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Functional anatomy of trigeminovascular pain

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1.1 Anatomy of the trigeminovascular system

The trigeminal system, consisting of afferent nerve fibers mostly arising from the trigeminal ganglion, conveys sensory information from extra- and intracranial structures to the central nervous system via the fifth cranial nerve. The term "trigeminovascular system" has been formed to describe the close morpho-functional relationship of trigeminal afferents with intracranial blood vessels, originally in the context of vascular headaches (Moskowitz, 1984). Nowadays, the term may be extended to extracranial tissues, as well as to the central projections of trigeminal afferents into the trigeminal nuclear brainstem complex, as specified below.

1.1.1 Vascularization and innervation of the dura mater encephali

Large arteries run in the outer (periosteal) layer of the dura mater, accompanied by one or two venous vessels. In the human dura, arterial branches form arterio-venous shunts and supply a rich capillary network of the inner (arachnoid-near) layer (Kerber and Newton, 1973; Roland *et al*., 1987). The remarkable dense vascularization of the dura mater is in contrast to the light red color of meningeal veins, suggesting very low oxygen consumption that leaves other functional interpretations, such as thermoregulation, open (Zenker and Kubik, 1996; Cabanac and Brinnel, 1985).

The meningeal innervation has been studied extensively in rodents, but there is general agreement that the findings conform, in principle, with the human meningeal system. The dura mater is innervated by bundles consisting of unmyelinated and myelinated nerve fibers (Andres *et al*., 1987), with diameters ranging from 0.1–0.4 μm (unmyelinated) and from 1–6 μm (myelinated including myelin sheath) in rat (Schueler *et al*., 2014).

Immunohistochemical observations indicate that most of the nerve bundles consist of mixed afferent and autonomic fibers, which split up into smaller branches and, finally, into single fibers. Trigeminal fibers, which originate in the ipsilateral trigeminal ganglion, and sympathetic fibers, predominantly arising from the ipsilateral superior cervical ganglion, form dense plexus around the middle, anterior and posterior meningeal

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artery, suggesting a vasomotor function (Keller and Marfurt, 1991; Mayberg *et al.*, 1984; Uddman *et al.*, 1989). An especially dense network of nerve fibers is found around dural sinuses (Andres *et al.*, 1987). In addition, a prominent system of cholinergic nerve fibers originating from the otic and sphenopalatine ganglia surrounds mainly large meningeal blood vessels (Amenta *et al.*, 1980; Edvinsson and Uddman, 1981; Artico and Cavallotti, 2001).

Ultrastructural analyses of trigeminal fibers reveal the typical details of non-corpuscular sensory endings, which can be extensively ramified, forming short bud-like extensions or longer branches at the vessel wall, but also within the connective tissue between blood vessels (Messlinger *et al.*, 1993). In addition, at sites where the cerebral (bridging) veins enter the sagittal superior sinus, non-encapsulated Ruffini-like receptor endings have been described (Andres *et al.*, 1987). Particular features of the sensory endings (von Düring *et al.*, 1990) are the free areas not covered by Schwann cells, and the equipment with vesicles and a specific fibrous plasma ("receptor matrix") accumulating adjacent to the cell membrane of the free areas (Andres *et al.*, 1987).

Functionally, the trigeminal and the parasympathetic fibers mediate arterial vasodilatation, and the postganglionic sympathetic nerve fibers mediate vasoconstriction (Jansen *et al.*, 1992; Faraci *et al.*, 1989). The vasodilatation of meningeal arteries induced by cortical spreading depression in rat was abolished after sphenopalatine ganglionectomy (Bolay *et al.*, 2002). There are multiple functional measurements of the meningeal vasoregulation, employing video microscopy and laser Doppler flowmetry, which all indicate regulation of meningeal arteries but obviously no venous vasoregulation (Gupta *et al.*, 2006; Kurosawa *et al.*, 1995; Fischer *et al.*, 2010; Williamson *et al.*, 1997).

The arterial vessels are accompanied by mast cells, arranged in a street-like manner frequently close to nerve fiber bundles, suggesting signaling functions (Dimlich *et al.*, 1991; Dimitriadou *et al.*, 1997; Keller *et al.*, 1991). In addition, extensive networks of dendritic cells with access to the cerebrospinal fluid and resident macrophages exist in all meningeal layers, suggesting competent immune functions within these tissue (McMenamin, 1999; McMenamin *et al.*, 2003).

1.1.2 Extracranial extensions of the meningeal innervation

Postmortem tracings with DiI show two systems of trigeminal fibers transversing the rat dura mater of the middle cranial fossa in a roughly orthogonal direction, one accompanying the middle meningeal artery (MMA), and the other running from the transverse sinus across the artery in a rostromedial direction (Strassman *et al.*, 2004). Recent neuronal tracing (Schueler *et al.*, 2014) has revealed that the MMA accompanying fiber plexus is formed by the spinosus nerve originating in the mandibular division (V3) of the trigeminal ganglion, while the MMA crossing plexus arises from the tentorius nerve originating in the ophthalmic division (V1). This innervation pattern conforms to the historical observations on the human meningeal system described by Luschka and Wolff's group (Luschka, 1856; Ray and Wolff, 1940).

Previous retrograde tracing studies in cat and monkey aimed at the question of whether intracranial structures may be innervated by divergent axon collaterals that also supply facial skin to explain pain referred to the surface of the head (Borges and

Moskowitz, 1983; McMahon *et al.*, 1985), but these studies brought no evidence for this hypothesis. Recently, however, it became clear that the rodent meningeal nerve fibers may traverse the cranium, and may communicate with extracranial structures such as the galea aponeurotica (Kosaras *et al.*, 2009). Postmortem anterogradely traced meningeal nerve fibers in rat and human preparations were found to split up in several branches, some of which pass through sutures and along emissary veins and innervate the periosteum and deep layers of pericranial muscles (Schueler *et al.*, 2014). *In vivo* retrograde tracing has confirmed this, and functional measurements have showed that at least some of the nerve fibers innervating pericranial muscles are collaterals of meningeal afferents innervating the dura mater (Schueler *et al.*, 2013; Zhao and Levy, 2014).

1.1.3 Neuropeptides and their receptors in meningeal tissues

Immunohistochemical studies have identified various neuropeptides in nerve fibers innervating the dura mater (O'Connor and van der Kooy, 1986; von Düring *et al.*, 1990; Keller and Marfurt, 1991; Messlinger *et al.*, 1993) and blood vessels of the pia mater in different species, including humans (Edvinsson *et al.*, 1988; You *et al.*, 1995). The peptidergic nerve fibers form a dense network around blood vessels, but can also be found in non-vascular regions of the dura mater (Messlinger *et al.*, 1993; Strassman *et al.*, 2004). Meningeal nerve fibers immunoreactive for calcitonin gene-related peptide (CGRP), substance P (SP) or neurokinin A (NKA) are considered to be afferents of the trigeminal sensory system. A few nerve fibers immunopositive for pituitary adenylate cyclase-activating polypeptide (PACAP) have been found in rat dura mater, some of them colocalized with CGRP, indicating two likely sources of PACAP-containing fibers: a minor sensory and a larger putatively parasympathetic one (Edvinsson *et al.*, 2001). SP-like immunoreactivity is found coexpressed with CGRP in a small proportion of thin unmyelinated nerve fibers. However, the CGRP-immunoreactive nerve fibers outnumber the SP-positive ones and, consequently, many CGRP-containing fibers display no SP-immunoreactivity. The majority of the CGRP-immunoreactive fibers are distributed to branches of the anterior and middle meningeal arteries, and to the superior sagittal and transverse sinuses (Keller and Marfurt, 1991; Messlinger *et al.*, 1993).

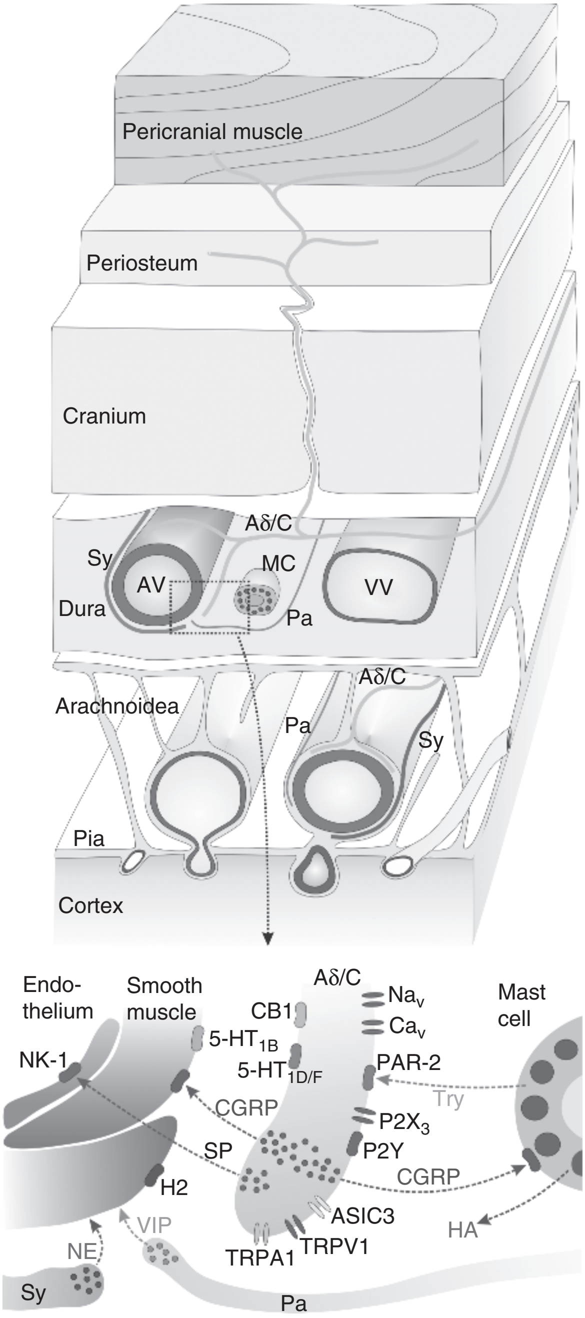
Nerve fibers immunoreactive for neuropeptide Y (NPY), which are most likely of sympathetic origin, are also found located around cerebral and dural blood vessels of human and rodents (Edvinsson and Uddman, 1981; Edvinsson *et al.*, 1998). These nerve fibers are similarly numerous in the cranial dura mater (Keller *et al.*, 1989). They form generally more intimate contact with the blood vessel wall than sensory peptidergic fibers (von Düring *et al.*, 1990; Keller and Marfurt, 1991; Edvinsson *et al.*, 1987). NPY potentiates the vasoconstrictor action of noradrenaline (Jansen *et al.*, 1992). In addition, a sparse innervation of nerve fibers immunoreactive for vasoactive intestinal polypeptide (VIP), most likely of parasympathetic origin, has been identified around dural and pial blood vessels in different species (Keller and Marfurt, 1991; Edvinsson *et al.*, 1998).

Release of VIP from the parasympathetic endings induces vasodilatation in meningeal tissues (Jansen *et al.*, 1992). Nitric oxide synthase (NOS) immunoreactivity has been identified in some trigeminal sensory neurons, and in parasympathetic postganglionic fibers innervating pial arteries and proximal parts of the anterior and middle cerebral arteries. In some of these neurons, NOS is colocalized with VIP, implying a modulatory role of nitric oxide (NO) on VIP-induced vasorelaxation (Nozaki *et al.*, 1993).

Antibodies raised against two components of the CGRP receptor, the calcitonin receptor-like receptor (CLR) and the receptor activity-modifying protein 1 (RAMP1), mark smooth muscle of dural arterial blood vessels, as well as mononuclear and Schwann cells (Lennerz *et al.*, 2008). Also, some thicker CGRP-negative A-fibers of rodent and human dura may express CLR and RAMP1 (Eftekhari *et al.*, 2013). Binding of CGRP to the vascular CGRP receptors in dural and pial tissues causes vasodilatation and increased meningeal blood flow (Edvinsson *et al.*, 1987; Kurosawa *et al.*, 1995). Endothelial cells of blood vessels in the dura mater and in cerebral blood vessels express the neurokinin-1 (NK-1) receptor. SP acting at the NK-1 receptor appears to be mainly responsible for plasma extravasation (Stubbs *et al.*, 1992; O'Shaughnessy and Connor, 1993), but intravascular SP may also cause dilatation of cerebral microvessels (Kobari *et al.*, 1996).

Blockade of NK-1 receptors effectively reduces the plasma protein extravasation in the rodent dura, acting most likely on postcapillary venules (Shepheard *et al.*, 1993; Lee *et al.*, 1994). Both CGRP and NK-1 receptors are also expressed on the surface of mononuclear cells, most of which may be mast cells (Ottosson and Edvinsson, 1997; Lennerz *et al.*, 2008). Release of CGRP and SP from peripheral terminals of meningeal afferents may thus degranulate dural mast cells and release their vasoactive mediator content, such as histamine (Schwenger *et al.*, 2007). In addition, application of the neuropeptide PACAP can degranulate mast cells, but the receptor type mediating this effect is not yet clear (Baun *et al.*, 2012). Mast cell degranulation is considered as a peripheral component of headache pathophysiology (Levy, 2009), but vasodilatation and neurogenic plasma extravasation induced by SP release seems to be negligible in the generation or maintenance of headaches (Dux *et al.*, 2012; see Figure 1.1).

Figure 1.1 Peripheral trigeminovascular structures of nociceptive transduction. Thin myelinated and unmyelinated afferent fibers (Aδ/C, yellow) of all trigeminal partitions and autonomic fibers, mostly postsynaptic sympathetic (Sy) and few parasympathetic fibers (Pa) innervate the cranial dura mater and cerebral arteries, which run on the cortical surface through the subarachnoidal space. Collaterals of meningeal Aδ/C fibers transverse the cranium and innervate also periosteum and deep layers of pericranial muscles. The inset shows multiple G-protein coupled receptors and ion channels involved in sensory transduction and efferent functions of Aδ and C fibers: Voltage-gated sodium and calcium channels (Na_v, Ca_v) cause excitation and release of neuropeptides like CGRP and substance P (SP), which can also be induced by opening of calcium conducting transient potential receptor channels (TRPV1, TRPA1) activated by thermal and chemical stimuli. TRPV1 and acid sensing ion channels (ASIC3) respond to low pH, purinergic receptor channels ($P2X_3$) and receptors (P2Y) to purines like ATP. CGRP activates CGRP receptors on arterial smooth muscle cells causing vasodilatation, which is supported by vasodilatory substances like VIP released from parasympathetic fibers (Pa), whereas vasoconstriction is caused by monoamines like norepinephrine (NE) released from sympathtic efferents (Sy). SP induces mainly plasma extravasation through endothelial NK-1 receptors. CGRP and SP can also degranulate mast cells (MC), thereby releasing tryptase (Try) that activates afferent PAR-2 receptors and histamine (HA) that causes arterial vasodilatation through H2 receptors. Vascular serotonin ($5\text{-}HT_{1B}$) and afferent $5\text{-}HT_{1D/1F}$ as well as cannabinoid (CB1) receptors are inhibitory, acting against vasodilatation and neuropeptide release.


Pericranial muscle
Periosteum
Cranium
Aδ/C
Sy
MC
AV
VV
Dura
Pa
Aδ/C
Arachnoidea
Pa
Sy
Pia
Cortex
Endo-thelium
Smooth muscle
Aδ/C
Na$_v$
Mast cell
CB1
Ca$_v$
5-HT$_{1B}$
NK-1
5-HT$_{1D/F}$
PAR-2
Try
CGRP
P2X$_3$
SP
P2Y
CGRP
H2
ASIC3
VIP
HA
NE
TRPV1
TRPA1
Sy
Pa

1.1.4 Transduction channels and receptors in the trigeminovascular system

Chemosensitive meningeal afferents express different members of the transient receptor potential (TRP) cation channel family. In rats, a dense network of TRP vanilloid 1 (TRPV1) channel expressing fibers has been identified (Huang *et al.*, 2012). TRPV1 immunoreactivity is colocalized with CGRP in most of the afferents (Hou *et al.*, 2002; Dux *et al.*, 2003), which has proved to be sensitive to capsaicin (Dux *et al.*, 2007). TRPV1 cannot only be activated by exogenous substances like capsaicin or resiniferatoxin, but also by noxious heat, acidic pH ($pH < 5.3$) and different endogenous compounds such as some membrane lipid metabolites (anandamide, N-arachidonoyl-dopamine; Price *et al.*, 2004).

The TRP ankyrin 1 (TRPA1) ion channel is another member of the TRP receptor family that is highly colocalized with TRPV1 receptors on trigeminal neurons innervating the dura mater and activated by substances like mustard oil and cannabinoids (Salas *et al.*, 2009; Jordt *et al.*, 2004). Recent observations indicate the activation of trigeminal TRPA1 receptors as a link between the two major vasodilator mechanisms. Vasodilatation induced by the production of NO in the vascular endothelium and by release of CGRP from trigeminal afferents (Eberhardt *et al.*, 2014) – that is, nitroxyl (HNO), the one-electron-reduced sibling of NO, modifies cysteine residues of the receptor, leading to activation of the ion channel and consequent release of CGRP. TRPA1 receptors can also be activated by environmental irritants or a volatile constituent of the “headache tree” – the umbellulone (Nassini *et al.*, 2012). Given that TRPA1 receptors are expressed not only on intracranial axons but also on their extracranial collaterals innervating (e.g., nasal mucosa, periosteum and pericranial muscles) (Schueler *et al.*, 2014), nociceptive stimulation of extracranial tissues may activate intracranial collaterals by an axon reflex mechanism, release vasoactive neuropeptides in meningeal tissue, increase intracranial blood flow, and contribute to the pathomechanisms of headaches (Schueler *et al.*, 2013).

Sensitization of meningeal nociceptors by a variety of blood- and tissue-borne agents may be an important peripheral mechanism in the initiation of headaches (Burstein *et al.*, 1998a). The proteinase activated receptor 2 (PAR-2), activated through cleavage by the serine protease tryptase released from stimulated mast cells, amplifies the vasodilatation caused by sensory neuropeptides (Bhatt *et al.*, 2010) and possibly also the central transmission of nociceptive signals (Zhang and Levy, 2008). The effect of PAR-2 activation is at least partly mediated by TRPV1 and TRPA1 receptor sensitization (Dux *et al.*, 2009).

Acid-sensing ion channels (ASICs), predominantly the ASIC3 subtype responding to low meningeal pH, has been identified on meningeal afferents (Yan *et al.*, 2011). ASICs are members of the ENaC/DEG (epithelial amiloride-sensitive Na^+ channel and degenerin) family of ion channels (Wemmie *et al.*, 2006). Acidic metabolites may be released by activated mast cells, or during ischemia developing as a consequence of cortical spreading depression linked to the aura phase of migraine.

Purinergic P2Y receptors and P2X receptor channels activated by ATP are richly expressed in trigeminal afferents, partly colocalized with TRPV1 receptors (Ichikawa and Sugimoto, 2004; Ruan and Burnstock, 2003).The majority (52%) of retrogradely labeled trigeminal ganglion neurons innervating the dura mater expresses either $P2X_2$ or $P2X_3$ or both receptors (Staikopoulos *et al.*, 2007). ATP enhances the proton-induced

CGRP release through P2Y receptors from the isolated rat dura mater (Zimmermann *et al.*, 2002). Conversely, CGRP caused delayed upregulation of purinergic P2X receptors in cultivated trigeminal ganglion neurons (Fabbretti *et al.*, 2006).

G-protein-coupled 5-HT$_{1D/1F}$ receptors are located on peripheral and central terminals of meningeal afferents (Amrutkar *et al.*, 2012; Buzzi and Moskowitz, 1991). Their activation inhibits the release of neuropeptides and transmitters from the trigeminal afferents, leading to attenuation of the central transmission of nociceptive signals. Recent findings indicate the presence of 5-HT$_7$ receptors on trigeminal terminals. Vasodilatation induced by the activation of trigeminal 5-HT$_7$ receptors seems to be the result of CGRP release from nerve terminals (Wang *et al.*, 2014).

In the trigeminal system, cannabinoid CB1 receptor immunoreactive neurons are found mainly in the maxillary and mandibular divisions of the trigeminal nerve (Price *et al.*, 2003). Activation of trigeminal CB1 receptors inhibits arterial blood vessel dilatation induced by electrical stimulation of the dura mater (Akerman *et al.*, 2004) and CGRP release induced by thermal stimulation in an *in vitro* dura mater preparation (Fischer and Messlinger, 2007). Activation of CB1 receptors may have a particular role in the regulation of CGRP release from TRPV1 expressing neurons, since both receptors can be activated by the same endogenous lipid metabolites as anandamide and N-arachidonoyl-dopamine, acting on both TRPV1 and CB1 receptors with different efficacies (Price *et al.*, 2004; Figure 1.1).

1.2 Trigeminal ganglion

The trigeminal ganglion is located extracranially in the Meckel's space and wrapped with a duplicature of the cranial dura mater. It is subdivided into the ophthalmic (V1), maxillary (V2) and mandibular (V3) division, and contains the cell bodies of the respective sensory trigeminal nerves. Furthermore, transition of nerve fibers of mesencephalic trigeminal neurons has been found in all three partitions within the trigeminal nerve (Byers *et al.*, 1986).

1.2.1 Types of trigeminal ganglion cells

The number of trigeminal ganglion cells varies considerably. In human trigeminal ganglia, 20–35 thousand neurons and about 100 times more non-neuronal cells have been counted (LaGuardia *et al.*, 2000). Each cell body is surrounded by satellite glial cells, other cell types are resident microglia-like macrophages (Glenn *et al.*, 1993) and fibroblasts. A functional crosstalk between neurons and macrophages and/or satellite glial cells is assumed, at least in pathological states (Franceschini *et al.*, 2012, 2013; Villa *et al.*, 2010).

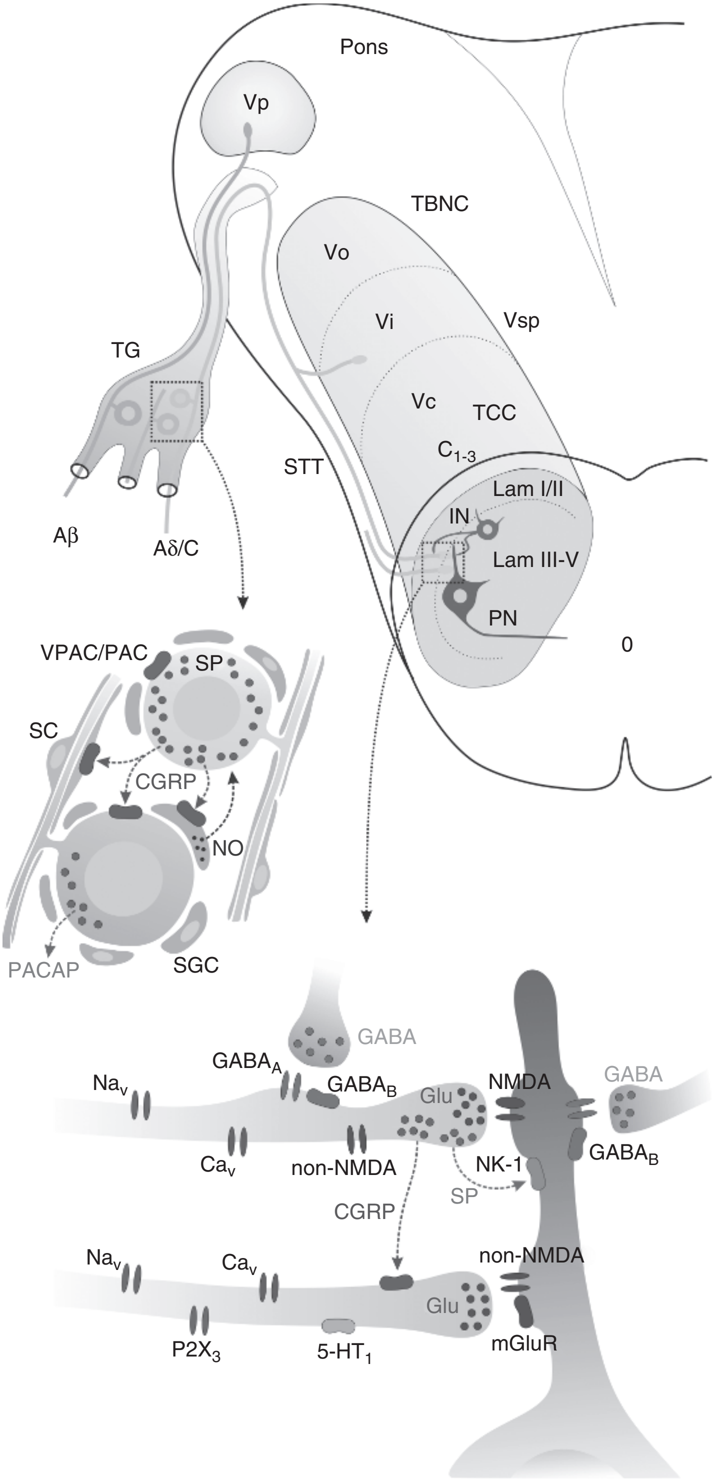
1.2.2 Neuropeptides and their receptors in the trigeminal ganglion

The largest peptidergic neuron population in the trigeminal ganglion expresses CGRP. In different species, including human, immunoreactivity for CGRP is found in 29–49% of trigeminal ganglion neurons (Alvarez *et al.*, 1991; Eftekhari *et al.*, 2010; Lennerz *et al.*, 2008), predominantly in small and medium-sized cells. Accordingly, CGRP immunoreactivity is preferably found in unmyelinated fibers of the trigeminal nerve (Bae *et al.*, 2015). In a minor group of neurons, CGRP is coexpressed with SP immunoreactivity (Lee *et al.*, 1985), which has been found in up to 33% of neurons (Del Fiacco *et al.*, 1990; Prins *et al.*, 1993; Prośba-Mackiewicz *et al.*, 2000). The isolectin IB4 from *Griffonia simplicifolia*, which binds to a subpopulation of small trigeminal ganglion neurons, stains less than 25% of CGRP or SP immunoreactive neurons (Ambalavanar and Morris, 1992). Immunoreactivity for PACAP is present in 29% of neurons, of which CGRP is coexpressed in 23% (Eftekhari *et al.*, 2015).

Immunoreactivity for the CLR and RAMP1 components of the CGRP receptor has been found in Schwann and satellite cells, and in a large proportion of neurons, but colocalization with CGRP is extremely rare (Alvarez *et al.*, 1991; Eftekhari *et al.*, 2010; Lennerz *et al.*, 2008). *In vitro* studies have provided evidence that CGRP release from neurons can stimulate surrounding satellite cells to increase intracellular calcium, which leads to an enhancement of purinergic (P2Y) receptors (Ceruti *et al.*, 2011), expression of different cytokines (Vause and Durham, 2010) and release of NO (Li *et al.*, 2008). In this way, CGRP could function as a paracrine factor to stimulate nearby glial cells and neurons (Figure 1.2).

Human trigeminal ganglia express all three receptor subtypes of the VIP/PACAP receptor family VPAC1, VPAC2 and PAC1 (Knutsson and Edvinsson, 2002). Provided that trigeminal ganglion neurons can release PACAP, the presence of PAC1 receptors on neuron somata suggests the possible existence of a signaling pathway for PACAP-mediated communication between neighboring trigeminal sensory neurons (Chaudhary and Baumann, 2002).

Figure 1.2 Trigeminal ganglion (TG) and structures in the trigeminal nuclear brainstem complex (TBNC) subserving nociceptive transmission. While the central processes of most mechanoreceptive Aβ fibers of the trigeminal ganglion (TG) project to the pontine subnucleus principalis (Vp), Aδ and C fibers run down the spinal trigeminal tract terminating in the spinal trigeminal nucleus (Vsp). Intracranial afferents terminate mainly in the trigemino-cervial complex (TCC), which is composed of subnucleus caudalis (Vc) and the dorsal horn of the first cervial segments (C1-3), and some also in the subnucleus interpolaris (Vi). The upper inset shows two trigeminal afferents with C fibers, wrapped by Schwann cells (SC), and somata, surrounded by satellite glial cells (SGC). The neuropeptides CGRP and PACAP are expressed by major proportions of TG neurons and may be released within the TG. VPAC and PAC receptors are present on neurons. CGRP receptors are present on neurons not producing CGRP, on SGC and SC, possibly enabling crosstalk between neurons and glia, which may include nitric oxide (NO) release from SGC. The lower inset shows important neuronal elements of transmission. Voltage-dependent conduction channels (Na_V, Ca_V) subserve depolarisation and neurotransmitter release. Glutamate (Glu), as the main transmitter, activates NMDA and non-NMDA receptor channels and metabotropic glutamate receptors (mGluR) on second-order neurons, among them projection neurons (PN) projecting to the thalamus and other nuclei involved in nociceptive processing. Glutamate receptors are also found presynaptically, possibly modulating neurotransmitter and neuropeptide release. The same function may apply to activating CGRP and purinergic ($P2X_3$) receptors and inhibiting $5\text{-}HT_1$ receptors, while SP may preferably act through postsynaptic NK-1 receptors. Inhibitory interneurons (IN) release GABA and other inhibitory neurotransmitters acting pre- and postsynaptically through $GABA_A$ receptor channels and $GABA_B$ receptors.


Pons
Vp
TBNC
Vo
Vi
Vsp
TG
Vc
TCC
C_{1-3}
STT
Lam I/II
IN
Lam III-V
Aβ
Aδ/C
PN
0
VPAC/PAC
SP
SC
CGRP
NO
PACAP
SGC
GABA
$GABA_A$
Na_v
$GABA_B$
Glu
NMDA
GABA
$GABA_B$
Ca_v
non-NMDA
NK-1
SP
CGRP
Na_v
Ca_v
non-NMDA
Glu
mGluR
$P2X_3$
$5\text{-}HT_1$

1.2.3 Representation of intracranial structures in the trigeminal ganglion

According to old anatomic observations in primates, all three divisions of the trigeminal nerve contribute to the innervation of the meninges (McNaughton, 1938), though not equally. Tracing experiments using application of horseradish peroxidase (HRP) to dural structures in the cat has confirmed this view (Steiger and Meakin, 1984). Afferents around the middle meningeal artery are found projecting predominantly to the ophthalmic division (V1) of the ipsilateral trigeminal ganglion, but to a minor extent also to the maxillary (V2) and mandibular (V3) divisions (Mayberg *et al*., 1984). The medial anterior cranial fossa and the tentorium cerebelli are represented mainly in V1, the orbital region of the anterior cranial fossa in V2 (Steiger and Meakin, 1984). In the rat, retrograde labeling with DiI of the dural spinosus nerve stains neuronal cell bodies, preferably in the V3 and, to a lesser extent, in the V2 division (Schueler *et al*., 2014). True blue application to the middle meningeal artery labels not only ipsilateral trigeminal ganglion cells, but also some neurons in the contralateral trigeminal ganglion and in the dorsal root ganglion at the C2 level (Uddman *et al*., 1989).

HRP labeled cell bodies innervating the intracranial carotid and the middle cerebral artery in the cat are located in the ophthalmic division of the trigeminal ganglion (Steiger and Meakin, 1984). Using Wallerian degeneration in monkey, the vessels of the circle of Willis are also found to be innervated by the V1 division, with a small maxillary contribution (Simons and Ruskell, 1988). In the rat, retrograde HRP labeling around basal intracranial arteries (Arbab *et al*., 1986) and true blue labeling of the middle cerebral artery (Edvinsson *et al*., 1989) is found not only in the trigeminal ganglion, but also in the first and preferably the second cervical spinal ganglion.

1.3 Trigeminal brainstem nuclear complex

1.3.1 Organization of the trigeminal brainstem nuclear complex

The trigeminal nerve enters the brain stem at the pontine level and projects to the trigeminal brain stem nuclear complex (TBNC), which is composed of the principal sensory nucleus (Vp) and the spinal trigeminal nucleus (Vsp). The bulk of myelinated mechanoreceptive afferents projects to the Vp, while both large diameter and small diameter fibers descend in the spinal trigeminal tract and project into the Vsp, which is subdivided into the rostral subnucleus oralis (Vo), the middle subnucleus interpolaris (Vi), and the caudal subnucleus caudalis (Vc) (Olszewski, 1950). The Vc is often referred to as the medullary dorsal horn (MDH), and some researchers emphasize its anatomic and functional transition to the cervical dorsal horn, terming the Vc, including the dorsal horn of the C1-C3 segments, trigeminocervical nucleus (TCN) (Goadsby *et al*., 2001; Hoskin *et al*., 1999). Using transganglionic tracing, central trigeminal terminals have been found throughout the TBNC and sparsely in the upper cervical dorsal horn, even contralaterally (Marfurt, 1981; Figure 1.2).

Gobel *et al*. (1977) proposed a laminar subdivision of the MDH similar to Rexed's nomenclature of the spinal dorsal horn (Rexed, 1952), in which lamina I corresponds to the marginal layer, lamina II to the substantia gelatinosa, and laminae III and IV to the magnocellular region. The most ventral lamina V merges with the medullary reticular formation without clear boundary (Nord and Kyler, 1968). Within the spinal trigeminal tract, some groups of neurons are termed interstitial islands of Cajal, or paratrigeminal or interstitial nucleus (Phelan and Falls, 1989). These cells may be homologues to laminae I and II neurons of the Vc, according to their nociceptive specific character and small receptive fields (Davis and Dostrovsky, 1988). Anatomical and electrophysiological studies have demonstrated that the Vp and the subnuclei of the Vsp are topographically organized in a largely ventrodorsal direction (Hayashi *et al.*, 1984; Shigenaga *et al.*, 1986; Strassman and Vos, 1993). Mandibular afferents terminate preferentially in the dorsal region, ophthalmic afferents terminate ventrally, and maxillary afferents terminate in between.

Early anatomical and neurophysiological studies suggest that each subnucleus receives information from all parts of the head (Kruger *et al.*, 1961; Torvik, 1956). The rostrocaudal axis of the face is represented from rostral to caudal in the TBNC in an “onion-leaf-like” fashion (Yokota and Nishikawa, 1980; Jacquin *et al.*, 1986). Labeling of various mandibular nerves in the rat with HRP has revealed that the oral afferents tend to terminate most heavily in the rostral TBNC, whereas the posterior perioral-auricular afferents terminate preferentially in the caudal aspect of the complex (Jacquin *et al.*, 1988).

It is not entirely clear if a similar somatotopic distribution in ventrodorsal and rostrocaudal directions exists for intracranial trigeminal structures.

1.3.2 Nociceptive afferent projections to the spinal trigeminal nucleus

The Vc is primarily responsible for processing nociceptive and temperature information, whereas the Vp is involved in processing tactile information. Trigeminal tractotomy (i.e., transection of the spinal trigeminal tract at the level of the obex) has been found to relieve facial pain (Sjoqvist, 1938). Isolated lesions of the Vc cause complete or partial loss of pain and temperature sensation on the ipsilateral side, whereas tactile sensations remain nearly intact (Lisney, 1983). These clinical data have been supplemented with a large body of neurophysiological evidence showing that the Vc is essential for the perception of pain in trigeminal tissues. Since the loss of facial pain sensation after trigeminal tractotomy is not complete, but frequently spares peri- and intraoral areas, rostral parts of the TNBC may contribute to trigeminal nociception in the oral region (Young, 1982). Similarly, behavioral responses to noxious orofacial stimuli may persist following tractotomy or Vc lesions in animals (Vyklický *et al.*, 1977) while, conversely, nociceptive responsiveness and intraoral pain may be diminished by more rostral lesions of the trigeminal complex (Broton and Rosenfeld, 1986; Graham *et al.*, 1988).

The projection of nociceptive facial afferents to the spinal trigeminal nucleus has been studied by a series of elegant experiments combining intraaxonal recordings in the Vsp and HRP injections to examine the central terminations of labeled axons. Hayashi (1985) found high-threshold mechanoreceptive Aδ afferents in the cat forming extensive terminal arbors in superficial layers of the Vi as well as in lamina I and, to a lesser extent, in outer lamina II of the Vc. Jacquin *et al.* (1986, 1988) confirmed these findings in the rat, and localized a second termination area in laminae III to V of the Vc. In line with the above findings, the sensory projection from the cornea, which is thought to be mainly nociceptive, has been shown to be focused in the outer laminae of Vc (Panneton and Burton, 1981).

The projection of intracranial trigeminal afferents to the TNBC has not been studied in detail by axonal tracing, but functional data suggest a similar distribution as for the facial nociceptive afferents.

1.3.3 Functional representation of meningeal structures in the spinal trigeminal nucleus

Electrophysiological studies in the cat have shown that the cranial meninges are mainly represented in Vc, but also in Vi and Vo (Davis and Dostrovsky, 1988). The neurons in Vc are preferentially located in the ventrolateral (ophthalmic) portion of the nucleus. Nearly all Vc neurons with meningeal afferent input evoked from the middle meningeal artery and the superior sagittal sinus (SSS) have facial receptive fields located in the ophthalmic division, whereas a considerable proportion of neurons in Vo and Vi have facial receptive fields in maxillary and mandibular areas. These neurons are typically nociceptive, responding either exclusively (nociceptive-specific) or at a higher rate of action potentials (wide-dynamic range) to noxious mechanical stimuli.

Another cluster of neurons with input from the SSS has been found to be located in the dorsal horn of the upper cervical spinal cord, particularly in C2 (Lambert *et al*., 1991; Storer and Goadsby, 1997). This meningeal representation is largely confirmed by measuring regional blood flow and metabolism, using the 2-deoxyglucose method, and by c-fos expression following electrical and mechanical stimulation of dural structures (Goadsby and Zagami, 1991; Hoskin *et al*., 1999; Kaube *et al*., 1993). Remarkably, two-thirds of the neurons in the upper cervical cord of the cat have convergent input from the superior sagittal sinus and the occipital nerve (Angus-Leppan *et al*., 1997), and a similar convergent input has been found in the rat (Bartsch and Goadsby, 2003).

In the rat, the number of neurons activated by electrical stimulation of dural sites (sinus transversus or parietal dura mater) peaks in the caudal Vc, but there is another cluster around the obex level corresponding to the Vi/Vc region (Burstein *et al*., 1998b; Schepelmann *et al*., 1999). Intracellular labeling has shown that such neurons give rise to an extensive axonal projection system that arborizes at multiple levels of the Vc and the caudal part of the Vi (Strassman *et al*., 1994a). The widespread meningeal representation extending from upper cervical to medullary levels has also been confirmed by immunocytochemical labeling for c-fos (Strassman *et al*., 1994b). As in the cat, most of these neurons have convergent cutaneous input, and their facial receptive fields are located in periorbital, frontal or parietal areas – that is, the same areas in which the patients of the early investigators like Ray and Wolff (1940) felt head pain elicited by stimulation of supratentorial dural structures. It appears possible that neurons in the Vc/C1-2 region are most important in signaling nociceptive information to higher centers of the CNS, whereas the Vi/Vc region may be more involved in autonomic and motor reflexes, as has been suggested for neurons with corneal afferent input (Meng *et al*., 1997).

1.3.4 Efferent projections from the spinal trigeminal nucleus

There have been numerous reports about efferent projections from the spinal trigeminal nucleus to higher centers of the CNS in various species (Stewart and King, 1963; Tiwari and King, 1973; Ring and Ganchrow, 1983; Van Ham and Yeo, 1992). Old data in the cat used reversible block of nuclei and antidromic stimulation to show that neurons in the Vc are mainly relayed in the contralateral ventroposteromedial nucleus (VPM) to neurons projecting into the somatosensory cortex (Rowe and Sessle, 1968), which has recently been confirmed (Lambert *et al.*, 2014). In addition, projections from the Vc to the contra- and ipsilateral nucleus submedius and the intralaminar nuclei centralis medialis and lateralis have been identified by HRP tracing in the rat (Peschanski, 1984).

Using tracing techniques, projections to the nucleus of the solitary tract (Menétrey and Basbaum, 1987), facial nucleus (Hinrichsen and Watson, 1983), the contralateral inferior olivar complex (Huerta *et al.*, 1983), the parabrachial and the Kölliker-Fuse nucleus (Cechetto *et al.*, 1985; Panneton *et al.*, 1994), the tectum and the cerebellar cortex (Steindler, 1985; Yatim *et al.*, 1996) and even the ventral cochlear nucleus (Haenggeli *et al.*, 2005) have been identified. Neurons with intracranial afferent input in the Vc and the cervical dorsal horn at the level of C1 have been found projecting to the hypothalamus, which may be of significance regarding endocrine and rhythmic disorders in migraine (Malick and Burstein, 1998; Malick *et al.*, 2000).

In addition to the ascending projections, spinal trigeminal neurons have been seen projecting ipsilaterally to all levels of the spinal cord and forming an extensive network of efferent connections, which may be important for motor reflexes associated with cranial pain (Ruggiero *et al.*, 1981; Hayashi *et al.*, 1984).

1.3.5 Neuropeptides and their receptors in the trigeminal nucleus

Corresponding to the distribution of nociceptive afferent terminals visualized by neuronal tracing, SP and CGRP immunoreactive nerve fibers are localized in different species, including humans, preferentially in Vc and in the caudal part of Vi, but less in Vo and Vp (Boissonade *et al.*, 1993; Helme and Fletcher, 1983; Pearson and Jennes, 1988; Tashiro *et al.*, 1991). The nerve fibers are mainly located in outer laminae I and II (substantia gelatinosa) of the Vsp, where CGRP immunoreactivity appears most dense (Lennerz *et al.*, 2008; Tashiro *et al.*, 1991). SP immunoreactivity is also found in deeper layers (IV/V; Salt *et al.*, 1983). Also in the human trigeminal tract, the proportion of nerve fibers immunoreactive for CGRP is higher than that immunoreactive for SP (Smith *et al.*, 2002).

In contrast, another study revealed a rich supply of SP and a moderate supply of CGRP- and PACAP-immunoreactive nerve fibers in the human Vc and dorsal horn at the C1-2 level (Uddman *et al.*, 2002). After trigeminal rhizotomy in the cat, most of the CGRP immunoreactive fibers disappeared throughout the TBNC, whereas a certain number of SP immunoreactive fibers remained intact (Henry *et al.*, 1996; Tashiro *et al.*, 1991), suggesting that these are of central origin. SP immunoreactive fibers originating from neurons in lamina I of the MDH have been found projecting into the hypothalamus (Li *et al.*, 1997) and to the solitary tract (Guan *et al.*, 1998).

Morphological and functional data suggest that neuropeptides are implicated in the trigeminal nociceptive processing within the Vsp. Following electrical stimulation of the trigeminal ganglion in the rat, depletion of CGRP, SP and NKA immunoreactivity have been observed in the ipsilateral medullary brainstem (Samsam *et al.*, 2000). Noxious stimulation causes CGRP release from medullary brainstem slices (Jenkins *et al.*, 2004; Kageneck *et al.*, 2014). Microiontophoretic injections of CGRP into the cat trigeminocervical complex at C1/2 level increases the firing of second order neurons to electrical stimulation of the dura mater or glutamate injection, reversed by CGRP receptor blockade (Storer *et al.*, 2004a).

Electron microscopy in the cat Vsp has revealed CGRP immunoreactivity within the substantia gelatinosa in axon terminals presynaptic to dendritic profiles (Henry *et al.*, 1996). Immunoreactivity for CLR and RAMP1, components of the CGRP receptor, has been observed associated with terminals of trigeminal afferents in the rat trigeminal tract entering Vsp (Lennerz *et al.*, 2008). Neither CGRP nor its receptor components have been identified in cell bodies of the Vsp. The functional interpretation of these findings is that CGRP-releasing terminals of primary afferents synapse at CGRP receptor-expressing central axons of trigeminal neurons. The action of CGRP within the trigeminal nucleus is most likely a presynaptic effect, whereby distinct terminals of primary afferents control the neurotransmitter release in other populations of primary afferents (Messlinger *et al.*, 2011; Figure 1.2).

Stimulation of the rat dura mater with acidic solution provokes release of immunoreactive SP in the rat medullary trigeminal brain stem measured with the microprobe technique (Schaible *et al.*, 1997). Henry *et al.* (1980) found that iontophoretical administration of SP in the cat Vc selectively activates nociceptive neurons. In the rat, iontophoretically applied SP has predominantly excitatory actions on both nociceptive and non-nociceptive nucleus caudalis neurons (Salt *et al.*, 1983). Selective blockade of the receptors for SP (NK-1) or NKA (NK-2), as well as NMDA and non-NMDA receptors (see below) reduced the expression of c-fos protein following corneal stimulation in the rat Vc (Bereiter and Bereiter, 1996; Bereiter *et al.*, 1998).

1.3.6 Channels and receptors involved in synaptic transmission in the trigeminal nucleus

Trigeminal afferents projecting to the spinal trigeminal nucleus release glutamate as primary excitatory neurotransmitter, binding to glutamate receptors of various types expressed pre- and postsynaptically. Activation of NMDA and non-NMDA receptors of second order neurons seems to play a dominant role in the transmission of nociceptive information (Leong *et al.*, 2000). Blockade of NMDA receptors reduces c-fos expression in the Vsp following stimulation of the superior sagittal sinus in the cat (Classey *et al.*, 2001). Ultrastructural data suggest that kainate receptors mediate nociceptive transmission postsynaptic to SP-containing afferents, but may also modulate the presynaptic release of neuropeptides and glutamate in the trigeminal nucleus (Hegarty *et al.*, 2007). Metabotropic glutamate receptors seem to be involved in the mechanisms of long-term potentiation in the Vsp (Youn, 2014). Recent expression studies show that glutamatergic neurons in the Vsp projecting to the thalamus differ from projecting neurons in the Vp by their exclusive equipment with vesicular glutamate transporter VGLUT2 (Ge *et al.*, 2014).

Agonists of the 5-HT$_{1B/1D/1F}$ receptors, which act on central terminals of meningeal afferents, modulate glutamate release (Choi *et al.*, 2012) that may play a central role in trigeminovascular activation, central sensitization and cortical spreading depression (Amrutkar *et al.*, 2012). Glutamatergic kainate receptors may also be targets of the migraine prophylactics topiramate (Andreou and Goadsby, 2011).

Immunohistochemical observations indicate that GABA receptors are involved in both pre- and postsynaptic inhibitory mechanisms of synaptic transmission in the

Vc (Basbaum *et al.*, 1986). GABA receptor activation has been shown to decrease c-fos expression in the Vc following intracisternal application of capsaicin (Cutrer *et al.*, 1995), and to attenuate the activity of neurons in the TNC, following electrical stimulation of cat sinus sagittalis (Storer *et al.*, 2004b).

Purinergic receptors have long been assumed to be involved in nociceptive transduction but also transmission in the spinal trigeminal system (Burnstock, 2009). Throughout the whole TBNC, thin nerve fibers immunoreactive for $P2X_3$ receptors are seen, mostly colocalized with the nonpeptidergic marker IB4, and sometimes with SP immunoreactivity (Kim *et al.*, 2008). The distribution is most dense in the superficial laminae of Vc, especially in the inner lamina II, and appears in electron microscopic sections presynaptic to dendrites or postsynaptic to axonal endings, suggesting different modes of nociceptive transmission (Figure 1.2).

A direct descending orexinergic projection, terminating in the spinal and trigeminal dorsal horn (Hervieu *et al.*, 2001; Marcus *et al.*, 2001), is considered to play a role in central pain modulation. Orexin is believed to have a major role in modulating the release of glutamate and other amino acid transmitters dependent on the wake-sleep rhythm (Siegel, 2004). In an animal model of trigeminovascular nociception, systemically administered orexin A was found to significantly inhibit nociceptive responses of neurons in the TNC to electrical stimulation of the dura mater surrounding the middle meningeal artery (Holland *et al.*, 2006).

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