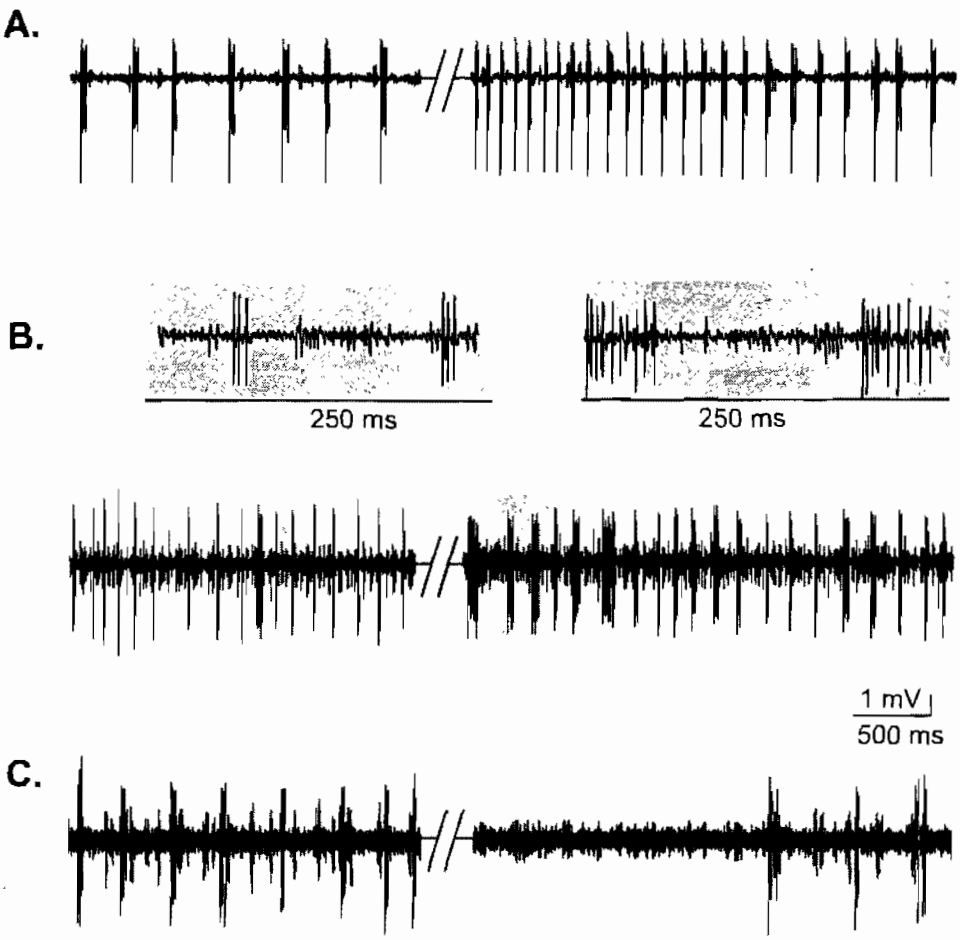


**Figure 5.**

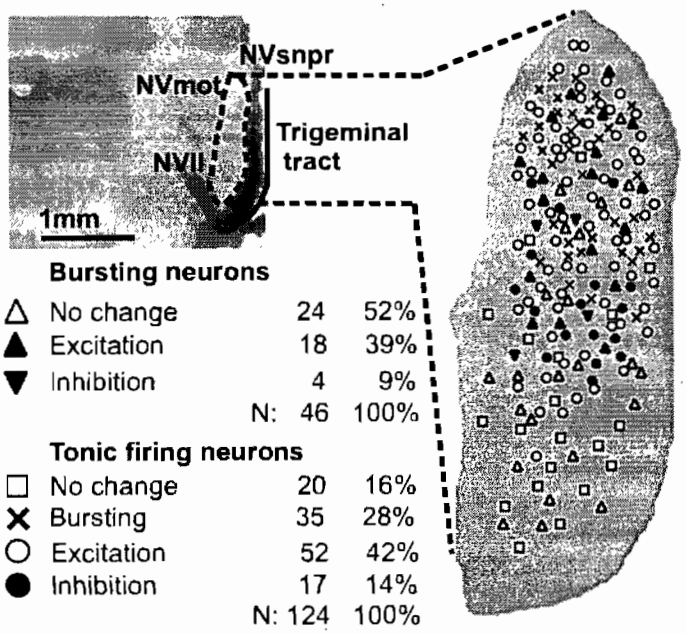

NVsnpr
NVmot
Trigeminal tract
NVII
1mm
Bursting neurons
No change 24 52%
Excitation 18 39%
Inhibition 4 9%
N: 46 100%
Tonic firing neurons
No change 20 16%
Bursting 35 28%
Excitation 52 42%
Inhibition 17 14%
N: 124 100%

**Figure 6.**

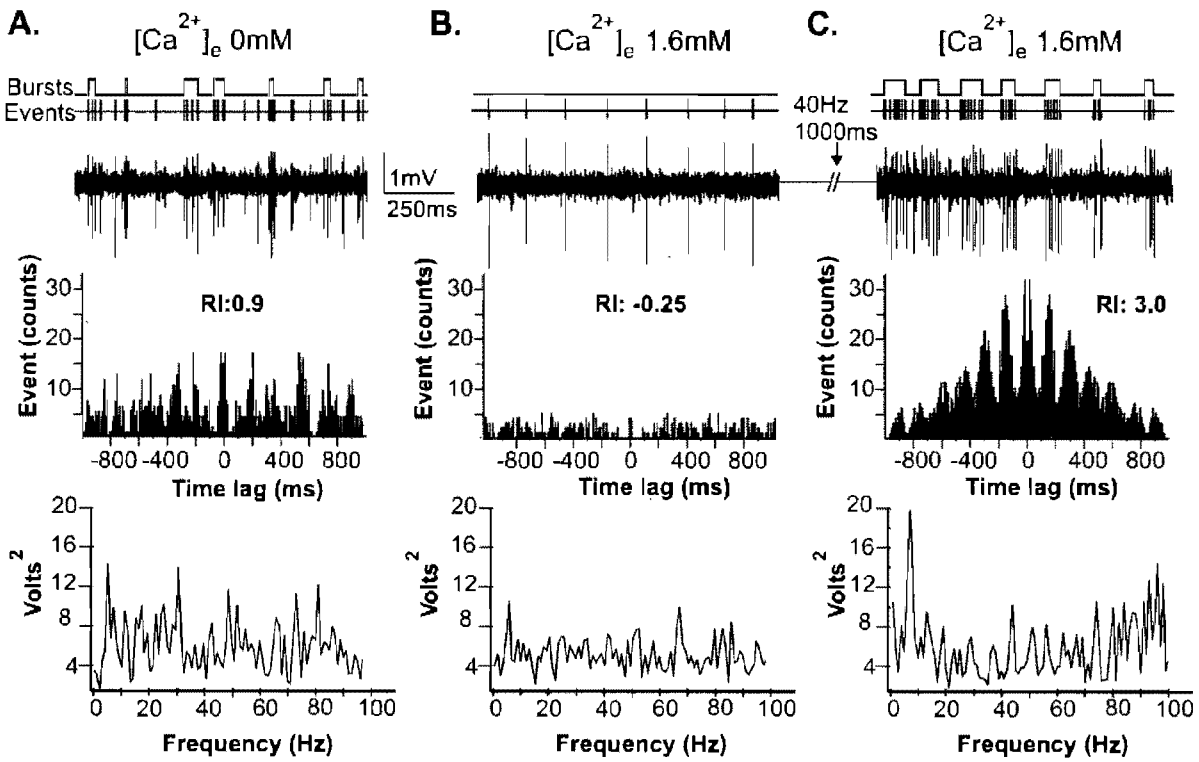

A.
[Ca2+]e 0mM
B.
[Ca2+]e 1.6mM
C.
[Ca2+]e 1.6mM
Bursts
Events
1mV
250ms
40Hz
1000ms
Event (counts)
RI:0.9
RI: -0.25
RI: 3.0
Time lag (ms)
Volts2
Frequency (Hz)

**Figure 7.**

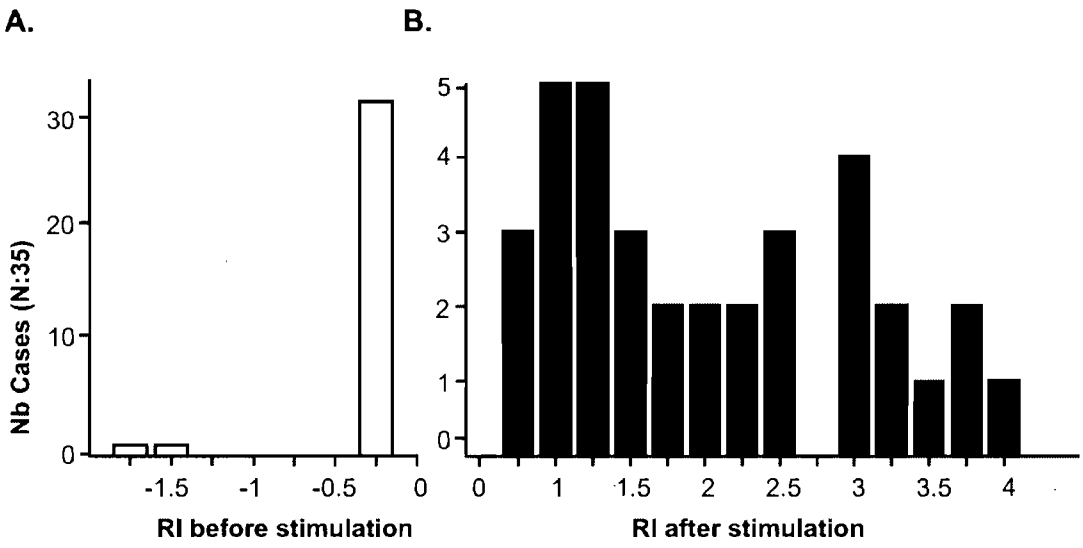

A.
B.
Nb Cases (N:35)
30
20
10
0
-1.5
-1
-0.5
0
RI before stimulation
5
4
3
2
1
0
0
1
1.5
2
2.5
3
3.5
4
RI after stimulation

**Figure 8.**

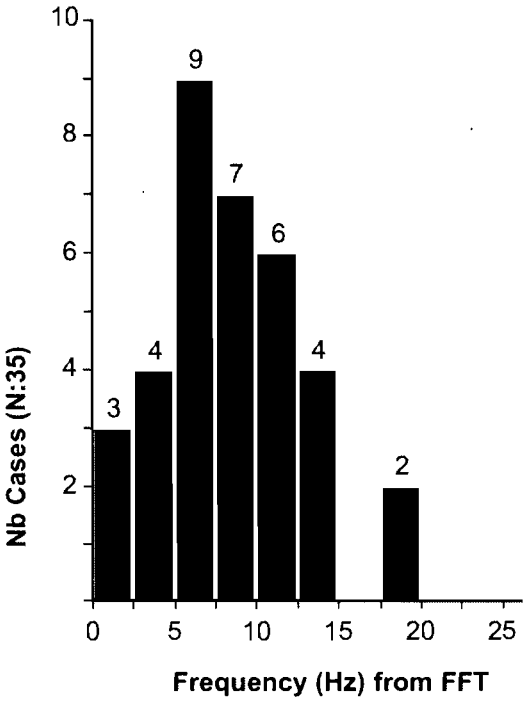

10
8
6
4
2
Nb Cases (N:35)
3
4
9
7
6
4
2
0
5
10
15
20
25
Frequency (Hz) from FFT

**Figure 9.**

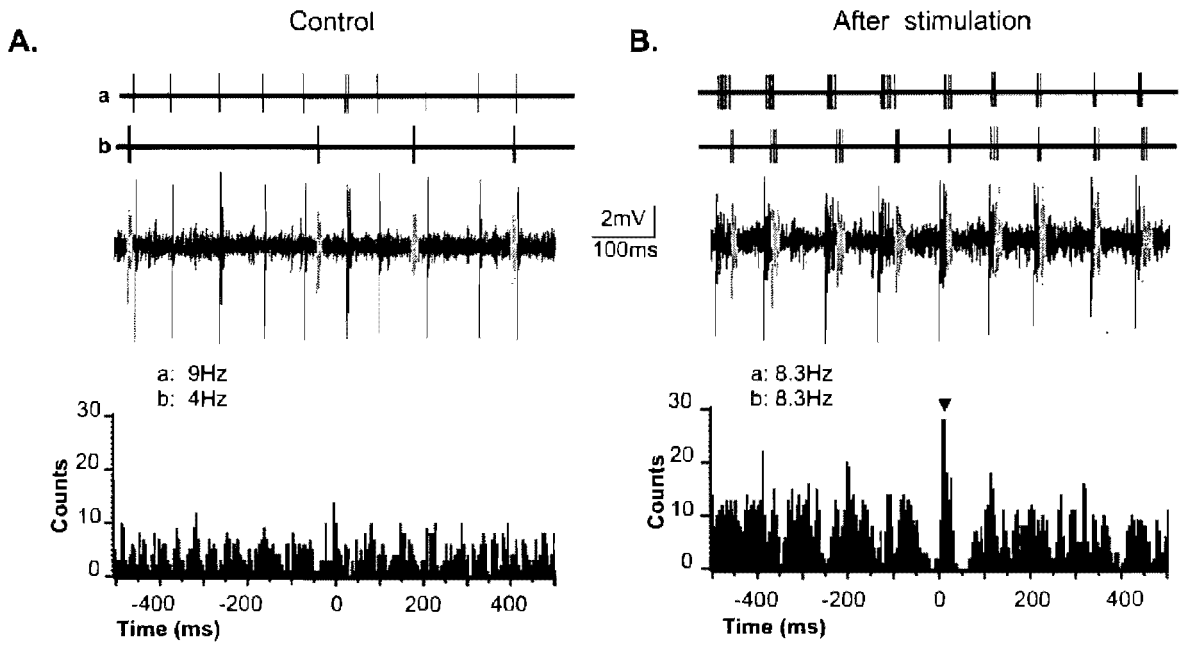

A.
Control
a
b
a: 9Hz
b: 4Hz
Counts
30
20
10
0
-400
-200
0
200
400
Time (ms)
2mV
100ms
B.
After stimulation
a: 8.3Hz
b: 8.3Hz
Counts
30
20
10
0
-400
-200
0
200
400
Time (ms)

**Figure 10.**

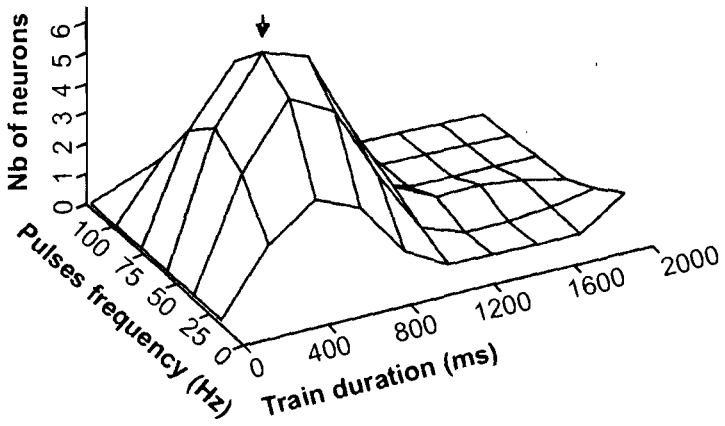

Nb of neurons
0 1 2 3 4 5 6
Pulses frequency (Hz)
100 75 50 25 0
0 400 800 1200 1600 2000
Train duration (ms)

**Figure 11.**

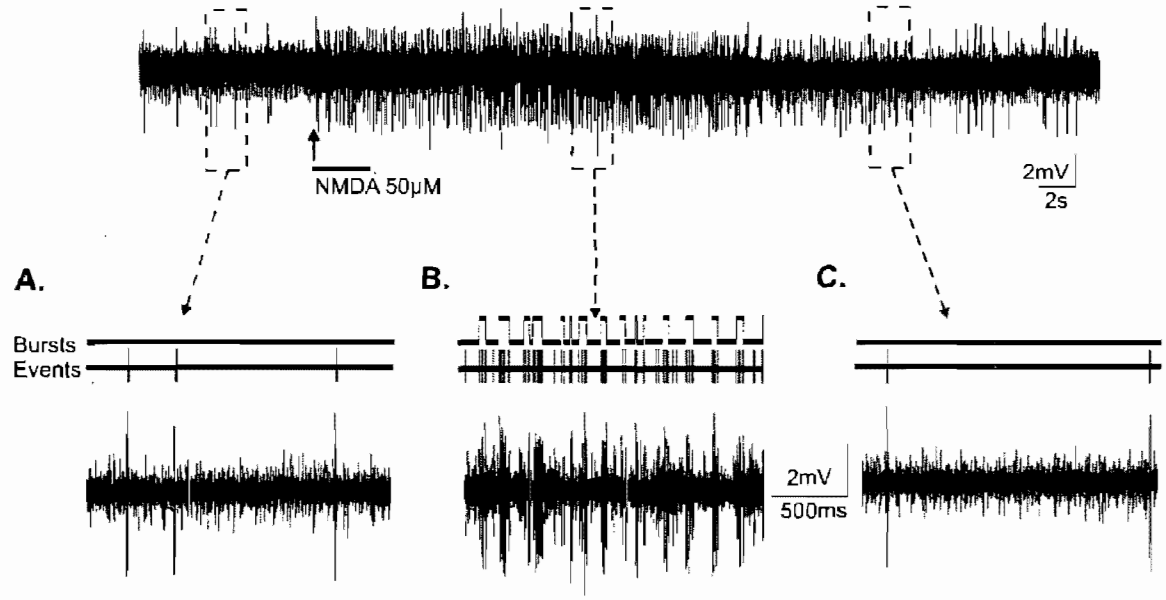
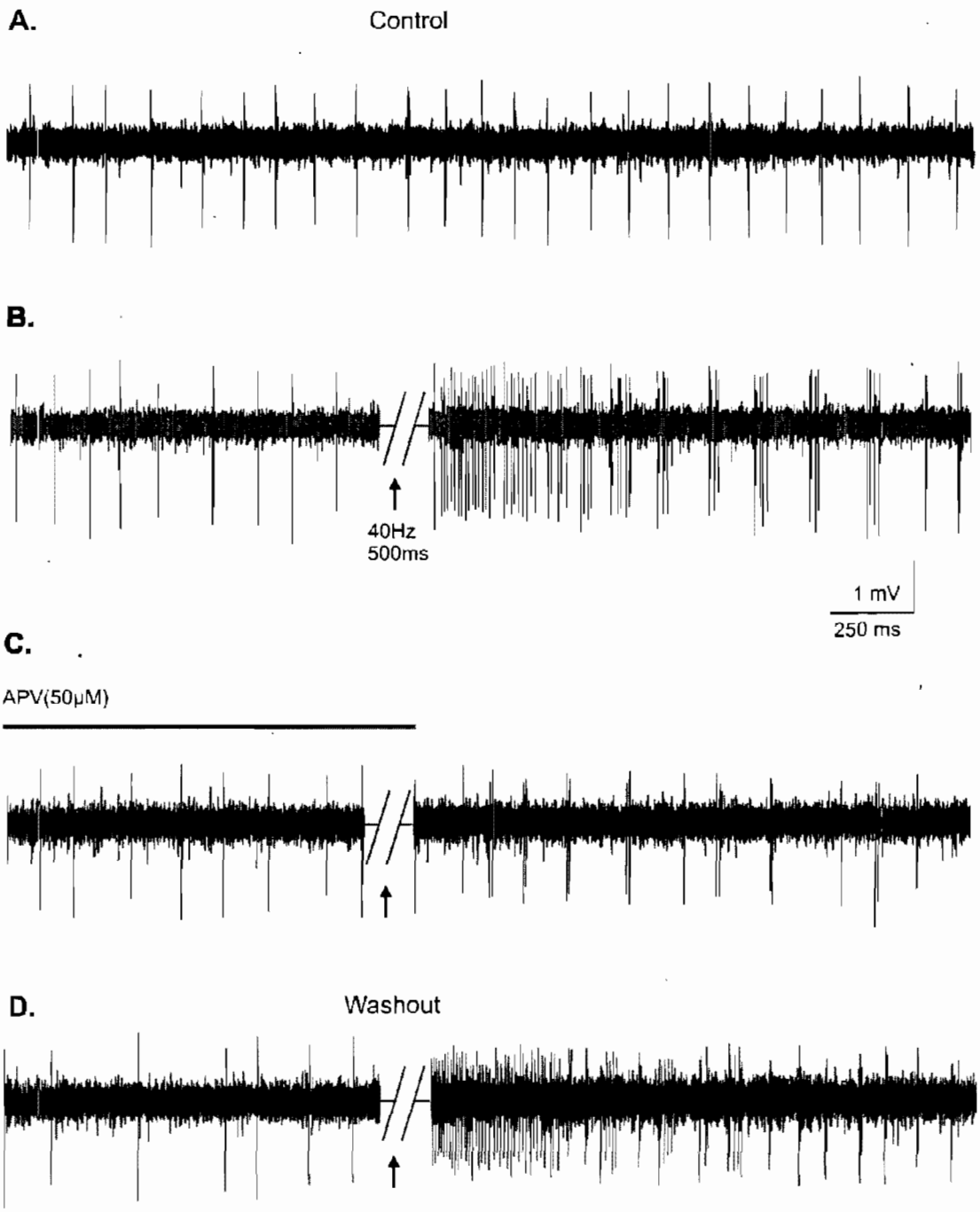


**Figure 12.**



# SECTION III

# DISCUSSION

## DISCUSSION

The data obtained from studies conducted in our lab during the last years suggest that NVsnpr is an interesting candidate to form the core of the CPG for mastication. One of the supporting evidence to this hypothesis is that we have found cells that burst rhythmically and intrinsically when the $[Ca^{2+}]_e$ diminishes. This rhythmical bursting relies on a persistent $Na^+$ conductance (INaP) that is modulated by $[Ca^{2+}]_e$. Our general hypothesis is that the level of activity of afferent fibers to the nucleus is instrumental and produces fluctuations of $[Ca^{2+}]_e$ which will help activate INaP and elicit rhythmic bursting activity in NVsnpr through activation of INaP (Brocard *et al.*, 2006). Thus, the main objective of this study was to test whether repetitive stimulation of afferent fibers can trigger rhythmic bursting in NVsnpr neurons in physiological concentrations of $Ca^{2+}$.

### 1. The pattern of activity of NVsnpr neurons depends on the age of the animals

As Brocard et *al.* (2006), we have found neurons that fire rhythmically even in presence of $Ca^{2+}$, $[Ca^{2+}]_e$ (1.6mM) and observed that the number of cases was particularly low in young animals and increased with age as reported previously. Brocard *et al.* (2006) reported a number of age-dependent changes in membrane properties such as input resistance, membrane time constant, duration of the after-hyperpolarization, (AHP), time dependent inward rectification, amplitude of the after-depolarization, (ADP), and rheobase. Some of theses changes such as lowering in input resistance and larger ADP could be related to morphological modifications

observed during development such as cell growth and increase in membrane channel density (Jacquin *et al.*, 1996; Cameron *et al.*, 2000).

For instance, bursting cells are normally larger than tonic firing cells and therefore have a lower input resistance (Larkman & Mason, 1990; Schwindt *et al.*, 1997; Brocard *et al.*, 2006). However, NVsnpr neurons develop features that can only be explained through changes either in composition or activation of particular membrane conductances. First, rheobase decreases with age indicating that NVsnpr neurons become more excitable in older animals (Brocard *et al.*, 2006). This could be due to a decrease in activation threshold of $Na^+$ channels or an increase in $Na^+$ currents such as INaP (Brocard *et al.*, 2006; Gao & Ziskind-Conhaim, 1998; Martin-Caraballo & Greer, 2000). Brocard *et al.* (2006) showed that the emergence of repetitive bursting in NVsnpr takes place around the second post-natal week and could be due to subthreshold activation of prominent INaP conductances (Crill, 1996). The age in which the emergence of bursting ability appears has been well documented in a number of studies. In neocortex and ventrobasal thalamus rhythmic bursting appears at the end of the second post-natal week (Franceschetti *et al.*, 1993; Kasper *et al.*, 1994a, b; Perez Velazquez & Carlen, 1996) whereas in the Pre-Bötzinger complex in the brainstem (Del Negro *et al.*, 2002; Del Negro *et al.*, 2005) it appears earlier (first postnatal week).

## 2. The pattern of activity of NVsnpr neurons depends on $[Ca^{2+}]_e$

In NVsnpr, bursting does not only depend on the emergence of INaP but also on $[Ca^{2+}]_e$. As we have seen in this and previous work (Brocard *et al.*, 2006) bursting persists in absence of $Ca^{2+}$ and is even potentiated by removal of $[Ca^{2+}]_e$. The effect of

$[Ca^{2+}]_e$ on bursting in NVsnpr persist with aging at least up to 30 days (Brocard *et al.*, 2006) in contrast to other neurons like pyramidal cells where this dependency on $[Ca^{2+}]_e$ is only transitory (disappears around the end of the third week) (Chen *et al.*, 2005). The concentration of $Ca^{2+}$ in the cerebrospinal fluid gradually drops from 1.6mM in fetal rat to 1.2mM in adult rat (Jones & Keep, 1988). This suggests that developmentally-related fluctuations in $[Ca^{2+}]_e$ might also contribute to changes in cell excitability and activation of INaP conductances. Interestingly bursting appears around the second postnatal week, in parallel to the emergence of masticatory jaw movements in the rat (Westneat & Hall, 1992).

**3. Rhythm analysis**

Our results indicate that the frequency of bursting in the cases of conversion is consistent with the frequency of jaw movements observed in adult animals (Westneat & Hall, 1992). We observe that this frequency is quite particular to mastication since the frequency of other rhythmic processes such as respiration (0.1-0.8Hz) (Suzue, 1984; Rybak *et al.*, 2003; Potts *et al.*, 2005; Fisher *et al.*, 2006; Janczewski & Feldman, 2006) or deglutition (0.1 to 0.5Hz)(Jean, 2001; Kogo *et al.*, 2002) fall in different ranges. Intrinsic bursting involving INaP can underly very different rhythmic activities in other systems than ours (Su *et al.*, 2001; Bikson *et al.*, 2002; van Drongelen *et al.*, 2006) suggesting that the frequency of bursting in NVsnpr neurons obtained after stimulation of the trigeminal tract is not due to invariant intrinsic properties but is related to mastication.

**4. Synchronization of NVsnpr neurons**

The role of synchronization is to provide a temporally correct pattern for NVmot activity during mastication. We have observed that in all cases of conversion where two units were detected, their firing became synchronous. In the example case shown in the article two neurons become synchronous after trigeminal stimulation. Different mechanisms could take account for the initiation of rhythmic activities and synchronization of bursting units. Recent experimental data from our lab suggests that trigeminal stimulation produces plateau potentials superimposed to an IPSP (Verdier *et al.*, 2007). This IPSP occurs normally at a shorter latency and a lower stimulation level than the plateau potential. It delays the plateau. This suggests that inhibitory effects of trigeminal stimulation can shape the plateau. Both plateau potentials and rhythmic bursting in NVsnpr depend on the activation of INaP. We suggest that inhibitions could also be underlying synchronization of neuron firing, as has been observed in other systems (Van Vreeswijk *et al.*, 1994; Elson *et al.*, 2002). Alternatively, a glial mechanism could underly cell synchronization (Cornell-Bell & Finkbeiner, 1991; Suadicani *et al.*, 2004; Scemes & Giaume, 2006). Trigeminal stimulation could activate glial cells syncitia in NVsnpr. It is well known that glial cells form extended syncitia of coupled cells in which calcium waves can propagate through GAP junctions (Cornell-Bell & Finkbeiner, 1991; Scemes & Giaume, 2006). Glial cells in NVsnpr could be able to produce a local change in $[Ca^{2+}]_e$ that would propagate onto target neurons, activating INaP and leading to synchronous activities.

**5. Functional implications of the location of rhythmic neurons in NVsnpr**

Tsuboi *et al.* (2003) reported that neurons modulated by stimulation of the cortical masticatory area, MA (either tonic firing or burst firing units) were located in dorsal NVsnpr, while unmodulated units were distributed everywhere in the nucleus. This is consistent with our results in the sense that the majority of modulatory effects in tonic or bursting units or changes in firing pattern from tonic to rhythmic bursting were also observed in the dorsal 2/3rds of the nucleus, while the cases where peripheral stimulation had no effect (unmodulated) were observed all over the nucleus and preferentially in its ventral part. Our results are also consistent with the investigation of Athanassiadis *et al.* (2005a) who also described rhythmic bursting units in the dorsal part of the nucleus.

**6. Effectiveness of peripheral stimulation**

In tonically firing neurons, peripheral stimulation caused excitation in 32% of cases, decrease in firing frequency in 11% and changes of firing pattern in 18% of cases. In the rabbit *in vivo* preparation, stimulation of the CMA at similar frequencies and train durations produced increase of firing frequency in 11% of neurons, no decreases in firing frequency and changes of firing pattern from tonic to rhythmic in 28% of neurons, but only in 11% was the rhythm in phase with mastication (Tsuboi *et al.*, 2003). These numbers differ from the obtained in our study, but not dramatically. The differences can be due to several factors including species, differences in the type of inputs (cortical vs peripheral) and circuits involved and conditions (*in vivo*; intact system vs *in vitro*; fragmented system), but the similarities may be due to the fact that

both types of afferents are glutamatergic. NMDA injections, which may be considered as a "stronger" stimulus (by acting directly on NMDA receptors) caused no decrease in firing frequency, an increase in firing frequency in 52% of neurons and induced rhythmic bursting in a larger percentage of cases (33%), closer to the proportion observed in the *in vivo* study of Tsuboi *et al.*, (2003). Interestingly the stimulation parameters found to be most efficient to induce rhythmic bursting are similar in our study to those that have been found to be most efficient *in vivo* (Lund *et al.*, 1984). This can be explained by our previous observations from intracellular recordings *in vitro* that showed that bursting of NVsnpr neurons is not only modulated by $[Ca^{2+}]_e$, but occurs only within a range of membrane potential that corresponds to the activation and inactivation potentials of INaP. Out of this range firing becomes tonic. Thus, we can hypothesize that in cases where firing switched from tonic to rhythmic, the stimulation was able to lower $[Ca^{2+}]_e$, but did not depolarize the cell recorded too much , which might have been the case of cells that increase their firing but remained tonic.

The type of effect elicited also seemed to vary with the position of the stimulating electrode. Stimulation in the ventral portion of the tract had fewer effects on cell firing and did not elicit rhythmic bursting. These results can be explained on the basis of the anatomical organization of primary afferents in NVsnpr. Jacquin *et al.* (1993) conducted HRP labeling and cytochrome-oxidase experiments staining primary afferent collaterals that project to NVsnpr in adult rat and found that the sensory fibers gave rise to transversely oriented collaterals in NVsnpr and NVspo. Therefore synaptic inputs are easier to obtain if stimulation is performed *vis-à-vis* the projecting areas. Since inputs from the intra-oral receptors are in the mandibular and maxillar divisions

of the nerve, which are more dorsal in the tract, it is more likely to obtain masticatory-like activities in the dorsal half of the nucleus than in the ventral half.

**7. Role of tonic versus phasic sensory afferent stimulation**

Tonic stimulation of periodontal and mucosal afferents can initiate mastication and fictive mastication in decerebrate or lightly anesthetized animals (Bremer, 1923; Bazett & Penfield, 1922; Van Willigen & Weijs-Boot, 1984; Juch *et al.*, 1985) or make subtreshold stimulations of CMA effective (Lund & Dellow, 1971; Lund & Dellow, 1973). This is in contrast with the phasic stimulation of sensory afferents that occur during mastication and which may trigger reflexes with important repercussions on the CPG (McGrath *et al.*, 1981; Di Francesco *et al.*, 1986; Chase & McGinty, 1970; Lund, 1991 for a review). For instance, the phasic feedback produced by periodontal afferent stimulation during mastication of hard foods evokes a jaw-opening reflex bilaterally (McGrath et al, 1981; Di Francesco et al., 1986). This reflex is actively supressed by the CPG as the food softens and is normally present during the first cycle of mastication only (Schwartz *et al.*, 1989; Lavigne *et al.*, 1987).

The CPG controls reflexes in several ways, by modulating the excitability of MNs, and premotor interneurons or by modulating the transmission from primary sensory afferents to CPG interneurons. Most NVsnpr neurons that fire during the jaw opening phase of fictive mastication receive periodontal feedback while those that fire during jaw closing are excited by spindle afferents (Tsuboi *et al.*, 2003). Thus, these inputs can alter the firing rate and pattern of NVsnpr neurons which in turn project to trigeminal MNs, premotor interneurons and even terminals of primary afferents (Kolta

*et al.*, 2000; Bourque & Kolta, 2001; Verdier *et al.*, 2003). This should allow the CPG to adapt its rhythmic output and/or to adjust the gain of reflexes, suppressing unnecessary ones and facilitating those that enhance motor performance.

**8. CPG converts tonic inputs into rhythmic bursts**

Tonic stimulation of sensory afferent receptors or the cortical masticatory area (CMA) is converted into a rhythmic command by the masticatory CPG. Little is known about how the CPG does this but there are several possibilities. Rhythmic activities could result from interactions within the network that forms the CPG, from intrinsic properties of some of its elements or a combination of the two factors (See Lund & Kolta, 2006 for a review). Network properties can explain the changes in the pattern of mastication (e.g grinding with the left molar or grinding with the right molar) that result from stimulation of different areas of the CMA (Westberg *et al.*, 1998). Changes in the boundaries of the CPG could account for the different patterns of mastication by the activation or silencing of different groups of neurons for each pattern. Westberg *et al.* (1998) have examined the firing pattern of trigeminal premotor interneurons during different patterns of fictive movements elicited in the rabbit by stimulating different sites of the CMA. More than one half of the neurons in NPontc and NVoralis that fire in one fictive pattern were silent during another. The remaining fired consistently across different patterns. These results indicate that some neurons participate in the shaping of masticatory patterns and are involved in the fine tuning of mastication, while other neurons could be involved in rhythm initiation and provide the basic masticatory rhythm.

## 9. Basic properties of the masticatory CPG

At least three types of commands are required to generate a minimal output from NVmot (Goldberg & Chandler, 1981; Kubo *et al.* 1981; Goldberg *et al.,* 1982): Inhibition of JC-motoneurons during JO (hyperpolarizing inputs), excitation of JO-motoneurons during JO (depolarizing inputs) and excitation of JC-motoneurons during JC (depolarizing inputs). Interestingly, JO-motoneurons are not inhibited during JC. According to Inoue and colleagues, the simplest CPG for mastication would require only two types of pre-motorneurons projecting to NVmot: The ones firing during the JO phase (inhibitors and excitators), and the ones firing during the JC phase (excitators) (Inoue *et al.*, 1994). In 1998, Yoshida's group conducted anatomical studies in the cat to label secondary order neurons in NVsnpr with HRP tracers. Their work indicated that the dorsal part of NVsnpr contains local circuit neurons projecting to dorsal NVmot, also corresponding to the area where the JC motoneuron pool is located. These results were confirmed by a number of other studies conducted in cat, rabbit and rat indicating that dorsal NVsnpr sends projections to the dorsal NVmot (Mizuno *et al.*, 1983; Landgren *et al.*, 1986; Li *et al.*, 1993; Kolta *et al.*, 2000; Arsenault *et al.,* 2004; Athanassiadis *et al.*, 2005a). Later, Tsuboi and colleagues (2003) showed that NVsnpr in rabbit contains a mixture of neurons bursting rhythmically in phase either with JO or JC motorneurons (50% in each category) suggesting that NVsnpr neurons could indeed shape the motor output. JO and JC neurons had a certain organization in NVsnpr. Both rhythmically active JO and JC premotorneurons were mostly located in the dorsal part of NVsnpr in the dorso-ventral axis. In addition, JC neurons were located in the anterior pole of NVsnpr, while JO

entire length of the rostro-caudal axis. It is interesting to observe that cells in NVsnpr that had receptive fields from intraoral- periodontal or jaw muscle spindles can accomplish motor functions. *In vivo,* more than 50% of JC neurons were excited by spindle afferents, while most JO neurons received periodontal feedback. Both the localization of the neurons in NVsnpr and their receptive fields is consistent with the peripheral input organization in NVsnpr described by Shigenaga and others (face-inverted primary afferent somatotopy) (Darian-Smith *et al.*, 1963a; Shigenaga *et al.*, 1986a).

**10. Putative mechanisms for burst generation**

Kolta *et al.* suggested in 2007 a mechanism leading to rhythmic bursting on the basis of observations from the work conducted by Brocard *et al.* (2006) in a rat brainstem slice preparation (Fig. 9). One assumption in our hypothesis regards the reduction in $[Ca^{2+}]_e$ after neuronal activity. Weak synaptic inputs to NVsnpr would cause a minor reduction in $[Ca^{2+}]_e$ and elicit low frequency tonic firing. At this stage only transient $Na^+$ and $K^+$ conductances would be involved. $Ca^{2+}$ would enter the cell through voltage dependent $Ca^{2+}$ conductances or neurotransmitter gated receptors activated by glutamate such as NMDA and AMPA receptors. $Ca^{2+}$ inflow would activate $Ca^{2+}$-dependent $K^+$ conductances such as small-$Ca^{2+}$ activated $K^+$ conductances (SK) and big-$Ca^{2+}$ activated $K^+$ conductances (BK) (Fig. 9). SK would be responsible for the after-hyperpolarization phase of the action potential, AHP, while BK would participate to the repolarizing phase of the action potential (Kolta *et al.*, 2007). BK function could be to limit burst duration. Brocard *et al.* (2006) showed that blocking SK with apamin reduces AHP; while blocking BK with charybdotoxin enhances after-depolarization

(ADP) promoting firing of several spikes more than in the control condition. Stronger synaptic inputs would reduce BK due to a greater depletion in $[Ca^{2+}]_e$. leading to smaller AHP and appearance of ADP or small plateaux (Step 2 in Fig. 9). Even stronger synaptic inputs should increase the depletion in $[Ca^{2+}]_e$ and depolarize the cell enough to activate INaP thereby producing the plateau potentials supporting rhythmic bursting (Kolta *et al.*, 2007) (Fig. 9).

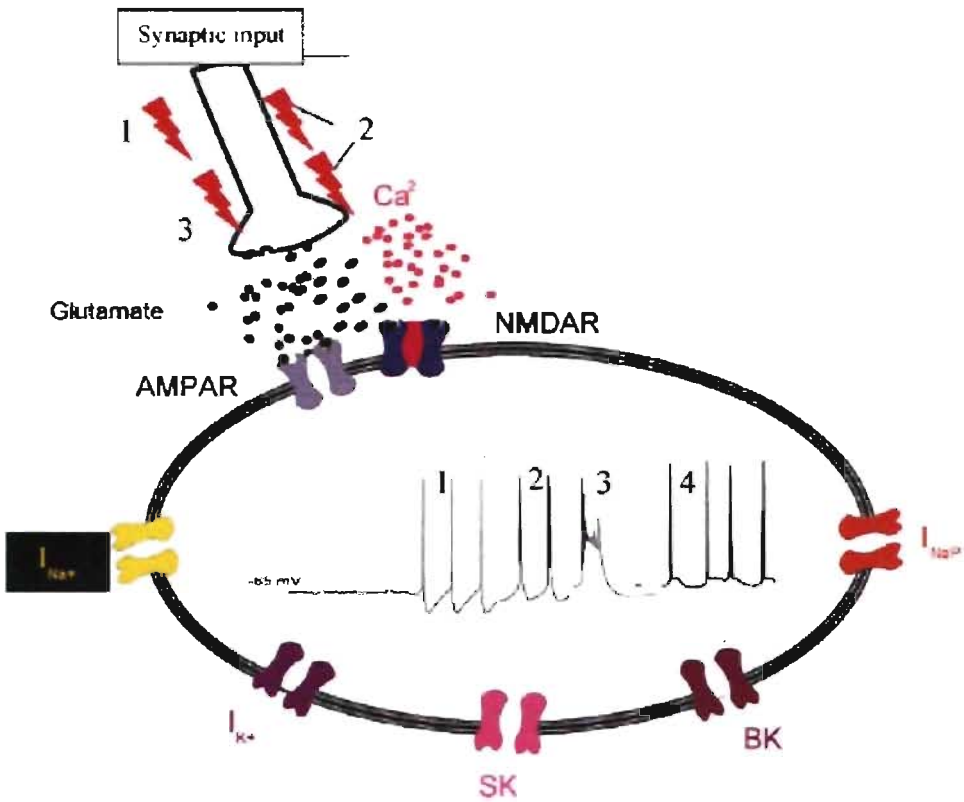



**Figure 9. Proposed mechanism underlying the initiation of rhythmic activity in NVsnpr.** Gradual intensity of stimulation correlates with $[Ca^{2+}]_e$ depletion, INaP activation and changes in the firing pattern of NVsnpr neurons. **1.** Weak stimulation generates small ionic changes in $[Ca^{2+}]_e$ and we obtain tonic responses. **2.** and **3.** Rhythmic bursting and plateau potentials arise with stronger stimulation and larger changes in $[Ca^{2+}]_e$ **4.** Further depolarization of the cell inactivates INaP and the neurons firing pattern becomes tonic. Adapted from Kolta *et al.* (2007).

It is still not known how the stronger inputs could mediate depletion of $[Ca^{2+}]_e$, but we have at least two different hypotheses to explain it. The first possibility would be that stronger sustained depolarization might remove the $Mg^{2+}$ block of NMDA receptors (Vargas-Caballero & Robinson, 2003; Hsiao *et al.*, 2002) and allow $Ca^{2+}$ to flow into the cells. In fact, our results show that NMDA receptors are involved in the initiation of rhythmic activities since local application of NMDA is able to elicit rhythmic bursting in NVsnpr neurons *in vitro*. NMDA receptor activation could take place before INaP activation and be required for synaptically-induced rhythmic-bursting in physiological conditions, since the application of APV blocks bursting activities elicited by stimulation in 60% of cases. The second possibility involves glial cells. Glial cells accomplish a number of metabolic functions including clearance of synaptic transmitters from the synaptic cleft (Bergles *et al.*, 1999; Danbolt, 2001; Araque & Perea, 2004). Even more importantly glia regulates $Ca^{2+}$ levels (Auld & Robitaille, 2003; Stringer *et al.*, 2007). Glial cells express a wide variety of receptors for neurotransmitter such as glutamate (including NMDA receptors), GABA, acetylcholine and other simple molecules like ATP, and are activated by $K^+$ released during neuronal firing (Porter & McCarthy, 1997; Verkhratsky *et al.*, 1998; Gallo & Ghiani, 2000). It is possible that $K^+$ and/or glutamate released during synchronous activation of afferent inputs to NVsnpr activate glial cells, which in turn may contribute to $Ca^{2+}$ depletion. It is well known that intracellular $Ca^{2+}$ rises in astrocytes as a consequence of glutamate receptor activation (Porter & McCarthy, 1996; Latour *et al.*, 2001). In addition, activated astrocytes can also release glutamate (Araque *et al.*, 1998a, b; Bezzi *et al.*, 1998) and this process is $Ca^{2+}$ dependent (exocytotic pathways) (Araque *et al.*, 1998 a, b; Pasti *et al.*, 2001); The glutamate released by glial cells evokes slow inward currents in adjacent neurons by activation of ionotropic glutamate

receptors (Araque *et al.*, 1998a, b; Parpura & Haydon, 2000). Such a mechanism could contribute to the depletion of $[Ca^{2+}]_e$. It has also been reported that stimuli that evoke intracellular $[Ca^{2+}]$ elevation in single astrocytes can propagate to adjacent ones and extend for hundreds of microns; (Charles *et al.*, 1991; Cornell-Bell & Finkbeiner, 1991; Dani *et al.*, 1992; Smith, 1994). $Ca^{2+}$-waves can propagate from one cell to another either through gap-junctions in the case of syncitium-coupled cells or through release of gliotransmitters (See Scemes & Giaume, 2006; Fiacco & McCarthy, 2006 for a review). Recent anatomical evidence indicate that glial cells in NVsnpr form a syncithia when activated (Arsenault *et al.,* 2007). Preliminary work in our lab has shown that coupling between glial cells increases with the stimulation of the trigeminal tract or with local application of NMDA in NVsnpr (Arsenault *et al.,* 2007). Such syncitia could play a role in synchronizing a population of neurons by depleting $Ca^{2+}$ in a circumscribed volume.

Another unknown aspect of the initiation of rhythmic activity in NVsnpr is related to the precise mechanism of activation of INaP in consequence of changes in $[Ca^{2+}]_e$. Su *et al.* (2001) conducted experiments in rat hippocampal pyramidal neurons and reported that $[Ca^{2+}]_e$ modulates current-dependent burst firing in CA1 neurons. Low $[Ca^{2+}]_e$ markedly increased incidence and reduced the threshold of spontaneous rhythmic activities in these cells. In their experiments, the concentrations of divalent cations were maintained constant in all perfusing solutions, and the induction of intrinsic bursting occurred in low $[Ca^{2+}]_e$ regardless of the species of divalent cations used to replace $[Ca^{2+}]_e$ ($Mg^{2+}$). This was also the case in our report and previous studies conducted in NVsnpr (Brocard *et al.*, 2006). According to Su *et al.* (2001), evidence suggest that $Ca^{2+}$ could decrease INaP by ion-selective binding to membrane

channels. $Ca^{2+}$ could bind to $Na^{+}$ channels (Armstrong & Cota, 1991) or to G-protein-coupled $Ca^{2+}$ sensing receptors (Yamaguchi *et al.*, 2000), such as metabotropic glutamate receptors (Kubo *et al.*, 1998) that might modulate INaP via second messenger cascades. Su *et al.* (2001) demonstrated that the latter was less likely in hippocampal pyramidal cells by showing that the application of neomycin and gadolinium, which activate $[Ca^{2+}]_e$-sensing receptors (Xiong & MacDonald, 1999), failed to reverse the induction of intrinsic bursting by low $[Ca^{2+}]_e$.

## CONCLUSION

This *in vitro* study proposes a methodological approach to investigate the initiation of rhythmic masticatory-like activities in NVsnpr at physiological $[Ca^{2+}]_e$. Our results support the hypothesis that NVsnpr could form the core of the masticatory CPG. Repetitive stimulation of the trigeminal tract elicits rhythmic activities in a subset of NVsnpr neurons and the frequency range of these activities is compatible with masticatory movements. In the cases where two units become rhythmic after stimulation, their activities were synchronous. Synchronization of CPG elements is important to obtain a coherent motor command from a population of MNs. In addition to these observations, rhythmic activities obtained with peripheral stimulation were mimicked by local applications of NMDA and blocked by APV suggesting that NMDA plays an important role in rhythmogenesis. Several mechanisms could explain how repetitive stimulation of sensory afferents can elicit rhythmic bursting. We suggest that NVsnpr acts as an interface that converts sustained tonic inputs into rhythmic outputs. Our general hypothesis being that sustained activity of peripheral afferents is responsible for cell depolarization and $[Ca^{2+}]_e$ depletion leading to rhythmic bursting activities in NVsnpr. The initiation of rhythmic bursting could take place through an INaP dependent mechanism to a certain extent. Above a certain depolarization level or at elevated $[Ca^{2+}]_e$ neuronal activity would become tonic again due to inactivation of INaP. This could be considered as a "feedback" mechanism that allows fine tuning of mastication by central or sensory inputs. NMDA receptor activation could contribute to INaP activation by amplifying the effect of $[Ca^{2+}]_e$ depletion in NVsnpr. Future work using $Ca^{2+}$-imaging and ion-sensitive recordings in brainstem *in vitro* preparations will be necessary to provide evidence that bursting is triggered by a fall in $[Ca^{2+}]_e$.

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