Mechanisms of trigeminal perception –
Characterization of the cellular properties of sensory neurons of the trigeminal system in rats and mice

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Erklärung


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Abstract

The trigeminal nerve is the major mediator of sensations from the mammalian head. With the ability to mediate chemosensation and pain it is one of the most important alarm organs of an animal and thus essential for its survival. Despite the significance of this system it has been much less examined compared to other sensory systems leaving the underlying mechanisms of signal coding and integration largely unexplored.

This work was designed to gain insight in the basic principles of chemosensory information perception and processing in the trigeminal system. This approach may also be termed “trigeminal function – from (odor) molecules to cognition” based on the range of posed questions: How are odorant molecules perceived by the trigeminal nerve at the molecular and cellular level? Which are the neuronal pathways mediating nasal trigeminal chemosensation? How is odor information coded at the level of the trigeminal ganglion, and what are the (cognitive) interpretations of this information?

In order to allow a differentiated exploration of trigeminal sensory functions, animal studies (comprising in vivo and in vitro investigations) as well as human psychophysical examinations were conducted.

I was able to construct different PrV based virus strains that represent important new tools for the functional exploration of the trigeminal system in order to enhance our understanding of mechanisms underlying differentiated trigeminal somatosensation. I could demonstrate the usability of the bidirectional tracer PrV-Kaplan for tracing defined sensory neuronal populations within the trigeminal ganglion and synaptically connected higher-order neurons in the brainstem. Moreover, infection had no influence on the biophysical properties of traced cells making PrV-Kaplan-labelled neurons ready for functional in vitro investigations. Additionally, I can report the generation of three PrV-Bartha derived recombinant virus strains expressing different fluorescent calcium indicator proteins (FCIPs) providing new tools for the functional analysis of whole circuits of synaptically connected neurons in vitro and in vivo.

I furthermore tried to resolve how odor cues are perceived by the trigeminal nerve in order to shed light on the still controversially discussed issue whether trigeminal chemosensation arises from a direct stimulation of intraepithelial free nerve endings, or if alternative signal transduction mechanisms involving other cells might play a role. I could demonstrate that despite the common knowledge that almost all odorants
exert a trigeminal component, most of the tested substances failed to activate trigeminal neurons in cell culture. However, some of the substances triggered trigeminal responses when neurons where cocultured with cells of the main peripheral trigeminal innervation target: skin derived keratinocytes. The presented data suggest that trigeminal neurons depend on a communication with cells of their peripheral innervation area for an entire evolvement of the chemosensory ability of the trigeminal nerve.

Coding of odor information at the level of the trigeminal ganglion was investigated by visualizing odor evoked trigeminal activity patterns. For this purpose an in vivo preparational approach was established, that allows high-resolution recording of optical signals arising from a large region of the rat trigeminal ganglion using voltage-sensitive dye imaging. For the first time the existence of odor specific activation patterns at the level of the trigeminal ganglion could be demonstrated. Moreover, the elicited activity patterns can be grouped dependent on stimulus quality: strong and maybe even painful trigeminal agonists like CO₂ and ethanol displayed activation patterns highly similar to each other. However, the ethanol pattern also included unique activation spots that might code for odor identity. In contrast, classical olfactory stimuli elicited activation patterns clearly distinct from those of strong trigeminal activators. This study provides first evidence that coding of odor information might not be a feature unique to the olfactory system, but to some extent also possible via the trigeminal nerve. The additionally performed psychophysical experiments point to the existence of similar trigeminal odor coding strategies in humans.

By working on both, the cellular and the systemic level of trigeminal perception, I contributed to the identification of nature’s principles of perceiving and encoding somatosensory stimuli.

The work presented here will essentially contribute to a better understanding of trigeminal chemoperception not only by the described results which in itself shed a new light on trigeminal stimulus perception and coding but also by the newly created viral tools and the established in vivo imaging approach that both allow a much more differentiated view on trigeminal function.
1. Introduction

"Cogito, ergo sum", "I think, therefore I am" René Descartes

Mental processes have always been in mankind’s main focus and are one of nature’s biggest mysteries. But before we “think” we have to learn how to “feel” philosophers like Auguste Comte argued, realizing that all knowledge is obtained through our sensory experience. Following up on his idea the starting point into all mental processes is actually provided by sensations and perceptions.

Although this concept has been partially challenged later mainly by Immanuel Kant, it still demonstrates the tremendous importance of our everyday life sensations like hearing, seeing, feeling, tasting and smelling.

Evolution created many different sensory structures and organs aimed at receiving environmental information since only well adapted life forms, able to detect relevant external factors and knowing how to react properly, represent the “best fit” and therefore have an evolutionary advantage.

How different these sensory systems may appear on the first look, they all share common attributes of perception: the need of an adequate stimulus which leads to an event of signal transduction mechanism and to the generation of a nerve impulse. Finally, the brain interprets this incoming information as a certain sensation.

Taken together, the study of sensory information processing is of fundamental relevance for understanding nature’s basic principles enabling us to perceive different stimuli and may be the most important way to gain deeper insight into our internal, mental representation of the outer world.

Chemosensation is thought to be one of the most ancient senses. During evolution different specialized subsystems have emerged: Vertebrate chemosensation comprises mainly the olfactory-system, the gustatory-system and the general chemical sense carried by trigeminal sensory neurons.

Olfactory as well as taste perception have gained more and more interest in recent years leading to extensive studies, also triggered by medically relevant issues like obesity, aging or neurodegenerative diseases. The outcome was an enormous progress in our understanding of the principle molecular and cellular mechanisms of these systems as well as their information processing in the brain. Though the Nervus trigeminus is the most important sensory facial nerve and involved in pain perception, much less is known about the the trigeminal system.
Trigeminal ganglia, located at the base of the skull containing sensory neuron somata and nerve fibers, detect somatic sensations from the mammalian head and transmit the information to the central nervous system. This somatic sense is not exclusively specialized in detecting chemical substances but also integrates stimuli like temperature and mechanical forces thus eliciting sensations of touch, heat or cold, proprioception or even pain.

Basic principles of trigeminal chemosensory information perception and processing are still very much unexplored what might be partially the result of the low accessibility into this system. Therefore we are far away from understanding the functional organisation of the sensory trigeminal system and the complex neuronal principles underlying its caused sensations.
1. Introduction

1.1. The Mammalian Trigeminal System

1.1.1. Functional Anatomy of the Mammalian Trigeminal System

Cranial nerves of the head are functionally homologues to the body’s spinal nerves. Just as spinal nerves provide the somatic and visceral sensory as well as the motor innervation of the body, cranial nerves do the same for the head.

The trigeminal nerve (Fig. 1-1) is the largest of all cranial nerves and provides sensory information of the head and face and the motor innervation of the muscles of mastication. This mixed nerve emerges from the side of the pons, by a small motor and a large sensory root.

The main trigeminal motor nucleus, innervating the masseter, temporalis, and pterygoids (muscles of mastication) lies at a midpontine level. Associated accessory trigeminal nuclei innervate the tensor tympani (muscle of the middle ear), tensor veli palatine (muscle of the palate) and the anterior belly of the digastric muscle (muscle of the upper neck).

Fig. 1-1: Anatomy of the human Nervus trigeminus
Overview of the three main branches (V1 – V3) of the trigeminal nerve and important sub branches (motor branches of the muscles of mastication are not considered)
Modified from Gray, 1918
Neurons with sensory function are typically organized in sensory ganglia. The sensory innervation of the body is provided by dorsal root ganglia neurons and accordingly all cranial nerves with sensory function (V, VII, IX, and X) have associated sensory ganglia. The sensory root of the trigeminal nerve passes into the trigeminal ganglion located in the base of the skull near the apex of the petrous part of the temporal bone. Sensory trigeminal ganglion neurons are pseudounipolar, sending out a single axon that bifurcates into two neurites; one innervating the periphery, the other one connecting to the brainstem. Three major branches emerge from the trigeminal ganglion. The first division (V1; ophthalmic nerve) exits the cranium through the superior orbital fissure, to innervate the structures of the orbit, the skin in the area above the eye, and forehead back to the vertex of the skull and parts of the nose. Some fibers from the ophthalmic division also innervate the meninges and blood vessels of the anterior and middle intracranial fossae. The second division (V2, maxillary nerve) exits through the foramen rotundum. It then re-enters a canal proceeding inferior to the orbit, the infraorbital canal, and exits through the infraorbital foramen, to innervate the skin below the eye and above the mouth. The third division (V3, mandibular nerve) exits the cranium along with the motor trigeminal branch through the foramen ovale. Sensory axons innervate the skin on the lateral side of the head, the tongue and the mucosal wall of the oral cavity. Some sensory axons re-enter a canal in the mandible to innervate the teeth and emerge from the mental foramen to innervate the skin of the lower jaw.

The trigeminal system maintains the topographic arrangement of sensory receptors in the periphery (Trepel, 1999; Saper, 2000). Anatomical and electrophysiological investigations discovered a clear somatotopic organization in the higher levels of the system, including brainstem, thalamus, and somatosensory cortex. For example, the spinal trigeminal nucleus located in the brainstem contains a map of the entire oral cavity as well as the surface of the face. The nucleus is organized topographically, with the forehead represented ventrally and the oral region dorsally. In this way the “where” information of sensory coding seems to be preserved in higher brain regions (Trepel, 1999; Saper, 2000).

Moreover, trigeminal ganglion neurons transmit information about the nature of a stimulus and therefore can be roughly categorized according to their sensory specialisation in proprioceptive, discriminative touch, pain, and temperature and chemical stimuli sensing neurons. Identification of functional classes of sensory
receptors ("molecular sensors") provided support to the concept that somatosensory modalities are subserved by separate populations of sensory receptor neurons. However, many ion channel molecules (in particular ion channels of the Transient Receptor Potential (TRP) superfamily) initially associated to the transduction of only one particular form of energy are also activated by stimuli of different quality, implying a limited degree of specificity in their transducing capacities. Moreover, molecular sensors associated with a stimulus quality may be concomitantly expressed in sensory receptor neurons functionally defined as specific for another stimulus quality. Therefore the capacity exhibited by the different types of primary sensory neurons to preferentially detect and encode the specific stimuli into a discharge of nerve impulses appears to result of a characteristic combinatorial expression of different molecular sensors in each neuronal type (reviewed in Belmonte and Viana, 2008).

The sensory specialisation of trigeminal subpopulations for a sub-modality is also preserved in higher brain areas (Saper, 2000). Neurons primary mediating information about touch terminate in the principal nucleus. The processes of second order neurons cross to the contralateral side of the central nervous system (CNS) and run through the medial lemniscus (Lemniscus medialis) to the Nucleus ventralis posterior of the thalamus. Trigeminal sensory neurons mediating information about pain and temperature project to the spinal trigeminal nucleus (Nucleus spinalis nervus trigemini, Sp5). Fibers of second order neurons run dorsolaterally to the medial lemniscus within the spinothalamic tract and terminate in the Nucleus ventralis posterior of the thalamus. Thus, in addition to the "where"-pathway of sensory information a "what"-pathway exists preserving stimulus identity.

However, much less is known about the somatotopic organization of the cells of the trigeminal ganglion itself. Although it is likely that the somatotopic organization of the somatosensory trigeminal system seen in higher brain areas originates with organization of the trigeminal nerve, it is still sparsely investigated. There is a general agreement that a gross somatotopic organization exists depending on the relative position of the branches of the trigeminal nerve: the somata of the mandibular nerve occupy the mandibular nerve branch region (the very posteriolateral portion of the ganglion), the cell bodies of the ophthalmic branches are located anteromedially, and the perikarya of the maxillary branches are interposed in between (in a central position of the ganglion) (Marfurt, 1981; Anton and Peppel, 1991; Schaefer et al., 2002;
Lazarov, 2002). Leiser and Moxon, 2006, who mainly focused on mapping vibrissae-responsive neurons in the ganglion, discovered a finer detail of somatotopy than previously reported. For example, they found cells innervating the dorsal whisker region located also more dorsally within in the trigeminal ganglion.

Peripheral innervation-site dependent ganglionic localization of trigeminal neurons in combination with innervations-site dependent differences in receptor expression pattern have been recently demonstrated in our lab (Damann et al., 2006). However, a potential preservation of this information in postsynaptic neurons in the brainstem could not be investigated due to impeded anterograde transsynaptic spread of used Pseudorabies virus-Bartha strain.
1.1.2. Nasal Trigeminal Chemosensation

Vertebrate chemosensation mainly comprises the olfactory and the gustatory system as well as the general chemical sense (trigeminal sense). These systems differ in functional and anatomical aspects but a key feature common to all of them is the detection of chemical cues in the environment. While the olfactory system is the main detector of volatile substances, the general chemical sense additionally contributes to the overall gustatory and olfactory sensation since most odorants also elicit trigeminal responses (Silver and Moulton, 1982). The trigeminal-mediated ability of odor-detection and discrimination has been proven by numerous animal studies in which the olfactory system has been lesioned, as well as in human psychophysical studies on anosmic patients, lacking olfactory nerve function (Doty et al., 1978; Mason and Silver, 1983).

From an evolutionary point of view the olfactory and the trigeminal systems appear to have evolved for different purposes. Olfaction not only informs about food availability and composition but also plays a crucial role in mate choice and the detection of predators. In contrast, the intranasal trigeminal system is thought to act as a sentinel of the airways, able to reflexively stop inspiration to prevent the inhalation of potentially life-threatening substances (Silver and Finger, 1991). Therefore it is not surprising that a predominant intranasal trigeminal perception is pain encompassing stinging, burning, or pungent sensations.

Only a few chemosensory stimuli produce exclusively olfactory or trigeminal sensation. For the activation of the trigeminal system CO\textsubscript{2} has been shown to be a useful irritant, since it selectively evokes pungency without any parallel olfactory sensation (Cain and Murphy, 1980). Doty et al., 1978, tested 47 odorants on anosmic patients and found only two of them, vanillin and decanoid acid, to possess no trigeminal activity even at undiluted concentration. However, vanillin has been recently shown to activate TRPV3 channels at high concentrations (10 mM; Xu et al., 2006). Hydrogen sulphide (H\textsubscript{2}S) is thought to selectively stimulate olfactory receptors, since it also cannot be perceived by anosmic patients (Hummel et al., 1991). In conclusion, the vast majority of volatile chemical stimuli possess both odor as well as some trigeminal characteristics. Therefore, what is commonly known as the sense of smell rather is a combination of olfactory and trigeminal sensations.

These two systems are not equivalent regarding their odor recognition, discrimination, or identification abilities. The olfactory system is very sensitive for different odorants
and in part even capable of distinguishing molecular stereoisomers. Anosmic patients, only relying on trigeminal function, have lost these high odor discrimination skills and are just able to roughly discriminate between different odor categories (Laska et al., 1997). However, for some molecules also the trigeminal system seems to be able to distinguishing molecular stereoisomers, since a different stereoselective activation of the trigeminal sensory system by R(+-) and S(-)-nicotine could be demonstrated (Thuerauf et al., 1999). Intranasal trigeminal fibers (Fig. 1-2) are distributed throughout the nasal cavity and are described as intraepithelial free nerve endings arising from Aδ and C fibres. The trigeminal innervation of the nasal cavity is given by the anterior ethmoidal nerve (N. ophthalmicus), the nasopalatine nerve (N. maxillaris), and internal nasal branches of the infraorbital nerve (N. maxillaris) (Bojsen-Moller, 1975). A large part of these fibers is supposed to be of nociceptive nature (Anton and Peppel, 1991; Sekizawa and Tsubone, 1994). Common nociceptor markers include e.g. mean somata size (small- and medium-diameter), neuropeptide expression, IB4 binding, kinetics of purine-induced currents (selective P2X3 expression) and sensitivity for the TRPV1 agonist capsaicin (discussed in 1.1.3). A special feature of these neurons is the so called “axon reflex”. Upon nerve stimulation the action potential does not only travel centripetally transmitting sensory information to the brain, but can also back-propagate causing the release of various neuropeptides, e.g. substance P and CGRP (Calcitonin Gene-Related Peptide) from peripheral collateral branches of the stimulated nerve terminal (Lundberg et al., 1987; Finger and Bottger, 1993). These peptides cause local changes of the innervated territory including vasodilatation, plasma extravasation and increased glandular secretion (Pernow, 1983; Stjarne et al., 1991; Rinder and Lundberg, 1996). All of these factors effect the aerodynamics of airflow pattern in both the upper and lower respiratory tracts (e.g. Ulrich et al., 1972).

Such responses are protective since prolonged exposure to some trigeminal stimulants produces expanded edematous and hemorrhagic lungs, dermal irritation, and dyspnea, as well as consolidation, distended alveoli, and ruptured alveolar septa (Doty, 1975). In many cases the ingenious defensive strategies of plants to ward off herbivores are based on the production of chemical agents such as capsaicin, isothiocyanates, and thiosulfimates that produce their behavioral effects by targeting excitatory TRP channels on primary afferent nerve fibers of the trigeminal pain pathway within the nasal and oral cavities. In high concentrations these substances produce irritation and
inflammation (Tewksbury and Nabhan, 2001; Jordt and Julius, 2002; reviewed in Wang and Woolf, 2005).

It is still controversially discussed whether trigeminal chemosensation arises from a direct stimulation of intraepithelial free nerve endings (Silver, 1992), or if alternative signal transduction mechanisms are involved (Finger et al., 2003; Lumpkin and Caterina, 2007; Lin et al., 2008). The apparent junctional barrier at the epithelial surface separating the sensory nerve fibers from potential stimuli in the nasal cavity has been dismissed as an obstacle to chemosensory transduction, since many trigeminal stimuli are lipid soluble and should easily diffuse across this junctional complex. The mechanism by which lipophobic trigeminal stimuli might reach the sensory nerve fibers is less clear but has been hypothesized to involve a paracellular pathway (Bryant and Silver, 2000).

Undoubtful a basic set of different receptors expressed on trigeminal neurons contribute to the chemosensory properties of this nerve therefore arguing for direct stimulation of free nerve endings: e.g. also the detection of menthol or mustard oil is mediated by TRP channels expressed on trigeminal free nerve endings. Several TRP channels are known to be expressed in trigeminal sensory neurons including TRPV1 (reviewed in Tominaga and Tominaga, 2005), TRPM8 (McKemy et al., 2002; Abe et al., 2005), TRPA1 (Story et al., 2003; Nagata et al., 2005), TRPV2 and TRPV4 (Caterina et al., 1999; Liedtke et al., 2000; Ma, 2001; Woodbury et al., 2004). Although TRPV3 could not be detected in sensory neurons by northern blot analysis (Peier et al., 2002b) recent data point to a functional TRPV3 channels expression in dorsal root ganglion neurons (Stotz et al., 2008). These disagreements in the literature concerning the distribution of TRPV3 could be partially due to its low to moderate abundance which renders a determination of ion channel distribution more reliable by electrophysiological technique rather than by immunohistochemistry. Additionally trigeminal neurons express a whole set of neurotransmitter receptors (glutamate, GABA, dopamine, serotonin, histamine, acetylcholine, peptide and neurotrophin receptors; reviewed in Lazarov, 2002), which can also be involved in trigeminal chemoperception via an interaction with their respective ligands, e.g. nicotine (Thuerauf et al., 2006) in cigarette smoke or GABA which is for example produced by morel mushrooms and reported to cause astringency perception (Rotzoll et al., 2006). Furthermore, these neurons express ATP-sensitive receptor types belonging to the P2X (ligand-gated cationic channels) and P2Y (G protein-coupled receptors) family.
and it has been shown that subunit-specific P2X-receptor expression defines some of the chemosensory properties of trigeminal neurons (Spehr et al., 2004). Additionally transduction systems involving different cell types could contribute to trigeminal chemoperception since specialized epithelial chemosensory cells so called “solitary chemosensory cells (SCCs)” forming synaptic contacts with trigeminal afferent fibers have been identified within the nasal respiratory epithelium (Finger et al., 2003) (Fig. 1-2, right). More recently, these cells have been shown to respond to a variety of chemicals at high concentrations levels typical of irritants and are positioned in the nasal cavity appropriately to monitor inhaled air quality (Lin et al., 2008).

Schaefer et al., 2002, could show that single trigeminal neurons innervating the nasal cavity possess dendritic collaterals to the olfactory bulb suggesting that nasal irritants could affect processing of coincident olfactory stimuli. Also psychophysical experiments point to an interaction between olfaction and the trigeminal system. Anosmic subjects show reduced trigeminal sensitivity when compared with healthy controls (Hummel et al., 1996; Gudziol et al., 2001; Kendal-Reed et al., 2001; Walker et al., 2001; Hummel et al., 2003) suggesting that in addition to the known mutual interactions between the olfactory and the trigeminal chemosensory systems in healthy
subjects (Stone et al., 1968; Cain and Murphy, 1980; Bouvet et al., 1987; Livermore et al., 1992; Livermore and Hummel, 2004) also the absence or presence of a functional olfactory system influences trigeminal perception. However, anatomical and functional characteristics of the underlying mechanisms are largely unknown. In consequence, trigeminal fibers within the nasal and oral mucosa appear to contribute to the overall chemosensory experience by detecting irritants, potentially noxious stimuli and odorants (Silver and Finger, 1991).
1.1.3. Trigeminal Somatosensation

Five major sub-modalities of somatic sensation can be differentiated: temperature sense (warmth and cold), proprioception (the sense of static position and movement of the limbs and the body), discriminative touch (required to recognize the shape, and texture of objects and their movements across the skin) nociception (the signaling of tissue damage or chemical irritation, typically perceived as pain or itch) and chemosensation. Only the somatosensory system mediates such diverse physical stimuli and the involvement in several submodalities mainly reflects the polymodal nature of individual TRP channels. Due to this polymodal nature it is not surprising that there is a partial overlap between sub-modalities.

Natural stimulation of trigeminal neurons occurs in the peripheral innervation area (e.g. nasal and oral cavity or the stratum granulosum and stratum spinosum of the skin (Zylka et al., 2005)), where terminals end as free (bare) nerve endings or may be encapsulated by non-neuronal structures. While encapsulated terminals typically mediate somatic modalities like touch and proprioception, neurons with free nerve endings mediate painful and thermal sensations. Also the degree of myelinisation is in part modality dependent: myelinated, fast conducting large diameter (Aα) axons innervate terminal capsules of mechanoreceptors and proprioceptors (Jessel and Basbaum, 2000a). However, neurons mediating temperature and nociception conduct impulses more slowly since they have small-diameter axons and are either unmyelinated or thinly myelinated (Aδ, C) (Hensel et al., 1974; Hensel, 1981; Jessel and Basbaum, 2000a; Heppelmann et al., 2001). Several types of sodium currents resistant to micromolar concentrations of TTX (Tetrodotoxin) have been identified in sensory neurons, and some evidence suggest that these channels play an important role in the transmission of nociceptive information (Ogata and Tatebayashi, 1992; Akopian et al., 1996; Akopian et al., 1999). Since trigeminal chemosensation has been extensively discussed in the last chapter (see 1.1.2), the next part will provide a brief summary of current knowledge about the remaining four sub-modalities of somatosensation.

Thermosensation

Within the wide range of temperatures perceived by the human body only those above 43 °C and below 15 °C not just evoke thermal sensations, but additionally cause a
predominant feeling of pain (LaMotte and Campbell, 1978; Tillman et al., 1995). Terminals of temperature-sensitive neurons are typically found in tissues exposed to the external environment (skin, nasal and oral musosa and cornea). However, the molecular mechanism of temperature detection has been a long lasting mystery. In general, all channels and enzymes are inherently temperature sensitive, but some channels of the TRP superfamily turned out to be real temperature sensing specialists: TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1 (reviewed in Patapoutian et al., 2003; Voets et al., 2004; Tominaga and Caterina, 2004; Voets et al., 2005; Patapoutian, 2005; Reid, 2005). TRPM8 and TRPA1 have been identified as cold sensitive, the others respond to warm temperatures.

The model of thermosensitive receptor protein expressing sensory neurons as the primary site of temperature sensation was challenged by studies demonstrating the ability of keratinocytes (the most abundant cell type within the epidermal skin layer) to sense temperatures as well (Moqrich et al., 2005). Recent data not only proved the heat responsiveness of keratinocytes but also indicate that these cells might transduce thermal stimuli. For example, the warm-activated ion channels TRPV3 and TRPV4 are more readily detectable in keratinocytes than in sensory neurons (Lee and Caterina, 2005; Zimmermann et al., 2005; Dhaka et al., 2006; Huang et al., 2008). The expression of functional TRPV3 and TRPV4 in keratinocytes coupled with the behavioral thermosensory defects in TRPV3 or TRPV4 deficient mice provides a strong circumstantial case for the contribution of the keratinocyte-expressed channels in heat sensation. Whether (and, if so, how) stimulation of these channels results in neuronal activation has not yet been identified. It has been shown that no synapses are apparent between free nerve endings and keratinocytes, however, the proximity of these cell types and even close membrane–membrane apposition (Hilliges et al., 1995; Chateau and Misery, 2004) provide ample opportunity for rapid paracrine communication.

Cold sensation

Although primary afferent neurons responding specifically to moderate, innocuous cool temperatures were identified rather early (Hensel, 1981), the underlying receptor termed TRPM8 was not identified before 2002 (McKemy et al., 2002; Peier et al., 2002a). This non-selective cation channel transduces stimuli like menthol and cooling
and is expressed in trigeminal neurons, especially in small diameter fibers. TRPM8 does not co-localize with any known marker of nociceptive fibers such as CGRP, substance P, TRPV1, or isolection B4 (IB4) (Peier et al., 2002a), and therefore is unlikely to be involved in noxious cool perception. Three recent knockout studies have firmly established the central importance of TRPM8 in cold sensing, and provided important insight into the effect of cold sensing on mouse behavior (Dhaka et al., 2007; Colburn et al., 2007; Bautista et al., 2007). In TRPM8 deficient mice the fraction of sensory neurons that respond to cooling was dramatically reduced (by at least 50%), indicating that TRPM8 is a principal but not the sole cold receptor in these cells.

Therefore the expression of TRPM8 cannot explain the broad range of temperature thresholds observed in the population of sensory neurons responding to cold e.g. many cold-sensitive neurons with a low threshold temperature lack TRPM8 expression (Nealen et al., 2003; Babes et al., 2004). A second member of the TRP family, TRPA1 was shown to be activated by much lower temperatures than TRPM8, and therefore is suggested to be important for the transduction of strong (painful) cooling stimuli below 15 °C (Story et al., 2003). However, the role of TRPA1 in cold sensation is highly debated. For example, a large proportion of trigeminal ganglion neurons (TGNs) that respond to strong TRPA1 agonists such as mustard oil lack a clear cold response (Jordt et al., 2004). By contrast, the large majority of menthol-sensitive neurons, which were until recently defined as TRPM8-expressing cells, respond to cold stimuli (Jordt et al., 2004). The interpretation of these results is complex since menthol has been recently shown to exert a bimodal effect on TRPA1, activating it at low micromolar concentrations (<100 mM) and blocking it in the high micro- to millimolar concentration range (Karashima et al., 2007). Also results from TRPA1 deficient mice were inconclusive: Kwan et al., 2006, reported significant deficits in assays for noxious cold sensation (ice-cold plate withdrawal latency, acetone cooling), whereas Bautista et al., 2006, found no significant difference between TRPA1 deficient and wildtype mice in all tested aspects of cold sensation.

However, also TRP-independent ionic mechanisms such as the closure of background potassium channels, may participate in cold sensing (Maingret et al., 2000; Viana et al., 2002; Kang et al., 2005). It is therefore likely that cold sensitivity in sensory
neurons depends on their respective set of expressed ion channels, resulting in a particular neuronal activation threshold.

Warm sensation

Heat activated currents in primary afferent neurons seem to be predominantly mediated by TRPV1 (Caterina et al., 1997). The absence of heat-induced currents in cultured DRG neurons of TRPV1 deficient mice underlines the thermosensory relevance of TRPV1 (Caterina et al., 2000; Davis et al., 2000). However, these knock-out mice respond almost normally to noxious heat suggesting the involvement of other transducing mechanism. Three additional TRP channels could be identified (TRPV2, TRPV3 and TRPV4) with activation thresholds of 52 °C, 36 °C and 32 °C, respectively (Caterina et al., 1999; Liedtke et al., 2000; Smith et al., 2002; Xu et al., 2002; Peier et al., 2002b; Chung et al., 2003). Whereas the expression of TRPV3 in sensory neurons is controversially discussed, TRPV2 and TRPV4 are unambiguously expressed in sensory neurons (Caterina et al., 1999; Liedtke et al., 2000; Ma, 2001; Woodbury et al., 2004). The capsaicin-insensitive homologue TRPV2 might explain the activation of large capsaicin-insensitive neurons at temperatures above ~50 °C, as well as the residual nocifensive response to noxious heat stimuli in TRPV1 deficient mice (reviewed in Caterina, 2007). As mentioned above, the expression of TRPV1, TRPV3 and TRPV4 has also been reported for keratinocytes (Lee and Caterina, 2005).

Proprioception and Mechanosensation

The proprioceptive sense mediates information about the position and movements of limbs and the whole body. Therefore it can be subdivided into the limb position sense (sense of stationary position of the limbs) and the sense of limb movement (kinesthesia). Three types of muscle and joint mechanoreceptors transduce information about the stationary position: muscle spindle receptors are stretch sensitive; golgi tendon organs sense contractile force; receptors in the joint capsule sense flexion or extension of the joint.

Trigeminal mechanoreceptive neurons have specialized end organs surrounding the nerve terminal which are capsule like structures with different physiological functions. Four major types of mechanoreceptors in the glabrous skin can be identified: The
Meissner’s corpuscle and the Merkel disk receptor are the two principal mechanoreceptors in the superficial layers of the skin. They sense deformation of the papillary ridges in which they reside and predominantly differ in their adapting kinetics. The Meissner’s corpuscle is a rapidly adapting receptor that confers to fine mechanical sensitivity. The Merkel disk is a slowly adapting receptor cell transmitting skin compression to sensory nerve endings.

The Pacinian corpuscle and Ruffini endings are larger subcutaneous receptors sensing deformation of an expanded skin area. The Pacinian corpuscle responds to rapid skin indentation as well as vibrations occurring several centimeters away but not to a steady pressure. Ruffini endings are slowly adapting receptors sensing stretch of the skin mainly contributing to the perception of object shape.

The hairy skin comprises the hair follicle receptor and the field receptor. The three separate classes of these rapidly adapting receptors (down, guard, and tylotrich hairs) differ in sensitivity to hair movement and conduction velocity.

In part an overlap between the mechanical sense and pain perception exists since nociceptors can be activated by mechanical stress as well resulting from direct pressure, tissue deformation or changes in osmolarity. One detection mechanism involves activation of mechanically gated proteins. However, stretch-induced signal transduction processes evoking the release of diffusible chemical messengers capable of exciting nearby located primary sensory nerve terminals have also been demonstrated (Koizumi et al., 2004). Mechanically stimulated keratinocytes in vitro cause ATP-release and signaling through the metabotropic ATP receptor P2Y2. P2Y2 activation mobilizes the release of intracellular Ca\(^{2+}\) stores, which in turn evokes the release of more ATP from the stimulated keratinocyte. The result is an intercellular relay that spreads across the culture. If sensory neurons are co-cultured with keratinocytes that were stimulated in this way, the neurons exhibit a delayed activation mediated by keratinocyte-derived ATP acting on their own purinergic receptors. Although this sequence of events has not been established in vivo, it provides a plausible picture of how a physical stimulus could result in sensory neuron excitation.
1. Introduction

Pain

Pain is not the result of an overstimulation of a generalized cutaneous receptor but is transmitted by specific sensory neurons that are activated by noxious stimuli (noxious heat, intense pressure, strong irritant chemicals) but not by innocuous ones such as warming or light touch (Zotterman, 1933; Burgess and Perl, 1967).

The population of nociceptive neurons mostly consists of cells with small- or medium-diameter cell bodies which give rise to unmyelinated, slowly conducting C-fibres or thinly myelinated, more rapidly conducting Aδ fibres. The rapid, acute, sharp pain termed first pain is predominantly mediated by Aδ fibers, whereas the delayed more diffuse or second pain is relayed by C-fiber nociceptors (Basbaum and Jessel, 2000).

C-fiber nociceptors can be classified as peptidergic and nonpeptidergic depending on CGRP / substance P expression. Peptidergic nociceptors express TrkA receptors for nerve growth factor (NGF) binding and depend on NGF for survival. Nonpeptidergic neurons express receptors for the glial cell line-derived neurotrophic factor (GDNF), depend on GDNF for survival, and express a surface carbohydrate group that binds IB4. A major subset of nonpeptidergic nociceptive neurons additionally express Mrgrpd (sensory neuron-specific GPCRs, called Mas related G protein-coupled receptors) (Zylka et al., 2005). Several studies report that IB4-positive neurons lack TrkA receptors (Silverman and Kruger, 1990; Averill et al., 1995; Molliver et al., 1995; Bennett et al., 1996; Zwick et al., 2002) while others show significant overlap between IB4 binding, TrkA or even neuropeptide expression (Wang et al., 1994; Kashiba et al., 2001).

Fig. 1-3: Sensory skin circuits

Nociceptive information is carried by molecularly distinct and parallel neuronal circuits (red and green). These parallel circuits terminate centrally within adjacent lamina of the spinal cord and terminate peripherally in different zones of the epidermis as free nerve endings. Peptidergic and nonpeptidergic fibers are occasionally intertwined, suggesting intercommunication peripherally. Zylka et al., 2005
The peripheral as well as the central innervation pattern is subclass-dependent (Fig. 1-3). Mrgprd-expressing fibers were shown to meander extensively amongst keratinocytes of the *stratum granulosum*, and ultimately terminate at the border to the *stratum corneum*, whereas CGRP-expressing (peptidergic) fibers terminate in the underlying *stratum spinosum* (Zylka et al., 2005). Peptidergic sensory neurons project predominantly to lamina I and outer lamina II of the SP5 (for DRGs dorsal horn of the spinal cord, respectively), whereas nonpeptidergic (IB4) neurons terminate in the inner lamina II (and outer lamina II).

Finally, also different functional properties can be attributed to the two distinct classes: IB4-negative neurons from non-injured mice are highly responsive to capsaicin and protons, whereas IB4-positive neurons are significantly less responsive (Dirajlal et al., 2003). It is thought that IB4-negative neurons specifically contribute to inflammatory pain and that IB4-positive neurons contribute to neuropathic pain (Mantyh and Hunt, 1998; Snider and McMahon, 1998).

Nociceptors have the ability to detect and integrate a wide range of stimulus modalities and therefore have to be equipped with a diverse repertoire of transduction mechanisms. TRPV1 seems to be a key mediator of inflammatory pain, since it is activated or positively regulated by a large range of inflammatory mediators. Furthermore, capsaicin sensitivity and TRPV1 expression both increase in peripheral neurons during experimental inflammation (Nicholas et al., 1999; Carlton and Coggeshall, 2001). Finally, mice lacking TRPV1 do not develop heat hyperalgesia (Caterina et al., 2000; Davis et al., 2000). However, also TRPA1 has been shown to be involved in pain perception, predominantly hyperalgesia caused by inflammation and nerve damage (Obata et al., 2005).

Nociceptors not only signal acute pain, but also contribute to persistent and pathological pain conditions (allodynia) that occur in the setting of injury, whereupon pain is produced by innocuous stimuli (Jessel and Basbaum, 2000b). Allodynia is not only thought to result from lowering the nociceptor activation threshold when nociceptor terminals become exposed to products of tissue damage and inflammation (peripheral sensitization). Also central sensitization processes have been demonstrated. For example Jürgen Sandkühler's group has identified a synaptic pain amplifier in the spinal cord that is turned on in mature animals by natural, asynchronous and irregular, low-rate discharge patterns in nociceptive C-fibers at synapses with spino-PAG (periaqueductal gray) neurons (Ikeda et al., 2006).
1. Introduction

1.2. The Skin – Structural Anatomy and Function

The skin is the body’s largest organs. Since it represents the outermost barrier to the external environment it has to fulfill multiple purposes: For example it protects the inner organs from harmful mechanical and chemical influences as well as from damaging UV radiation. Additionally, it precludes pathogens like bacteria, fungi, or viruses from entering the body. Through sweat secretion and changes in blood flow (vasoconstriction; vasodilation) the skin is also deeply involved in thermoregulation and prevents from dehydration. Moreover, skin glands are thought to secret sexual attractants, underlining its importance in social communication.

The skin is composed of three primary layers: the epidermis, the dermis and the hypodermis. The epidermis is composed of a stratified squamous epithelium with an underlying basal lamina. It contains nerve fibers but no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries of the dermis. The dermis mainly consists of fibroblasts and additionally provides anchoring and waste removal of the epidermis. The hypodermis consists of loose connective tissue and elastin. It serves as a heat insulator and attaches the skin to underlying bone and muscles.

1.2.1. The Epidermis

The epidermis constitutes the waterproof, protective surface of the body. It mainly consists of keratinocytes with Merkel cells, melanocytes, Langerhans cells and lymphocytes residing in between this predominant cell type. The epidermis can be subdivided into four layers (starting from the outermost layer): stratum corneum, stratum granulosum, stratum spinosum, stratum basale. Cells are formed from cubic epidermal stem cells in the stratum basale that divide every 20 days. Progenitors produce keratinocytes that migrate in the course of differentiation towards the suprabasal layers thereby changing shape and protein composition. Keratin proteins start to accumulate in cornified keratinocytes, called corneocytes. Finally, they die due to isolation from blood supply. The nucleus disappears and cell cytoplasm is released. This process is known as keratinization and takes place within weeks. The keratinized layer of the skin fulfills protective functions preventing the body from dehydration and harmful chemicals and even pathogens. The outermost layer of the epidermis consists of 25 to 30 layers of dead cells (30-300µm) varying with age.
1.2.2. Keratinocytes

The major epidermal cell type is represented by keratinocytes which account for 85% of living epidermal cells. Keratinocytes which built the stratified squamous epithelia are named according to their cytoskeleton mainly consisting of keratin proteins. The basal epidermal layer (stratum basalis) consists of a single layer of undifferentiated cells that are anchored in the basal lamina via hemidesmosomal junctions. The stratum spinosum is characterized by the presence of extensive desmosomal connections between cells. Granules, present in the stratum granulosum, contain products of keratinocyte differentiation that are used to assemble various terminal keratinocyte structures, including the corneocyte membrane or marginal band. Granular layer cells are still living albeit they approach the final steps in differentiation. The transition to the stratum corneum separates the living from the dead epidermal layers. In the latter, cellular organelles and nucleic acids are destroyed by the action of proteases, nucleases, and other enzymes. The resulting cornified cells (corneocytes) represent the terminal stage in keratinocyte differentiation and consist of a stabilized array of keratin filaments. Lipids secreted from cells in the stratum granulosum surround these corneocytes, thus avoiding dehydration. Disulfide bonds stabilize keratin intermediate filament bundles, making them resistant and flexible at the same time. Adjacent corneocytes are held together by modified desmosomes and by an interlocking system of ridges and grooves (Holbrook and Wolff, 1987). Taken together, keratinocyte proliferation, differentiation, and apoptosis are a complex and carefully choreographed process (Eckert et al., 2002).

Apart from this classical view on skin function as a passiv protective barrier, it is well established that keratinocytes can communicate among themselves via gap-junctions (Salomon et al., 1988). This communication phenomenon is not unique to keratinocytes, since it is well known that a lot of non excitable cells can transmit calcium waves from one cell to the other (e.g. astrocytes (Cornell-Bell et al., 1990), hepatocytes (Thomas et al., 1991), other epithelial cells (Hansen et al., 1993), and endothelial cells (Demer et al., 1993)). Keratinocytes generate intracellular calcium waves upon mechanical stimulation, as already discussed above (Koizumi et al., 2004). In this respect it is hypothesized that keratinocytes can communicate also with other cell types including sensory neurons. Recently, sensors which respond to mechanical stress, osmotic pressure, temperature, and chemical stimuli have been found to be not exclusively expressed in sensory neurons, but also in epidermal...
keratinocytes. For example TRPV1, TRPV3 and TRPV4 were shown to be present in epidermal keratinocytes (Denda et al., 2001; Inoue et al., 2002; Chung et al., 2004b). TRPV1 is activated by heat (>43 °C), acidic conditions (pH < 6.6) and capsaicin (Caterina et al., 1997). TRPV3 is activated by heat (>35 °C), mechanical stress, camphor, and 2-aminoethoxydiphenyl borate (2APB) (Chung et al., 2004a; Moqrich et al., 2005). TRPV4 is also activated by heat >35 °C and osmotic pressure (Dhaka et al., 2006). TRPV1 seems to be most strongly expressed in the upper and basal layers of the epidermis. High expression of the receptor at the surface of the epidermis would be consistent with a role in detecting external temperature. The reason for the high expression at the basal layer is not clear, but TRPV1 may also have some role in epidermal–dermal interaction. Another interesting family are ATP receptors. Two distinct families of ATP receptors are known (Burnstock and Williams, 2000). One is the ATP-activated purinergic receptor (P2X) family, which is a ligand-gated ion channel, and the other is the P2Y family of metabotropic, heptahelical G-protein-coupled receptors. P2X3 is expressed in human epidermal keratinocytes (Denda et al., 2002; Inoue et al., 2005), with the strongest expression in the upper area of the epidermis. P2X3 is produced during terminal differentiation in a human keratinocyte culture system (Inoue et al., 2005). Additionally, an differentiation phase dependent expression of P2X1, P2X2, P2X4, P2X5, and P2X7 as well as P2Y1 and P2Y2 in cultured human keratinocytes has also been demonstrated (Greig et al., 2003; Inoue et al., 2005). ATP is released from epidermal keratinocytes not only after mechanical stimulation (Koizumi et al., 2004), but also by tapestripping or exposure to air (Denda et al., 2002; Denda and Denda, 2007). The latter observations point to an potential involvement of the epidermal ATP system in transducing itch of the skin in the case of dermatoses (e.g. atopic dermatitis), which is characterized by barrier dysfunction or skin surface dryness. However, the list of substances that could potentially be secreted by keratinocytes / skin cells is large (glutamate (Fuziwara et al., 2003), dopamine (Fuziwara et al., 2005), melatonin and serotonin (Slominski et al., 2002), GABA (Ito et al., 2007), PGE2 (Huang et al., 2008), NGF and artemin (reviewed in Albers and Davis, 2007)). Additionally, a variety of endocrinological receptors and their agonists are expressed in epidermal keratinocytes (Slominski et al., 2000; Slominski and Wortsman, 2000; Slominski et al., 2001; Slominski, 2005) as well as a series of receptors, which were originally found in the central nervous system as neurotransmitter receptors including
ionotropic (P2X, NMDA, GABA$_A$, glycine) and G-protein coupled receptors (P2Y, adrenergic $\beta_2$, dopamine-2-like, serotonin) (reviewed in Denda et al., 2007).
Thus, keratinocytes appear to be equipped with a sensing system similar to that of peripheral neurons potentially enabling a “skin sensory perception” (reviewed in Denda et al., 2007).
1.3. Tracing the Trigeminal System with Pseudorabies Viruses

1.3.1. Retrospect: Tracing History

Neurons make use of specialized synaptic structures to communicate among each other, or with peripheral structures like muscle cells. Neuronal connectivity and wiring are essential for correct information processing within the nervous system. In order to decode neuronal network connectivity, several different methods have been developed within the recent years:

Already in the 18th century Cajal and Golgi visualized neurons using the silver-staining method. Limitations of these Cajal and Golgi staining are the limited numbers of labeled neurons (approximately 1% of all neurons) and the lacking possibility of neuronal network investigations. The first reconstruction of small neuronal networks was possible using the histochemical labeling of myelin degradation products after antero- and retrograde degeneration. The discovery of HRP (horseradish peroxidase) tracer back in the sixties/seventies was the milestone for the development of a new kind of tracer. After peripheral or central injection these tracers make use of the cellular axonal transport mechanism therefore reaching axonal and dendritic nerve endings. Dynein and Kinesin are the most important motorproteins involved in microtubule associated cellular transport mechanism. Kinesin typically travels from the cell soma to the terminals and Dynein in the opposite direction. Therefore tracers using these transport mechanism can be divided into two groups. Those ones interacting with Kinesin-like transport mechanism are transported to the cell terminals, and those interacting with Dynein-like transport mechanism are transported to the cell soma. Finally, HRPs enzymatic activity is used for visualization. Additionally, radioactive marked aminoacids have been used for autoradiography based visualisation.

A fundamental breakthrough in tracing history was the discovery of transneuronal tracers. In contrast to conventional tracers they are transported via synapses from one neuron to another. Thus, synaptically connected neurons could be specifically visualized heralding the decoding of neuronal network connectivity. WGA (wheat-germ agglutinin) (Broadwell and Balin, 1985; Fabian and Coulter, 1985) is probably one of the most prominent members of this tracer family.
Via intranasal application the connectivity of the rodent olfactory system starting from
the olfactory epithelium to the *Bulbus olfactorius* up to olfactory cortex could be
elucidated (Shipley, 1985; Baker and Spencer, 1986; Itaya, 1987). However, these
tracers travel relatively slow and their concentration decreases with every crossed
synapse causing detection limit problems. The development of liposoluble DiI / DiO based tracing techniques allowed the
analysis of neuronal networks also in fixed tissue. Within the recent years particularly promoter driven expression of enzymatic (e.g. β-
galactosidase) or fluorescent (e.g. green fluorescent protein, GFP) reporter proteins in
transgenic animals increased in popularity. For the olfactory system, for example, a
transgenic mouse line has been created expressing a fusion protein of the microtubule
associated protein, Tau, and β-galactosidase under the control of the OMP (olfactory
marker protein) promotor which enabled the reconstruction of axonal projections of
olfactory receptor neurons (Mombaerts *et al.*, 1996).

1.3.2. Advantages of Viral Tracers

Using viral constructs genes can easily be delivered into infected cells, e.g. allowing
viral mediated expression of marker proteins. Since several virus types not only
evolved the ability to infect neurons but are additionally able to cross synapses and
therefore travel within circuits of the nervous system, viruses are excellent transneural
traces. These so called neurotrophic viruses travel within defined neuronal circuits
after peripheral or central application. They start to reproduce themselves in every
infected cell and this infectious offspring is able to be transferred to synaptically
connected neurons (transneuronal spread) thus labeling higher order neurons. Using
the inherent propagation properties of different virus types, anterograde as well as
retrograde tracing is possible. Virology terms a tracer that selectively travels in the
direction of neuronal information flow an anterograde tracer. Retrograde tracers are
those ones just able to travel in opposite direction. Besides Rabies Virus (predominantly traveling in retrograde direction (Kelly and
Strick, 2000; Graf *et al.*, 2002)) also some DNA based viruses of the Herpesvirus
family belong to the neurotrophic viruses. Herpes Simplex Virus-1 (HSV-1[H129]) a member of the subfamily of alpha-
**Herpesviridae**, predominantly traveling in anterograde direction was used to trace
trigeminal pain afferents after inoculation to the murine tooth pulp (Barnett et al., 1995).

Pseudorabis-Virus (PrV), also a member of the subfamily of $\alpha$-Herpesviridae, is a transneuronal tracer with a broad host spectrum and well characterized attenuated strains with reduced pathogenesis (e.g. PrV-Bartha, vaccination strain). After injection of PrV-Bartha into a series of peripheral organs including stomach (Yang et al., 1999), heart (Standish et al., 1994; Wang et al., 2001), intestine (Vizzard et al., 2000) and pankreas (Streefland et al., 1998) viral antigens could be detected immunohistochemically in exactly the brain region already known from conventional tracing studies to innervate the according organs. An injection of PrV-Bartha into central structures of the nervous system also leads to high specific transneuronal infections (Jansen et al., 1995). However, in contrast to HSV-1 and Rabies, PrV is only a weak human pathogen.

Since viruses are self replicating transneuronal tracers, propagation within the nervous system and labeling of higher order neurons is not accompanied with a reduction in the detectable viral antigens. Consequently, detection limit problems are nearly absent.

Unlike the generation of transgenic animals, viral tracing experiments only require minor prefabrication time since genetically unaltered animals can be used. The broad host spectrum of PrV provides the opportunity of transferring the tracing approach to a different model organism using the same viral constructs.

Moreover, a combination of transgenic animal models and advanced viral tracing techniques opens the door to complete new application possibilities. For example a recombinant PrV-strain was used to investigate the neuronal network of the feeding center in the hypothalamus (DeFalco et al., 2001). The virus strain used in this study is Cre-deficient and therefore only replicates in neurons expressing Cre recombinase and in neurons in synaptic contact with the originally infected cells. The virus was injected into transgenic mice expressing Cre under the neuropeptide Y or the leptin receptor promotor. Hypothalamic neurons expressing the leptin receptor and neuropeptide Y have previously been shown to be involved in food intake regulation. After injection into the hypothalamus the virus only infects relevant neurons and selectively retrogradly labels brain areas innervating the feeding center. In this way a selective labeling of defined neuronal population can be combined with a reconstruction of their innervation area.
1.3.3. Functional Neuronal Network Exploration using Viral Tracing Tools

Within the recent years research has more and more focused on the exploration of neuronal networks. Essential for a decryption of neuronal network information processing is the ability of performing simultaneous measurement of neuronal activity at multiple locations at a high temporal and spatial resolution. Originally this has been achieved by using electrophysiological recordings. However, patch clamp recordings are limited to simultaneous measurement from only a limited number of neurons while most microelectrode arrays suffer from low spatial resolution of the recorded field potentials. Therefore many studies tried to overcome these problems by using optophysiological approaches. A gross overview about the advantages and disadvantages of these techniques has been assembled in Tab. 1-1.

Two of these imaging methods depend on endogenous changes in optical tissue properties due to neuronal activity: intrinsic signal imaging is based on changes in blood oxygen levels in activated regions and autofluorescence imaging is derived from NADH (reduced form of nicotinamide adenine dinucleotide) or flavoproteins. Both methods do not require any dye loading. However, in most cases these techniques suffer from a poor temporal and spatial resolution as well as low signal to noise ratios. In contrast, calcium- or voltage-sensitive dye imaging displays a high temporal and spatial resolution and especially the application of calcium sensitive dyes results in strong signals changes. Thus, Ca-Imaging presents one of the most advantageous imaging approaches, combining a decent temporal and excellent spatial resolution with high fractional changes of fluorescence intensity.

Despite all the advantages of calcium sensitive dyes, cell type specific labeling cannot be achieved using bulk loading approaches, leading to high background fluorescence values and decreased signal to noise ratios. Additionally, some adult neuronal tissues do not take up the dyes in sufficient amounts. A real breakthrough in this respect was the development of fluorescent calcium indicator proteins (FCIP). Several FCIPs have been described in the recent years (e.g. inverse pericam (Nagai et al., 2001) camgaroo (Baird et al., 1999) and GCaMP (Nakai et al., 2001)). Using appropriate promoters, these genetically encoded calcium sensors allow for a cell type specific targeting, thereby dramatically increasing calcium sensitivity especially in tissue slices and in vivo preparations. These advantages led to the creation of several different transgenic animals including worms (Kerr et al., 2000; Suzuki et al., 2003), fruitflies (Fiala et al.,...
1. Introduction

2002; Reiff et al., 2002; Yu et al., 2003; Liu et al., 2003; Wang et al., 2003), zebrafish (Higashijima et al., 2003), and mice (Hasan et al., 2004; Ji et al., 2004).

Especially the construction of transgenic mice is a slow and expensive process since each cell system of interest requires a separate transgenic line. Thus, for a fast and reliable introduction of FCIPs into various mammalian cell systems a viral vector would represent the perfect tool for the functional analysis of whole circuits of synaptically connected neurons in vitro and in vivo.

Tab. 1-1: Simplified overview of advantages and disadvantages of different imaging techniques.

<table>
<thead>
<tr>
<th>Method</th>
<th>Intrinsic signal imaging</th>
<th>Auto-fluorescence imaging</th>
<th>Ca²⁺ Imaging</th>
<th>Voltage-sensitive dye (VSD) imaging</th>
<th>Fluorescent calcium indicator proteins (FCIP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basis</td>
<td>blood oxygen level-dependent</td>
<td>endogenous fluorescence derived from NADH or flavoproteins</td>
<td>Cytosolic Ca²⁺ increase</td>
<td>Voltage dependent fluorescence changes of VSD</td>
<td>Virus mediated expression of FCIPs</td>
</tr>
<tr>
<td>Temporal resolution</td>
<td>- -</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Spatial resolution</td>
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<td>Yes</td>
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A Pseudorabies virus mediated expression of these construct with all the advantages of viral tracers mentioned above, would therefore have a broad field of application in different neuroscience areas.

1.3.4. The Pseudorabies Virus

Pseudorabies virus (PrV), a swine herpesvirus of the Alphaherpesvirinae subfamily, is the causative agent of Aujeszky's disease. PrV has a broad host range which includes nearly all mammals (except higher primates and humans), as well as other vertebrate species (reviewed in Mettenleiter, 1994). However, pigs are considered to be the natural host of PrV and are the only animals that are able to survive a productive infection. Natural infection with PrV occurs via the oro-naso-pharyngeal route. The nasal cavity is innervated predominantly by four nerves: (I) the olfactory nerve (I
cranial), (II) the trigeminal nerve (V cranial), as well as (III) parasympathetic, and (IV) sympathetic efferents. After primary infection of epithelial cells, the viral particles invade peripheral neurons via nerve endings innervating the mucosae and ascend toward the central nervous system (CNS), resulting in a non-suppurative meningoencephalitis (Pensaert and Kluge, 1989; Enquist, 1994). In mice, the infection spreads through only three of the four main neuronal pathways innervating the nasal cavity (Flamand et al., 2001): via anterograde spread in the trigeminal circuit and via retrograde spread in the sympathetic and parasympathetic circuit. However, PrV does not propagate in the olfactory system after intranasal application (Sabin, 1938; Babic et al., 1994; Damann, 2002).

The spreading of an infection from a site in the periphery to the central nervous system is called neuroinvasion. The efficient spread of herpesviruses from the peripheral nervous system (PNS) to the CNS upon infection of many non-natural hosts was recognized over 60 years ago and signaled the possibility of using herpesviruses for transneuronal tracing, to identify chains of synaptically linked neurons within a neuronal circuit (Card and Enquist, 2001; Enquist, 2002; Song et al., 2005). The most commonly used PrV tracers are derived of an attenuated (reduced pathogenesis) PrV strain termed Bartha (originally called “Bartha’s K” strain, (Enquist et al., 1998). This “life vaccine” strain was initially developed to prevent propagation of PrV in pig populations. PrV-Bartha exhibits gene deletions in the Us region encompassing the gI, gE, Us9 and Us2 genes and mutations in gC, UL10, and UL21 genes (Mettenleiter, 2000).

In recent years native PrV-Bartha as well as marker protein-expressing variants, have been effectually used to define the organization of CNS circuits after intracerebral (Jasmin et al., 1997; DeFalco et al., 2001; Krout et al., 2003; Willhite et al., 2006) or peripheral (Jansen et al., 1995; Jansen et al., 1997; Smith et al., 2000; Billig et al., 2000; Cano et al., 2001; Irnaten et al., 2001; Horvath et al., 2003) injection. In contrast to wild type PrV, PrV-Bartha has lost its capability to spread in anterograde direction of information processing within the nervous system, and therefore allows selective tracing in retrograde direction (Enquist, 2002). Identification of CNS circuits involved in processing of sensory information requires transneuronal tracing from peripheral sensory organs in the anterograde direction towards the CNS. Both retrograde and anterograde spread can be accomplished by wild type α-herpesviruses (Sabin, 1938), providing a potential tool for tracing sensory neuronal systems.
Morphology and Genomic Organization

PrV has a unique virion architecture which is common to all *Herpesviridae* family members. The infectious viral particle consists of three morphologically distinct structural components: The icosahedral capsid (I) contains the viral genome (linear double-stranded DNA; approx. 150 kbp) and is embedded in a proteinaceous tegument (II). The tegument is surrounded by the envelope (III), a lipid bilayer membrane containing several virally encoded glycoproteins.

The major capsid protein is encoded by UL19 and assembles into 162 capsomers (150 hexons and 12 pentons) arranged in a $T = 16$ icosahedral lattice (Newcomb *et al.*, 1993). The resulting capsids have a diameter of approximately 125 nm (Pomeranz *et al.*, 2005). The PrV genome is characterised by two unique regions $U_L$ (unique long) and $U_S$ (unique short). The $U_S$ region is flanked by the internal and terminal repeat sequences (IR and TR, respectively). Moreover, the complete DNA sequence of Pseudorabies virus has been identified (Klupp *et al.*, 2004). The PrV genome consists of 70 different genes, each with specific functions (Pomeranz *et al.*, 2005). All PrV genes have homologous in one or more related α-herpesviruses and none of them is unique to PrV. A basic set of 40 herpesvirus genes are furthermore conserved among all α-, β-, and γ-herpesvirinae. These genes encode proteins that are fundamental for virus replication, packaging, and entry into as well as egress from infected cells.

Infection and Viral Replication

Viral entry begins with an attachment or binding of the virus particle to the cell surface. In the case of PrV, this initial binding step is mediated by an interaction between the virions envelope glycoprotein C (gC) and cell surface heparan sulphates. Subsequently the viral glycoprotein D (gD) binds to specific cellular receptors to stabilize the virion-cell interaction. Finally, PrV glycoproteins B, H, and L (gB, gH and gL) mediate the fusion of the viral envelope and the cellular plasma membrane. The viral capsid and tegument proteins are released into the cell. The capsid is transported along microtubules to the cell nucleus. For HSV-1 virions it has been shown that the capsids interact with Dynein for the transport along microtubules from the cell periphery to the nuclear pore (Sodeik *et al.*, 1997; Dohner *et al.*, 2002). After capsid docking at the nuclear pore the PrV genomic DNA is released into the nucleus (Granzow *et al.*, 1997; Granzow *et al.*, 2005).
The VP16 tegument protein localizes to the nucleus independent of the capsid and transactivates cellular RNA polymerase II transcription of the only immediate-early protein of PrV, IE180. As VP16 is part of initial infecting virions, immediate-early gene expression does not require new viral protein synthesis. IE180 protein expressed in the cytoplasm is transported back to the nucleus, where it transactivates RNA polymerase II transcription of the early genes. Since early gene expression requires this viral transactivator encoded by the immediate-early gene, their transcription is sensitive to protein translation inhibitors such as cycloheximide. Viral transcription activators propel the transcription cascade forward by activating transcription of the next set of viral genes. Early proteins are involved in viral DNA synthesis and also act themselves as transactivators of transcription. Onset of DNA synthesis signals the start of the late stage of the PRV replication cycle and synthesis of true late proteins. True late genes require viral DNA replication for efficient transcription and their expression is severely impaired in the presence of phosphonoformic acid (FO; foscarnet), an inhibitor of DNA replication. Late genes encode proteins required for virion assembly and egress. Newly synthesized capsid proteins are transported to the nucleus, where they assemble around a scaffold and the genomic DNA is inserted. Subsequently, the fully assembled nucleocapsid buds out of the nucleus where it gains its final envelope by associating with tegument and envelope proteins and budding into the trans-Golgi apparatus. Finally, the mature virus is brought to the cell surface within a sorting vesicle.

The duration required to complete the PrV growth cycle varies according to cell type; typically viral progeny can be detected within 4 to 5 h after infection. If infected animals survive a productive infection, the herpesvirus genome resides in host’s nuclei for the remainder of the lifetime. Reactivation from latency allows spread to naive hosts and maintains the presence of the virus in the population (Enquist, 1994).

1.3.5. Viral Influence on Neuronal Physiology

After fusion of the viral envelope and the cellular plasma membrane the viral capsid and tegument proteins are released into the cell. Immediately after cell entering and even before viral protein synthesis, α-herpesviruses start influencing cell physiology. Viral tegument proteins immediately begin to take over the host cell protein synthesis machinery. One of these tegument proteins of HSV-1 is called virion host shutoff.
(VHS) protein. It causes the inhibition of host protein synthesis by degradation of cellular mRNAs after infection (Fenwick and Clark, 1982). The VHS protein of PrV has also been identified and its ribonucleolytic activity was confirmed in in vitro studies using recombinant VHS protein (Lin et al., 2004).

A whole bunch of viral proteins including transcription transactivators and viral structure proteins are produced upon viral gene expression. Some of these proteins are known to directly interact with host cell signal transduction cascades. For example the US3 protein kinase, which in conserved among all α-herpesviruses appears to be one of the most potent HSV encoded anti-apoptotic proteins (Mori et al., 2004; Geenen et al., 2005). During infection of a cell, most viruses trigger apoptotic signals leading to cell death. A classical concept of virology defines this virus induced cell death only as a pathological event underlying the process of viral diseases. Nowadays, virus-induced apoptosis has been postulated to represent an active cellular and host response to infection which curtails viral replication and spread by eliminating infected cells before completion of the viral replication cycle (Fazakerley and Allsopp, 2001).

Mitogen-activated protein kinases (MAPKs) play a central role in transducing a wide variety of extracellular stimuli to the nucleus. Among well established MAPKs the c-Jun N-terminal protein kinase (JNK) and the p38 MAPK signal transduction pathways function to communicate apoptotic signals in the cell. Accumulating evidence has suggested that activation of the JNK / c-Jun signal transduction cascade mediates apoptosis of neurons in central and peripheral populations via the actions of a proapoptotic BH3-only Bel-2 family member, Bim (Putcha et al., 2001). JNK cascade is activated by stressors including oxidative stress, ultraviolet irradiation, and deprivation of trophic factors.

Small RNA viruses, such as poliovirus, might have evolutionally coped with such a cell suicide mechanism by completing their replication cycle very rapidly for survival before the cell death occurs. Such viruses have no requirements for antiapoptotic genes. In contrast, large RNA and DNA viruses that take a longer time for replication, might have learned to possess antiapoptotic genes which may function to keep infected cells alive long enough for progeny virus production. This function seems to be mediated via the US3 protein. Virus infections of neurons activate the JNK cascade to induce apoptosis. The US3 protein kinase has been shown to attenuate JNK cascade activation thereby inhibiting cell apoptosis and enabling viral propagation in the
infected organism. These examples demonstrate the diverse impact that viral infection may exert on cell physiology.

Early experiments already investigated a possible influence on electrophysiological properties of infected cells. For example a loss or reversal of membrane potential in epithelial and secretory cells infected with herpes viruses has been demonstrated (Van Horn et al., 1970; Fritz and Nahmias, 1972; Fritz et al., 1974; Weigel et al., 1981). However, subsequent experiments on dorsal root and sympathetic ganglion neurons did not reveal changes in resting membrane potential following infection with herpes viruses (Fukuda and Kurata, 1981; Oakes et al., 1981; Kiraly and Dolivo, 1982) pointing to cell specific viral effects.

Herpes simplex virus types 1 and 2 causes a loss of excitability in dorsal root ganglion neurons infected with these viruses (Fukuda and Kurata, 1981; Oakes et al., 1981). Further studies showed that loss of excitability results from the selective, precipitous, and complete internalization of voltage-activated sodium channel proteins from the plasma membrane of HSV-1 infected DRG neurons (Storey et al., 2002). In contrast, sympathetic ganglia infected with Pseudorabies virus develop spontaneous electrical activity (Kiraly and Dolivo, 1982). These opposing effects of Pseudorabies and herpes simplex viruses on electrical excitability appeared to be virus specific.

In order to test the usability of PrV-Bartha as a “live-cell”-tracing tool a detailed electrophysiological characterization of in vivo infected, in vitro infected and uninfected TGNs was preformed (Damann et al., 2006). To prevent viral replication in cell culture, the pyrophosphate analogue foscarinet (phosphonoformic acid, FO) a direct noncompetitive inhibitor of viral DNA-polymerase was added to the culture medium. FO is known to inhibit the propagation of several types of viruses, including HSV-1 and CMV. A concentration of 400 µg/ml culture medium proved to be sufficient to inhibit viral spread for at least three days, allowing reliable identification of traced cells. None of the criteria tested (membrane potential; threshold for activation of voltage gated sodium channels; maximal amplitude of APs; threshold of current injection for eliciting APs; membrane potential due to current injection eliciting APs; width of AP at 75% of amplitude; maximal amplitude of APs due to current injection; I_h current (-130 mV); amplitude of "sag"; threshold of current injection for I_h-activation) gained significance for changes in electrophysiological properties due to viral infection or FO treatment of cultured TGNs.
Virulent wild type PrV strains (PrV-Kaplan) induce violent pruritus (itching) in a wide range of hosts, while infection with PrV-Bartha does not. It is likely that viral effects on neuronal firing patterns contribute to the pathology and clinical symptoms associated with infection. Recordings of spontaneous activity of superior cervical ganglion (SCG) neurons infected with wild type Kaplan and attenuated Bartha strains revealed Kaplan-infected neurons fire spontaneously and continuously, in contrast to silent uninfected neurons. Bartha-infected neurons do not display this spontaneous firing phenotype (McCarthy et al., 2007). However, in comparison to the study of Damann et al., 2006, no antiviral drug has been used. Although closely related, wild type PrV and PrV-Bartha seem to be able to differentially influence neuronal physiology. This underlines the demand on detailed electrophysiological investigations on potential PrV-Kaplan mediated changes on neuronal physiology under antiviral treatment before also taking this anterograde tracer into account as a possible “live-cell”-tracing tool.
2. Objectives

The trigeminal nerve is the major mediator of sensations from the mammalian head and comprises neurons that transduce mechanical, thermal and chemical stimuli. Single trigeminal neurons mediate sensory input from selective areas of the head (meninges, cornea and conjunctiva of the eyes, facial skin, mucous membranes of the oral and nasal cavities).

The trigeminal ability of mediating such different sensations from diverse innervation areas is not only thought to require complicated signal uptake mechanism at the reception site but also complex encoding strategies for the “what” and “where” information of sensory processing. Despite or maybe because of this complexity, the trigeminal system is not well investigated.

My work aimed at shedding light on the perception and encoding of sensory information in the trigeminal system, especially focussing on some of the general assumptions and questions about trigeminal chemosensation.

On the basis of my previous work with Pseudorabies virus (Rothermel, 2005), the investigation of viral strains that could be used for a decryption of trigeminal neuronal pathways mediating nasal chemosensation was of major interest. Therefore a viral strain would be required that is able to trace the entire trigeminal pathway, starting with an innervation site dependent labelling of trigeminal neurons and synaptically connected higher order neurons in the brainstem (4.1). The construction of viral vectors expressing fluorescent calcium indicator proteins would additionally provide a new tool for the functional analysis of whole circuits of synaptically connected neurons in vitro and in vivo (4.2).

Beside the decryption of sensory circuits mediating nasal trigeminal chemosensation, I was interested in the uptake of sensory information by the trigeminal nerve: it is still controversially discussed whether trigeminal chemosensation arises from a direct stimulation of intraepithelial free nerve endings, or if alternative mechanisms like signal transduction from other cells could be involved. Shedding light on this issue is of major importance since the outcome could modify our general view on how the peripheral sensory nervous system is able to take-up somatosensory information. As a first approach to that issue, chemosensory abilities of individual trigeminal neurons should be analyzed in primary neuronal cell cultures using the calcium imaging technique (4.3). Within the recent years skin derived keratinocytes, where shown to be
equipped with a sensing system similar to that of peripheral neurons, potentially enabling a “skin sensory perception” (reviewed in Denda et al., 2007). Since the trigeminal nerve innervates large parts of the facial skin furthermore a coculture of trigeminal neurons and human keratinocytes should be established as a model system in order to investigate the possible contribution of cells of the peripheral innervation area in transducing chemosensory information (4.3).

Gaining more knowledge about the uptake of sensory information of course leads to the question how this sensory information is encoded within the trigeminal system. In contrast to the olfactory system where different odorants are known to evoke unique patterns of activity in the olfactory bulb (which in turn are thought to play a primary role in coding odor information), nothing is known about the olfactory stimulus representation at the level of the trigeminal ganglion. Trigeminal ganglia, containing sensory neuron somata and nerve fibers which detect somatic sensations in the periphery and transmit information to the central nervous system, are located at the base of the skull. Due to the difficulty in accessing trigeminal ganglia experimentally a direct in vivo visualisation of evoked population activity in the trigeminal ganglion in response to sensory stimuli has not been achieved so far and basic principles of spatiotemporal activity pattern formation remain elusive. In order to shed light on olfactory stimulus representation at the level of the trigeminal ganglion I planned to establish a preparational approach that allows high-resolution recording of optical signals arising from a large region of the rat trigeminal ganglion in vivo using voltage-sensitive dye imaging. On the basis of testing different odor subcategories (substances with different trigeminal potencies) the elicited activity pattern at the ganglionic level should be analysed and compared (4.4). As a result I wanted to answer the controversially discussed topic if trigeminal chemoperception is only based on a graduated activation of trigeminal (pain) fibers or if odor specific activity patterns could exist at the level of the ganglion. If proven odor specific activity patterns would provide an explanation for the relatively high discriminative abilities of anosmic patients. In addition to the proposed experiments, human psychophysical examinations should contribute to shed light on some of the (cognitive) interpretations of the tested stimuli (4.5).
3. Material and Methods

3.1. Animals

The study was conducted using 0 - 5 days-old (primary trigeminal cell culture; coculture approach) CD1 mice (Charles River Laboratories WIGA, Germany). Data for the trigeminal ganglion in vivo preparation were acquired from 35 adult male Wistar rats (Charles River Laboratories WIGA, Germany). Exclusively male rats were used in order to rule out any potential effect of the hormonal status on the measurements. Additional only adult rats were used (>6 month, 400-500 g) to ensure the ganglion is fully maturated (Lagaes et al., 2007).

The animals were caged, with water and commercial food ad libidum. All animal experiments were carried out in accordance with the European Union Community Council guidelines, approved by the German Animal Care and Use Committee (AZ 9.93.2.10.32.07.022) in accordance with the Deutsche Tierschutzgesetz and the NIH guidelines. The virus-infected animals were kept separated, and biosafety level 2 precautions were applied.

3.2. Establishment of the Trigeminal Ganglion in vivo Preparation

Anesthesia was introduced with Chloralhydrat (4% solution in saline, 1 ml/100 g bodyweight = 400 mg/kg bodyweight) injected intraperitoneally. Lidocaine (1%, s.c. injection) was used as a local anesthetic and applied to all pressure points and incisions. The femoral artery was cannulated for NaCl infusion (0,5–2 ml/h). A double tracheotomy was performed and animals were artificially ventilated (50–70 cycles/min, 4–6 ml tidal volume; BASILE, Italy). Anesthesia was maintained with Isoflurane (1–2%). Animals were placed in a stereotaxic apparatus and core temperature was held at 37.5 °C with a heating pad. Heart rate was monitored by electrocardiogram (ECG). A craniotomy was performed to expose the cerebral hemispheres, which were then gently aspirated to visualize the trigeminal ganglia at the base of the skull. After decerebration the isoflurane level was decreased to less than 1% to rule out influences on nociceptive ion channels (Matta et al., 2008). At the end of the experiment, the rat was killed with an overdose of anesthetic.
3.2.1. *In vivo* voltage-sensitive dye loading of the Trigeminal Ganglia

A wide tip patch pipette was filled with a standard pipette solution containing the VSD (RH-1691) at an OD of 5-7. This pipette was inserted into the ganglion and a weak manual pressure pulse was used to inject a small amount of the solution. The procedure was repeated at multiple penetration sides (total injected volume ~500-1000 nl). The quality of the staining was checked before each measurement and only animals showing a homogenous distribution of the dye were included in the study.

3.2.2. Optical Imaging and Electrical Recordings

Optical imaging using voltage-sensitive dyes has been proven extremely useful for the real time exploration of neuronal network dynamics (Grinvald and Hildesheim, 2004; Jancke et al., 2004; Sharon et al., 2007; Grabska-Barwinska et al., 2009). The voltage-sensitive dye binds to the external surface of excitable membranes and acts as a molecular transducer that transforms changes in membrane potential into optical signals. The resulting changes in the absorption or the emitted fluorescence occur in microseconds and are linearly correlated with the membrane potential changes of the stained cell. These changes are monitored using a CCD camera (DalStar, Dalsa, Colorado Springs, USA; data acquisition rate 200 Hz) placed above the ganglion. Voltage-sensitive dye imaging permits the visualization of neuronal activity with a millisecond time resolution and a spatial resolution of ~50-100 micrometer. At a given recording site the optical signal strongly correlates to changes in membrane potentials measured with intracellular electrodes (Salzberg et al., 1973; Salzberg et al., 1977; Grinvald et al., 1977; Grinvald et al., 1981). However, the optical signal represents the sum of membrane potential changes, in both pre- and post-synaptic neuronal elements, as well as a possible contribution from the depolarization of neighboring glia cells. Therefore, since the optical signals measure the integral of the membrane potential changes, subthreshold potentials in extensive dendritic arborization are detected by optical recordings. Real-time optical imaging of neuronal network activity is a particularly attractive technique for providing new insights to the spatio-temporal aspects of the function of the mammalian brain. In summary the three major advantages of this method are: 1) direct recording of the summed intracellular activity of neuronal populations (including dendritic and axonal processes) 2) the possibility of
3. Materials and Methods

repeated measurements from the same animal with different stimulus conditions 3) high temporal recording resolution of neuronal population activity pattern.

For detection of intrinsic signals, the trigeminal ganglion was illuminated with red light (630 nm). Data acquisition consisted of 22 frames (400 ms each). Stimulus onset was synchronized with the end of the second frame (800 ms).

For electrophysiological experiments, single- / multiunit recordings were carried out using tungsten electrodes. Penetrations under a microscope were guided by an image of the ganglion taken under green illumination to emphasize the blood vessel pattern. The signal was filtered between 0.1 and 3 kHz and digitized at 25 kHz via a PCI-6601 National Instruments board. Data acquisition was controlled with in-house programs, and spike sorting was performed using Multi Spike Detector (Alpha-Omega, Israel).

3.2.3. Stimulus Delivery

Figure 4.4.1 schematically illustrates olfactometer construction. All olfactometer tubes were built entirely of Teflon. The olfactometer is divided into two parts: One part continuously delivers a clean air background at 1 l/min. The other part (operated at 0.4 l/min) consists of a control line (saturator tube containing *aqua dest.*) and saturated vapor lines called odor lines (saturator tubes containing different odorants) which all open out to the constant background stream in a small mixing chamber. The mixing chamber ends in a single odor tube right in front of the animal. To prevent other than nasal trigeminal afferent stimulation (e.g. corneal or oral) this odor tube was tightly adjusted to the nostril ipsilateral to the imaged ganglion. The reverse tracheotomy was used to control for a smooth constant air flow through the nasal cavity.

To avoid cross-contaminations the olfactometer uses dedicated lines for each odorant. A simultaneous switch between the control line and one of the saturated vapor lines ensures a constant total volume flow rate of 1.4 l/min. Instead of an artificial sniffing rhythm, a constant air flow was applied in order to avoid any other than odor related trigeminal stimulations. This is of special importance since it cannot be excluded that the stop flow of an artificial sniffing rhythm would cause mechanical trigeminal activation.

For automated stimulus delivery the valves (Buerkert, Germany) controlling the olfactometer were coupled via TTL pulses to the optical imaging interface. 200 ms after the start of the recording the stimulus was switched on and stayed on during the
whole imaging period. Simuli were presented in random fashion. An interstimulus
time of 20 sec between different conditions and approximately 3 min before returning
to the same condition should be more than sufficient to avoid adaptation, since it has
been previously shown, that the nasal trigeminal system regains sensitivity also with
very brief stimulus interruptions (300-500 ms) (Wise et al., 2003).

3.2.4. Odor Concentration
The control line passed through a saturator tube filled with distilled water. Odor lines
1 and 2 were passing through stimulus-filled saturator tubes. The stimulus-saturated
air stream (and control air stream) mixed with clean background air in the mixing
chamber. The sum of the flow through the two olfactometer parts was held constant at
1.4 l/min. Following stimulus concentration were used in the saturator tubes: Ethanol
either undiluted or diluted 50% in distilled water; citral, 1 mM (20 mM); vanillin, 1
mM. CO₂ was used at a final concentration of ~30%.

3.2.5. *In vivo* Trigeminal Ganglion Drug Application

*In vivo* drug application to the trigeminal ganglion was performed in analogy to the
loading with voltage-sensitive dyes. However, the total amount of injected solution
was less than 500 nl. Drugs were dissolved in a standard pipette solution at indicated
concentrations. After drug application the VSD staining was controlled.

3.2.6. Nasal Drug Application
The odor tube was removed and the nasal cavity of the rat was flushed with a 20 mM
acetazolamid (Sigma) solution (dissolved in saline). The flushing procedure was
repeated several times for at least 20 min. In control measurements, the same
procedure was applied with saline lacking the drug.

3.2.7. Data Analysis
To remove slow common noise, we obtained the time courses of evoked activity by
subtracting the average value at each pixel before stimulus onset from each pixel
value. To correct for the fast heart beat noise and respiration noise and to obtain the activation maps (in units of fractional change of fluorescence intensity $\Delta F/F$), we divided the resulting values by the activity recorded during the ‘no-stimulus’ condition. Such data reflect evoked population neuronal activity, as confirmed by intracellular recordings (Sterkin et al., 1998; Grinvald et al., 1999). To calculate statistical significance (z-score), we divided each pixel by its standard deviation calculated over the blank conditions, after subtracting its mean.
3. Materials and Methods

3.3. Cell Cultures

3.3.1. Primary Cell Culture of Trigeminal Ganglion and Trigeminal Brainstem Neurons

Primary cell cultures of trigeminal neurons were established as described in Spehr et al., 2004. Primary cell cultures of trigeminal brainstem neurons were newly established. New-born mice (P0 - P5) were decapitated and the trigeminal ganglia as well as the trigeminal brainstem complex were excised under a binocular. The tissue was washed in phosphate buffered saline solution (PBS, Invitrogen) and collected in cold Leibowitz Medium (L15, Invitrogen). For infected animals ganglia / brainstem cells ipsilateral to the site of inoculation were kept separately from the contralateral ones. The tissue was cut into small pieces and incubated (37 °C, 95% air, 5% CO₂) for 45 min (20 min) in warm Dulbecco’s modified essential medium (D-MEM) containing 0,025% collagenase (type IA, Sigma) or Trypsin / EDTA (Invitrogen) for trigeminal ganglion neurons and trigeminal brainstem neurons, respectively. The tissue was gently titrurated with a fire-polished glass pipette and the suspension was centrifugated at 200 g for 8 min. The obtained pellet was resuspended in culture medium with the following composition: D-MEM/F-12 (1:1) with Glutamax (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 µg/ml penicillin / streptomycin and 400 µg/ml foscarnet (FO). Foscarnet supplement was omitted for all trigeminal mono- and cocultures of the keratinocyte project.

For calcium imaging experiments and patch clamp recordings cells were plated on poly-L-lysine (Sigma) (0,01%) coated petri dishes (Falcon, BD Biosciences, Heidelberg, Germany). Cells were kept in a humidified atmosphere (37 °C, 5% CO₂). One hour after plating, 2 ml culture medium was added to each dish.

Long-term maintenance of neurons in culture (several days to weeks) and presence of NGF has been shown to significantly alter the phenotype of neurons, particularly with respect to capsaicin sensitivity (Bevan and Winter, 1995; Winston et al., 2001). Therefore, the neurons in this study were maintained for the shortest period possible to keep the experiments close to in vivo conditions. Neurons were grown for 1 day up to 3 days before being used. Most recordings were made within 36 h of preparation. Cells were cultured without addition of NGF to the cell medium.
3.3.2. Primary Human Keratinocyte Cell Culture / Coculture Approach

Primary human keratinocytes were kindly provided by Prof. Dr. L. Steinsträßer and Dr. F. Jacobsen (Klinik für Plastische Chirurgie und Schwerbrandverletzte, BG-Kliniken Bergmannsheil, RUB). Locally prepared cell culture material was obtained from human surgery in which parts of the skin had to be removed (e.g. amputations). Cultivated cells were then transferred to the Dept. of Cellphysiology.

Keratinocytes were kept in flasks (T-75, Gibco, Germany) in an incubator (Heraeus) at 37 °C, 5% CO₂ and 95% air with 10 ml of the accordant culture medium. Growth of cells was controlled using a conventional light microscope. At 70% confluency, cultures were trypsinated (Gibco, 1 ml 1x trypsin + EDTA, 10 min) splitted 1:10 and disseminated into new flasks.

For calcium imaging experiments cells were plated on petri dishes (Falcon, BD Biosciences, Heidelberg, Germany) and kept in a humidified atmosphere (37 °C, 5% CO₂). For trigeminal neurons / keratinocyte cocultures, two days after keratinocyte plating, the culture medium was removed from each dish and 750 µl D-MEM/F-12 containing a high density of trigeminal neurons (from a fresh ganglion preparation) was added. One hour later, 1,25 ml culture medium was added to each dish. Control keratinocyte monocultures were treated in the same way, omitting trigeminal neurons.

3.3.3. Cell Lines: HEK293, PK15 and MDBK

Cell cultures were processed in sterile environment using a clean bench and autoclaved instruments as well as sterile filtered media and buffers. HEK293, PK15, and MDBK cells were kept in flasks (T-75, Gibco, Germany) in an incubator (Heraeus) at 37 °C, 5% CO₂ and 95% air with 10 ml of accordant culture medium. Growth of cells was controlled using a conventional light microscope. At 90% confluency, cultures were trypsinated (Gibco, 1 ml 1x trypsin + EDTA, 10 min) splitted 1:10 and disseminated into new flasks.

To establish cryo stocks of HEK293, PK15 and MDBK cells, confluently grown cells were detached from the flasks by trypsin treatment (see above) and solubilised in 10 ml minimal essential medium. Cells were centrifuged (Minifuge RF, Heraeus) for 5 min at 800 U/min and the supernatant was removed. The cell pellet was resuspended...
in 900 µl culture medium. 100 µl DMSO was added. Each cryo tube was filled with 500 µl of the cell suspension and was kept in styrofoam at -72 °C for 24 h. Permanent storing was done in liquid nitrogen (-196 °C). When necessary, cryo tubes were thawed at room temperature and cells were washed in 10 ml culture medium. After centrifugation for 5 min at 800 U/min the supernatant was removed and cells were placed with 10 ml fresh culture medium into a flask.

3.3.4. Transient Transfection of HEK293 Cells

For heterologous protein expression HEK293 cells were plated in cell culture dishes (Falcon, BD Biosciences, Heidelberg, Germany) with 2 ml growth medium 3 - 4 days before transfection. Cells were transiently transfected with expression plasmids containing inverse perecam, GCaMP2 or cmgaroo-2 cDNA using calcium phosphate precipitation method (see below). Before transfection, 650 µl of medium were removed from each culture. 100 µl of the transfection reagent was applied drop wise to each culture dish. 3 – 4 h later, cells were washed 2 times with PBS ++ and 2 ml of fresh culture medium was applied.

**Transfection reagent for 5 cell culture dishes**

- H₂O sterile: 219 µl
- Plasmid: 6 µg
- 2,5 M CaCl₂: 25 µl
- 2xHBS: 250 µl

Incubate for 10 min at room temperature before use.
3.3.5. Growth Media and Buffers for Cell Cultures

**Minimal-Essential-Medium - PK15 / MDBK**

Minimal-Essential-Medium (Eagle) including Hanks-Salts + non-essential AA/
Minimal-Essential-Medium (Eagle) including Earl-Salts + non-essential AA
1:1  
(Sigma)

- NaHCO$_3$ 1,25 g/l
- Na-Pyruvat 120 mg/l
- pH 7.2 – 7.4
- sterile filtering
- FBS 10%
- Antibiotic / Antimycotic Solution* 1:100
- keep at 4 °C

*10000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml

**Minimal-Essential-Medium - HEK293**

- D-MEM (Gibco)
- MEM: non-essential AA (Gibco) 1:100
- FBS 10%
- Penicillin / Streptomycin (Gibco) 1:100

**Keratinocyte Medium (100 ml)**

- D-MEM(Gibco) 59 ml
- F-10 30 ml
- FBS 10%
- Penicillin/Streptomycin (Gibco) 1%
- Isoproterenol 1 µM
- Adenin 24,3 µg/ml
- Hydrocortison 0,8 µg/ml
- hEGF 20 ng/ml
- Triiodothyronin 1,346 ng/ml
- Insulin 5 µg/ml
### Methocel Medium

Methyl cellulose (Fluka) 10 g

Autoclavable minimal essential medium (Gibco) 3.76 g

A. bidest 390 ml

Resuspending and autoclaving, cooling to room temperature

200 mM L-glutamin 4 ml

NaHCO3 880 mg in 6 ml a. bidest, sterile

keep at 4 °C

For use, the methocel medium is mixed 1:4 with the growth medium

### PBS⁻

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<td>Na₂HPO₄</td>
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pH 7.2

### PBS⁺⁺

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pH 7.3 – 7.5
3. Materials and Methods

3.4. The Pseudorabies Virus

3.4.1. PrV Strains

For an anterograde transsynaptic tracing in the murine somatosensory system up to the trigeminal brainstem complex a wild type related strain, PrV-KaΔgGgfp was generated according to PrV-ΔGCam used in an earlier study (Damann et al., 2006). Expression of the reporter gene (green fluorescent protein; [GFP]) was controlled by the human cytomegalovirus immediate-early 1 promoter / enhancer (PhCMV), inducing transcription of DNA in the early stages of infection. For tracing primary TGNs from the nose, no transsynaptic passage of viral particles was needed and therefore, data obtained with PrV-KaΔgGgfp infection could additionally be compared with data obtained following tracing neurons with the retrograde tracer PrV-Bartha (PrV-ΔGCam). PrV-ΔGCam comprises the gene for the Yellow Cameleon (YC2.1) (Miyawaki et al., 1997). YC2.1 was inserted into the gG locus thereby substituting a 196bp BamHI fragment in the nonessential gG gene. Three different PrV-Bartha based strains were generated (also according to PrV-ΔGCam) for fluorescent calcium indicator protein (FCIP) expression. PrV-Bartha_IP carries DNA for inverse pericam (Nagai et al., 2001). Inverse pericam was generated from a circularly permuted green fluorescent protein (cpGFP) in which the amino and carboxyl portions had been interchanged and reconnected by a short spacer between the original termini. The cpGFP was fused to calmodulin and its target peptide, M13. The chimeric protein, named “pericam”, is fluorescent and its spectral properties change reversibly with the amount of Ca$^{2+}$, probably because of the interaction between calmodulin and M13 leading to an alteration of the environment surrounding the chromophore. Different types of pericam could be obtained by mutating several amino acids adjacent to the chromophore. Of these, “flash pericam” became brighter with Ca$^{2+}$, whereas “inverse pericam” dimmed.

PrV-Bartha_GCaMP2 carries DNA for GCaMP2 (Nakai et al., 2001). GCaMPs are high-affinity Ca$^{2+}$ probes composed of a single GFP. The N-terminus of cpEGFP is connected to the M13 fragment of myosin light chain kinase which is a target sequence of calmodulin, whereas the C terminus of cpEGFP was connected to CaM (calmodulin). When Ca$^{2+}$ binds to CaM conformational changes due to the Ca$^{2+}$–CaM–M13 interaction induce a subsequent conformational change in cpEGFP so that the fluorescence intensity changes.
PrV-Bartha_Cam2 carries DNA for camgaroo-2 (Baird et al., 1999; Griesbeck et al., 2001). Camgaroo-1 is a genetically encoded Ca$^{2+}$ indicator consisting of *Xenopus* calmodulin inserted in place of residue 145 of EYFP-Q69K. However, camgaroo-1 unfortunately expressed poorly at 37 °C. Camgaroo-2 was generated by a random mutation in the cDNA encoding camgaroo-1. Sequencing of the brightest clones (camgaroo-2) revealed just one new mutation, replacement of residue 69 (Gln in wild type, Lys in EYFP V68L/Q69K) by Met.

The reporter genes were controlled by the human cytomegalovirus immediate early (IE) 1 promoter / enhancer (PhCMV) inducing marker protein expression in early stages of infection.

The FCIP construct inverse pericam was kindly provided by Dr. A. Miyawaki (Laboratory for Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan). The FCIP construct GCaMP2 was kindly provided by Dr. J. Nakai (Laboratory for Memory and Learning, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan). The FCIP construct camgaroo-2 was kindly provided by Dr. O. Griesbeck (AG Zelluläre Dynamik, Max-Planck-Institut für Neurobiologie, Am Klopferspitz 18, 82152 Martinsried, Germany) and Nobel laureate R. Y. Tsien (Department of Pharmacology and Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093-0647; United States)

All viral constructs were generated and kindly provided by Prof. Dr. Thomas Mettenleiter and Dr. B. Klupp (Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Insel Riems, Germany)

### 3.4.2. Production of Viral Stocks

The PrV strains were propagated in monolayers of PK15 cells, allowing production of high titered viral suspensions (Card and Enquist, 2001). For the production of viral suspensions from PrV-DNA, PK15 cells were grown in T-75 flasks to a confluency of 80% and were transfected with viral DNA using calcium phosphate precipitation. 500 µl of the transfection reagent containing the viral DNA was prepared according to the protocol for transfecting HEK293 cells (see 3.3.4). 4 ml of the 10 ml growth medium were removed from PK15 cells and 500 µl of the transfection reagent was added. After
4 h incubation (37 °C, 5% CO₂) the supernatant was replaced by fresh growth medium. Two days after transfection slight cytopathic effects indicated initiation of viral replication and the growth medium was replaced by fresh medium. Five to six days after transfection CPE (cytopathogen effect) was at 100%. Viral titres sufficient for inoculation were achieved after a second round of replication. Therefore, 1 to 2 ml of the supernatant was applied to a new T-75 flask containing PK15 (80% confluent). High titered suspensions could be harvested from these cell cultures after three days and were aliquoted into 100 μl stocks.

In order to produce high titered suspensions from frozen stocks, PK15 cells were grown in T-75 flasks to a confluency of 80%. The growth medium was removed. Frozen PrV aliquots were removed from the freezer and kept on ice until usage. 1 ml viral suspension providing a MOI (multiplicity of infection) of 0.1 to 10, was applied to the PK15 cell culture. Every 15 min flasks were moved to ensure uniform distribution of virus on the cell monolayer. After 1 h of incubation (37 °C, 5% CO₂) the supernatant was removed, cells were washed with PBS++ and 10 ml of fresh medium was added. 2 to 3 days after infection, cells revealed complete CPE and showed round and lysed cells. To release intracellular virions, cells were frozen and thawed 3 time (-72 °C; 37 °C). Cell debris were centrifugated 5 min at 3000 U/min, 4 °C and the supernatant, containing ~5x10⁸ PFU/ml, was aliquoted in 100 μl volumes and stored at -80 °C.

3.4.3. Plaque Assay and Determination of Viral Titre

A plaque assay was used for quantitative detection of infectious virus particles (titer calculation). Determination of viral titres was performed using 90% confluent MDBK cells in 6-well cell culture dishes (Nunc). 6 diluted frozen stocks ranging from 10⁻³ to 10⁻⁸ were generated in 2 ml Eppendorf tubes. MDBK cells were incubated for 1 h with 500 μl of the diluted suspensions and slightly moved every 15 minutes. Subsequently, the medium was taken from the cell culture, unabsorbed virus was removed by washing with PBS++ and 2 ml viscose methocel medium was added. Three days later, cells were fixed for 15-30 minutes at room temperature with 4% PFA, washed with water and stained using crystal violet solution. After repeated washing with water, dishes were dried and plaques were counted in those dishes with the highest number of countable plaques. The plaque sum was divided by the volume that was applied and
multiplied with the reciprocal value of the dilution step. The titre was calculated in PFU (plaque forming units)/ml and determined two times. Only stocks with a titre between $10^8$ and $10^9$ were used in further experiments.

3.4.4. Inoculation Procedure

For intranasal inoculation, animals were placed on their backs and a 2 µl (P0 - P5 mice)/5 µl (adult rats) drop of viral suspension (~$10^6$ PFU) was applied to the right nostril. Adult animals were slightly anesthetised for this procedure by a small amount injection of Chloralhydrat (intraperitoneal). Viral suspension was sucked up into the nasal cavity by regular breathing. Animals were kept in that position for a few minutes to ensure contact of the inoculum with the nasal epithelium.

Experimental in vitro infection of neuronal cultures was performed 24 h after plating at a multiplicity of infection of 100 PFU/cell, using a 1 h incubation at 37 °C, followed by two changes with growth medium containing 10% serum to remove unabsorbed virus. Infected and uninfected cultures were incubated in growth medium containing foscarinet (FO, Fluka) (400 µg/ml) at 37 °C for up to 2 days before experiments were performed.

3.5. Histology

3.5.1. Whole-Mount Preparation

At various time-points after infection, rats were sacrificed by an overdose of anaesthetics and the brain was removed from the skull. Ganglia remained in the base of the skull. Tissue was stored in 4% PFA in 0,1 M PBS at 4 °C until examination (epi-fluorescence, confocal or multiphoton laser-scanning microscopy).

3.5.2. Cryosections

At various time-points after infection, mice (P1 - P5) were killed by decapitation. Skin as well as the lower jaw and front teeth were removed and the head was brought into 4% PFA immediately. To rid the nasal cavity from residual air that might prevent proper fixation of the olfactory epithelium the PFA solution containing the mouse
heads was degassed in a vacuum incubator for 45 minutes. For cryoprotection tissue was placed in 10, 20 and 30% sucrose solution for 30 minutes respectively. After that it was embedded in Tissue Tec freezing medium onto specimen stages. Embedded heads were stored at -70 °C until they were processed further. Cryosectioning was performed with a Leica CM3050S cryomicrotome. Coronal sections of 10-12 µm thickness were mounted on superfrost microscope slides (Menzel) and dried at 42 °C for 30 minutes. Sections were stored at 4 °C until examination.

3.5.3. Fixatives

*Paraformaldehyde (4%)*

- A. bidest 400 ml
- Paraformaldehyde (PFA) 40 g
- NaOH 2 pills

solve at 70 °C, filter after cooling

pH adjustment (HCl) to 7,4

PB 0,2 M 500 ml

A. bidest ad 1 l

can be stored for 1 week at 4 °C

3.6. Epifluorescence, Confocal and Multiphoton Laser-Scanning Microscopy

Markerprotein fluorescence was detected using epifluorescence (exciter: 470/40; dichroic mirror: 495 DCLP; postfilter: 515 LP) or confocal microscopy (LSM510 Meta, Zeiss) using a Argon laser at 488 nm emission. The laser was used in combination with a HFT 488 mirror. Fluorescence from the excited specimen was filtered by BP 500-550 mirrors.

Two-photon fluorescence within whole mount preparations of ganglia was excited by a mode-locked Ti:Sapphire laser (Mai Tai, 100 fs, 80 MHz; pumped by a 5-W CW laser; 860 nm; Spectra Physics; Germany) coupled to a laser scanning microscope (LSM510 Meta, Zeiss) fitted with a 40x objective lens (Zeiss). A dichroic mirror (HFT KP 650) was inserted to the back aperture of the objective to reflect emitted light through detection optics and an emission filter (BP 500–550 IR) onto the
photomultiplier. Image acquisition was controlled by custom software (Carl Zeiss AIM).

3.7. Imaging of Intracellular Calcium Levels

To study stimulus-induced changes of the intracellular calcium concentration, cells were loaded for 45 minutes at 37 °C with 3 µM Fura-2 AM (Molecular Probes) solved in Ringer’s solution. This step was omitted for trigeminal neurons infected with the PrV-FCIP constructs.

Fura-2 is a widely used UV-excitable fluorescent calcium indicator. Upon calcium binding, the excitation maximum of the indicator dye undergoes a blue shift from 363 nm (Ca\(^{2+}\)-free) to 335 nm (Ca\(^{2+}\)-saturated), while the fluorescence emission maximum is relatively unchanged at ~510 nm. The indicator is excited at 340 nm and 380 nm respectively and the ratio of the fluorescent intensities corresponding to the two excitation wavelengths can be used to visualize shifts in the intracellular calcium concentration.

Cell culture dishes were placed under the microscope and continuously perfused with Ringer’s solution (standard assay buffer, 450-500 µl /min). Perfusion could be switched to stimulus / drug application to transiently superfuse the cells. Chemicals were also dissolved in assay buffer.

Measurements were performed using the Olympus Cell^R Imaging Station consisting of an Olympus IX71 microscope, a MT20 illumination system with a 150W Xenon arc burner and a F-view II CCD camera (all Olympus Biosystems GmbH, Munich, Germany) connected to a computer. Pictures were taken using an Olympus CPLFLN 10xPH objective that allowed a wide field of view in the measurements combined with good fluorescence characteristics. For acquisition of data points region of interest (ROI) were defined in the phase contrast pictures. Only cells exhibiting a typical neuron / keratinocyte morphology were included in the analysis. One sampling cycle consisted of one frame imaged with an excitation wavelength of 340 nm and one with 380 nm (1-2 Hz). Fluorescence ratios of both frames were calculated and ratios of defined ROIs were presented as online kinetics.

Stimuli were applied for 15 s followed by an interstimulus interval of 45 s or 2 min 45 s for screening and blocker experiments, respectively. At the end of each measurement cells were exposed to a 300 µM ATP solution and, separated by a brief interstimulus interval, additionally to a 45 mM potassium chloride solution, to test for cell viability.
Data acquisition and analysis were performed using Olympus Cell^R/Cell^M Software, while traces were calculated using Origin. Imaging traces represent 340/380 ratios.

Functional FCIP expression in HEK cells as well as functional virally mediated FCIP expression in infected trigeminal neurons was investigated using the laser scanning microscope (2.5-5 Hz; laser-excitation: 488 nm; emission detection range: 504 to 536 nm). Image acquisition was controlled by custom software (Carl Zeiss AIM).

**Standard Assay Buffer**

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<th>Solvent</th>
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**Stock solutions**

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**Chemicals (used in 4.3)**

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<tr>
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</tr>
<tr>
<td>Helional</td>
<td>3-(1,3-benzodioxol-5-yl)-2-methylpropanal</td>
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3.8. Patch Clamp Recordings

Electrophysiological recordings were performed using the whole-cell mode of the patch clamp technique. Cells were maintained in an extracellular recording solution equivalent to the standard assay buffer used in calcium imaging (see 3.7). Patch electrodes were pulled from borosilicate glass (1.2 mm O.D. x 1.17 mm I.D., Harvard apparatus, Edenbridge, Kent, UK) and fire polished to 6 - 8 MΩ tip resistance using a horizontal pipette puller (Zeitz Instr., Munich, Germany). Patch clamp recordings of trigeminal and brainstem neurons were carried out at room temperature. For recordings a HEKA EPC9 amplifier was used. Adjustments to the capacity and series resistance were made by using the build in compensation algorithm of the amplifier. Membrane potential of the examined neurons was held at -60 mV. Data were acquired using Pulse software and were filtered using a 2.9 kHz Bessel filter.

Pipette solution for patch clamp recordings from trigeminal and brainstem neurons

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3.9. Statistical Analysis

Statistical significance (*: p < 0.05; **: p < 0.01) was assessed by Student’s t test. For statistical calculation WinStat was used. Unless stated otherwise, “±” following numbers of values or percentages, indicates the standard deviation.
3. Materials and Methods

3.10. Human Psychophysical Experiments

The human psychophysical tests represent a collaboration project with Prof. Dr. Thomas Hummel (Department of Otorhinolaryngology [Smell and Taste Clinic] University of Dresden Medical School; Fetscherstrasse 74; D-01307 Dresden, Germany). All psychophysical experiments were performed in Dresden by Prof. Dr. Thomas Hummel and Artin Arshamian (Stockholm University; SE-106 91 Stockholm). Permission for publication of the data in this PhD thesis was kindly granted by Prof. Dr. Thomas Hummel and Artin Arshamian.

3.10.1. Experiment 1: Threshold Tests for CO\(_2\) and Citral Combination

Subjects

Twenty four subjects attended this study (20 healthy subjects of which 12 women, mean age 26, 1 and 4 anosmic subjects of which one women, mean age 41, 7). Ten of the healthy subjects performed one experiment while the remaining ten healthy and four anosmic patients performed two.

Procedure

During experiment one, the thresholds for CO\(_2\) and citral were determined for each subject individually by using ascending and descending concentrations for each substance. Staircase concentrations were used starting from 10% while adding 5% to every step (i.e. 10%, 15%, 20%, 25% etc.).

For example, when a subject was tested for CO\(_2\) and notified that the 25% concentration gave a trigeminal sensation then that concentration (25%) was stated as the threshold level for that subject. Subsequently, 20% CO\(_2\) was affirmed as the subthreshold and 30% as the suprathreshold concentration for that subject. The same procedure was carried out with citral. For this respective subject 25% was the subthreshold, 30% the threshold, and 35% the suprathreshold concentration for citral. For determining the citral threshold healthy subjects were trained to separate between the odor of citral and a potential trigeminal activation.

After the individual thresholds were set, three different CO\(_2\) and citral combinations were tested.
3. Materials and Methods

For example (subject above):

1. Subthreshold CO₂ (20%) in combination with subthreshold citral (25%).
2. Subthreshold CO₂ (20%) in combination with threshold citral (30%).
3. Subthreshold CO₂ (20%) in combination with suprathreshold citral (35%).

Subjects were asked to notify the experimenter when they felt a trigeminal sensation, and each combination was tested several times.

The stimulus duration was always 250 ms in every healthy subject. For the anosmic subjects the duration time of the stimulus was considerably longer than compared to healthy subjects. For both CO₂ and citral a 1000 ms duration time had to be applied until threshold values were reached.

3.10.2. Experiment 2: Suprathreshold CO₂ Exposure Followed by Suprathreshold Citral

Subjects

Fourteen subjects (4 anosmic) attended this experiment.

Procedure

Establishing Suprathreshold concentration

A 60% suprathreshold citral concentration (the highest possible (stabile) concentration) was used as the anchor to which the individual CO₂ concentrations had to be adjusted. Each subject rated the intensity of the 60% citral using an ascending trigeminal intensity scale from 1 to 10. After deciding the intensity of citral, subjects were given a series of CO₂ concentrations, and were told to match the citral intensity to one of the CO₂ concentrations and subsequently rate it on the same intensity scale. To obtain high trigeminal activity, stimulus duration of 1000 ms was used for both citral and CO₂.
Suprathreshold delivery

Subjects were given the (individual concentration) 1000 ms CO\textsubscript{2} stimulation and directly after that the 60% citral concentration. They were asked to only rate the intensity of the trigeminal part of citral using the same scale as above. This procedure was carried out several times.

The time period from the delivery of the CO\textsubscript{2} to the notification that citral had arrived was approximately 9 seconds. As a control condition the opposite procedure and order was used, i.e. citral was delivered for 1000 ms and was followed by a 1000 ms deliverance of CO\textsubscript{2} and a subsequent intensity rating of the latter.
4. Results

4.1. Anterograde Transsynaptic Tracing in the Murine Somatosensory System using PrV

PrV’s neurotropic nature and its property to spread within synaptically connected neurons have been exploited in numerous tract tracing studies in mammals, in order to define functional neuronal circuits. PrV-Bartha, an attenuated live-vaccine strain proved to be particularly appropriate for this purpose because of its reduced virulence and specific transneuronal spread in the retrograde direction of information processing within the nervous system (Enquist, 2002). However, identification of CNS circuits involved in processing of sensory information requires anterograde transneuronal tracing from peripheral sensory organs towards the CNS. Both retrograde and anterograde spread can be accomplished by wild-type α-herpesviruses (Sabin, 1938), like PrV-Kaplan, providing a potential tool for anterograde tracing in sensory neuronal systems.

Single trigeminal neurons mediate sensations from selective areas of the head (receptive fields) and are specialized for different qualities of somatosensory information (modalities) (Patapoutian et al., 2003). Our current knowledge about differentiated processing of this “where” and “what” information on the cellular level is limited due to the problem of identification of and accessibility to single neurons of a defined destination. Recently, it has been shown that PrV-Bartha strains could be utilized to identify TGNs that innervate the mucous membranes of the murine nasal cavity or the epithelial cells of the facial skin (Damann et al., 2006). Electrophysiological analysis of these neurons in cell culture could solve the puzzle of their chemosensory capabilities, displaying neuronal features which depend on their peripheral innervation pattern. However, postsynaptic neurons in the brainstem, the first relay for trigeminal information processing, could not be identified due to impeded anterograde transsynaptic spread of Bartha strains. Identification of synaptically connected higher order neurons in vitro would allow easy access to these cells and would facilitate a detailed physiological characterization in order to further our understanding of mechanisms underlying differentiated trigeminal somatosensation.

This study was designed to assess the suitability of PrV-Kaplan for transsynaptic tracing within somatosensory circuits in the trigeminal system. The data are compared
to results from a tracing approach using PrV strain Bartha, which already proved to be an appropriate “live-cell”-tracing tool (Damann et al., 2006). Basic electrophysiological properties of identified neurons in primary cell culture were analysed.

0-5 day old Swiss CD-1 mice were unilaterally intranasally inoculated with 2 µl of high titered PrV-KaΔgGgfp (resulting in ~10⁶ pfu). Cryosections through the head of infected animals sacrificed at different time points after infection (6, 8, 10, 12, 16, 20, 24, 36, 42, 47, 51 and 55 hours post infection (hpi)) were analyzed for GFP fluorescence in order to determine the onset of marker protein expression at different levels of the trigeminal system. GFP fluorescence was detected in the ipsilateral trigeminal ganglion at 12 hpi and in the brainstem at 36 hpi (Fig. 4-1 A,D). The time indicated represents the earliest time point of incidence of fluorescent cells. These findings indicate transsynaptic spread of PrV-Kaplan to neurons of higher order and underline the capability of PrV wild-type strains to ascend in the anterograde direction within the nervous system of mice. At later stages, marker protein fluorescence indicated presence of virus also at the contralateral side of infection (after 24 and 47 hpi in the trigeminal ganglion and the brainstem, respectively; data not shown).

Next, the appropriate time windows for preparation of trigeminal and brainstem neurons, that allow identification of virally labelled neurons in vitro, were determined. Primary cell cultures of trigeminal and brainstem neurons were prepared at 12 hpi and 36 hpi, respectively (Fig. 4-1 B,E). Immediately after plating, fluorescence was observed in few trigeminal neurons allowing identification of nasal TGNs, similar to recent findings using PrV-Bartha strains (Damann et al., 2006). Preparing mice at 36 hpi, cultures of brainstem neurons contained few cells showing GFP fluorescence, identifying higher order neurons that are involved in trigeminal sensory processing. The number of fluorescent neurons within trigeminal and brainstem cell cultures further increased within the next hours of culturing, a fact that is most likely due to emerging GFP expression in neurons that were reached by PrV very shortly before tissue preparation. In order to prevent secondary viral infection in the culture dish, the cell culture medium for trigeminal and brainstem cell cultures were supplemented with the direct non-competitive viral DNA-polymerase inhibitor foscarnet (400 µg/ml) as shown previously (Damann et al., 2006). Under these conditions, plating of dissociated trigeminal and brainstem cells allowed reliable in vitro-identification of in vivo-traced fluorescent cells.
The general appearance of traced cultured neuronal cell bodies in phase contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only (Fig. 4-1 C,F).

**Fig. 4-1: Tracing of trigeminal neurons and synaptically connected higher order neurons in the brainstem.**
A) Representative cryosection through the head of an intranasally infected mouse (P3; Bregma ~ 0 mm). Red square indicates area of marker protein expression in the ipsilateral trigeminal ganglion. Enlarged view: earliest incidence of infected trigeminal neurons in cryosections through the head of an animal sacrificed 12 hpi. At 42 hpi a massive infection of the trigeminal ganglion could be detected.
B) Phase contrast light microscopy of trigeminal neuron in primary cell culture 2 days after plating.
C) Fluorescence image showing traced trigeminal neuron 3 days after plating. General appearance of traced cultured neuronal cells in phase contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only.
D) Representative cryosection through the head of an infected mouse (P4; Bregma ~5.8 mm). Red square indicates area of marker protein expression in the ipsilateral trigeminal brainstem complex. Enlarged view: earliest incidence of infected brainstem neurons in cryosections through the head of an animal sacrificed 36 hours post infection. At 51 hours post infection a massive infection in the trigeminal brainstem complex could be detected.
E) Phase contrast light microscopy of brainstem neurons in primary cell culture 4 days after plating.
F) Fluorescence image showing a traced brainstem neuron 5 days after plating. General appearance of traced cultured neuronal cells in phase contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only. Bars in A: 1000 µm (overview); 20 µm, 250 µm (enlarged view; left and right, respectively); B, C and E: 20 µm; D (overview): 600 µm; 10 µm, 100 µm (enlarged view; left and right, respectively); F: 10 µm
To investigate a possible influence of viral infection on cellular physiology, whole-cell patch-clamp recordings of labelled TGNs and brainstem neurons were performed. Experiments were designed to compare traced cells with non-infected control neurons. For tracing primary TGNs from the nose, no transsynaptic passage of viral particles was needed and therefore, data obtained with PrV-KaΔgGfp infection could additionally be compared with data obtained following tracing neurons with the retrograde tracer PrV-Bartha (PrV-ΔGCam). Cells were recorded from one to four days *in vitro* (div). Within one experimental group, data obtained from different time points revealed no significant differences and therefore could be combined for statistical analysis. Electrophysiological data obtained from TGNs of different experimental groups (traced with PrV-Bartha, traced with PrV-KaΔgGfp, non-infected control) were analyzed and tested for significance using an unpaired Student’s t-test. Data from brainstem neurons traced with PrV-KaΔgGfp were compared with recordings from uninfected control neurons of the same brain region. Significance was defined for p < 0.05. Exemplary recordings from traced and uninfected control cells are illustrated in Fig. 4-2 A and B for trigeminal and brainstem neurons, respectively.

Fig. 4-2: Electrophysiological recordings of infected and un-infected trigeminal and brainstem neurons.
A) Representative traces from patch-clamp recordings of trigeminal neurons. Traced TGNs (PrV-Kaplan, left) could not be distinguished from uninfected control neurons (right). Upper picture: Action potentials induced by current injection (50 pA). Lower picture: Ih channel activation in current-clamp mode. Lower picture insert: injection of currents ranging from −20 pA to −80 pA; duration 2s; Bars: upper picture: 50 mV/20 ms; lower picture: 40 mV/650 ms.
B) Representative traces from patch-clamp recordings of brainstem neurons. Traced brainstem neurons (PrV-Kaplan, left) could not be distinguished from uninfected control neurons (right). Action potentials induced by current injection (50 pA). Bars: 25 mV/10 ms.
The resting membrane potential of infected and non-infected trigeminal and brainstem neurons did not differ significantly. The same was true for the activation threshold of voltage-dependent sodium channels (Na_v) measured in voltage-clamp experiments. There was also no significant difference in the amplitude of the resulting inward current. In addition, recordings in current-clamp mode indicated no detectable influences of PrV on electrophysiological properties. The threshold potential to elicit action potentials was not changed between control and traced neurons. Action potential (AP) overshoots had almost equal maximal amplitudes. The width of APs at 75% of maximum amplitude was also within the same range. The threshold for activation of I_h-channels in current-clamp recordings and the amplitude of the sag (I_h-induced change in membrane potential) after hyperpolarisation to -100 mV was not significantly altered in trigeminal neurons. The data are summarized in Tab. 4-1 (trigeminal neurons) and Tab. 4-2 (brainstem neurons). In summary, none of the electrophysiological properties tested differed significantly between groups, indicating that under the used cell culture conditions (including foscarnet), PrV-KaΔgGfp-traced neurons show no detectable alterations in physiological properties when compared to uninfected or PrV-Bartha-infected neurons.
Tab. 4-1: Electrophysiological analysis of traced and un-infected trigeminal neurons.
Electrophysiological characterization of traced trigeminal neurons using either PrV-Bartha (Ba) or PrV-Kaplan (Ka), and uninfected (control, c) TGNs revealed no significant differences. APs: Action potentials; SD: Standard deviation; SEM: Standard error of the mean

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<td>33</td>
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<td>40</td>
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<tr>
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<tr>
<td></td>
<td>SD</td>
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</tr>
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<td>2.45</td>
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<td></td>
<td>range</td>
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<td>3.00</td>
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<td>Maximal amplitude (overshoot) of APs due to current injection/ mV</td>
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<td></td>
<td>mean</td>
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<td>37.9</td>
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<tr>
<td></td>
<td>SD</td>
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<td>15.3</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
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<td>11</td>
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<tr>
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<td>3.7</td>
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<td></td>
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Tab. 4-2: Electrophysiological analysis of traced and un-infected brainstem neurons using PrV-Kaplan.
Electrophysiological characterization of traced brainstem neurons using PrV-Kaplan (Ka), and uninfected (control, c) brainstem neurons revealed no significant differences. APs: Action potentials; SD: Standard deviation; SEM: Standard error of the mean

<table>
<thead>
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<th>c</th>
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<th>Membrane potential due to current injection eliciting APs/ mV</th>
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<td>mean</td>
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<td>Membrane potential/ mV</td>
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<td>Threshold for activation of VGSCs/ mV</td>
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<tr>
<td>Maximal sodium current amplitude/ nA</td>
<td>20</td>
<td>-1.92</td>
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</tbody>
</table>

SD: Standard deviation; SEM: Standard error of the mean

APs: Action potentials; SD: Standard deviation; SEM: Standard error of the mean
4. Results

4.2. PrV mediated functional Expression of Fluorescent Calcium Indicator Proteins

Pseudorabies virus strains like PrV-Bartha and its marker expressing variants have been used in numerous studies as retrograde transneuronal tracing tools, defining the synaptic organization of mammalian neuronal circuits. However, the possibilities for functional examination of virus infected neurons are limited to electrophysiological approaches or bulk loading strategies using calcium dyes. Here I report the production of recombinant viral strains using three different functional calcium indicators. As a viral system neurotropic PrV strain Bartha was chosen, since this virus has been established as a “live-cell”-tracing tool allowing a functional analysis of infected neurons (Damann et al., 2006).

Three different FCIPs were utilized: inverse pericam (Nagai et al., 2001), camgaroo-2 (Griesbeck et al., 2001) and GCaMP2 (Nakai et al., 2001). These FCIPs were selected according to their respective molecular weight and their reported magnitude of stimulus-induced fluorescence changes. The Yellow-cameleon construct was excluded from this study since in preliminary experiments the calcium dependent FRET (fluorescence resonance energy transfer) effect described for YC2.1 (Miyawaki et al., 1997) could not be observed in our heterologous expression systems (Damann et al., 2006).

Functionality of FCIP constructs was verified in HEK 293 cells. All constructs showed robust expression and a homogenous cytosolic fluorescence distribution (Fig. 4-3). Functionality of the expressed constructs was tested by stimulating the cells using ATP, which resulted in fluorescence changes for all of the three tested FCIPs. Relative fluorescence changes (ΔF/F) of up to -35% (mean -15,3 +/- 0,55 SEM n = 200 cells) for inverse pericam, up to 23,9% (mean 9,5 +/- 0,97 SEM n = 29 cells) for camgaroo-2 and up to 166,7% (mean 54,4 +/- 4,1 SEM n = 96 cells) for GCaMP2, could be observed (Fig. 4-3).
Since functionality of all calcium sensitive proteins was confirmed, three different virus strains containing the respective constructs were generated (in collaboration with Barbara G. Klupp and Thomas C. Mettenleiter; Friedrich-Loeffler-Institut, Bundesforschungsinstutit für Tiergesundheit, Insel Riems, Germany).

No differences in the kinetics of viral neuroinvasion into trigeminal ganglion neurons after nasal inoculation of mice were observed comparing the newly created viral constructs with previously used PrV-Bartha strains (data not shown).

For functional analysis of the virally encoded FCIPs, primary murine neuronal trigeminal cell cultures were in vitro infected with the different viral constructs: 24 h after plating, neuronal cell cultures were infected at a multiplicity of infection of 100 PFU/cell, using a 1 h incubation at 37 °C, followed by two changes with growth medium containing 10% serum to remove unabsorbed virus. Infected cultures were

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Fig. 4-3: Expression and functional tests of fluorescent calcium indicator proteins (FCIP) in cell culture
A-C) HEK cells transfected with inverse pericam (A), camgaroo-2 (B) or GCaMP2 (C) constructs were imaged by confocal microscopy. Top: Resting fluorescence (left) and fluorescence after ATP application (right) for the accordant FCIP constructs. Bottom: representative FCIP responses of ATP stimulated HEK cells; fluorescence changes were recorded from depicted cell somata (top row) and are indicated as ΔF; black solid line: ATP (300 µM) application; grey solid line: ringer control; scale bar: 20µm
D) Bottom: Quantification of FCIP signal amplitudes in HEK cells. Averaged fractional fluorescence changes of the tested FCIP constructs (ΔF/F). bars represent mean ΔF/F; error bars = SEM;
incubated in growth medium containing the direct non-competitive viral DNA-polymerase inhibitor foscarnet (FO, Fluka) (400 µg/ml) at 37 °C for up to 4 days before experiments were performed.

Each newly created viral strain (PrV-Bartha_IP for inverse pericam, PrV-Bartha_Cam2 for camgaroo-2 and for PrV-Bartha_GCaMP2 for GCaMP2) showed a robust FCIP expression in infected trigeminal neurons, indicated by basic fluorescence that was homogenously distributed within the cytosol of the respective cells (Fig. 4-4). In phase contrast light microscopy, the general appearance of infected neuronal cell bodies did not differ from non-infected neurons; therefore infected cells could only be identified using fluorescence microscopy. Markerprotein expressing cells displayed a typical TGN morphology with spherical and pseudounipolar shape and long processes. Emission spectra of infected cells displayed the construct specific maxima, arguing for a correct virally mediated FCIP expression (data not shown).

Functionality of virally expressed FICPs was tested by neuronal depolarization using 45 mM potassium chloride. Neuronal stimulation resulted in fluorescence changes in a reliable way for all of the three tested virally expressed FCIPs. Fractional changes in fluorescence intensity observed for the construct were up to - 31,5% (mean -18,9 +/- 1,4 SEM n = 21 neurons), for PrV-Bartha_IP, up to 21,9% (mean 16 +/- 1,5 SEM n = 8 neurons) for PrV-Bartha_Cam2 and up to 133,6% (mean 79,1 +/- 5,9 SEM n = 33 neurons), for PrV-Bartha_GCaMP2 (Fig. 4-4).
These results demonstrate that all constructed viral strains caused a functional expression of the calcium indicator proteins in infected cells. Moreover, PrV-Bartha’s innate neurotropic tracing abilities seem to be completely preserved.
4. Results

4.3. In vitro Investigation of Chemosensory Properties of Trigeminal Ganglion Neurons

Human psychophysical examinations (Doty et al., 1978) and animal studies (Silver and Moulton, 1982) support the hypothesis that nearly all odorants are not exclusively perceived by the classical olfactory system, but also exhibit a trigeminal component.

In order to investigate the chemosensory properties of trigeminal ganglion neurons a number of different chemicals were tested concerning their ability to stimulate trigeminal neurons in vitro using the calcium imaging technique. All test substances elicit a clear odor sensation and were additionally chosen for comprising different substance classes, including alcohols and aldehydes which are generally thought to be trigeminal activators (Inoue and Bryant, 2005) (benzyl alcohol; benzyl benzoate; cinnamal; cinnamyl alcohol; hexylcinnamal; amylbutyrat; citronellal; citronellool; helional) and chemicals whose trigeminal potency has been described in human psychophysical examinations (Doty et al., 1978) (amyl acetate; citral; eugenol). Chemicals were typically tested at a concentration of 200 µM.

In contrast to the expected outcome, most of the tested substances did not elicit Ca\(^{2+}\) responses in trigeminal monocultures at the used concentration. To test for concentration dependent activation of trigeminal ganglion neurons by the given stimuli, some of the substances were additionally tested at a concentration of 1 mM. Substances at this concentration did not elicit any responses in trigeminal neurons in the monoculture as well. Fig. 4-5 shows example traces of trigeminal neurons in monoculture stimulated with different substances. These screening results are summarized in Tab. 4-3.

In contrast to the inability of trigeminal neurons to react to most of the tested substances previous experiments suggested that primary human keratinocytes can be stimulated by several chemicals (Sisnaiske, 2007). Therefore all substances tested on trigeminal neurons were additionally tested on keratinocyte monocultures. Indeed most of these chemicals were able to trigger an increase in intracellular calcium concentration in keratinocytes upon odor stimulation (Tab. 4-3).
Fig. 4-5: Example traces of calcium imaging measurements of trigeminal monocultures
Substances were tested for their trigeminal potency using a 200 µM concentration. Stimuli were applied for 15 seconds (interstimulus time 45 seconds). At the end of each experiment ATP and a 45 mM potassium solution was applied to control for cell viability of the entire cell population and neurons, respectively. Odorous stimuli were applied in random order. None of the trigeminal neurons shows an increase in intracellular calcium concentration upon stimulus application.

Tab. 4-3: Summary of monoculture calcium imaging experiment
A) Screening substances for their trigeminal potency. At used concentrations (up to 1 mM) most of the tested substances did not elicit responses in trigeminal neurons.
B) Screening substances for their ability to stimulate keratinocytes. Most of the chemicals elicit an increase in intracellular calcium concentration in keratinocytes; number of cell reacting / total number of cells
C,D) Phase contrast light microscopy of trigeminal neuron (C) and human keratinocytes (D) in primary cell culture; scale bar: 20 µM

<table>
<thead>
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<th>A) Mono-Cultures</th>
<th>B)</th>
<th>C)</th>
<th>D)</th>
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<tr>
<td>Concentration</td>
<td>n</td>
<td>Trig</td>
<td>n</td>
</tr>
<tr>
<td>Amyl Acetate</td>
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<td>-</td>
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<tr>
<td>Citral</td>
<td>200 µM</td>
<td>324</td>
<td>-</td>
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<tr>
<td>Eugenol</td>
<td>200 µM</td>
<td>9 / 46</td>
<td>+ (20%)</td>
</tr>
<tr>
<td>Benzyl Benzoate</td>
<td>200 µM</td>
<td>253</td>
<td>-</td>
</tr>
<tr>
<td>Benzyl Alcohol</td>
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<td>203</td>
<td>-</td>
</tr>
<tr>
<td>Cinnamal</td>
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<td>374</td>
<td>-</td>
</tr>
<tr>
<td>Cinnamyl Alcohol</td>
<td>200 µM</td>
<td>253</td>
<td>-</td>
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<td>Hexyl-Cinnamal</td>
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<td>-</td>
</tr>
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<td>261 (116)</td>
<td>-</td>
</tr>
<tr>
<td>Citronellal</td>
<td>200 µM (1 mM)</td>
<td>40 (110)</td>
<td>-</td>
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<td>215 (116)</td>
<td>-</td>
</tr>
<tr>
<td>Helional</td>
<td>200 µM (1 mM)</td>
<td>227 (22)</td>
<td>-</td>
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Since a communication of keratinocytes with other cell types, including sensory neurons, is thought to be involved in mechanical and thermal signal uptake and transduction, a coculture of trigeminal neurons and keratinocytes has been established in order to investigate if this holds also true for chemosensory signal transduction. The general appearance of trigeminal neurons and keratinocytes in coculture was not different from their morphology in monoculture (Tab. 4-3, Tab. 4-4). Also no changes in responsiveness to ATP or high potassium could be observed (Fig. 4-6). Keratinocytes moreover maintain their ability to respond to tested chemicals in the coculture approach (Tab. 4-4).

For the first time also responses of trigeminal neurons could be observed (Fig. 4-6 and Tab. 4-4). These events were mostly detected after preceding keratinocyte activation (Fig. 4-6). Typically, only a few keratinocytes were activated in one field of view, therefore facilitating an allocation. Trigeminal neurons in monoculture which were cultivated in conditioned keratinocyte medium for 1-4 days were also not responsive to the stimuli (data not shown).

Fig. 4-6: Possible communication between keratinocytes and trigeminal neurons in coculture. The keratinocyte reacts with an increase in intracellular calcium application upon citronellol stimulation. 4.2 seconds later also the trigeminal neuron shows a detectable activation.
Several of these substances (e.g. citral; cinnamal) have been recently found to activate different sets of TRP channels in a concentration-dependent manner (Story et al., 2003; Macpherson et al., 2006; Stotz et al., 2008) and many of these TRP channels are actually expressed in keratinocytes and trigeminal ganglion neurons. In contrast, olfactory receptors do not seem to be present in trigeminal neurons (Spehr et al., 2004). Therefore the further project focussed on two substances (citronellol and helional) whose corresponding olfactory receptor (citronellol [OR1A1, OR1A2]; helional [OR17-40]) is suggested to be expressed in keratinocytes (unpublished data, RT-PCR analysis, (Sisnaiske, 2007)). Additionally, and in comparison to other stimuli, these substances elicited keratinocyte responses in a repetitive and more reproducible way (Fig. 4-7).

Neuronal responses in most cases were detected after preceding keratinocyte activation (Fig. 4-6, Fig. 4-7). The latency between keratinocyte and neuronal response

<table>
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<tr>
<th>Substance</th>
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<th>Kera</th>
<th>n</th>
<th>Trig</th>
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<td>2 / 146</td>
<td>+/- (1%)</td>
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<td>(13%)</td>
<td>6 / 46</td>
<td>+ (13%)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>200 µM</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<td>Benzyl Benzoate</td>
<td>200 µM</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Benzyl Alcohol</td>
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<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<td>(11%)</td>
<td>1 / 46</td>
<td>+/- (2%)</td>
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<td>(16%)</td>
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<tr>
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<td>7 / 96</td>
<td>+/- (7%)</td>
<td>0 / 32</td>
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<td>n.q.</td>
<td>+</td>
<td>1 / 40</td>
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<td>Citronellol</td>
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<td>(42%)</td>
<td>28 / 104</td>
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<tr>
<td>Helional</td>
<td>200 µM</td>
<td>108 / 282</td>
<td>(38%)</td>
<td>29 / 126</td>
<td>+ (23%)</td>
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</table>
was quantified across all experiments. Therefore onset of the neuronal / keratinocyte response was determined (2 times SD above basiline) and the time between these two onsets was calculated. The mean latency between keratinocyte and neuronal responses was 16 seconds (SD 11.8) for helional and 13.4 seconds (SD 12.2) for citronellol (n = 27).

Since keratinocytes release ATP upon mechanical stimulation (Koizumi et al., 2004) the role of ATP as a possible communication mediator released from keratinocytes upon chemical stimulation was investigated. The used drug PPADS (Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate) is an antagonist of P2X and P2Y receptors and blocks a large number of P2X (P2X₁, P2X₂, P2X₃, P2X₂/₃, P2X₄, P2X₅, P2X₆, P2X₇) and P2Y (P2Y₁, P2Y₄, P2Y₆, P2Y₁₃) subtypes (reviewed in von Kügelgen, 2008). In control experiments PPADS did not seem to have any influence on keratinocyte or neuronal physiology at the used concentration (100 µM). 90% of the ATP- (300 µM) mediated responses in trigeminal neurons were affected by PPADS (n = 135), demonstrating sufficient blocker potency.

Cocultures were repetitively stimulated with the same odorant. After first odor application, PPADS was washed in for 2:45 minutes. The next odorant was applied in the presence of PPADS. After 2:45 minutes washout time the odorant alone (without PPADS) was applied again. Fig. 4-7 shows an example trace of repetitive stimulation with helional in coculture. The selected keratinocyte is reacting to each odor application with an increase in intracellular calcium concentration. The selected neuron reacts with a constant delay relative to the keratinocyte response. The assumed communication upon helional stimulation was not affected by PPADS (n = 7). Preliminary experiments also point to the absence of ATP involvement in mediating the assumed communication upon citronellol stimulation (n = 2).
4. Results

Fig. 4-7: Repetitive stimulation of cocultures with helional.
The keratinocyte reacts to each odor application with an increase in intracellular calcium concentration. The depicted neuron reacts with a constant delay to the keratinocyte response. The assumed communication upon helional stimulation is not affected by PPADS. However, neuronal ATP-receptors seem to be blocked since no ATP evoked response can be observed in the neuron. Stimuli were applied for 15 seconds (odor interstimulus time 2.45 min)
4.4. Spatiotemporal Dynamics of Odor Representation in the Trigeminal Ganglion \textit{in vivo} Visualized by voltage-sensitive dye Imaging

4.4.1. \textit{In vivo} voltage-sensitive dye Recording of the Trigeminal Ganglion

The establishment of the trigeminal ganglion \textit{in vivo} preparation was one of the most time consuming steps in this study. Crucial steps include e.g. the selection of the best anesthetic and especially the determination of the accurate decerebration level. Finally, the animal could be kept stable after decerebration for up to 10 hours, in some cases even up to 24 hours with no significant changes in heart rate or exhaled CO$_2$ concentration. Directly before the measurement the previously to the surface of the ganglion adjusted camera was focused into the ganglion (250 to 300 µm). Fig. 4-8 illustrates the experimental setup and the location of the trigeminal ganglia at the base of the scull.

\textbf{Fig. 4-8: In vivo voltage-sensitive dye recording of the rat trigeminal ganglion}

A) Schematic experimental setup: The head fixed animal was held under isoflurane anesthesia. After decerebration the olfactometer was adjusted and the position of the camera was trimmed to visualize one ganglion.

B) Schematic dorsal view of the rats head demonstrating trigeminal ganglia location at the base of the scull (after decerebration). The white outlined region represents the drilling window. \textit{Bottom:} Schematic illustration of the scull-base anatomy (modified from Greene, 1935). The trigeminal ganglion is marked in orange. \textit{Top:} Image of the trigeminal ganglion under green illumination (535 nm) as seen under preparation conditions; scale bar = 1 mm
dotted black line = midline of the animal; ba, basilar artery; fc, falx cerebri; ica, internal carotid arteries; man, mandibular branch of the trigeminal nerve; mca, middle cerebral artery; oa, ophthalmic artery; on, optic nerve; oph-max, ophthalmomaxillary branch of the trigeminal nerve; ov, ophthalmic vein; pca, posterior communicating arteries; soh, stalk of hypophysis; t, tentorium, cut; tb, tympanic bulla
4.4.2. *In vivo* voltage-sensitive dye Imaging of Odor Evoked Activity Patterns in the Trigeminal Ganglion

The region of the ganglion was outlined (Fig. 4-9, BF, orange dotted line) and only fluorescence changes within this area were calculated. The model patterns of response to odors were evaluated from the time averaged change of VSDI luminance ($\Delta F/F_0$, averaging from 2-5 s). Mean $\Delta F/F_0$ response evoked by odor application was compared to the $\Delta F/F_0$ recorded in the "blank" condition. The difference of the two means was tested for significance across trials, i.e. z-score values were achieved according to the following equation:

$$
(\Delta F_1/F_0 - \Delta F_B/F_0)/\sqrt{SE(\Delta F_1/F_0)^2 + SE(\Delta F_B/F_0)^2},
$$

where SE is a trial-wise standard deviation normalized to the square root of the trial number. Significant thresholds were set to +/- 1 sd, respectively, justified by the large variances in signal onset and frequency seen in the single trial analysis (Fig. 4-11). Across animals comparison was achieved by overlaying activity maps from different animals. Individual green images were aligned to the ganglion midline and the mandibular branch region (Fig. 4-9, BF, white and green dotted line, respectively). The overlay color code indicates number of animals showing activity / suppression at the same trigeminal region (Fig. 4-9, A). Representative z-score maps and local time courses (LTC) of activity ($\Delta F/F$) derived from one animal are shown in Fig. 4-9 B and C, respectively. LTCs were obtained from the same region across all conditions (colorcode as indicated in Fig. 4-9, BF). In order to measure response onset latency, the model patterns were correlated with z-score values, performed on data from a much smaller time window (100 ms). Pearson's correlation coefficients between each 100 ms frame to the corresponding model patterns were then studied across animals. The onset latency was defined as a time point at which the correlation coefficients crossed the threshold of 0.8. Vertical red line indicate stimulus onset. The application of CO$_2$ (30%) to the nose of the animals typically elicited an activation in the posterior central / posterior lateral region of the ganglion (Fig. 4-9, A-C, CO$_2$). Contours plotted around significantly activated regions of individual animals demonstrate the stability and reproducibility of the observed pattern. In addition to these very local spots of activity a large area of the ganglion was suppressed following CO$_2$ application. The calculated mean response onset latency was 1.2 s (SEM = 0.47 s; n = 6).
Application of ethanol to the nose of the animal elicited activity spots at the posterior central / posterior lateral region that largely overlap with the activity spots seen after CO₂ application (Fig. 4-9, A B, Ethanol). Also here, the stability and reproducibility of the signal was confirmed by across animal comparisons (Fig. 4-9, A). Response shapes differed comparing the two conditions: The VSD activity pattern caused by ethanol application typically displays a “pulse-like” waveform (Fig. 4-9, C) not observed in the CO₂ condition. However, ethanol also caused activity in the anterior region of the trigeminal ganglion where no activation was observed following CO₂ application (Fig. 4-9, A-C, Ethanol). In addition to this very local spots of activity a large area of the ganglion was also suppressed due to ethanol application (Fig. 4-9, A-C, Ethanol). The calculated mean response onset latency was 1,8 s (SEM = 0,38 s; n = 9). Mean response onset latencies of the CO₂ and ethanol patterns were not significantly different (unpaired Student’s t-test).

Application of citral to the nose of the animal elicited an activity pattern clearly distinct from that observed in the ethanol or CO₂ condition: Citral elicited an overall activity of the ganglion. No focal spots of activity could be identified as well as no suppression (Fig. 4-9, A-C, Citral). The calculated mean response onset latency was 0,32 s (SEM = 0,2 s; n = 5).

Also vanillin, an odorant previously reported to have no trigeminal component (Doty et al., 1978) elicited an activity pattern similar to that caused by citral application: no focal spots of activity and no suppression could be observed (Fig. 4-9, A-C, Vanillin). The calculated mean response onset latency (0,1 s SEM = 0,2 s; n = 4) was not significantly different compared to the citral condition (unpaired Student’s t-test).

The calculated mean response onset latency in the ethanol condition was significantly different from the mean response onset latency of the citral and the vanillin condition (unpaired Student’s t-test, p > 0,05).

An intra-animal comparison of maximal signal amplitudes (mean ΔF/F₀ ratios) evoked by the different chemicals revealed the following ranking: Ethanol > CO₂ > citral > vanillin (Fig. 4-9, C).
Fig. 4-9: In vivo voltage-sensitive dye imaging of odor evoked activity patterns in the rat trigeminal ganglion
A) Across animal comparison of averaged z-score maps (2000-5000 ms). Activity maps derived from different animals were overlaid by aligning individual green images to the ganglion midline and the mandibular branch region (see BF). Significant thresholds for activation / suppression were set to +/- 2 (upper row) or +/- 1 (lower row), respectively. The overlay color code indicates number of animals showing significant activity / suppression at the same trigeminal region. Contours in the CO2 condition outline activated regions of individual animals; (n); Scale bar, 1 mm.
B) Representative averaged (2000-5000 ms) z-score maps illustrating odor evoked trigeminal ganglion activity pattern derived from the one animal. Maps were created out of randomized stimulus application; Color scale indicates z-score values; Scale bar, 1 mm.
BF: Image of the trigeminal ganglion under green illumination (535 nm) containing color coded regions used for local time course calculation. Two landmarks: midline of the ganglion (dotted white line) and the mandibular branch region (dotted green line) were used for alignm ent of green images across animals; Scale bar, 1 mm.
C) Local time course (∆F/F) of activity from the highlighted regions in the bright field (BF) image evoked by the different odorants (red trace = local time course obtained from the position of the red circle, light red surrounding area = SD, etc.). Vertical red line indicates stimulus onset. 5 trials per condition; recording duration = 5 s
D) Response onset latency quantification. In order to measure response onset latency, the model patterns were correlated with z-score values, performed on data from a much smaller time window (100 ms). Pearson's correlation coefficients between each 100 ms frame to the corresponding model patterns were then studied across animals. The onset latency was defined as a time point, at which the correlation coefficients crossed the threshold of 0.8; bars represent mean response onset latencies; error bars = SEM;
P, posterior; L, lateral
4.4.3. Cellular Localization of the voltage-sensitive dye Signal

In order to localize trigeminal ganglion neurons innervating the rat’s nasal cavity, a Pseudorabies virus based tracing approach was performed. 5µl high titered ($5 \times 10^8$ PFU/ml) Pseudorabies virus Bartha strain (PrV-Cam) was applied to one nostril of the rat. Animals were sacrificed at different time points after infection in order to analyze viral mediated marker protein expression. An *ex in vivo* preparation of the base of the skull containing the two ganglia was used for epifluorescence and 2-Photon based marker protein detection in trigeminal ganglion neurons. 48 h post infection marker protein expressing cells could be exclusively detected in the ipsilateral trigeminal ganglion pointing to a specific labeling of trigeminal ganglion neurons (TGN) innerving the nasal cavity (nasal TGN) (Fig. 4-10; n = 5).

The major cluster of nasal TGNs could be identified in the posterior region of the trigeminal ganglion on both sides relative to the midline of the ganglion (Fig. 4-10, B). The location of infected cells is consistent with the posterior central activity spot seen in the CO$_2$ and ethanol condition (Fig. 4-9). Marker protein expressing cells could also be detected at different locations however not that tightly clustered (Fig. 4-10, B).

![Fig. 4-10: Cellular localization of the voltage-sensitive dye signal](image)

A) *Ex in vivo* preparation of the base of the skull containing the trigeminal ganglia of an intranasally PrV-Bartha infected animal sacrificed 48 h post infection. The left trigeminal ganglion is outlined (orange dotted line). The two yellow squares point to areas where large amounts of infected cells were detected. Fewer infected cells could also be seen at different locations. Scale bar, 1 mm; dotted white line = midline of the ganglion; P, posterior; L, lateral

B) Fluorescence images showing traced nasal trigeminal neuron located in the areas marked in (A). Scale bar, 40 µm
4.4.4. The voltage-sensitive dye Signal Correlates with Odor Evoked Spikes

The voltage-sensitive dye signal reports changes in membrane potentials integrated across several millimeters of cortex with high temporal accuracy, however, single spike events are not detectable by this method (Sterkin et al., 1998; Petersen et al., 2003; Jancke et al., 2004). In order to investigate to which extend the measured VSD signal reflects spike related activity, extracellular recordings were performed. Insertion of the recording electrode into the posterior central / posterior lateral trigeminal region (where large amplitudes of activity were observed in the VSD measurements following CO₂ and ethanol application) revealed spikes after application of CO₂ (Fig. 4-11, B) and ethanol (Fig. 4-11, D). The Peri-Stimulus-Time-Histogram (PSTH) showed temporal spike patterns closely resembling the stimulus specific VSD activity patterns obtained at the same location (Fig. 4-11, A C) and even start at the same time the first VSD signal could be detected.

Single trial analysis of the PSTH showed discrete bursts of activity that differ in onset, number and frequency comparing the two conditions (Fig. 4-11, B D). Single trial analysis of the voltage-sensitive dye signal revealed separated bursts of activity differing in the same parameters (Fig. 4-11, A C). Comparing single trial PSTH with VSD single trials, striking similarities of these responses are apparent, arguing for the variations in VSD activity being caused by variations in spike timing.

Ethanol application elicited sharply separated high frequency spike trains, varying in onset and silent period duration (mulitphasic). Therefore it is not surprising that when averaged VSD signals (Fig. 4-11, A C) show increased standard deviations, impeding significance calculations.

CO₂ application did not elicit sharply separated spike trains, but rather an asynchronous firing pattern with individual spike bursts, reflected in a gradual increase over time in the averaged PSTHs and VSD signals.

Taken together, high amplitudes of the voltage-sensitive dye signal (arising from the trigeminal ganglion) seem to correlate with suprathreshold activity.
Fig. 4-11: **The voltage-sensitive dye signal correlates with odor evoked spikes**

A,C) *left* Local time course ($\Delta F/F$) of activity evoked by CO$_2$ (A) or ethanol (C) application (obtained from the same region highlighted in red in the bright field image (Fig 4.4.2 BF); however from of a different animal). Red trace represents averaged VSD signal of 4 trials (light red area = SD). Note the differences in VSD signal shape, with a typical “pulse like” waveform in the ethanol condition. *right* Single trial analysis of the VSD signal. The color scale indicates fractional changes in fluorescence intensity ($\Delta F/F$).

B,D) *left* Peri-Stimulus-Time-Histogramm (PSTH) of spike responses evoked by CO$_2$ (B) or ethanol (D) application (electrode penetration site located within the typically activated area) (4 trials; bin factor 100 ms) blue = spontaneous activity; black = stimulus evoked activity; Note that spikes resemble the activity pattern seen in the VSD measurements (A,C) and even set in at the same time the first VSD signal could be detected. *right* PSTH seen in (B,D) split into single trial PSTHs; Note that bursts of activity differ in onset, number and frequency comparing the two conditions. The onset of spiking activity is consistent with the onset of the VSD signal.

Vertical red line indicates stimulus onset; Recording duration = 5 s
4.4.5. Suppression in the voltage-sensitive dye Signal Correlates with Trigeminal Ganglion Spontaneous Activity Suppression

For a closer investigation of the prominent suppression observed in the VSD signal following CO$_2$ and ethanol application (Fig. 4-12, VSD traces), extracellular recordings were also performed at posterior central / posterior medial trigeminal ganglion areas (where a main suppression in the VSD signal was observed). Insertion of the recording electrode at this area revealed a high degree of rhythmic spontaneous activity (Fig. 4-12, PSTH, blue bars). This is remarkable since these recording sides were relatively close to the ones in the posterior medial / posterior lateral regions where almost no spontaneous activity was observed (Fig. 4-11). The spontaneous activity was neither related to heartbeat nor to respiration. This spontaneous activity was suppressed following CO$_2$ or ethanol application (Fig. 4-12, PSTH, black bars). In contrast no reduction of spontaneous activity could be observed after citral application (Fig. 4-13).

Therefore the suppression in the VSD measurements following CO$_2$ and ethanol application are likely mediated by an odorant specific reduction of spontaneous electrical activity in this area.
4. Results

Fig. 4-12: Suppression in the voltage-sensitive dye signal correlates with trigeminal ganglion spontaneous activity suppression
A, B) top: Local time course (ΔF/F) of activity evoked by CO₂ (A) or ethanol (B) application (extracted from the same region highlighted in blue in the bright field image (Fig 4.4.2 BF); however from of a different animal). Blue trace represents averaged VSD signal of 4 trials (light blue area = SD). Note the suppression in the voltage sensitive dye signal in both experimental conditions; Recording duration = 5 s
bottom: Peri-Stimulus-Time-Histogramm (PSTH) of spike responses to CO₂ (A) or ethanol (B) application (electrode penetration site located within the typically suppressed posterior medial area) (4 trials; bin factor 100 ms) blue = spontaneous activity; black = stimulus evoked activity; Note the rhythmic spontaneous activity found in this region. This spontaneous activity is suppressed after CO₂ and ethanol application. The suppression of spontaneous activity sets in at the same time the first suppression in the VSD signal can be detected; Recovery from suppression is observed after several seconds; Recording duration = 20 s; Vertical red line indicates stimulus onset;

Fig. 4-13: Citral application does not change spontaneous trigeminal ganglion activity.
A, B) Top: Peri-Stimulus-Time-Histogramm (PSTH) of spike responses to citral application (electrode penetration site located within the typically suppressed area) (4 trials; bin factor 100 ms) blue = spontaneous activity; black = stimulus evoked activity; Note the rhythmic spontaneous activity found in this region. This spontaneous activity is not influenced by citral application.
A, B) Bottom: PSTH split into single trial PSTHs; Vertical red line indicates stimulus onset; Recording duration = 20 s
4.4.6. Modulation of the voltage-sensitive dye Signal

In order to investigate if spatial trigeminal activity patterns are stimulation site dependent an alternative oral odor application was tested. Focal activation- as well as suppression-spots at the level of the trigeminal ganglion could also be detected following oral ethanol application. However, the spatial localization of these spots was altered compared to the nasal application site (Fig. 4-14). Oral application elicits a broad activity spot in the anterior medial region of the ganglion (n = 3). However, there was no activation observed in this area following nasal ethanol application (intra animal comparison). Also a shift of the main suppression area was observed. In the depicted animal, for example, the main suppressed ganglion area following oral ethanol stimulation seem to be significantly activated following nasal ethanol application.

Taken together, these results underline the trigeminal ability of coding the “where” information of sensory processing.

![Fig. 4-14: Spatial trigeminal activation pattern are stimulus site dependent.](image)

Averaged (2000-5000 ms) z-score maps of odor evoked trigeminal ganglion activity pattern. Color scale indicates z-score values. Green solid lines outline significantly activated areas (z-score >1). Gray solid lines outline significantly suppressed areas (z-score <−1) Changing the odor application site elicits a clear shift of the main activity and suppression spot.

Scale bar, 1 mm. Dotted white line = midline of the ganglion;
In order to investigate influences of stimulus concentration on trigeminal ganglion activity pattern vapor phases of 50% ethanol as well as a saturated citral concentration (~20 mM) were tested. In all preceding experiments undiluted ethanol as well as 1 mM citral was used. No differences in the activity patterns evoked by the altered ethanol or citral concentrations could be observed (Fig. 4-15) compared to the patterns elicited with standard concentrations (Fig. 4-9, A). While the response onset latency of reduced ethanol concentration was strikingly prolonged (3.8 s) compared to the standard ethanol concentration (1.8 s; SEM = 0.38 s; n = 9) no drastic changes in the calculated mean response onset latencies of the two citral concentration could be observed (standard: 0.32 s; SEM = 0.2 s; n = 5) (20 mM: 0.45 s; SEM = 0.25; n = 2).

In conclusion, stimulus specific activation patterns seem to be to some extent present at the level of the trigeminal ganglion. Therefore these results imply a certain trigeminal ability for coding stimulus identity, the “what” of sensory information processing.

Fig. 4-15: Trigeminal activation pattern are stimulus specific
Averaged (2000-5000 ms) z-score maps of odor evoked trigeminal ganglion activity pattern. Color scale indicates z-score values. Green solid lines outline significantly activated areas (z-score >1). Gray solid lines outline significantly suppressed areas (z-score <1). Since uninfluenced by stimulus concentration (reduction for ethanol; increase for citral), trigeminal ganglia activity patterns seem to be to some extent stimulus specific.
Scale bar, 1 mm. Dotted white line = midline of the ganglion;
4. Results

To gain first insights into the signal detection / transduction processes related to trigeminal activity patterns the influence of the carbonic anhydrase (CA) enzymatic inhibitor acetazolamid on the CO₂ evoked trigeminal activity patterns was investigated. CA has been shown to be important for CO₂ detection in GC-D (guanylyl cyclase D) neurons in the olfactory epithelium (Hu et al., 2007). Moreover CA has been shown to be expressed in trigeminal ganglion neurons (Wong et al., 1983; Tanimoto et al., 2005) and it is hypothesized that carbonated water excites lingual nociceptors via carbonic anhydrase-dependent processes (Simons et al., 1999).

The nose of the animals was flushed with a high blocker concentration (20 mM) to maximize the drugs likelihood of reaching thin trigeminal free nerve endings innervating the nasal cavity. Fig. 4-16 shows averaged z-score maps of odor evoked trigeminal ganglion activity pattern before and after drug application. Acetazolamid had no major influence on trigeminal ganglion activity caused by ethanol application: areas showing strong activation in the control condition were also present after drug application. However, suppression seems to be less prominent after acetazolamid block. In contrast, drug application had a huge impact on the activity spots elicited after CO₂ application. No significantly activated regions could be detected in the CO₂ condition after drug application (n = 3) whereas suppressed areas seem to even increase in size. These results point to the importance of carbonic anhydrase in trigeminal mediated CO₂ detection.

Fig. 4-16: Involvement of carbonic anhydrase in trigeminal CO₂ detection
Averaged (2000-5000 ms) z-score maps of odor evoked trigeminal ganglion activity pattern. Color scale indicates z-score values. Green solid lines outline significantly activated areas (z-score >1). Gray solid lines outline significantly suppressed areas (z-score < -1). Trigeminal ganglion activity patterns before and after nasal acetazolamid application. Note the lack of significantly activated regions in the CO₂ condition after drug application. In contrast suppressed areas seem to increase in size. In contrast, drug application had no major influence on trigeminal ganglion activity caused by ethanol application. Scale bar, 1 mm. Dotted white line = midline of the ganglion;
4.5. Human Psychophysical Experiments

In the rat, nasal application of either CO$_2$ or ethanol not only caused local spots of trigeminal activation but also a suppression of large ganglionic areas. In contrast, no suppression could be observed when using citral as a stimulus. Citral application elicited an overall trigeminal ganglion activity. Interestingly, the ganglionic area activated by citral also included areas that were suppressed by CO$_2$ and ethanol. This led to the initial hypothesis for the human psychophysical experiments: If CO$_2$ application to the nose of humans (e.g. anosmic patients or a trigeminal focus group), would also cause a suppression of large areas of the ganglion, this suppression should be able to influence the trigeminal detection threshold for citral. Therefore anosmic patients should be able to detect citral alone in lower concentration than in a mixture with CO$_2$. Results of these psychophysical experiments could therefore give a hint whether similar trigeminal odor coding strategies could also be present in humans.

4.5.1. Experiment 1: Threshold Tests for CO$_2$ and Citral Combination

Three different CO$_2$ and citral combinations were tested.

1. Subthreshold CO$_2$ in combination with subthreshold citral.
2. Subthreshold CO$_2$ in combination with threshold citral.
3. Subthreshold CO$_2$ in combination with suprathreshold citral.

Nineteen out of twenty healthy subjects (95%) reported a trigeminal perception in the subthreshold CO$_2$ and citral combination. The trigeminal effect subsequently increased in the threshold citral and suprathreshold citral combinations in each of the nineteen subjects.

In one subject the answers were random, i.e. sometimes no trigeminal perception in the citral subthreshold and threshold combination and sometimes trigeminal perception in both were reported. However a trigeminal perception in the suprathreshold citral combination was always reported. All of the anosmic subjects reported a trigeminal perception in the subthreshold CO$_2$ and citral combination.
4.5.2. Experiment 2: Suprathreshold CO\textsubscript{2} Exposure Followed by Suprathreshold Citral

A paired t-test for the healthy subjects showed that there was no significant difference between the perceived trigeminal intensity of citral tested separately or tested directly after CO\textsubscript{2} administration, \( t_9 = -0.432 \) (\( p > 0.05 \)), \( M \) (tested separate) citral = 4.3, SD = 1.89; \( M \) (tested after) 4.5, SD = 1.5.

In contrast, the control condition, i.e. the difference of the perceived intensity of CO\textsubscript{2} tested separately or tested directly after citral administration, showed a significant effect. Subjects rated CO\textsubscript{2} perception as significantly more intense after citral administration as compared to tested separately, \( t_9 = -3.147 \), (\( p < 0.05 \)), \( M \) (tested separate) CO\textsubscript{2} = 4.3, SD = 1.65, \( M \) (tested after) CO\textsubscript{2} = 5.3 SD = 2.

For the anosmic subjects there was no difference between the perceived trigeminal intensity of citral tested separately or tested directly after CO\textsubscript{2} administration, nor was there any difference in the control condition. Anosmic patients differed from healthy subjects in the sense that they consistently gave significantly lower intensity ratings for the suprathreshold citral and CO\textsubscript{2} concentrations.
5. Discussion

*Anterograde Transsynaptic Tracing in the Murine Somatosensory System using PrV*

Research on PrV has accelerated rapidly in the past 20 years. More recently, PrV-Bartha has been extensively used in laboratory animal models to study viral pathogenesis. Its remarkable propensity to infect synaptically connected neurons has led to the use of PrV as a tracer of neuronal circuits. Moreover, recombinant PrV strains expressing fluorescent proteins can be used as “live-cell”-tracing tools for *in vitro* investigations of selectively labelled neurons (Smith *et al.*, 2000; Irnaten *et al.*, 2001; van denTop *et al.*, 2003; Damann *et al.*, 2006). However, its application is limited due to its selective transport in the retrograde direction. For labelling sensory pathways from the periphery up to the brain, a transsynaptic tracer must have the capacity to invade the nervous system in the anterograde direction.

This work demonstrates the usability of the bidirectional tracer PrV-Kaplan for tracing defined sensory neuronal populations within the trigeminal ganglion and synaptically connected higher order neurons in the brainstem. Patch-clamp analysis revealed that under the used cell culture conditions, infection of neurons by PrV-Kaplan had no influence on the biophysical properties of traced cells when compared with virus-free cell cultures, making PrV-Kaplan traced neurons ready for functional *in vitro* investigations.

In conclusion, PrV-Kaplan enables rapid labelling and *in vitro* identification of neurons of the trigeminal system of mice. In contrast to infected TGNs grown with conventional culture medium, the functionality of infected cells was retained for several days under foscarnet treatment. Under these conditions, labelled neurons displayed physiological properties highly similar to uninfected control neurons. In respect to earlier publications reporting HSV-mediated reduction of neuronal excitability of cultured DRG neurons (Mayer *et al.*, 1986; Storey *et al.*, 2002), my detailed electrophysiological characterization of PrV-infected and foscarnet-treated neurons has demonstrated unchanged excitability [foscarnet treatment itself has also no effect on the physiology of neurons as shown before (Damann *et al.*, 2006)]. This methodological approach now allows experiments to be carried out, with a focus directed on intrinsic properties of brainstem neurons that receive information from the nasal cavity. A former study already demonstrated unique features of barrelette cells in the brainstem that might have a function in determining the temporal resolution of...
tactile related responses along the trigeminothalamic pathway (Lo et al., 1999). In future, recombinant PrV-Kaplan variants expressing different fluorescent marker proteins may be used for double tracing studies, similar to studies using variants of PrV-Bartha (reviewed in Song et al., 2005; Damann et al., 2006). Inoculation of PrV-Kaplan at two distinct areas of trigeminal innervation and subsequent physiological analysis of two neuronal populations should bring us a step closer to the understanding of differentiated trigeminal somatosensation. This technique may also be transferred to other mammalian primary somatosensory afferent neurons to combine fast tract tracing with physiological in vitro analysis.

**PrV mediated Functional Expression of Fluorescent Calcium Indicator Proteins**

The first viral project evaluated PrV-Kaplan as a “live-cell”-tracer in somatotopically defined and synaptically connected neurons. However, the possibilities for functional examination of virus infected neurons are limited to electrophysiological approaches or bulk loading strategies using calcium dyes. Since, in a distant prospect, *in vivo* investigation of trigeminal ganglion activity pattern formation should also be realized at the cellular level, PrV-Bartha derived recombinant virus strains that express fluorescent calcium indicator proteins (FCIP) were generated and functionally characterized.

All three generated virus strains were virulent and expressed their according FCIP (GCaMP2, camgaroo-2, inverse pericam) in infected murine trigeminal ganglion neurons. Functionality of these virally expressed constructs was verified by confocal Ca$^{2+}$-Imaging. These FCIPs expressing virus strains provide a new tool for the functional analysis of whole circuits of synaptically connected neurons *in vitro* and *in vivo*.

The chosen stimulus, 45 mM potassium chloride, should strongly depolarize the neurons and thus elicit a maximal calcium response tapping the full potential of the FCIPs. Viral mediated expression does not seem to influence FCIP functionality since fractional changes of virally expressed FCIPs displayed the same tendency compared to fractional changes of FCIPs transfected into HEK cells. Comparing the different virally expressed FCIPs revealed PrV-Bartha_GCaMP2 showing the highest fractional changes. PrV-Bartha_IP and PrV-Bartha_Cam2 elicited only medium to low sized
fractional changes, though PrV-Bartha_IP clearly revealed a better signal to noise ratio.

Camgaroo-2 has been described to produce fractional changes of up to 170% (Hasan et al., 2004). Why these levels could not be achieved is not clear. Remarkably also the transfected construct in HEK cells never elicited more than 24% fractional changes in fluorescence, arguing against a misexpression caused by the virus.

To allow functional neuronal network examinations neurotopic Pseudorabies viruses seem to be highly appropriate:

1) The usability of PrV strains as "live-cell"-tracing tool allowing a functional analysis of identified neurons has been demonstrated in recent studies. A main argument against the application of viral tracers for functional investigations has been the possibility that viral infection itself could alter cell physiology. However, in contrast to reported herpes simplex virus induced changes in neuronal excitability of cultured dorsal root ganglion neurons (Mayer et al., 1986; Storey et al., 2002), detailed electrophysiological characterization of PrV-Bartha (Damann et al., 2006) as well as PrV-Kaplan (Rothermel et al., 2007) (wild type related PrV) infected trigeminal neurons have demonstrated unchanged neuronal physiology.

2) Pseudorabies viruses have the property to spread within synaptically connected neurons, which has been exploited in numerous tracing studies. PrV-Bartha, an attenuated live-vaccine strain, proved to be particularly appropriate for this purpose because of its reduced virulence and specific transneuronal spread in the retrograde direction of information processing (Enquist, 2002).

3) PrV has a broad host spectrum providing the opportunity of transferring the tracing approach to different model organisms using the same viral constructs. Thus, preliminary experiments performed on rat trigeminal neurons, in vitro infected with the newly created virus strains, demonstrated that all constructs are also functionally expressed in these neurons (data not shown).

In recent years native PrV-Bartha as well as marker protein-expressing variants, have been effectually used to define the organization of CNS circuits after intracerebral (Jasmin et al., 1997; DeFalco et al., 2001; Krout et al., 2003; Willhite et al., 2006) or peripheral (Jansen et al., 1995; Jansen et al., 1997; Smith et al., 2000; Billig et al., 2000; Cano et al., 2001; Irnaten et al., 2001; Horvath et al., 2003) injection. The newly generated FCIP expressing viral constructs represent a powerful tool for
combining fast tract tracing with physiological analysis applicable in multiple methodological approaches including slice or in vivo imaging studies. In vivo two-photon calcium imaging provides the opportunity to simultaneously monitor the activity in multiple components of neural circuits. For this kind of approach an expression of virally encoded FCIPs would render complicated bulkloading techniques or time consuming transgenic animal line constructions largely unnecessary and moreover provide the possibility to study synaptically connected neuronal circuits in a wholistic approach. In future, the generation of recombinant FCIP expressing PrV-Kaplan strains will additionally allow anterograde neuronal tract tracing. These results demonstrate that virally encoded FCIPs are functionally expressed in infected murine neurons, enabling the exploration of calcium level changes in infected cells. In conclusion, these newly generated FCIPs expressing viral strains represent a perfect tool for the functional exploration of different neural systems in vivo and in vitro.
In vitro Investigation of Chemosensory Properties of Trigeminal Ganglion Neurons

It is still controversy discussed whether trigeminal chemosensation arises from a direct stimulation of intraepithelial free nerve endings (Silver, 1992), or if alternative signal transduction mechanisms are involved (Finger et al., 2003; Lumpkin and Caterina, 2007; Lin et al., 2008). Undoubtful, a basic set of different receptors expressed on trigeminal neurons (see 1.1.2) contributes to the chemosensory properties of this nerve, therefore arguing for a direct stimulation of free nerve endings: e.g. the detection of menthol, capsaicin or mustard oil is mediated via TRP channels. Furthermore, these neurons express ATP-sensitive receptor types belonging to the P2X (ligand-gated cationic channels) and P2Y (G protein-coupled receptors) family and it has been shown that subunit-specific P2X-receptor expression defines some of the chemosensory properties of trigeminal neurons (Spehr et al., 2004).

However, this complex receptor expression pattern does not explain all chemosensory features associated with the trigeminal nerve, since many odorants, known to activate trigeminal neurons in vivo, fail to do so in vitro. Only one (eugenol) out of three tested substances (amyl acetate; citral; eugenol) whose trigeminal activity has been confirmed in anosmic patients (Doty et al., 1978) could elicit responses in our trigeminal monocultures. Regarding these psychophysical examinations it is surprising that eugenol elicits such robust responses in cell culture, since it has been classified as one compound of the rare group of substances detected by less than 15% of the anosmic subjects (including vanillin, decanoic acid, phenyl ethyl alcohol and geraniol; only 5 out of 47 tested substances). From a cell biological view, however, it is not that surprising since eugenol is known to activate TRPA1 (Bandell et al., 2004), TRPV1 (Yang et al., 2003), TRPV3 (Xu et al., 2006) and at least TRPA1 and TRPV1 are expressed in trigeminal neurons. Moreover, eugenol potentiates ionotropic γ-aminobutyric acid (GABA_A) receptors, (Aoshima and Hamamoto, 1999) but inhibits N-methyl-D-aspartate (NMDA) receptors (Wie et al., 1997) and TTX-S and TTX-R Na⁺ currents (Cho et al., 2008). By inducing translocation of the AhR (arylhydrocarbon receptor; basic helix-loop-helix transcription factor) to the nucleus, intracellular eugenol is also thought to be involved in regulating gene expression and inhibition of the cell cycle progression in HaCaT (human adult low calcium high temperature keratinocytes) cells (Kalmes et al., 2006).
The reason why citral fails to elicit trigeminal responses \textit{in vitro} is not clear: Although the used concentration (200 µM) is not sufficient for TRPV1 activation (activation $K_D$ 465 µM) and may even be insufficient for TRPA1 activation (threshold ~200 µM), TRPM8 channels should be activated (activation $K_D$ 33.5 µM; inhibition $K_D$ between 188µM and 241 µM) (Stotz \textit{et al.}, 2008). However, the authors also propose that at higher agonist concentration, inhibition can develop before activation reached a steady state. Therefore one explanation might be that TRPM8 activation was too weak or too short to be recognized as a calcium influx in our imaging experiments. Interestingly, even at a concentration of 1 mM, citral exclusively activates a subpopulation of capsaicin-responsive and menthol-insensitive dorsal root ganglion neurons (Stotz \textit{et al.}, 2008).

Also other substances like cinnamal have been reported to exert their chemosensory sensation by targeting excitatory TRP channels (Macpherson \textit{et al.}, 2006) and many TRP channels are actually expressed in keratinocytes and trigeminal ganglion neurons. In contrast, olfactory receptors do not seem to be present in trigeminal neurons (Spehr \textit{et al.}, 2004). Therefore the project focused on two substances (citronellol and helional) whose corresponding olfactory receptors are suggested to be expressed in keratinocytes (citronellol [OR1A1, OR1A2]; helional [OR17-40]; unpublished data, RT-PCR analysis, (Sisnaiske, 2007)). Like most of the tested chemicals also these substances were unable to stimulate trigeminal neurons in monoculture. However, they do stimulate keratinocytes in mono- and coculture and the elicited responses were even more reproducible compared to most other substances tested.

Primary human keratinocytes are a heterogeneous cell population: keratinocytes used in this study were obtained from different skin areas (facial, body and genital skin) and donors of various ages. Furthermore, primary human keratinocytes continue to differentiate in culture, making it also difficult to compare results even from one donor. Therefore, it is not surprising that keratinocytes did not respond to every odorant in each cell charge.

Since coculture experiments using citronellol and helional as stimuli revealed trigeminal responses downstream of keratinocyte responses, the presence of a communication between the skin and the somatosensory system is hypothesized. Trigeminal neurons in monoculture cultivated in conditioned keratinocyte medium were also unresponsive to the stimuli, making it unlikely that a diffusible innate
medium factor or substances constantly released from cultured keratinocytes alter trigeminal odor responsiveness.

The nature of the possible communication remains unclear. There are no synapses apparent between free nerve endings of peripheral neurons and keratinocytes (Ullmann et al., 2007). This is in perfect agreement with our own results which showed that pre- and postsynaptic markers could not be detected in the coculture system (data not shown). Taken together, this is a strong hint for a communication based on transmitter / mediator release from keratinocytes (like previously demonstrated for mechanical stimulated keratinocytes (Koizumi et al., 2004)). If true, the huge latency variations in keratinocyte to neuronal responses may be explainable by the distance variation of these cell types in the coculture. Additionally, due to the limited field of view (10x objective) it is not surprising that for some neuronal responses seen in the coculture, no pioneered keratinocyte response could be detected since the corresponding keratinocyte might be not included in the observed area. The list of substances that could potentially be secreted by keratinocytes / skin cells is large (glutamate (Fuziwara et al., 2003), dopamine (Fuziwara et al., 2005), melatonin and serotonin (Slominski et al., 2002), GABA (Ito et al., 2007), PGE$_2$ (Huang et al., 2008), NGF and artemin (reviewed in Albers and Davis, 2007)). Additionally, a variety of endocrinological receptors and their agonists are expressed in epidermal keratinocytes (Slominski et al., 2000; Slominski and Wortsman, 2000; Slominski et al., 2001; Slominski, 2005) as well as a series of receptors, which were originally found in the central nervous system as neurotransmitter receptors including ionotrophic (P2X, NMDA, GABA$_A$, glycine) and G-protein coupled receptors (P2Y, adrenergic $\beta_2$, dopamine-2-like, serotonin) (reviewed in Denda et al., 2007). The role of ATP as a possible communication mediator was taken into account since keratinocytes have been shown to release ATP upon mechanical stimulation. The performed blocker experiments, however, argue against an ATP-mediated communication induced by odorants, at least in the case of helional stimulation.

In conclusion, the complex receptor expression pattern observed in trigeminal neurons (1.1.2) does not explain all chemosensory features associated with the trigeminal nerve. For evolving its entire chemosensory ability, the trigeminal nerve rather seems to depend on communication with cells of its peripheral innervation area.

In this respect, stimulus-dependent differences in keratinocyte substance release would - if true - enable the skin to encode information about stimulus identity.
Spatiotemporal Dynamics of Odor Representation in the Trigeminal Ganglion in vivo Visualized by voltage-sensitive dye Imaging

Vertebrate chemosensation mainly comprises the olfactory and the gustatory system as well as the general chemical sense (trigeminal sense). These systems differ in functional and anatomical aspects but a key feature common to all of them is the detection of chemical cues in the environment. While the olfactory system is the main detector of volatile substances, the general chemical sense additionally contributes to the overall gustatory and olfactory sensation since most odorants also elicit trigeminal responses (Silver and Moulton, 1982). The trigeminal mediated ability of odor-detection and -discrimination has been proven by numerous animal studies in which the olfactory system has been lesioned, as well as human studies on anosmic patients (Doty et al., 1978; Mason and Silver, 1983).

Glomerular structures, representing the first level of information processing in the olfactory system, are relatively easy to access and large numbers of imaging studies have broadened our understanding about the functional organization of the olfactory bulb (Wachowiak et al., 2004; Spors et al., 2006; Yaksi et al., 2007). Trigeminal ganglia, located at the base of the scull containing sensory neuron somata and nerve fibers, detect somatic sensations in the periphery and transmit the information to the central nervous system. The trigeminal nerve is the major mediator of sensations from the mammalian head and comprises neurons transducing mechanical, thermal and chemical stimuli. Single neurons of this system mediate sensory input from selective areas of the head (meninges, cornea and conjunctiva of the eyes, facial skin, mucous membranes of the oral and nasal cavities, and even from the rodent vibrissa pad).

As a result of the difficulty in accessing these structures experimentally, a direct in vivo visualisation of evoked population activity in the trigeminal ganglion in response to sensory stimuli has not been achieved so far. Therefore basic principles of spatiotemporal activity pattern formation in trigeminal ganglia remain elusive. As a result, it is still controversially discussed if trigeminal chemoperception is only based on a graduated activation of trigeminal (pain) fibers or odor specific activity patterns could exist at the level of the ganglion.

In the present work a decerebration approach of the rat was established optimized for gaining large-area optical access to the base of the scull containing both trigeminal ganglia. A small-pressure ejection-based delivery protocol was used for fast delivery of voltage-sensitive dyes into the trigeminal ganglion.
These preparations enable high-resolution *in vivo* recordings of optical signals arising from a large region of the rat trigeminal ganglion. Thus, odor-related trigeminal activity patterns could be demonstrated for the first time. This imaging approach is not limited to a certain ganglion area but rather offers a broad view on the ongoing activity of nearly the whole ganglionic tissue.

The population activity of trigeminal ganglia in response to nasal chemical stimulation was investigated in the here presented study. Lateral asymmetries in the trigeminal ganglion of the male rat have been described previously by Lagares *et al.*, 2007. However, in this study a comparison of the voltage-sensitive dye activity pattern of the right and left trigeminal ganglion in response to the different stimuli revealed no differences, and therefore data were pooled.

The four tested stimuli could be grouped into two subcategories:

1) Substances with a strong trigeminal component: including CO$_2$ as a pure pain activator without any parallel olfactory sensation (Cain and Murphy, 1980) and ethanol that also elicits a pungent sensation but additionally exhibits a clear olfactory component.

2) Typical olfactory stimuli: Citral, a substance with a lemon-like smell previously shown to also elicit trigeminal responses (Doty *et al.*, 1978) and vanillin one of the rare odorants thought to cause no trigeminal activation (Doty *et al.*, 1978).

Clear differences in the trigeminal activity pattern elicited by these two subcategories of stimuli could be observed. First activity patterns elicted by subcategory 1 will be discussed:

Strong trigeminal activators like CO$_2$ and ethanol caused distinct activity spots with a neighbouring broader area being simultaneously suppressed. Focussing on the areas that showed strong activation following stimulus application I asked if the localisation of these spots is somehow explainable with a trigeminal somatotopic organisation.

There is a general agreement that a gross somatotopic organization exists at the level of the trigeminal ganglion: the somata of the mandibular nerve occupy the mandibular nerve branch region (the very posterolateral portion of the ganglion), the cell bodies of the ophthalmic branches are located anteromedially and the perikarya of the maxillary branches are interposed in between, in a central position of the ganglion (Marfurt, 1981; Anton and Peppel, 1991; Schaefer *et al.*, 2002; Lazarov, 2002). The trigeminal innervation of the nasal cavity is given by the anterior ethmoidal nerve (*N. ophthalmicus*), the nasopalatine nerve (*N. maxillaris*), and internal nasal branches from
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the infraorbital nerve (*N. maxillaris*) (Bojsen-Moller, 1975). CO₂ and ethanol cause a shared posterior central / posterior lateral activity spot. With respect to the somatotopic organization, this spot is likely to arise from nasopalatine nerve and / or infraorbital nerve stimulation. The anterior activity spots unique to the ethanol condition may therefore be triggered by ethmoidal nerve stimulation.

In conclusion, the main trigeminal activity spots observed for this stimuli subcategory are in accordance with the gross somatotopic trigeminal organization. This finding was confirmed by viral tracing experiments which revealed a good overlap between activation spots seen in the VSD signal and the ganglionic localization of trigeminal neurons innervating the nasal cavity.

Another question is why these substances elicit common as well as unique activation spots (unique anterior activity, observed in the ethanol condition) since they are both known to be strong trigeminal agonists. In order to rule out insufficient dye loading at the anterior central region in animals treated with CO₂, ethanol and CO₂ were tested in the same animal (Fig. 4-9). The anterior ganglionic region, activated following ethanol application (yellow trace) showed no significant activation in the CO₂ condition, demonstrating that the differences in the activity patterns cannot be attributed to an inhomogenity in dye loading.

These differences in activity patterns following ethanol and CO₂ application may be therefore explainable with a differential activation of subcategories of trigeminal neurons: It is well established that single trigeminal neurons are equipped with different combinations of mechano- (incl. proprio-), thermo-, and chemoreceptor-proteins that potentiate a differentiated perception of environmental influences. It has been shown that ethanol activates the transient receptor potential channel TRPV1 (Trevisani *et al.*, 2002) and inhibits the cold-menthol receptor TRPM8 (Weil *et al.*, 2005; Benedikt *et al.*, 2007) which are both expressed in subpopulations of trigeminal neurons. However, ethanol is also known to effect a large number of voltage and ligand-gated channels (*GABA*<sub>A</sub> and glycin receptors (Mihic *et al.*, 1997); G-protein-coupled inwardly rectifying potassium channels (GIRKs) (Lewohl *et al.*, 1999); voltage-gated Na<sup>+</sup>-channels (Shiraishi and Harris, 2004); large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK) (Crowley *et al.*, 2005; Liu *et al.*, 2006); purinergic and serotonin receptors (Davies *et al.*, 2006; Hu *et al.*, 2006) as well as NMDA receptors (Xu and Woodward, 2006)).
Theories for trigeminal CO₂ detection mechanisms involve the stimulation of free nerve endings through tissue acidification (Hummel, 2000; Shusterman and Avila, 2003): CO₂ reaches the cell cytoplasm by diffusing through the cell membrane. Within the cell carbonic anhydrase (CA) metabolizes CO₂ into H⁺ and HCO₃⁻ and the released protons activate pH-sensitive TRP or ASIC (acid-sensing ion channel) channels. CA has been shown to be expressed in subpopulations of trigeminal ganglion neurons (Wong et al., 1983; Tanimoto et al., 2005) and it is hypothesized that carbonated water excites lingual nociceptors via carbonic anhydrase-dependent processes (Simons et al., 1999).

CO₂ responses in a subpopulation of olfactory sensory neurons (GC-D cells) also requires the activity of carbonic anhydrase to catalyze the conversion of CO₂ to bicarbonate (HCO₃⁻). Bicarbonate is then thought to activate cGMP-producing ability of guanylyl cyclase-D (GC-D), a membrane GC exclusively expressed in the CO₂-responsive OSNs. cGMP in turn opens the cGMP-sensitive CNG (cyclic nucleotide-gated) channels which leads to Ca²⁺-influx into GC-D⁺ neurons (Hu et al., 2007; Sun et al., 2009).

Therefore mechanisms of trigeminal and olfactory mediated CO₂ detection seem to be completely different from the recently discovered molecular basis of insect CO₂ reception: In Drosophila two members of a large family of Drosophila gustatory seven-transmembrane-domain chemoreceptor genes (Gr21a and Gr63a), are coexpressed in chemosensory neurons and together are sufficient for olfactory CO₂-chemosensation in Drosophila (Jones et al., 2007; Kwon et al., 2007).

The typical activation spot in the posterior central region elicited by intranasally administered CO₂ was completely abolished after application of the CA inhibitor acetazolamid, revealing the importance of carbonic anhydrase for trigeminal mediated CO₂ detection. A general drug influence on cell excitability was excluded by demonstrating unchanged ethanol activation pattern after drug application (Fig. 4-16) (citral and vanillin evoked activity patterns were also unaltered following acetazolamid application; data not shown). These results strongly support the idea of chemosensory activation patterns being mediated by different neuronal subpopulations, since blocking CO₂ evoked activity did not affect ethanol trigeminal activation.
Taken together, the local spots of activity seen in the CO$_2$ and ethanol condition are likely to be mediated by discrete neuronal populations differing in receptor expression (e.g. TRPV1 and CA) as well as affiliation to the ophthalmic or maxillary branch of the trigeminal nerve. The unique activity spot in the ethanol condition associated to an activation of ethmoidal nerve fibers would therefore argue for a TRPV1 expressing population of trigeminal neurons in the anterior central area of the ganglion. The activity spot shared by the ethanol and the CO$_2$ condition associated to a stimulation of the nasopalatine and/or infraorbital nerve, points to TRPV1 and CA expressing (maybe also to some extent coexpressing) cells located in the posterior central portion of the ganglion.

But do these findings really point to a trigeminal odor coding?

The differences in the activity patterns observed upon CO$_2$ and ethanol application were remarkably constant. Stability and reproducibility of the patterns was demonstrated by the across animal comparison (Fig. 4-9, A). However, a potential influence of stimulus concentration could not be excluded meaning that activity pattern differences may also reflect different stimulus intensities. An intensity matching of CO$_2$ and ethanol is quite difficult, since it is nearly impossible to determine the odor concentration in the nasal mucus and especially in deeper layers of the olfactory epithelium where the free nerve endings of the trigeminal nerve are located. In case the trigeminal activity pattern would be based on an activation of subpopulations of neurons, depending on their receptor expression pattern, a change in stimulus concentration should have only a minor effect on the elicited activity map, since still the same neurons will be activated. However, if the trigeminal activity pattern is based on stimulus concentration a reduction of concentration would dramatically alter the shape of the elicited trigeminal activation pattern.

Therefore different ethanol and citral concentrations were tested (vapor phase of undiluted ethanol and 50% diluted ethanol; 1 mM and 20 mM citral; [20 mM citral represents a saturated solution]). Ethanol and citral were chosen since they elicit very distinct activity patterns (the citral pattern will be discussed later). No differences in the activity patterns evoked by the altered ethanol or citral concentrations could be observed (Fig. 4-15) compared to the pattern elicited with standard concentrations (Fig. 4-9). These results demonstrate that chemosensory pattern formation at the level of the trigeminal ganglion is largely independent of stimulus concentration.
conclusion, stimulus specific activation patterns seem to be to some extent present at the level of the trigeminal ganglion. Therefore these results might suggest a certain trigeminal ability for coding stimulus identity, the “what” of sensory information processing.

But what about the “where” of sensory information processing?

In order to investigate if spatial trigeminal activity patterns are stimulation site dependent, an alternative oral odor application was tested (Fig. 4-14). Comparing the activity spots of nasally and of orally administrated ethanol revealed clearly shifted spatial patterns. Oral ethanol application elicited a broad activity spot in the anterior medial region of the ganglion. However, there was no activation observed at this area following nasal ethanol application. Therefore spatial trigeminal activation patterns seem to be stimulus site dependent likely arising from subpopulations of trigeminal neurons associated with different trigeminal (sub-) branches.

Taken together, these results underline the trigeminal ability of also coding “where” information of sensory processing.

An unexpected finding in this study was the discovery of the suppression of large trigeminal ganglion areas following CO$_2$ and ethanol application. For verifying these results all potential artifacts that could interfere with the detected VSD had to be excluded:

1) The camera and filter settings used in this study are in principal also capable of detecting an intrinsic BOLD (Blood Oxygen Level Dependent) signal that might interfere with the recorded VSD signal. Since the shape of the BOLD signal is species and tissue dependent, we also performed intrinsic BOLD imaging in the trigeminal ganglion. The BOLD signal in response to nasal ethanol application predominantly consisted of the main bold response peaking after 5 s. No fast response (initial dip) or post stimulus undershoot were detected (Fig. 5-1). These results demonstrate the unlikelihood of a BOLD signal related response being responsible for the observed decline in the signal interpreted as suppression.
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2) Indirect evidence pointing to inhibition, as indicated by suppression of activity compared to baseline levels, is provided by the acetazolamid blocking experiments. Drug application had a large impact on CO$_2$ evoked activity spots (Fig. 4-16): No significantly activated regions could be detected in the CO$_2$ condition after drug application, whereas suppressed areas seemed to increase in size. In conclusion, excitatory drive seems not be required for the observed suppression (and activity does not automatically trigger suppression; see citral and vanillin conditions). These results suggest that excitation and suppression are two independent mechanisms probably mediated by discrete neuronal populations.

3) The most striking observation arguing for a substantial suppressive effect is given by the electrophysiological measurements: Electrode recordings from locations near the center of the suppressed region revealed a high degree of rhythmic spontaneous activity (Fig. 4-12, PSTH; blue bars). This spontaneous extracellular activity was almost completely abolished after CO$_2$ or ethanol application (Fig. 4-12, PSTH; black bars). In contrast no reduction of spontaneous activity could be observed after citral application (Fig. 4-13).

In conclusion, a subpopulation of trigeminal neurons exerts spontaneous activity which is suppressed during CO$_2$ and ethanol stimulation. However, the underlying mechanisms leading to the suppressive response are unclear. Regarding the olfactory

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Fig. 5-1: Intrisic BOLD signal imaging in the trigeminal ganglion
Global time course ($\Delta R/R$) of whole ganglionic activity evoked by ethanol application. Vertical red line indicates stimulus onset. 5 trials; recording duration = 8.8 s
system, a lot of centrifugal inputs have been shown to modulate information processing at the level of the olfactory bulb (Price and Powell, 1970; Pinching and Powell, 1972). In analogy, it might be possible that these inhibitory responses may arise from centrifugal brainstem inputs into the trigeminal ganglia and therefore could not be observed in cell cultures experiments. However, there are currently no reports available demonstrating a direct trigeminal ganglion activity modulation by higher brain areas. The only modulation descript so far is taking place in a related system at primary sensory brainstem synapses of dorsal root ganglion neurons. Measurements with isolated DRG neurons have shown that in almost all neurons Cl⁻ is accumulated into the cytoplasm, supporting a Cl⁻ equilibrium potential near -40 mV (Alvarez-Leefmans et al., 1988; Rocha-Gonzalez et al., 2008). The opening of Cl⁻ permeable channels therefore induces a depolarizing Cl⁻ efflux. Presynaptic GABA_A receptors in the dorsal horn conduct Cl⁻ efflux and cause primary afferent depolarization (PAD) following GABAergic interneuron activation (Cervero et al., 2003). PAD mediates presynaptic inhibition of nociceptive afferents but intense afferent stimulation can cause excitatory Cl⁻ efflux which contributes to dorsal root reflexes, hyperalgesia, and neurogenic inflammation (Rudomin and Schmidt, 1999).

High amplitudes of the VSD signal reflect suprathreshold activity: Electrophysiological confirmation.

Electrophysiological recordings from the posterior central / posterior lateral site where a main activity spot can be seen in VSD experiments following CO₂ and ethanol stimulation, also caused spikes after CO₂ (Fig. 4-11, B) and ethanol (Fig. 4-11, D) application. Single trial analysis of the PSTH showed discrete bursts of activity that differ in onset, number, and frequency comparing the two conditions (Fig. 4-11, B D). Single trial analysis of the voltage-sensitive dye signal revealed separated bursts of activity differing in the same parameters (Fig. 4-11, A C).

Taken together, the voltage-sensitive dye signal arising from the trigeminal ganglion seems to originate to a large extend from suprathreshold trigeminal activity. This is not trivial, since the VSD loading approach is not selective for trigeminal ganglion neurons and also satellite glia cells are likely to take up the dye. However, the spiking activity seen in electrophysiological measurements points to a predominantly neuronal origin of the VSD signal.
What could be the origin of the multiphasic responses?

Ethanol application elicits sharply separated spike trains, varying in onset and silent period duration. CO₂ application did not elicit that sharply separated spike trains but rather an asynchronous firing pattern with individual spike bursts, reflected in a gradual increase over time in the averaged PSTHs and VSD signals. These signal oscillations are somewhat surprising, since the olfactometer is creating a continuous air flow through the nose of the animal. Also no stimulus induced whisker movements were observed which possibly could trigger multiphasic trigeminal responses. One explanation might be that the breathing rhythm caused contractions within the nasal cavity which could affect the continuous odor air flow through the nasal cavity. However, the in-trial variations in silent period durations between individual spike trains argue against this hypothesis.

It is more likely that the observed multiphasic responses are due to intrinsic firing properties of individual trigeminal neurons, since surprising similarities to response properties of trigeminal brainstem neurons are apparent: trigeminal brainstem neurons are able to fire spike bursts, spontaneously, as well as at the onset of depolarizing, and offset of hyperpolarizing current pulses (Sandler et al., 1998). Therefore the differences in trigeminal neuron spike rate after ethanol or CO₂ application could be considered as an analogue to the modulation in spike rate in trigeminal brainstem neurons by different input parameters which is thought to code for sensory information in the trigeminal system (Sandler et al., 1998).

Are the calculated response onset times physiological?

The calculated response onset times for ethanol and CO₂ are rather slow (e.g. CO₂ mean = 1,2 s, SEM = 0,47s) and not comparable to detection times known from the olfactory system where odor information arrives at the olfactory bulb over a range of 80–160 ms (Wesson et al., 2008). These slow response kinetics of the trigeminal nerve are somewhat unexpected given that these two test substances have a strong trigeminal component and the Nervus trigeminus is also known for its protecting function against harmful stimuli. However, the determined response onset times are within the same range as onsets times from time–intensity curves of psychophysical studies in which subjects were asked to track intensity of nasal irritation during continuous (7-10 s) presentation of CO₂. Using 35,5% CO₂, the time latency from stimulus onset to first non-zero rating was 1,63 s (range = 1,34-1,98 s, (Wise et al., 2003)). Differences in
the response onset times in our study to the onset times observed in the psychophysical time-intensity examinations are likely to be attributable to species differences as well as to the peripheral versus central component of odor detection examined in these two studies. The partially high standard deviation seen in the calculated response onset times are in agreement with previously reported large but stable individual differences in temporal trigeminal detection dynamics (Wise et al., 2003). Differences in the response kinetics of the olfactory system vs. the trigeminal system are also likely of anatomical nature: in contrast to trigeminal free nerve endings olfactory sensory neurons have a direct contact to the nasal lumen which facilitates stimulus contact. The involvement of different trigeminal channels / detection mechanism could contribute to condition specific differences in response onset latencies.

Elsberg et al., 1935, suggested that the trigeminal system functions as a total mass detector, responding according to the total quantity of material inhaled (concentration X duration). This observation was partially contradicted by other studies (Wise et al., 2004; Wise et al., 2005; Wise et al., 2006) rather favoring a model of an imperfect mass-integrator. A general influence of the stimulus concentration on the response onset time could also be detected in our measurements: The response onset latency of the reduced ethanol concentration was strikingly prolonged (3.8 s) compared to the standard ethanol concentration (1.8 s; SEM = 0.38 s; n = 9). However, no drastic changes in the calculated mean response onset latencies of the two citral concentration could be observed (standard: 0.32 s; SEM = 0.2 s; n = 5) (20 mM: 0.45 s; SEM = 0.25; n = 2). CO₂ and ethanol patterns are more complex compared to the ones elicited by citral or vanillin. The correlation analysis used for calculating the response onset latencies might however easier correlate “simpler” patterns, potentially leading to false positive results (emerging as fast detection times) and therefore impeding correct onset calculation.

The second subcategory of tested odorants (citral / vanillin) elicited very different activation pattern from the ones observed in the ethanol or CO₂ conditions.

Citral elicits an overall activity of the trigeminal ganglion with no detectable focal activity spots or suppressed areas. Citral is known to activate a large number of different TRP channels found in sensory neurons (TRPV1 and TRPV3, TRPM8, and
TRPA1; (Stotz et al., 2008)). Citral’s broad receptor spectrum may therefore be one explanation for the observed homogenous and broad activation. Surprisingly, also the application of vanillin, an odorant previously reported to have no trigeminal component (Doty et al., 1978), elicited an activity pattern relatively similar to that caused by citral application. Vanillin which was used at a concentration of 1 mM in the saturator tube has been recently shown to activate TRPV3 channels, however only at a much higher concentration (10 mM (Xu et al., 2006)). On average vanillin elicited the lowest signal amplitudes in the VSD measurements. One might speculate that the weak trigeminal activity evoked by vanillin is too low to reach perceptual thresholds. That would explain the inability of anosmic patients to perceive vanillin as a trigeminal odorant.

Studies within the trigeminal system (e.g. research on tactile information coding in the barrel field cortex or chemosensory studies on the somatosensory system), often only refer to the trigeminal ganglion as being a simple relay station, detecting somatic sensations in the periphery and transmitting it to the central nervous system. However, recent studies demonstrate that this picture might be too simplistic: It has been shown that trigeminal ganglion neurons directly communicate with satellite glia cells using gap-junctions (Thalakoti et al., 2007) and these gap-junctions are thought to facilitate not only neuron-glia but also glia-glia and neuron-neuron communication. Other hints for potential intraganglional communication mechanism were already discovered quite early demonstrating ATP release from stimulated sensory neurons (HOLTON, 1959). In this respect a possible explanation for the observed overall activity patterns following citral and vanillin application would involve direct intraganglionic neuron-glia, glia-glia and / or neuron-neuron communication using e.g. gap-junctions. In order to investigate the involvement of gap-junctions in the vanillin evoked activity pattern formation we injected small amounts (500-1000 nl; 10 mM) of the commonly used gap-junction blocker carbenoxolone into the ganglion. Preliminary experiments revealed no differences in vanillin evoked activity patterns after blocker application. These results imply the unlikelihood of an involvement of gap-junctions in response to mild trigeminal odor stimulation, however, they do not exclude other intraganglionic communication mechanism.

In respect to the gross somatotopic organization of the trigeminal ganglion and the exclusive nasal stimulus application, one would expect the citral and vanillin response to originate from a trigeminal ganglion area where nasal trigeminal neurons are
located. Therefore future studies are aimed at unraveling these special trigeminal ganglion activity patterns and their potential underlying intraganglional communication mechanism in more detail.

The lack of suppressive responses elicited by nasal application of citral and vanillin might argue for a specific feature of strong (and maybe classified as painful) trigeminal stimuli to elicit suppression responses in the trigeminal ganglion. Since the trigeminal system can reflexively stop inspiration to prevent inhalation of potentially threatening substances, one could also speculate about the involvement of suppressive responses in mediating trigeminal reflexes. Certainly, a larger set of substances would have to be tested in order to prove these assumptions. In this respect the observation that citral at maximal solvable concentration (20 mM) also failed to cause trigeminal suppression is remarkable. On the one hand it could well be that this maximal citral concentration is still not comparable to the used CO₂ and ethanol concentration in terms of trigeminal potency. On the other hand it demonstrates that pattern stability seems to exist at the ganglion level across a large stimulus concentration range, thereby enabling a real trigeminal odor coding.

Taken together, this study provides evidence that coding odor information might not be a feature unique to the olfactory system, but to some extent is also possible via the trigeminal nerve. The here presented data indicate a prototypical activation pattern related to a painful CO₂ stimulus. Ethanol, also a strong trigeminal agonist, produced a map showing high similarity to the CO₂ pattern but included unique activation spots. Reducing the ethanol concentration revealed independency of the spatial map from stimulus intensity. Citral and vanillin elicited activation patterns clearly distinct from that ones seen for CO₂ and ethanol. Therefore this study provides first evidence that trigeminal chemoperception is not only based on a graduated activation of trigeminal fibres since different chemicals are capable of evoking distinct spatiotemporal trigeminal activity patterns. These patterns might provide an explanation for the relatively high odor discrimination ability of anosmic patients.

The observed activity patterns are likely to be the result of an activation of subpopulations of trigeminal neurons expressing different sets of receptor proteins. However, the ganglion appears to be more than a simple relay station since unexpected suppressive as well as intraganglional communication phenomena could be observed.
Unraveling the underlying mechanism will be the aim of future studies. Since trigeminal ganglion neurons are polymodal and transduce very different sensations it is also of special interest how other modalities are coded at the level of the ganglion. In this respect the herein described preparational approach should be easily transferrable to other areas including barrel field and trigeminal pain research.
Human Psychophysical Experiments

The psychophysical experiments were conducted in order to investigate if (indirect) evidence can be found, supporting the hypothesis that suppressive effects are also present in human trigeminal chemoperception:

In the rat, nasal application of either CO$_2$ or ethanol not only caused local spots of trigeminal activation but also a suppression in large ganglionic areas. In contrast, no suppression could be observed when using citral as the stimulus. Citral application elicited an overall trigeminal ganglion activity. Interestingly, the ganglionic area activated by citral also included areas that were suppressed by CO$_2$ and ethanol. This led to the initial hypothesis for the human psychophysical experiments:

If CO$_2$ application to the nose of humans (e.g. anosmic patients or a trigeminal focus group) would also cause a suppression of large areas of the ganglion, this suppression should be able to influence the trigeminal detection threshold for citral. Therefore anosmic patients should be able to detect citral alone in lower concentration than in a mixture with CO$_2$.

The results from the first experiment show that in both healthy and anosmic subjects the combined citral and CO$_2$ administration did not result in any higher trigeminal threshold for citral. Instead subjects experienced a trigeminal activation when the two stimuli - CO$_2$ and citral - were combined at subthreshold concentration. The second experiment showed that a prolonged exposure to CO$_2$ did not decrease the trigeminal intensity of citral. However, the CO$_2$ intensity was rated as significantly more intense after citral administration.

The observed difference in trigeminal perception between healthy (normosmic) and anosmic subjects, i.e. the much higher trigeminal threshold, could be attributed to the interactions between the intranasal trigeminal and the olfactory systems (reviewed in Kobal and Hummel, 1988; Hummel et al., 1996; Frasnelli and Hummel, 2007). For example Hummel et al., 1996 showed that the amplitudes of the chemo-somatosensory event-related potentials to trigeminal stimuli were significantly smaller in patients with olfactory loss, like hyposmia and anosmia, than healthy controls. A possible explanation for this could be that brain areas important for the olfactory system to some degree also are activated during trigeminal stimulation. The lack of olfactory
input in anosmic patients would then result in a loss of central-nervous interactions which in turn could lead to a reduced trigeminal sensitivity.

There are different explanations that could account for the observed phenomena in the two experiments but those that are most probable are either the perceptual consequences of temporal summation (wind-up) or the outcome of changed neuronal pattern, i.e. changed activation of the ganglion that is not equivalent to the sum of CO$_2$ and citral. Both of the hypothesized explanations are described below.

**The Perceptual Consequences of Temporal Summation (wind-up)**

The wind-up mechanism, in humans more known as perceptual wind-up or temporal summation, can be most easily described as the mechanism of increased pain perception due to repetitive stimulation with constant intensity. There has been a lot of research concerning the underlying mechanisms of wind-up and some like Mendell, 1966, suggest that this phenomenon could be explained by the reverberatory activity evoked by afferent C-fibers in interneurones of spinal cord lasting for 2-3 s. If in this period another stimulus would reach the spinal cord, that stimulus would be added to the activity in progress, thereby producing a stronger discharge in the interneurons than the stimulus before (Fig. 5-2). There is a general agreement that there are still many questions left to be answered before the whole picture of the mechanisms underlying wind-up can be completely explained.

![Original illustration of the wind-up recorded in a single motor unit in a α-chloralose anaesthetised rat.](image)

Sixteen stimuli were applied in the most sensitive area of the cutaneous receptive field of the unit. The number of spikes recorded after each of the stimuli increase progressively until reaching a saturation point at stimulus number 12. Note the gap between the early and late responses, presumably A- and C-fibre mediated responses; after (Herrero et al., 2000).
Most of the studies in spinal wind-up (both in humans and rodents) have used electrical inputs as the painful stimulus (reviewed in Herrero et al., 2000). But in the trigeminal system other painful stimuli like CO\(_2\) have also been used to demonstrate the effects of perceptual wind-up. For example Hummel et al., 1994, showed that chemo-somatosensory event-related potentials and pain ratings were modified by repetitive painful stimulation. When subjects received stimuli every two seconds the intensity rating of the first stimulus was significantly lower than to the second or third stimulus. If on the other hand the duration was eight seconds the intensity estimates decreased with successive stimuli when given consecutively. The reason for this result is most likely a summation of the stimulus response at the spinal level when the time interval is short enough.

The effects of wind-up on sensory processing can also be found in rodents. Coste et al., 2008, tested different intensities and frequencies of painful stimuli on rat trigeminal spinal dorsal horn wide dynamic range (WDR) nociceptive neurons. Electrophysiological recordings of the rat WDR neurons in vivo revealed that all of them showed signs of wind-up following constant stimulation at C-fibre threshold and suprathreshold intensities. More importantly, they could show that repeated stimulation with subthreshold stimuli, which initially did not evoke C-fibre responses, triggered C-fibre latencies. This result could point to the possibility that wind-up mechanisms amplify subthreshold excitatory inputs to WDR neurons.

A similar observation can also be made in humans. Stimuli that initially are perceived as being only warm, produce a very high pain sensation upon repeated delivery (Kleinbohl et al., 2006). This phenomenon can also be seen using transcutaneous electrical stimulation, where low, non-painful intensities after repetitive stimulation sum up to a harsh pain (Arendt-Nielsen et al., 2000). Using central integration of the neural responses, the subliminal neural activity in the nociceptors sums up to a painful trigeminal sensation.

Regarding the threshold tests for the CO\(_2\) and citral combination it could well be that despite the potential CO\(_2\) suppression of some trigeminal ganglion neurons in humans, the activity caused by simultaneous CO\(_2\) and citral application leads to spinal and central integration processes triggered by temporal summation and therefore results in a clear trigeminal perception. Hence, this could be a new way of showing the importance of spinal wind-up.
Experiment 2 (suprathreshold CO$_2$ exposure followed by suprathreshold citral and vice versa) shows a crucial impact of application order on wind-up establishment. Healthy subjects showed no significant difference between the citral trigeminal intensity tested alone or directly after CO$_2$ administration. However, the CO$_2$ intensity was rated as significantly stronger after citral administration as compared to CO$_2$ tested separately. These results are reasonable assuming that parts of the human trigeminal ganglion are suppressed due to CO$_2$ application. This suppression could prevent a wind-up phenomenon in the CO$_2 \rightarrow$ citral application order leading to equal citral intensity ratings. However, if citral is applied first large parts of the ganglion are activated including areas that will be activated by the following CO$_2$ application which may account for the observed wind-up in this condition.

The wind-up phenomenon as a possible explanation therefore provides indirect hints for suppressive effects caused by CO$_2$ application.

**Changed Pattern Activation and hence Changed Neural Information.**

As described before the idea for the experiment was derived from the observation that CO$_2$ suppressed part of the areas that were activated by citral.

We therefore expected for the first experiment that the simultaneous administration of CO$_2$ and citral would result in an increased trigeminal threshold for citral.

In the other experiment the preceding administration of a strong CO$_2$ stimulus should result in a decrease of the intensity ratings of the subsequently administered citral.

We observed in the first experiment that the combination of CO$_2$ and citral resulted in a significant change of trigeminal perception (nineteen out of twenty healthy subjects (95%) reported a trigeminal perception in the subthreshold CO$_2$ and citral combination). In the other experiment we observed no significant differences in trigeminal intensity ratings for citral.

One possible explanation for the difference in the outcome of the two experiments might be the presumably altered ganglionic activity pattern when CO$_2$ and citral are combined in different configurations. What may happen is not only a suppression of some ganglion areas, but rather the establishment of a new pattern which could be caused by the molecular sensors detecting the stimuli:
The capacity exhibited by the different types of primary sensory neurons to preferentially detect and encode the specific stimuli into a discharge of nerve impulses appears to result of a characteristic combinatorial expression of different molecular sensors in each neuronal type (reviewed in Belmonte and Viana, 2008). Both CO₂ and citral activate different ion channels that mediate different information. For example, citral, like capsaicin, menthol, allicin and eugenol, activates and modulates TRP channels (Stotz et al., 2008). Many TRP channel proteins are expressed in sensor fibres of the trigeminal ganglia (Caterina et al., 1999; Xu et al., 2005) and nasal epithelium (Xu et al., 2006). The influence of citral on the caused sensation by other TRP modulating stimuli like capsaicin shows an interesting effect: a combination of capsaicin and citral is initially perceived as a very hot sensation that diminishes soon. The reason could be that citral acts as a partial agonist of TRPV1, TRPV3, TRPM8, and TRPA1 but with longer activation it inhibits TRPV1–3 and TRPM8. Similar results can also be seen upon co-superfusion of anandamide and capsaicin, both agonist of the same receptors which results in much smaller currents in the mouse trigeminal neurons than currents evoked by capsaicin alone (Roberts et al., 2002). Therefore the co-application of CO₂ and citral might lead to a new trigeminal activity pattern since e.g. citral modulated TRPV1 is also thought to play a role in trigeminal CO₂ detection via its proton sensitivity.

Fig. 5-3 is a very simple schematic illustration of possible chemical stimulus evoked trigeminal activation patterns (the size of the coloured areas are arbitrary).

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**Fig. 5-3: Schematic illustration of possible chemical stimulus evoked trigeminal activation patterns**
Top: Activation (yellow) caused by citral application; Activation (red) and suppression (blue) caused by CO₂ application
Bottom: two possible combination pattern
(the size of the coloured areas are arbitrary)
Apart from the reception side, superimposing activity and suppression does not automatically imply that the information sent to the CNS is exactly the same as before only “weaker”, it could as easily mean that the information itself is changed. What occurs at the CNS level, the integration of information from different cells to form a perception, not only depends on signal strength but also on the kind of stimuli that processed. CO₂ elicits a distinct trigeminal sensation often described as sharp and stinging. For citral the trigeminal perception has been described initially as cooling followed by a burning sensation. The combination of these two stimuli might therefore not only result in a simple addition or subtraction of ganglion activity but could have created a distinctly new trigeminal sensation which was not accessed in the given tasks of the psychophysical experiment.

These psychophysical examinations clearly demonstrated that the initial hypotheses of this collaboration project might have been too simplistic, since perceptions and sensation are always brain-made interpretations of the outer world.

In future, rat experiments will demonstrate if co-application of CO₂ and citral leads to a new trigeminal activity pattern and psychophysical experiments will clarify if the co-application of these two stimuli results in a new trigeminal sensation. However, only a combination of different approaches will help to unravel the molecular and cellular mechanisms as well as the peripheral vs. central components underling trigeminal sensation.
6. Conclusion

This work presents a great advancement in the research of trigeminal perception. I was able to create and establish new tools for the exploration of the trigeminal system, like the anterograde “live-cell”-tracing tool PrV-Kaplan, the functional fluorescent calcium indicator proteins expressing PrV-Bartha variants and last but not least a method allowing high-resolution recordings of optical signals arising from a large region of the rat trigeminal ganglion \textit{in vivo}.

Using PrV-Kaplan for the identification of postsynaptic brainstem neurons (the first relay for trigeminal information processing) synaptically connected to trigeminal neurons innervating the nasal cavity, will allow easy access to these cells and facilitate a detailed physiological characterization in order to further our understanding of mechanisms underlying differentiated trigeminal somatosensation. The completely new generated FCIP expressing viral strains PrV-Bartha_IP, PrV-Bartha_Cam2 and PrV-Bartha_GCaMP2 represent capable new tools for the functional analysis of whole circuits of synaptically connected neurons \textit{in vitro} and \textit{in vivo}.

The imaging results of dissociated trigeminal neurons revealed that many odorants known to exhibit trigeminal sensation \textit{in vivo} fail to elicit responses \textit{in vitro}. Therefore the complex receptor expression pattern observed in trigeminal neurons does not explain all chemosensory features associated with the trigeminal nerve. For evolving its entire chemosensory ability, the trigeminal nerve rather seems to depend on information from cells of its peripheral innervation areas. These results could also be of medical relevance, since they point to alternative prospects of modulating the system.

The \textit{in vivo} imaging approach of the trigeminal ganglion demonstrates that odor specific spatiotemporal activation patterns, at least to some extent, exist at the level of the trigeminal ganglion. Therefore trigeminal chemoperception does not seem to be only based on a graduated activation of trigeminal (pain) fibers, as previously assumed. Odor specific trigeminal activation patterns might be one explanation for the relatively high odor discrimination ability of anosmic patients. Studies comprising the trigeminal system (e.g. research on tactile information coding in the barrel field cortex
or chemosensory studies on the somatosensory system) often only refer to the trigeminal ganglion as being a simple relay station, detecting somatic sensations in the periphery and transmitting it to the central nervous system. However, with respect to the data, it cannot be excluded that a part of sensory information processing already takes place at the level of the trigeminal ganglion.

For the first time olfactory stimulus representations in the trigeminal ganglia could be visualized and both activation as well as suppressive mechanisms significantly contribute to the observed patterns. Although the underlying mechanisms mediating this suppression are still elusive, these finding allows speculation about more complex trigeminal coding strategies than previously expected. In addition, the psychophysical experiments provide indirect evidence for the potential existence of suppressive effects also in the human trigeminal system.

In conclusion, this work demonstrates that trigeminal mediated odor perception and processing is much more complex than previously assumed and the newly developed methodological approaches should be more than useful for getting deeper insight into this system in future research.

Moreover, these results may not only be important for a better understanding of trigeminal chemoperception but should in general help to expand our knowledge about information processing in peripheral sensory systems.
Zusammenfassung


Vorangegangenen Studien über das trigeminalen System (z.B. die Erforschung von taktiler (Schnurhaar vermittelter) Informationsverarbeitung in Nagern oder chemosensorische Studien im somatosensorischen System) schrieben dem trigeminalen Ganglion meist nur eine informationsweiterleitende Funktion zu, indem der trigeminalen Nerv verschiedenste Stimuli in der Peripherie detektiert und diese Information an das zentrale Nervensystem weiterleitet. Durch die neuen Daten der optischen Ableitungen trigeminaler Aktivitätsmuster kann es jedoch nicht mehr ausgeschlossen werden, dass ein gewisser Teil der sensorischen Informationsverarbeitung bereits auf der Ebene des Ganglions selbst stattfindet.

Zum ersten Mal konnten neuronale duftstoffvermittelte Aktivitätsmuster im trigeminalen Ganglion *in vivo* und in Echtzeit visualisiert und damit gezeigt werden, dass diese sowohl aus Aktivierung sowie aus Suppression von Nervenzellaktivität bestehen. Obwohl die zugrundeliegenden Mechanismen der Suppression noch nicht vollständig aufgeklärt werden konnten, erlauben die bisher gesammelten Daten jedoch eine Spekulation über weit komplexere trigeminale Kodierungsmechanismen als bisher angenommen. Zudem deuten die psychophysischen Untersuchungen indirekt auf die Möglichkeit ähnlicher Kodierungsstrategien im menschlichen trigeminalen System.


Die in dieser Arbeit gewonnenen Ergebnisse könnten daher nicht nur zu einem besseren Verständnis der trigeminalen Chemoperzeption beitragen, sondern auch einen generellen Beitrag zur Aufklärung der Informationsverarbeitung peripherer sensorischer Systeme leisten.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>ASIC</td>
<td>acid-sensing ion channel</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>β-Gal</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>BHV-1</td>
<td>bovine herpes virus -1</td>
</tr>
<tr>
<td>BO</td>
<td><em>Bulbus olfactorius</em>, olfactory bulb</td>
</tr>
<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin-gene-related peptide</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CPE</td>
<td>cytopathogen effect</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td>DMEM</td>
<td>dulbecco’s modified essential medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol bis(amoënbylether) tetraacetic acid</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FLI</td>
<td>Friedrich-Loeffler-Institute</td>
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<tr>
<td>FO</td>
<td>foscarnet, phosphonoformic acid</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<td>GABA</td>
<td>gamma-aminobutyric-acid</td>
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<tr>
<td>gB</td>
<td>glycoprotein B</td>
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<td>GC-D</td>
<td>guanylyl cyclase D</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>gG</td>
<td>glycoprotein G</td>
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<tr>
<td>GG</td>
<td>gasserian ganglion (=Trigeminal ganglion)</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cell line 293</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>hpi</td>
<td>hours post infection</td>
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<tr>
<td>HSV-1</td>
<td>Herpes simplex virus-1</td>
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<tr>
<td>IB4</td>
<td>Isolectin B4</td>
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<td>MDBK</td>
<td>Madin-Darby bovine kidney cell line</td>
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<td>mRFP1</td>
<td>monomeric red fluorescent protein 1</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>OB</td>
<td>olfactory Bulb</td>
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<td>OE</td>
<td>olfactory epithelium</td>
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<tr>
<td>ORN</td>
<td>olfactory receptor neuron</td>
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<tr>
<td>PAD</td>
<td>primary afferent depolarization</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<tr>
<td>PK15</td>
<td>porcine kidney cell line 15</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PrV</td>
<td>Pseudorabies virus</td>
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<tr>
<td>PSTH</td>
<td>Peri-Stimulus-Time-Histogram</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RUB</td>
<td>Ruhr-University Bochum</td>
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<tr>
<td>SCC</td>
<td>solitary chemosensory cells</td>
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<tr>
<td>SCG</td>
<td>superior cervical ganglion</td>
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<tr>
<td>Sp5</td>
<td>spinal trigeminal nucleus</td>
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<td>Sp5C</td>
<td>spinal trigeminal nucleus <em>caudalis</em></td>
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<td>TG</td>
<td>trigeminal ganglion</td>
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<td>trigeminal ganglion neuron</td>
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<td>TM</td>
<td>transmembrane</td>
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<tr>
<td>TrkA</td>
<td>tyrosine kinase A</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<td>VSD</td>
<td>voltage-sensitive dye</td>
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<td>WDR</td>
<td>wide dynamic range</td>
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<tr>
<td>YC2.1</td>
<td>Yellow Cameleon 2.1</td>
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Reference List


ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, 112, 819-829.


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Curriculum Vitae

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„Mechanisms of trigeminal perception – Characterization of the cellular properties of
sensory neurons of the trigeminal system in rats and mice”

Supervisors:
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Curriculum Vitae


10/2008 - 2/2009 scholarship holder of the Wilhelm and Günther Esser-Foundation Ruhr-University Bochum

Academic practical courses:

- method course “Biostatistics”, Ruhr-Universität-Bochum, Bochum, Germany
- cell culture course “in vitro skin model”, Heidelberg, Germany
- method course “trigeminal wholemount explants”, Department of Anatomy and Neurobiology, University of Maryland, School of Medicine, Baltimore, Maryland, United States
- method course “Microscopy”, Ruhr-Universität-Bochum, Bochum, Germany
- method course “working with laboratory animals” FELASA (Federation of European Laboratory Animal Science Association) Ruhr-Universität-Bochum, Bochum, Germany
- workshop “research management” (official GERMAN RESEARCH FOUNDATION workshop) Ruhr-Universität-Bochum, Bochum, Germany
- workshop “Getting published in science - Strategies for writing journal articles“, Ruhr-Universität-Bochum, Bochum, Germany
- workshop “Advanced Presentation Techniques”, Ruhr-Universität-Bochum, Bochum, Germany
- workshop “Science College 2008”, Ruhr-Universität-Bochum, Research School, Germany
- workshop „Perspektiven und Chancen für Nachwuchswissenschaftler“, EuroConsult Research & Education Universität Bonn Universitätssclub Bonn, Germany

Foreign Languages:

English fluent in speaking and writing
French basic knowledge
Computer Literacy:

• Experiences in handling Word, Excel, Powerpoint, Adobe Photoshop and Coreldraw
• Experiences in handling the common data acquisition and analysis – software for the patch clamp-technique (Pulse, Pulse Fit)
• Basic knowledge MATLAB software

Collaborations (within RUB)

• Jun.-Prof. Dr. Dirk Jancke, Department of General Zoology and Neurobiology, Cognitive Neurobiology, Faculty of Biology
• Dr. Heike Benecke, Department of Cellphysiology, Faculty of Biology
• Prof. Dr. K.-P. Hoffmann, PD Dr. C. Distler, Department of General Zoology and Neurobiology, Faculty of Biology

Collaborations (outside RUB)

• Dr. Hartwig Spors, Department of Molecular Neurogenetics, Max Planck Institute of Biophysics, Frankfurt/Main, Germany
• Prof. Dr. Thomas Mettenleiter, Dr. Barbara G. Klupp, Friedrich-Loeffler-Institut, Insel Riems, Germany
• Jun. Prof. Dr. L. Steinsträßer, Dr. F. Jacobsen, Bergmannsheil Bochum, Germany
• BDF Beiersdorf AG, Hamburg, Germany
• PD Dr. Anja Horn-Bochtler, Institute of Anatomy, Ludwig-Maxmilians University, Munich, Germany
• Prof. Dr. Thomas Hummel, Department of Otorhinolaryngology, University of Dresden Medical School, Germany

Travel Awards

• Ruth und Gert Massenberg Stiftung Travel Award 2004 “AChemS Meeting“
• participant of the 57th Meeting of Nobel Prize Winners (Physiology or Medicine) from July 1st to 6th in Lindau 2007 (Graduiertenkolleg GRK 736 nominee; accepted by the Review Panel of the Lindau Council “The Review Panel of the Lindau Council conducted a peer review among all candidates proposed to them in order to select the participants for the restricted number of places”
• GlaxoSmithKline Stiftung Travel Award 2007 “AChemS Meeting“
• AChemS Travel Award 2008
• GlaxoSmithKline Stiftung Travel Award 2008 “Neuroscience 2008“
• AChemS Travel Award 2009
Publication list

Articles


**Rothermel M, Ng B, Jancke D, Hatt H.** Spatiotemporal dynamics of odor representation in the trigeminal ganglion *in vivo* visualized by voltage-sensitive dye imaging. *in preparation*

**Rothermel M, Brunert D, Klupp BG, Mettenleiter TC, Hatt H.** Advanced tracing tools: Functional neuronal expression of virally encoded fluorescent calcium indicator proteins (FCIP). *in preparation*


Talks


**Published abstracts:**

Damann N, Klopfleisch R, Teifke JP, Klupp B, Dörner JF, **Rothermel M**, Hatt H, Mettenleiter TC, Wetzel CH. **Viral tracing of murine trigeminal neurons innervating the nasal cavity.** *Olfactory Bioresponse* III 40, Dresden, Germany, (Poster award)


**Rothermel M**, Damann N, Mettenleiter TC, Hatt H, Wetzel CH. **Pseudorabies virus mediated live-cell tracing: a useful tool for innervation dependent labelling and functional characterization of trigeminal neurons.** *3rd Neuron satellite meeting*
“Neurons and sensory systems” (Neuroscience 2005), Washington DC, Abstract book, p. 59

Damann N, Rothermel M, Klupp BG, Mettenleiter TC, Hatt H, Wetzel CH, Different nociceptive and thermosensory capacities of nasal and cutaneous trigeminal neurons identified in vitro by pseudorabies virus mediated tracing. 3rd Neuron satellite meeting “Neurons and sensory systems” (Neuroscience 2005), Washington DC, Abstract book, p. 60

Rothermel M, Schöbel N, Damann N, Mettenleiter TC, Hatt H, Wetzel CH, Tracing the trigeminal system with Pseudorabies viruses. 36th annual meeting of the Society for Neuroscience (Neuroscience 2006), Atlanta, Georgia, 50.1/J14.


Rothermel M, Benecke H, Sisnaiske JI, Jacobsen F, Steinsträßer L, Steinau HU, Hatt H. What does the skin tell the brain? – Investigating possible interaction mechanism between the skin and neurons of the peripheral nervous system. 6th International Symposium, Neuronal mechanisms of Vision, NEUROVISION, Sonderforschungsbereich 509, Ruhr Universität Bochum, Bochum. Abstract book, p. 4

Rothermel M, Benecke H, Sisnaiske JI, Jacobsen F, Steinsträßer L, Steinau HU, Hatt H. Chemosensory information processing between skin and neurons of the peripheral sensory system. 37th annual meeting of the Society for Neuroscience (Neuroscience 2007), San Diego, California, 824.6/HH23.

Menge M, Distler C, Rothermel M, Hoffmann KP. Darstellung der Verbindung vom Colliculus superior (CS) zu den Schulter- und Armmuskeln. Undergraduate Poster Session Hoffmann Lab (30.0.08)

Rothermel M, Ng B, Jancke D, Hatt H. Spatiotemporal dynamics of odor representation in the trigeminal ganglion in vivo visualized by voltage-sensitive dye imaging. International Symposium on Olfaction and Taste (ISOT) / Annual Meeting of the Association for Chemoreception Sciences (AChemS) Meeting 2008, San Francisco, California,

**Rothermel M., Ng B., Hatt H., Jancke D.** In vivo voltage-sensitive dye imaging of odor representation in the trigeminal ganglion. *38th annual meeting of the Society for Neuroscience (Neuroscience 2008)*, Washington DC

Schulze C., **Rothermel M., Lienbacher K., Curie T., Klupp BG., Mettenleiter TC., Distler C., Hatt H., Hoffmann KP., Horn A.** Transsynaptic retrograde labelling in the oculomotor system in rodent using tetanus toxin fragment C and Pseudorabies virus: opportunities and limitations. *8th Meeting of the German Neuroscience Society 2009*