Involvement of kainate glutamate receptors in the modulation of neuronal transmission in brain areas involved in migraine pathophysiology

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Declaration

I, Anna Andreou, confirm that the work in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Abstract

Migraine pathophysiology is thought to involve activation of the trigeminal fibres which innervate dural structures. The nociceptive inflow from the meninges is relayed to the trigeminocervical complex (TCC), before ascending to higher brain areas, including the thalamus. Glutamate is implicated in the transmission of the nociceptive information and thus an increased understanding of the nature and effects of glutamate receptors activation has major implications in migraine pathophysiology and treatment. Here the role of kainate receptors, a member of the ionotropic glutamate receptors subfamily, was investigated in relaying sensory information upon activation of the trigeminovascular system.

In order to study the role of kainate receptors on the periphery, we used the neurogenic dural vasodilation (NDV) model, in which electrical stimulation of the dura mater causes reproducible vasodilation, due to calcitonic gene-related peptide (CGRP) release. In this set of experiments kainate receptor activation but not blockade was effective in inhibiting NDV. Vasodilation induced by systemic administration of CGRP was not changed by administration of a kainate receptor agonist.

In the TCC, local application by microiontophoresis of a selective kainate receptor antagonist on second order neurons which were excited by meningeal electrical stimulation, caused dual effects; 50% of the neurons tested were inhibited, whereas in a second subpopulation, activation in response to meningeal stimulation was facilitated. However, in all neurons tested, post-synaptic activation in response to kainate receptor agonists application was selectively inhibited.

Microiontophoretic ejection of a kainate receptor antagonist in the ventroposteromedial thalamus (VPM) was able to inhibit cell firing in response to dural stimulation, as well as post-synaptic firing in response to kainate receptor activation. Both effects were reversed when the kainate receptor antagonist was co-ejected with a 5-HT_{1B} receptor antagonist.

We also carried electrophysiology studies in both the TCC and the VPM nucleus in order to compare the effects of the clinically active kainate receptor antagonist LY466195. Systemic and local application of LY466195 was able to inhibit cell firing in response to dura mater stimulation in both the TCC and VPM nucleus. Moreover, further to the kainate binding, a significant action of the compound on *N*-methyl-D-aspartate receptors was observed.

Contents

Figures and Tables

Published Abstracts and Papers

Papers

Andreou AP, Holland PR & Goadsby PJ (2008). Activation of GluR5 kainate receptors inhibit neurogenic dural vasodilation in an animal model of trigeminovascular activation. Br J Pharmacol., In press

Abstracts

Andreou AP, Storer RJ, Holland PR, Goadsby PJ (2006) CNQX inhibits trigeminovascular neurons in the rat: a microiontophoresis study. Cephalalgia 26:p1383

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Glossary

3V,	third ventricle
5-HT,	5-hytroxytryptamine
ACC,	antireor cingular cortex
AChE,	acetylcholinesterase
AMPA,	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
ASICs,	acid-sensing ion channels
ATPA,	(RS)-2-alpha-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid
B2,	Bradykinin 2 receptor
BDNF,	brain-derived neurotrophic factor
CB1,	cannabinoid receptor 1
CCL3,	Chemokine (c-c motif) ligand 3
CGRP,	calcitonin gene-related peptide
CNQX,	6-cyano-7-nitroquinoxaline-2,3-dione
CNS,	central nervous system
CSD,	cortical spreading depression
CSF,	cerebrospinal fluid
DHE,	dihydroergotamine
DOR,	delta opioid receptor
DRG,	dorsal root ganglia
DRN,	dorsal raphe nucleus
EAA,	excitatory amino acids
EAAC,	excitatory amino acid carrier
EAAT,	excitatory amino acid transporter
EP,	E series of prostaglandin receptors
EP,	Entopeduncular nucleus
EPSC,	excitatory post-synaptic current
ES,	electrical stimulation
FHM,	familial hemiplegic migraine
Fos,	Fos proteine-like immunoreactivity
GABA,	γ-aminobutyric acid
GDH,	glutamate dehydrogenase
GLAST,	glutamate/aspartate transporter

GLT,	glutamate transporter
GlyR,	glycine receptors
GPCRs,	G-protein-coupled receptors
GR127935,	N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methy-l-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride
GS,	glutamine synthetase
GTP,	guanosine 5′-triphosphate
GYKI 52466,	4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-y l)-benzenamine hydrochloride
IACUC,	Institutional Animal Care and Use Committee
iGluR,	ionotropic glutamate receptor
IHS,	international headache society
kainate,	2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine
LTM,	low threshold mechanosensitive
LY293558,	(3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1*H*-tetrazol-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid monohydrate
LY382884,	3S, 4aR,6S, 8aR)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid
LY466195,	(3*S*,4*aR*,6*S*,8*aR*)-6-[[(2*S*)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]decahydro-3-isoquinolinecarboxylic acid
MDH,	medullary horn
mGluR,	metabotropic glutamate receptor
MK 801,	dizocipline maleate
MMA,	middle meningeal artery
MOR,	mu-opioid receptor
MRI,	magnetic resonance image
NA,	noradrenaline
NAS-181,	(2R)-2-[[[3-(4-Morpholinylmethyl)-2H-1-benzopyran-8-yl] oxy]methyl]morpholine dimethanesulfonate
Nav,	voltage-gated sodium channel
NBQX,	2,3-Dioxo-6-nitro-1,2,3,4-

	tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NK,	neurokinin
NMDA,	N-methyl-D-aspartic acid
NO,	nitric oxide
NOS,	nitric oxide synthase
NPY,	neuropeptide Y
NRM,	nucleus raphe magnus
NS,	nociceptive specific
NS-102,	5-nitro-6,7,8,9-tetrahydrobenzo[g]indole-2,3-dione-3-oxime
NSAID,	non-stereoidal anti-inflammatory drugs
P2,	purinergic receptors
PACAP,	pituitary adenylate cyclase-activating peptide
PAG,	periaqueductal gray
PET,	positron emission tomography
PHI,	peptide histidine
POm,	posterior medial thalamus
PPE,	plasma protein extravasation
PVP,	Paraventricular nucleus
RE,	Reunions nucleus
RT,	reticular nucleus
SP,	substance P
SSS,	superior sagittal sinus
SYM 2081,	(2S,4R)-4-Methylglutamic acid
TAC's,	trigeminal autonomic cephalalgias
TASK,	two-pore domain weak inward rectifying K+ channels – related arachidonic acid-stimulated K+ channel
TBOA,	DL-*threo*-β-Benzyloxyaspartic acid
TCC,	trigeminocervical complex
TM,	transmembrane domain
TNC,	trigeminal nucleus caudalis
TREK,	two-pore domain weak inward rectifying K+ channels-related K+ channel
TRPA,	transient receptor potential ankyrin
TRPM,	transient receptor potential melastatin

TRPV,	transient receptor potential vanilloid
VB,	ventrobasal complex
Vc,	subnucleus caudalis
VGCC,	voltage-gated calcium channels
VGLUT,	vesicular transporter
VIP,	vasoactive intestinal peptide
VL,	ventrolateral nucleus
vlPAG,	ventrolateral column of the PAG
VM,	Ventromedial nucleus
Vp,	principal trigeminal nuclei
VPI,	ventral posterior inferior nucleus
VPL,	ventral posterior lateral nucleus
VPM,	ventroposteromedial thalamic nucleus
VPMpc,	ventral posterior medial parvicellular nucleus
Vsp,	spinal trigeminal nucleus
WAY 100135,	(S)-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride
WDR,	wide dynamic range
ZI,	zona incerta
α-KG,	α-Ketoglutarate

Chapter 1: Introduction

1.1 Headache classification

Headache disorders are very common among the population and include disorders with head pain characteristics. In 1988 the International Headache Society (IHS) developed the widely used headache classification system, which was subsequently revised in 2004 (Headache Classification Committee of the International Headache Society, 2004), with the aim to provide an accurate headache diagnostic tool to facilitate the management of patients with headache disorders.

The IHS classification divides headache in three groups:

A. Primary headaches; classification is based on headache symptoms and the headache itself is the primary problem, with no identifiable underlying cause.

B. Secondary headaches; classification is based on their cause and headache symptoms are due to an underlying condition.

C. Cranial neuralgias, facial pain and other headaches which are more uncommon than the other two categories.

Headaches are classified in 14 major categories (table 1) and each one is divided in different subcategories depending on the specific symptoms. Migraine is the most common and well studied type of primary headache.

Table 1: Headache classification

	IHS code	Classification
Primary headaches	1.	Migraine
	2.	Tension-type headache
	3.	Cluster headache and other trigeminal autonomic cephalalgias
	4.	Other primary headaches
Secondary headaches	5.	Headache attributed to head and/or neck trauma
	6.	Headache attributed to cranial or cervical vascular disorder
	7.	Headache attributed to non-vascular intracranial disorder
	8.	Headache attributed to a substance or its withdrawal
	9.	Headache attributed to infection
	10.	Headache attributed to disorder of homoeostasis
	11.	Headache or facial pain attributed to disorder of cranium, neck, eyes, ears, nose, sinuses, teeth, mouth or other facial or cranial structures
	12.	Headache attributed to psychiatric disorder
Central neuralgias, central and primary facial pains, and other headaches	13.	Cranial neuralgias and central causes of facial pain
	14.	Other headache, cranial neuralgia, central or primary facial pain

Adapted from (Headache Classification Committee of the International Headache Society, 2004)

1.2 Migraine classification

Migraine is one of the most disabling chronic disorders (Menken et al., 2000). It is defined as a chronic disorder that results in severe, normally unilateral, pulsating headaches which can involve aura that results in neurological symptoms (Headache Classification Committee of the International Headache Society, 2004). The IHS classification of migraine is divided in six sub-categories (table 2) and each one is divided into a variety of subtypes including familial hemiplegic migraine (FHM) and chronic migraine. Migraine without aura is the most common form which accounts for 70-90% of attacks (Rasmussen and Olesen, 1992) and it is defined as:

- An idiopathic, recurring headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity, and association with nausea, photo- and phonophobia.

Migraine with aura symptoms are typically seen in about 15-20% of patients (Rasmussen and Olesen, 1992) and it is defined as:

- An idiopathic, recurring disorder manifesting with attacks of neurological symptoms unequivocally localisable to cerebral cortex or brain stem, usually gradually developed over 15-20 minutes and usually lasting less than 60 minutes. Headache, nausea and/or photophobia usually follow the neurological aura symptoms directly or after a free interval of less than an hour. The headache usually lasts 4-72 hours.

According to the IHS diagnostic criteria for primary headaches, a migraine diagnosis requires that certain clinical features must be present for attacks to qualify as migraine (table 3).

Table 2: International Headache Society classification of Migraine

IHS code	Migraine type
1.1	Migraine without aura
1.2	Migraine with aura
	1.2.1 Typical aura with migraine headache
	1.2.2 Typical aura with non-migraine headache
	1.2.3 Typical aura without headache
	1.2.4 Familial hemiplegic migraine (FHM)
	1.2.5 Sporadic hemiplegic migraine
	1.2.6 Basilar-type migraine
1.3	Childhood periodic syndromes that are commonly precursors of migraine
	1.3.1 Cyclical vomiting
	1.3.2 Abdominal migraine
	1.3.3 Benign paroxysmal vertigo of childhood
1.4	Retinal migraine
1.5	Complications of migraine
	1.5.1 Chronic migraine
	1.5.2 Status migrainous
	1.5.3 Persistent aura without infarction
	1.5.4 Migrainous infarction
	1.5.5 Migraine-triggered seizure
1.6	Probable migraine
	1.6.1 Probable migraine without aura
	1.6.2 Probable migraine with aura
	1.6.3 Probable chronic migraine

Adapted from (Headache Classification Committee of the International Headache Society, 2004)

Table 3: Diagnostic criteria for migraine

migraine without aura	migraine with aura
A. At least 5 attacks fulfilling criteria B–D	A. At least 2 attacks fulfilling B
B. Headache attacks lasting 4–72 hours	B. At least three of the following characteristics:
C. Headache has at least two of the following characteristics: 1. unilateral location 2. pulsating quality 3. moderate or severe pain intensity 4. aggravation by or causing avoidance of routine physical activity	1. one or more fully reversible aura symptoms indicating focal cerebral cortical and/or brainstem dysfunction 2. at least one aura symptom develops gradually over more than 4 minutes or two or more symptoms occur in succession. 3. aura symptoms last no more than 60 minutes 4. headache follow aura with a free interval of less than 60 minutes, but may begin before or simultaneously with the aura
D. During headache at least one of the following: 1. nausea and/or vomiting 2. photophobia and phonophobia	
E. Not attributed to another disorder	

Adapted from (Headache Classification Committee of the International Headache Society, 2004)

1.3 Migraine prevalence

Headache disorders are the most prevalent neurological conditions (Rasmussen et al., 1991; Leonardi et al., 1998) and migraine is a very common condition worldwide. Estimates of its prevalence have varied widely, ranging from 3% to 23% (figure 1) and these differences can, in the main, be accounted for the differing definitions and methodologies employed. Population based-studies carried out using the IHS classification (Headache Classification Committee of the International Headache Society, 2004) in United States and European countries, suggest a lifetime prevalence of

16% with a female to male ratio of approximately 3:1 and the one year overall prevalence to be 6% in males and 15% to 18% among females (Launer et al., 1999; Lipton et al., 2002; Steiner et al., 2003; Lipton and Bigal, 2005b; Rasmussen, 2006). Onset of migraine is most common in the 2nd and 3rd decade of life (Breslau et al., 1991; Russell et al., 1995).

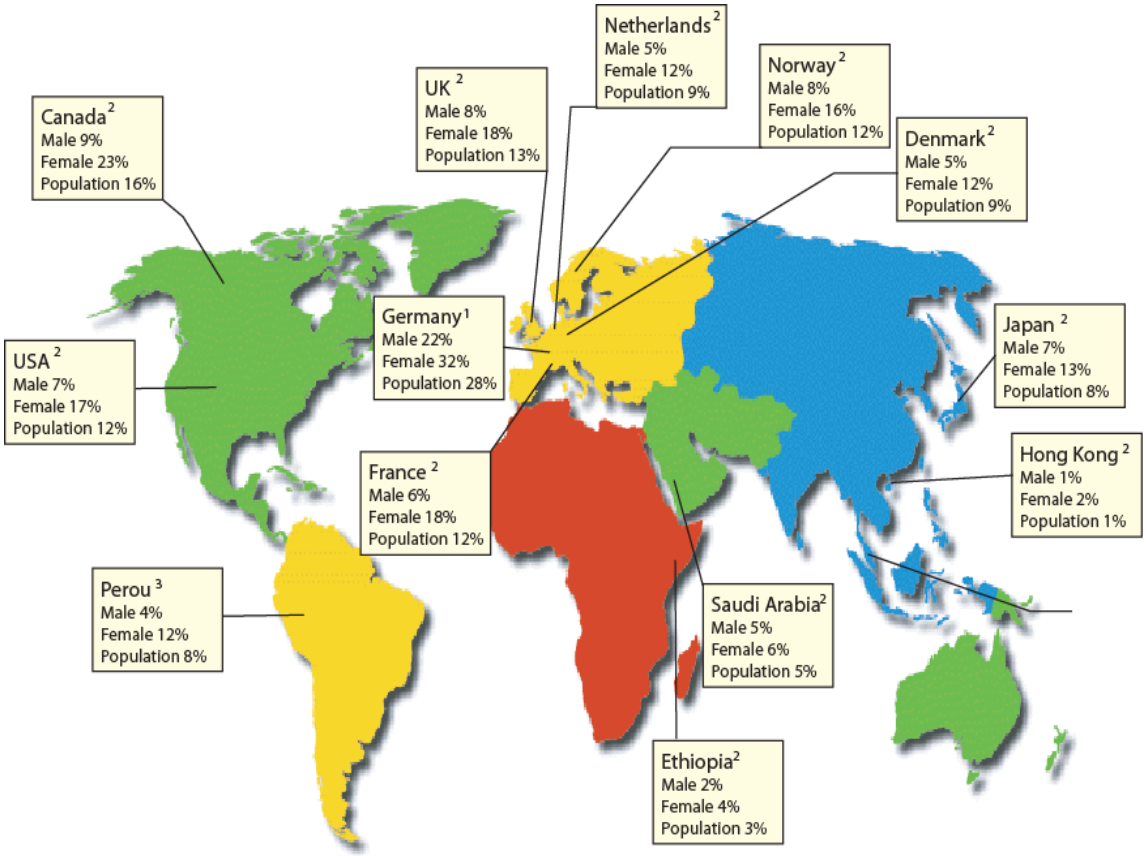


Figure 1: Estimates of migraine prevalence in studies using the diagnostic criteria of the International Headache Society

1 lifetime prevalence; 2 one-year prevalence; 3 not available period for prevalence
Adapted from (Peres, 2005)

Migraine prevalence is age and gender dependent (Russell et al., 1995). Before puberty, migraine is slightly more common in boys, with the highest incidence between 6-10 years of age, but this is reversed (Russell et al., 1995) with the onset of female's cyclic hormonal changes (Epstein et al., 1975; Stewart et al., 2000). In general, women are more commonly affected than men with a lifetime prevalence of 12-17% and 4-6%, respectively (Russell et al., 1995; Peres, 2005). The male to female ratio in Europe is similar to that in America and Africa although the exact percentages differ. America has a prevalence of 5.9% and 17.6% respectively (Lipton et al., 2001; Lipton et al., 2002; Lipton and Bigal, 2005a), while Africa has a much lower prevalence of 1.7% in males and 4.2% in females (Tekle Haimanot et al., 1995). A more recent survey carried out over five countries across Europe and America found an overall prevalence of between 5% and 12% in agreement with previous epidemiologic studies (MacGregor et al., 2003).

1.4 The clinical picture of migraine

The clinical picture of a migraine attack can be divided into three or four distinct stages depending on the presence of aura (figure 2) (Blau, 1992) and each phase may occur alone or in combination with any other phase.

1.4.1 Premonitory symptoms

Premonitory symptoms are neurological, behavioral or physiological symptoms that in some patients can precede a migraine attack. Preceding symptoms can be forewarning of a migraine attack by 2-48 hours before the aura in migraine with aura and before the onset of pain in migraine without aura (Giffin et al., 2003).

Until recently accurate data on premonitory symptom prevalence was lacking (Rossi et al., 2005). Recent studies using patients who fulfill the IHS criteria, described premonitory symptoms in 87% of subjects and the presence of premonitory symptoms

in 84% of migraine attacks. The symptoms were more frequent in patients experiencing migraine with aura (Quintela et al., 2006; Schoonman et al., 2006). Premonitory symptoms vary between individuals and in general neuropsychiatric were the most frequent (82%), followed by sensory (65%) and other general symptoms (58-63%) (Quintela et al., 2006). In an electronic diary-based study the most common premonitory symptoms reported were tiredness (72%), poor concentration (51%) and stiff neck (50%) (Giffin et al., 2003).

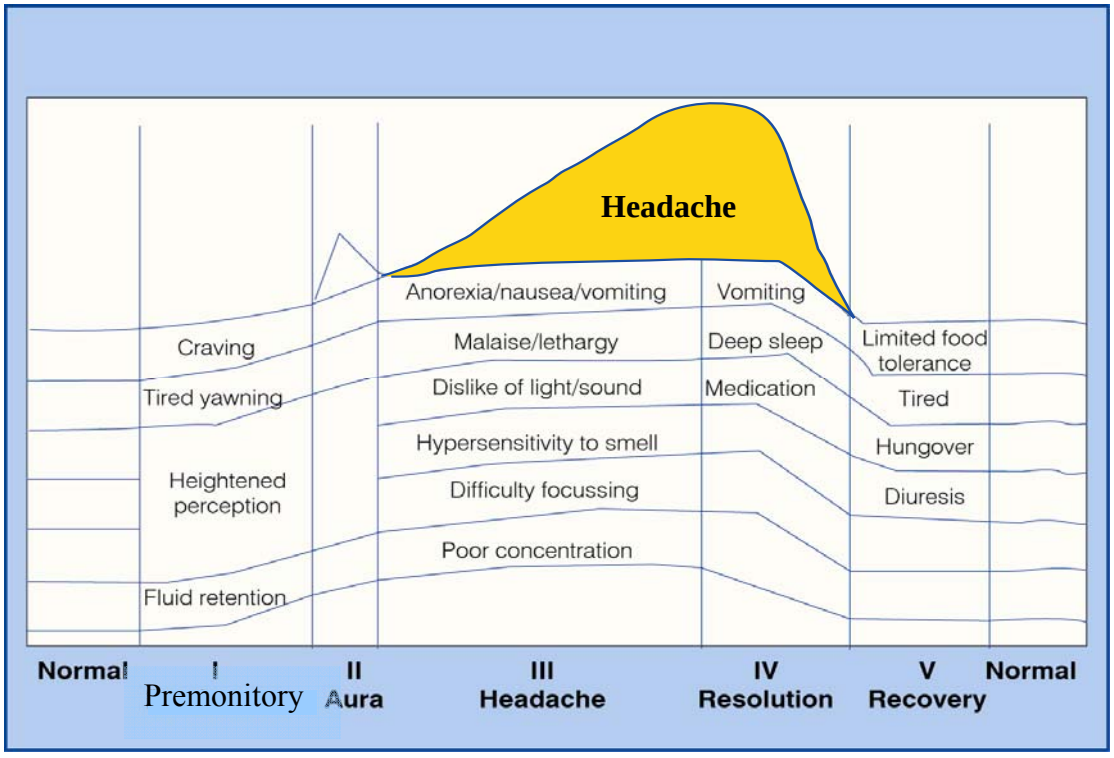


Figure 2: Stages of a migraine attack and possible symptoms during the different phases

The clinical picture of a migraine attack can be divided into distinct phases and each phase may occur alone or in combination with any other, while characteristic symptoms occur during each phase. Adapted from (Blau, 1992).

The diverse nature of symptoms associated with the premonitory phase, suggests that central disturbances occur at both cortical and subcortical levels. Changes in water metabolism or appetite would implicate hypothalamic involvement (Zurak, 1997), as the hypothalamus is the main centre for the integration of endocrine functions, autonomic responses and behaviour, and governs the rythmicity of many physical functions such as body temperature, feeding behaviour and sleep-wake regulation (Saper, 1990; Swaab, 1997). A recent study using positron emission tomography (PET) scanning of spontaneous migraine attacks revealed hypothalamic activation during the attack (Denuelle et al., 2007). The presence of cognitive slowing during the premonitory phase suggests cortical level disruption (Reeves, 1976; Farmer et al., 2003). However, the pathophysiology of the premonitory phase is largely speculative due to the lack of direct studies specifically investigating this phenomenon and demonstrating the actual activation of any brain area during this phase. Dopamine upregulation is likely to be involved in the development of these symptoms in brainstem pathways (De Marinis et al., 2003), as dopamine antagonists have been shown to eliminate the premonitory symptoms of migraine (Peroutka, 1997).

1.4.2 The Aura

The aura symptoms consist of reversible visual, sensory, language or less frequently motor symptoms that precede or accompany the migraine-like headache (table 4). Typically aura develops over 5-20 minutes and last for less than 1 hour. Headache follows aura about 80% of the time and usually commences while cerebral blood flow remains diminished (Olesen et al., 1990).

Visual symptoms are the most common, and are usually described as zigzag or scintillating figures (fortification spectrum), scotomata, flashing lights, distortions in shape and size and they mostly affect one hemifield of both eyes, although subjectively they often appear to affect only one eye. The most frequent visual auras start as a small uncolored but bright flickering spot in the visual field, which becomes more apparent as it begins to expand into a 'C' or inverted 'C' shape zigzag-shaped line. This continues to travel across the visual field, eventually breaking up and disappearing. Occasionally in some patients, sensory symptoms affecting the hand and gradually spreading to the whole arm and the perioral region occur in conjunction with visual aura. These symptoms are frequently described as a sensation of pins and needles, usually affecting

one arm, which travels up from the hand over several minutes to affect the face and tongue (Russell and Olesen, 1996). Motor aura is less frequent and not well recognized by patients and is usually described as motor weakness (Silberstein et al., 2001).

The pathology of aura is now believed to be due to a wave of cortical spreading depression (CSD; paragraph 1.7) which spreads out from the occipital lobe across the cortex, resulting in an initial hyperaemic phase followed by a oligaemic phase (Olesen et al., 1990; Lauritzen, 1994; Olesen, 1998).

1.4.3 The Headache phase

The headache phase lasts between 4-72 hours if untreated. The pain can be severe and in about 80% of the patient the head pain is described as throbbing, as compared to pressing or bursting-like. The head pain of a migraine is more often unilateral than bilateral and may be accompanied by sensitivity to light and sound and difficulties in concentrating and focusing. Movement usually aggravates the pain and nausea, especially in patients suffering from migraine without aura (Zagami and Bahra, 2006). Many migraine patients develop cutaneous allodynia- a painful response to a stimulus that is ordinarily not painful- during a migraine headache, characterised by increased skin sensitivity, mostly within the referred area of pain of the ipsilateral head, which could spread to the other side of the head or the forearm (Burstein et al., 2000). The appearance and spread of cutaneous allodynia is a due to the development and spread of neuronal sensitization from first order trigeminal peripheral neurons to second and third order central neurons in the brainstem and thalamus (Dodick and Silberstein, 2006).

1.4.4 The Resolution phase

The resolution phase or the postdrome is the phase following the severe headache, before full recovery of migraine symptoms (Blau, 1982). In a recent study, Quintela et al. (2006) found resolution symptoms after a migraine attack in 80% of patients and the most common resolution symptoms reported were asthenia, tiredness, somnolence and concentration difficulties. In the same study the severity of the headache was significantly associated with a higher frequency of resolution symptoms.

Table 4: Summary of different types of aura

Aura	Characteristics
Visual	Scotoma, photopsia or phosphenes, rotation, oscillation, shimmering of objects, excessive brightness
Visual hallucinations or distortions	Metamorphosia, macropsia, zoom or mosaic vision
Sensory	Paresthesias, often migrating, and can become bilateral olfactory hallucinations
Motor	Weakness or ataxia
Language	Dysarthria or aphasia
Delusions and disturbed consciousness	Déjà vu, multiple conscious trance-like states

Adapted from (Silberstein et al., 2001).

1.5 Migraine Triggers

A wide range of events and conditions have been proposed to alter conditions in the brain that may trigger migraines. Any single trigger may not be enough to enhance the probability of a migraine attack on its own and migraine triggers vary greatly from one person to another. Triggers often appear to be cumulative, reaching a threshold above which migraine is initiated. Triggers can be both external and internal stimuli including hormonal changes, dietary factors, environmental changes, sensory stimuli and stress (figure 3).

In a recent study Kelman (2007) found that 76% of the patients could recognise the presence of one or more triggers that could initiate their migraine attacks. The most frequent triggers reported are (figure 3): hormonal changes in women (33.3%), stress (25.5%), missing a meal (12%), weather changes (11.3%), perfume smells (10.7%), neck pain (10.6%), alcohol (9.5%) and sleep disturbance (8.6%).

Hormonal change as seen in menstrual migraine is a reliable trigger of attacks in women and this additional hormonal trigger may account for the increased prevalence of migraine in women compared with men in the reproductive years (Silberstein and Merriam, 1991). Migraine in women usually declines after the onset of menopause (Neri et al., 1993; Fettes, 1999), indicating the influence of hormonal changes on migraine occurrence.

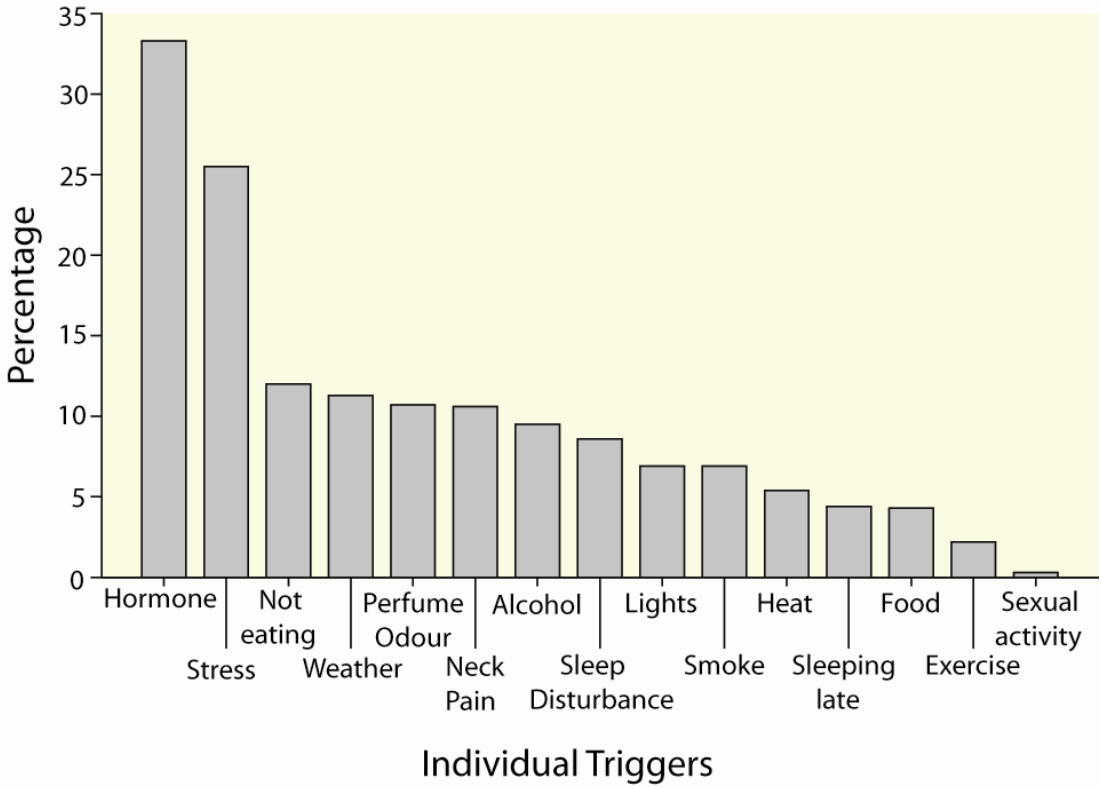


Figure 3: Common migraine triggers identified by migraineurs
Percentage frequency of individual migraine triggers occurring at least occasionally (>33% of headaches). Hormone trigger refers only to women. Adapted from (Kelman, 2007).

Nitroglycerin, which is a nitric oxide (NO) donor is now used in human experimental models for headache induction (Iversen et al., 1989; van der Kuy and Lohman, 2003; Afridi et al., 2004; Afridi et al., 2005a). Red wine is also believed to act via a similar mechanism, as it can cause significant increases in NO release (Leikert et al., 2002), though other studies suggest ethanol causes vasodilation of meningeal vessels by transient vanilloid 1 receptor (TRPV1) activation (Nicoletti et al., 2007). It is important to note that certain migraine triggers may not be a contributory factor, but a result of the migraine premonitory symptoms, giving the impression of a triggering function (Headache Classification Committee of the International Headache Society, 2004).

How different triggers act to initiate a migraine attack is not known. A rather complex hypothesis has been suggested implying that different triggers can activate

hypothalamic, limbic and cortical areas that can impinge on parasympathetic neurons innervating the meninges resulting in vasodilation and local release of molecules that activate meningeal nociceptors (Burstein and Jakubowski, 2005). Recently in a series of experiments Lambert et al. (2008) suggested that cortical activation can indirectly through inhibition of the nucleus raphe magnus (NRM) induce pain responses. In this study it was shown that stimulation of the NRM can suppress trigeminovascular activation in the brainstem. This NRM-mediated inhibition of trigeminovascular activity was antagonised by cortical activation (CSD and light were used as triggers of cortical neuronal activation), through inhibition of neurons in the NRM. Thus migraine triggers could cause cortical activation, which disinhibits trigeminovascular sensation through the NRM (Lambert et al., 2008). As this area consists of both descending inhibitory systems and facilitatory descending pathways to the spinal cord and trigeminocervical complex, it is likely that stimulation of the NRM induces inhibition of trigeminovascular activity through activation of the inhibitory descending pathway in the NRM. Cortical activation could in turn utilise the facilitatory descending pathway in the NRM, which is hypothesised to facilitate trigeminovascular nociceptive transmission, or suppress the inhibitory descending pathway. A more detailed discussion on the descending inhibitory and facilitatory descending pathways of the NRM and the interrelated region of the ventromedial medulla follows in section 1.8.7.

1.6 Genetics of migraine

Migraine is a multifactorial disorder and genetic factors play an important role in the development of the disorder. Studies examining the genetic basis of migraine are complicated by the heterogeneous nature of the condition and the lack of objective clinical or diagnostic tests. Family and twin studies showed evidence for an increase risk in the family members of migraineurs (Noble-Topham et al., 2003) and a higher concordance rate in monozygotic twins for migraine (Ziegler et al., 1998), indicating that genetic factors are a major contribution to the pathogenesis of both migraine with or without aura.

Familial Hemiplegic Migraine (FHM) is a rare subtype of migraine with prominent aura symptoms which is inherited in an autosomal dominant manner. The molecular linkage of FHM involves three mutations which form the only establish molecular knowledge of migraine so far (van den Maagdenberg et al., 2007):

A) FHM 1 locus affecting the *CACNA1A* calcium channel gene (mapped to chromosome 19p13) (Ophoff et al., 1996). The *CACNA1A* gene encodes the pore-forming A1 subunit of $Ca_v2.1$ (P/Q-type) voltage-gated neuronal calcium channels that modulate release of neurotransmitters at peripheral and central synapses. Over 50 *CACNA1A* mutations have been associated with a wide range of clinical phenotypes including FHM, and also sometimes associated with ataxia or even fatal coma, episodic ataxia type 2 and spinocerebellar ataxia type 6 (Wessman et al., 2004; van den Maagdenberg et al., 2007).

B) FHM 2 locus affecting the *ATP1A2* gene (on chromosome 1q23) (De Fusco et al., 2003). The *ATP1A2* gene encodes the A2 subunit of sodium-potassium pump ATPases. Sodium potassium pumps transport sodium ions out of the cell while importing potassium ions. ATPases modulate the re-uptake of potassium and glutamate from the synaptic cleft into glial cells. *ATP1A2* mutations have been associated with a range of clinical phenotypes of FHM with and without cerebellar ataxia, and also with basilar migraine and alternating hemiplegia of childhood. Many patients also suffer from epilepsy (De Vries et al., 2006).

C) FHM 3 affecting the *SCN1A* gene (on chromosome 2q24) (Dichgans et al., 2005). The *SCN1A* gene encodes the A1 subunit of neuronal voltage-gated sodium ($Na_v1.1$) channels that play an important role in the generation and propagation of action potentials. Mutations in this gene have been associated with epilepsy syndromes, but migraine has not been reported in these epilepsy syndromes (Meisler and Kearney, 2005). A Q1489K mutation in three German FHM families of common heritage (Dichgans et al., 2005) and a L1649Q mutation in a North-American FHM family (Vanmolkot et al., 2007) confirm the relationship between *SCN1A* and FHM 3. Mutation scanning of a large number of other FHM families, however, suggests that the *SCN1A* gene is a rare cause of FHM (Dichgans et al., 2005; Vanmolkot et al., 2007).

Interestingly a common consequence of these mutations is an increase in glutamate availability at the synaptic cleft of cells (Goadsby, 2007). The FHM 1 mutation can have as a consequence enhanced glutamate release due to enhanced calcium flux at the presynaptic terminal (Schneggenburger and Neher, 2005). FHM 2 results in a smaller electrochemical gradient for Na^+. One effect of this is the reduction or inactivation of astrocytic glutamate transporters, leading to a build-up of synaptic glutamate (De Fusco et al., 2003). The FHM 3 mutation can result in facilitation of high-frequency discharges that might also increase synaptic glutamate levels (Dichgans et al., 2005). Thus, the neurons at glutamatergic synapses can fire at a higher frequency than they do under normal conditions and this might explain the increased susceptibility to CSD (van den Maagdenberg et al., 2004; Wessman et al., 2007).

Unlike FHM, there are very few genetic breakthroughs for common types of migraine and no genes have been identified yet, although genome-wide linkage studies have identified several chromosomal regions with significant or suggestive linkage for nonhemiplegic migraine (van den Maagdenberg et al., 2007), including 1p36, 6q25, 9q34, 11q24, 19p13, 4q24 and Xq. Interestingly, the 11q24 locus which has demonstrated significant linkage to migraine with aura (Cader et al., 2003) maps, among other candidates, the *GRIK4* gene which codes for the KA1 kainate receptor subunit (Mayer, 2007).

If these mutations are directly responsible for the pathogenesis of migraine it is unlikely that they would have effects specific to one neural process, such as CSD. In agreement with this, new evidence suggests that FHM 1 mutation in mice reduces the expression of calcitonin gene-related peptide (CGRP) in trigeminal ganglia neurons (Mathew et al., 2007), further supporting that a systemic dysfunction of sensory neural function at multiple levels is a more plausible explanation for the triggering and symptomatology of migraine. FHM shares many phenotypical similarities with common types of migraine, indicating common neurobiological pathways, and someone would expect a more prominent role of CGRP cells in the trigeminal ganglia in these mutations, considering the importance of CGRP in migraine pathophysiology (Goadsby et al., 1990). Instead the reduction of CGRP in trigeminal neurons of FHM 1 mutant mice points to a loss of balance of the vasoactive peptides. In agreement with this, is evidence coming from a study in which FHM patients with known mutations in the CACNA1A and ATP1A2 genes do not show hypersensitivity to CGRP infusion, as

characteristically seen in migraine patients without aura. This indicates that the pathophysiologic pathways underlying migraine headache in FHM may be different from the common types of migraine and further questions whether CGRP antagonists would be effective in the treatment of FHM patients (Hansen et al., 2008).

1.7 Cortical spreading depression and migraine

Cortical spreading depression (CSD), discovered by Leão in 1944, is a self-propagating depolarisation of neurons and glia linked with depressed neuronal electrical activity (Leão, 1944) that moves at a rate of about 2-3 mm/min across the cerebral cortex. Leão first observed that CSD leads to transient dilatation of pial arteries (Leão, 1944). Following this transient hyperperfusion, hypoperfusion ensues, which persists long after CSD waves have passed. Spreading depression has been demonstrated in almost all the grey matter regions of the central nervous system (CNS) (Somjen, 2001), although the cortex of primates, especially in humans, is relatively more resistant to CSD. Early observations from Lashley (Lashley, 1941) suggested an association between CSD and the migraine aura and several imaging studies of patients during migraine with aura showed unilateral regions of occipital hypoperfusion that tend to spread rostrally from the occipital cortex and persist into the headache phase (Sanchez-del-Rio and Reuter, 2004). The aura is now believed to be a wave of CSD which spreads out from the occipital lobe across the cortex, resulting in an initial hyperaemic phase followed by an oligaemic phase (Olesen et al., 1990; Lauritzen, 1994; Olesen, 1998). Strong evidence supporting that an electrophysiological event such as CSD generates the aura in human visual cortex came only recently with the use of high-field functional MRI with near-continuous recording during migraine visual aura in humans. With this method Hadjikhani and colleagues (2001) observed blood oxygenation level-dependent (BOLD) signal changes that demonstrated characteristics of CSD as time-locked to percept onset of the aura (figure 4).

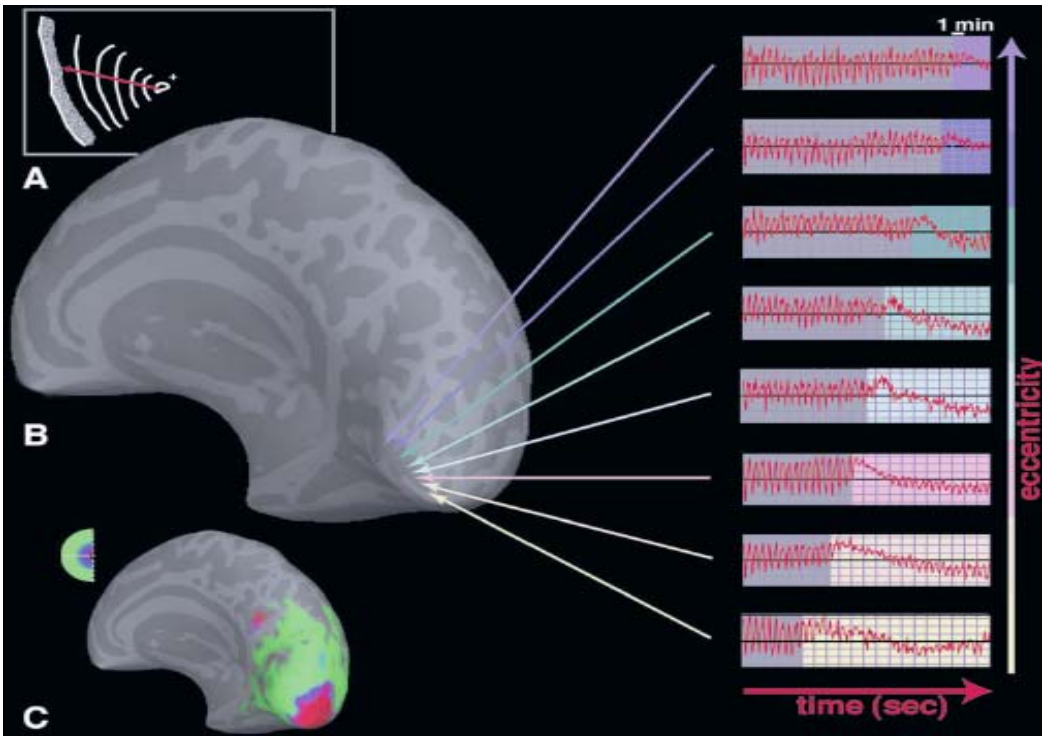


Figure 4: Spreading suppression of cortical activation during migraine aura

(*A*) A drawing showing the progression over 20 minutes of the scintillations and the visual field defect affecting the left hemifield, as described by the patient. (*B*) A reconstruction of the same patient's brain, based on anatomical MR data. The posterior medial aspect of occipital lobe is shown in an inflated cortex format. MR signal changes over time are shown to the right. Each time course was recorded from one in a sequence of voxels that were sampled along the calcarine sulcus, in the primary visual cortex, from the posterior pole to more anterior location, as indicated by arrowheads. A similar BOLD response was found within all of the extrastriate areas, differing only in the time of onset of the MR perturbation. The MR perturbations developed earlier in the foveal representation, compared with more eccentric representations of retinotopic visual cortex. This finding was consistent with the progression of the aura from central to peripheral eccentricities in the corresponding visual field (*A* and *C*). (*C*) The MR maps of retinotopic eccentricity from this same subject, acquired during interictal scans. As shown in the logo in the upper left, voxels that show retinotopically specific activation in the fovea are coded in red (centered at 1.5° eccentricity), parafoveal eccentricities are shown in blue, and more peripheral eccentricities are shown in green. Adapted from (Hadjikhani et al., 2001).

It is not yet clear how CSD is triggered in human cortex during migraine aura. A number of diverse stimuli trigger CSD including direct cortical trauma, exposure to high concentrations of excitatory amino acids or K^+, direct electrical stimulation, inhibition of Na^+/K^+-ATPase and energy failure (Somjen, 2001). As previously mentioned genetic predispositions and environmental factors may modulate individual susceptibility by lowering the CSD threshold (van den Maagdenberg et al., 2004), and cortical excitation may cause sufficient elevation in extracellular K^+ and glutamate to initiate CSD (De Fusco et al., 2003).

A serious debate has occurred over the years as to whether CSD and the resultant aura are responsible for the pain in migraine. Bolay et al (2002) showed that CSD, evoked in the rat cerebral cortex by a pinprick or local electrical stimulation, activates trigeminal nerve terminals on cerebral blood vessels and reflex middle meningeal vasodilation and increases neural activity in the ipsilateral trigeminal nucleus. It was also demonstrated that the trigeminal activation produced by CSD causes inflammation in the meninges that occurs after the CSD has subsided (Iadecola, 2002). Previous studies have also shown that CSD induces Fos-like immunoreactivity in the rat cerebral cortex (Herrera et al., 1993) and in the ipsilateral trigeminal nucleus caudalis (TNC), (Moskowitz et al., 1993; Bolay et al., 2002). CSD also causes plasma protein extravasation (PPE) in the dura mater and *c-fos* induction in the TNC, events that seem to be dependent on intact branches of the trigeminal and superior salivatory nuclei (Bolay et al., 2002).

Contradicting preclinical data to these previous findings also exists. Ebersberger et al (2001) induced CSD in the rat cerebral cortex and assessed plasma extravasation in the dura mater and neuronal activity in deep laminae of the trigeminal nucleus *in vivo*. In this series of experiments CSD did not alter dural plasma extravasation compared to the CSD-free contralateral side and ongoing neuronal activity or receptive field stimulation-evoked responses were not altered by a single CSD or repeated CSDs. In an *in vitro* model, the application of KCl to the dura at concentrations found extracellularly during CSD did not alter the release of CGRP and prostaglandin E2 from the dura (Ebersberger et al., 2001). It has been further questioned whether the c-*fos* induction by CSD (Moskowitz et al., 1993) is an actual downstream event or if it is an artefact due to hyperosmolar activation of trigeminal fibres (Ingvardsen et al., 1997), caused by application of potassium chloride (Moskowitz et al., 1993).

Further to the preclinical data that do not support links between CSD and migraine headache, clinical evidence supporting that migraine aura, may not be the trigger for the pain and other symptoms also exist (Goadsby, 2001):

- Aura does not necessarily precede headache (Goadsby, 2001), as aura symptoms (and by extension CSD) occur in only a minority of migraine patients (Rasmussen and Olesen, 1992; Russell and Olesen, 1996).
- Aura without headache is not uncommon (Goadsby, 2001) and therefore aura does not necessarily lead to head pain.
- Migraine aura is not always contralateral to the headache (Goadsby, 2001).

Strong evidence suggests that CSD is responsible for the clinical symptoms of aura and this phenomenon may provide important insights into some elements of the pathogenesis of migraine and therapeutic developments might shed more light on the relationship between aura and headache (Goadsby, 2007).

1.8 Pathophysiology of migraine

Migraine is a form of sensory processing disturbance with wide implications within the CNS (Goadsby, 2007). In order to understand the known pathophysiology of migraine, further to the genetics (paragraph 1.6) and the physiological basis of the aura (paragraph 1.7), the elements to be considered are the physiology and pharmacology of the pain-producing structures of the head. These include the dura mater and cranial vessels, and more appropriately their innervation which forms the trigeminovascular system. The central projections of the fibres innervating the cranial vessels to the caudal brainstem and upper spinal cord, and diencephalic modulatory systems that influence trigeminal pain transmission are of great importance (figure 5).

Our initial understanding of the pathophysiology of migraine came from the early observations by Wolff, Ray and Penfield (Penfield, 1932, 1934; Ray and Wolff, 1940; Wolff, 1948) who identified cranial pain-producing structures, by observing that mechanical stimulation of the meninges and the meningeal and cranial vessels caused

severe headache in awake patients during cranial surgery. The importance of these structures in migraine pathophysiology is their rich innervation by primary afferents from neurons located mainly in the trigeminal ganglia as well as from the upper cervical ganglia (Mayberg et al., 1984; Arbab et al., 1986). As the key pathway for migraine pathophysiology is the trigeminovascular input from the meningeal vessels, it is essential to refer to the anatomy of the trigeminal ganglion and its peripheral (trigeminovascular) and central projections (trigeminocervical), to further understand the pathophysiology of migraine.

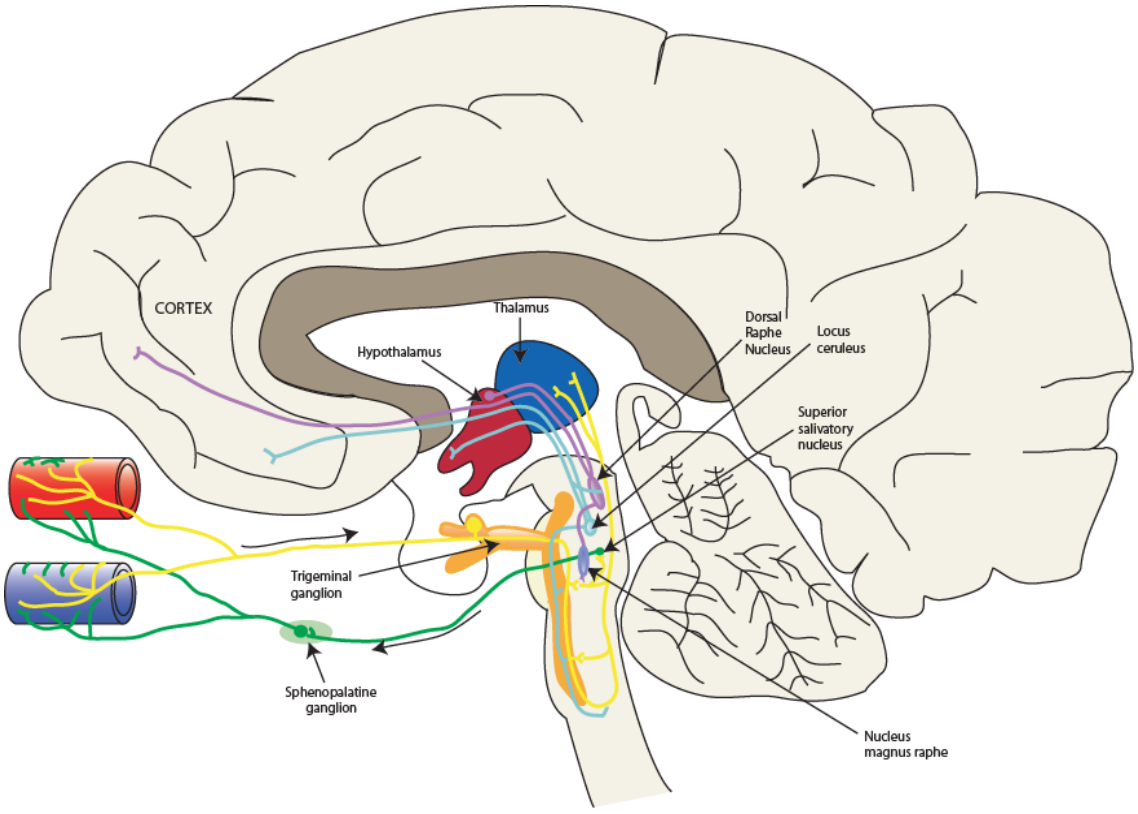


Figure 5: Pathophysiology of the trigeminovascular system

Sensory fibres innervating the cranial vessels arise from neurons which have their cell bodies within the trigeminal ganglion. Sensory inputs from the dural blood vessels (such as the superior sagittal sinus and middle meningeal artery) synapse on second order neurons in the trigeminocervical complex. These in turn project to the thalamus in the quinto/trigemino-thalamic tract. There are also connections between pontine neurons and the superior salivatory nucleus which results in a reflex activation of parasympathetic fibres (with resultant vasodilation), and these are relayed in the pterygopalatine (sphenopalatine) ganglion (Goadsby et al., 2002).

1.8.1 Trigeminal ganglion and trigeminal nerve

The following is adapted from published reviews (Schaltenbrand and Walker, 1997; Crossman and D., 2000; Shankland, 2000; Go et al., 2001; Kandel et al., 2000).

The trigeminal ganglion is the sensory ganglion of the trigeminal nerve and occupies the Meckel's cavity (cavum Meckelii) in the dura mater covering the trigeminal impression near the apex of the petrous part of the temporal bone. The trigeminal ganglion consists of pseudounipolar primary sensory neurons (the dendrite of these neurons are located in the trigeminal nerve, the cell bodies are located in the trigeminal ganglion and the axons protrude through the sensory root and into the ventrolateral midpons) and is analogous to the dorsal root ganglia (DRG) of the spinal cord, which contain the cell bodies of incoming sensory fibres from the rest of the body (Tandrup, 1995).

The trigeminal ganglion gives rise to the trigeminal nerve (Vth cranial nerve), which is the largest of the cranial nerves and it has three major branches:

- the ophthalmic nerve (V1)
- the maxillary nerve (V2)
- the mandibular nerve (V3)

The ophthalmic and maxillary nerves are purely sensory, whereas the mandibular nerve has both sensory and motor functions. These three branches converge on the trigeminal ganglion from which a single large sensory root enters the brainstem at the level of the pons. Immediately adjacent to the sensory root, a smaller motor root emerges from the pons at the same level, and thus the trigeminal nerve is a mixed nerve containing both motor and sensory components.

The motor root runs in front of and medial to the sensory root, and passes beneath the ganglion. The cell bodies of the motor fibres are located in the motor nucleus of the Vth nerve within the pons. Motor fibres are distributed (together with sensory fibres) in branches of the mandibular nerve and supply the muscles of mastication and the tensor tympani and tensor veli palatine muscles.

The sensory fibres of the ophthalmic, maxillary and mandibular nerves supply the cutaneous exteroceptors of the face, the mucous membranes of the nasal and oral

cavities, and a large portion of the intracranial dura mater and vessels. The sensory fibres, also called nociceptors, convey information regarding pain, temperature, touch, and proprioception. The areas of cutaneous distribution (dermatomes) of the three branches of the trigeminal nerve have sharp borders with relatively little overlap, unlike dermatomes in the rest of the body, which show considerable overlap (figure 6).

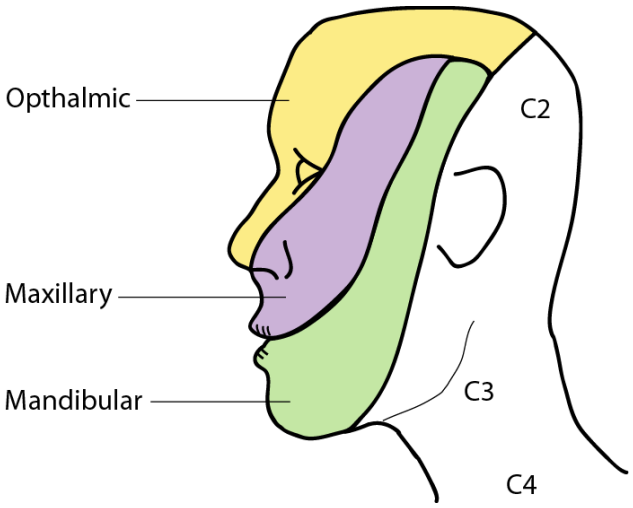


Figure 6: Superficial distribution of sensory fibres in the three divisions of the trigeminal nerve

The trigeminal nerve has three divisions: ophthalmic, mandibular, and maxillary. Anterior structures of the head and face are innervated by the ophthalmic division. Posterior regions are innervated by the upper cervical (C) nerves. Adapted from (Crossman and D., 2000).

1.8.2 Primary sensory afferents

Early anatomical studies provided evidence for the meningeal representation in the trigeminal ganglion by using horseradish peroxidase histochemistry (Messlinger et al., 2006). Most of the nociceptors around meningeal vessels were found to project mainly to the ophthalmic division of the ipsilateral trigeminal ganglion and to a minor degree to the maxillary and mandibular divisions (Mayberg et al., 1984; Steiger and Meakin, 1984). Further to the trigeminal ganglion projections to meningeal structures, neurons located in the upper cervical ganglia were also shown to contribute to the these projections (Mayberg et al., 1984; Arbab et al., 1986).

The nociceptors that run adjacent to the blood vessels transmit nociceptive information mainly through Aδ- (thinly myelinated) and C- (unmyelinated) fibre types (Strassman et al., 1986; Schepelmann et al., 1999; Bartsch and Goadsby, 2003b) although other types of primary afferents transmitting somatic sensations have also been characterised (Millan, 1999) (table 5). The somatic pain associated with the Aδ-fibres is characterised by an initial extremely sharp pain and is referred to as the "first" pain. The "second" pain is referred to the more prolonged and delayed feeling of dull ache or burning pain as a result of C-fibre activation.

The peripheral terminal of the nociceptor is where noxious stimuli are detected and transduced into inward currents that, if sufficiently large, begin to drive action potentials along the axon to the CNS and set a train of events that ultimately lead to a conscious awareness of the noxious stimulus (Woolf and Ma, 2007). The sensory specificity of the nociceptor is established by expression of ion channels which respond with a high threshold only to particular features of the mechanical, thermal, and chemical environment (Ramsey et al., 2006; Woolf and Ma, 2007) (figure 7). The high threshold of these transducers differentiates nociceptors from sensory neurons that respond to innocuous stimuli by expressing transducers with low thresholds (Woolf and Ma, 2007). Transmission of nociception occurs in response to calcium influx at the central terminal and releasing glutamate as well as multiple synaptic modulators and signalling molecules, which will activate postsynaptic receptors on second order neurons (Millan, 1999) (table 5).

Table 5: Characteristics of primary afferent sensory fibres

Fibre type	Aβ	Aδ	C
Diameter (μm)	>10	2-6	0.4-1.2
Conduction velocity (m/sec)	30-100	12-30	0.5-1.2
Myelination	Thickly myelinated	Thinly myelinated	Unmyelinated
Activation threshold	Low	High	High
Principal neurotransmitters released	EAA	SP, NKA, CGRP, EAA	SP, NKA, CGRP, EAA
Receptors activated post-synaptically	AMPA	NK1+2,CGRP1+2, NMDA/AMPA, mGlu	NK1+2,CGRP1+2, NMDA/AMPA, mGlu
Laminae in which neurons terminate	IIi, III, IV, V	I/IIo, V	I/IIo, V, X
Types of second order neurons contacted	LTM, WDR	NS, WDR, LTM	NS, WDR
Sensory modality transmitted	Innocuous: light touch, vibration and pressure	Noxious: sharp, pricking pain Sharp, stinging pain "First pain"	Noxious: thermal, mechanical and chemical irritation Dull, burning pain "Second pain"

AMPA; α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid, CGRP; calcitonin gene-related peptide, EAA; excitatory amino acids, LTM; low threshold mechanosensitive, mGlu; metabotropic glutamate, NK; neurokinin, NKA; neurokinin A, NMDA; *N*-methyl-*D*-aspartic acid, NS; nociceptive specific, SP; substance P, WDR; wide dynamic range. Adapted from (Millan, 1999).

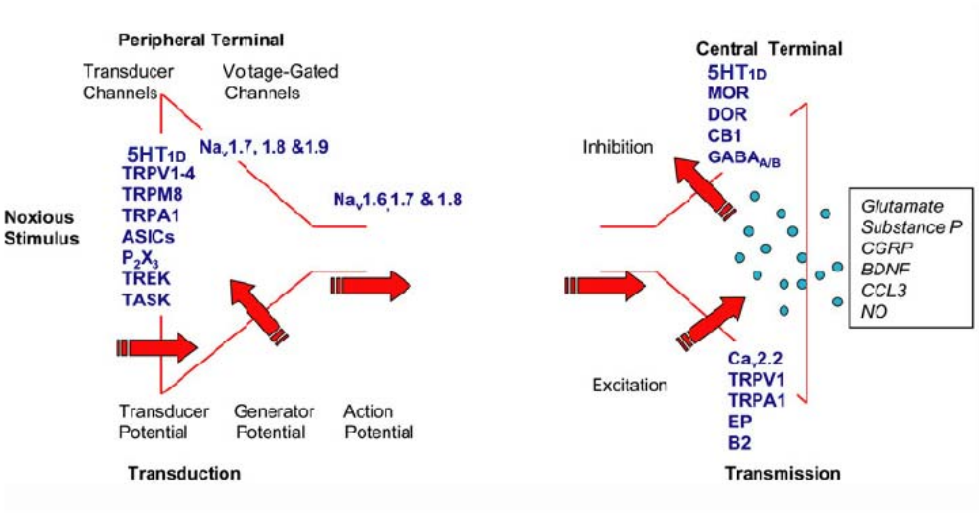


Figure 7: Transduction and transmission of noxious stimuli on the peripheral and central terminal of the nociceptor

Transduction is mediated by high-threshold transducer ion channels which depolarize the peripheral terminal activating voltage-dependent sodium channels. Transmission occurs in response to calcium influx at the central terminal releasing glutamate as well as multiple synaptic modulators and signalling molecules and is subject to both excitatory and inhibitory influences. Adapted from (Woolf and Ma, 2007)

TRPV, transient receptor potential vanilloid family; TRPM, transient receptor potential melastatin family; TRPA, transient receptor potential ankyrin family; ASICs, acid-sensing ion channels; P2, purinergic receptors; TREK, two-pore domain weak inward rectifying K^+ channels-related K^+ channel; TASK, two-pore domain weak inward rectifying K^+ channels -related arachidonic acid-stimulated K^+ channel; Na_v, voltage-gated sodium channel; MOR, mu-opioid receptor; DOR, delta opioid receptor; CB1, cannabinoid receptor 1; EP, E series of prostaglandin receptors; B2, Bradykinin 2 receptor; CGRP, calcitonin gene-related peptide; BDNF, brain-derived neurotrophic factor; CCL3, Chemokine (c-c motif) ligand 3; NO, nitric oxide.

Absence of kainate receptors from "Millan's table"– it's all about history

Upon activation of primary afferents, post-synaptic receptors on second order neurons are activated and transmit the nociceptive information to higher brain areas. The glutamate receptors, *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA), as well as some of the metabotropic glutamate receptors have been shown to be post-synaptically activated during nociceptive transmission (Millan, 1999). Although the presence of kainate receptors in the spinal dorsal horn was known since the early 1990's (Bettler et al., 1990; Gu and Huang, 1991; Bahn et al., 1994; Petralia et al., 1994), the lack of selective, pharmacological tools had hitherto hampered analysis of their putative role in the spinal transmission of nociceptive information (Fletcher and Lodge, 1996). In his extensive review Millan (1999) refers to the plausible role of kainate receptors in transmitting nociception based on studies in which the kainate ligand (2S,4R)-4-Methylglutamic acid (SYM 2081) was shown to desensitise kainate receptors and attenuate the mechanical allodynia and thermal hyperalgesia provoked by nerve injury (Sutton et al., 1999). However, it was unclear whether the kainate receptors possible involvement were engaged by small and/or large afferent fibres (Huettner, 1990). The presence of kainate receptors on central primary afferent terminals in the dorsal horn was not definitively demonstrated, and it was uncertain whether they were localised on intrinsic dorsal horn neurons or on the central terminals of small primary afferents (Bettler et al., 1990; Mitchell and Anderson, 1991). In the late 1990's Li et al. (1999) demonstrated for the first time post-synaptic activation of kainate receptors in the dorsal horn by activation of sensory primary afferents, and since then the pivotal role of kainate receptors in nociception has emerged (Ruscheweyh and Sandkuhler, 2002; Wu et al., 2007). A more detailed review on kainate receptors follows in section 1.13.

1.8.3 Anatomy and pharmacology of the neural innervation of the dura mater and dural and intracerebral blood vessels

Free unencapsulated nerve endings of Aδ and C fibres have been shown by histological examination to surround most of the dura mater as well as dural vascular structures (Andres et al., 1987). These fibres arise from the trigeminal ganglion and the supratentorial part of the dura mater is innervated by fibres from all three branches of the trigeminal nerve, whereas midline structures, which include the large sinuses, are innervated by fibres which arise from the ophthalmic division. The anterior cranial fossae are mainly supplied by fibres from the maxillary branch. The posterior cranial fossae are innervated by branches arising from the mandibular branch of the trigeminal nerve and by branches from the upper cervical segments and the vagus and glossopharyngeal nerves (Steiger and Meakin, 1984).

Using transganglionic neuronal tract tracing techniques on the middle meningeal artery (MMA), the superior sagittal sinus (SSS), the middle cerebral arteries and the basilar arteries, labelled cell bodies have been found in both the trigeminal ganglia (mainly in the ophthalmic division) and upper cervical DRG (Arbab et al., 1986) (Mayberg et al., 1984; Liu et al., 2003; Liu et al., 2004). Sympathetic nerve fibres that arise from the superior cervical ganglia were shown to innervate both the dura mater and dural vasculature (Edvinsson and Uddman, 1981; Keller et al., 1989; Uddman et al., 1989). Parasympathetic fibres arise from the pterygopalatine (sphenopalatine) and otic ganglia (Uddman et al., 1989).

Both the sensory and autonomic fibres that surround the cranial structures contain a variety of neuropeptides and neurotransmitters which can mediate contraction or dilation of the blood vessels (Edvinsson and Goadsby, 1994) (table 6). It has been shown that migraine attacks involve changes in the regulation of tone of intra- or extracranial blood vessels (Olesen, 1991). In human dural vasculature, noradrenaline and neuropeptide Y (NPY) cause potent vasoconstriction, while vasoactive intestinal peptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP) are potent vasodilators.

The functional importance of these peptides in migraine has been studied in depth (Edvinsson et al., 1983b; Edvinsson et al., 1983a; Edvinsson and Goadsby, 1995; Edvinsson, 1998). In migraine, there is a clear association between the headache and the

release of CGRP. During migraine attacks with or without aura in humans, CGRP levels are elevated in the extracerebral circulation, while other peptides studied, including SP, were unaltered (Goadsby et al., 1990). Treatment with sumatriptan aborts CGRP release in parallel with treating migraine (Goadsby and Edvinsson, 1993), indicating the concomitance between the headache and the release of CGRP. Intravenous infusion of human α-CGRP has been shown to cause a delayed migraine-like headache in patients (Lassen et al., 2002) and the CGRP receptor antagonist BIBN 4096 BS (olcegepant) (Doods et al., 2000) was effective in treating acute attacks of migraine (Olesen et al., 2004b). Thus the development of further CGRP receptor antagonists for the treatment of migraine receives increasing interest (Herbert and Holzer, 2004; Rudolf et al., 2005; Recober and Russo, 2007; Salvatore et al., 2008) .

Table 6: Pharmacology of the three systems of perivascular nerve fibres innervating the cranial circulation

Perivascular fibres	Sensory	Sympathetic	Parasympathetic
Originated ganglia	Trigeminal ganglia	Superior cervical ganglia	Sphenopalatine ganglion, otic ganglion, carotid miniganglia
Pharmacology	SP, CGRP, NKA, PACAP	NA, NPY, ATP	AChE, VIP, PH, PACAP, NOS
Vasomotor action	dilation	contraction	dilation

NA, noradrenaline; NPY, neuropeptide Y; ATP, adenosine; VIP, vasoactive intestinal peptide; PHI, peptide histidine; AChE, acetylcholinesterase; PACAP, pituitary adenylate cyclase-activating peptide; NOS, nitric oxide synthase; SP, substance P; CGRP, calcitonin gene-related peptide; NKA, neurokinin A

Evidence for the importance of CGRP in migraine also comes from experimental animal models. Stimulation of the cat superior sagittal sinus led to increased release of CGRP and VIP levels while there was no change in SP or NPY (Zagami et al., 1990). When the dura mater is electrically stimulated in rats it causes dilation of dural blood vessels (Williamson et al., 1997a), caused by CGRP release from trigeminal sensory nerves that innervate the cranial blood vessels since this effect is abolished by the rat CGRP receptor antagonist $CGRP_{8-37}$ (Williamson et al., 1997a). Significant attenuation of the neurogenic meningeal vasodilator response is similarly seen with triptans, such as sumatriptan (Williamson et al., 1997c). Intravenous administration of CGRP also causes dural blood vessel dilation that is similarly abolished by the CGRP receptor antagonist $CGRP_{8-37}$. CGRP-induced dilation however is not abolished by sumatriptan, indicating that it is likely the triptans act pre-junctionally to prevent CGRP release, rather than on the smooth muscles of the blood vessels (Williamson et al., 1997c). In the TCC, CGRP receptor antagonists inhibited trigeminovascular neurons activated by L-glutamate, demonstrating a possible central site of action for CGRP receptor antagonists (Storer et al., 2004a).

Although SP and CGRP are largely colocalised, there is a selective release of CGRP rather than SP and no clinical evidence support the involvement of SP in migraine pathophysiology (Goadsby et al., 1990). Trigeminal sensory C fibres arise from neurons in the trigeminal ganglia in which CGRP and SP are colocalised, whereas sensory Aδ fibres arise from trigeminal neurons containing predominantly CGRP and glutamate (Uddman et al., 1989; Edvinsson and Hargreaves, 2000). The detection of CGRP in the extracerebral circulation of migraine patients during an attack may reflect a greater density of Aδ fibres innervation of the cerebral circulation or it could reflect preferential activation of Aδ over C fibres or differential release of CGRP from C fibres pools (Edvinsson and Hargreaves, 2000). Released CGRP is a potent vasodilator of the pain producing intracranial blood vessels. The marked changes of CGRP levels during migraine and the abundant presence of CGRP carrying afferents from the trigeminal ganglion in the TCC (figure 8), indicate activation of the trigeminal system (Goadsby and Edvinsson, 1998).

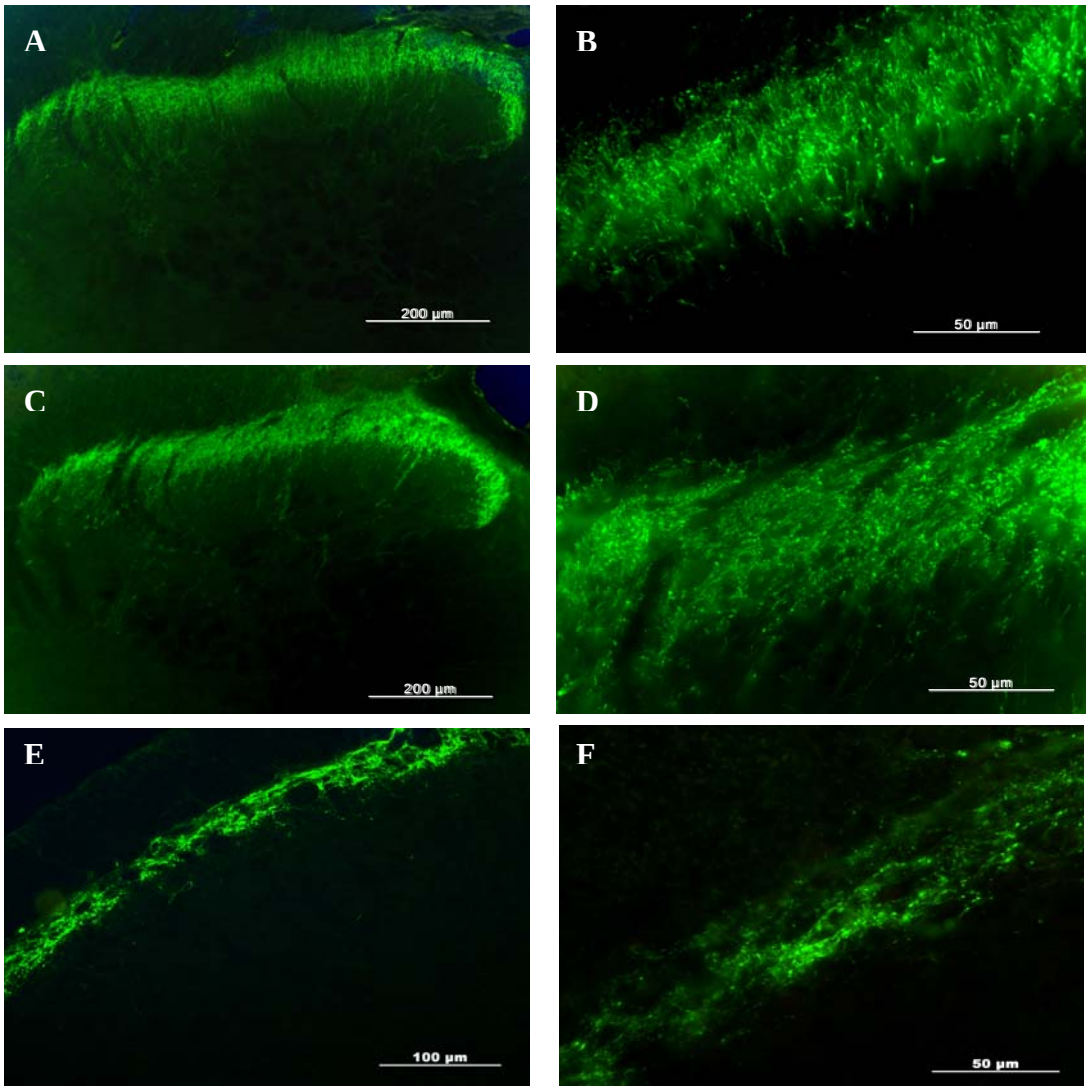


Figure 8: Localisation of calcitonin gene-related peptide-like in the trigeminocervical complex

Representative examples of the distribution calcitonin gene-related peptide (CGRP; labelled with fluorescein isothiocyanate) fibres in the dorsal region of the cervical spinal levels C2 (A, B), C1 (C, D) and trigeminal nucleus caudalis (TNC; E, F) are shown. CGRP staining showed fibres in lamina I and outer lamina II of the C1-C2 levels as well as strong immunoreactivity in fibres in the superficial layers of TNC (Andreou et. al, unpublished data).

1.8.4 Anatomy of the trigeminal brainstem nuclear complex and trigeminal projections

The pseudounipolar neurons of the trigeminal and upper cervical ganglia that innervate the pain producing cranial structures project centrally and terminate on second order neurons in the TCC. The sensory root of the trigeminal nerve enters the lateral pons where it terminates in the sensory nuclei of the TCC on second order neurons. The TCC extends from the rostral pons down to the upper cervical spinal cord levels and is composed of the principal trigeminal nuclei (Vp) and the spinal trigeminal nucleus (Vsp) (subdivided into the nucleus oralis-Vo, the subnuclear interpolaris-Vi, and the nucleus caudalis-Vc). The TCC is organised somatotopically, with the three trigeminal divisions being represented in a sequence from ventrolateral to dorsomedial (Strassman et al., 1994) (figure 10).

The subnucleus caudalis (Vc), also known as the trigeminal nucleus caudalis (TNC) or medullary dorsal horn (MDH) extends from the obex to the cervical spinal cord and is analogous to the dorsal horn of the spinal cord (Olszewski, 1950). It is composed of separate layers similar in appearance to the spinal cord dorsal horn with the outermost layer, the subnucleus marginalis corresponding to lamina I. Ventral to this lies the subnucleus gelatinosus (lamina II), and the subnucleus magnocellularis which corresponds to laminae III and IV. The TCC extends from the trigeminal nucleus caudalis to the segments of C2–C3 in the rat, cat, and monkey (Bartsch and Goadsby, 2003a).

The TNC, as well as the other nuclei of the spinal trigeminal nucleus and the principal trigeminal nuclei, are organised in a ventrodorsal direction (figure 9):

- Mandibular afferents are mainly represented on the dorsal part of each subnucleus.
- Ophthalmic afferents terminate ventral in the trigeminal subnuclei or on the ventrolateral aspect of the TNC (Hu et al., 1981).
- Maxillary afferents terminate between the mandibular and ophthalmic representations in the trigeminal subnuclei.

Primary afferent sensory fibres converge on second order neurons in laminae I–VI, which constitute the dorsal horn, and on second order neurons in the TNC. According to their responses when activated by different stimuli, these second order neurons have been classified into three categories (Millan, 1999) and all three have been identified in the TCC (Hu, 1990):

1. Nociceptive-specific (NS) neurons are silent at rest and become activated in response to high intensity, noxious stimuli and receive inputs from Aδ- and C-fibres.
2. Non-nociceptive low-threshold (LT) neurons that respond to innocuous stimulation only.
3. Wide-dynamic range (WDR) neurons exhibit a dynamic response over a broad stimulus range eliciting an incremental response to both innocuous and noxious stimuli. WDR neurons also receive considerable convergent inputs from extracranial cutaneous and intracranial visceral structures and may respond to C-, Aδ- and Aβ- fibres.

An organisation pattern of cutaneous, primary afferent inputs to the dorsal horn of the spinal cord has been suggested with C-fibres projecting in lamina I, outer lamina II and laminae VI and X, Aδ-fibres terminating in lamina I, outer lamina II and laminae III-V, and Aβ-fibres terminating in laminae II(inner)-VI and X. This is however not a strict organisation, as it does not quantitatively differentiate between various laminae as concerns primary afferent input, and the cell types (NS, WDR or LT) are also qualitatively, rather than quantitatively represented (Hu, 1990; Millan, 1999).

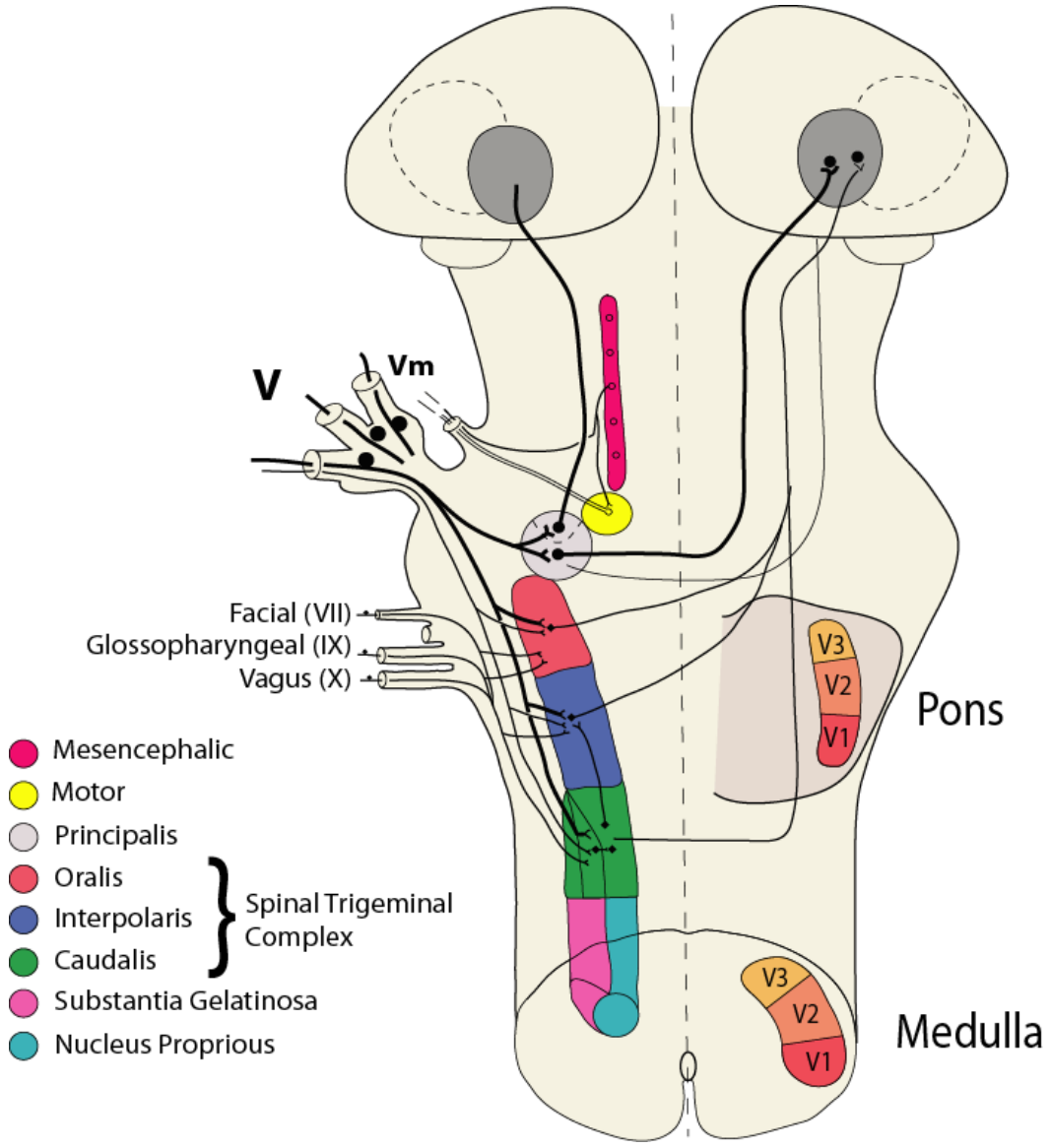


Figure 9: Cytoarchitecture of the trigeminal brainstem nuclear complex and the somatotopical organisation of the spinal trigeminal nucleus

The TCC extends from the rostral pons down to the upper cervical spinal cord levels and consists of a complex of sub nuclei divided into the principal sensory nucleus (Vp), at which a major part of the trigeminal nerve terminates, and the spinal trigeminal nucleus (Vsp). The Vsp is further divided into three sub-nuclei; the nucleus oralis (Vo), interpolaris (Vi) and caudalis (Vc) arranged in a rostrocaudal manner. V3, mandibular; V2, maxillary; V1, ophthalmic division (Wilkinson, 1986; Nieuwenhuys et al., 1988).

Earlier studies have shown that the spinal trigeminal nucleus, especially the TNC, has an important role in the mediation of pain and temperature sensations from the head and facial regions (Mosso and Kruger, 1973; Dubner and Bennett, 1983). Sensory inputs from the dural blood vessels (such as the superior sagittal sinus and the middle meningeal artery) synapse on second order neurons in the TCC and nociceptive electrical and mechanical stimulation of the superior sagittal sinus results in Fos expression in this nuclei complex (Kaube et al., 1993b; Hoskin et al., 1996; Goadsby and Hoskin, 1997). The sensory central projections of the superficial temporal artery and the SSS in rats terminate in the TNC, the trigeminal nucleus interpolaris and the dorsal horn in the segment C1-C3 (Liu et al., 2003; Liu et al., 2004). Stimulation of the superior sagittal sinus or certain other dural components increases neuronal activity in the TNC (Davis and Dostrovsky, 1986; Strassman et al., 1986; Bartsch and Goadsby, 2003b; Bolton et al., 2005) and most of these also have facial receptive fields located in the ophthalmic division (Davis and Dostrovsky, 1986). Electrical stimulation of the SSS causes increased metabolic activity and blood flow in the TNC and in C1 and C2 of the spinal dorsal horn (Goadsby and Zagami, 1991). CGRP-like immunoreactivity, which represents CGRP carrying afferents from the trigeminal ganglion (Henry et al., 1993), is abundant in the TCC (figure 9) and stimulation of the trigeminal ganglion causes increase release of CGRP and SP (Goadsby et al., 1988).

Clinical correlates, that indicate an important role of the brainstem in migraine, come from imaging studies, which showed activation of the brainstem during migraine attacks (Weiller et al., 1995), and this activation is migraine specific (Bahra et al., 2001). Both experimental and clinical evidence suggest that abnormal neuronal modulation at the level of the brainstem is clearly implicated in migraine pathophysiology (Goadsby et al., 2002).

1.8.5 Ascending pathways of the trigeminal system

The majority of the secondary neurons in the TCC decussate at the level of the medulla and travel up the brainstem through the ventral trigeminal tract, which ascends in close relationship with the contralateral medial lemniscus, and carry sensory information from the face and the meninges to higher brain areas. Their role is not only to facilitate the perception and detection of noxious stimuli, but also to communicate with cognitive

circuits which control mood associated with pain, the attention to and memory of pain as well as the tolerance of pain (Craig et al., 1994).

The most important ascending pathways in migraine pathophysiology are:

- The trigeminothalamic tract (also called the quintothalamic tract). The axons transmitting information from the trigeminal nerve synapse at the ventral posteromedial nucleus (VPM) of the contralateral thalamus (ipsilateral projections have been reported in some species, associated with the spinothalamic tract carrying sensory information from the body (Gaze and Gordon, 1954).

- The trigeminohypothalamic tract. These neurons are located bilaterally in the TCC and their axons synapse mainly to the lateral preoptic, anterior, lateral, perifornical, and caudal hypothalamic nuclei (Burstein et al., 1998).

- The spinomesencephalic and spinoreticular tract. The mesencephalon modulates painful input before it reaches the level of consciousness. The reticular formation is responsible for the automatic orientation of the body to painful stimuli (Kandel et al., 2000).

1.8.6 The thalamus and trigeminal nociception

The following discussion has been adapted from published work (Sherman and Guillery, 2001; Percheron, 2003; Jones, 2006).

The thalamus is a nuclear complex located in the diencephalon and is composed of functionally and histologically distinct nuclei (figure 10). The two major components of the thalamus are the dorsal thalamus and the ventral thalamus. The dorsal thalamus is comprised of roughly 15 nuclei with relay cells that project to the cortex. The ventral thalamus, the major portion of which is the thalamic reticular nucleus, consists of reticular GABAergic cells that project into the dorsal thalamus to inhibit relay cells.

The internal medullary lamina cells divide the thalamus into the lateral, medial and anterior nuclei. The ventral posterior nuclear group contains the main somatosensory relay nuclei and it lies within the ventral part of the lateral nuclei area. In primates the ventral posterior nuclear group can be divided into the ventral posterior medial (VPM) nucleus, ventral posterior medial parvicellular (VPMpc) nucleus, ventral posterior lateral (VPL) nucleus and the ventral posterior inferior (VPI) nucleus. The ventral posterior thalamic area in rats and mice differ from primates with the major differences being:

- The absence of the VPI nucleus in rats and mice. In some cases the term ventrobasal complex (VB) is more commonly used to describe both the VPM and VPL nuclei (Yokota, 1989).

- The absence of intrinsic interneurons within the VB complex of rats and mice (Barbaresi et al., 1986; Harris, 1986). In primates, interneurons (mainly GABAergic) are located amongst the relay cells and they participate in feed-forward inhibitory circuits, contributing to the modulation of afferent sensory inputs destined for the cortex. In the mouse and rat, interneurons are essentially missing from all thalamic nuclei except the lateral geniculate nucleus (Arcelli et al., 1997).

- In canines and primates some ipsilateral thalamic projections from trigeminal or spinal neurons have been reported (Gaze and Gordon, 1954), whereas only contralateral projections have been found in rodents (Matsushita et al., 1982; Kemplay and Webster, 1989).

- The rat VPM has a much larger volume dedicated to the vibrissal representation (Vahle-Hinz and Gottschaldt, 1983), than either canines or primates. In rats the vibrissae serve an important sensory function of exploring the environment. There is a strict somatotopic organization within the VPM with the different horizontal arrangements of vibrissae represented at different rostrocaudal levels (Waite, 1973).

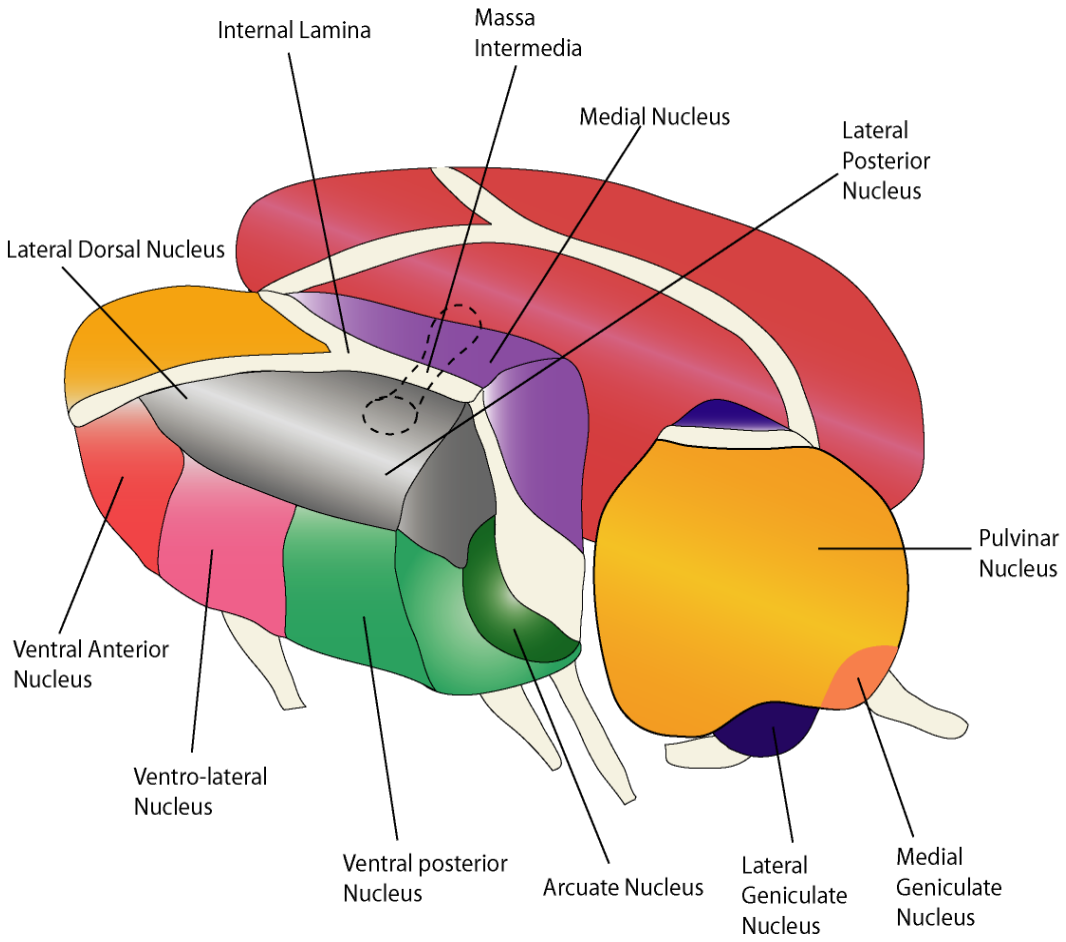


Figure 10: Anatomy of the dorsal thalamus

The dorsal thalamus is a complex structure which is divided into three nuclei complexes by the Y-shaped internal medullary lamina. The ventral posterior nucleus (VPM) is the main relay for sensory tracts conveying discriminative sensation to the primary (SI) somatosensory cortex. The VPM nucleus is located in its medial aspect and conveys sensory information from the cranio-facial region. Adapted from: http//mind-brain.com/thalamus.php.

At the level of the thalamus, a large portion of cells in the VPM nucleus display trigeminocervical convergence (Raboisson et al., 1989; Shields and Goadsby, 2005, 2006). This fact combined with evidence of thalamic activation in many functional studies of migraine (Afridi and Goadsby, 2006), indicates an important role for the thalamus as a relay for trigeminovascular nociception. It is believed that the thalamus has a special role in "regulating" the flow of nociceptive information to the cortex (Aguilar and Castro-Alamancos, 2005).

Anatomical studies in rats showed direct projection of neurons located in spinal trigeminal nuclei to the contralateral VPM (Kemplay and Webster, 1989; Cliffer et al., 1991; Iwata et al., 1992). Neurons in VPM receive convergent trigeminal viscerosomatic inputs classified by cutaneous receptive fields (Shields and Goadsby, 2005, 2006). Recordings of facial receptive fields in VB demonstrated a somatotopic organisation of neurons with craniofacial structures represented medially in the VPM and the remaining somatic structures represented medially in the VPL (Emmers et al., 1965; Waite, 1973; Angel and Clarke, 1975; Ohye, 1990) (figure 11). Neurons with inputs from the ophthalmic dermatome are located mainly at the dorsal aspect of VPM (Cropper and Eisenman, 1986; Rhoades et al., 1987; Dallel et al., 1988) and relay neurons can be LTM responding to tactile stimulation of the skin and vibrissae, as well as NS or WDR (Guilbaud et al., 1980; Guilbaud et al., 1981; Cropper and Eisenman, 1986). Normal responses to facial receptive fields of individual VPM neurons represent the integration of input activity transmitted through both the Vp and the Vsp pathways (Friedberg et al., 2004). A large number of neurons in the VPM receive contralateral projections from the TNC (Tiwari and King, 1974; Ganchrow, 1978; Hu et al., 1981). In cat and primates the VPM receives projections from all trigeminal nuclei (Whitlock and Perl, 1961; Burton and Craig, 1979; Matsushita et al., 1982) and as in the rat, NS and WDR neurons are found mainly in the "shell" region of the VB complex (Perl and Whitlock, 1961; Tiwari and King, 1974; Ganchrow, 1978; Willis et al., 1979; Honda et al., 1983; Yokota et al., 1985; Yokota, 1989). Unlike the rat trigeminothalamic projections, in cats and primates an ipsilateral projection from Vp to the medial aspect of VPM has been demonstrated (Burton and Craig, 1979; Jones et al., 1986). In all species the ventral posterior nucleus is the principal thalamic relay conveying nociceptive information to primary somatosensory cortex (Rowe and Sessle, 1968; Abe, 1978; Patrick and Robinson, 1987; Percheron, 2003).

The VB complex is also the main thalamic area at which somatic and visceral nociception converges (Angel and Clarke, 1975; Guilbaud et al., 1980; Berkley et al., 1993; Zhang and Davenport, 2003). Noxious heat is also encoded by VB nociceptive neurons which display the same thermal thresholds as those activating second order neurons and cause withdrawal responses (Mitchell and Hellon, 1977; Peschanski et al., 1980).

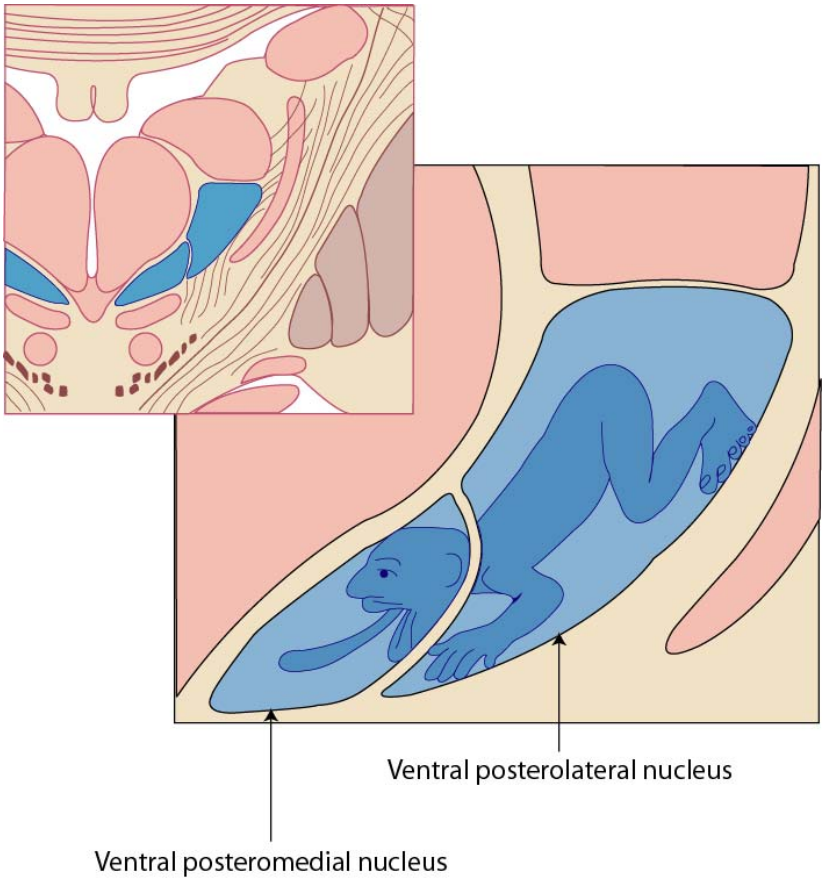


Figure 11: Somatic sensory map in the ventral posterior thalamic nucleus
Adapted from (Ohye, 1990).

In vivo electrophysiological studies in experimental animals demonstrate that trigeminovascular nociceptive stimulation activates neurons in the VPM nucleus, in addition to other areas. Stimulation of the cat SSS increased blood flow and metabolic activity in the thalamus (Lambert et al., 1988; Goadsby et al., 1991). Metabolic activity increases substantially in the VPM but remains unchanged in the VPL while more subtle increases were also observed in posterior thalamic nuclei (Goadsby et al., 1991). Ablation of the trigeminal ganglion blocked the increased metabolic activity in the thalamus (Goadsby et al., 1991). Electrical stimulation of the SSS and the MMA in cats and rats results in increase firing in the VPM and the majority of these cells have

cutaneous receptive fields located on the head or face (Zagami and Lambert, 1990; Shields and Goadsby, 2005; Shields et al., 2005; Shields and Goadsby, 2006). Receptive fields of these neurons were generally small and responsive to innocuous and nociceptive stimulation (Zagami and Lambert, 1990). Topical application of capsaicin and bradykinin to the SSS and MMA also resulted in increased firing of thalamic neurons (Davis and Dostrovsky, 1988a, b; Zagami and Lambert, 1990, 1991). Systematic studies of the thalamus reveal that trigeminovascular nociceptive information is relayed in several thalamic nuclei of which the VPM is of major importance. The remaining nuclei include the posterior medial (POm) thalamus, the zona incerta (ZI), intralaminar complex and ventrolateral nucleus (VL) (Zagami et al., 1990).

The pharmacology of the VPM also shows interesting insights into migraine pathophysiology. Both microiontophoretically applied and intravenous naratriptan reversibly modulates nociceptive neurotransmission by trigeminovascular thalamic neurons in the VPM in response to stimulation of the SSS and indicate the VPM nucleus as a target for the triptans (Shields and Goadsby, 2006). Propranolol (a β blocker) reversibly inhibits the response to SSS stimulation through blockade of β_1 adrenoceptors and both propranolol (Shields and Goadsby, 2005) and valproate (anti-convulsant) were able to inhibit responses to L-glutamate ejection, (Shields et al., 2003; Shields and Goadsby, 2005). These results suggest that the VPM may be an additional target for anti-migraine medications.

Functional magnetic resonance imaging (fMRI) studies of nociceptive neurotransmission in human demonstrated that thermal stimulation of the trigeminal dermatomes activates the VPM nucleus (DaSilva et al., 2002). Functional imaging studies in humans show consistent thalamic activation in spontaneous attacks of migraine and in human models of trigeminal nociceptive stimulation (Kobari et al., 1989; Bahra et al., 2001; DaSilva et al., 2002; Afridi and Goadsby, 2006). Additional to the pain symptoms, sensitivity to both light and sound; symptoms that accompany the migraine headache- have been reported during thalamic injury (Cummings and Gittinger, 1981).

Thalamocortical neurons in the VPM receive modulatory inputs from several sources. An important component of the thalamic involvement is that it receives modulatory

inputs from the brainstem regions implicated in migraine as shown by functional imaging (Weiller et al., 1995; Bahra et al., 2001; Afridi et al., 2005a) and in particular monoaminergic centres, such as the dorsal raphe nucleus (DRN) and the locus coeruleus (Goadsby, 2002; Goadsby et al., 2002). Modulatory inputs in the thalamus activate metabotropic and ionotropic receptors and are further distinguished by their inputs to the reticular nucleus, which itself has modulatory input to VPM neurons, and possibly a high degree of convergence onto relay cells (Sherman and Guillery, 2001). Sources of modulatory inputs to the sensory thalamus are shown in table 7 and some of the nuclei with interest in migraine and nociception are discussed below. It should be noted that other modulatory inputs also exist in the thalamus but their importance is not well characterised. One example is dopaminergic innervation, the origin of which is diverse, and thus more complex. Dopaminergic neurons of the hypothalamus, periaqueductal gray, ventral mesencephalon, and the lateral parabrachial nucleus all project bilaterally to the monkey thalamus (Sanchez-Gonzalez et al., 2005).

Table 7: Sources of modulatory inputs and major neurotransmitters to the sensory thalamus

Modulatory input	Neurotransmitter
cortex	glutamate
reticular nucleus	GABA
raphe nuclei	serotonin
locus coeruleus	noradrenaline
pontine tegmental nuclei	acetylcholinesterase
hypothalamus	histamine

The Reticular nucleus and GABAergic modulation

The reticular nucleus (RT) is a thin layer of cells on the dorsolateral and ventral aspects of the thalamus. The VB complex of rodents, unlike that of higher mammals is largely devoid of GABAergic interneurons (Barbaresi et al., 1986; Porter et al., 2001). The principal source of GABAergic modulatory inputs in rodents therefore arises from the RT and the nucleus serves as an important modulatory input in primates brain (Ross et al., 1993). All of the cells in the RT are GABAergic (Houser et al., 1980) and make multiple contacts with VB neurons (Peschanski et al., 1983), while RT neurons receive glutaminergic axons from VB neurons. This circuit is responsible for the stimulus induced feed-back inhibition observed in VPM neurons and it has been shown that activation of RT neurons causes inhibition of spontaneous and evoked activity in VB complex neurons (Mushiake et al., 1984).

GABA agonists in animal models of migraine seem to mimic GABAergic modulation in the VPM, mainly through $GABA_A$ ionotropic receptors (Shields et al., 2003) and the effective anti-convulsants in migraine therapy valproate is thought to have a GABAergic action (Loscher, 1999; Shields et al., 2003).

The rostral raphe nuclei and serotonergic modulation

The raphe nuclei are a moderate-size cluster of nuclei found in the brainstem. Imaging studies are consistent with activation of monoaminergic brain stem regions, including raphe nucleus comprising serotonergic neurons and the locus coeruleus comprising noradrenergic neurons, and the periaqueductal gray (PAG) during migraine (Weiller et al., 1995). Serotonergic fibres project to the thalamus from the rostral raphe complex including the dorsal raphe nucleus (Consolazione et al., 1984) and the nucleus raphe medianus (Consolazione et al., 1984; Peschanski and Besson, 1984). The adjacent PAG has also been shown to have serotonergic inputs to the thalamus (Consolazione et al., 1984).

Electrical stimulation of the DRN can modulate both spontaneous and nociceptive induced activity in thalamic parafascicular neurons (Storozhuk et al., 1995), while electrical stimulation of the dorsal and median raphe nuclei elicits metabolic changes in several thalamic and cortical regions in rats, some of which are involved specifically in

the processing of pain from the head and face (Cudennec et al., 1987; Cudennec et al., 1988). Microinjection of opiates into the PAG results in elevated levels of 5-HT metabolites in the telencephalon (Algeri et al., 1980) and depresses the responses of ventrobasal neurons to noxious stimulation (Kayser et al., 1983), in a similar fashion as when injected into the DRN (Kayser et al., 1983). Stimulation of the PAG in rats both facilitates and inhibits nociceptive transmission on VB neurons (Emmers, 1979) which probably reflects differential effects of 5-HT_1 and 5-HT_2 receptor activation. Microiontophoretically applied serotonin inhibits the spontaneous firing of rat parafascicular neurons in a dose dependent manner, as well as the responses to noxious stimuli (Andersen and Dafny, 1982). 5-HT has both facilitatory and inhibitory actions when microiontophoresed in the VB complex (Phillis and Tebecis, 1967; Eaton and Salt, 1989), likely due to different receptor mechanisms.

Noxious trigeminal stimulation with capsaicin results in Fos expression in the DRN along with other brainstem nuclei (Ter Horst et al., 2001). Microiontophoresis of naratriptan onto thalamocortical neurons within the VPM nucleus inhibited the response to SSS stimulation and L-glutamate ejection. In addition to its action on serotonin 5-$HT_{1B/1D}$ receptors, this inhibition was also mediated by activation of 5-HT_{1A} receptors (Shields and Goadsby, 2006), further supporting that the thalamus may be a therapeutic target in migraine.

Hypothalamus

Although a number of studies show modulation of trigeminal and spinal responses by hypothalamic inputs (paragraph 1.8.7), little is known about the hypothalamic modulation of the thalamus. Morimoto et al., (Morimoto et al., 1988) showed that neuronal activity of the VB complex, responding to skin warming, is affected by alterations of hypothalamic temperature. Thus thermal information from the peripheral thermoreceptors is modulated by hypothalamic temperature at the level of the relay nuclei of the thalamus (Morimoto et al., 1988).

1.8.7 Descending nociceptive projection to the TCC related to migraine

The trigeminal nucleus has been shown to receive projections from a variety of nociceptive modulatory structures in the brainstem (figure 12), some of which are briefly discussed below. It is noteworthy that other areas outside the brainstem also send descending axons to the TCC, including the somatosensory cortex and the thalamus. For example, the spinal cord is the largest target for dopaminergic projections from the subparafascicular thalamic nucleus (Commissiong et al., 1978; Takada et al., 1988; Moriizumi and Hattori, 1992; Takada, 1993). Furthermore a major component of the spinal dorsal horn and the trigeminal system is the network of intrinsic local interneurons.

Nucleus Raphe Magnus and reticular nuclei

The nucleus raphe magnus (NRM) and the adjacent reticular formation are known to modulate sensory responses of neurons in the spinal trigeminal nucleus (Sessle et al., 1981) and have been shown to send serotonergic and non-serotonergic projections to both the trigeminal nucleus oralis and caudalis and to the spinal cord in the cat and rat (Lovick and Robinson, 1983; Lovick and Wolstencroft, 1983). These projections mediate sensory modulation of both spinal and trigeminal responses simultaneously. Stimulation of this area can either suppress or facilitate nociceptive spinal neurons and nociceptive reflexes (Zhuo and Gebhart, 1990) whereas lesioning or inactivation of the NRM attenuates both the suppression and the facilitation of nociceptive transmission (Behbehani and Fields, 1979). These results led to the idea that NRM and the adjacent reticular formation contain two populations of neurons that, when activated have opposing efferent effects. Two physiological classes of putative nociceptive modulatory cells in the NRM were first identified by Fields et colleagues (Fields et al., 1983; Fields et al., 1991). One population of cells was hypothesized to mediate nociceptive inhibition (OFF cells), whereas activation of a distinct neuronal population was thought to mediate nociceptive facilitation (ON cells). Both ON and OFF cells are non-serotonergic (Potrebic et al., 1994; Gao and Mason, 2000) . ON cells are excited by noxious stimulation, whereas increase in OFF cell discharge is associated with nociceptive suppression. Serotonergic neurons within the NRM comprise a distinct physiological and functional class of neurons (Gao and Mason, 2000) and it is believed that tonically released serotonin contributes to anti-nociception (Sorkin et al., 1993; Mason, 2001),

and these neurons seem to have a further differential function depending on the awake/sleep cycle (Mason, 2001). The primary effect of serotonin on nociceptive dorsal horn neurons and nocifensive movements is inhibitory (Yaksh and Wilson, 1979). During waking serotonergic cell discharge is highest, and therefore high levels of serotonin in the dorsal horn would be expected to suppress nociceptive transmission. During slow wave sleep serotonergic cell discharge is greatly reduced, and thus lowered levels of serotonin in the dorsal horn would be expected to result in a relative decrease in the suppression of nociceptive transmission that would appear as a disinhibition (Mason, 2001).

Stimulation of the NRM has been shown to produce potent inhibition of TNC nociceptive and non-nociceptive neuronal responses (Chiang et al., 1994; Lambert et al., 2008), possibly via the OFF cells pathway. NRM has been also shown to be modulated by cortical mechanisms, as the inhibitory effects of this nucleus on trigeminal neurons can be antagonised by multiple waves of CSD, further suggesting an indirect modulation of the TCC by cortical activation via the cortico-NRM-trigeminal neuraxis (Lambert et al., 2008). Cortical activation could result in activation of the ON cells pathway and/or inhibition of the OFF cell pathway in the NRM.

Parabrachial Area

The parabrachial area, including the A7 adrenergic cell nucleus and the Kölliker-Fuse nucleus send direct adrenergic or noradrenergic fibres to the TCC. Stimulation of the parabrachial area has been shown to produce potent inhibition of TNC nociceptive and non-nociceptive neuronal responses (Chiang et al., 1994).

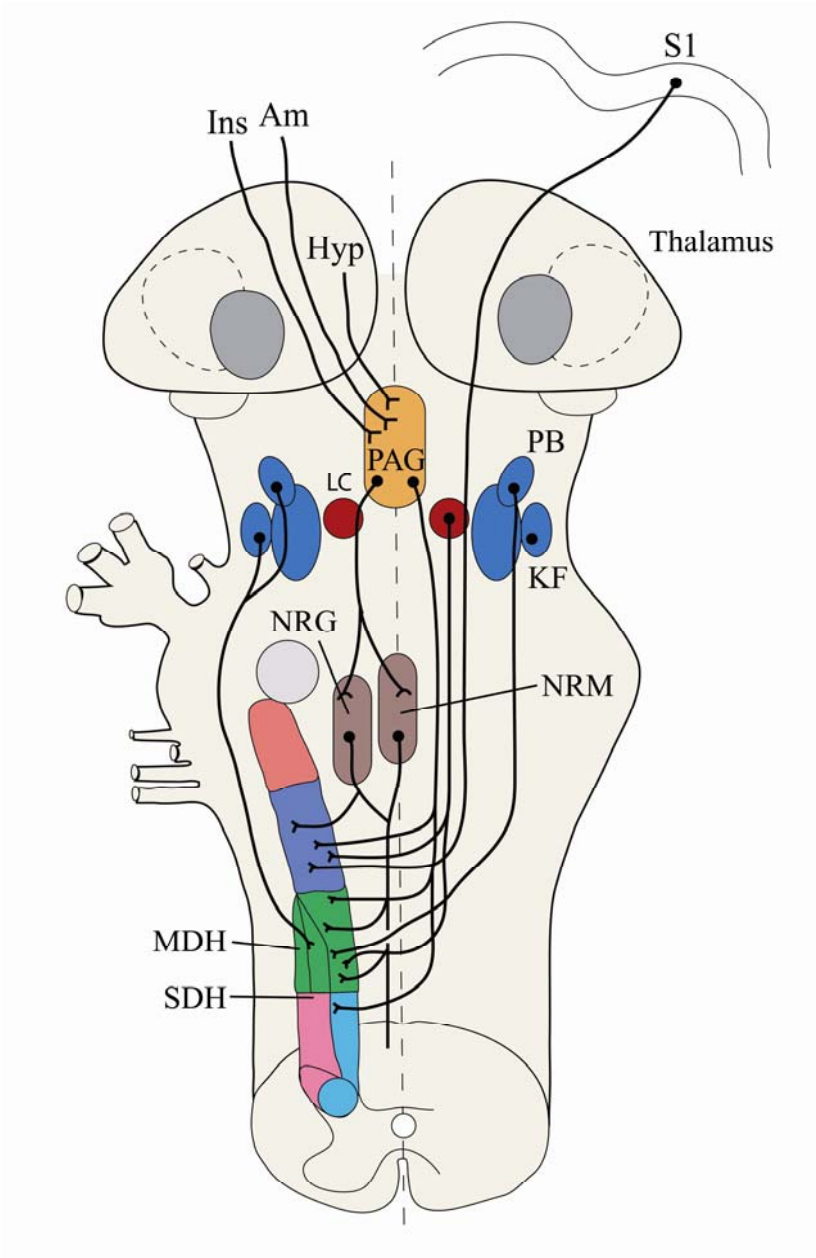


Figure 12: Schematic representation of the descending inhibitory pathways to the trigeminal nucleus caudalis related to nociception

The periaqueductal gray (PAG) receives input from the insular cortex (Ins), the amygdala (Am), and the hypothalamus (Hyp) and projects to the nucleus raphe magnus (NRM) and the adjacent reticular formation, including the nucleus reticularis gigantocellularis (NRG) within the rostral ventromedial medulla. Serotonergic pathways then descend to the medullary dorsal horn (MDH) and spinal dorsal horn (SDH). Other descending pathways arise bilaterally from the parabrachial (PB). Locus coeruleus (LC) and Kölliker-Fuse (KF) nuclei, and there is a direct projection from the somatosensory cortex (SI) to the subnucleus interpolaris. Adapted from (Messlinger and Burstein, 2000)

Periaqueductal gray

Electrical stimulation of the PAG and lesions in the region of the PAG in humans have been shown to treat somatic pain, and in some cases this has been shown to trigger migraine-like headache in previously headache-free individuals (Raskin et al., 1987; Haas et al., 1993; Veloso et al., 1998; Goadsby, 2002). Imaging studies have further shown increased activation in the rostral brainstem during spontaneous and triggered migraine attacks in imaging studies and the activated area in the brainstem includes the PAG (Weiller et al., 1995). In animal models relevant to migraine it has been shown that electrical and chemical activation of the ventrolateral column of the PAG (vlPAG), can inhibit trigeminovascular specific nociception in the cat and rat (Knight and Goadsby, 2001; Knight et al., 2002; Knight et al., 2003; Knight et al., 2005). Interestingly it has also been demonstrated that microinjection of the 5-$HT_{1B/1D}$ receptor antagonist naratriptan into the vlPAG selectively inhibits responses to dural electrical stimulation in the TCC raising the possibility that the triptans may exert part of their anti-migraine efficacy within the PAG (Bartsch et al., 2004).

Hypothalamus

To date numerous hypothalamic nuclei have been implicated in the descending modulation of pain and nociceptive processing. It is widely accepted that the hypothalamus plays a major role in the group of primary headaches resulting in pain and autonomic involvement termed trigeminal autonomic cephalalgias (TAC's) (Goadsby and Lipton, 1997). Clinical evidence indicating endocrine abnormalities have identified altered production of melatonin, cortisol, testosterone, lutenising-hormone and prolactin during cluster headache. Similar findings in chronic migraine support the involvement of the hypothalamus in the pathophysiology of both cluster headache and chronic migraine (Peres et al., 2001).

Experimental evidence for a role of the hypothalamus in migraine has been provided from a variety of studies. Malick *et al.* (Malick et al., 2001) demonstrated that stimulation of the dura mater in the rat produced Fos expression in the ventromedial, paraventricular and dorsomedial hypothalamic nuclei resulting in suppression of appetite and increases in arterial blood pressure. Stimulation of the SSS in the cat has also demonstrated hypothalamic activation with up-regulation of Fos in the supra-optic

and posterior hypothalamic nucleus consistent with a role for hypothalamic structures in modulation of nociceptive processing (Benjamin et al., 2004).

1.9. Central sensitization and migraine

Sensitization is the process whereby afferent activity is increased for an unchanged stimulus (Goadsby, 2005c). Central sensitization refers to this process occurring on second order neurons and can be defined as altered behavioural of neurons, characterized by increased excitability, increase synaptic strength and enlargement of their receptive fields (McMahon et al., 1993; Woolf and Doubell, 1994; Woolf and Salter, 2000). Clinically, central sensitization is manifested as a state of either hyperalgesia- an exaggerated pain in response to a stimulus that normally causes mild pain, or allodynia- a pain response to a normally non painful stimulus, and exaggerated pain response referred outside the original pain site (Dodick and Silberstein, 2006).

During a migraine headache about 80% of migraine patients develop cutaneous allodynia, characterised by increased skin sensitivity, mostly within the referred area of pain of the ipsilateral head (Selby and Lance, 1960; Burstein et al., 2000). The underlying mechanism of cutaneous allodynia is believed to involve sensitization of the TCC (Goadsby, 2005c). As trigeminocervical neurons receive convergent inputs from both dural structures and from the skin of the periopthalmic area, sensitization of these neurons will alter the perception of pain of normal skin or scalp (Bartsch and Goadsby, 2003a). Stimulation of nociceptive afferent of the dura mater leads to a sensitization of second-order neurons receiving cervical input. This mechanism might be involved in the referral of pain from trigeminal to cervical structures and might contribute to the clinical phenomena of cervical hypersensitivity in migraine (Bartsch and Goadsby, 2003b).

Central sensitization is associated with abnormal neuronal hyperexcitability in the TCC, due to an increase of the sensory inputs arriving from nociceptors on peripheral trigeminal fibres that supply the affected area, which is a consequence of peripheral sensitization (Strassman et al., 1996). Topical application of inflammatory agents on the rat dura, which induces long-lasting activation of the trigeminovascular pathway

(Ebersberger et al., 1997; Burstein et al., 1998; Schepelmann et al., 1999), provokes long lasting sensitization in trigeminocervical neurons that receive convergent inputs from the intracranial dura and extracranial periorbital skin. This neuronal sensitization is manifested as increased responsiveness to mechanical stimulation of the dura, to mechanical and thermal stimulation of the skin, and expansion of dura and cutaneous receptive fields (Burstein et al., 1998). These changes are parallel to an increase of the extracellular glutamate concentration of second order neurons in the TCC (Oshinsky and Luo, 2006), while CGRP does not seem to mediate the excitability of trigeminovascular neurons (Levy et al., 2005), and suggest an important contribution of glutamate and its receptors in allodynia (Oshinsky and Luo, 2006). At least, NMDA receptor activation seems to be pivotal for the induction of central sensitization in dural neurons (Woolf and Thompson, 1991).

About two thirds of the patients developing cutaneous allodynia report that untreated migraine attacks will result in a spread of allodynia to the other side of the head or the forearm (Selby and Lance, 1960; Burstein et al., 2000), indicating the involvement of higher extra-trigeminal processes. The limb or upper body allodynia seen in migraineurs, and the extend of cutaneous allodynia could be due to the development and spread of neuronal sensitization from second order neurons in the TCC, to third order neurons in the thalamus (Burstein et al., 2000; Dodick and Silberstein, 2006), and these consideration led to the study of thalamic processing in experimental animals of trigeminovascular activation (Zagami and Lambert, 1990; Goadsby and Gundlach, 1991; Zagami and Lambert, 1991).

1.10 Treatment of migraine

Efficient treatment of migraine depends on correct diagnosis and different approaches are suitable for individual patients, depending on the presence of secondary factors, the identification of migraine triggers, such as hormones and the frequency of attacks. Generally pharmacotherapeutic treatment of migraine can be acute or preventive depending on the frequency of attacks and individual needs and in severe cases of chronic sufferers both approaches can be used.

1.10.1 Acute treatment

Acute treatment is used to stop further progression of headache symptoms once they have started and is sufficient when attacks are more infrequent. Attacks should be treated at the earliest possible stage, as it has been shown that this approach can prevent the headache from progressing and reduces the need for multiple medications (Mathew, 2003; Burstein et al., 2004; Burstein and Jakubowski, 2004; Goadsby et al., 2008). Optimisation of the treatment of acute attacks of migraine also involves the use of effective doses of medications, the avoidance of medications that could cause medication overuse headaches and the treatment of associated symptoms. Acute treatment can be achieved either by non-specific analgesics, which can be efficient for the treatment of headache pain and analgesic for other biological symptoms, such as nonsteroidal anti-inflammatory drugs, or by specific acute headache treatments, which specifically treat migraine attacks and include ergot derivatives and the triptans.

Nonsteroidal anti-inflammatory drugs (NSAID) are known to have anti-inflammatory, analgesic and antipyretic properties and their effect is thought to be due to the inhibition of the synthesis of prostaglandins involved in the development of pain that accompanies injury or inflammation. Although prostaglandins have vasodilatory actions, they do not cause migraine-like headache when given by infusion to humans (Peatfield et al., 1981). The absence of any pathology of an inflammatory response in humans during migraine (Nissila et al., 1996) and the poor efficacy of some anti-inflammatory drugs in migraine, such as trachykinin and endothelin receptor antagonists (Narbone et al., 2004; Peroutka, 2005), limits the hypothesis supporting the involvement of inflammation in migraine. Thus, it is likely that the treatment of migraine attacks of NSAID is due to their effect on brain areas involved in migraine pathophysiology (Jurna and Brune, 1990; Kaube et al., 1993a). The NSAID ibuprofen, diclofenac and indomethacin were shown to attenuate nociceptive activation in the thalamus (Jurna and Brune, 1990). Acetylsalicylic acid, one of the most commonly used substances in the treatment of headache and other pain syndromes, as well as the cyclooxygenase inhibitor ketorolac, reduced trigeminovascular activity in the cat TCC. The results suggest that a central action might contribute to the analgesia produced by these non-steroid anti-inflammatory agents.

Ergot alkaloits or their derivatives have being used for the acute treatment of migraine for many years and the most widely used in clinical practice have been ergotamine tartrate and dihydroergotamine (DHE). Both ergotamine and DHE have vasoconstrictor properties and binding affinities to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_2, α_1 and α_2 adrenoceptors and D_2 dopamine receptors. Ergots have been shown to bind on receptors in areas involved in migraine pathophysiology (Goadsby and Gundlach, 1991; Hoskin et al., 1996) and to inhibit trigeminovascular nociception in the trigeminocervical complex (TCC) following intravenous and microiontophoretic administration (Lambert et al., 1992).

The triptans are 5-hydroxytryptamine (5-HT; serotonin) agonists with high affinity to 5-$HT_{1B/1D}$ receptors (Humphrey et al., 1990; Goadsby, 2000). Most of the members of the triptans family also display some binding affinity for 5-HT_{1F} (i.e. sumatriptan) (Beer et al., 1993) and 5-HT_{1A} receptors (Shields and Goadsby, 2006). The triptans were developed on the basis of clinical observations which indicated the involvement of serotonin in migraine pathophysiology. These observations include:

- increased 5-hydroxyindoleacetic acid (the main metabolite of serotonin) in the urine of patients during migraine attacks (Sicuteri et al., 1961; Curran et al., 1965)
- decreased platelet 5-HT levels at the onset of migraine (Curran et al., 1965)
- intravenous 5-HT could abort headache (Kimball et al., 1960; Anthony et al., 1967)

Sumatriptan and its precursor were the first molecules developed that could mimic some of the actions of serotonin without many of its side effects, and sumatriptan is now a commonly used triptan for the acute treatment of migraine (Humphrey et al., 1990). Other triptans have also been developed and used in migraine treatment including almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan and zolmitriptan. The triptans are the most effective migraine abortive agents and can treat not only the headache but also relieve the nausea, vomiting, photophobia and phonophobia associated with an attack. They are effective against migraine with and without aura, though if taken during the aura they are less effective against the headache (Bates et al., 1994; Olesen et al., 2004a).

The triptans have modulatory actions at several sites along the trigeminovascular nociceptive pathway (Goadsby et al., 2002; Tepper et al., 2002) (figure 13). 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptor mRNA are expressed in the human trigeminal ganglia and afferents (Rebeck et al., 1994; Bouchelet et al., 1996; Hou et al., 2001). The use of 5-HT_{1B} and 5-HT_{1D} receptor specific antibodies demonstrated a differential distribution of these receptor subtypes within the human trigeminovascular system. Only 5-HT_{1B} receptor protein was detected on dural arteries. In contrast, only 5-HT_{1D} receptor protein was detected on trigeminal sensory neurons including peripheral and central projections to dural blood vessels and to the medulla, confined to discrete areas associated with the trigeminal sensory system (Longmore et al., 1997). The localisation of the 5-HT_{1F} receptor to the trigeminal nucleus caudalis (TNC) and the differential distribution of the 5-$HT_{1B/1D}$ receptors make them prime candidates for involvement in the pathophysiology of migraine (Bruinvels et al., 1994; Longmore et al., 1997) (figure 4). Triptans may act as vasoconstrictors, activating 5-HT_{1B} receptors on smooth muscle of cranial arteries (Tfelt-Hansen et al., 2000). Alternatively 5-HT_{1D} receptors are located on trigeminal ganglion cells (Bonaventure et al., 1998). Activation of these receptors partially explains the ability of triptans to inhibit the release of calcitonin gene-related peptide (CGRP), a potent vasodilator from stimulated nerve endings (Goadsby and Hargreaves, 2000). Triptans also act centrally to modulate firing of second (Goadsby and Hargreaves, 2000; Goadsby et al., 2002) and third (Shields and Goadsby, 2006) order neurons in response to trigeminovascular nociceptive stimulation. Further to their action on different loci of the ascending pathway involved in migraine, activation of 5-$HT_{1B/1D}$ receptors in descending pain modulatory pathways from the periaqueductal gray has also been shown as a potential mechanisms of action (Bartsch et al., 2004).

1.10.2 Preventive treatment

Preventive therapy aims to reduce the frequency and severity of migraine attacks and is normally recommended when:

- incidence of attacks is more than two or three per month
- attacks are severe and impair normal activity
- acute treatment has failed or produces unwanted side effects
- the patient is unable to cope with the severity and frequency of attacks

How prophylactic treatments work is not clear but it appears to modify the sensitivity of the brain that underlies migraine (Goadsby et al., 2002). Drugs given for preventive therapy include β-adrenergic-receptor antagonists (such as propranolol), serotonin antagonists (such as pizotifen), γ-aminobutyric acid (GABA) modulators (such as valproate) and topiramate. The later is also thought to have at least partial action on kainate receptors (Gryder and Rogawski, 2003; Diener et al., 2007). Some anti-convulsants also seem to provide some promising results in nociception prevention (Dickenson and Ghandehari, 2007).

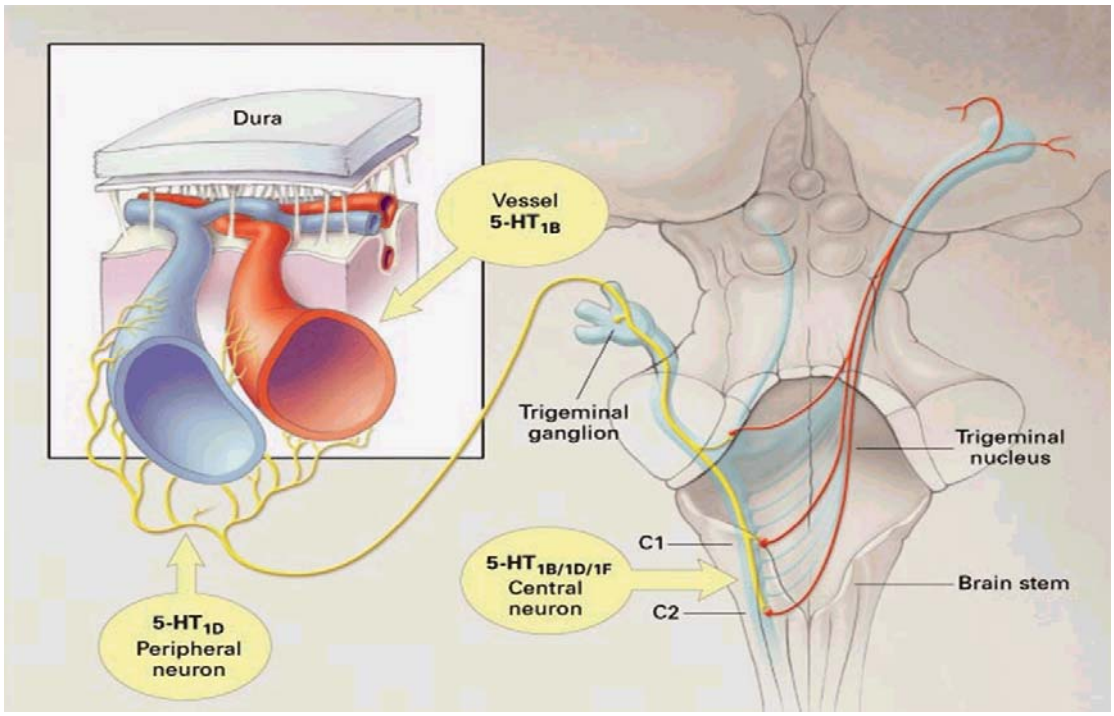


Figure 13: Possible sites of triptan action on the trigeminovascular system
Possible sites of action of triptans on intracranial blood vessels, first and second order trigeminovascular neurons. Adapted from (Goadsby et al., 2002).

1.11 Glutamate as a neurotransmitter in the central nervous system

Glutamate (figure 14) is the major excitatory neurotransmitter in the mammalian CNS and as such glutamate receptors play a vital role in the mediation of excitatory synaptic transmission and it's involvement in nociception is well documented (Carpenter and Dickenson, 2001).

Excitatory synaptic transmission is mediated primarily through ionotropic glutamate receptors (iGluR), which are ligand gated ion channels and metabotropic glutamate receptors (mGluR) which act by coupling to G-proteins. To date there is significant evidence suggesting that both iGluR and mGluRs play multiple roles in synaptic plasticity and some are involved in migraine pathophysiology (Mitsikostas et al., 1999; Storer and Goadsby, 1999; Sang et al., 2004; Vikelis and Mitsikostas, 2007).

Glutamate in the brain is synthesized *de novo* by astrocytes and neurons, since the blood-brain-barrier limits the entry of the plasma glutamate in most regions of the brain (Hawkins et al., 1995; Hertz et al., 1999; Smith, 2000). Glutamate is synthesised in neural tissues by two major pathways:

a. from α-Ketoglutarate (α-KG), taken from the tricarboxylic cycle of the glucose metabolism, and donor amino acids by means of transamination
b. from glutamine by the action of the glial-specific enzyme glutaminase

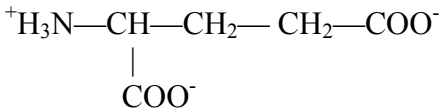


Figure 14: Glutamate chemical structure

Vesicles accumulate glutamate to a very high concentration and it is released to synapses by calcium-dependent exocytosis. After release, synaptic glutamate is very rapidly inactivated by cellular uptake (Clements et al., 1992). Presently, five different glutamate transporters have been cloned, at least one of which is astrocyte-specific and accounts for a major part of glutamate removal from synaptic clefts, although a minor fraction of released glutamate is re-accumulated into the neurons from which it was released (Hertz et. al, 1999; Rothstein et al., 1996). Glutamate in the astrocytes is stored in much lower levels, due to the presence of the enzyme glutamine synthetase or glutaminase. Glutamine synthetase is a glial-specific enzyme (Tansey et al., 1991) that converts glutamate into glutamine as part of the recycling of synaptically released glutamate (Schousboe et al., 1997).

Glutamine has no known neurotransmitter action and a substantial amount of glial glutamine is released extracellularly. Accordingly, the glutamine concentration in the extracellular space between astrocytes and neurons is relatively high. Glutamine is a very effective precursor for glutamate, which undergoes re-uptake from the extracellular space into neurons. Hydrolysis of glutamine to glutamate requires no energy and is catalyzed by phosphate-activated glutaminase, an enzyme different to glutamine synthetase. Therefore, while neurons cannot generate glutamine from glutamate, both neurons and glia can form glutamate from glutamine (Schousboe et al., 1997; Hertz et al., 1999). Excess of glutamate in neurons and astrocytes can be also catabolised and one route is the oxidative deamination by the enzyme glutamate dehydrogenase (Kugler, 1993) (figure 15).

The restriction of glutamate entry from the blood circulation into the brain (Hawkins et al., 1995), the rapid uptake of released glutamate by glutamate transporters on the glial cells that surround the glutamatergic synapses (Rothstein et al., 1996; Lehre and Danbolt, 1998) and the presence of the glial glutamine synthetase which converts glutamate into the non-neurotransmitter glutamine (Tansey et al., 1991), are well developed systems, used to prevent neuronal damage due to the neurotoxic action of excess extracellular glutamate concentration (Choi, 1988).

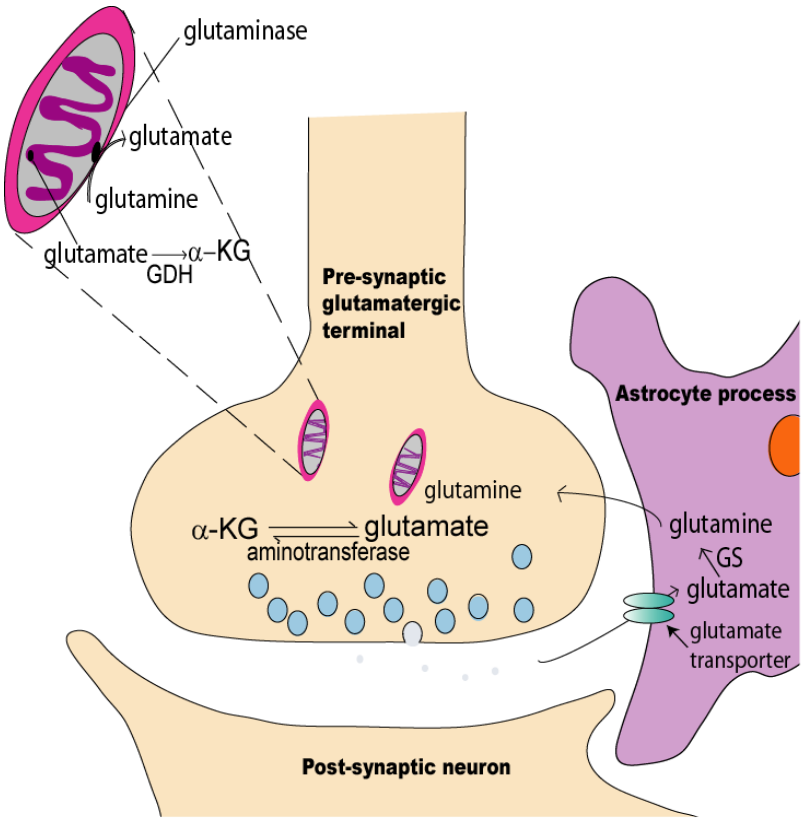


Figure 15: Metabolic interactions that occur at glutamatergic synapses between neurons and astrocytes

Glutamate can be synthesised in neurons from α-Ketoglutarate (α–KG), taken from the tricarboxylic cycle of the glucose metabolism, and donor amino acids by means of transamination. Transmitter glutamate is accumulated in vesicles containing a very high glutamate concentration and released to the synapses by calcium-dependent exocytosis. After release, synaptic glutamate is extremely rapidly inactivated by cellular uptake by glutamate transporters, from which the astrocyte-specific accounts for a major part of glutamate removal from synaptic clefts. In the astrocytes, glutamate is converted to glutamine by the action of the glial-specific enzyme glutamine synthetase (GS). Glutamine has no neurotransmittion actions and it is up taken from the extracellular space into neurons. Hydrolysis of glutamine to glutamate requires no energy and is catalyzed by glutaminase. Glutamate in the neurons can be catabolised by the enzyme glutamate dehydrogenase (GDH).

1.11.1 Glutamate transporters

High affinity excitatory amino acid transporters (EAATs) are essential to terminate glutamatergic neurotransmission and to prevent excitotoxicity. So far, five structurally distinct transporters have been identified from animal and human tissues: glutamate/aspartate transporter (GLAST; EAAT1 in human), glutamate transporter-1 (GLT-1; EAAT2 in human), excitatory amino acid carrier-1 (EAAC1; EAAT3 in human), excitatory amino acid transporter 4 (EAAT4) and excitatory amino acid transporter 5 (EAAT5). EAAT family members display about 50-55% homology. The regional and cellular distributions of the EAATs differ in the CNS. Rothstein et al. (1994) demonstrated that GLAST is expressed in astroglia, as well as in a small number of neurons. GLT-1 is an astroglia-specific transporter and is distributed throughout the brain and the spinal cord. EAAC1 was found to be presented in the cortex, hippocampus, caudate-putamen and spinal cord and be confined to pre- and post-synaptic neurons. EAAT4 is localized mainly in cerebellar Purkinje cells, whereas EAAT5 is retina-specific.

In the cervical spinal cord, it was shown that EAAC1 like-immunoreactivity was present in lamina II. GLAST like-immunoreactivity was also present in lamina II, in both astroglia and neurons and around the central canal (lamina X). GLT-1 was highly expressed in astroglial cells in laminae I-III and the area around the central canal (figure 16) (Tao et al., 2005), and finally, though EAAT4 was initially found to be neuronal in the brain, it has been co-localized with astroglia in the spinal cord. (Rothstein et al., 1994; Hu et al., 2003). EAAC1, in addition to its expression in the spinal cord neurons, is detected in the DRG and distributed predominantly in small DRG neurons (Tao et al., 2005). Some of these EAAC1-positive DRG neurons are positive for CGRP or are labelled by isolectin B4 (Tao et al., 2005), a marker of non-peptidergic neurons.

Recent studies have identified three different vesicular transporters (VGLUT1-3) (Fremeau et al., 2002). VGLUT1 and VGLUT2 are expressed throughout the central nervous system. Their complementary expression pattern in the brain defines two district classes of glutamatergic synapses (Fremeau et al., 2001). Fremeau et al. (Fremeau et al., 2001), suggested that VGLUT1 is expressed in terminals with low probability of release and activity-depended potentiation, whereas VGLUT2 is expressed in terminals displaying high probability of release and high-fidelity

neurotransmission. The explanation of such a difference is not clear, but it may be related to differences in the cytoplasmic C-termini of VGLUT1 and VGLUT2, resulting in different protein-protein interactions that could affect the mode and rate of synaptic vesicle recycling (Persson et al., 2006). VGLUT3 is expressed in a variety of cells including GABAergic, monoaminergic neurons as well as astrocytes and its role is not yet very clear (Fremeau et al., 2002).

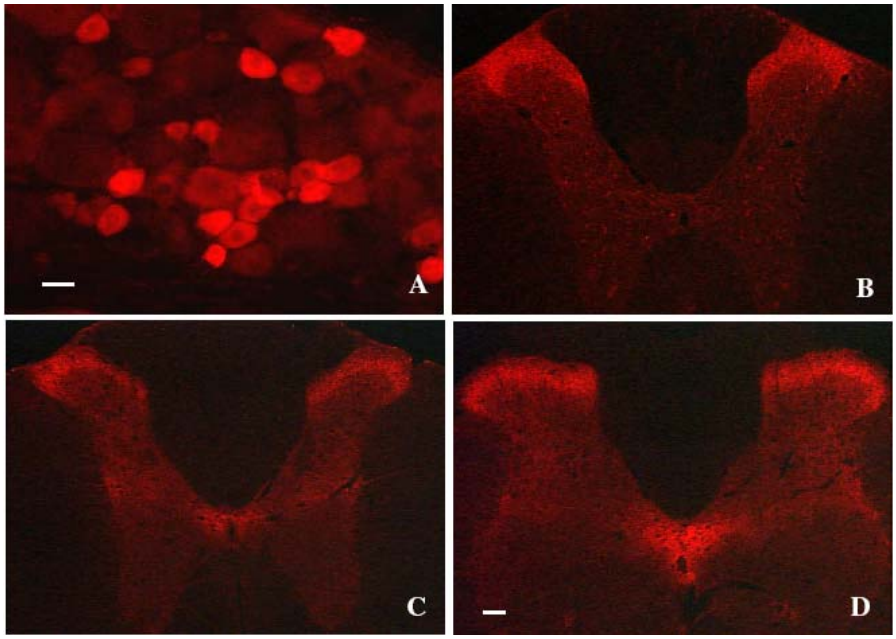


Figure 16: Localisation of glutamate transporters in the dorsal root ganglion and the spinal cord

EAAC1 is expressed mainly in small dorsal root ganglion cells (A) and distributed predominantly in the superficial dorsal horn of the spinal cord (B). GLAST (C) and GLT-1 (D) are expressed highly in the superficial dorsal horn and the region around the central canal. Scale bars: 10 μm in A and 125 μm in B, C, and D. Taken from (Tao et al., 2005).

In the spinal cord both VGLUT1 and VGLUT2 are expressed, though the spinocervical tract was found to contain dense labeling for VGLUT2 (Persson et al., 2006). This suggests that VGLUT2 is the transporter responsible for the vesicular accumulation of glutamate at the spinocervical tract terminals, and thus most glutamatergic fibre systems in the spinal cord, should display high probability of release, because of their use of VGLUT2 as vesicular transporter (Persson et al., 2006).

The VGLUTs are currently considered as the best glutamate markers for staining glutamatergic cells; their presence is a strong indication that glutamate is accumulated in vesicles from which it can be released. Staining studies found these transporters in populations of axons that are known to be glutamatergic and their expression in cultured cells results in glutamate uptake and the subsequent conversion of neurons to a glutamatergic phenotype (Bellocchio et al., 2000; Todd et al., 2003). In agreement with this, Todd et al (2003) demonstrated that in the rat lumbar spinal cord, glutamate transporters were not co-localised in glycinergic, serotonergic, cholinergic or GABAergic axons.

Recent evidence suggests that spinal glutamate transporters might play an important role in normal sensory transmission. Intrathecal application of the selective glutamate transporter blocker DL-*threo*-β-Benzyloxyaspartic acid (TBOA) resulted in significant and dose-dependent spontaneous nociceptive behaviours, and in remarkable hypersensitivity in response to thermal and mechanical stimuli (Liaw et al., 2005). TBOA on the dorsal surface of the spinal cord also resulted in a significant elevation of extracellular glutamate concentrations (Liaw et al., 2005). These findings indicate that a decrease of spinal glutamate uptake can lead to excessive glutamate accumulation in the spinal cord, which might, in turn, result in over-activation of glutamate receptors, and production of spontaneous nociceptive behaviours and sensory hypersensitivity (Tao et al., 2005). However, the glutamate transporters seem to also have opposing actions in pathological pain in animal models. Inhibition or transient knockdown of spinal GLT-1 led to a significant reduction of nociceptive behaviour in the formalin model, whereas different glutamate transporter inhibitors (TBOA, dihydrokainate, threo-3-hydroxyaspartate) reduced formalin-induced nociceptive responses and complete Freund's adjuvant-evoked thermal hyperalgesia (Tao et al., 2005). Different potential mechanisms by which glutamate transporters are involved in pathological pain have been suggested; however their exact function is not completely understood.

1.11.2 Glutamate and migraine

Findings from both animal and human studies suggest a link between glutamate and migraine and further suggest that glutamate plays a key role in migraine mechanisms (Vikelis and Mitsikostas, 2007). Migraine pain-relay centres, including the trigeminal ganglion, TNC, and thalamus, contain glutamate-positive neurons (Greenamyre et al., 1984; Kai-Kai and Howe, 1991) and glutamate activates neurons in the TNC (Hill and Salt, 1982; Salt and Hill, 1982).

Migraineurs have elevated levels of neuronal amino acids in the plasma, including glutamate, glutamine, glycine, cysteic acid and homocysteic acid, and glutamate levels are further increased during a migraine attack (Ferrari et al., 1990; Alam et al., 1998), although contradictory evidence also exists (Cananzi et al., 1995). Increased peripheral glutamate, if correlated with increased brain levels, suggests that migraineurs have a persistent neuronal hyperexcitability that becomes heightened during a migraine attack. In support of this theory is the finding that migraineurs exhibit signs of central sensitization during an attack (Burstein et al., 2000) which is a process of excessive activation of neurons in the dorsal root ganglion or the TNC following peripheral sensory stimulation (Woolf and Decosterd, 1999). Glutamate release and glutamate receptor activation mediates central sensitization (Burstein, 2001).

Glutamine levels are also increased in the cerebrospinal fluid (CSF) during migraine attacks and CSF concentrations of glutamate were higher in migraineurs than in controls, suggesting an excess of neuroexcitatory amino acids in the CNS (Martinez et al., 1993). The increased levels of glutamate, particularly in migraine with aura patients may be relevant to their neurological symptoms (D'Andrea et al., 1991). This is further supported by the mutations linked to FHM, which result in an increase in glutamate availability at the synaptic cleft of cells and may explain the increased susceptibility to CSD (van den Maagdenberg et al., 2004; Wessman et al., 2007). Furthermore a case report detailed the history of a patient with hemiplegic migraine, seizures, and episodic ataxia whose genetic screening revealed the SLC1A3 mutation on the excitatory amino acid transporter EAAT1. Functionally, this mutation reduces the glial cell's ability to clear glutamate from the synaptic cleft, which would contribute to post-synaptic hyperexcitation (Ramadan and Buchanan, 2006). CSD in the neocortex of a variety of species including man has been demonstrated to be dependent on activation of the *N*-methyl-D-aspartate (NMDA) receptor (Faria and Mody, 2004). Topiramate a preventive

given for migraine treatment increases the cortical levels of glutamine, possibly by acting on the metabolic pathway of glutamate and GABA (Moore et al., 2006).

In vivo studies using microdialysis and blood flow measurements demonstrated increased levels of glutamate in the TNC (Bereiter and Benetti, 1996) during and post stimulation of dural structures (Goadsby and Classey, 2000). This paralleled changes in sensory thresholds of facial receptive fields as recorded from secondary neurons in the rat TNC (Oshinsky and Luo, 2006), thus further supporting the involvement of glutamate in central sensitization (Ramadan, 2003). Triptans seem to act post-synaptically in the TNC (Goadsby et al., 2001) and pre-synaptically by decreasing glutamate release from primary afferents (Travagli and Williams, 1996). Glutamatergic neurons in the trigeminal ganglion are 5-$HT_{1B/1D/1F}$ receptor positive and this may indicate that 5-HT_1 receptor activation could possibly inhibit the release of glutamate pre-synaptically (Ma, 2001).

Glutamate plays a crucial role in the transmission of nociceptive information in the VB complex. It is involved in signalling from spinothalamic tract and leminiscal pathways and corticothalamic afferents (Broman and Ottersen, 1992). Extracellular levels are increased following experimentally produced pain (Silva et al., 2001). It triggers post-synaptic excitatory potentials by activating multiple glutamate receptors. (McCormick and von Krosigk, 1992; Salt and Eaton, 1995; Dougherty et al., 1996; Li et al., 1996; Salt and Turner, 1998; Salt et al., 1999b; Salt et al., 1999a).

The presence of glutamate in the transmission of sensory information, implicates the involvement of glutamate receptors that modulate glutamate responses, both in the trigeminal ganglia, the TCC, thalamus and sensory cortex in migraine pathophysiology (Vikelis and Mitsikostas, 2007).

1.12 Classification of glutamate receptors

Glutamate receptors (GluR) are categorised in two distinct classes, ionotropic and metabotropic receptors. The ionotropic glutamate receptors (iGluRs) contain cation-specific ion channels, whereas the metabotropic glutamate receptors (mGluRs) are

coupled to guanosine 5′-triphosphate (GTP)-binding proteins (also known as G-proteins) and act through the production of second messengers (figure 17). It is thought that glutamate activates post-synaptic ionotropic receptors which mediate fast synaptic transmission, and metabotropic receptors which modulate glutamate's release, post-synaptic responses, as well as the activity of non-glutamatergic synapses (Dingledine et al., 1999).

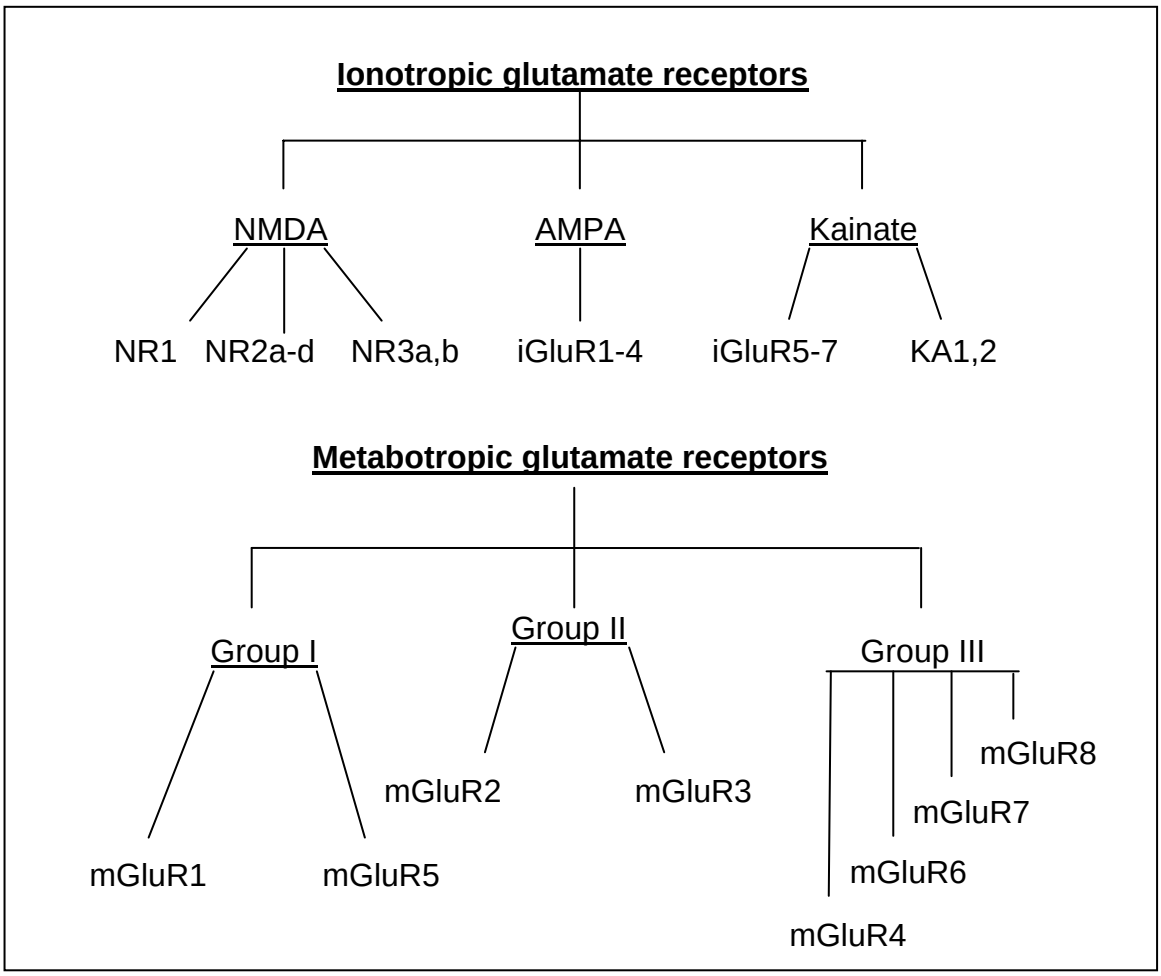


Figure 17: Classification of glutamate receptors

1.12.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) have been divided into three subtypes (figure 17) and named after the agonists which were first identified to selectively activate them:

- *N*-methyl-D-aspartate (NMDA)
- α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA)
- 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate)

AMPA and kainate receptors are also referred to as non-NMDA receptors, since notably AMPA receptors are activated by kainate, and kainate receptors with certain subunit compositions by AMPA (Kew and Kemp, 2005). In recent years, cloning studies have demonstrated that AMPA and kainate receptors are distinct receptor complexes and several agonists and antagonist that differentially act on AMPA or kainate receptors have been developed (Ozawa et al., 1998; Kew and Kemp, 2005).

Expression cloning in *Xenopus* oocytes, homology cloning and polymerase chain reaction (PCR)-based strategies were used to clone cDNAs encoding iGluR. So far 16 cDNAs have been isolated: 4 for AMPA receptor subunits (GluR1-4), 5 for kainate receptor subunits (iGluR5, 6, 7, KA1 and 2), and 7 for NMDA receptor subunits (NR1, 2A, 2B, 2C, 2D, 3A and 3B; figure 17), (Ozawa et al., 1998; Kew and Kemp, 2005).

Although the overall amino acid characteristics of iGluR subunits across the three families are only 20-30% homologous, they share common structural features. The subunits have an extracellular amino terminal domain, followed by a first transmembrane domain (TMI) and then a pore forming membrane-residing domain that forms a re-entrant loop entering from and exiting to the cytoplasm. The second (TMII) and third (TMIII) transmembrane domains are linked by a large extracellular loop and the TMIII domain is followed by an intracellular carboxy terminus. The iGluR ligand binding domains comprise polypeptides in both the amino terminus (S1 domain) and the extracellular loop between TMIII and TMIV (S2 domain) (figure 18).

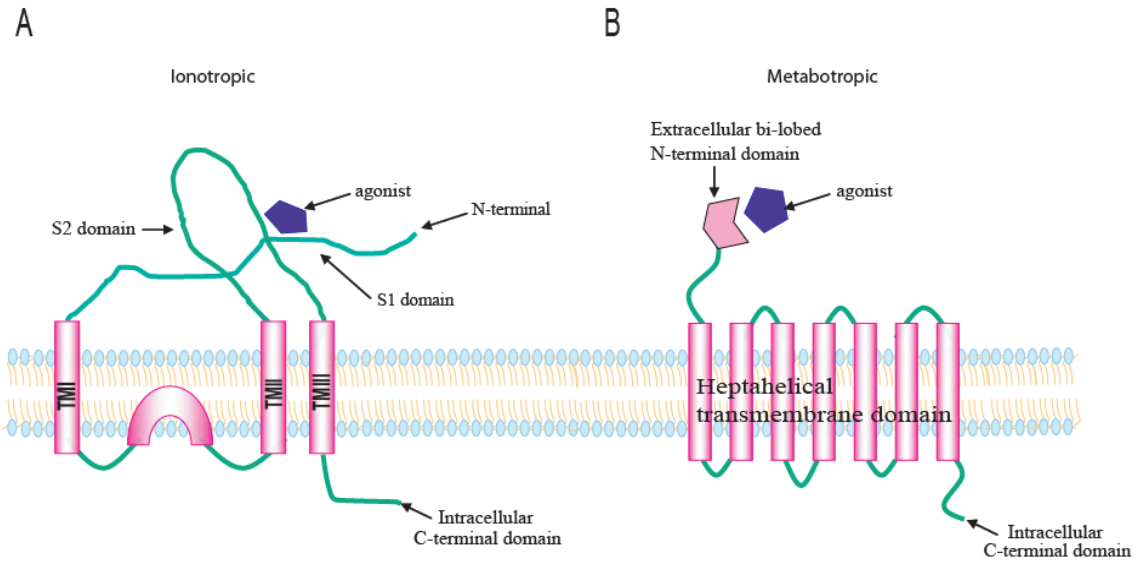


Figure 18: Ionotropic and metabotropic glutamate receptor subunits schematic representation

A. Schematic representation of an ionotropic glutamate receptor's (iGluR) family subunit. The extracellular amino terminal domain is followed by a first transmembrane domain (TMI) and then a pore forming membrane-residing domain that does not cross the membrane. The second (TMII) and third (TMIII) transmembrane domains are linked by a large extracellular loop and the TMIII domain is followed by an intracellular carboxyl terminal. The iGluR ligand binding domains, comprise polypeptides in both the amino terminus (S1 domain) and the extracellular loop between transmembrane domains 3 and 4 (S2 domain). B. Schematic representation of a metabotropic flutamate receptor's (mGluR) family member illustrating the extracellular bi-lobed N-terminal agonist binding domain connected via a cysteine-rich region to the transmembrane heptahelical domain with an intracellular C-terminus. Glutamate binds within the bi-lobed N-terminal domain whilst the transmembrane heptahelical domain contains the binding sites for both positive and negative allosteric modulators.

1.12.2 Metabotropic glutamate receptor

Metabotropic glutamate receptors (mGluRs), modulate glutamate release, post-synaptic response, as well as the activity of other synapses (Dingledine et al., 1999). They are G-protein-coupled receptors (GPCRs). Eight subtypes have been identified and classified into three groups (I-III) based upon their sequence homology, transduction mechanism and pharmacological profile (figure 17). Group I includes mGluR1 and 5 receptors, which couple to Gq and activate phospholipase C. Group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8) receptors couple to Gi/Go and inhibit adenylyl cyclase. Group I receptors are mostly located post-synaptically, thus their activation

increases excitability. On the other hand, group II/III receptors are generally pre-synaptic and their activation reduces glutamate release (Kew and Kemp, 2005).

mGluRs belong to family 3 of the GPCR superfamily and similar to all GPCRs, mGluR contain a heptahelical domain in the membrane region (figure 18). In addition, like all members of family 3, mGluR are characterised by a large extracellular amino terminal domain where the glutamate binding site is found (Kew and Kemp, 2005). Moreover, it was shown that the closed conformation of this domain is required for receptor activation (Bertrand et al., 2002; Bessis et al., 2002).

1.13 Kainate receptors

Kainate receptors, originally described by Watkins group (Davies et al., 1979; Watkins and Evans, 1981), constitute a separate group from the NMDA and AMPA receptors, although they share many structural characteristics. Like the other ionotropic glutamate receptor subunits they possess an extracellular N-terminus that, together with a loop between TMII and TMIII, forms the ligand binding domain, and a re-entrant loop that forms the lining of the pore region of the ion channel (figure 18).

The kainate receptor is constituted by the iGluR5, 6, 7, KA1 and 2 subunits, which form tetrameric assemblies giving rise to functional receptor-channels (Dingledine et al., 1999). iGluR5-7 subunits are also known as “low-affinity subunits” due to the low binding affinity that they show (in the range of 50-100 nM), whereas the KA1 and 2 subunits are known as the “high affinity subunits”, since they display high affinity [3H] kainate binding (in the range of 5-15 nM) (Pinheiro and Mulle, 2006). iGluR5-7 are of similar size of 900 amino acids and KA1 and 2 are slightly larger proteins of approximately 970 amino acids (Ozawa et al., 1998). There is 75%-80% homology between iGluR5, 6 and 7, and 68% between KA1 and 2, whereas the two subclasses share only 45% homology (Pinheiro and Mulle, 2006). The kainate receptor subunits share less than 40% homology with the AMPA iGluR1-4 subunits and less than 20% with NMDA receptors (Huettner, 2003).

From studies in which kainate receptors were expressed in *Xenopus* oocytes or in transfected HEK 293 cells, it is known that the iGluR5-7 can form functional homomeric receptors. The high-affinity KA2 subunit can only form functional receptors when it forms tetramers combined with the GluR subunits, whereas not much work has been done for the KA1 subunit. Several studies have also demonstrated that the iGluR5-7 subunits can co-assemble into heteromeric glutamate receptors (Huettner, 2003). Bahn and colleagues (1994) demonstrated by using *in situ* hybridisation that KA1 and 2 subunits coexist with functioning iGluR5-7 subunits in the same population of cells, suggesting that the native kainate receptors may be heteromeric tetramers composed of different combinations of dimmers of these subunits (table 8).

Table 8: Functional kainate receptors

	iGluR5	**iGluR6**	**iGluR7**	**KA1**	**KA2**
iGluR5	iGluR5/iGluR5	iGluR5/iGluR6	iGluR5/iGluR7	NKRs	iGluR5/KA2
iGluR6	iGluR6/iGluR5	iGluR6/iGluR6	iGluR6/IGluR7	NKRs	iGluR6/KA2
iGluR7	iGluR7/iGluR5	iGluR7/iGluR6	iGluR7/IGluR7	NKRs	iGluR7/KA2
KA1	NKRs	NKRs	NKRs	NFRs	NFRs
KA2	KA2/iGluR5	KA2/iGluR6	KA2/iGluR7	NFRs	NFRs

Functional kainate receptors that can be formed by combination of the different kainate subunits. The nine different colours indicate the nine known combinations of functional receptors.

IGluR5 homomeric receptors, IGluR6 homomeric receptors,
IGluR7 homomeric receptors, IGluR5/IGluR6 heteromeric receptors,
IGluR5/IGluR7 heteromeric receptors, IGluR5/KA2 heteromeric receptors,
IGluR6/IGluR7 heteromeric receptors, IGluR6/KA2 heteromeric receptor,
IGluR7/KA2 heteromeric receptors, NKRs, not known receptors;
NFRs, not functional receptors

The iGluR5-7 subunits undergo both splice variation at the C-terminal domain and RNA editing, which increase the number of subunit isoforms, giving rise to a large number of possible receptors with different pharmacological and functional properties (Chittajallu et al., 1999). There are three known splice variance for iGluR5 (iGluR5a, b and c), two for iGluR6 (iGluR6a and b) and two for the iGluR7 subunit (iGluR7a and b) (Bettler and Mulle, 1995; Jaskolski et al., 2005). The KA1 and 2 subunits do not undergo any known process of alternative splicing or RNA editing (Pinheiro and Mulle, 2006). The physiological properties of the iGluR5-7 subunits are not affected by alternative splicing (Jaskolski et al., 2004). The genes coding for the different kainate receptors have been identified in both humans and animals, and human molecular genetic studies have demonstrated alterations that might occur on these genes and their association to diseases (Szpirer et al., 1994; Pinheiro and Mulle, 2006) (table 9).

Table 9: Kainate receptors and human genetics of disease

Subunit	Gene	Locus	Associated disease	Reference
iGluR5	GRIK1	21q22.1	• Juvenile absence epilepsy • region implicated in Down's syndrome	(Gregor et al., 1994; Sander et al., 1997)
iGluR6	GRIK2	6q16.3-q21	• Schizophrenia • Early-onset Huntington's disease • Autism	(Paschen et al., 1994; Rubinsztein et al., 1997; Motazacker et al., 2007)
iGluR7	GRIK 3	1p34-p33	• Schizophrenia	(Puranam et al., 1993)
KA1	GRIK4	11q22.3	• Depression • Migraine	(Cader et al., 2003; Paddock et al., 2007)
KA2	GRIK5	19q13.2	• Schizophrenia	(Szpirer et al., 1994)

Kainate receptors are not permeable to Ca^{2+}, but they display high permeability to intracellular Cl^- and extracellular Na^+. K^+ also leaves the intracellular space through the kainate channels and finally polyamine interaction with the receptors is prevented (Chittajallu et al., 1999; Huettner, 2003). These channel properties have been studied in homomeric iGluR5 and 6 receptors and heteromers with KA2 receptors, and are due to the introduction of the amino acid arginine at the Q/R(glutamamine/arginine) RNA editing site, in the second membrane domain of iGluR5 and 6 subunits. Thus, unedited iGluR5 and 6 kainate receptors display permeability to Ca^{2+}, Na^+ and K^+ and polyamine molecules interactions are allowed (Chittajallu et al., 1999). The Q/R site editing is incomplete during development and significant amounts of both edited and unedited versions of the iGluR5 and 6 subunits co-exist in the adult brain (Ozawa et al., 1998). The Q/R RNA editing does not occur in the iGluR7 subunits. iGluR6 subunit's permeability to Ca^{2+} also depends on the I/V (isoleukin/valine) and I/Y (tyrosine/cystaine) sites and fully edited subunits are impermeable to Ca^{2+} (Kohler et al., 1993). Another important property of kainate receptors is that they do not desensitise as rapidly as AMPA receptors.

1.13.1 Localisation of kainate receptors

Kainate receptors are found throughout many regions of the brain, as shown by immunostaining and *in situ* hybridisation. iGluR6/7 and KA2 showed moderate staining in the olfactory bulb, cortex, caudate putamen, hypothalamus, brainstem, thalamus and amygdala, and light to moderate staining in the hippocampus and the cerebellar layers (Petralia et al., 1994). Similar results were obtain with radioligand binding studies using [3H]kainate (Ozawa et al., 1998). Localisation of specific subunits at the mRNA level by *in situ* hybridisation revealed functional differences between neuronal kainate receptors in different areas, that may reflect their distinct subunit composition and their diverse roles in synaptic transmission (Ozawa et al., 1998). Despite the moderate presence of kainate receptors demonstrated by immunocytochemistry and *in situ* hybridisation, electrophysiological recordings provided greater understanding of the presence and pharmacology of kainate receptors in different areas (Kullmann, 2001; Huettner, 2003; Lerma, 2003). For the purpose of this thesis focus will be given on structures involved in ascending pain pathways.

Presence of kainate receptors in ganglia and their function

The presence of kainate receptors in DRG was the first indication of their existence, which was shown after depolarization of dorsal root fibres by exposure to kainate (Davies et al., 1979). Treatment of DRG with kainate also produced selective blockade of action potential conduction along C-fibres, suggesting that certain primary afferent C-fibres possess kainate receptors which may be activated physiologically by released glutamate at their central terminations (Agrawal and Evans, 1986). The blockade of action potential conduction along C-fibres following treatment of DRG with kainate is very likely due to the rapid desensitisation of kainate receptors with prolonged agonist exposure (Huettner, 1990). Native kainate receptors were pharmacologically characterised in the DRG by Huettner (1990), confirming the expression of functional kainate receptors on the small diameter sensory neurons that comprise of Aδ- and C-fibres. *In situ* hybridisation and Northern blot analysis showed the presence of kainate receptors in DRG and demonstrated that the iGluR5 subunit is the predominant subunit expressed (Bettler et al., 1990; Partin et al., 1993; Sato et al., 1993). In agreement with these results, functional kainate receptors were eliminated by iGluR5 deletion in DRG cells from iGluR5 knockout mice exhibiting an absolute requirement for the iGluR5 subunit (Kerchner et al., 2002). Recording from cells expressing kainate receptors suggest that homomeric iGluR5 receptors predominate in DRG (Bettler et al., 1990), although in a study with iGluR5 and iGluR6 mice the significance of the iGluR6 subunit on Aδ- and C-fibres was also shown (Youn and Randic, 2004). In the trigeminal ganglia, reverse transcriptase-PCR and recordings from kainate expressing cells, demonstrated the presence of heteromeric iGluR5/KA2 receptors (Sahara et al., 1997).

It has been proposed that kainate receptors are transferred from the DRG neurons along the primary afferents and function as autoreceptors in the dorsal horn (Agrawal and Evans, 1986; Hwang et al., 2001) and may be activated physiologically by released glutamate at their central terminations (Agrawal and Evans, 1986; Huettner et al., 2002). Evidence from immunocytochemical studies also exists for peripheral transport of kainate receptors on sensory, both myelinated and unmyelinated axons (Carlton et al., 1995; Coggeshall and Carlton, 1998). It has been shown that activation of these receptors on peripheral axons leads to increased activity along the sensory root and thus to peripheral nociceptive transduction (Agrawal and Evans, 1986; Ault and Hildebrand, 1993).

Presence of kainate receptors in the spinal cord and their function

Anatomical studies demonstrated a low presence of kainate receptors in the spinal cord (Tolle et al., 1993; Petralia et al., 1994). More recent evidence, demonstrated the presence of different kainate subunit mRNA in cultured spinal neurons (Dai et al., 2002) and the localisation of all kainate receptors' subunits at both pre- and post-synaptic sites in the rat dorsal horn by confocal and electron microscopy (Hwang et al., 2001; Lu et al., 2003; Lucifora et al., 2006). Several electrophysiological studies have also provided physiological evidence for functional pre- and post-synaptic kainate receptors in spinal neurons (Budai and Larson, 1994; Li et al., 1999; Kerchner et al., 2001b; Kerchner et al., 2001a; Wilding and Huettner, 2001). Primary afferent synapses in the dorsal horn of the spinal cord represent an example where both pre- and post-synaptic kainate receptors may play a role in transmission at individual contacts (Huettner, 2003).

Original evidence for post-synaptic kainate receptors in the spinal cord, was obtained by Li et al. (1999) in studies of rat spinal cord slices and confirmed from electrophysiological recordings from dorsal horn neuronal cultures (Lee et al., 1999; Kerchner et al., 2001a). Whole-cell recording in layer II of the dorsal horn revealed that high-intensity single-shock stimulation of primary afferent sensory fibres produces a fast, kainate-receptor-mediated excitatory post-synaptic current (EPSC). This kainate receptor-mediated component of transmission was most pronounced for stimulation intensities that were sufficient to activate high threshold Aδ- and C-fibres. Activation of low-threshold afferent fibres generates typical AMPA-receptor-mediated EPSCs only, indicating that kainate receptors may be restricted to synapses formed by high-threshold nociceptive (pain-sensing) and thermoreceptive primary afferent fibres (Li et al., 1999). The presence of post-synaptic receptors in the spinal cord, activated by kainate was also shown *in vivo* by microiontophoretic application of kainate (Budai and Larson, 1994). Kainate evoked responses, as well as noxious and innocuous receptive fields were inhibited by the 2,3-benzodiazepine compound, 4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride (GYKI 52466), supporting the possible involvement of these receptors in spinal nociception. However, the pure selectivity of kainate agonists on kainate receptors versus AMPA receptors, and the non-selectivity of GYKI 52466 do not lead to clear conclusions.

The presence of pre-synaptic receptors in primary afferents in the superficial laminae of the spinal cord, has been demonstrated by light and electron microscopy, on both myelinated and non myelinated primary afferent fibres (Hwang et al., 2001; Lucifora et al., 2006). The dominant subunit expressed on pre-synaptic terminals in laminae I-III is the iGluR5 (Lucifora et al., 2006). Pre-synaptic kainate receptors are largely formed in DRG and transferred in primary nerve terminals that project mainly in outer laminae of the spinal cord (Kerchner et al., 2001a). Kerchner et al. (2002), using knockout mice, provided evidence that both iGluR5 and 6 subunits contribute to the receptors expressed by DRG and spinal neurons. In cultured dorsal horn neurons isolated from iGluR5 knockout mice, responses to kainate were still present and were completely insensitive to iGluR5 selective antagonists. In neurons from iGluR6 knockout mice, functional kainate receptors expression was reduced, suggesting that iGluR6 is more important than iGluR5 for the assembly of functional kainate receptors in dorsal horn neurons. However, application of iGluR5 kainate antagonists in wild-type and iGluR6 cells blocked post-synaptic responses produced by kainate, indicating the existence of iGluR5 kainate receptors on dorsal horn neurons, but probably in lower stoichiometry than the iGluR6 subunit (Kerchner et al., 2002).

For many years the function of kainate receptors on primary afferents in the spinal cord was not known (Agrawal and Evans, 1986). More recently, Kerchner et al., (2001a) proved *in vitro* that presynaptic kainate receptors regulate spinal sensory transmittion, by applying kainate and the iGluR5 subunit agonist (RS)-2-alpha-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid (ATPA) in DRG-dorsal horn neuronal cultures and spinal cord slices. More specifically, it was shown in the presence of AMPA antagonist, that kainate suppressed spontaneous NMDA receptor-mediated EPSCs in cultures of spinal dorsal horn neurons and EPSCs in dorsal horn neurons evoked by stimulation of synaptically coupled DRG cells in DRG-dorsal horn neuron co-cultures. In addition ATPA was able to suppress DRG-dorsal horn synaptic transmission to a similar extent as kainate, but it had no effect on excitatory transmission between dorsal horn neurons. In recordings from dorsal horn neurons in spinal slices, kainate and ATPA were able to suppress NMDA and AMPA receptor-mediated EPSCs evoked by dorsal root fibre stimulation. Together, these data suggest that kainate receptor agonists, acting at a pre-synaptic locus, can reduce glutamate release from primary afferent sensory synapses (Kerchner et al., 2001a; Kerchner et al., 2002).

Further studies provided evidence for expression of pre-synaptic kainate receptors by spinal cord inhibitory interneurons (Kerchner et al., 2001b; Lu et al., 2005). Confocal and electron microscopy revealed the presence of kainate receptors on 20-35% of GABAergic terminals in laminae I-III of the dorsal horn of the spinal cord (Lu et al., 2005). In spinal cord slices endogenous glutamate released upon stimulation of primary afferent fibres was shown to reduce evoked inhibitory post-synaptic currents (IPSCs) via the activation of kainate receptors and this suppression of inhibition was prevented by $GABA_B$ receptor antagonists (Kerchner et al., 2001b). As glutamate-containing sensory fibres come into close proximity with the GABA/glycine terminals of local interneurons, diffused glutamate from primary afferents will activate pre-synaptic kainate receptor on neighbouring interneurons. It has been suggested that kainate receptors activation on interneurons enhances GABA/glycine release via an action potential-independed action, leading to a negative feedback pathway on GABAergic synapses mediated by subsequent activation of $GABA_B$ autoreceptors (Kerchner et al., 2001b). In such a scheme, pre-synaptic kainate receptors exert a facilitatory action on GABA release (figure 19) and highlight a novel role for these receptors in regulating sensory transmission.

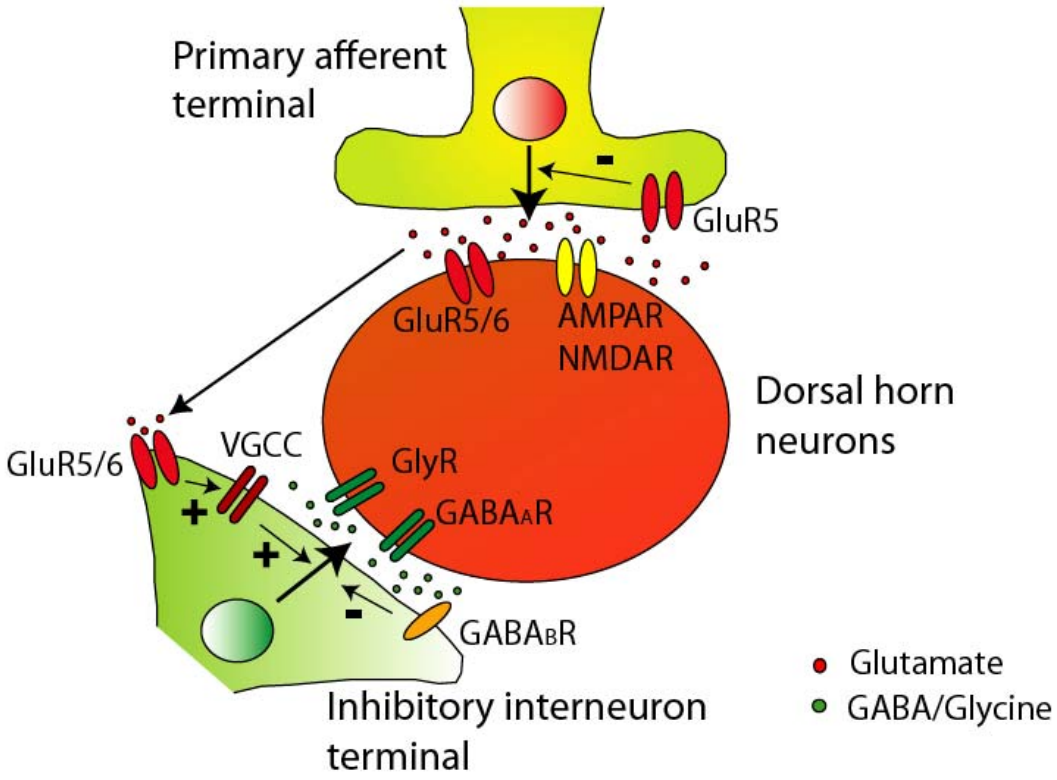


Figure 19: Location and function of kainate receptors on dorsal horn neurons

Superficial spinal dorsal horn kainate receptors are strategically placed to regulate sensory transmission. (1) Glutamate acting on iGluR5-carrying kainate autoreceptors on primary afferent terminals has been shown to inhibit further glutamate release. (2) Glutamate released from primary afferents following high-intensity, but not low intensity stimulation, acts on kainate receptors on superficial dorsal horn neurons, inducing an excitatory post-synaptic potential, thus promoting the transmission of nociceptive information. In addition, the kainate receptor-mediated depolarization may remove the magnesium blockade from neighbouring NMDA receptors, allowing Ca^{2+} entry that is necessary for the induction of long-term potentiation. (3) Glutamate released from primary afferents diffuses to neighbouring terminals of inhibitory interneurons. There, glutamate acts on kainate receptors, inducing a membrane depolarization that allows Ca^{2+} entry through voltage-gated calcium channels. Elevated Ca^{2+} levels in the terminal promote spontaneous release of glycine and GABA that by acting on $GABA_B$ autoreceptors inhibits release of inhibitory transmitter. Figure adapted from (Kerchner et al., 2001b).

GluR, Glutamate receptor subunit; AMPAR, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptors; NMDA, *N*-methyl-*D*-aspartic acid receptors; GABA/R, γ-aminobutyric acid/receptors; GlyR, glycine receptors; VGCC, voltage-gated calcium channels.

Presence of kainate receptors in the thalamus and their function

Kainate receptors in the thalamus have been shown with [^{3}H]kainic acid autoradiography studies, *in situ* hybridisation and immunocytochemistry (Monaghan and Cotman, 1982; Bettler et al., 1990; Wisden and Seeburg, 1993; Paschen and Djuricic, 1994; Petralia et al., 1994). However, the exact synaptic location of kainate receptors is still uncertain, although they are known to be present in both the ventrobasal thalamus and the thalamic reticular nucleus (Salt, 2002; Binns et al., 2003). In the primate thalamus, *in situ* hybridisation showed that only iGluR6 transcripts were expressed at detectable levels (Ibrahim et al., 2000b), while all five kainate receptor subunits' mRNA were expressed in the human thalamus (Ibrahim et al., 2000a). The possible presence of the iGluR5 subunit in the thalamus was also shown by *in situ* hybridisation (Bettler et al., 1990; Paschen and Djuricic, 1994). By comparing results from [^{3}H]kainic acid autoradiography and kainate mRNA expression Ibrahim et al., (2000a) suggested both post- and pre-synaptic localisation of kainate receptors.

Recently, it was shown that iGluR5-containing kainate receptors in neurons of the ventrobasal thalamus play a critical role in sensory function (Salt, 2002; Binns et al., 2003). It has been shown that iGluR5-selective agonists applied by microiontophoresis activate post-synaptic relay neurons, but the selective iGluR5-antagonist 3S, 4aR,6S, 8aR)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid (LY382884) failed to reduce these responses; thus it has been suggested that kainate receptors do not appear to mediate post-synaptic responses in these neurons (Binns et al., 2003). In the same study however, application of the same antagonist inhibited responses to receptive field mechanical stimulation, and thus suggested that pre-synaptic kainate receptors regulate GABAergic transmission (Binns et al., 2003). Therefore, iGluR5-mediated disinhibition appears to be important in sensory processing in the ventrobasal thalamus (Salt, 2002).

Presence of kainate receptors in the cortex and their function

Both pre- and post-synaptic kainate receptors are present in thalamocortical synapses in the somatosensory cortex (Wu et al., 2007). In genetically modified mice with deletions of iGluR5 and/or 6 it has been shown that both subunits are involved in synaptic transmission in the antireor cingulated cortex (ACC) (Wu et al., 2005), which is critically involved in nociception and pain perception (Wu et al., 2007). Functional iGluR5-carrying receptors have also been found on interneurons in the ACC and activation of interneuronal iGluR5-carrying receptors caused action potential dependent GABA release, showing that iGluR5 is mainly somatodendritic but not pre-synaptic on interneurons (Wu et al., 2006).

1.13.2 Kainate receptors in nociception

The localisation of the kainate receptors along the spinothalamic pathway, especially in both spinal cord and DRG neurons, has implicated these receptors in spinal nociception and in the transmission of pain perception. Evidence has begun to accumulate indicating that iGluR5 glutamate receptors play an important role in nociception and central sensitization (Ruscheweyh and Sandkuhler, 2002; Wu et al., 2007). In iGluR5 and 6 knockout mice, it has been shown that deletion of any of the subunits does not affect nociceptive responses in the tail-flick reflex and hot-plate test (Ko et al., 2005). However, the same study reported that in iGluR5-deficient mice, responses to capsaicin and formalin-induced inflammatory pain were substantially reduced, whereas no significant differences were observed in iGluR6 knockout mice (table 10). These evidence point to a relatively selective activation of kainate receptors in chronic, but not acute pain. This might reflect a specific function of kainate receptors at synapses with high glutamate release.

Table 10: Kainate receptors in nociception on genetic models

		Targets	Pain Model	Phenotype
Knockout	iGluR5 -/-	GluR5	Hot plate/Tail flick	No effect
			Capsaisin hyperalgesia	Analgesia
			Formalin inflammation	Analgesia
			CFA inflammation	No effect
	iGluR6 -/-	GluR6	Hot plate/Tail flick	No effect
			Capsaisin hyperalgesia	No effect
			Formalin inflammation	No effect
			CFA inflammation	No effect

Adapted from (Wu et al., 2007).

Although electrophysiological studies have implicated iGluR5 agonists as potential anti-nociceptive drugs, mixed results have been reported for the effects of kainate receptor antagonists in animal pain models (Ruscheweyh and Sandkuhler, 2002; Wu et al., 2007) (table 11). Non-selective AMPA/kainate receptor antagonists, including 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) have been shown to produce anti-nociception in animal models of peripheral neuropathy and Fos expression (Mao et al., 1992; Mitsikostas et al., 1999). Stanfa and Dickenson (1999) demonstrated that the relatively selective iGluR5 receptor antagonist LY382884 as well as the non-selective AMPA/kainate receptor antagonists NBQX and (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1*H*-tetrazol-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid monohydrate (LY293558), produced anti-nociception in the carrageenan inflammation model in rats, as well as reducing C-fibre evoked response in normal animals. In neuropathic primate models, LY382884 was also effective in reducing the increased responses of spinothalamic tract cells to weak mechanical stimuli and to thermal stimuli. In normal animals it preferentially inhibited the responses of spinothalamic tract neurons to weak mechanical stimuli over those induced by noxious mechanical stimuli. The increased effectiveness of the iGluR5 receptor antagonist on the pathological activity could be related to the increase in

kainate receptors in the spinal cord dorsal horn that occurs after induction of peripheral neuropathy and peripheral inflammation (Palecek et al., 2004). In addition, the mixed AMPA/iGluR5 receptor antagonist LY293558 produced analgesia in the capsaicin model in humans (Sang et al., 1998). A more recent study, which used a range of new decahydroisoquinoline iGluR5-selective competitive antagonists, demonstrated that these selective compounds can reverse capsaicin-induced mechanical allodynia and carrageenan-induced thermal hyperalgesia (Jones et al., 2006). However, similar results in these models were obtain by administration of the kainate ligand (2S,4R)-4-methyl glutamic acid (SYM 2081) (Turner et al., 2003). Anti-nociceptive effects were also observed by intrathecal administration of the iGluR5 receptor agonist ATPA, which reduced responses to thermal and mechanical stimulation in rats (Wu et al., 2003). Taken together, these data suggest that iGluR5 receptors play an important role in persistent, but not acute pain. However, most of the studies to date have been conducted using compounds that lack a high degree of selectivity for iGluR5 versus AMPA or iGluR6 receptors (Pinheiro and Mulle, 2006), therefore limiting the strength of this conclusion. In addition, most of these studies used intraperitoneal drug application and it is not clear if the anti-nociceptive effects of kainate receptor antagonists are due to actions on kainate receptors in the spinal cord or elsewhere.

Table 11: Pharmacological manipulation of kainate receptors in nociceptive animal models

		Targets	Drug Administartion	Pain Model	Phenotype	Species
Agonist	Kainic acid	AMPA/ Kainate receptors	Intraperitoneal	Hot plate/ Tail flick	Hyperalgesia	Mice
			Intrathecal		No effect	
			Subcutaneous		Hyperalgesia	
			Intraperitoneal	Mechanical stimulation	Hyperalgesia	Rats
				Acetic acid nociception	No effect	Mice
	ATPA	iGluR5	Intraperitoneal	Hot plate	No effect	Mice
			Intrathecal	Hot plate	Analgesia	Rats
				Mechanical stimulation		
Antagonist	CNQX	AMPA/ Kainate receptors	Intrathecal	Hot plate/Tail flick	Analgesia	Rats
				Cold plate	No effect	
	NBQX	AMPA/ Kainate receptors	Intraperitoneal	Formalin inflammation	Analgesia	Rats
			Intrathecal	CFA inflammation	Analgesia	Rats
			Intraperitoneal	Hot plate	No effect	Rats
				Formalin inflammation	No effect	
				Chronic injury	Analgesia	
	NS-102	kainate receptors	Intrathecal	CFA inflammation	Analgesia	Rats
	SYM 2081	kainate receptors	Intrathecal	Hot plate/Tail flick	Analgesia	Rats
				Cold plate	No effect	
			Intraperitoneal	Chronic injury	Analgesia	Rats
			Intraperitoneal	Freeze injury	Analgesia	Rats
			Intraperitoneal/ Intathecal	Capsaisin hyperalgesia	Analgesia	Rats
			Subcutaneous		No effect	
			Intraperitoneal	Carrageenan inflammation	Analgesia	
	NS1209	AMPA/ iGluR5	Intraperitoneal	Hot plate	Analgesia	Rats
				Formalin inflammation		
				Chronic injury		
	LY293558	AMPA/ iGluR5	Intraperitoneal	Formalin inflammation	Analgesia	Rats
			Intavenous	Capsaisin hyperalgesia	Analgesia	Humans
			Intavenous	Migraine	Analgesia	Humans
	LY382884	iGluR5	Intraperitoneal	Formalin inflammation	Analgesia	Rats
			Intrathecal	CFA inflammation	Analgesia	Rats
	LY467711/ LY525327	iGluR5	Oral	Capsaisin hyperalgesia	Analgesia	Rats
				Formalin inflammation		
				Carrageenan inflammation		

Adapted from (Wu et al., 2007)

1.13.3 Kainate receptors in trigeminovascular nociceptive processing and migraine

Although a variety of studies have indicated the involvement of kainate receptors in nociception, very few studies have investigated their possible involvement in migraine pathophysiology.

Sahara et al. (1997), using patch clamp techniques, showed that in trigeminal ganglia cell cultures, small diameter neurons responded to both L-glutamate and kainate. In addition, reverse transcriptase-PCR showed that although all five known kainate receptor subunits are expressed on trigeminal ganglia neurons the dominant receptors are possibly heteromeric iGluR5/KA2 receptors. In the TCC and caudal brainstem the presence of kainate receptors was demonstrated at both pre- and post-synaptic sites (Hill and Salt, 1982; Salt and Hill, 1982; Petralia et al., 1994; Onodera et al., 2000; Hegarty et al., 2007) and functional responses from kainate receptors responding to kainate receptor agonists and/or antagonists have been recorded (Hill and Salt, 1982; Salt and Hill, 1982).

Non-selective AMPA/kainate receptor antagonists have been shown to produce anti-nociceptive effects in both animal and human migraine studies (Mitsikostas et al., 1999; Storer and Goadsby, 1999; Sang et al., 2004), suggesting a possible role of kainate receptors in migraine. The non-NMDA receptor blocker GYKI 52466 lead to a dose-dependent inhibition of trigeminovascular-evoked responses in the TCC. In another study where Fos expression was assessed in the TCC following intracisternal capsaicin administration, the AMPA/kainate receptor antagonists CNQX and NBQX reduced Fos expression (Mitsikostas et al., 1999). The relatively higher selectivity of these antagonists for AMPA over kainate receptors however, limited any conclusions for the specific role of kainate receptors in the trigeminovascular system (Lester et al., 1989; Paternain et al., 1995; Lee et al., 1999; Kong and Yu, 2006). Indirect evidence connecting kainate receptors with migraine also comes from a small human study at which the AMPA/iGluR5 antagonist LY293558 given intravenously was effective in treating headache pain in two thirds of patients and also relieved nausea, photophobia and phonophobia symptoms during migraine attacks (Sang et al., 2004). This compound, now called tezampanel has completed the Phase IIb trials and in a randomized, double-blind, placebo-controlled, parallel-group trial LY293558 was comparable to sumatriptan by injection at providing pain relief, and elicited fewer side effects (Murphy et al., 2008). However, due to the AMPA action of the LY293558

antagonist, it is not clear if the observed effects are due to specific actions on kainate receptors or due to combined AMPA/kainate effects.

With the recent discovery of more selective kainate agents, kainate receptor and particularly iGluR5 subunit, involvement in migraine pathogenesis has received fresh impetus. The competitive iGluR5-selective receptor antagonist (3*S*,4*aR*,6*S*,8*aR*)-6-[[(2*S*)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]decahydro-3-isoquinolinecarboxylic acid (LY466195) and the Ethyl (3S,4aR,6S,8aR)-6-(4-Ethoxycarbonylimidazol-1-ylmethyl)decahydroisoquinoline-3-carboxylic ester were effective in blocking dural plasma protein extravasation and in reducing Fos expression in the TCC after stimulation of the trigeminal ganglion (Filla et al., 2002; Weiss et al., 2006). Important clinical correlates also exist from a double-blind study, in which the selective iGluR5 antagonist LY466195 was effective in relieving head pain in migraine patients (Johnson, KW, International Headache Research Seminar, Copenhagen, 25 March 2007). Topiramate is a clinically effective migraine preventive (Bussone et al., 2006; Mulleners and Chronicle, 2008) and it has been shown to reduce trigeminovascular activation and to inhibit CSD (Akerman and Goadsby, 2005a, b). It has been suggested that the pharmacology of topiramate includes modulation of kainate-evoked neural responses (Rosenfeld, 1997), further supporting the kainate receptor as a target for future migraine therapeutics.

"Non-kainate" receptors in trigeminovascular nociceptive processing and migraine

Since glutamate plays a key role in migraine mechanisms (Ramadan, 2003) it is accepted that pharmacological manipulation of its receptors will play a key role in migraine mechanisms. Part of this section has been adapted by Vikelis and Mitsikostas (2007) and includes studies on NMDA, AMPA and mGluRs, while the combined kainate/AMPA studies have been mentioned above.

Glutamate receptors and especially NMDA and AMPA are abundant throughout the CNS (Ozawa et al., 1998; Kew and Kemp, 2005) and similar to kainate receptors their pharmacological manipulation demonstrated effectiveness in anti-nociception (Fundytus, 2001; Bleakman et al., 2006).

NMDA pharmacology seems to underline CSD mechanisms. Scheller and colleagues (2000) reported that application of NMDA induced CSD (Scheller et al., 2000). NMDA blockade prevented CSD, whereas AMPA receptor antagonists had no effect (Lauritzen and Hansen, 1992; Nellgard and Wieloch, 1992). Interestingly magnesium depletion, which releases the voltage-dependent block of the NMDA receptor channel has been shown to induce CSD (Mody et al., 1987).

NMDA, AMPA and group III mGlu receptors were found to modulate noxious responses within second order trigeminal neurons after meningeal irritation by capsaicin (Mitsikostas et al., 1998; Mitsikostas et al., 1999; Mitsikostas and Sanchez del Rio, 2001). It was concluded that since this model may be predictive for treatment of vascular headaches (Moskowitz and Macfarlane, 1993) these receptors may also serve as potential targets for the development of new anti-migraine drugs. Fos expression induced after electrical stimulation of SSS was reduced after treatment with the NMDA anatagonist dizocipline maleate (MK 801) in cats (Classey et al., 2001) further suggesting that NMDA receptors provide a potential therapeutic target for migraine due to trigeminovascular activation from meningeal afferents. The role of NMDA receptors can be more complex in central pain pathways. It has been shown that NMDA antagonists can increase Fos in the NRM, DRN and PAG, which are part of the descending anti-nociceptive system (Hattori et al., 2004). In another study the NMDA receptor's antagonist MK 801, and the AMPA receptor's antagonist GYKI 52466 significantly and dose-dependently decreased neuronal activity induced by electrical stimulation of the SSS within the TCC (Storer and Goadsby, 1999). NMDA receptors are found throughout the TCC and mainly on second order neurons that come in close proximity to CGRP-carrying primary afferents (figure 20).

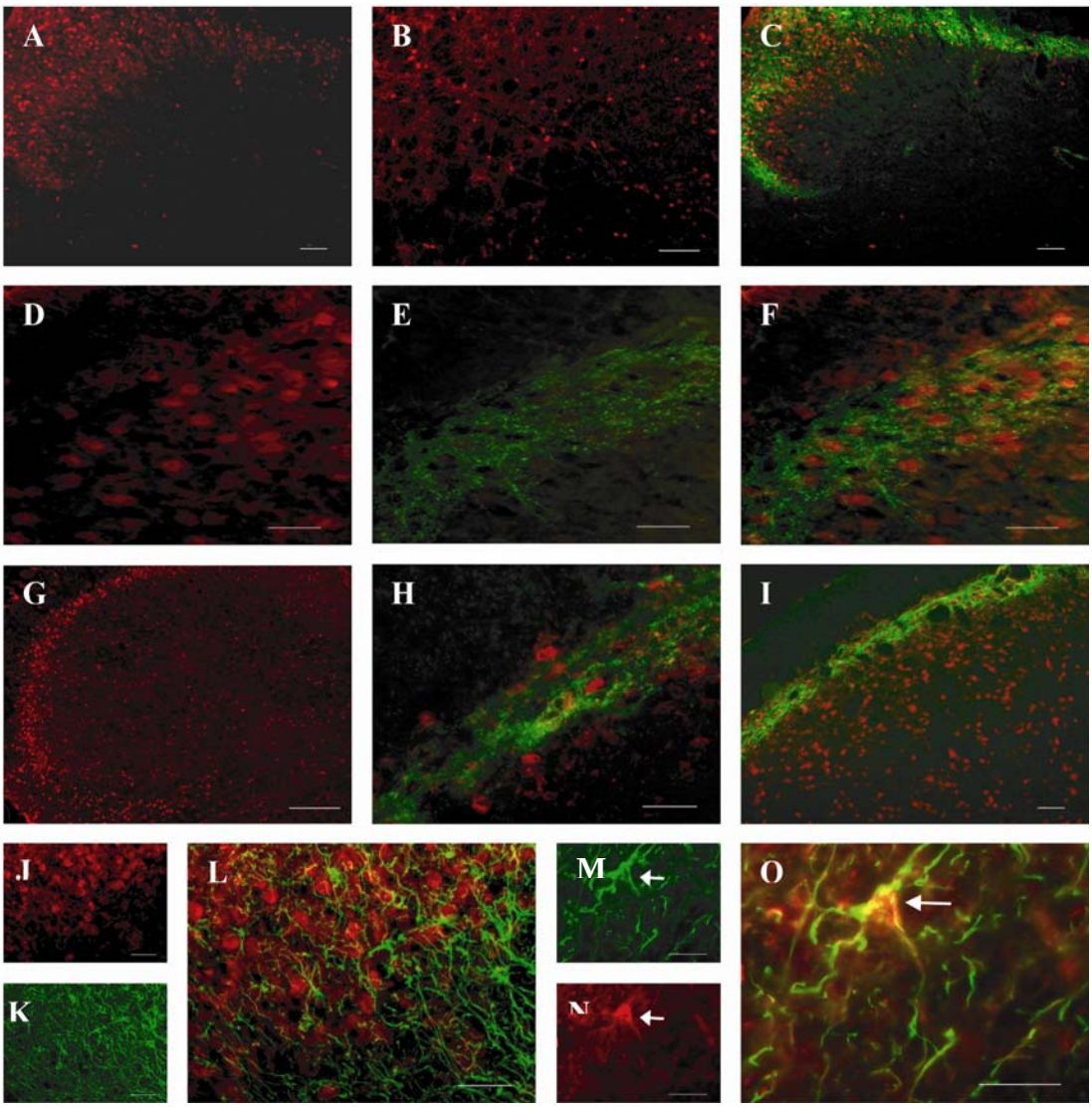


Figure 20: Presence of NMDA receptors in the trigeminocervical complex
Representative examples of the distribution of NR1-like NMDA receptors staining (labelled with texas red-avidin) in the dorsal region of the cervical spinal levels C2 (A, B, C), C1 (D, E, F) and trigeminal nucleus caudalis (TNC; G, H, I) are shown. Photomicrographs show NR1-like receptors in C2 (A) and a higher magnification indicates scattered cells in deeper laminae-IV-V of C2 (B). NR1 positive cells are localised in a close proximity to calcitonin gene-related peptide (CGRP; labelled with fluorescein isothiocyanate) fibres in C2 (C). High magnification photomicrographs show the intracellular aspect of NR1-like NMDA receptor in laminae I and II in C1 cervical level (D; note the punctuate appearance of the staining restricted to the cytoplasm in the soma), the CGRP-like staining in laminae I and outer lamina II, and their co-location in these laminae (E). Photomicrographs show NR1-like staining in the trigeminal nucleus caudalis (TNC; G) and their co-location with CGRP in the superficial laminae of the TNC (H). An example from TNC section stained for CGRP and NeuN (labelled with texas red; I), indicates that not all neurons express the NR1 protein. (J–O). Double labelling of NR1 and GFAP positive astrocytes in green. Only few astrocytes were shown to express NR1-like NMDA receptor (M–O; white arrows) Scale bars = 50 μm (D–F, H, J–O), 100 μm (A–C, I), 500 μm (G). (Andreou et al.; Unpublished data).

Additional evidence for a link between glutamate and migraine arises from human studies. Kaube *et al.* (2000) studied the effect of ketamine, a non-competitive antagonist at the NMDA receptor in patients with severe, disabling auras resulting from FHM, demonstrating efficacy in about 50% of the patients. ADX10059 is a potent, selective, negative allosteric modulator of the mGluR5 receptor and in a study for the acute treatment of migraine ADX10059 showed a statistically significant higher number of patients pain-free 2 hours after dosing compared to placebo (http://www.addexpharma.com/press-releases/news-releases/3-january-2008-adx10059/).

1.14 Proposed experiments

Migraine pathophysiology involves activation or the perception of activation of the trigeminovascular system. Glutamate, the major excitatory neurotransmitter in the CNS, is implicated in elements of the pathophysiology of the disorder, including trigeminovascular activation, central sensitization and cortical spreading depression. The pharmacology of glutamatergic trigeminovascular responses in brain areas involved in migraine pathophysiology is relevant to the development of new therapies for this disabling condition. The kainate receptor, a member of the iGluRs family, is present in key structures of the ascending trigeminovascular pathway, including the trigeminal ganglia, the TCC and the thalamus. Kainate receptors have been implicated in the transmission of pain perception and evidence suggests a potential role of these receptors at synapses with excess glutamate release. Kainate receptor acting antagonists were effective in treating headache pain in clinical trials with mild side effects.

The aim of the studies presented in this thesis was to investigate further the possible involvement of the iGluR5 kainate receptor in the pathophysiology of migraine, both at the peripheral site of the trigeminovascular system and at central synapses, and to investigate their role in varying aspects of the trigeminal nociceptive processing in animal models of trigeminovascular activation.

Chapter 2: Methods

2.1 Animals

Male Sprague-Dawley rats (n = 173), of weight 280-405 g were used in the experiments reported in this thesis. The animals were supplied by B&K Universal, UK and from Charles River Laboratories, USA. All animals were maintained on a 12-hour light/dark cycle, with food and water freely available in compliance with UK Home Office and Institutional Animal Care and Use Committee (IACUC) regulations. All experiments were conducted under a personal and project licence issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and in accordance with guidelines of the University of California, San Francisco IACUC.

2.2 Anaesthesia

In all experiments anaesthesia was induced with 60 $mgkg^{-1}$ intraperitoneal pentobarbitone sodium (Sigma-Aldrich Ltd., St. Louis, MO, U.S.A or Ovation Pahrmaceuticals, Inc., IL, USA) and maintained by intravenous injections of 10-20 $mgkg^{-1}$ α-chloralose (Sigma-Aldrich) in a solution of 2-hydroxy-β-cyclodextrin (Sigma-Aldrich) or infusion of pentobarbitone sodium (25-35 $mgkg^{-1}h^{-1}$) through the cannulated femoral vein, as previously reported in our group (Storer et al., 2004a; Holland et al., 2006). A sufficient depth of anaesthesia was judged from the absence of withdrawal reflexes, and gross fluctuations in blood pressure and respiratory parameters. When neuromuscular blocker (pancuronium bromide – 1 $mgkg^{-1}$ initially, maintenance with 0.4 $mgkg^{-1}$) was used, only fluctuations in blood pressure and respiration were used to asses the level of anaesthesia. All experiments were conducted under terminal anaesthesia and at the end of each experiment animals were given a lethal dose of pentobarbital sodium (Lethobarb®, 200 $mgml^{-1}$ – 1 ml, Fort Dodge Animal Health, Southampton, UK; or Euthasol, 600 $mgml^{-1}$ – 0.2 ml, Virbac AH, Texas, USA).

2.3 Surgery

All surgical procedures took place with the aid of a dissecting microscope (Opmi 99, Zeiss, UK).

2.3.1 Cannulation of blood vessels and trachea

The left femoral artery and vein were exposed and cannulated with polythene tubing (external diameter 0.96 mm, Smiths Medical International Ltd., Kent, UK) for blood pressure recordings and intravenous administration of anaesthetic and neuromuscular blocker. In those experiments where intravenous administration of test compounds was used, the right femoral vein was also cannulated. The cannula was secured with braided silk sutures and its patency was confirmed by a free backflow of blood and maintained by periodic flushes of isotonic saline. The trachea was exposed by blunt dissection and intubated with endotracheal polythene tubing (external diameter 2.7 mm) for artificial ventilation. Upon cannulation of the trachea and femoral vessels, rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA – Model 1600).

2.3.2 Animal Maintenance

Throughout the surgery and experimental observation the blood pressure was continuously monitored via the femoral arterial line which was connected to a saline filled transducer (Ohmeda DTX Plus pressure transducer with PM-1000 transducer amplifier from CWE Inc.) and maintained at 80-130 mmHg. Through the saline filled transducer animals received a constant rate of saline at 3 mlh^{-1}. Animals were artificially ventilated with oxygen-enriched air, 2-2.5 ml, 80 to 100 strokes per minute (Small Rodent Ventilator model 7025; Ugo Basile, Varese, Italy). The end-tidal CO_2 was monitored (Capstar-100; CWE Inc., Ardmore, PA) and kept between 3.5 and 4.5% by altering either the rate of respiration or stroke volume. Arterial blood gas parameters were measured (GEM Premiere 3000, Instrumentation Laboratory, UK) at regular intervals during the experiment and maintained within the normal physiological parameters: pH; 7.35–7.45, pCO_2; 4.5-6.0 pKa. Temperature was maintained with a rectal thermometer at 37.5 ºC via a homoeothermic blanket system (TC-1000 Temperature Controller; CWE). Outputs from the blood pressure transducer amplifier,

the CO_2 analyser, and the homeothermic blanket were connected to a data recorder (Vetter Analog, SDR Clinical Technology, Middle Cove, Australia) and then to an analogue-to-digital converter (Power 1401; CED, Cambridge, UK). Physiological parameters were displayed with an online data analysis system (Spike2 version 5; CED).

2.3.3 Cranial surgery for the intravital microscopy studies

Exposure of the middle meningeal artery

A midline incision of approximately 2.5 cm was made in the skin covering the dorsal surface of the skull and the skin and the right or left masseter muscle were retracted to expose the underlying parietal bone. A small cranial window, approximately 5 x 5 mm was drilled using a sharp-tipped dental drill (Volvere GX®, Nakanishi Dental, Japan), until the blood vessels of the dura mater were clearly visible through the intact thinned skull. The drilling was performed in the presence of cooling saline to prevent any damage due to overheating. Any bleeding from the skull was halted with bone wax (Ethicon; Livingston, UK) and the closed cranial window was bathed in warm mineral oil (Sigma-Aldrich) to prevent dehydration. Drilling was followed by an hour of rest period.

2.3.4 Cranial surgery and laminectomy for electrophysiological experiments in the trigeminocervical complex

Exposure of the middle meningeal artery

For the electrophysiological experiments an identical procedure as described in section 2.3.3 was followed. The final layer of the thinly drilled bone however was gently peeled away using forceps in order to expose the underlying MMA (figure 21). Prevention of dehydration of the exposed dura mater was also ensured by bath application of warm mineral oil (Sigma-Aldrich).

Laminectomy

Electrophysiological recordings were performed in the TCC at the level of the cervico-medullary junction and thus a C1 laminectomy was performed. The skin and muscles of the dorsal neck were incised and separated. Any bleeding was halted by gentle application of small cotton wool pellets (Richmond Dental, Charlotte, NC, USA). The bone of the first cervical vertebrae was exposed and partially drilled away on the ipsilateral side to the exposed MMA (figure 21). The drilling was performed in the presence of cooling saline to prevent any damage due to overheating. The atlanto-occipital membrane and underlying dura mater was incised (ophthalmic scissors, Fine Scientific Tools, UK) and removed to expose the underlying dorsal cervical spinal cord immediately before electrode placement.

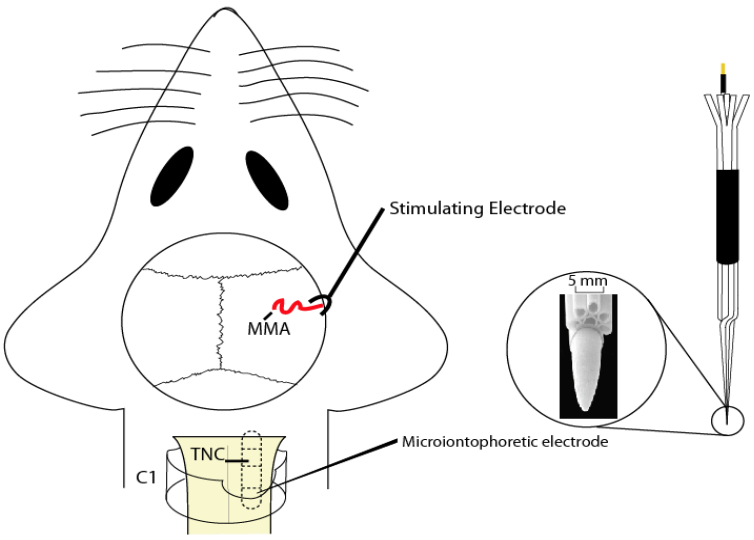


Figure 21: Surgical preparation and experimental setup for electrophysiological experiments in the trigeminocervical complex

MMA; middle meningeal artery, TNC; trigeminal nucleus caudalis, C1; cervical level 1

2.3.5 Cranial surgery for electrophysiological experiments in the ventroposteromedial thalamic nucleus

Exposure of superior sagittal sinus

The skin of the dorsal surface of the skull was divided and the parietal bone was exposed as described in section 2.3.3. A rectangular window (approximately 2 x 7 mm) was drilled along the midline suture and its margins were approximately 1 mm caudal and rostral of the bregma and lamda reference points (Paxinos et al., 1985). The bone was cooled by constant irrigation with isotonic saline during drilling to prevent heat damage to the underlying sinus. The rectangular section of bone was then gently lifted away from the intact dura exposing the superior sagittal sinus (SSS; figure 22). The site was covered with warm mineral oil.

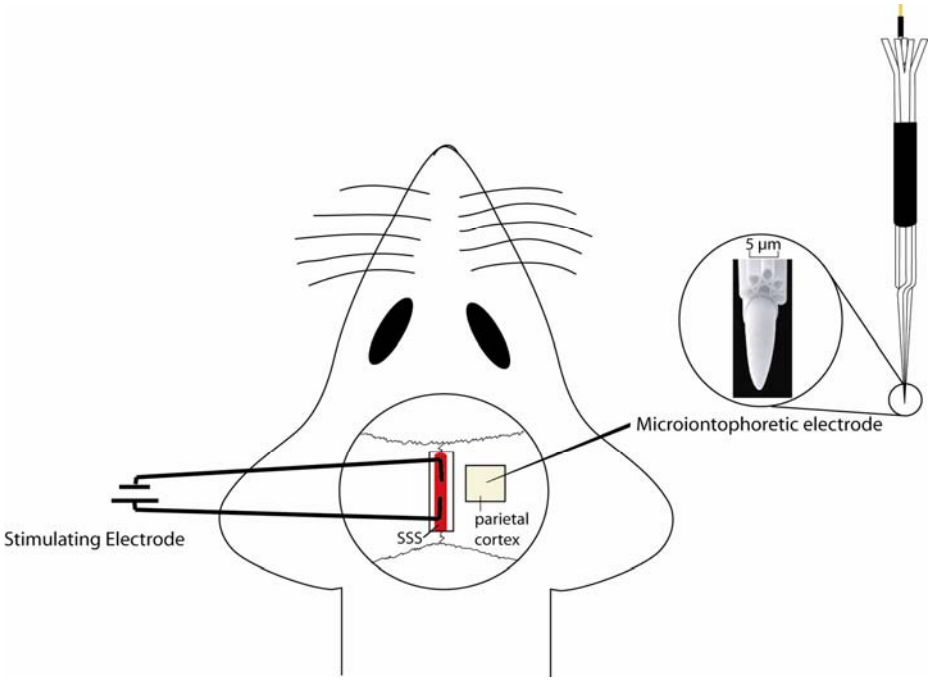


Figure 22: Surgical preparation and experimental setup for electrophysiological experiments in the ventroposteromedial thalamic nucleus
SSS; superior sagittal sinus

Exposure of the parietal cortex overlying the ventroposteromedial thalamic nucleus

A small window (approximately 2 x 2 mm) was drilled in the presence of cooling saline on the superior surface of the right parietal bone. Its rostral margin was approximately 2 mm caudal to bregma and its mediolateral margin was 2 mm from midline, thus approximately 1 mm lateral to the cranial bone along the exposed SSS (figure 21). This thin "bone wall" separating the SSS and the parietal cortex served as a "natural insulation" between the stimulating electrode placed later along the SSS and the cortex. The final layer of the thinned bone was gently lifted away from the intact dura mater exposing the parietal cortex (figure 22). Any bleeding was halted by gentle application of small cotton wool pellets. Isotonic saline was used to prevent dehydration instead of mineral oil, as the later could block the iontophoretic electrode (section 2.5.1) upon contact. The dura mater was carefully incised and retracted to expose the underlying parietal cortex just before the microiontophoretic recording electrode was lowered into the thalamus.

2.4 Placement of stimulating electrodes and stimulating parameters

2.4.1 Placement of stimulating electrode for the intravital microscopy studies

To study neurogenic vasodilation (see section 3.1), a platinum bipolar stimulating electrode (NE-200x, Clark Electromedical Instruments, UK) was placed on the surface of the thinned cranial window, approximately 200 μm from a branch of the MMA using a micromanipulator (Narishige, Japan). The electrode was positioned carefully so that it was neither over the MMA or depressing the skull, thus preventing any blood flow impairment. The electrode was connected to a Grass S88 stimulator (Grass Instrumentation, West Warwick, RI, USA) and the surface of the cranial window was stimulated for 10 s using electrical square wave stimuli at a frequency of 5 Hz, duration of each pulse 1 ms (figure 23), with increasing voltage until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using that voltage (Williamson et al., 1997a; Akerman et al., 2002c).

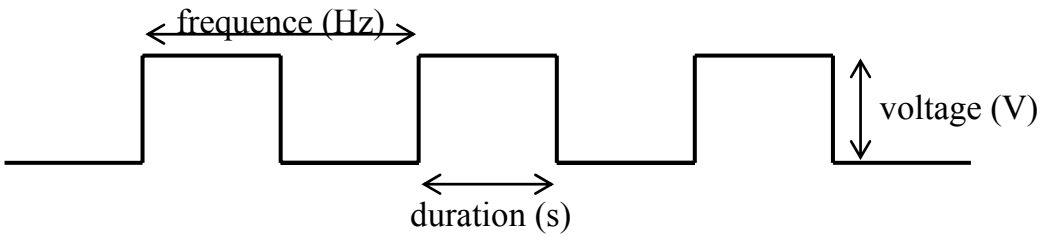


Figure 23: Square wave electrical pulses and stimulating parameters

2.4.2 Electrode placement and stimulation of the middle meningeal artery

A platinum bipolar stimulating electrode was placed on the dura mater straddling the MMA using a micromanipulator. Square-wave stimuli (0.5 Hz) of 100 μs duration (S88 stimulator) was applied to activate trigeminal afferents. To allow optimal recordings from neurons in the TCC the stimulus intensity (voltage) was adjusted down to just supra-maximal levels, so as not to over stimulate the neuron, and varied from 10 to 16 V.

2.4.3 Electrode placement and stimulation of the superior sagittal sinus

Two platinum wire stimulating electrodes, with horizontally levelled tips of approximately 2 mm length each, were placed onto the SSS using a micromanipulator (David Kopf). Every effort was taken to minimise contact between the cortex and stimulating electrodes and fresh insulating mineral oil was applied. Trigeminovascular afferents were activated by stimulating the SSS with square-wave pulses. Supra-maximal stimulating voltages were initially used but once a suitable unit was identified this was reduced to threshold levels to reduce the risk of current spread to the cortex. The stimulating parameters used in these experiments were: 0.5 Hz , 100 - 250 μs, 12 - 30 V.

2.5 Preparation and placement of recording electrodes

2.5.1 Microiontophoretic electrode filling

In all electrophysiological studies carbon-fibre seven-barrelled microiontophoretic electrodes were used (Carbostar 7S®; Kation Scientific, Minneapolis, U.S.A.). These consisted of a seven-barrelled glass pipette incorporating a carbon fibre recording electrode (Impedance @ 1 KHz: 0.4 - 0.8 MΩ) (Gottschaldt et al., 1988), with an exposed tip length of 15 - 20 μm and diameter of 2-3 μm (figure 24). The general condition of an electrode tip was checked by careful microscopic examination before filling. To avoid blocking of the tip of the micropipette all solutions were filtered (Anotop 10®, 0.02 μm inorganic membrane filter, Whatman Int. Ltd., UK) to remove particulates. Barrels were filled at least one hour prior to use with non-metallic syringe needles (Microfil® 34G, WPI, Sarasota, USA). Internal glass filaments within the iontophoresis barrels allowed self filling of aqueous solutions. The proximal ends were covered (Parafilm M®, American National Can, Chicago, USA) to reduce the risk of retrograde tracking of solutions and contamination of barrels prior to use, as well as the formation of salt bridges between barrels (Shi 1990). After ensuring complete filling under a microscope (Opmi 99), the electrode was lowered 1 mm into a saline bath in order to avoid microcrystal formation at the tip. The electrical impedance of the filled barrels was then measured (Impedance Check, FHC, ME, USA) and the resulted impedance values depended on the solution used (table 12).

Table 12: The impedance of various barrels containing different solutions measured at 1 KHz to outward currents

Solutions filling the barrels	Impedance (MΩ)
Electrolyte solutions	10-15
Amino acid solutions	20-40
Amine or alkaline solutions	50-100

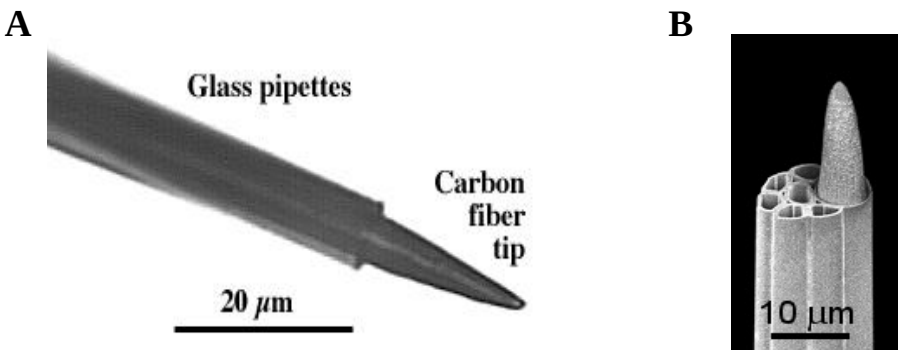


Figure 24: Light and scanning microscopic images of the carbostar-7S electrode

A. Light microscopic image showing the carbon fibre tip protruding from the glass shank of 6 fused-together iontophoresis barrels. The stiffness of the carbon fibre provides good mechanical stability for the electrode and the sharp tip allows easy tissue penetration. B. Ultrastructure of the carbostar-7S electrode as revealed by scanning electron microscopy. The fused-together glass micropipettes form a seal around the protruding carbon fibre.

2.5.2 Placement of electrodes in the trigeminocervical complex

Electrodes were fitted to a hydraulic microdrive (Model 650 Micropositioner**,** Burleigh) mounted on a micromanipulator, which was in such rotation to form approximately 90° angle with the exposed spinal cord. Upon the connection of the electrodes to a head-stage amplifier and an iontophoretic pump (section 2.6), they were lowered into the region of the cervico-medullary junction at 5 μm increments. A small current of approximately 20 nA was passed through the barrels as the electrode was lowered to reduce the risk of barrel blocking. At a depth of 100 μm, the iontophoretic current was switched off and the electrode was left for 20 minutes at rest to allow normalization of the equilibrium at the tip of each barrel. The microdrive was then used to advance the electrode in 5 μm steps while searching for trigeminovascular nociceptive responsive neurons within the TCC. Throughout electrophysiological recordings the frame was rested on a vibration free air table (TMC, Peabody, MA, USA).

2.5.3 Stereotaxis and identification of the ventroposteromedial thalamic nucleus

Electrodes were fitted to a hydraulic microdrive mounted on a micromanipulator, which was positioned exactly 90° to the horizontal axis and then lowered into the region of the VPM according to the stereotaxic co-ordinates of Paxinos and Watson (1997). The coordinates used were: 3.12-3.5 mm caudal to bregma, 3.3-3.6 mm from midline and 5-5.2 mm dorsal to the parietal cortex. As before, a small current of approximately 20 nA was passed through one of the barrels as the electrode was lowered into the brain (using the micromanipulator). At this depth receptive fields on the head and face were tested as the electrode was slowly advanced using the micromanipulator. When it became apparent that the electrode was in the VPM due to the responses to stimulation of the facial receptive field, a rest period of approximately 20 minutes was allowed. The microdrive was then used to advance the electrode in 5 μm steps while searching for trigeminovascular nociceptive responsive neurons within the VPM nucleus.

2.6 Equipment and settings

2.6.1 Electrophysiological recordings

The signal from the recording electrode was fed via a head-stage amplifier (NL100AK, Neurolog, Digitimer, Herts, U.K) to an AC preamplifier (gain x1000; Neurolog NL104A, Digitimer, UK). The head-stage amplifier was grounded to the animal by a reference silver electrode inserted either into the dorsal neck muscles or subcutaneously. The signal was then filtered through Neurolog filters (bandwidth approximately 500 Hz - 10 kHz; Neurolog NL125) and next through a 50/60 Hz noise eliminator (Humbug, Quest Scientific, North Vancouver, BC, Canada). The filtered signal was then further amplified through a second stage variable amplifier (gain approximately x50 - 90; Neurolog NL106), from which the output was split in two. One output was fed to a gated amplitude discriminator (Neurolog NL201), then displayed on an oscilloscope (OS 7020A, Goldstar Precision Co., Korea) and an audio amplifier (Neurolog NL120) to assist with the discrimination of single unit activity from background noise. The second output was fed to an analogue-to-digital converter (Power 1401), and then to a personal computer (Dell Computer Corporation, Berks, UK) running Spike® 2, version

5 software (Cambridge Electronic Design) which was used to collect and analyse data (figure 25). Outputs from the microiontophoresis current generator (Dagan 6400, Dagan Corporation, MN, U.S.A.) and the Grass stimulator were also connected to the analogue-to-digital converter and then displayed on the computer running the Spike software. A second oscilloscope was used to display the captured signal upon stimulation with the Grass stimulator. The stereotaxic frame, as well as the table on which it was mounted, were grounded to the earth of the mains electrical supply.

Equipment and settings used for microiontophoresis in electrophysiological experiments

A microiontophoresis current generator provided ejection and retention currents (Bloom, 1974). Barrels were connected to the current generator (Dagan 6400, Dagan Corporation, MN, U.S.A.) through a head-stage by individual teflon coated silver wires (0.25 mm, WPI). The distal end of the wire – which was placed in the drug solution – was stripped of 1 cm of coating. Care was taken to avoid damage to the teflon coat while inserting wires into the barrels. The head-stage was grounded to the stereotaxic frame. The polarity of the ejecting and retaining current was then set on the microiontophoresis pump and a retaining current of 5 nA was applied in each barrel. Upon placement of the electrode into the neuronal tissue the resistance of each barrel was measured and values of between 16 and 120 MΩ were recorded.

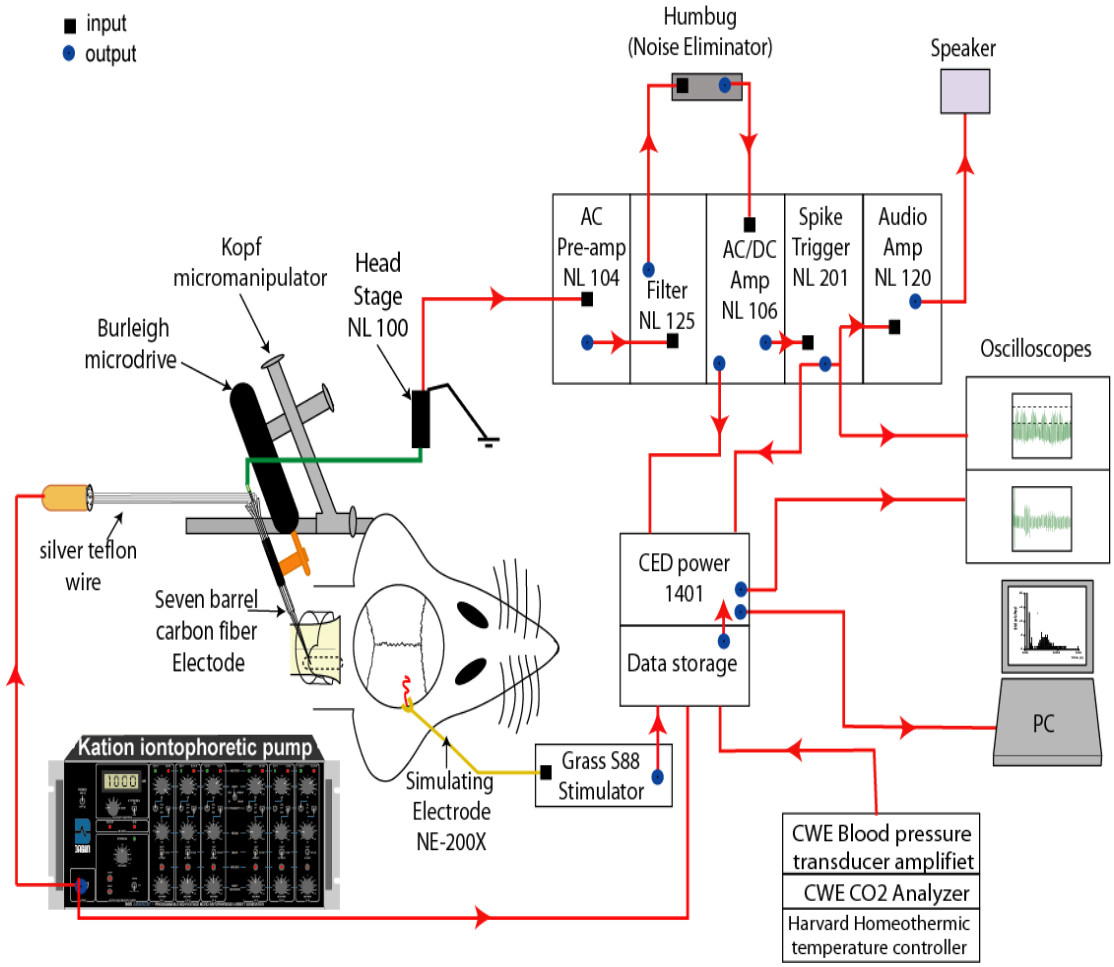


Figure 25: Schematic diagram of the preparation and equipment used in the electrophysiological experiments

A recording electrode is lowered into the trigeminocervical complex, and the electrical signal is fed through a series of amplifiers and filters to a pair of oscilloscopes and a personal computer for analysis. A bipolar stimulating electrode is placed on the middle meningeal artery and connected to a Grass S88 stimulator. Silver teflon wire was used to connect the iontophoretic pump with the microiontophoretic electrode. The same setup was used for electrophysiological recordings in the ventroposteromedial thalamic nucleus.

NL, Digitimer neurolog product number; NE, Clark Electromedical product; CWE, Instruments for physiology and respiration; CED, Cambridge Electronic Design products

2.6.2 Intravital microscopy experiments

A branch of the middle meningeal artery was viewed through the thinly drilled cranium with an intravital microscope (Microvision MV2100; Finlay Microvision, Warwickshire, UK), and the image displayed on a monitor. The vessel diameter was constantly measured using a video dimension analyser (Living Systems Instrumentation, Burlington, VT) and displayed on an online data analysis system (Spike2v5 software) To measure the current passing through the cranial window during stimulations, the stimulating electrode was connected through a resistor (1 KΩ) to an oscilloscope (Goldstar Precision), which displayed the passing current through the electrode (figure 26).

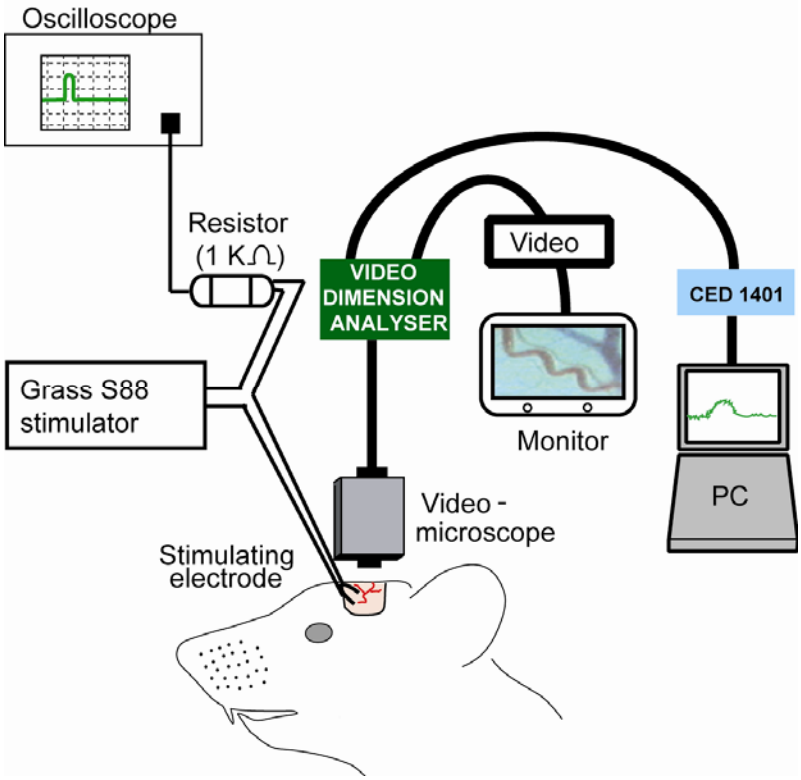


Figure 26: Overview of the intravital set-up in the rat
The magnified image of the dural artery is displayed on a monitor and recorded. The diameter of the blood vessel is constantly measured via the video dimension analyser. A second output from the video dimension analyser passes to the CED 1401, which converts the signal to enable on-line analysis via the Spike software on the computer. The stimulating electrode was connected in parallel through a resistor (1 KΩ) to an oscilloscope, which displayed the current passing though the electrode.

2.7 Principles and limitations of intravital microscopy

Intravital microscopy is a well developed animal model relevant to migraine (Bergerot et al., 2006), which permits the direct study of the peripheral end of the trigeminovascular system (also see section 3.1). The model was developed by Williamson and colleagues and utilises video microscopy to visualise dural blood vessels and a video dimension analyser to measure directly blood vessel diameter through a thinned closed cranial window (Williamson et al., 1997a; Williamson et al., 1997b). Electrical stimulation of the cranial window causes reproducible dural and pial blood vessel dilation via activation of the trigeminal nerve, which is thought to be via the release of CGRP from presynaptic trigeminal nerve endings (neurogenic dural vasodilation; section 3.1) (Williamson et al., 1997a; Akerman et al., 2002b). This method is suited to studying the peripheral component of the trigeminovascular pharmacological system and mainly uses systemic administration of potential anti-migraine compounds. Intravital microscopy has proved highly predictive of anti-migraine efficacy, although not all anti-migraine drugs are useful in this model (Akerman et al., 2001; Akerman et al., 2002a; Akerman et al., 2003b). As the method does not investigate the central component of the trigeminovascular system, the efficacy of many anti-migraine compounds that act centrally may not be seen using this model. In addition, any minor changes at the peripheral site of the trigeminal nerve might be masked by the intense electrical stimulation that is used to drive CGRP release and subsequent vasodilation.

2.8 Principles and limitations of microiontophoresis in electrophysiological experiments

The following summary has been adapted from published work (Curtis, 1964; Bloom, 1974; Stone, 1985).

Microiontophoresis utilises the flow of electric charge through an aqueous solution which contains an ionised compound to eject drugs with a high degree of anatomical precision. The solution which fills each barrel of the microiontophoretic electrode is connected to the iontophoresis machine by a suitable lead. Establishing a potential difference between a drug solution and the external medium surrounding the barrel tip causes the movement of ions through the solution resulting in drug ejection. By convention the direction of current flow is taken to indicate the flow of positive charge – outward and inward refer to the transport of positive charges out of or into a micropipette, respectively. If an outward current is applied to a solution containing positively charged drug molecules (cations), the cationic (+) molecules will flow in the same direction as the current because of electrostatic forces. Alternatively if an inward current is applied to the same solution, cations will be retained in the barrel, while anions (-) will move in the opposite direction (figure 27). The use of a multibarrelled electrode and a multichannel iontophoretic generator allows a number of different drugs to be released near the same neuron and, in addition, barrels for control and a dye for marking the recording site can be used in the same experiment. The iontophoretic technique is suitable for applying chemical substances close to receptors upon neurons within the nervous system.

This technique will only work with compounds that are both water soluble and ionisable. In the case of compounds that are available as salts and readily dissociate in solution iontophoretic ejection is easily applied. Although many of the biologically active substances are in the ionic form, some of them may not be dissolved in aqueous solutions. With more complex organic molecules it is often necessary to add small quantities of acid or alkali to the solution. This exploits the presence of specific proton donor or acceptor moieties on the parent molecule - for example if a compound has an amine side-group ($-NH_2$), this basic moiety will act as a proton-acceptor in an acidic environment. It is then protonated forming a charged amide ($-NH_3^+$) side-group - rendering the whole molecule more amenable to microiontophoretic ejection (and also more water soluble). Adjusting of the pH however might affect the compound's

stability. Additionally, for any substance the individual electrical charge carried by the molecule must be present at a pH that does not cause significant biological effects on neurons' excitability. For example, topiramate is fully polarised as an anion in an aqueous solution at pH 12. Ejection of control (H_2O, OH^-) however at this pH results in increase in firing rate (figure 28).

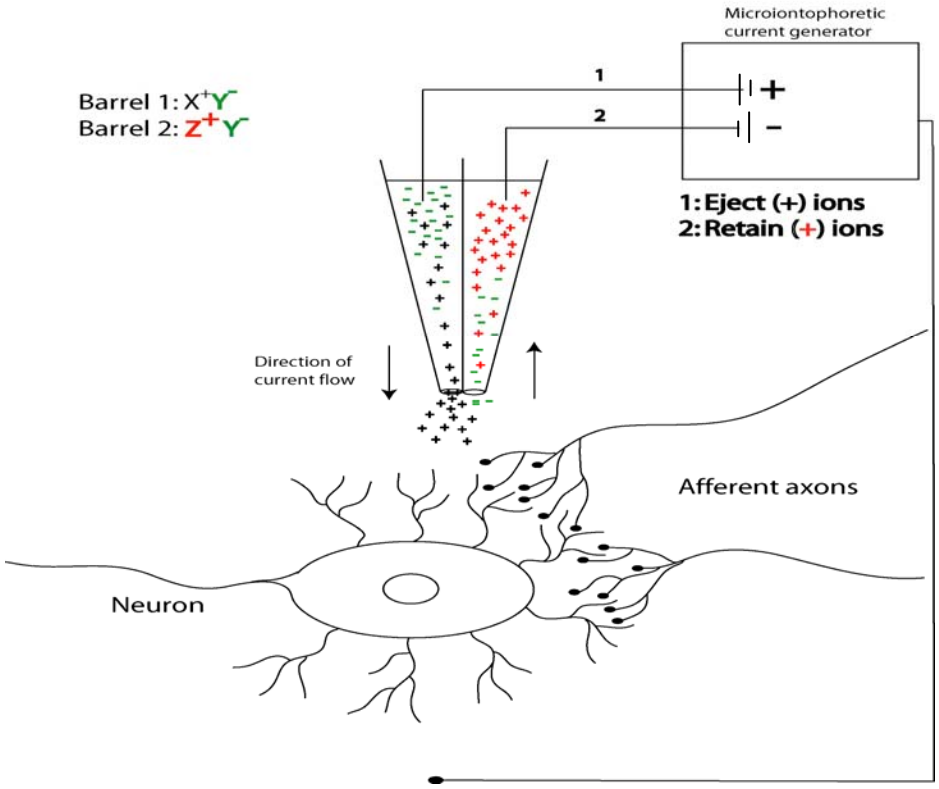


Figure 27: The principles of microiontophoresis

Two micropipette barrels are shown, each containing an ionic solution. The tips of both barrels are in close proximity to the cell body of the neuron under study. In barrel 1, X^+ ions are being ejected by the outward flow of current (10-100 nA) (current is by convention taken to mean the flow of (+) charge), while Y^- anions are retained. In barrel 2, Z^+ ions are retained by a smaller (-5 nA) retaining current flowing in the opposite direction. This retaining current however results in the ejection of Y^- anions from the barrel. It is assumed that this has a negligible biological effect, due to the small retaining current used.

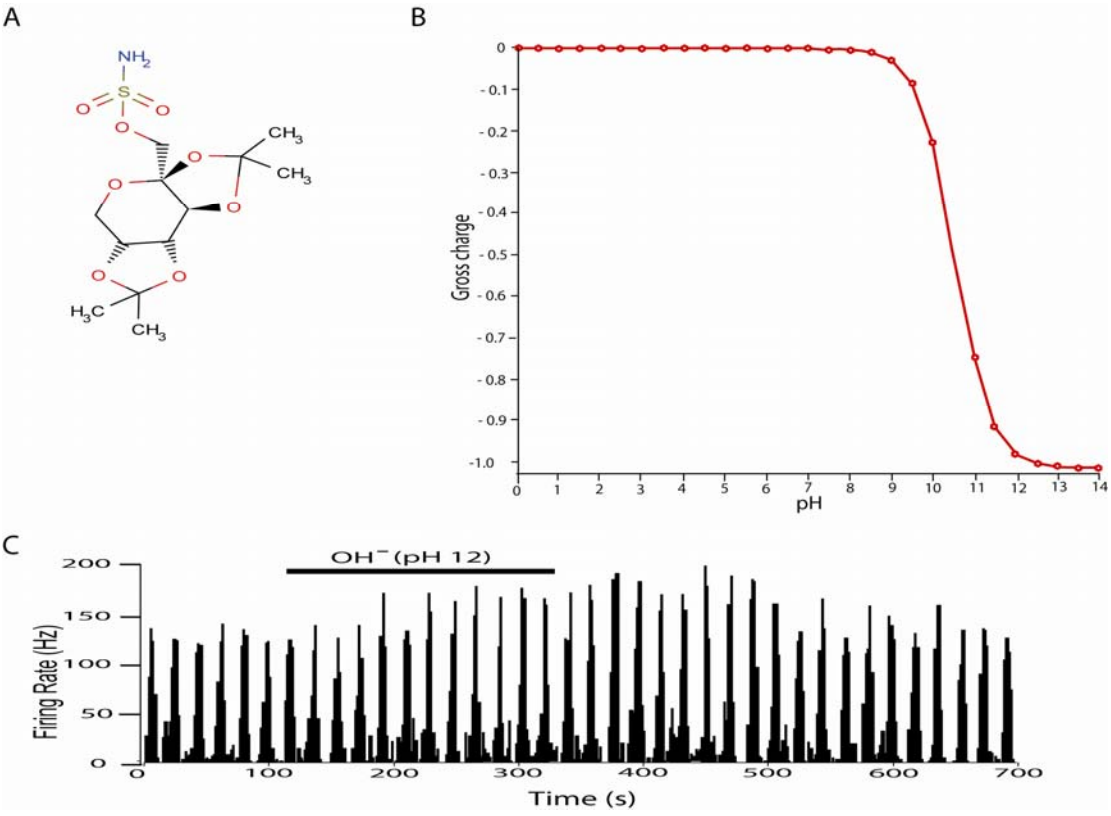


Figure 28: Effect of pH on the electrical charge carried by topiramate and pH effects

A. Topiramate's chemical structure. B. Graph illustrating the gross charge of topiramate as function of pH. C. Effect of control H_2O (OH^-) at pH 12 on L-glutamate-evoked activity.

When no active iontophoretic ejection of molecules is used, drug efflux could naturally occur from the tip of the micropipette into the surrounding environment and thus small amounts of the molecules may diffuse down a concentration gradient. This effect is minimized by application of a small current of the opposite polarity to the ejecting current and is known as the retaining or holding current (figure 27). Though these currents will retain the desired molecule, they will result in the ejection of oppositely charged ions in the solution. As individual retaining currents are small (no more than 5-10 nA) it is assumed that this ejection has minimal biological effects. In the iontophoretic current generator this is achieved by utilising a second battery in parallel

to the first having a reverse polarity, providing a retaining and ejecting voltage (figure 29).

When multi-barrelled pipettes are used however, neurons are exposed to retaining and ejecting currents from several barrels and the sum of these currents may influence the excitability of the neuron. In an attempt to counter this, a barrel containing 200 mM NaCl is used for "current balancing". A current is continuously applied to the balance barrel and this current is equal in magnitude to the algebraic sum of all currents being passed through the drug-containing barrels, but of opposite signs (figure 30).

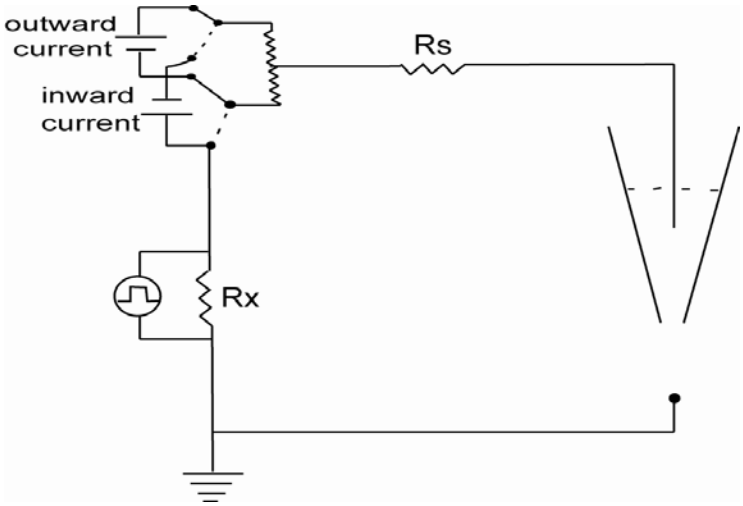


Figure 29: Iontophoretic circuit permitting ejecting and retaining current
The circuit includes a crossover facility which allows through a switch retaining as well as ejecting currents. A series of resistors Rs is also included to minimize the effects of fluctuation of electrode resistance and a current monitoring device is placed across a small series resistor Rx. Adapted from (Stone, 1985)

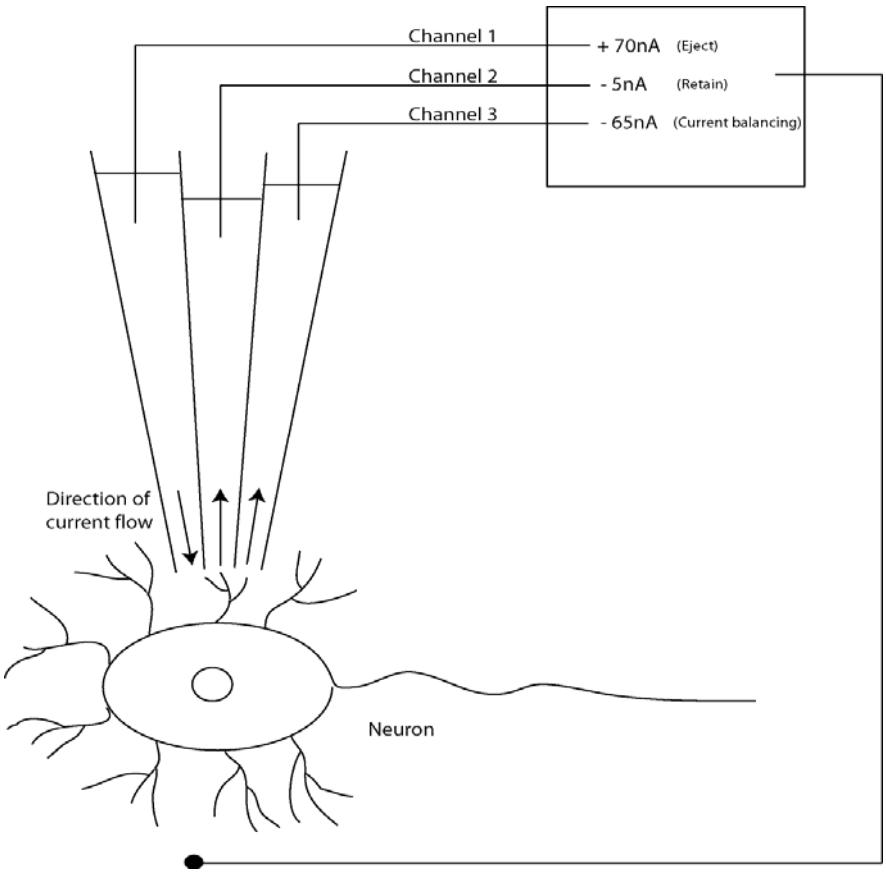


Figure 30: The principle of "current balancing".
In this schematic diagram barrels 1 and 2 are being used for drug ejection and barrel 3 for current balancing. The direction and magnitude of the current in barrel 3 (-65 nA) is adjusted automatically to counter the net current flowing from the other barrels (+65 nA). Theoretically the neuron under study should not be influenced by current flow.

'Osmotic artefact' is a phenomenon that may occur with the bulk flow of the solution which carries the active ion, in case the solution in the pipette is present at a significantly lower concentration than the biological fluids, which are in contact with the tip. To minimise this phenomenon iso-osmotic ionised compounds are usually used. The compounds are at a concentration of 150-200 mM, since the solution which is iso-osmolar with body fluids is approximately 165 mM. If the substances are not sufficiently soluble, a solution of sodium chloride is normally used so that the total solute concentration is approximately 165 mM. Despite the concentration of the solution within each barrel, the amount of ions ejected for a given current is

independent of the concentration (see section 2.8.1). Additionally, drug leakage could be also caused by flow of fluid due to the hydrostatic pressure of the column of the solution within the barrel. This is minimized by using micropipettes with reduced tip diameter of 2-3 μm.

As discussed above, if a microiontophoretic electrode is located extracellularly very close to a neuronal membrane, the passage of current through a barrel to ground and the solution's concentration may be sufficient to cause a change of neuronal excitability. Thus it is important to use a control to estimate the effect of these factors. This contains a suitable ionic solution. This is ejected with a current of the same direction and magnitude as the drug under study. As the pH of the drug solutions may be adjusted by the addition of H^+ or OH^- ions, and these will also be ejected with the drug, ejection of H^+ or OH^- ions may directly affect neuronal excitability. Changes in pH also affect ionotropic glutamate receptors. For example, protons inhibit most native and recombinant kainate receptors except from iGluR6/KA1 heteromers (Mott et al., 2003). This must be taken into account in any studies and thus the pH of the control should be the same as the test compounds.

One of the great strengths of microiontophoresis is the anatomical precision that it provides in pharmacological studies. It allows drugs to be delivered to small numbers of – or even individual – cells. The relatively short length of the recording electrode tip has important electrophysiological properties. Short electrode tips are superior at discriminating the electrical activity of individual neurons. As it is unlikely that the ejected drugs diffuse over large distances, it can be assumed that the drug is present in a confined area and that the electrode is recording electrical activity from this same area. It has been calculated that the extracellular action potentials of a single neuron can be recorded within a distance of approximately 50 μm of the cell body, depending on the size of the soma and of the dendritic field and the type of cell (Mountcastle et al., 1957). Further to that the distance from which the drug is being applied cannot exceed 40-60 μm (Curtis, 1964). The relative ease with which the diffusion and the ejection of substances are controlled, aids the application of drugs upon localised areas, while mechanical displacement and tissue damage remains minimal by the extremely small volume of solvent which is ejected. It is reasonable to assume therefore that any alteration in the excitability of a neuron is due to the effects of the ejected drug on that cell. When drugs are administered systemically, such as intravenously or intrathecally,

it is not possible to localise their effects. This is particularly a problem in thalamic studies, as the drugs may have actions at more distal sites, such as the terminals of primary trigeminal nerves or in the TCC.

2.8.1 Quantifying the microiontophoretic drug ejection and diffusion after ejection

One of the difficulties of microiontophoresis is to calculate the quantity of the molecule that gets ejected. The number of ions ejected from a solution in a micropipette is proportionate to the charge flux through the solution. In its most simple form, the molar flux (Q) of an ion produced by an ejection current is:

Q = It / FZ

Q: molar flux (number of ions ejected from a solution)
I: current
t: transport number (the ability of an ion to carry charge)
F: Faraday constant (96485 coulomb/mole)
Z: valency of the ion (the number of electrons that an atom donates or accepts to form the duplet state or octet state)

Several other factors, however, influence the molar flux including the molecular weight (M) of the ion. The total flux of ions produced by a given quantity of charge is proportionate to M – as the molecular weight of a molecule increases, less will be ejected per unit of charge. The transport number (t) of any particular ion in a solution is the fraction of the applied current carried by that ion. Unfortunately the value of t for a drug may vary dramatically between micropipettes and even different barrels of the same multi-barrelled pipette. It is affected by a diverse range of factors including: the age of the micropipette, the pH of the solution, the concentration of small ions (such as Na^+ and Cl^-) in the solution, electrostatic interactions between the ion and the glass wall of the pipette and the nature of the receiving medium. The value of t can only be estimated for a particular compound and even if it can be given a value, calculating the molar flux is further complicated by drug ejection by non-microiontophoretic means. These include drug ejection by electro-osmosis and hydration effects (section 2.8.2).

What is important to note is that the solutions concentration does not influence the molar flux and thus the concentration of the compound into the barrel will have no effect on its ejecting potency. From the definition of transport number from the above

equation, a change of the drug concentration does not affect the amount of compound ejected by iontophoretic current, as there is no term involving the solution's concentration in the equation. This is because the assumption is often made that all the charge passing through the micropipette tip is carried by the drug ion. It has been further calculated that for concentrations between 20 mM and 1 M, as well as for a current above 2 nA (barrel resistance up to 100 MΩ) the amount of drug ejected is linear of to the ejecting current (Purves, 1981). Some deviations might however appear in practice. For example when a solution contains Na^+ and Cl^- ions, these will also carry some of the ejecting current. The same occurs when the pH is adjacent since H^+ and OH^- ions will also carry some of the charge. Another factor that may contribute to iontophoretic ejection is the absorption of molecules to the glass wall within the micropipette, due to ionic charges. This is more obvious when passing current through a peptide solution (positively charged) which is in a low concentration (3 mM); and can be avoided by using higher concentration. Since concentration plays no role on the actual amount of ion ejected with microiontophoresis, it is better to use iso-osmotic solutions in order to avoid osmotic artefacts.

As can be seen it is very difficult to quantify the amount of drug ejected by this technique. The concentration of a drug that any given neuron is exposed to cannot be known. One partial exception is when a micropipette is used to study the effects of a drug on the same cell. In this case all variables should remain constant and the quantity of drug ejected will be proportionate to the ejecting current. Experiments thus provide qualitative information concerning the receptors and ion channels involved in modulating trigeminovascular nociceptive neurotransmission**.**

Upon ejection of a compound, any further movement of the ejected molecules is dependent on diffusion. That is because the electrical force that drives microiontophoretic ejection is limited to the micropipette barrel and the immediate environment of its tip. According to Ohm's Law (R = V/I), the flow of electrical current (I) requires a potential difference (V) between two points in a circuit. As the electrical resistance (R) of the external medium – in this case nervous tissue – is generally substantially less than the internal environment of the micropipette, only a small potential gradient exists beyond the micropipette tip and current will therefore not flow. The tip may be considered to act as a point source of the drug and any further movement is dependent on diffusion. Diffusion constants differ between molecules and

the conditions determining the extent of diffusion will differ between experiments – even for the same molecule. This introduces a further variable in any attempt to quantify the results of drug studies.

2.8.2 Drug ejection by electro-osmosis and hydration effects

Electro-osmosis is essentially the opposite of microiontophoresis. When an aqueous solution is in contact with a glass surface anions are tightly adsorbed onto the surface of the glass. A fixed layer of negative charge - an electrical "double layer" – is formed on the glass surface leaving the bulk of the fluid volume carrying a positive charge. The passage of current therefore causes the flow of the fluid volume in the direction of the current. The dissolved compound (especially if (+) charged) then moves with the fluid. This obviously favours compounds that are positively charged – it may even counter microiontophoretic ejection of anions. Electro-osmosis may contribute significantly to drug ejection from a micropipette – especially in dilute solutions or when the drug is poorly ionised (figure 31).

Hydration effects also influence the ejection of a compound from a micropipette. Water molecules are electrostatically attracted to any ion in aqueous solution. They form a hydration shell surrounding the ion. A drug – even one that is poorly ionised – that is soluble in water will be dissolved in this hydration cell. If an electric current flows through the solution the ion will move under its influence. As the ion migrates so does its hydration shell - and any drug dissolved within it. Microiontophoresis in this case is only indirectly responsible for drug ejection (figure 31).

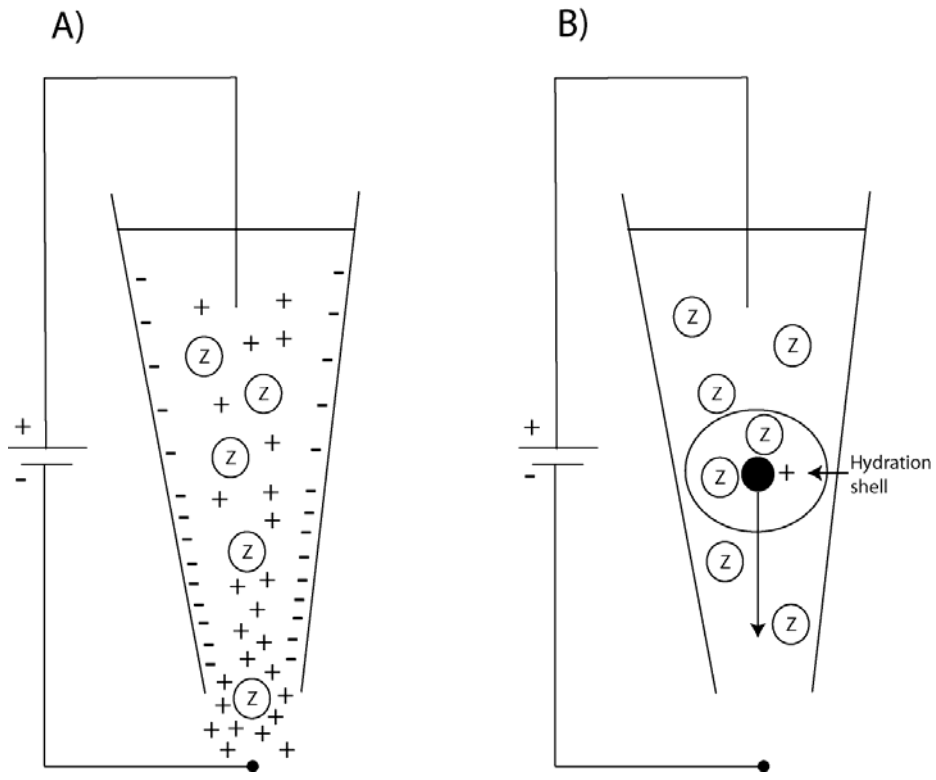


Figure 31: Principles of drug ejection by electro-osmosis and hydration

A. In electro-osmosis anions are adsorbed onto the surface of the glass micropipette. The solution containing the drug molecule Z has a net positive charge. The fluid then moves en-mass in the direction of the current (in this case outward) carrying molecules of Z with it. If Z is positively charged electro-osmosis will enhance its ejection – the opposite may occur if Z is an anion. B. Hydration effects may also result in drug ejection. A shell of water molecules surrounds ions in solution. Molecules of a drug, Z, may become trapped in this shell and be ejected along with the ions. Adapted from (Stone, 1985).

2.9 Drugs used in electrophysiological studies

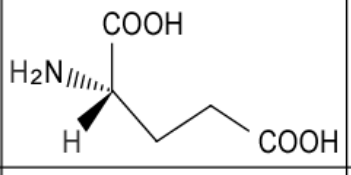
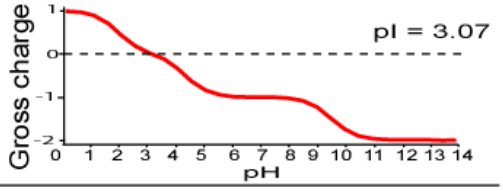
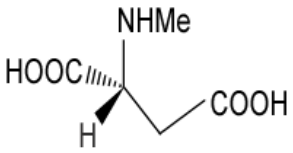
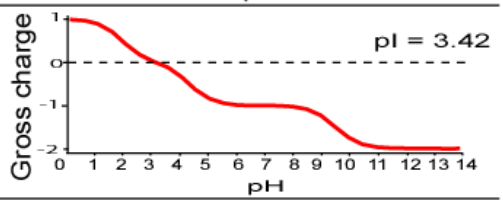
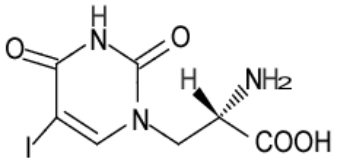
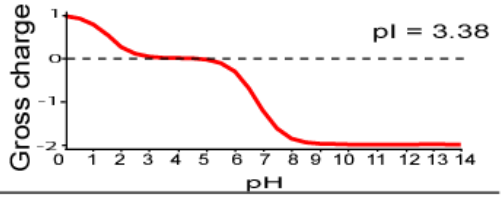
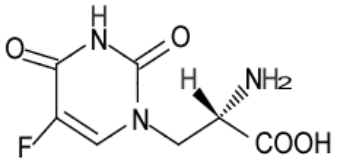
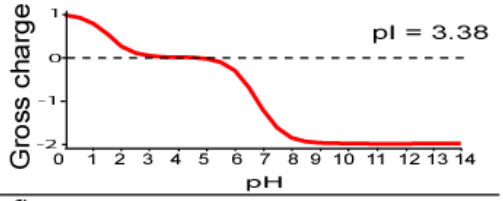
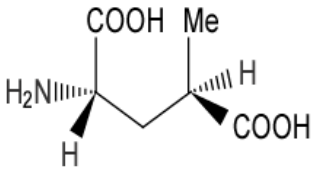
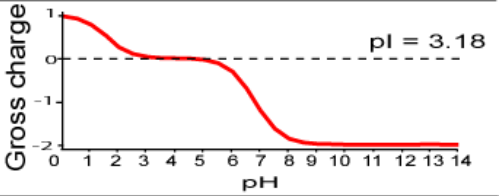
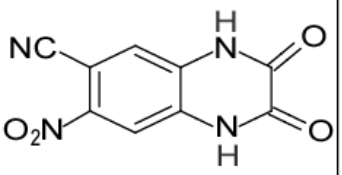
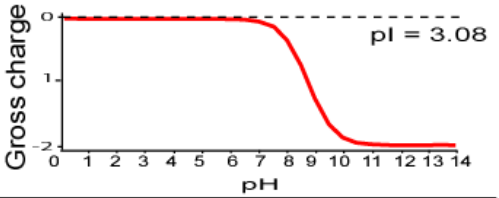
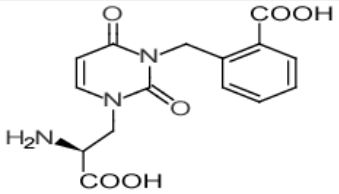
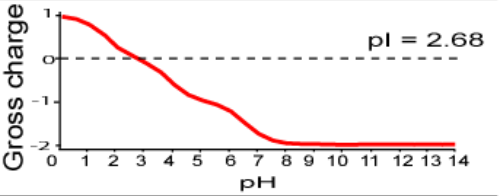
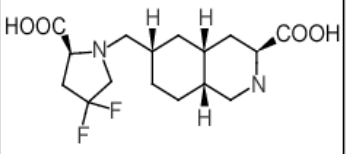
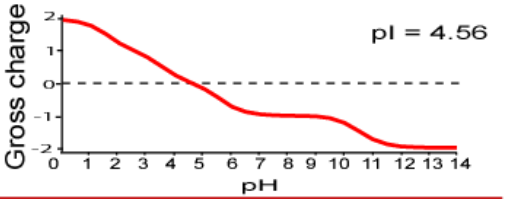
A detailed description of the drugs used in all studies is provided below. The specific drugs used in each study will be discussed in each chapter separately.

2.9.1 Drugs used by microiontophoresis

All active drugs used by iontophoresis were dissolved either in sterile deionised water or 100 mM of NaCl. Pontamine Sky Blue which was used for marking the site of recordings was dissolved in 100 mM sodium acetate (pH 6.5). The pH was adjusted by adding small amounts of 100 mM NaOH to the solutions, thus increasing their ionisation, making them more amenable to microiontophoretic ejection and in some cases also improving their solubility. The isoelectric point (pI; the pH value at which the molecule carries no charge) and the gross charge of molecules along the pH range was estimated with the Marvin version 5 software (ChemAxon Ltd, Budapest, Hungary). The drugs used in the microiontophoretic studies are listed in table 13 and the gross charge of the molecules along the pH range is given. The barrel concentration, pH and ejection polarity for each compound as used in these experiments are given in table 14. When selecting a pH which confers an adequate charge upon a particular drug, the dissociation constants and the stability of the compound were considered, together with the possibility that direct pharmacological effects may be produced by H^+ or OH^- ions.

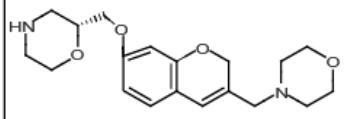
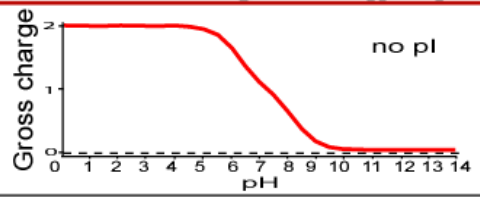
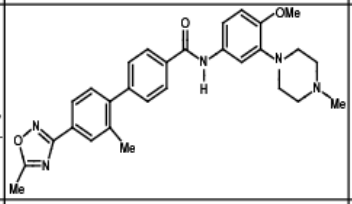
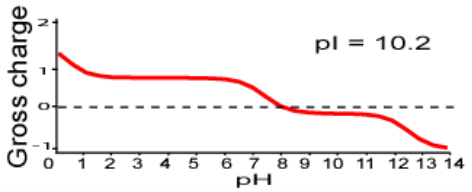
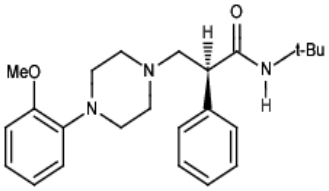
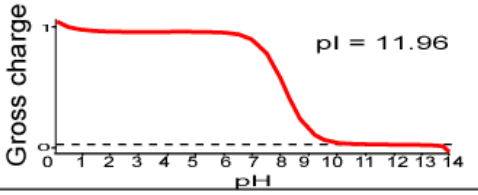
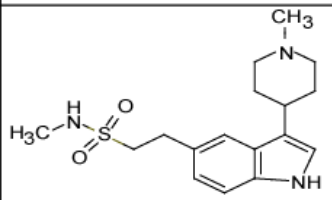
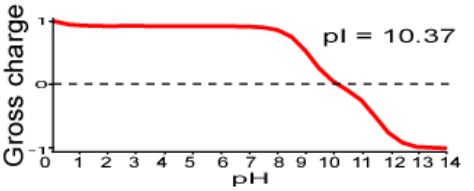
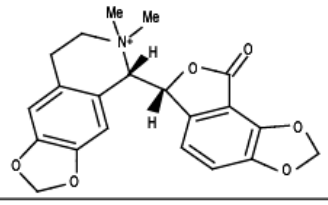
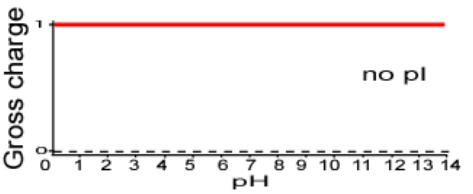
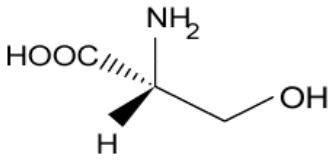
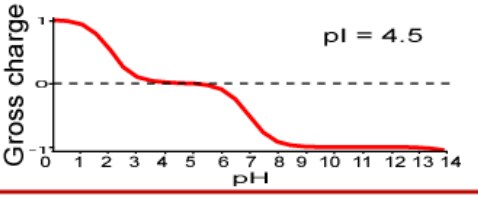
Anions (-) were retained in their barrels with small positive currents (5 nA) while cations (+) were retained with negative currents (-5 nA). Ejection currents in directions opposite to the retaining currents were used, ranging from 5 to 100 nA. As any effect following microiontophoretic drug ejection on cell firing rate could be a current or pH artefact, the effects of suitable control were also tested. The control consisted of ejection of either Na^+ or Cl^- ions – matching the polarity of the ejected drug – and also H^+ or OH^- ions in those cases where pH alterations had been made to the solutions. The control was ejected at the same parameters as the drug under investigation.

Tables 13: Structure and gross charge of drugs used by microiontophoresis

Compound	Structure	Gross charge = f(pH)
L-Glutamate (L-Glutamic acid)		
NMDA (*N*-methyl-D-aspartic acid)		
Iodowillardiine [(S)-(-)-5-Iodowillardiine]		
Fluorowillardiine [(S)-(-)-5-Fluorowillardiine]		
SYM 2081 [(2S,4R)-4-Methylglutamic acid]		
CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione)		
UBP 302 [(S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl) pyrimidine-2,4-dione]		
LY 466195 [(3S,4R,6S,8R)-6-[[(2S)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl] decahydro-3-isoquinoline-carboxylic acid)]		

Chemicals' IUPAC (International Union of Pure and Applied Chemistry) Name is provided in brackets. pI; isoelectric point

Tables 13 (continued): Structure and gross charge of drugs used by microiontophoresis

Compound	Structure	Gross charge = f(pH)
NAS-181 [(2R)-2-[[[3-(4-Morpholinyl-methyl)-2H-1-benzopyran-8-yl]oxy]methyl]morpholine dimethanesulfonate]		
GR 127935 [N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methy l-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide]		
WAY 100135 [(S)-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl) -2-phenylpropanamide dihydrochloride]		
Naratriptan [N-methyl-2-[3-(1-methylpiperidin-4-yl)-1H-indol-5-yl] ethanesulfonamide]		
Bicuculline methiodide [[R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g] isoquinolinium iodide]		
D-Serine		

Chemicals' IUPAC (International Union of Pure and Applied Chemistry) Name is provided in brackets. pI; isoelectric point

Table 14: Parameters of compounds used in all microiontophoretic studies

Drug	Concentration	pH	Ejection Polarity	Source
L-Glutamate monosodium	200 mM	8.0	(-)	Sigma-Aldrich
Iodowillardiine	50 mM	8.0	(-)	Tocris Cookson
Fluorowillardiine	50 mM	8.0	(-)	Tocris Cookson
SYM 2081	50 mM	8.0	(-)	Tocris Cookson
NMDA	50 mM	8.0	(-)	Sigma-Aldrich
CNQX	50 mM	8.0	(-)	Tocris Cookson
UBP 302	50 mM	8.0	(-)	Tocris Cookson
LY466195	50 mM	7.5	(-)	Lilly Laboratories
NAS-181	50 mM	5.5	(+)	Tocris Cookson
GR127935 hydrochloride	20 mM	5.5	(+)	Tocris Cookson
Naratriptan hydrochloride	25 mM	5.5	(+)	Glaxo Wellcome Research and Development
(S)-WAY100135	10 mM	5.5	(+)	Tocris Cookson
(-)-Bicuculline methiodide	100 mM	5.5	(+)	Tocris Cookson
D-Serine	50 mM	8.0	(-)	Tocris Cookson
NaCl	200 mM	7.5-8	(+/-) Automated Current Balancing	Sigma-Aldrich
Pontamine Sky Blue	2.5% (w/v)	6.5	(-)	BDH Laboratory Supplies

Sigma-Aldrich Ltd., St. Louis, MO, U.S.A.
Tocris Cookson, Avonmouth, U.K; Ellisville, Missouri ,USA
BDH Laboratory Supplies, Poole, UK
Lilly Laboratories, Eli Lilly and Company, Indianapolis, Indiana, USA
Glaxo Wellcome Research and Development, Herts., UK
(+): cation (-): anion

2.9.2 Intravenous administration of LY466195 and topiramate

The effects of intravenous doses of LY466195 (kindly provided by Lilly Laboratories) on the responses following dural vessels' stimulation and on the glutamate agonists NMDA, Iodowillardiine and Fluorowillardiine-evoked firing were studied in the thalamus. The effects of iv doses of LY466195 in the TCC were tested only on cell firing in response to dural vessels' stimulation. LY466195 was dissolved in de-ionised sterile water and doses of 10, 50 and 100 μgkg^{-1} were used for recordings in the VPM and doses of 50, 100 and 200 μgkg^{-1} were used for recordings in the TCC.

Topiramate (kindly provided by Johnson & Johnsons) was only tested on cell firing of third order neurons in response SSS stimulation in the VPM at a dose of 30 $mgkg^{-1}$. Topiramate was dissolved in de-ionised sterile water and sonication was used to help the solubility of the molecule. All solution were filtered (0.02 μm inorganic membrane filter) and given by a slow intravenous injection over one minute, following by flushing of the cannula with saline. Vehicle control ejections were achieved by intravenous injection of 0.3 ml de-ionised water for injection.

Binding affinities of the major compounds used

As the antagonists and the glutamate receptor agonists used in this thesis were chosen according to their selectivity over different glutamate receptors and receptor subunits (Jane et al., 1997; More et al., 2004; Weiss et al., 2006), binding affinities are shown in table 15. Due to the different approaches used for calculating the binding affinities for each drug, affinity is expressed as either K_i – the molar concentration of a competitive ligand that would occupy 50% of the receptor, or as K_d – the molar concentration of a radio-labelled ligand which occupies 50% of the receptor.

Table 15: Receptor binding affinities of the major kainate compounds used at human recombinant glutamate receptors

		Agonists		Antagonists	
Receptor	Subunit	Fluorowillardiine (Ki; nM)	Iodowillardiine (Ki; nM)	UBP 302 (Kd; nM)	LY466195 (Ki; μM)
kainate	iGluR5	1820 ± 416	0.24 ± 0.06	402	0.05 ± 0.02
	iGluR6	none	none	36180	> 100
AMPA	iGluR1	14.7 ± 1.3	163 ± 42	104520	75 ± 7
	iGluR2	25.1 ± 5.2	176 ± 29		269 ± 22
	iGluR4	305 ±107	972 ± 155		312 ± 62
NMDA	NR1/NR2	none	none	none	2.5 ± 0.9

2.10 Drugs used for intravital microscopy

Calcitonin gene-related peptide (CGRP, rat; Tocris Cookson Inc.) induced vasodilation was achieved via intravenous infusion of 1 μgkg^{-1} CGRP (Tocris Cookson). CGRP was initially dissolved in distilled water, aliquoted, and frozen. Subsequent dilutions were made in 0.9% saline before injection at a dose of 1 μgkg^{-1}. Iodowillardiine and UBP 302 were dissolved in saline with pH corrected to 8.0-8.5. Doses of 5, 10 and 20 $mgkg^{-1}$ of Iodowillardiine and a 50 mgkg μgkg^{-1} of UBP 302 were used. When more than one drug was given intravenously, an interval time of at least 5 minutes was set before administering the second compound. In these experiments the line was always flushed with saline prior to administration of the second compound. As stated above, all solutions were filtered and given by slow intravenous injection over one minute, followed by flushing of the cannula with saline. Vehicle control ejections were achieved by intravenous injection of 0.3 ml of pH adjusted saline.

2.11 Electrophysiological recordings

2.11.1 Receptive fields and cell characterisation in the trigeminocervical complex

Receptive fields were sought on the ipsilateral craniofacial region for all cells. The cutaneous receptive field was assessed in all three divisions of the trigeminal innervation and identified as the electrode was lowered in the spinal cord. The receptive field was characterised for non-noxious (gentle brushing with a blunt probe) and noxious (pinch with toothed forceps) responses. Once a neuron sensitive to stimulation of the ophthalmic dermatome of the trigeminal nerve was identified it was tested for convergent input from the dura mater in response to electrical stimulation. Cells were classified as LTM if they responded only to non-noxious stimuli, NS if they responded to noxious stimulation only, and WDR if they responded to both (Hu et al., 1981).

2.11.2 Receptive fields and cell characterisation in the ventroposteromedial complex

Receptive fields were sought on the contralateral craniofacial region for all cells. For those cells with a receptive field not involving the vibrissae, non-noxious and noxious stimuli were provided as in the TCC and cells were classified as LT, NS and WDR. The majority of cells however had receptive fields limited to the vibrissae and furry buccal pad, which is not surprising given the large volume dedicated to the representation of vibrissae within the rat VPM (Vahle-Hinz and Gottschaldt, 1983). With such cells each vibrissa was manipulated with a fine needle mounted on a probe, taking care not to deflect surrounding vibrissae, to find the whisker(s) that triggered a response upon deflection.

2.11.3 Analysis of the neuronal responses to electrical stimulation of the middle meningeal artery (MMA) and the superior sagittal sinus (SSS)

To record the response of units following electrical stimulation of the MMA, post-stimulation histograms were constructed (figure 32). Each histogram was constructed by calculating the number of spikes recorded per 1 ms bin (with a total sweep length of 50 ms) elicited by 25 electrical stimuli. The main body of the response was found to occur

with a latency of 8-20 ms. Neurons firing with such latency were classified as receiving inputs from Aδ-fibres. Calculations of neuronal conduction velocities, were estimated by measuring the distance between the MMA stimulating site and the cervico-medullary recording site (25-30 mm). An additional 1 ms was included for the central synaptic delay (Schepelmann et al., 1999; Bartsch and Goadsby, 2003b).

The baseline response probability was calculated from up to three trials. Sufficient time was allowed to elapse between each trial to allow the cell to recover. A minimum response probability of 30% was required for a cell to be considered responsive to trigeminovascular nociceptive stimulation and suitable for further study (Nagler et al., 1973). Post-stimulus histograms following stimulation of the SSS were constructed in the same way over 50 electrical stimuli as previously described (Shepheard et al., 1999; Shields and Goadsby, 2005, 2006). The main body of the response was found to occur with a latency of 10-20 ms in both the TCC and the VPM.

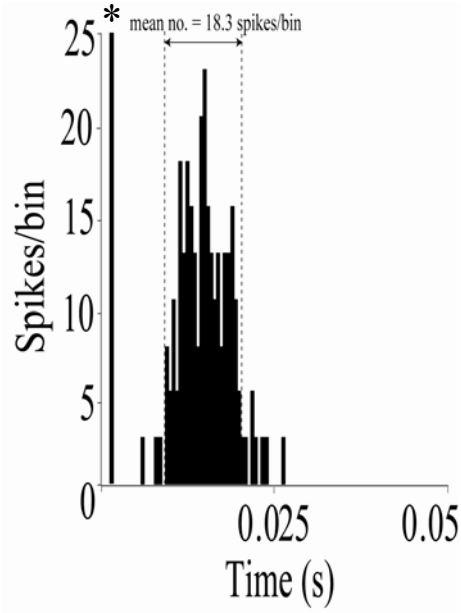


Figure 32: Post-stimulus histograms –calculation of response probability

Post-stimulus histogram demonstrating the response of a representative second order neuron in the trigeminocervical complex, to electrical stimulation of the middle meningeal artery. The number of spikes per 1 ms bin was collected over 25 stimuli. The mean number of spikes per bin was then calculated. * stimulus artefact

2.11.4 Analysis of the neuronal responses to microiontophoretic ejection of glutamate receptor agonists

Neuronal action potential firing in response to microiontophoretic ejection of glutamate agonists was analysed as cumulative rate histograms. The data was collected into successive one second bins. The ejection current of each agonist was titrated for each cell to produce a sustainable firing rate - comparable to the response elicited by stimulation of the receptive field. Response profiles of different receptors were taken into account and in experiments where more than one agonist was ejected, care was taken to produce similar firing activity during baseline periods, as activation of different receptors produces different profiles of response (Stone, 1985) (figure 33).

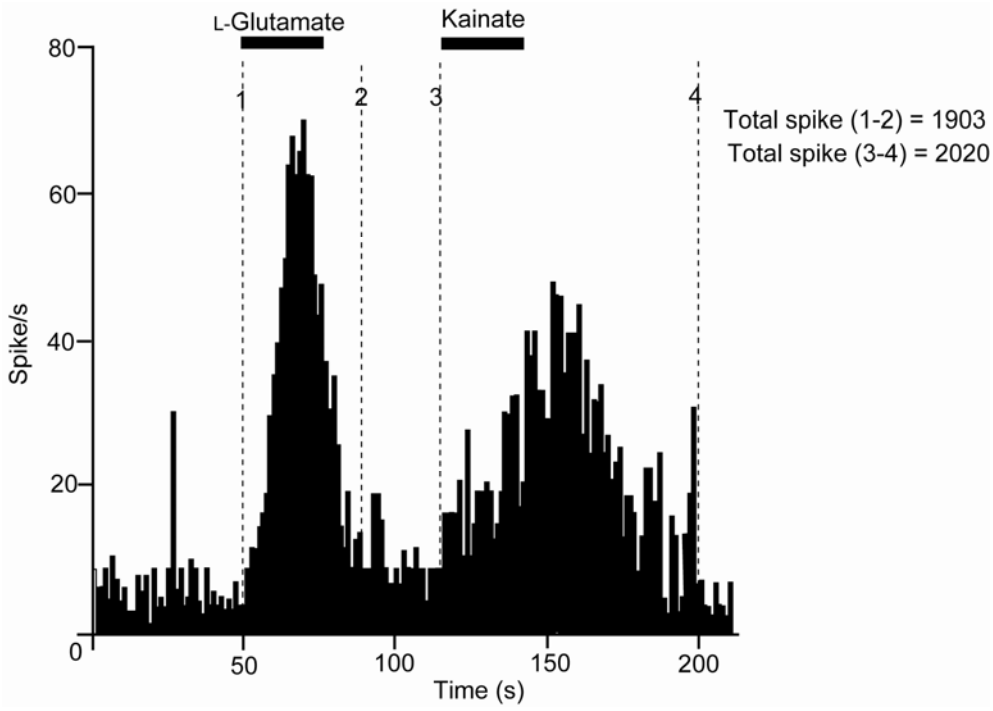


Figure 33: Titration of glutamate receptor agonists' responses
A cumulative histogram composed of successive responses to L-glutamate (40 nA) and kainate (15 nA) is used as an example. The total spike count between cursors 1 and 2 was 2020 and that was comparable to the total spike count between cursors 3 and 4 (1903). These figures indicate that peak height cannot be used to reflect the total activity if the response profiles are appreciably different (Stone, 1985).

Each agonist was ejected for approximately 7 seconds. An interval period of approximately 40 seconds, at which holding current was applied before the subsequent ejection, was used when more than one agonist were ejected. An interval period of approximately 30 seconds was used when only one agonist was ejected. Interval period was judged on the response profiles of the different agonists used such as enough recovery period was allowed and the slow decline responses of some receptors was not affecting the responses of the next test compound (Stone, 1985). At least five pulses of a glutamate agonist or five cycles of three different glutamate agonists ejected in a random order were recorded during baseline period. Five cycles were used as the firing rate could naturally vary between pulses – this was used to reduce the possibility of registering a spurious effect. The mean firing rate during each ejection period was calculated using the "Spike®" software package. The mean background firing rate – during the retention phase – was calculated and subtracted from the firing rate during the preceding ejection phase to calculate the net effect of each glutamate agonist (figure 34). The AC/DC amplifier channel was used as a guide for the start and end point of each increase in firing rate (figure 34). This was done as the increase in firing rate in response to microiontophoretic ejection of glutamate agonists, is not limited to the ejection duration, as this among others depends on the drug diffusion from the end of the tip, the distance of the electrode from the cell and the receptor's response profile, for example, kainate receptor activation produces a prolonged increase in firing rate (figure 33).

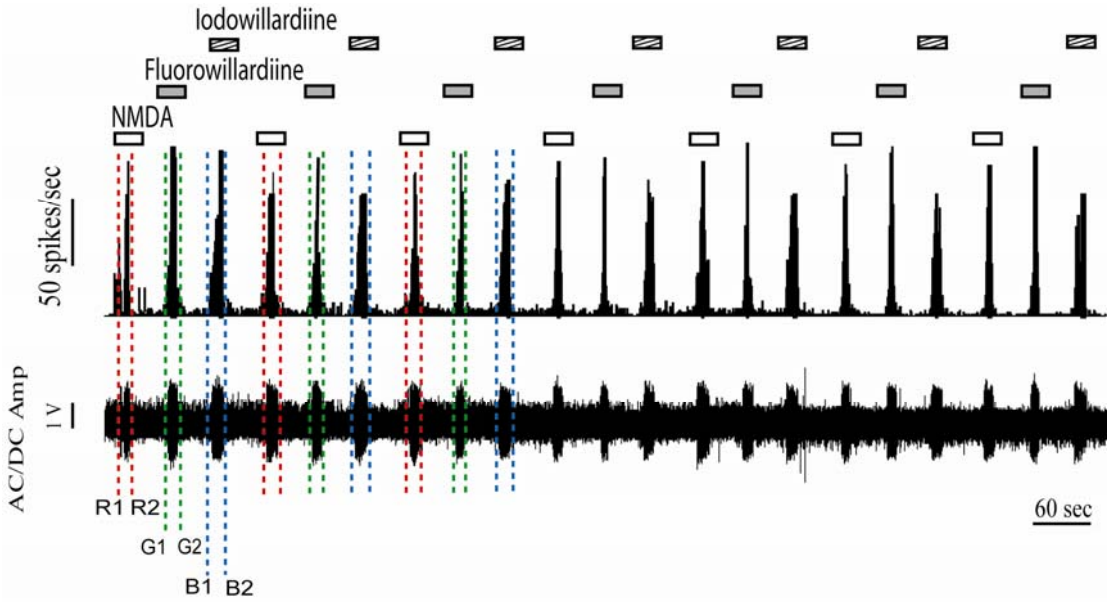


Figure 34: Peri-stimulus histograms – calculation of glutamate agonists-evoked responses

The mean firing rate of a thalamocortical neuron during each 7 seconds ejection of NMDA, Fluorowillardiine and Iodowillardiine was calculated by measuring the rate during the ejection phase (for example NMDA: between red cursors R1-R2; Fluorowillardiine: between green cursors G1-G2; Iodowillardiine: between blue cursors B1-B2) and subtracting the corresponding rate of the retention phase (for example Fluorowillardiine: between cursors R2-G1; Iodowillardiine between cursors G2-B1). The increase in cell firing was calculated separately for each agonist (for example NMDA: between red cursors; Fluorowillardiine: between green cursors; Iodowillardiine: between blue cursors). The mean firing rate of trigeminocervical neurons was calculated using the same approach.

2.11.5 Selection criteria used to identify cells suitable for microiontophoretic study

Both trigeminocervical and thalamocortical neurons were required to display the following criteria before they were selected for microiontophoretic study:

- Electrical stimulation of the MMA or the SSS was the primary method for identifying suitable neurons. Neurons had to respond to electrical stimulation of the vessel with a response probability of at least 30% before further characterisation was undertaken. This minimum required level of response ensured that only neurons activated by a nociceptive trigeminovascular input were studied.

- Each cell was required to have a V1 ophthalmic cutaneous receptive field (TCC neurons) or a cranio-facial receptive field (VPM neurons). Modulation of the cutaneous inputs however was only studied in the TCC. Generally neurons in both the TCC and VPM activated by convergent viscerosomatic inputs from dural vascular structures rarely had receptive fields located on the body.

- Once a suitable unit was identified on the basis of its response to dural electrical stimulation glutamate agonists were ejected. As glutamate receptors are predominantly located on the cell body of neurons, an increased firing rate in response to agonist ejection was taken as evidence that the electrode was recording from the soma of a neuron and not from an axon. Five repeated stable baselines had to be recorded for a cell to be considered for further examination.

- Microiontophoretic ejection of an inhibitory drug had to be followed by recovery of both dural responses to electrical stimulation and glutamate agonist-evoked activation, comparable to baseline recordings. This allowed neuron recovery to be monitored and ensured that the effect was not due to the cell drifting away from the electrode tip.

2.11.6 Histological confirmation of recording sites

The location of each recording site was verified by two methods. The first involved direct marking of the recording site by ejection of Pontamine Sky Blue. A microiontophoretic pump (BAB-350 Iontophoresis Pump, Kation Scientific, Minneapolis, USA) was used to eject Pontamine Sky Blue using a current of 3 μA for 10 minutes. Alternatively the depth of each recording site was recorded from the microdrive and two reference points were then marked along the electrode tract dorsal and ventral to the recording sites. The recording sites were then reconstructed using these reference points. At the end of each experiment a 5 mm thick coronal section encompassing the thalamus and electrode tract (for recordings in the VPM) or an upper spinal cord section encompassing the C1 spinal cord level and electrode tract (for recordings in the TCC) were removed, and placed immediately in neutral buffered 10% formalin (Sigma-Aldrich) for 24 hours. This was slowly replaced by 30% sucrose solution (BDH Laboratory Supplies) over subsequent days. Once the tissue block had

fully equilibrated in 30% sucrose it was cut using a freezing microtome into 40 μm sections and placed on microscope slides. Sections were then examined (Axioplan microscope, Zeiss, Germany at x 25 magnification) to locate Pontamine spots. Those sections in which blue marks were found were stained with a combination of neutral red and crysel violet and photographed (AxioCam MRc5 microscope camera, Zeiss, Germany). Recording sites were then reconstructed, approximating their original positions into coronal planes for diagrammatic purposes.

2.12 Statistical Analysis

All statistical analysis was carried out using SPSS v 16 (SPSS, Chicago, USA).

2.12.1 Electrophysiological studies

The mean net firing rate was calculated for each of the successive cycles of glutamate agonists during each of the test conditions. Five pulses were analysed to avoid variations of the responses of a cell between individual pulses and the reliability of the measurements was tested using Cronbach's alpha. The average of the three baselines obtained following stimulation of dural structures was used for further comparisons. A repeated measures ANOVA for both responses evoked by glutamate agonists and dural stimulation were computed with two factors: Drugs and Repeats, to determine intra- and inter- drug effects and interactions. Bonferroni corrections were applied and when the assumption of sphericity with regards to the factor of Repeats was violated, adjustments were made for the degrees of freedom and P values according to the Greenhouse-Geisser correction. The results from all cells were analysed together and compared by a series of Student's paired *t*-tests to examine the effect of each intervention in turn. An independent *t* test was used for group comparisons to compare the effects of antagonists over two different neuronal subpopulations. This approach was taken to make sure that any artefact caused by current ejection was not mistaken for a drug effect. Significance was assessed at the $P < 0.05$ level (Field, 2005). All data are expressed as the mean value and the standard error of the mean (SEM) for each treatment group.

2.12.2 Intravital microscopy

The effects of electrical stimulation and CGRP administration on dural vessel diameter were calculated as a percentage increase from the pre-stimulation baseline diameters. The nature of the experimental set-up, where the magnification of the dural vessel visualised was different in each animal by virtue of selecting an appropriate target vessel, resulted in dural vessel diameter being measured in percentage change from baseline. The typical vessel diameter measured was 115 ± 7 μm. All data are expressed as mean ± SEM. Statistical analysis was performed using an ANOVA for repeated measures with Bonferroni *post-hoc* correction for multiple comparisons followed by Student's paired *t*-test. To compare the effect of Iodowillardiine only and Iodowillardiine and UBP 302 an independent samples *t*-test was used. Significance was assessed at the $P < 0.05$ level (Field, 2005) and all data are expressed as the mean value and the standard error of the mean (SEM) for each treatment group.

Chapter 3: Activation but not blockade of the iGluR5 kainate receptor attenuates neurogenic dural vasodilation in an animal model of trigeminovascular nociception

3.1 Introduction

A key manifestation of migraine is activation, or the perception of activation, of trigeminal afferents mainly in the ophthalmic division of the trigeminal nerve, which innervate the pain-producing intracranial structures, such as the dura mater (Goadsby, 2005b). Stimulation of the dura mater and blood vessels in humans is known to produce headache-like pain referred to the ophthalmic division of the trigeminal nerve (Penfield, 1932, 1934; Penfield and McNaughton, 1940; Wolff, 1948; McNaughton and Feindel, 1977). Migraine pathophysiology is believed to involve the release of neuropeptides from trigeminal afferents innervating pain producing intracranial structures (Edvinsson and Goadsby, 1994). Electrical stimulation of dura mater causes CGRP release from the pre-junctional nerve fibres innervating the dural blood vessels, resulting in reproducible vasodilation (Williamson et al., 1997c). Trigeminal nerve activation causes dural blood vessel dilation (Williamson et al., 1997b) and given that dural blood vessel distension is painful in humans (Ray and Wolff, 1940), the reaction of dural blood vessels is used as an indication of trigeminal nerve activation from a peripheral perspective and it enables us to model some aspect of the migraine attack.

The development of intravital microscopy permitted the direct study of the peripheral branch of the trigeminovascular system by means of neurogenic dural vasodilation (NDV). NDV is substantially due to the release of CGRP following electrical stimulation of the dura from pre-junctional trigeminal nerve fibers innervating the dural blood vessels (Kurosawa et al., 1995; Williamson et al., 1997a; Akerman et al., 2002b, c). Upon release, CGRP binds to CGRP receptors on dural vessels and results in vasodilation (figure 35). Intravital microscopy uses a thinned closed cranial window combined with a video microscopy device that allows the visualisation and measurement of cranial (dural and pial) blood vessels in real time (Williamson et al., 1997a, c; Petersen et al., 2004; Gozalov et al., 2005; Petersen et al., 2005b; Petersen et al., 2005a).

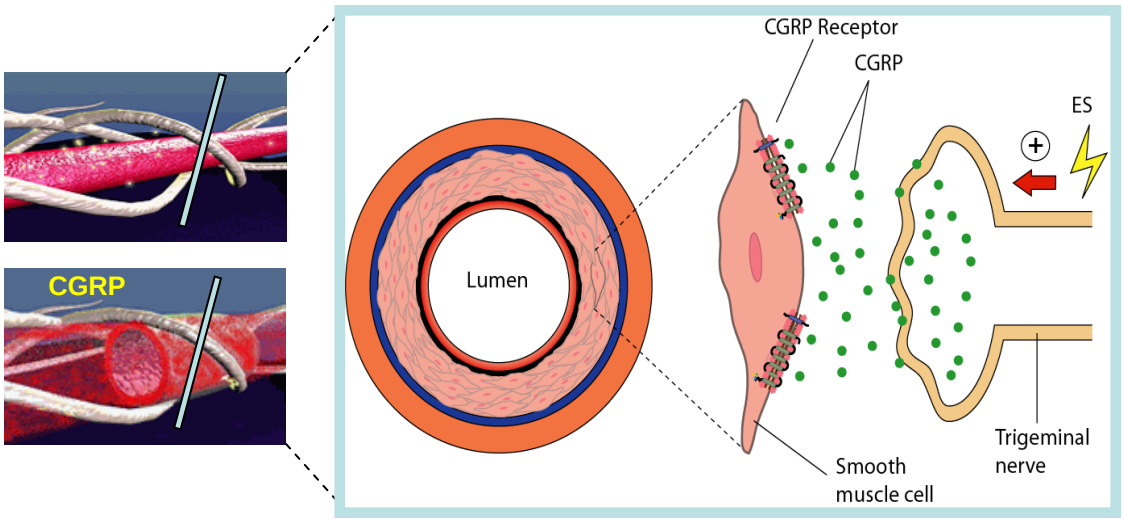


Figure 35: Summary of neurogenic dural vasodilation
The diagram shows a schematic representation of a dural artery innervated by a trigeminal sensory nerve. Electrical stimulation (ES) causes CGRP release from the pre-junctional activated trigeminal neurons. CGRP activates CGRP receptors in the smooth muscle of the dural artery causing the vessel to dilate.

The CGRP antagonist $CGRP_{8-37}$ is able to inhibit completely NDV, whereas substance P is not implicated in the development of the reproducible neurogenic vasodilation as NK1 receptor antagonists were unable to inhibit NDV (Williamson et al., 1997c), as well as demonstrating similarly poor results in clinical trials (Goldstein et al., 1997; Goldstein et al., 2001). This further indicates the importance of CGRP in neurogenic vasodilation and the good utility of NDV in modelling partly the pharmacology of the trigeminovascular system. Why SP plays no role in migraine pathophysiology is not well understood. As CGRP and SP are colocalised mainly in C fibres arising from the trigeminal ganglion, someone will expect a contribution of both neuropeptides in the observed vasodilation. The importance of CGRP over SP might reflect a greater density of Aδ fibres innervation of the cerebral circulation or it could reflect preferential activation of Aδ over C fibres or it could reflect differential release of CGRP from C fibres pools (Edvinsson and Hargreaves, 2000). The measurement of dural vessel dilation due to electrical stimulation-evoked release of CGRP, which is present in trigeminal afferents innervating dural structures, including the MMA (Keller and Marfurt, 1991), is an established method of characterising trigeminovascular activation.

Moreover, since CGRP receptor antagonists are clearly effective in acute migraine (Olesen et al., 2004b; Ho et al., 2008), the pharmacology of the control mechanisms for dural CGRP release are of direct relevance to the development of newer migraine therapies.

The NDV model has proven ability to predict anti-migraine therapeutic potential as compounds with anti-migraine efficacy in the clinic, such as BIBN4096BS, topiramate, rizatriptan and sumatriptan were effective in inhibiting NDV (Williamson et al., 1997c; Williamson et al., 1997b; Williamson et al., 2001; Petersen et al., 2004; Akerman and Goadsby, 2005b; Recober and Russo, 2007). There are also a series of compounds which includes calcium channel blockers and cannabiods, that are able to inhibit NDV, as well as trigeminovascular activation in other models of migraine and thus represent potential new migraine therapeutics (Akerman et al., 2001; Akerman et al., 2003a; Akerman et al., 2004b; Shields et al., 2005; Akerman et al., 2007).

CGRP and NO, are potent vasodilators and are able to cause an immediate headache and a delayed migraine in patients (Iversen et al., 1989; Iversen and Olesen, 1994; Lassen et al., 2002; Afridi et al., 2004; Afridi et al., 2005b). Similarly when used in intravital microscopy by systemic administration, are able to cause reproducible dural blood vessel dilation. CGRP is acting on vascular CGRP receptors (Akerman et al., 2002b, c; Akerman et al., 2004a). Due to a direct action of exogenous CGRP on post-synaptic CGRP receptors on the smooth muscle of dural arteries drugs that can inhibit CGRP-induce dilation demonstrate at least their partial action on the smooth muscles of blood vessels, whereas compounds that can inhibit NDV but not CGRP-induced dilation, have a direct action on the pre-junctional site of the trigeminal fibres innervating the dural vessels. Thus, intravital microscopy further helps to dissect the pharmacology of the trigeminovascular system.

The presence of iGluR5 subunits in trigeminal ganglion neurons (Sahara et al., 1997) and at the pre-synaptic sites of primary afferents (Hwang et al., 2001; Lucifora et al., 2006; Hegarty et al., 2007) indicates a possible role of kainate receptors in trigeminovascular physiology. Evidence from immunocytochemical studies also exists for peripheral transport of kainate receptor on sensory, both myelinated and unmyelinated axons (Carlton et al., 1995; Coggeshall and Carlton, 1998), suggesting its possible presence on the pre-junctional site of the trigeminal peripheral branch. It has

been shown that activation of these receptors on peripheral axons leads to increased activity along the sensory root and thus to peripheral nociceptive transduction (Agrawal and Evans, 1986; Ault and Hildebrand, 1993).

In the present study we investigated the possible involvement of pre-junctional iGluR5 kainate receptors in a model employing NDV using the selective iGluR5 receptor agonist Iodowillardiine (Swanson et al., 1998) and the selective receptor antagonist UBP 302 (More et al., 2004). As iGluR5 subunits are located on trigeminal nerve endings (Sahara et al., 1997), a demonstration of an effect would provide further evidence of a potential opportunity for treating migraine through this site.

3.2 Methods

3.2.1 Animals

Male Sprague-Dawley rats (280–370 g; n = 37) were anaesthetised with pentobarbitone (60 $mgkg^{-1}$) and cannulated for measurement of blood pressure, experimental drug administration and maintenance of anaesthesia (15 $mgkg^{-1}hr^{-1}$).

3.2.2 Drugs

The effects of the specific iGluR5 antagonist UBP 302 (50 $mgkg^{-1}$) and the specific iGluR5 agonist Iodowillardiine (5, 10 and 20 $mgkg^{-1}$) were investigated on neurogenic and CGRP induced vasodilation. Both Iodowillardiine and UBP 302 were dissolved in saline (pH 8) and administrated intravenously. In control experiments equal volumes of vehicle only was administered (section 2.10).

3.2.3 Experimental protocol

Neurogenic dural vasodilation and CGRP- induced vasodilation

A thinned closed cranial window was performed allowing the visualisation and measurement of the MMA in real time, using a video microscopy device. NDV was achieved by electrical stimulation of the cranial window as described in section 2.4.1. In brief, a bipolar stimulating electrode was placed on the thinned cranial window, close to the MMA. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Grass S88 Stimulator) with increasing voltage until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using that voltage. CGRP induced dilation was achieved via the administration of CGRP (Tocris Cookson Ltd., UK) 1 μgkg^{-1} intravenous bolus injection via the cannulated femoral vein. This dose of CGRP has been shown repeatedly to produce maximal vessel dilation (Williamson et al., 1997c; Akerman et al., 2002c).

Effect of iGluR5 receptor agonist Iodowillardiine on Evoked Dural Vessel Dilation

Two control responses to dural electrical stimulation were performed (interval time between stimulations: 15 minutes), and at least 10 minutes later, Iodowillardiine, at doses of 5, 10, and 20 (n = 6 for each dose) $mgkg^{-1}$ was administered intravenously. Electrical stimulation was then repeated at 5, 15, 30, 45, 60 and 90 minutes after drug administration. For those doses, at which no full recovery was observed after 90 minutes of Iodowillardiine treatment, CGRP 1 μgkg^{-1} was administered intravenously at least 10 minutes after the 90 minute response to dural electrical stimulation (figure 36). In the experiments where the antagonist UBP 302 was co-administrated with Iodowillardiine (n = 6), two control responses to dural electrical stimulation were performed, and at least 10 minutes later UBP 302 50 $mgkg^{-1}$ was administrated intravenously, followed by intravenous administration of Iodolwillardiine 10 $mgkg^{-1}$, 5 minutes later. Electrical stimulation was then repeated at 5, 15, 30, 45, 60 and 90 minutes after Iodowillardiine administration (figure 36).

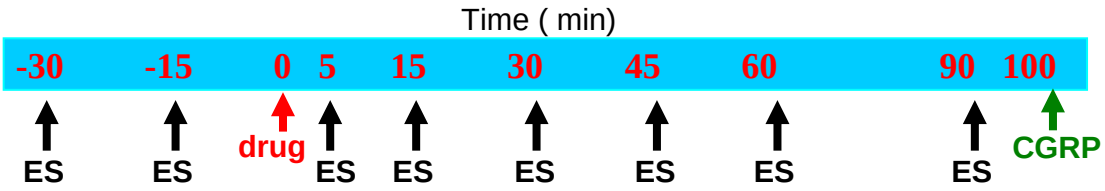


Figure 36: Electrical stimulation experimental protocol
Two control responses to electrical stimulation (ES) were performed before administration of Iodowillardiine or UBP 302 or control. ES was repeated at 5, 15, 30, 45, 60 and 90 minutes. When Iodowillardiine was administrated, CGRP (1 $mgkg^{-1}$) was given after the 90 minute, to test the vessel vitality.

Effect of the iGluR5 receptor antagonist UBP 302 on Evoked Dural Vessel Dilation

Two control responses to dural electrical stimulation were performed, and at least 10 minutes later, UBP 302, at a dose of 50 $mgkg^{-1}$ (n = 5) was administered intravenously. Electrical stimulation was then repeated at 5, 15, 30, 45, 60 and 90 minutes after drug administration (figure 36).

Effect of Iodowillardiine on CGRP-Evoked Dural Vessel Dilation

Two control responses to CGRP (1 μgkg^{-1}) induced dilation were performed, and at least 10 minutes later 10 $mgkg^{-1}$ Iodowillardiine (n = 6) was administered intravenously and then the CGRP induced dilation was repeated at 5, 15, 30, 45, 60 and 90 minutes after the agonist treatment (figure 37).

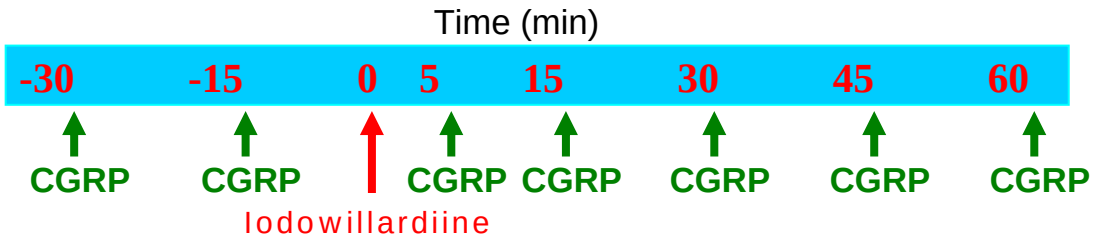


Figure 37: CGRP bolus experimental protocol
Two control responses to CGRP (1 $mgkg^{-1}$) dilation were performed before administration of Iodowillardiine (10 $mgkg^{-1}$) and then followed for 60 minutes.

3.3 Results

Baseline blood pressure and respiratory parameters were within normal limits for all animals.

Visualised branches of the MMA through the closed cranial window with diameter ranging from 90 to 140 µm were studied. Electrical stimulation (50-150 µA) of the cranial window, produced transient control responses in the range of 50 to 180% increase in dural blood vessel diameter (n = 31), compared to baseline diameter at rest (figure 38). Each vasodilation was calculated as the maximum difference in vessel diameter following electrical stimulation compared to the diameter at rest. For each animal, the mean ± SEM of the control neurogenic dural vasodialations was set as maximum baseline dilation- normalised to 100%. Subsequent dilations were expressed as a percentage of the control response.

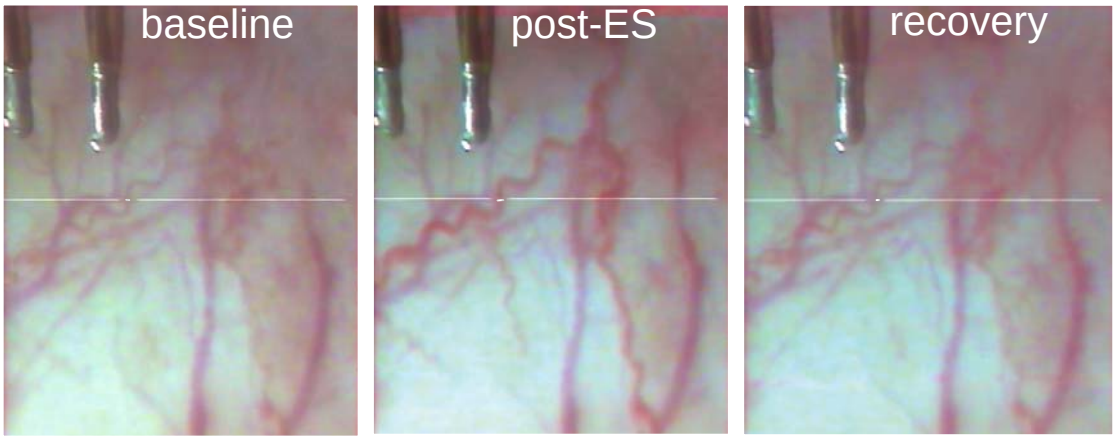


Figure 38: Neurogenic dural vasodilation
Examples indicating the measurement of the MMA diameter before electrical stimulation (ES) of the dura mater, 30 seconds post ES which resulted in vessel dilation and during recovery period. The two levelled lines (white) define the vessel under study and its diameter is measured through the video dimensional analyser.

3.3.1 Effects of Iodowillardiine on neurogenic dural vasodilation

The iGluR5 receptor agonist Iodowillardiine given intravenously at 5 $mgkg^{-1}$ had no effect on the responses to electrical stimulation of the cranial window, but a significant attenuation was observed at 10 ($F_{3,12}$ = 8.2; P < 0.005; figure 39; table 16) and 20 $mgkg^{-1}$ ($F_{3,15}$ = 14.3; P < 0.001; figure 39; table 16). At both doses Iodowillardiine produced its maximal effect after 15 minutes and responses were not fully recovered 90 minutes after drug administration. Iodowillardiine at 20 $mgkg^{-1}$ inhibited neurogenic dural vasodilation by 50% (t_5 = 5.2; P < 0.005; n = 6) and 90 minutes after treatment with Iodowillardiine NDV had not fully recovered to baseline. CGRP (1 μgkg^{-1}) intravenous administration, at least 10 minutes after the 90 minute neurogenic-induced dilation, caused maximum dilation to baseline levels. The CGRP induced-dilation was significantly different (t_5 = 4.2; P < 0.05) from the neurogenic dilation recorded at 90 minutes after Iodowillardiine treatment, indicating that the vessel was still responding. Control vehicle injections demonstrated no significant effect (figure 39).

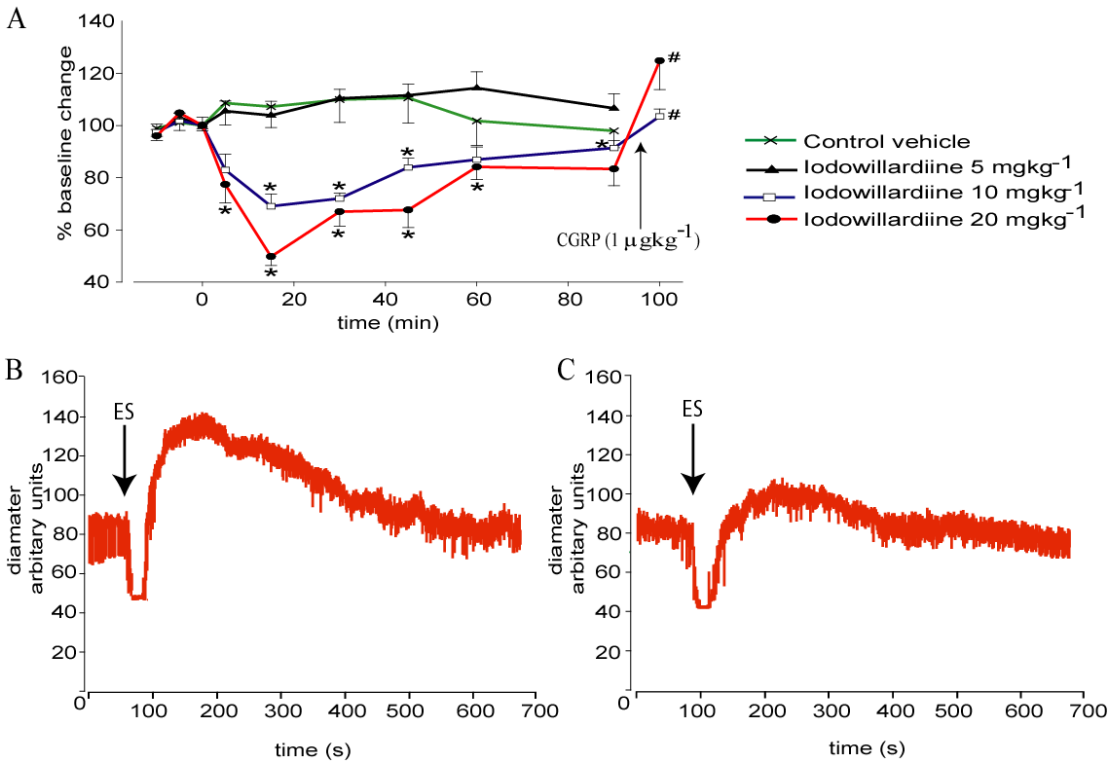


Figure 39: Effect of intravenous administration of Iodowillardiine (5, 10, and 20 mgkg^{-1}) on neurogenic vasodilation

A. Following control responses to electrical stimulation, rats were injected with Iodowillardiine at 5, 10 or 20 mgkg^{-1} and electrical stimulation was repeated after 5, 15, 30, 45, 60 and 90 min. Both 10 and 20 mgkg^{-1} doses of Iodowillardiine inhibited NDV. CGRP 1 µgkg^{-1} was given intravenously after the 90 min neurogenic induced dilation and the CGRP induced dilation was significantly different from the neurogenic dilation recorded 90 min after Iodowillardiine treatment. B, C. Example of neurogenic dural vasodilation tracing (Spike2 v5) prior any pharmacological treatment (B) and at 15 minutes after 20 mgkg^{-1}odowillardiine injection (C). The initial reduction immediately after electrical stimulation (ES) of the dura is due to spasm reaction to ES of the MMA. *, $P < 0.05$ significance compared with the control response. #, $P < 0.05$ significance compared with the 90 minute response to electrical stimulation.

3.3.2 Effect of UBP 302 on neurogenic dural vasodilation

The iGluR5 receptor antagonist UBP 302 given intravenously at 50 mgkg^{-1}, elicited no change in NDV ($F_{1,5}$ = 0.6; P = 0.5; n = 5; figure 40). UBP 302 administration did not alter the diameter of the vessels under observation at rest prior to electrical stimulation and at the dose of 50 mgkg^{-1} did not elicit any significant blood pressure changes. Control vehicle injections demonstrated no significant effect.

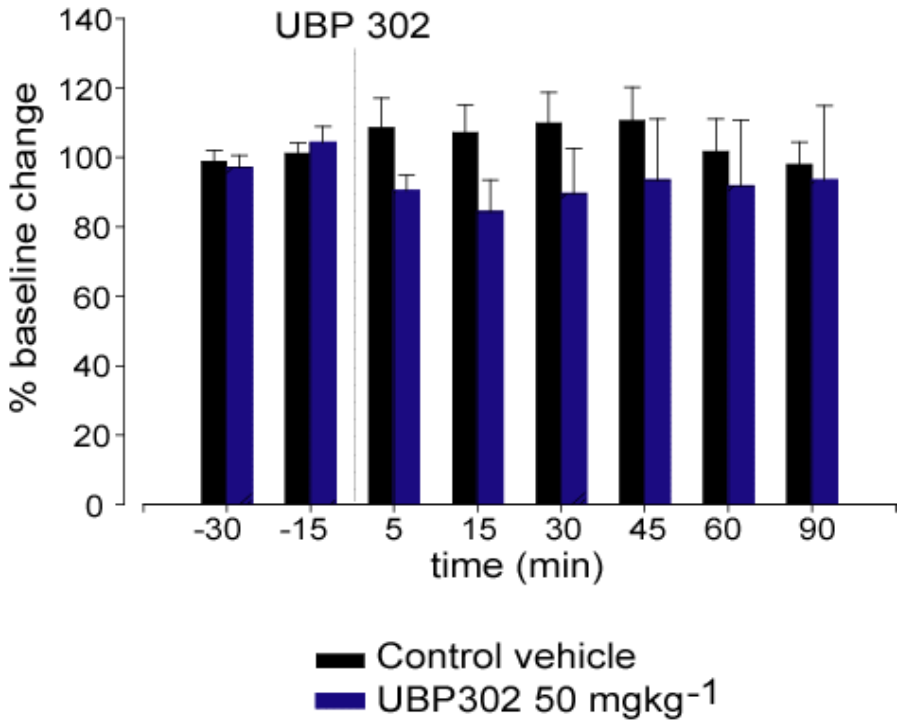


Figure 40: Effect of UBP 302 50 mgkg^{-1} on neurogenic vasodilation
Following control responses to electrical stimulation, rats were intravenously injected with UBP 302 at 50 mgkg^{-1} (n = 5) and electrical stimulation was repeated after 5, 15, 30, 45, 60 and 90 min. No significant effect was observed.

3.3.3 Effects of Iodowillardiine and UBP 302 on neurogenic dural vasodilation

Iodowillardiine given alone at 10 $mgkg^{-1}$ significantly inhibited NDV by 31% ($n = 6$; $t_5 = 7.2$; $P < 0.001$; figure 39, 41; table 16). Ninety minutes after Iodowillardiine treatment, full recovery was not observed after electrical stimulation and CGRP 1 μgkg^{-1} was given intravenously to test vessel response. The iGluR5 receptor antagonist UBP 302 (50 $mgkg^{-1}$) given alone did not cause any significant changes in vessels response to electrical stimulation of the cranial window ($F_{1,5} = 0.6$; $P = 0.5$; $n = 5$; figure 40). However, pre-treatment with UBP 302 50 $mgkg^{-1}$ 5 minutes prior to Iodowillardiine 10 $mgkg^{-1}$ administration reversed the inhibition of NDV ($F_{2,11} = 0.6$; $P = 0.6$; $n = 6$; figure 41) caused by Iodowillardiine when administered alone. Grouped comparisons of UBP 302 when administered alone (figure 40) and UBP 302 given intravenously pre-Iodowillardiine treatment showed no significant difference between the two experimental setups.

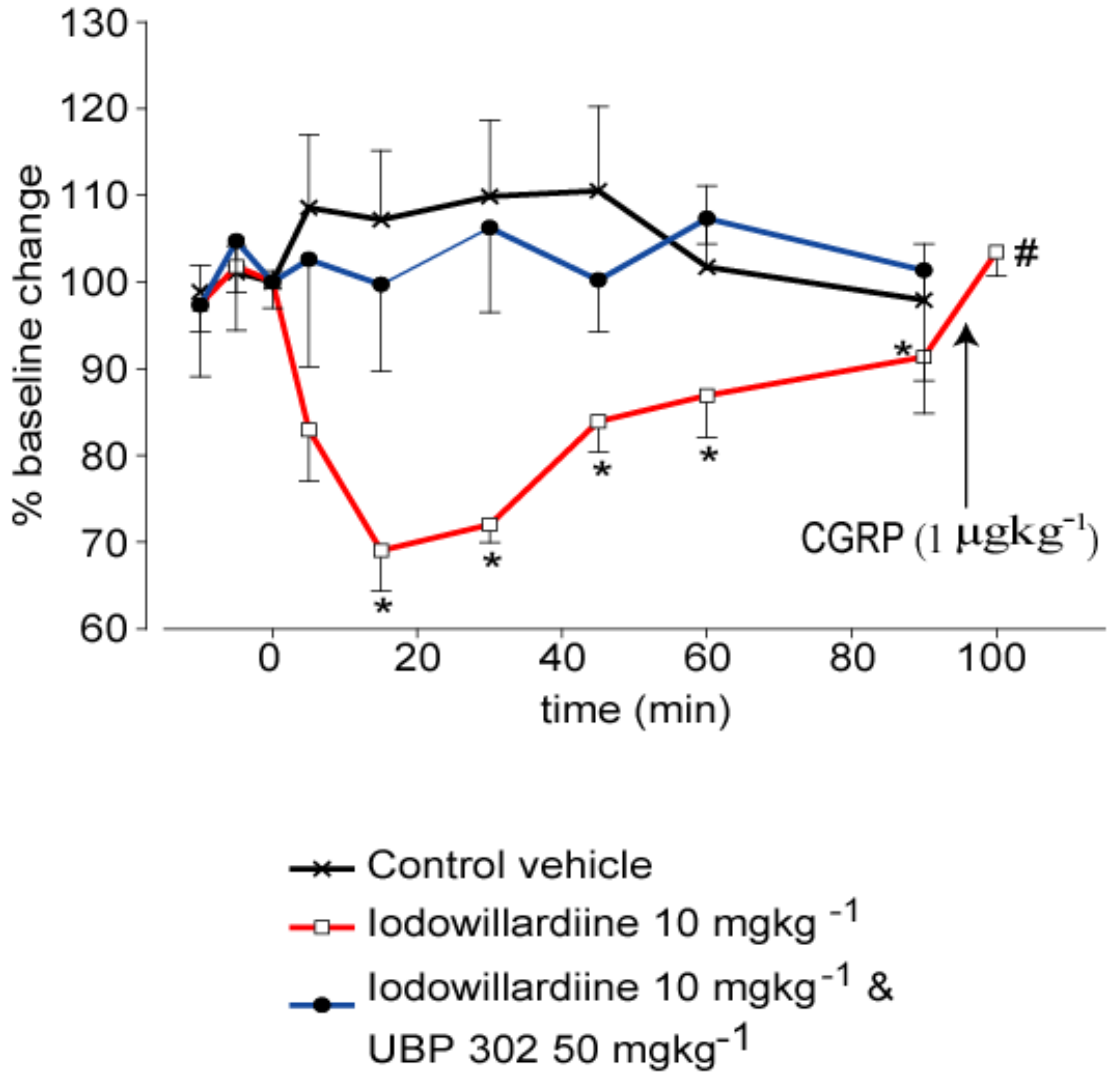


Figure 41: Effect of intravenous administration of Iodowillardiine 10 mgkg^{-1} on neurogenic dural vasodilation and the effect of pre-treatment with UBP 302 at 50 mgkg^{-1}

After control responses to electrical stimulation, Iodowillardiine at 10 mgkg^{-1} was either injected or pre-treated with UBP 302 at 50 mgkg^{-1} 5 minutes before Iodowillardiine and electrical stimulation was repeated at 5, 15, 30, 45, 60 and 90 minutes. Inhibition of NDV is seen in response to Iodowillardiine, which is not fully recovered after 90 min and CGRP injection at 1 µgkg^{-1} after the 90 min electrical stimulation produce maximum dilation. The inhibition in response to Iodowillardiine was reversed by the iGluR5 antagonist UBP 302.

3.3.4 Effects of Iodowillardiine on CGRP-induced dural vasodilation

CGRP bolus injections were used to induce dural vasodilation in response to activation of CGRP receptors on the vessel walls. Systemic administration of CGRP at 1 μgkg^{-1} was able to produce reproducible dural blood vessel dilation in the range of 75 to 155% (n = 6) and resulted in a transient decrease in blood pressure of 29 ± 4%, lasting 1-2 minutes, compared to baseline levels at rest. For each animal, mean control responses were set as maximum dilation of 100% ± SEM. Intravenous administration of Iodowillardiine (10 $mgkg^{-1}$) had no significant effect on vessel dilation in response to CGRP (n = 6; $F_{2,11}$ = 2.8; P = 0.1; figure 42).

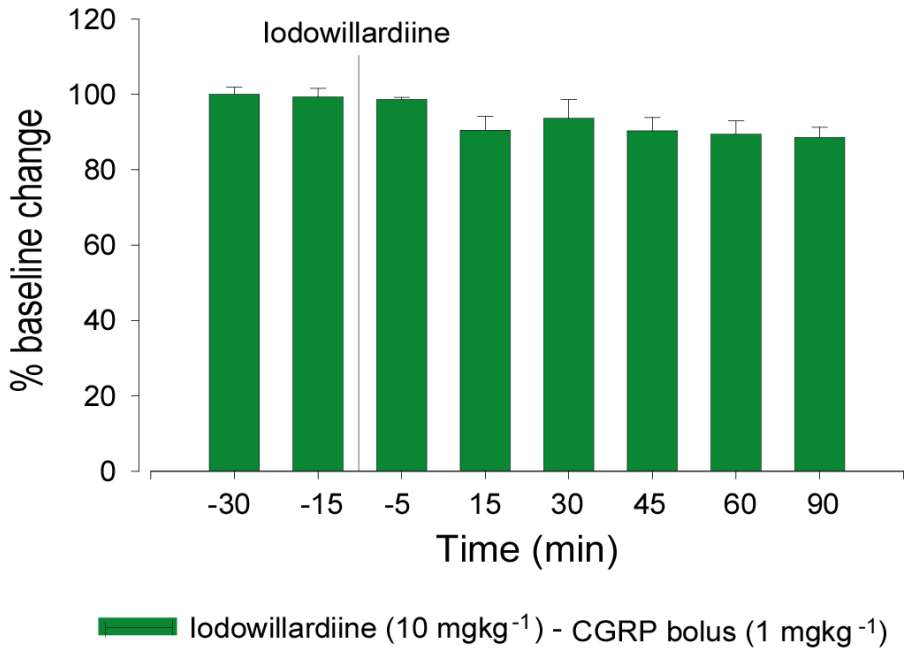


Figure 42: Effects of intravenous Iodowillardiine (10 $mgkg^{-1}$) on CGRP bolus induced vasodilation

Following control responses to CGRP administration, rats were injected with Iodowillardiine at 10 $mgkg^{-1}$ and CGRP injection was repeated after 5, 15, 30, 45, 60 and 90 minutes. Overall, no significant effect of Iodowillardiine on CGRP induced vasodilation was observed.

Table 16: Effects of intravenous injection of Iodowillardiine 10 mgkg^{-1}, Iodowillardiine 20 mgkg^{-1}, UBP 302 50 mgkg^{-1}, co-administration of 10 mgkg^{-1} Iodowillardiine and 50 mgkg^{-1} UBP 302 and control vehicle on neurogenic dural vasodilation

	% Increase in dural vessel diameter				
Time point	Iodowillardiine 10 mgkg^{-1}	Iodowillardiine 20 mgkg^{-1}	UBP 302 50 mgkg^{-1}	Iodowillardiine 10 mgkg^{-1} & UBP 302 50 mgkg^{-1}	Control vehicle
Control response	179 ± 7	197 ± 25	168 ± 7	180 ± 14	165 ± 12
5 min	165 ± 8	178 ±24 *(t_5 = 2.7, n = 6)	166 ± 7	184 ± 20	170 ± 12
15 min	150 ± 5 *(t_5 = 7.2, n = 6)	152 ± 17 *(t_5 = 5.2, n = 6)	158 ± 3	181 ± 18	168 ± 11
30 min	149 ± 6 *(t_5 = 9.9, n = 6)	164 ± 17 *(t_5 = 3.1, n = 6)	154 ± 5	186 ± 16	171 ±13
45 min	151 ± 6 *(t_5 = 5.3, n = 6)	167 ± 22 *(t_5 = 3.2, n = 6)	157 ± 8	181 ± 16	170 ±10
60 min	160 ± 4	183 ± 22 *(t_5 = 3.4, n = 6)	158 ± 14	186 ± 16	164 ± 7
90 min	165 ± 4 *(t_5 = 1.0, n = 6)	181 ± 21	157 ± 13	189 ± 19	164 ± 11

* $P < 0.05$

3.4 Conclusions

The kainate iGluR5 selective receptor agonist Iodowillardiine was able to significantly attenuate NDV at doses of 10 and 20 $mgkg^{-1}$ by a maximum of 50%, and this inhibition was blocked by pre-treatment with the selective iGluR5 receptor antagonist UBP 302. When the antagonist UBP 302 was administrated alone it caused no inhibition or further dilation of the blood vessels under investigation. These data indicate that activation of the iGluR5 carrying kainate receptors inhibits neurogenic vasodilation at the peripheral terminal of trigeminal nerve and its vasculature. Blockade of these receptors with the antagonist UBP 302 does not elicit any significant effects. Iodowillardiine failed to inhibit vasodilation induced by systemic administration of CGRP. As CGRP is likely acting directly on CGRP receptors on the smooth muscle of dural arteries to induce dural vasodilation (Williamson et al., 1997a, c), failure of Iodowillardiine to block this effect at concentrations that were able to inhibit significantly NDV, suggests that Iodowillardiine is acting at the pre-junctional site. The data are compatible with the existence of a pre-synaptic iGluR5 kainate receptor on trigeminovascular nerve terminals in the dura mater (figure 43).

Although information about the presence of kainate receptors on trigeminal peripheral processes is not available from anatomical studies, data from this study favours the existence of iGluR5 kainate receptors at this site. iGluR5 receptors have been found in the trigeminal ganglia and on primary afferents in the spinal cord and caudal brainstem (Sahara et al., 1997; Hegarty et al., 2007). The trigeminal ganglion is bipolar, with its peripheral afferent fibres innervating the dural structures and centrally projecting to the TCC. The pharmacology of the peripheral and central projections of the trigeminal ganglion in general mirror each other; thus, work demonstrating the presence of kainate receptors on central primary afferents may also apply on the neuronal mechanisms involving the peripheral trigeminal afferents. In agreement with this, there is evidence of functional kainate receptors on peripheral sensory axons (Carlton et al., 1995; Coggeshall and Carlton, 1998; Kinkelin et al., 2000).

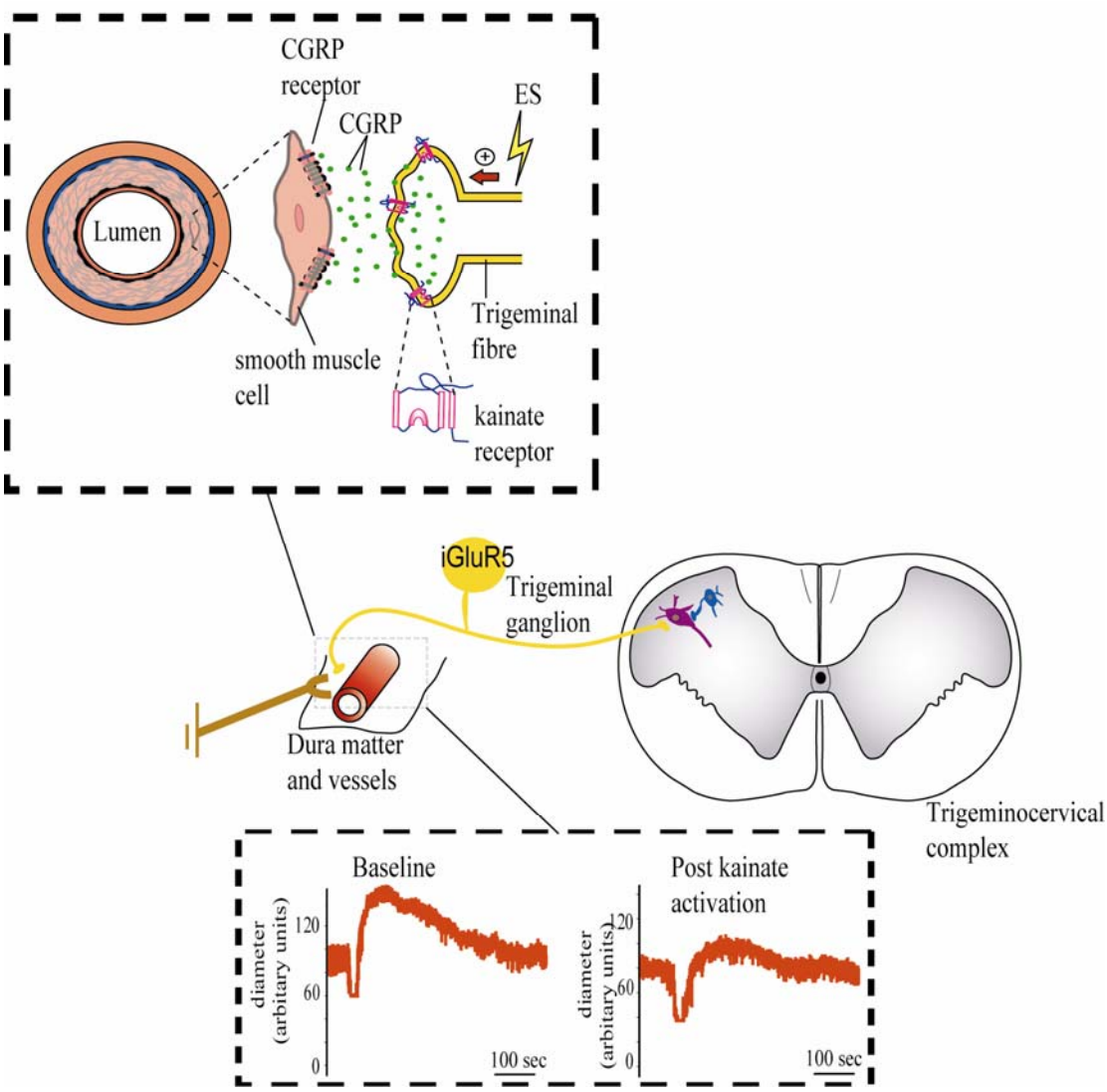


Figure 43: Overview of the effects of systemic administration of the iGluR5 kainate receptor agonist Iodowillardiine

Selective activation of iGluR5 receptors was shown to result in inhibition of neurogenic dural vasodilation suggesting the existence of a pre-synaptic iGluR5 kainate receptor on trigeminovascular nerve terminals in the dura mater. ES; electrical stimulation

The blockade of NDV by Iodowillardiine, may involve either pre- or post-synaptic receptors, or both. Iodowillardiine at concentrations that were able to inhibit significantly NDV was unable to block dilation induced by exogenous CGRP. Due to a direct action of exogenous CGRP on post-synaptic CGRP receptors on the smooth muscle of dural arteries, it is likely that Iodowillardiine has no effect on dural vessels. There is no current anatomical evidence for the presence of kainate receptors on dural vessels, although the complete absence or expression of non-functional kainate receptors on cerebral vessels has been suggested (Faraci et al., 1994; Morley et al., 1998; St'astny et al., 2002). In addition, detection of kainate receptors on the heart and cardiac nerve terminals has been demonstrated but a functional role has not been shown (Gill et al., 1998; Gill et al., 2007). Results from the present study, as shown with both the iGluR5 kainate agonist and antagonist, support the absence of at least functional iGluR5 carrying kainate receptors on dural vessels.

Neurogenic dural vasodilation has been shown to be almost completely blocked by the CGRP antagonist $CGRP_{8-37}$ and as such the observed dilation is due to the release of CGRP from the trigeminal peripheral pre-junctional site, acting on CGRP receptors on the vessels (Williamson et al., 1997a). The data presented in this study indicate a pre-junctional effect for Iodowillardiine on the trigeminovascular system, probably from a reduction of CGRP release from peptidergic trigeminal afferents. As Iodowillardiine was able to reduce the degree of meningeal vasodilation by 50%, the new data suggest that iGluR5 activation does not completely block the presynaptic release of CGRP. The mechanism of Iodowillardiine activation of iGluR5 kainate receptors resulting in reduction of CGRP release is not known. It has been proposed that presynaptic spinal kainate receptors function as autoreceptors at primary afferent synapses in the dorsal horn (Agrawal and Evans, 1986) and Kerchner and colleagues (2001a) demonstrated that activation of presynaptic kainate receptors carrying the iGluR5 subunit, by specific agonists acting at a presynaptic locus, reduces glutamate release from primary afferent sensory synapses (Kerchner et al., 2001a). In our study it is possible that the selective iGluR5 receptor agonist Iodowillardiine inhibits transmitter release from the pre-junctional site in a similar manner as with central primary afferents, and thus partially reduces CGRP release from the peripheral end of the trigeminal nerve. The absolute mechanism of regulation of neurotransmitter release by kainate receptors remains to be established (Kerchner et al., 2001a; Lerma, 2003). Previous studies on presynaptic kainate receptors in central brain synapses, indicate that strong activation of kainate

receptors leads to inhibition of glutamate release (Huettner, 2003; Lerma, 2003), possibly by inactivating calcium or sodium channels, or both (Kamiya and Ozawa, 2000). As release of both glutamate and CGRP from trigeminal neurons is controlled by calcium channels (Xiao et al., 2008), it is likely that strong activation of kainate receptors, which could be achieved by systemic administration of selective agonists, inactivates calcium channels and reduces neurotransmitter release. Metabotropic mechanisms have also been suggested to play a role in central presynaptic kainate receptor mediated reductions neurotransmitter (Negrete-Diaz et al., 2006). In the hippocampus, biphasic effects of kainate receptors have been reported, showing that weak activation enhances neurotransmitter release, and both ionotropic and metabotropic mechanisms have been suggested (Kamiya, 2002; Lerma, 2003; Rodriguez-Moreno and Sihra, 2004). Such bidirectional effects however, have not been reported for pre-synaptic kainate receptors on sensory fibres. Inhibition of glutamate and neuropeptides release following activation of kainate receptors is in agreement with the proposed role of AMPA and NMDA glutamate receptors on sensory fibres (Lee et al., 2002; Bardoni et al., 2004). The fact that blockade of the iGluR5 receptors with UBP 302 did not cause any baseline dilation or neurogenic induced dilation changes, could suggest that kainate receptors at the pre-junctional site are not tonically activated, but when selectively activated can regulate dural vessel diameter. UBP 302 is not degraded in the blood, so when co-applied with Iodowillardiine it blocked the Iodowillardiine induced inhibition of NDV. The possibility of iGluR5 activation by endogenous glutamate release and subsequent regulation of glutamate and CGRP release at the peripheral trigeminal end cannot be excluded (deGroot et al., 2000; Huettner et al., 2002).

Since ganglionic sympathetic efferents in normal rats largely do not express kainate receptors (Carlton et al., 1998; Coggeshall and Carlton, 1999), it is unlikely that the kainate receptor agonist caused norepinephrine or vasoconstrictor peptide release in our experiments. The fact that NDV is not affected by either α_1/α_2-adrenoreceptor agonists and antagonists, as well as β-adrenergic antagonists further illustrates that the adrenergic system does not play a significant role in the pharmacology of neurogenic trigeminovascular responses in the dura mater (Akerman et al., 2001). Our data are consistent with the common feature of compounds that successfully inhibit NDV with a likely effect on Aδ trigeminal afferents to inhibit the release of CGRP.

The selectivity of the compounds used over kainate receptors has been demonstrated on native and recombinant kainate receptors. Iodowillardiine showed high selectivity on native iGluR5 dorsal root ganglia kainate receptors (EC_{50} 140 nM) over hippocampal AMPA receptors (EC_{50} 19 mM) (Wong et al., 1994) and is one of the most potent kainate receptor agonists defined to date. Iodowillardiine potently activates homomeric iGluR5 and heteromeric iGluR5/KA2 kainate receptors but shows no activity on homomeric iGluR6 or iGluR7 kainate receptors and only weak activity on heteromers of iGluR6 and 7 with KA2 (Jane et al., 1997; Swanson et al., 1998). UBP 302 demonstrates similar affinity for homomeric and heteromeric iGluR5 kainate receptors, but very little affinity on iGluR6 kainate receptors (More et al., 2004). The demonstration that kainate receptors in trigeminal ganglia neurons are heteromeric iGluR5/KA2 assemblies (Sahara et al., 1997) further supports that the compounds used were selectively acting on iGluR5 carrying kainate receptors.

Results from this study support a role of iGluR5 receptor agonists as a potential approach to the development of anti-migraine therapies consistent with previous work (Filla et al., 2002; Sang et al., 2004; Weiss et al., 2006), although suggesting other possible mechanisms of action, as the anti-nociceptive effects in these models in which kainate receptors were blocked, are more likely due to at least central actions of the compounds used. In a recent clinical study the highly selective kainate receptor antagonist LY466195 was effective in aborting migraine attacks (reported by Johnson, KW International Headache Research Symposium, Copenhagen 2007), as well as inhibit trigeminovascular activation in an animal model (Weiss et al., 2006). Consistent with our data LY466195 failed to inhibit NDV as well as CGRP and capsaicin-induced dilation (Chan et al., 2009), further suggesting that kainate receptor antagonists do not exhibit their anti-nociceptive effects via peripheral mechanisms. In this context anti-nociceptive effects of iGluR5 activation have been previously reported, in which the iGluR5 specific agonist (RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid increased the hind paw withdrawal latency to thermal and mechanical stimulation in rats (Wu et al., 2003).

In vivo studies in which intravital microscopy techniques were used, showed that topical application on the cranial window of kainate or the iGluR5 specific agonist (RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid, could cause a small but significant dilation of the underlying dural vessels, an effect blocked by a nitric oxide

synthase inhibitor (Faraci et al., 1994) or by heme oxygenase (Robinson et al., 2002). Results from these studies suggest that kainate has no direct effect on dural blood vessels and that kainate-induced dilation on both dural and cerebral arterioles is due to the formation of nitric oxide or carbon monoxide from an extra-vascular source (Faraci et al., 1994; Robinson et al., 2002). In our studies both the iGluR5 receptor agonist and antagonist used at high doses by intravenous administration, had no effects on vessel dural tone at rest and their action was tested on neurogenic induced dilation, which involves low intensity brief electrical stimulation of the cranial window. These differences could be due to the different drugs used, as both UBP 302 and Iodowillardiine are highly selective for iGluR5 and derivatives from the same precursor, willardiine (Swanson et al., 1998; More et al., 2004), as well as due to the different technique that was applied. Furthermore, the absence of an effect on vessel tone by both willardiine derivatives used, is unlikely to be due to the anaesthetic as it is a well established regimen that has not been observed to alter blood vessel diameter or blood flow studies (Akerman et al., 2001; Akerman and Goadsby, 2005c; Holland et al., 2005). Using the same anaesthesia and methods other compounds have been shown to affect vascular tone (Akerman et al., 2001; Akerman and Goadsby, 2005c), and this is usually as a consequence of blood pressure changes. These observations suggest that pentobarbital itself has no actions on vascular tone.

Neurogenic dural vasodilation is an established model of trigeminal activation and has proved highly predictive of anti-migraine efficacy (Williamson et al., 1997c; Williamson et al., 1997b; Akerman and Goadsby, 2005b). As the method does not investigate the central component of the trigeminovascular system however, the efficacy of many anti-migraine compounds that act centrally may not be seen using this model. To our knowledge both the iGluR5 receptor agonist and antagonist used in our study have not been previously described in *in vivo* models and no data exists for blood-brain barrier penetration, although the existence of carboxylic groups on both compounds might suggest decreased lipophylicity of the molecules. The restricted blood brain barrier penetration of the agonist used, as suggested from its structure, makes it unlikely that the compound had any effects on central medullary nuclei that control cardiovascular reflexes. As NDV is based on local stimulation of peripheral trigeminal afferents and thus compounds that can inhibit the vasodilatory response have the potential to inhibit trigeminovascular nociceptive transmission at this site of action. This might explain the mode of action of iGluR5 receptor activation seen in the current study,

and this is believed to be the site of action of well characterized anti-migraine compounds that do not cross the blood-brain barrier (Williamson et al., 1997c). Since CGRP receptor antagonists are effective in acute migraine (Olesen et al., 2004b; Ho et al., 2008), the pharmacology of the control mechanisms for dural CGRP release by kainate receptors are of direct relevance to the development of newer migraine therapies. The intravital microscopy models, whilst helping to identify compounds that may be of therapeutic value in the clinic are also able to help us dissect the pharmacology of the trigeminovascular system, and therefore the mechanisms underlying migraine and the actions of therapeutic compounds.

The role of kainate receptors on trigeminal afferents under normal physiological conditions is not known, although studies of kainate receptors on other peripheral sensory afferents suggest an action of these receptors in detecting normal sensory responses and upon their activation they participate in peripheral nociceptive transduction (Agrawal and Evans, 1986; Ault and Hildebrand, 1993). A similar mechanism might exist on trigeminal afferents. Although direct evidence from animal models of migraine showing increased glutamate release in the periphery following trigeminovascular activation do not exist, increased glutamate release is seen following stimulation of Aδ and C fibres from peripheral sensory neurons (deGroot et al., 2000). These results indicate that stimulation of Aδ and C fibres can result in peripheral release of glutamate, providing a major source of ligand for the glutamate receptors localised on peripheral primary afferents (deGroot et al., 2000). This might account for trigeminal afferents as well, as during migraine attacks it has been shown that glutamate and glutamine levels are elevated in the plasma and in the CSF (Ferrari et al., 1990; Martinez et al., 1993; Alam et al., 1998). Lam et al, (2009) showed that peripheral application of glutamate activates trigeminal nociceptive afferents innervating craniofascial structures and can induce peripheral sensitization, reflected by changes to the mechanical activation threshold. It is remained to be determined whether peripheral kainate receptors on trigeminal afferents could be activated by the increased levels of glutamate under such abnormal conditions.

In conclusion, this study demonstrated that activation of the peripheral iGluR5 kainate receptors with the selective agonist Iodowillardiine is able to inhibit neurogenic dural vasodilation as monitored by intravital microscopy. This effect is likely to result from inhibition of pre-junctional release of CGRP from trigeminal neurons. From these data

kainate iGluR5 receptor agonists acting peripherally may be novel approaches to anti-migraine therapy, although their central effects on this receptor class require further studies with appropriate methods and compounds.

Chapter 4: Pre- and post-synaptic modulation of nociceptive dural input to the trigeminocervical complex via the iGluR5 kainate receptor

4.1 Introduction

Migraine pathophysiology involves activation, or the perception of activation of trigeminal afferents that innervate the meninges (Goadsby et al., 2002; Goadsby, 2007). The nociceptive inflow from the meninges to the spinal cord is relayed on second order neurons in the TCC, before ascending to higher brain areas. Stimulation of dural vessels produces Fos immunoreactivity, as well as increased neuronal activity in the trigeminal nucleus caudalis and the dorsal horn at the C1 and C2 spinal cord levels. Further to the convergent inputs from dural structures including the MMA, second order trigeminal neurons also receive inputs from the ophthalmic dermatome of the cutaneous receptive field, and the cervical afferents innervating the neck (Bartsch and Goadsby, 2002; Piovesan et al., 2003). This neural connectivity in the TCC plays a fundamental role in the perception of head pain, sensitivity to sensory information and allodynia of the facial skin seen during migraine attacks (Selby and Lance, 1960; Burstein et al., 2000; Goadsby et al., 2002). Thus an increased understanding of the mechanisms and the pharmacological profile of trigeminocervical second order neurons has major implications for the pathophysiology and treatment of migraine.

Trigeminocervical nociceptive transmission involves release of glutamate from primary afferents and there is substantial evidence that glutamate receptors are involved in trigeminovascular nociceptive processing (Vikelis and Mitsikostas, 2007). The presence of functional kainate receptors in DRG (Agrawal and Evans, 1986; Huettner, 1990), trigeminal ganglion (Sahara et al., 1997) and in both pre- and post-synaptic sites in superficial laminae of the spinal cord and caudal brainstem (Li et al., 1999; Hwang et al., 2001; Hegarty et al., 2007), indicate the importance of characterising their function in the TCC. Kainate receptors participate in post-synaptic sensory transmission in the spinal cord (Li et al., 1999) and regulate post-synaptic firing (Bortolotto et al., 1999) and pre-synaptic neurotransmitters' release (Kerchner et al., 2002). Growing evidence has further illustrated activation and modulation of kainate receptors by nociceptive stimuli (Ruscheweyh and Sandkuhler, 2002; Wu et al., 2007).

Competitive iGluR5-selective receptor antagonists were effective in reducing Fos expression in the TCC after stimulation of the trigeminal ganglion (Filla et al., 2002; Weiss et al., 2006), and along with the previous intravital study (chapter 3), the possibility of a central effect of kainate receptor antagonists acting directly on trigeminal

neurons in the TCC is raised. In the present study, we used electrophysiology in combination with microiontophoresis to investigate the potential *in vivo* effects of iGluR5 agents on second order neurons in the TCC, utilizing the willardiine-derived iGluR5 receptor antagonist UBP 302, in order to clarify to some degree the role of iGluR5 kainate receptors in trigeminovascular nociceptive processing (figure 44). The responses of neurons in the TCC were examined in relation to dural stimulation and nociceptive and non-nociceptive activation of the cutaneous facial receptive field.

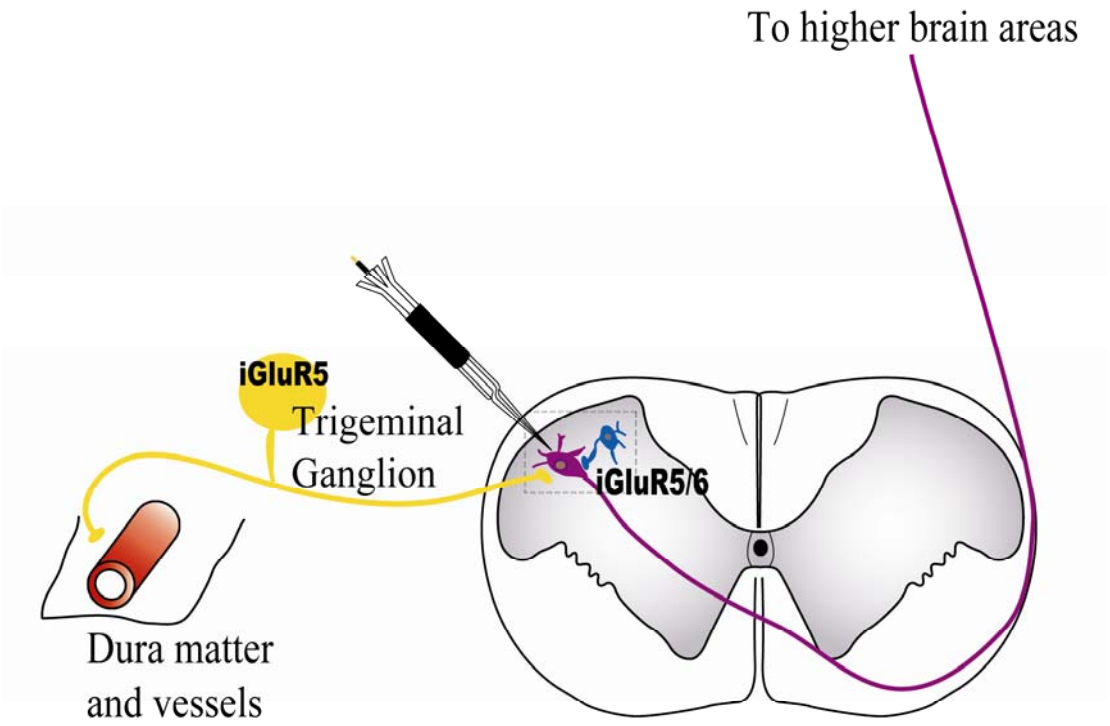


Figure 44: Electrophysiology of neurons in the trigeminocervical complex–microiontophoresis

Electrophysiological recordings were made from second order neurons in the trigeminocervical complex, which received convergent inputs from dural vessels and the ophthalmic dermatome of the facial skin.

4.2 Methods

4.2.1 Animals

Thirty six male Sprague-Dawley rats (300–370 g) were anesthetised with 60 $mgkg^{-1}$ pentobarbital intraperitoneally and then maintained with 10-20 $mgkg^{-1}h^{-1}$ α-chloralose made up with 2-hydroxy-β-cyclodextrin by intravenous injections. The surgical preparation was as described previously (section 2.3). Arterial blood gas parameters were measured regularly during the experiment and were within physiological limits.

4.2.2 Drugs and microiontophoresis

Seven-barrel carbon-fibre electrodes were used to deliver microiontophoretically freshly prepared solutions of glutamate receptor agents. For each electrode, the central barrel was filled with 200 mM NaCl for automated current balance. The test compounds used by microiontophoresis in this study were: the iGluR5 antagonist UBP 302, the unselective ionotropic glutamate receptors antagonist CNQX, saline control which represents the ejection of both Cl^- and OH^- ions, since the pH of the saline was adjusted by the addition of 0.01 M NaOH, the ionotropic glutamate agonists L-glutamate monosodium, Iodowillardiine, Fluorowillardiine, SYM 2081, NMDA. Pontamine sky blue dye was used for marking recording sites. All the iGluR tested compounds were chosen according to their pharmacological activity (table 17) and were ejected as anions and retained with small positive currents. Microiontophoretic barrels had resistances of 9-100 MΩ.

Table 17: Characteristics of test compounds used by microiontophoresis

	Polarity	Ejection current (nA)	pH	Pharmacological activity
L-glutamate	(-)	25 - 65	8.0	non-selective glutamate receptor agonist (Watkins and Jane, 2006)
SYM 2081	(-)	50 - 100	8.0	selective iGluR5, iGluR6 kainate receptors ligand (Jones et al., 1997)
Fluorowillardiine	(-)	10 - 20	8.0	selective iGluR1,2 and 4 AMPA receptor agonist (Jane et al., 1997)
Iodowillardiine	(-)	5 - 25	8.0	selective iGluR5 kainate receptor agonist (Jane et al., 1997; Swanson et al., 1998)
NMDA	(-)	10 - 20	8.0	selective NMDA receptor agonist (Watkins and Jane, 2006)
CNQX	(-)	20	8.0	potent AMPA/kainate receptor antagonist (Watkins and Jane, 2006) and weak antagonist at the glycine modulatory site on NMDA receptors (Lester et al., 1989; Watkins and Jane, 2006)
UBP 302	(-)	10, 20, 30, 40, 50, 100	8.0	selective iGluR5 kainate receptor antagonist (More et al., 2004)

SYM 2081, (2S,4R)-4-Methylglutamic acid; NMDA, *N*-methyl-D-aspartic acid; CNQX, -cyano-7-nitroquinoxaline-2,3-dione; UBP 302, (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrim idine-2,4-dione

4.2.3 Experimental protocol

Neurons were identified by their response to ophthalmic (V1) facial and corneal receptive field stimulation. Once neurons were identified, they were tested for a stable response to electrical MMA stimulation and an increased firing rate to all microintophorised glutamate agonists, as described in section 2.11.5. Neurons were selected based on the above criteria. Three baseline responses to MMA stimulation were collected 5 minutes apart. The glutamate agonists tested for each experiment were ejected in a random order and once five stable baseline cycles were recorded, then UBP 302 or vehicle control was ejected at increasing currents (10, 20, 30, 40, 50 and 100

nA), each current applied during one individual cycle of agonists ejection. A post-stimulus histogram was collected during each UBP 302 ejecting current and at the end of the 30 minutes recovery period. CNQX was applied at 20 nA over three cycles of glutamate agonists and a post-stimulus histogram was collected at the end of the third cycle. To assess the effects of the receptor antagonists on noxious and non-noxious stimulation of the cutaneous facial receptive field, light touch, noxious pinch and corneal brush were randomly applied during baseline cycles, during each UBP 302 (for all currents) or CNQX or vehicle control ejection and following the recovery period. At the conclusion of the receptor antagonist or vehicle control ejection, receptor agonist cycles were continued for 30 minutes and the neuronal responses were observed.

Upon termination of the experiment, the location of the recording site was established as described previously (section 2.11.6).

4.3 Results

4.3.1 Localisation and neuronal characteristics

A total of 61 units were studied from the TCC. Extracellular recordings were made from WDR neurons responding in a reversible excitatory manner to all glutamate agonists tested and to MMA electrical stimulation with latencies consistent with Aδ fibres (on average 8-12 ms). Overall cells were located at a depth of 489 ± 39 µm below the dorsal cervical spinal cord surface (figure 42). Cells tested for evoked responses to SYM 2081 (iGluR5/6 subunit agonist; $n = 35$) but not to Iodowillardiine were found in laminae III-IV. Cells tested for evoked responses to Iodowillardiine (iGluR5 agonist; $n = 31$) with or without evoked responses to SYM 2081 were found in superficial laminae (II-III). neuronal subpopulations did not elicit any differences in their response probability to MMA or receptive field stimulation ($P \geq 0.32$). Thus, no further analysis was contacted to further investigate these subpopulations.

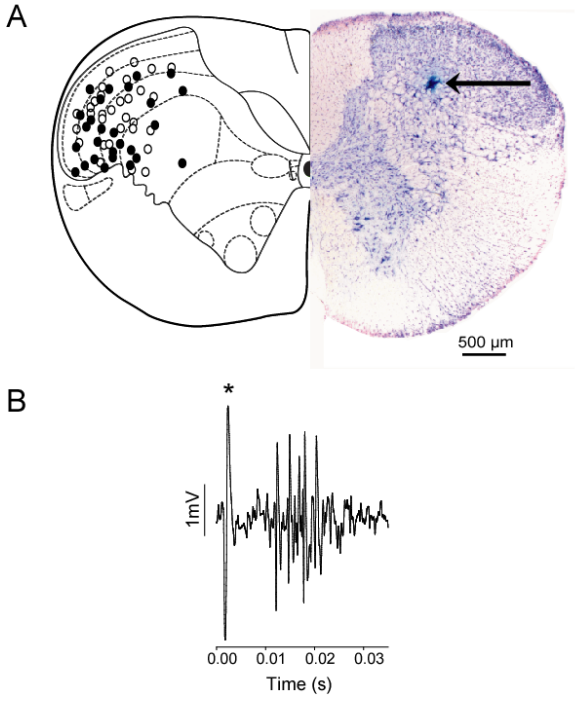


Figure 45: Localisation of recording sites within the trigeminocervical complex
A. Reconstruction of recording sites within the C1 spinal cord level, plotted after Paxinos and Watson (1997), indicating recording sites identified histologically (solid circles) and by microdrive readings (open circles). A photomicrograph demonstrating a recording site marked by ejection of pontamine sky blue (arrow) is shown. B. An original trace showing a cluster of cells in the TCC, firing in response to stimulation of the MMA. * stimulus artefact

4.3.2 Effect of iGluR5 kainate receptor antagonist UBP 302 on trigeminocervical neurons

The effects of UBP 302 at currents 10, 20, 30, 40, 50 and 100 nA were tested on a total of 27 WDR trigeminovascular neurons, and compared to the effects of saline at the same currents and duration (n = 15). The main body of the response to MMA stimulation was found to have a latency of 10-20 ms following the stimulus, representing Aδ fibres.

Effect of UBP 302 on middle meningeal artery-evoked stimulation

UBP 302 produced a differential response when microintophorised into the TCC. It significantly inhibited in a dose dependent manner evoked firing to MMA stimulation in 15 out of 27 neurons ($F_{3,35}$ = 13.70; P < 0.001; figure 46). The maximum recorded inhibition of 69% (t_{12} = 7.57; P < 0.001) was obtained at the highest ejection current of 100 nA. In 12 of the 27 neurons tested evoked responses to MMA stimulation were significantly facilitated ($F_{7,39}$ = 6.74; P < 0.001; figure 46). In some cases further to the facilitation observed from the Aδ fibres inputs, a decrease in the threshold to C fibre activation was also observed during application of UBP 302. The firing returned to baseline within 30 minutes in both groups (figure 46). Vehicle control ejection had no effect (n = 15; $F_{4,50}$ = 0.46, P = 0.75).

Based on the responses of the two groups of cells to UBP 302 ejection, these units were further analysed separately and will be referred to as the inhibitory group and facilitatory group.

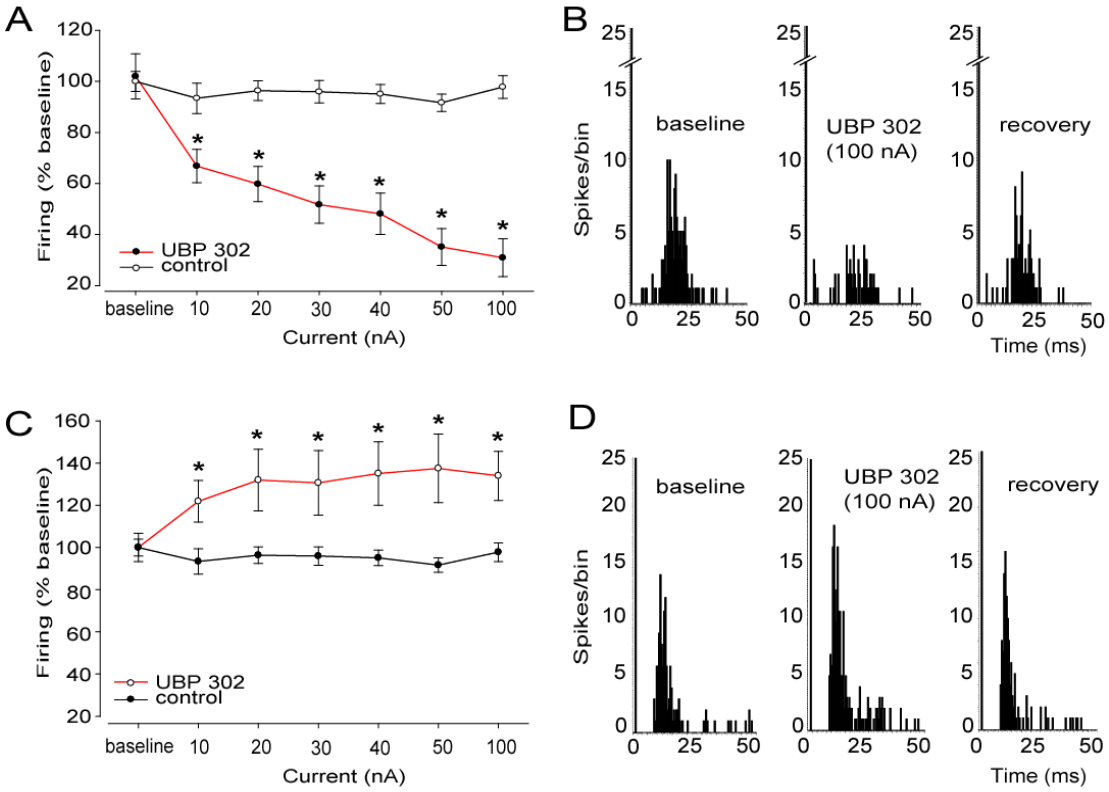


Figure 46: Effect of microiontophoretically administered UBP 302 on middle meningeal artery evoked firing

Ejection of UBP 302 produced two opposing responses. A. In 15 units ejection of UBP 302 demonstrated a dose dependent inhibition of the cell firing, maximally at 100 nA (inhibitory group). C. In 12 neurons UBP 302 significantly potentiated cell firing (facilitated group). Control vehicle had no effect. B, D. Post-stimulus histograms from a representative cluster of neurons in the two groups, recorded before UBP 302 ejection, during ejection of UBP 302 at 100 nA, and 30 minutes after the cessation of ejection. * $P < 0.05$

Effect of UBP 302 on glutamate receptor agonist evoked activity

For all agonists tested there was no difference across the mean firing of the five repeated epochs recorded during baseline (L-glutamate: $n = 33$, $F_{3,83} = 1.90$, $P = 0.14$; Fluorowillardiine: $n = 25$, $F_{2,40} = 0.34$, $P = 0.68$; SYM 2081: $n = 33$, $F_{2,78} = 2.08$, $P = 0.12$; Iodowillardiine: $n = 26$, $F_{3,78} = 0.24$, $P = 0.88$; NMDA: $n = 6$, $F_{4,20} = 1.68$, $P = 0.20$) and all responses were highly reliable (Cronbach's $\alpha \geq 0.95$).

The effect of UBP 302 on Fluorowillardiine evoked firing was tested in 17 units and UBP 302 failed to inhibit significantly neuronal firing across time ($F_{4,54} = 1.99$; $P = 0.12$; figure 47). Saline control ejections produced no significant effects across the pulses of Fluorowillardiine ($n = 8$; $F_{2,16} = 0.52$, $P = 0.63$). Based on the MMA stimulation evoked responses recorded during UBP 302 application, as previously described, 12 of the units were clustered in the inhibitory group and 5 were clustered in the facilitatory group. Neurons in both groups exhibited similar responses to UBP 302 ejection and there was no significant difference between these units (independent *t* test: inhibitory group vs facilitatory group; $P \geq 0.41$; table 18).

All neurons which displayed NMDA-evoked firing were grouped in the inhibitory group. UBP 302 ($n = 6$; $F_{3,13} = 2.75$, $P = 0.09$) or control ($n = 6$; $F_{2,9} = 1.36$, $P = 0.30$) did not alter firing in response to NMDA.

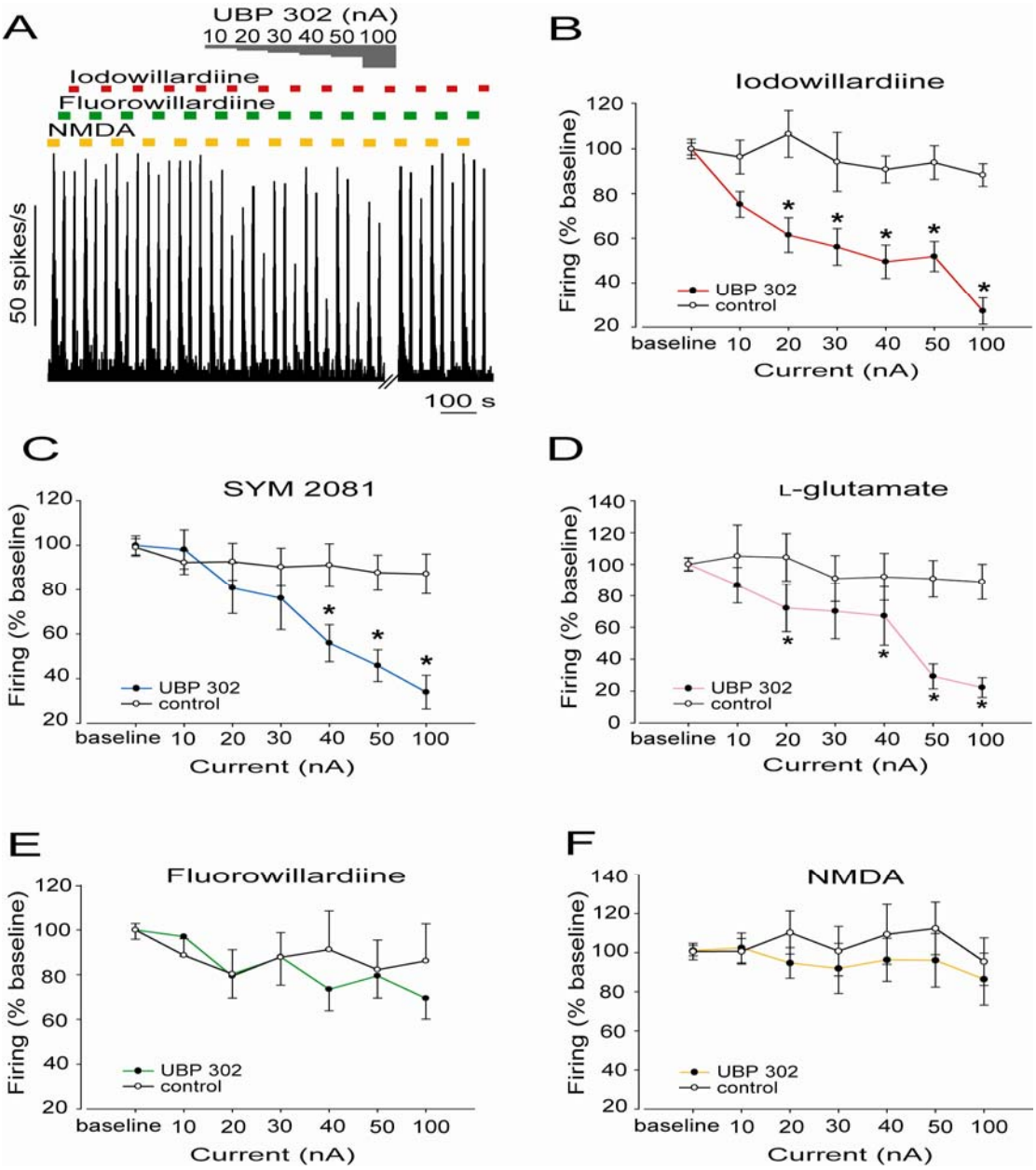


Figure 47: Effects of microiontophoretically delivered UBP 302 on the firing rates of second order neurons to pulsed ejections of the agonists L-glutamate, Iodowillardiine, Fluorowillardiine, NMDA and SYM 2081

A. Example of the effects of UBP 302 on the firing rates to pulsed ejections of Fluorowillardiine, Iodowillardiine and NMDA. Cell firing in response to the ejected agonists returned to baseline levels within 30 min after UBP 302 microiontophoresis ceased. B-F. Comparison of the current response curves for UBP 302 and control (Cl^- and OH^-) on Iodowillardiine (B), SYM 2081 (C), L-glutamate (D), Fluorowillardiine (E) and NMDA (F) evoked responses. B-D. Cell firing in response to Iodowillardiine (n = 19), SYM 2081 (n = 18) and L-glutamate (n = 18) was significantly inhibited by microiontophoretically administered UBP 302. E-F. Cell firing in response to Fluorowillardiine (n=17) and NMDA (n=6) was unaffected by microiontophoretically delivered UBP 302. Vehicle control had no significance on any agonist-evoked firing. * $P < 0.05$

Table 18: Effects of UBP 302 on the responses to Fluorowillardiine-evoked firing on the neurons of the inhibitory and facilitatory groups

	UBP 302 doses					
	10 nA	20 nA	30 nA	40 nA	50 nA	100 nA
Inhibitory group (n = 12) (paired t test vs baseline)	91± 10% (t_{11} = 0.86; P = 0.41)	78 ± 12% (t_{11} = 1.73; P = 0.11)	92 ± 17% (t_{11} = 0.50; P = 0.63)	74 ± 12% (t_{11} = 2.80; P = 0.02)	79 ± 13% (t_{11} = 1.97; P = 0.07)	71 ± 11% (t_{11} = 3.25; P = 0.01)
Facilitatory group (n = 5) (paired t test vs baseline)	113 ± 22% (t_4 = 1.13; P = 0.32)	82 ± 17% (t_4 = 0.73; P = 0.95)	74 ± 6% (t_3 = 4.41; P = 0.02)	72 ± 18% (t_3 = 0.52; P = 0.64)	81 ± 17% (t_3 = 0.91; P = 0.43)	66 ± 20% (t_3 = 0.77; P = 0.50)
independent t test (inhibitory vs facilitatory)	t(15) = 0.85; P = 0.41	t(15) = 0.25; P = 0.81	t(14) = 0.44; P = 0.67	t(14) = 0.11; P = 0.92	t(14) = 0.001; P = 1.0	t(14) = 0.33; P = 0.97

Comparison of the UBP 302 effects at different currents on neurons clustered in the inhibitory group and the facilitatory group. Neurons in both groups were not significantly sensitive to microiontophoretically administered UBP 302 and there was no difference between the two groups. Data are presented as the mean firing in percentage (± standard error of the mean) of baseline firing.

UBP 302 reversibly and significantly inhibited trigeminal firing in response to microintophorised Iodowillardiine and demonstrated dose dependency (n = 19; $F_{3,40}$ = 16.22; $P < 0.001$; figure 47). The inhibition reached its maximum at the higher ejection current of 100 nA (figure 47). Overall in all 19 units tested, UBP 302 significantly decreased the firing rate at 20, 30, 40, 50 and 100 nA ($P < 0.05$ for 30 nA; $P < 0.01$ for 20, 40 and 50 nA; $P < 0.001$ for 100 nA), while cell firing in response to control ejection of control produced no significant effects across the pulses of Iodowillardiine (n = 7; $F_{1,2}$ = 1.0, P = 0.42). Iodowillardiine evoked firing returned to baseline 15-30 minutes after UBP 302 ejection ceased. Based on the responses of the MMA evoked activity to UBP 302, 9 units displayed inhibitory responses and 10 were clustered in the facilitatory group. Iodowillardiine evoked firing was inhibited by UBP 302 in both groups during UBP 302 ejection and there was no significance between the two groups (independent t test: inhibitory group vs facilitatory group; $P \geq 0.41$; table 19).

Table 19: Effects of UBP 302 on the responses to Iodowillardiine evoked firing on the neurons of the inhibitory and facilitatory groups

	UBP 302 doses					
	10 nA	20 nA	30 nA	40 nA	50 nA	100 nA
Inhibitory group (n = 9) (paired t test vs baseline)	63 ± 9% (t_8 = 3.05; P < 0.05)	49 ± 13% (t_8 = 3.06; P < 0.05)	46 ± 12% (t_8 = 3.49; P < 0.01)	40 ± 12% (t_8 = 3.76; P < 0.01)	52 ± 11% (t_7 = 2.97; P < 0.05)	24 ± 8% (t_7 = 4.98; P < 0.01)
Facilitatory group (n = 10) (paired t test vs baseline)	86 ± 6% (t_9 = 2.86; P < 0.05)	73 ± 8% (t_9 = 3.37; P < 0.01)	65 ± 11% (t_9 = 3.41; P < 0.01)	59 ± 9% (t_9 = 4.56; P < 0.01)	52 ± 9% (t_8 = 4.73; P < 0.01)	30 ± 9% (t_8 = 6.38; P < 0.001)
independent t test (inhibitory vs facilitatory)	t(11) = 0.05; P = 0.96	t(17) = 0.59; P = 0.57	t(17) = 0.35; P = 0.73	t(13) = 0.52; P = 0.61	t(15) = 0.85; P = 0.41	t(15) = 0.59; P = 0.57

Comparison of the UBP 302 effects at different currents on neurons clustered in the inhibitory group and the facilitatory group. Neurons in both groups were significantly inhibited by microiontophoretically administered UBP 302 at all currents. Data are presented as the mean firing in percentage (± standard error of the mean) of baseline firing.

SYM 2081 evoked firing was reversibly reduced by UBP 302 in a dose dependent manner (n = 18; $F_{3,46}$ = 9.15; P < 0.001; figure 47) and in a related way as with Iodowillardiine evoked activity, especially at high currents (independent t test Iodowillardiine vs SYM 2081: P > 0.05). In all 18 units tested, UBP 302 significantly decreased the firing rate at 40, 50 and 100 nA (P < 0.05 for 40, 50 and 100 nA). Saline control ejection had no effects on SYM 2081 evoked firing (n = 15; $F_{2,19}$ = 1.78; P = 0.20). UBP 302 reduced firing in both inhibitory (n = 9) and facilitatory (n = 9) grouped cells with no significant difference between the two groups (table 20).

Table 20: Effects of UBP 302 on the responses to SYM 2081-evoked firing on the neurons of the inhibitory and facilitatory groups

	UBP 302 doses					
	10 nA	20 nA	30 nA	40 nA	50 nA	100 nA
Inhibitory group ($n = 9$) (paired t test vs baseline)	100 ± 13% ($t_8 = 0.90$; $P = 0.39$)	76 ± 19% ($t_8 = 1.08$; $P = 0.31$)	69 ± 26% ($t_8 = 1.17$; $P = 0.28$)	39 ± 10% ($t_8 = 2.94$; $P < 0.05$)	35 ± 8% ($t_8 = 3.28$; $P < 0.05$)	24 ± 7% ($t_8 = 3.92$; $P < 0.05$)
Facilitatory group ($n = 9$) (paired t test vs baseline)	91 ± 12% ($t_8 = 0.65$; $P = 0.54$)	82 ± 11% ($t_8 = 1.45$; $P = 0.19$)	81 ± 9% ($t_8 = 1.52$; $P = 0.17$)	72 ± 9% ($t_8 = 3.03$; $P < 0.05$)	56 ± 10% ($t_8 = 4.76$; $P < 0.05$)	45 ± 12% ($t_8 = 4.90$; $P < 0.05$)
independent t test (inhibitory vs facilitatory)	$t(16) = 0.62$; $P = 0.55$	$t(16) = 0.84$; $P = 0.42$	$t(16) = 0.98$; $P = 0.34$	$t(16) = 2.44$; $P < 0.05$	$t(16) = 1.95$; $P = 0.69$	$t(16) = 1.80$; $P = 0.91$

Comparison of the UBP 302 effects at different currents on neurons clustered in the inhibitory group and the facilitatory group. Neurons in both groups were significantly inhibited by microiontophoretically administered UBP 302 at 40, 50 and 100 nA. Data are presented as the mean firing in percentage (± standard error of the mean) of baseline firing.

L-glutamate evoked firing was significantly decreased by UBP 302 ejection ($n = 18$; $F_{3,40} = 3.64$; $P < 0.05$; figure 47). The antagonist was able to reduce significantly and reversibly the probability of neuronal firing in response to L-glutamate ejection when compared to control ($P < 0.05$ for 20 and 40 nA; $P < 0.001$ for 50 and 100 nA). UBP 302 was able to reduce firing on both inhibitory and facilitatory grouped cells in a similar fashion (independent t test: inhibitory group vs facilitatory group: $P > 0.05$ for 10, 30, 40, 50 and 100 nA; $P < 0.05$ for 20 nA; table 21). Compared to baselines, cells clustered in the inhibitory group were significantly inhibited by UBP 302 at all currents, whereas neurons clustered in the facilitatory group were significantly inhibited by UBP 302 only at higher currents (table 21). Microintophorised controls did not affect cell firing in response to L-glutamate ejection ($n = 15$; $F_{3,35} = 2.01$, $P = 0.13$).

Table 21: Effects of UBP 302 on the responses to L-glutamate-evoked firing in the inhibitory and facilitatory groups

	UBP 302 doses					
	10 nA	20 nA	30 nA	40 nA	50 nA	100 nA
Inhibitory group (n = 9) (paired t test vs baseline)	65 ± 12% (t_8 = 3.53; P <0.05)	69 ± 25% (t_8 = 2.54; P < 0.05)	59 ± 21% (t_8 = 2.10; P = 0.07)	58 ± 28% (t_8 = 2.59; P < 0.05)	37 ± 14% (t_7 = 3.86; P < 0.05)	22 ± 10%[4] (t_7 = 4.95; P < 0.05)
Facilitatory group (n = 9) (paired t test vs baseline)	101 ± 18% (t_8 = 0.32; P = 0.76)	69 ± 16% (t_8 = 1.42; P = 0.19)	83 ± 26% (t_8 = 1.75; P = 0.12)	70 ± 23% (t_8 = 2.20; P = 0.06)	26 ± 6% (t_8 = 5.46; P < 0.05)	38 ± 17% (t_8 = 3.81; P < 0.05)
independent t test (inhibitory vs facilitatory)	t(16) = 1.82; P < 0.05	t(10) = 2.29; P = 0.15	t(16) = 1.57; P = 0.15	t(16) = 1.71; P = 0.11	t(12) = 1.34; P = 0.21	t(10) = 2.12; P = 0.06

Comparison of the UBP 302 effects at 10, 20, 30, 40, 50 and 100 nA on neurons clustered in the inhibitory group and the facilitatory group. Neurons in the inhibitory group were significantly inhibited by microiontophoretically administered UBP 302 at a variety of currents. Neurons in the facilitatory group were significantly inhibited only at high currents compared to baseline; however there was no difference between the two groups. Data are presented as the mean firing in percentage (± standard error of the mean) of baseline firing.

Effect of UBP 302 on receptive fields

The effects of UBP 302 on responses to innocuous (brush) and noxious (pinch and corneal brush) mechanical stimulation of the V1 facial cutaneous receptive field were tested in 26 WDR neurons (figure 48). Based on the responses of the MMA evoked activity to UBP 302 14 of the neurons recorded were clustered in the inhibitory group and 12 were clustered in the facilitatory group, and neurons were analysed separately based on their group.

Microiontophoretic ejection of UBP 302 had no effect on firing rate in response to non-noxious stimulation of the V1 ophthalmic cutaneous area ($n = 26$; $F_{2,53} = 2.35$, $P = 0.10$) and there was no difference between the inhibitory and facilitatory groups ($P \geq 0.07$).

UBP 302 significantly reduced in a dose-dependent manner evoked firing to both pinch and corneal brush stimulation in all neurons clustered in the inhibitory group (pinch: $F_{3,30} = 11.09$, $P < 0.001$; corneal: $F_{3,31} = 10.16$, $P < 0.001$), and noxious stimulation evoked firing returned to baseline within 30 minutes after the occlusion of UBP 302 ejection. Evoked responses to noxious stimuli were unchanged by UBP 302 in the cells that exhibited facilitation of the MMA (pinch: $F_{7,36} = 0.76$, $P = 0.53$; corneal: $F_{3,61} = 1.03$, $P = 0.38$). For both responses to pinch and corneal stimulation, there was a significant difference between the inhibitory and facilitatory groups ($P < 0.05$).

Vehicle control ejection at the same currents and duration did not change the firing rate evoked by innocuous ($F_{3,34} = 1.05$; $P = 0.39$) and noxious (pinch: $F_{7,37} = 0.62$; $P = 0.63$; corneal: $F_{2,24} = 1.16$; $P = 0.34$) stimulation of receptive field.

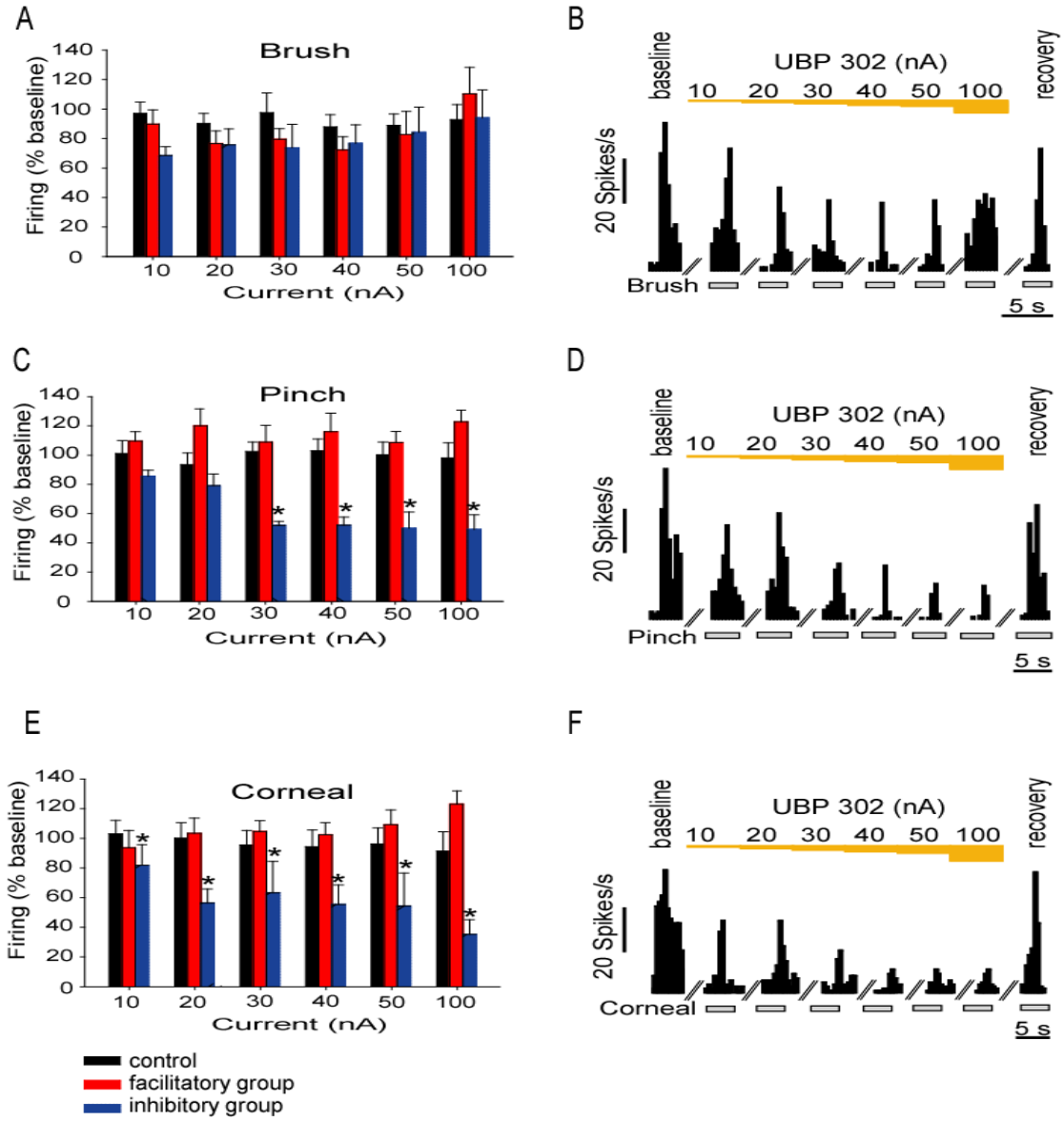


Figure 48: Effects of microiontophoretically delivered UBP 302 on the firing rates of second order neurons in response to receptive field characterisation

A. Cell firing in response to light brush was inhibited by UBP 302 at low currents in the inhibitory group. C, E Cell firing in response to pinch (C) and to corneal stimulation (E) were significantly inhibited by UBP 302 in the inhibitory group, but noxious evoked responses to both pinch and corneal stimulus was unchanged by UBP 302 in the facilitatory group. Control ejection at the same currents and duration did not change the firing rate evoked by innocuous and noxious stimulation of receptive field. Data represent the mean firing in percentage (± standard error of the mean) of baseline firing. * $P < 0.05$

B, D, F. Example of the effects of UBP 302 on the firing rates to light brush (B), pinch (D) and corneal brush (F) from a representative neuron clustered in the inhibitory group. Cell firing returned to baseline levels within 30 min after UBP 302 microiontophoresis stopped. Break lines indicate the period during which cell firing in response to stimulation of the middle meningeal artery and to ejected glutamate agonists was tested.

4.3.3 Effects of CNQX on trigeminocervical neurons

To test if the facilitatory effects of UBP 302 were due to unselective binding of UBP 302 to other iGluR subunits, the unselective iGluR antagonist CNQX was microintophorised on WDR neurons that displayed increased firing rate in response to random ejection of L-glutamate, NMDA and SYM 2081 and received inputs from the dura mater after electrical stimulation of the MMA. Due to the high potency of CNQX, the antagonist was microejected at a current of 20 nA over three repeated cycles of the agonists. The effects of CNQX were tested on a total of 10 neurons, and compared to the effects of saline at the same current and duration ($n = 9$).

Effect of CNQX on middle meningeal artery-evoked stimulation

Application of CNQX resulted in significant inhibition of MMA electrical stimulation to 43 ± 13% compared to baseline levels ($n = 10$; $t_9 = 3.97$; $P < 0.005$) and control ($t_{16} = 4.21$; $P < 0.005$), and this effect was ubiquitous among all cells (figure 49). Control ejection at the same duration did not affect responses to MMA stimulation ($n = 9$; $F_{2,16} = 0.74$, $P = 0.49$).

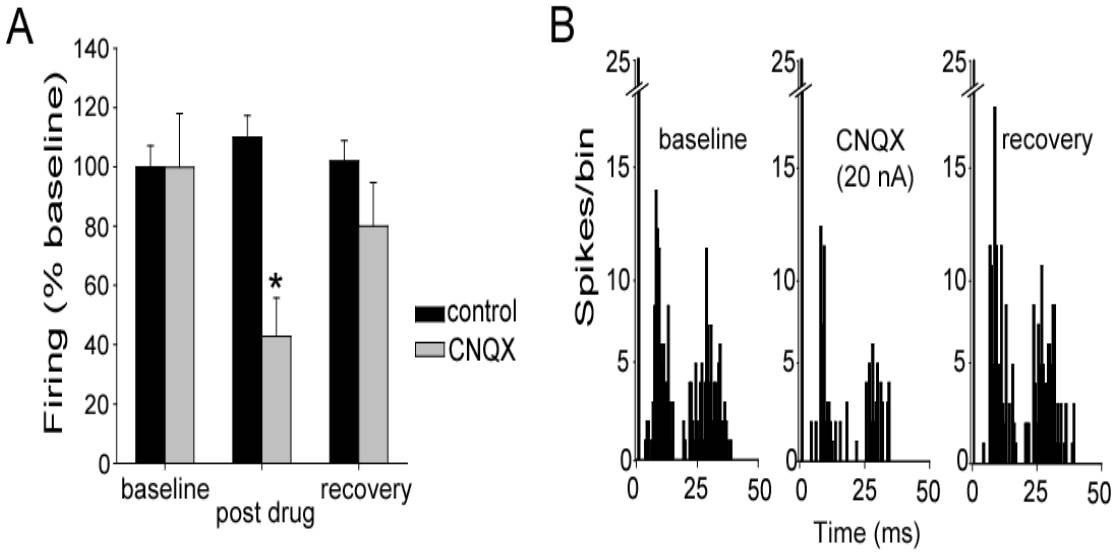


Figure 49: Effect of microiontophoretically administered CNQX on middle meningeal artery evoked firing

A. Ejection of CNQX significantly inhibited cell firing in response to MMA stimulation, returning to baseline 30 minutes after CNQX ejection stopped. Vehicle control had no effect. B. Post-stimulus histograms from a representative neuron. The post stimulus histograms were recorded before CNQX ejection, during ejection of CNQX and 30 minutes after ejection. * $P < 0.05$

Effect of CNQX on glutamate agonists-evoked activity

The baseline responses to glutamate receptor agonist application demonstrated good stability and there was no difference across the mean firing of the five repeated epochs (L-glutamate: $n = 19$, $F_{3,46} = 1.62$, $P = 0.20$; NMDA: $n = 19$, $F_{4,72} = 0.53$, $P = 0.72$; SYM 2081: $n = 19$, $F_{2,32} = 0.26$, $P = 0.75$). Responses for all the glutamate agonists across time demonstrated significant reliability (Cronbach's $\alpha \geq 0.92$).

Cell firing in response to L-glutamate, SYM 2081 and NMDA was significantly reduced by CNQX and due to the increasing inhibition that CNQX produced during each cycle, each one of the three cycles was analysed individually (figure 50). CNQX inhibited L-glutamate evoked responses to 16 ± 9% ($t_7 = 5.50$, $P < 0.005$) and abolished SYM 2081 evoked firing ($t_7 = 4.21$, $P < 0.005$). NMDA evoked firing was significantly reduced to a lesser degree than to L-glutamate and SYM 2081 ($F_{4,28} = 10.96$, $P < 0.001$). Ejection of

Cl^- and OH^- ions at the same current as the CNQX, had no significant effect on glutamate receptor agonist evoked firing ($n = 9$; $P \geq 0.12$).

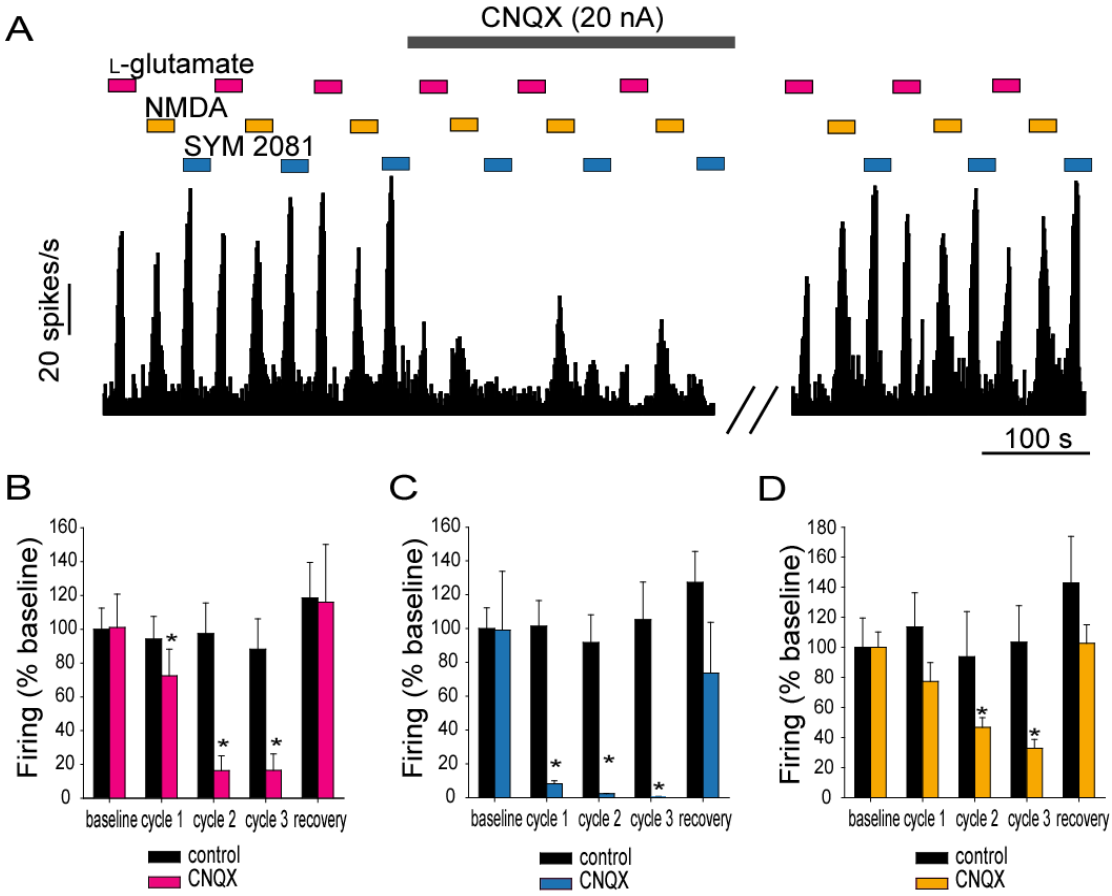


Figure 50: Effects of microiontophoretically delivered CNQX on the firing rates of second order neurons to pulsed ejections of the glutamate receptor agonists L-glutamate, NMDA and SYM 2081

A. Example of the effects of CNQX on the firing rates to pulsed ejections of the L-glutamate, Iodowillardiine and SYM 2081. Cell firing in response to the ejected agonists returned to baseline levels within 30 min after CNQX microiontophoresis ceased. B-E. Effects of CNQX on cell firing in response to L-glutamate (B), SYM 2081 (C) and NMDA (D), separately for each cycle. CNQX strongly inhibited L-glutamate ($n = 8$) and SYM 2081($n = 8$) evoked firing. NMDA-evoked firing ($n = 8$) was significantly reduced to a lesser degree compared to L-glutamate and SYM 2081. Ejection of control ($n = 9$) had no significant effects on glutamate agonists-evoked firing. * $P < 0.05$

Effect of CNQX on receptive fields

CNQX significantly inhibited both non-noxious receptive fields to 24 ± 7% (t_9 = 2.9; P < 0.05) and pinch (noxious) evoked firing to 40 ± 9% (t_9 = 5.7; P < 0.005) and to a lesser degree noxious corneal mechanical stimulation 45 ± 17% (t_4 = 2.72; P = 0.053; figure 51). No facilitation was observed on any of the neurons tested with CNQX. Ejection of control did not produce any inhibition (n = 9; $P \geq 0.5$).

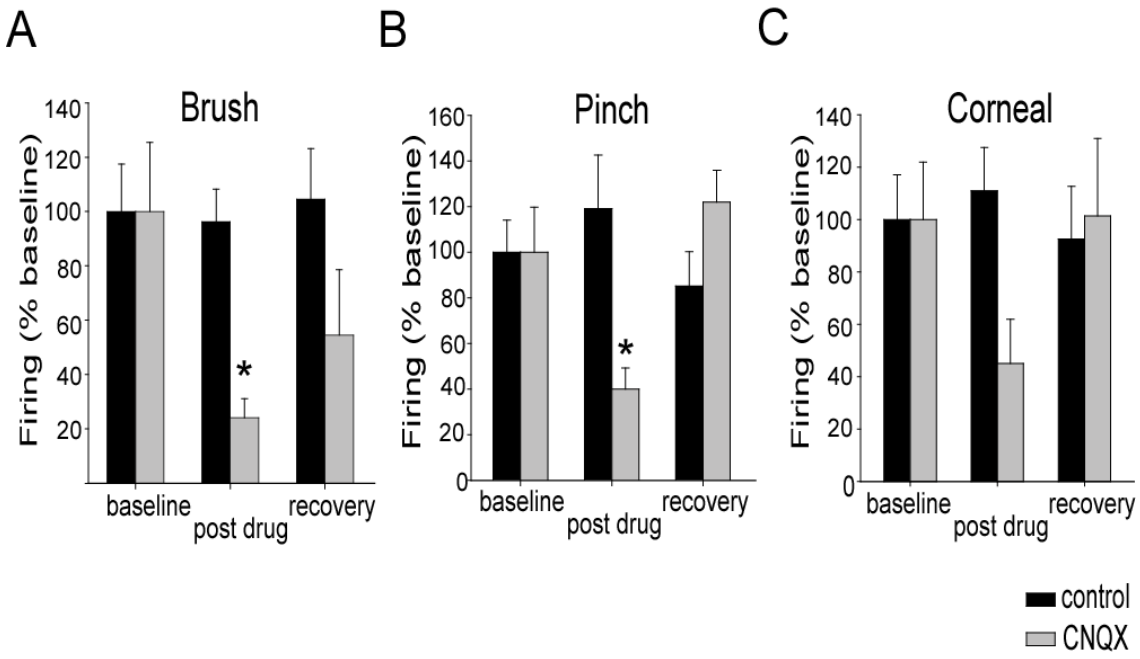


Figure 51: Effects of microiontophoretically delivered CNQX on the firing rates of second order neurons to receptive field characterisation

A-C. Comparison of the current-response charts for CNQX and saline on the cell firing in response to light brush (A), pinch (B) and corneal (C). CNQX significantly inhibited both non-noxious and noxious pinch receptive field and to a lesser degree noxious corneal mechanical stimulation. No inhibition was observed during ejection of control. * P < 0.05

4.4 Conclusions

The current study provides evidence that kainate receptors in the TCC can regulate trigeminovascular transmission, probably pre- and post- synaptically, and this is the first study, to our knowledge, to suggest a pre-synaptic modulatory role for spinal kainate receptors at the level of individual neurons *in vivo*. By using specific agonists the study demonstrated the presence of post-synaptic kainate receptors in the TCC. Selective antagonism of iGluR5 receptors was shown to result in inhibition of trigeminovascular processing in some synaptic locations, which was also accompanied by inhibition of cell firing in response to noxious mechanical stimulation of the ophthalmic cutaneous receptive field (figure 52). In a further neuronal subpopulation, ejection of the selective receptor antagonist UBP 302 facilitated trigeminovascular processing, perhaps by differentially targeting iGluR5 receptors at pre-synaptic locations (figure 52). In contrast to the dual effects of UBP 302, application of CNQX resulted only in inhibition of nociceptive transmission in the TCC.

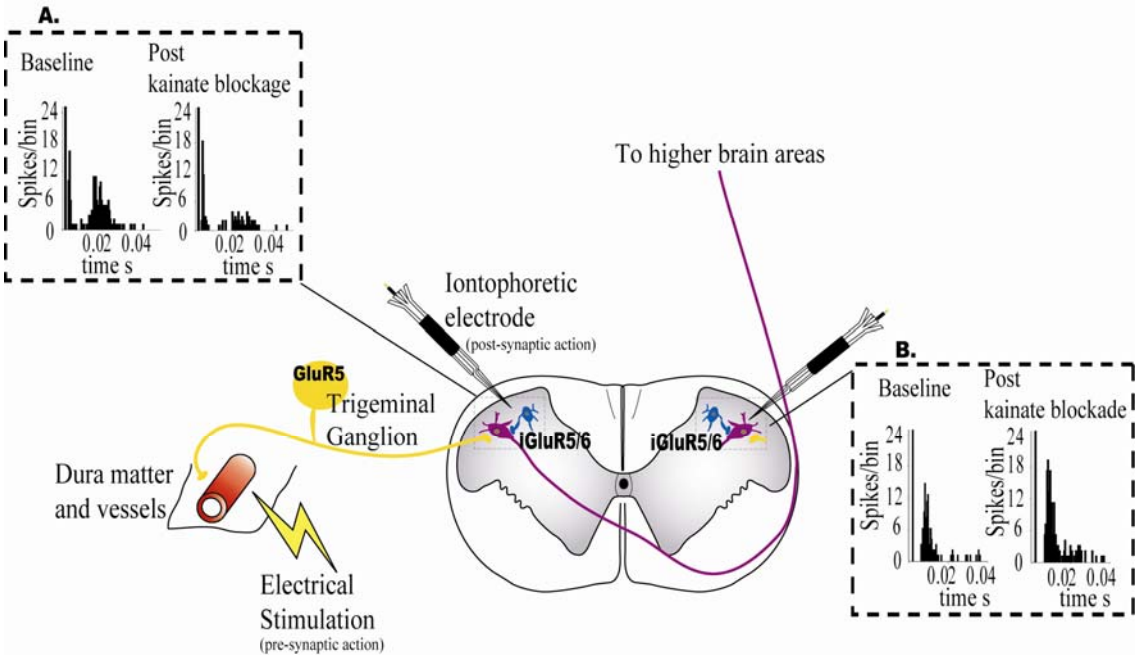


Figure 52: Overview of the effects of microintophorised UBP 302 on second order trigeminovascular neurons

Selective antagonism of iGluR5 receptors was shown to result in inhibition of trigeminovascular processing in some synaptic locations (A), and in facilitation of trigeminovascular processing in a different neuronal subpopulation (B).

By using selective receptor agonists and the selective iGluR5 receptor antagonist UBP 302, the study has demonstrated the presence of post-synaptic kainate receptors in the TCC and particularly the presence of functional iGluR5 kainate receptors. Recent anatomical evidence, demonstrated the presence of different kainate subunit mRNA in cultured spinal neurons (Dai et al., 2002) and the localisation of all subunits at both pre- and post- synaptic sites in the rat dorsal horn by confocal and electron microscopy (Lu et al., 2005). Further to the latest anatomical studies, our data are in agreement with electrophysiological evidence showing activation of spinal cord neurons by kainate (Li et al., 1999; Kerchner et al., 2001a). The present evidence of selective activation of iGluR5 receptors in dorsal horn neurons is in contrast to studies implying that kainate receptors in the spinal cord were predominantly insensitive to the iGluR5 agonist ATPA (Kerchner et al., 2001a). It is possible that prolonged activation of kainate receptors as described in Kerchner's study (2001a), converts agonist actions into antagonist effects (Chittajallu et al., 1999). In our studies, we used the receptor agonist Iodowillardiine which has a higher selectivity over iGluR5 subunits than ATPA (Swanson et al., 1998) and for the microejection period used no desensitisation was observed. As shown in dorsal horn neurons from iGluR5 or iGluR6 knockout mice, the iGluR5 subunit is expressed in these neurons and probably forms heteromeric assemblies with the iGluR6 subunit (Kerchner et al., 2002). Consistent with the anatomical evidence on the presence of kainate receptors, reconstruction of the recording sites in our studies provided evidence for the presence of iGluR5/6 subunits in superficial laminae of the TCC. In our studies, in experiments in which only SYM 2081 (iGluR5/6 agonist) was used as a kainate agonist- in addition to the NMDA and AMPA agonists or L-glutamate used, cells with evoked responses to SYM 2081 were mainly found in laminae III-V. In experiments in which Iodowillardiine was used as a kainate agonist (iGluR5 agonist), cells with evoked responses to Iodowillardiine were found in more superficial laminae. In consideration of the laminae localisation of the iGluR5-preferentially activated neurons, it is plausible that iGluR5 receptors could be found mostly in superficial laminae (inner lamina II and III). However the fact that cells activated by SYM 2081 were found at a greater depth (laminae III-V) indicates that the kainate evoked responses from these neurons could at least partly count for iGluR5/6 kainate receptors and not exclusively for the iGluR5 subunit. However, the experimental methods do not allow for a quantitative analysis of the presence of different subunits. These subpopulations did not elicit any differences in their response probability to MMA or receptive field stimulation ($P \geq 0.32$) and equally inhibitory and facilitatory responses were recorded

from these neurons. Thus, no further analysis was contacted to further investigate these subpopulations.

The selectivity of UBP 302 for iGluR5 kainate receptors over AMPA and NMDA receptors was proven by its failure to block Fluorowillardiine-evoked firing in the same neurons where responses to kainate agonists' were inhibited. Further to the blockade of kainate receptor evoked firing, UBP 302 was able to block partially L-glutamate evoked responses suggesting that kainate receptors carrying the iGluR5 subunit could possibly participate in the generation of post-synaptic currents (Lee et al., 1999; Li et al., 1999; Kerchner et al., 2001a). The inhibition of the noxious stimulation evoked firing is possibly produced by the post-synaptic inhibition of iGluR5 kainate receptors on these neurons. Whole-cell currents in dorsal horn neurons are significantly reduced in iGluR6 knockout mice, suggesting a prominent role for this subunit in the assembly of functional kainate receptors that also include the iGluR5 subunit (Kerchner et al., 2002). In the current study blockade of the iGluR5 kainate receptor in a subpopulation of neurons, reduced post-synaptic firing in response to trigeminovascular activation, suggesting an important contribution of the iGluR5 subunit in post-synaptic sensory neurotransmission on second order neurons, at least at the level of TCC.

It has been shown that high intensity, low frequency stimulation of the dorsal root nerve, which is sufficient to activate Aδ and C fibres, activates post-synaptic kainate receptors on spinal dorsal horn neurons. In contrast, low intensity stimulation that activates only non-nociceptive primary fibres is insufficient (Li et al., 1999). This suggests that kainate receptors are post-synaptically placed, largely at synapses that receive inputs from nociceptive primary afferent fibres, although an opposing study showed no significant contribution of kainate receptors to C fibre-evoked excitatory post-synaptic currents (Dahlhaus et al., 2005). In our study, in the same subpopulation of neurons where trigeminovascular activation was inhibited by UBP 302, the iGluR5 selective antagonist preferentially inhibited noxious over non-noxious receptive field responses. This may further support the hypothesis that innocuous sensory transmission is not mediated by iGluR5 kainate receptors on WDR neurons in the TCC.

Interestingly, the iGluR5 receptor selective antagonist in addition to the inhibition of trigeminovascular activation also facilitated trigeminovascular processing in a subset of neurons. These facilitated responses were accompanied by reduction in responses to

selective kainate receptor agonists, while responses of the same neurons to Fluorowillardiine were unaffected. As the post-synaptic kainate evoked currents were inhibited by UBP 302, it is unlikely that the observed facilitation was due to non-binding modality phenomena of the UBP 302. The post-synaptic inhibition of Iodowillardiine/SYM 2081-evoked firing further suggests that the observed facilitation was due to post-synaptic activation of other excitatory receptors, by pre-synaptically released excitatory neurotransmitters. The increased released of excitatory neurotransmitters has likely occurred during noxious stimulation, and thus UBP 302 failed to inhibit noxious evoked firing in these neurons. The classical iGluR receptor antagonist CNQX produced only inhibition of cell responses to glutamate receptor agonists, receptive field mechanical stimulation and MMA electrical stimulation, so it is unlikely that the facilitatory effects of UBP 302 were due to unselective actions of the antagonist on other iGluR receptors.

It has been shown that iGluR5 subunits are expressed in the trigeminal ganglion (Sahara et al., 1997) and iGluR5 subunits are pre-synaptically located on primary afferents in the dorsal spinal cord, as well as on GABAergic interneuronal terminals (Lu et al., 2005). Although multiple mechanisms by which kainate receptors modulate cerebral neurotransmission have also been described (Kullmann, 2001), it is believed that in the spinal cord pre-synaptic kainate receptors on primary afferents act as autoreceptors and occlude further glutamate release when activated (Kerchner et al., 2001a) (figure 19). When kainate heteroreceptors on GABAergic terminals are activated by glutamate spillover from neighbouring excitatory synapses they occlude GABA/glycine release via a complex mechanism which involves activation of $GABA_B$ autoreceptors (Kerchner et al., 2001b) (figure 19), and thus promote a possible pro-nociceptive mechanism in the spinal cord. Based on the in vitro observations by Kerchner and his colleagues (2002), the facilitation observed in a subpopulation of trigeminovascular neurons by antagonism of iGluR5 kainate receptor is more likely to be due to blockade of pre-synaptic kainate autoreceptors, resulting in enhanced glutamate release in response to electrical stimulation of the Aδ and C fibres surrounding the MMA. The released glutamate could then act on "non-kainate" GluRs, resulting in facilitated sensory transmission. Given that in these neurons post-synaptic kainate evoked firing was reduced by UBP 302, it is also likely that post-synaptic iGluR5 kainate receptors do not mediate sensory

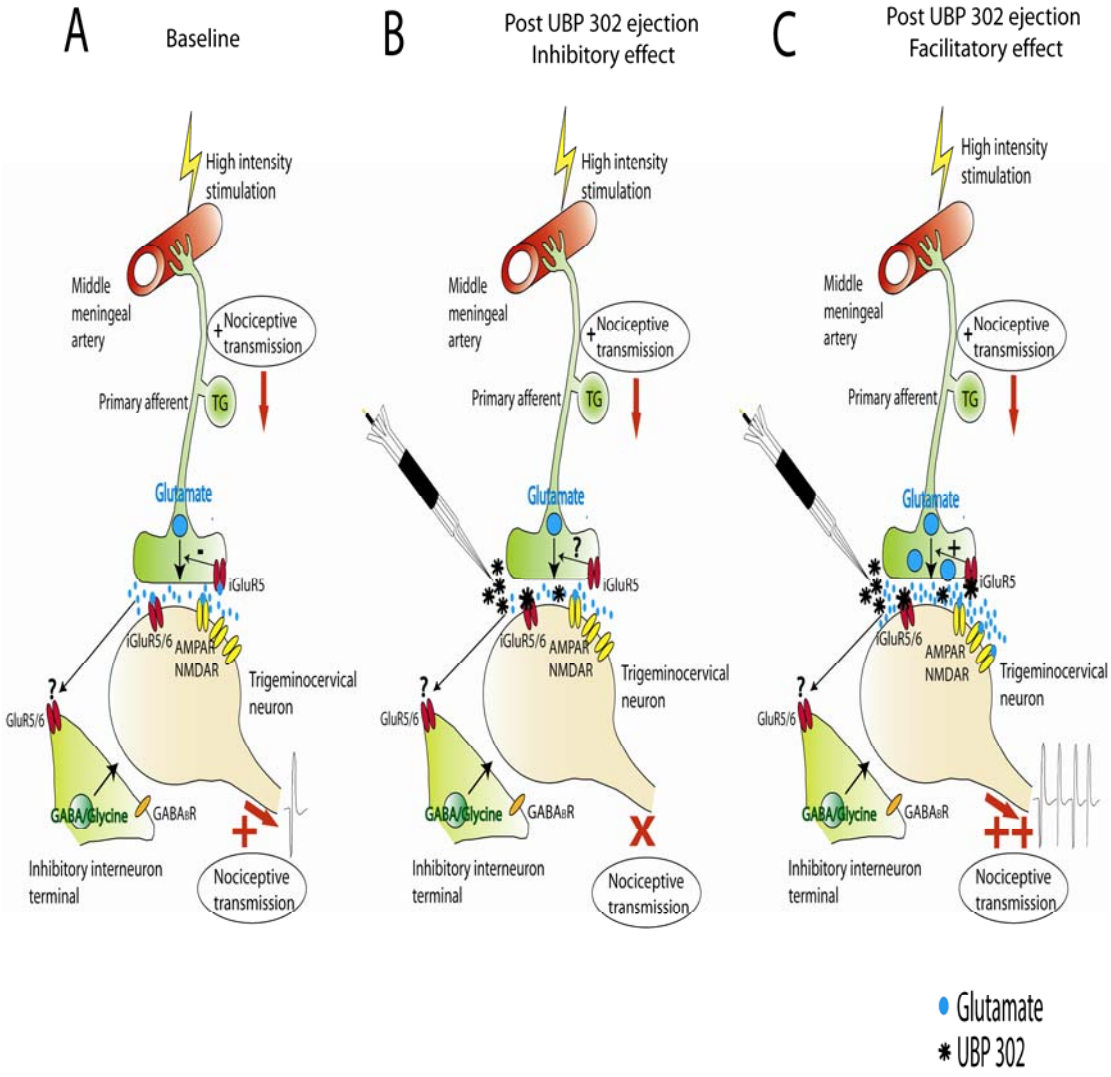


Figure 53: Proposed mechanism of action of microintophorised UBP 302 on trigeminovascular activation

A. During baseline electrical stimulations of the middle meningeal artery, activation of primary afferents innervating the adjacent dura causes glutamate release. Glutamate activates post-synaptic kainate receptors in addition to AMPA and NMDA receptors activation, promoting nociceptive transmission from second order neurons in the trigeminocervical complex. Pre-synaptic kainate receptors on primary afferents could be activated by glutamate and control glutamate release, whereas activation of kainate receptors on inhibitory interneurons is also possible. B. Local application of UBP 302 by microiontophoresis can selectively block post-synaptic kainate receptors and inhibit nociceptive transmission, whereas blockade of pre-synaptic kainate receptors might not occur, or its effects are less important. C. Local application of UBP 302 by microiontophoresis can selectively block post-synaptic kainate receptors. In addition blockade of pre-synaptic kainate receptors might inhibit the negative control feedback of glutamate release by kainate receptors. This results in increased glutamate release, which by acting on a bigger scale on AMPA and NMDA receptors facilitates nociceptive transmission. Further more it is also possible that post-synaptic iGluR5 kainate receptors do not mediate sensory transmission in all second order neurons.

transmission in all second order neurons. With the current known action of kainate receptors on inhibitory interneurons, it is unlikely to explain the observed facilitation as a plausible action of UBP 302 on kainate heteroreceptors (figure 53).

The role of kainate receptors in trigeminovascular processing has been relatively obscure due to the lack of selective antagonists. Although it has been shown that the so called non-NMDA receptors are involved in trigeminocervical activation (Mitsikostas et al., 1999; Storer and Goadsby, 1999), kainate receptors have only recently been linked with migraine headache (Filla et al., 2002; Weiss et al., 2006). Inhibition of neurons that modulate sensory input in the TCC is a plausible target for the development of anti-migraine compounds. The inhibition of trigeminovascular neurons from both UBP 302 and the classical AMPA/kainate antagonist CNQX are in agreement with the positive clinical results from the use of the AMPA/kainate receptor antagonist LY293558 (Sang et al., 2004) and the iGluR5 selective antagonist LY466195 (oral presentation by Johnson KW, International Headache Research Seminar, Copenhagen, 25 March 2007). However, the facilitated trigeminovascular processing observed in some neurons raises the question of whether the TCC is the site of action of the clinically active kainate compounds. The presence of kainate receptors in other brain structures involved in migraine pathophysiology (Binns et al., 2003; Eyigor et al., 2005), raises the possibility that the clinical actions may be on other brain areas or might reflect the sum of responses in the TCC and/or of responses in other structures.

Results from the current study provide evidence for the regulation of trigeminovascular transmission by both post- and pre- synaptic kainate receptors and indicate a modulatory role of kainate receptors in trigeminovascular nociceptive processing, at the level of TCC. Differential activation of kainate receptors defines novel roles of this receptor in pro- and anti- nociceptive mechanisms in the TCC.

Chapter 5: Modulation of nociceptive dural input to the ventroposteromedial thalamic nucleus via the iGluR5 kainate receptor

5.1 Introduction

The ventroposteromedial thalamic nucleus (VPM) is the site of convergence of trigeminocervical nociceptive inputs in the thalamus (Ohye, 1990; Angus-Leppan et al., 1995). Thalamic activation is known to occur in spontaneous attacks of migraine (Afridi and Goadsby, 2006) and trigeminovascular stimulation in animals excites neurons in the VPM (Davis and Dostrovsky, 1988a; Zagami and Lambert, 1990) as well as induces blood flow changes in the thalamic region (Lambert et al., 1988). The VPM has been shown to be a site of action of triptans (Shields and Goadsby, 2006) and β-blockers that are effective in migraine treatment (Shields and Goadsby, 2005).

The preceding chapters examined the role of kainate receptors at a peripheral trigeminal site and in the TCC. At these sites, the results indicated a possible role of kainate receptors in anti- and pro- nociceptive mechanisms, although the complete detail of the role of iGluR5 receptors in migraine pathophysiology, specificly in the VPM, remains to be determined. Only limited studies have examined the role of kainate receptors in synaptic transmission in the thalamus and it has been demonstrated that iGluR5 kainate receptors are present in the thalamus at both pre- and post-synaptic locations (Ibrahim et al., 2000a). *In vitro* electrophysiological evidence suggests that although kainate receptors are present post-synaptically and can produce small inward currents following bath application of kainate, they do not contribute to the development of post-synaptic currents when electrical stimulation of corticothalamic fibres is applied (Bolea et al., 2001). However, the role of post-synaptic kainate receptors in the thalamus in *in vivo* conditions remains unknown, as well as their role in the transmission of nociceptive information to higher brain areas.

Under physiological conditions of sensory stimulation of the whiskers in rats, pre-synaptic kainate receptors on GABAergic terminals of the thalamic relay nucleus (TRN) seem to have a disinhibitory effect via a reduction of the recurrent GABAergic inhibition, and this suggests an important, novel role of kainate receptors in the physiology of extracting sensory information from background neuronal activation (Salt, 2002; Binns et al., 2003). Whether a similar role for kainate receptors on serotonergic synapses in the VPM nucleus exists is however unknown. The VPM thalamic nucleus receives serotonergic inputs from the rostral raphe complex including the dorsal raphe nucleus (DRN) (Consolazione et al., 1984) and the nucleus raphe median (Consolazione

et al., 1984; Peschanski and Besson, 1984). The PAG has also been shown to have serotonergic inputs to the thalamus (Consolazione et al., 1984).

In the present study, we used electrophysiology in combination with microiontophoresis to investigate the potential *in vivo* effects of iGluR5 agents on third order neurons in the VPM, activated in response to electrical stimulation of the SSS (figure 54). To test if GABAergic influences occur with kainate receptor blockade on neurons responding to trigeminovascular stimulation, we tested whether blockade of $GABA_A$ receptor-mediated transmission would alter sensory responses during microiontophoretic application of a selective kainate receptor antagonist. The possible role of kainate receptors on serotonergic synapses in the VPM was also tested. In order to investigate if modulation of serotonergic receptors in the VPM can alter the inhibitory responses to kainate receptor antagonism, we co-ejected $5\text{-}HT_{1A/B/D}$ receptor compounds with a selective kainate receptor antagonist.

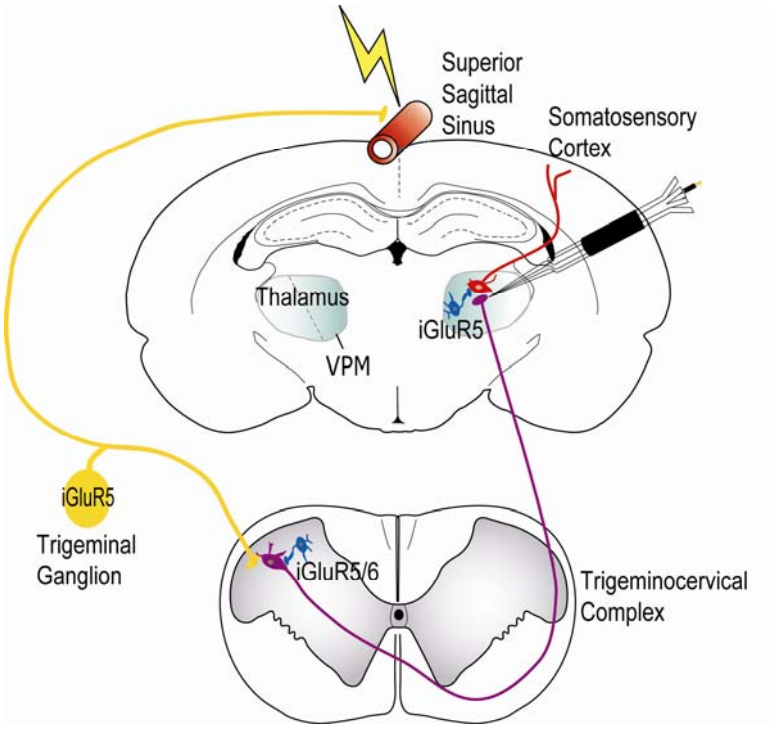


Figure 54: Electrophysiology of neurons in the ventroposteromedial thalamic nucleus – microiontophoresis

Electrophysiological recordings were made from third order neurons in the ventroposteromedial (VPM) thalamus that received trigeminal convergent inputs from dural vessels.

5.2 Methods

5.2.1 Animals

Thirty-six male Sprague-Dawley rats (280–405 g) were anesthetised with 60 $mgkg^{-1}$ pentobarbital intraperitoneally and then maintained with a 25-35 $mgkg^{-1}h^{-1}$ infusion (section 2.2). The surgical preparation was as described in section 2.3.5.

5.2.2 Drugs and microiontophoresis

Micropipette barrels were filled with 200 mM NaCl for automated current balance, pontamine sky blue dye, UBP 302, Iodowillardiine, NMDA, Fluorowillardiine, Bicuculline methiodide, naratriptan hydrochloride, WAY100135, GR127935, NAS-181 and NaCl at pH 8 which was used as control (Na^+ for cations or Cl^- and OH^- for anions). All tested compounds were chosen according to their pharmacological activity (table 22), and were ejected either as anions or cations as shown in table 22. Microiontophoretic barrels had resistances of 16-120 MΩ.

Table 22: Characteristics of test compounds used by microiontophoresis

	Polarity	Ejection current (nA)	pH	Pharmacological activity
Fluorowillardiine	(-)	5 - 25	8.0	iGluR1, 2 and 4 AMPA receptor agonist (Jane et al., 1997)
Iodowillardiine	(-)	5 - 25	8.0	iGluR5 kainate receptor agonist (Jane et al., 1997; Swanson et al., 1998)
NMDA	(-)	10 - 50	8.0	NMDA receptor agonist (Watkins and Jane, 2006)
UBP 302*	(-)	20, 40, 80	8.0	iGluR5 kainate receptor antagonist (More et al., 2004)
Bicuculline methiodide**	(+)	80	5.5	$GABA_A$ receptor antagonist (Olsen et al., 1976)
Naratriptan hydrochloride**	(+)	60	5.5	$5\text{-}HT_{1B/D/F}$ receptor agonist (Humphrey et al., 1990; Hoyer et al., 1994)
WAY100135**	(+)	60	5.5	$5\text{-}HT_{1A}$ receptor antagonist (Cliffe et al., 1993; Fletcher et al., 1993)
NAS-181**	(+)	60	5.5	$5\text{-}HT_{1B}$ receptor antagonist (rat specific)(Berg et al., 1998)
GR127935**	(+)	60	5.5	$5\text{-}HT_{1B/D/F}$ receptor antagonist (Clitherow et al., 1994)

* Control: Cl^- and OH^-

** Control: Na^+

NMDA, *N*-methyl-D-aspartic acid; UBP 302, (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrim idine-2,4-dione; Bicuculline methiodide, [R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium iodide; WAY100135, (S)-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride; GR127935, N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methy-l-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride; NAS-181, (2R)-2-[[[3-(4-Morpholinylmethyl)-2H-1-benzopyran-8-yl]oxy]methyl]morpholine dimethanesulfonate

5.2.3 Experimental protocol

Neurons were identified stereotaxically in the region of the VPM by their response to facial receptive field stimulation. All neurons displayed a stable response to electrical stimulation of the SSS and an increased firing rate to microintophorised glutamate receptor agonist(s), as described in section 2.11.5. Three baseline responses to SSS stimulation (50 stimuli were displayed in post-stimulus histograms) were collected 5 minutes apart. When more than one agonist was tested, the agonists tested for each experiment were ejected in a random order. Once five stable baseline cycles were recorded, UBP 302 or vehicle control were ejected at currents 20, 40 and 80 nA (each current applied in a random) over 5 cycles of Iodowillardiine, or at 80 nA over 3 cycles of Iodowillardiine/Fluorowillardiine/NMDA ejection. A post-stimulus histogram was collected at the maximum ejection current of UBP 302 and during recovery period (15 minutes). To test the effect of the $GABA_A$ and 5-HT_1 receptors compounds, each drug was co-ejected with Iodowillardiine, as was the control at the same current and duration. These compounds were co-ejected with UBP 302, upon establishment of an action of UBP 302 on cell firing, and their responses were compared to that of UBP 302 at 80 nA and control alone (figure 55). Post-stimulus histograms were recorded during each condition. Upon termination of the experiment, the tissue was fixed in order to identify the location of the recording site (section 2.11.6).

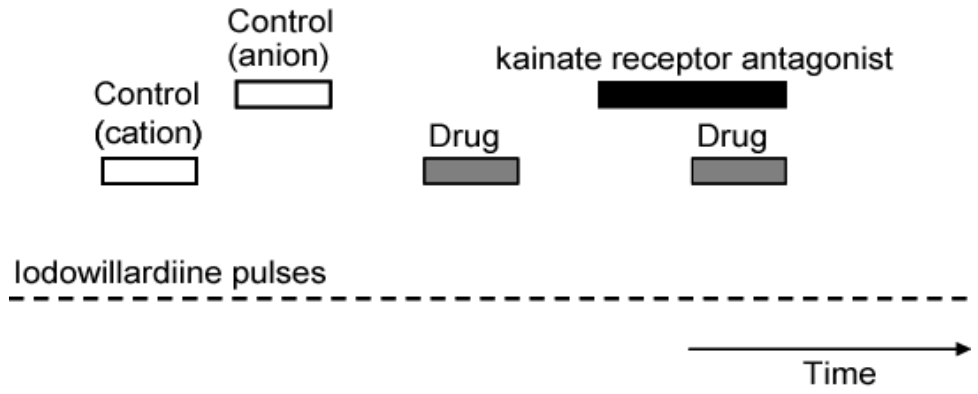


Figure 55: Drug ejection protocol
Diagram demonstrating schematically the protocol for drug ejection during microiontophoretic experiments in the VPM. Due to restrictions on the number of available barrels not all studies could be conducted on individual cells.

5.3 Results

5.3.1 Localisation and neuronal characteristics

A total of 74 cells were studied in the VPM (figure 51), and each displayed convergent trigeminal viscerosomatic inputs classified by cutaneous receptive fields as shown in table 23. Cells responded with an increased probability of firing to electrical SSS stimulation with an average latency of 12 ± 0.6 ms to the onset. To be included in the study, a probability of firing of greater than 30% was required. Only cell bodies were recorded, which were characterised by their biphasic action potential morphology and increased firing response to glutamate receptor agonist(s).

Cells responding to nociceptive stimulation of the cutaneous receptive field (WDR and NS) displayed similar probability of firing in response to SSS stimulation and exogenous application of the kainate receptor agonist Iodowillardiine, with LTM cells ($P \geq 0.28$), and thus responses from both subpopulations were considered homogenous and included in the same group for further analysis. Although neurons in both groups displayed a probability of firing to electrical SSS stimulation of greater than 30%, a slightly higher voltage of 27 ± 3 V was used to excite LTM neurons compared to nociceptive responding neurons (21 ± 2 V).

Table 23: Receptive field characteristics

Receptive field characteristics	Number of cells
Low threshold mechanosensitive (LTM)	26
Nociceptive specific (NS)	1
Wide dynamic range (WDR)	11
Vibrissal (# of vibrissae activated/cell: median)	36 (2)

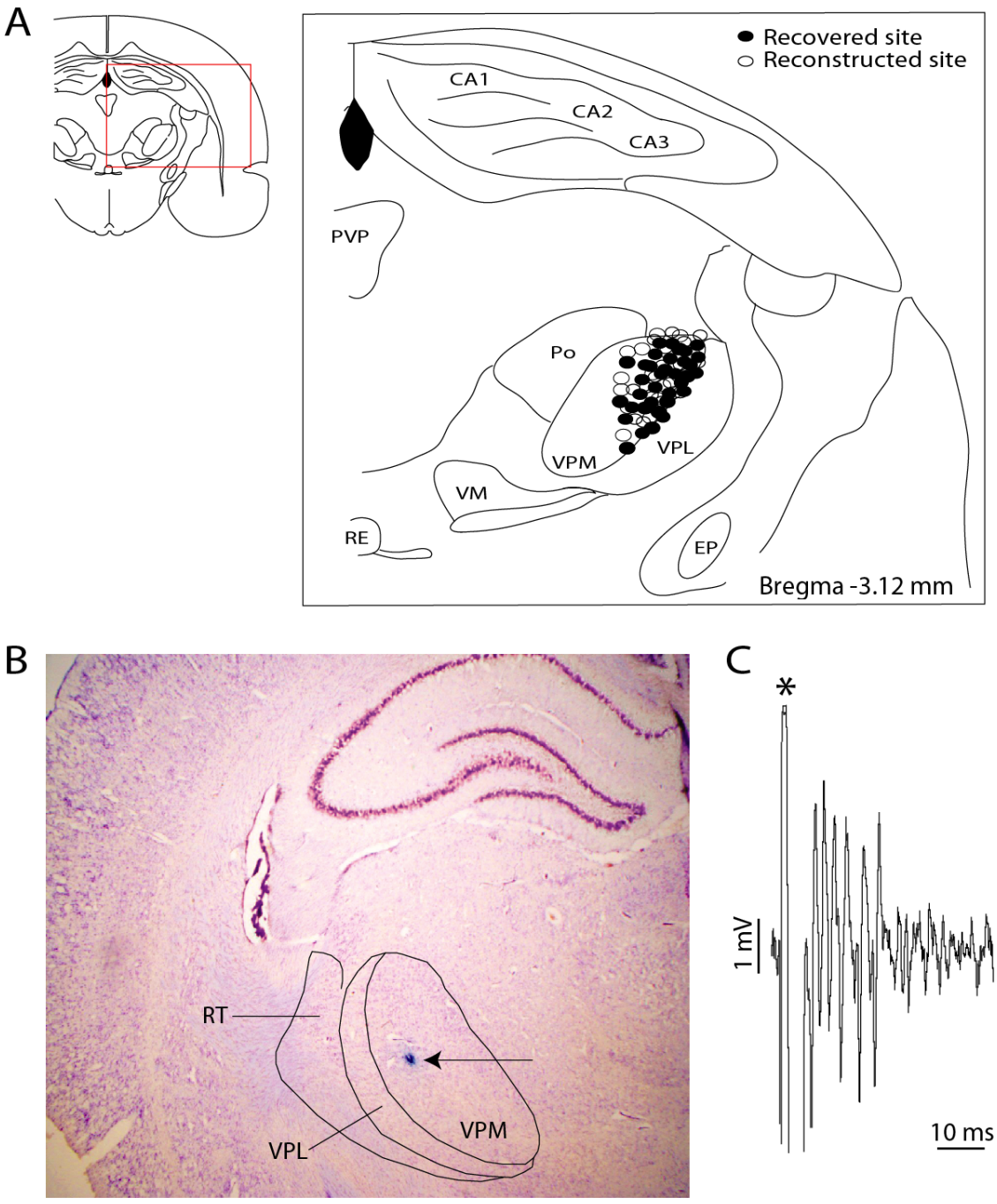


Figure 56: Localisation of recording sites within the ventroposteromedial thalamus

A. Reconstruction of recording sites within the ventroposteromedial thalamus (VPM) projected onto a representative section from Paxinos and Watson (1997). Sites directly identified from the ejection of pontamine sky blue are marked with solid circles (●) and sites reconstructed from the microdrive recordings with open circles (○). B. A photomicrograph demonstrating a recording site marked by ejection of pontamine sky blue (arrow) is shown. C. An original trace showing a cluster of cells in the VPM firing in response to stimulation of the superior sagittal sinus. * stimulus artefact

CA1, CA2, CA3, CA1-3 fields of Ammon's horn; EP, Entopeduncular nucleus; Po, Posterior complex; PVP, Paraventricular nucleus; RE, Reunions nucleus; VM, Ventromedial nucleus; VPL, Ventroposterolateral nucleus; VPM, Ventroposteromedial nucleus; RT, reticular nucleus

5.3.2 Selectivity of UBP 302 over Iodowillardiine, Fluorowillardiine and NMDA evoked firing

Although the selectivity of the antagonist UBP 302 for iGluR5 receptors over AMPA and NMDA evoked firing was previously shown in the TCC (section 4.3.2), we tested the effects of UBP 302 on firing in response to microiontophoretic ejection of Iodowillardiine, Fluorowillardiine and NMDA on five VPM neurons.

For all three agonists tested there was no difference across the mean firing of the five repeated epochs recorded during baseline (Fluorowillardiine: $F_{4,16} = 0.99$, $P = 0.44$; Iodowillardiine: $F_{2,7} = 0.74$, $P = 0.49$; NMDA:, $F_{4,16} = 1.23$, $P = 0.34$) and all responses were reliable (Cronbach's $\alpha \geq 0.9$).

The effects of UBP 302 were tested under the highest current used in this study (80 nA) over 3 repeated cycles of agonist ejection. Ejection of UBP 302 significantly inhibited firing in response to Iodowillardiine in all five neurons tested ($F_{2,8} = 25.03$, $P < 0.001$; figure 52), but not to NMDA ($F_{2,8} = 4.21$, $P = 0.60$; figure 52) and Fluorowillardiine ($F_{2,8} = 1.99$, $P = 0.20$; figure 57).

Upon verification of the selectivity of UBP 302 for the iGluR5 agonist evoked firing at the level of the VPM, further tests where conducted by using only the agonist Iodowillardiine, thus assuring the presence of iGluR5 carrying kainate receptors on all neurons tested.

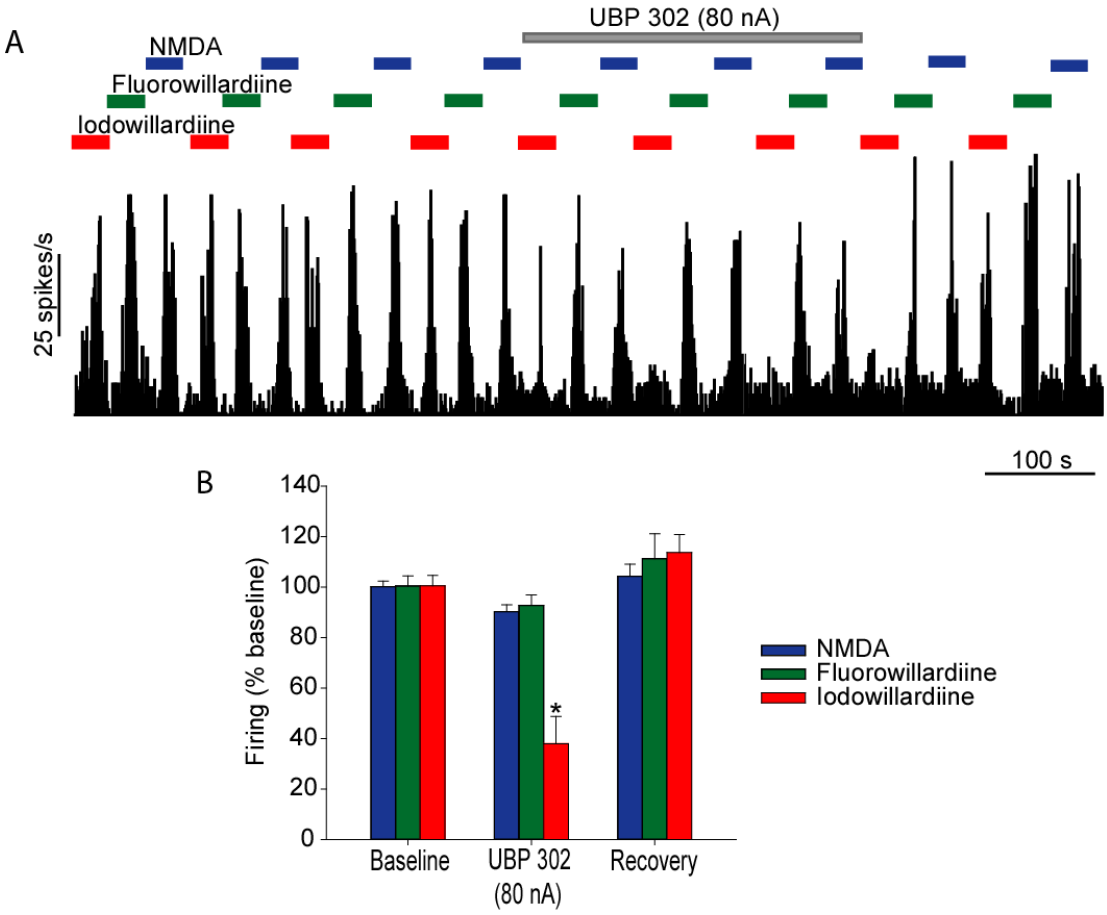


Figure 57: Effects of microiontophoretically delivered UBP 302 on the firing rates of third order neurons to pulsed ejections of the glutamate receptor agonists Iodowillardiine, Fluorowillardiine and NMDA

A. Example of the effects of UBP 302 on the firing rates to pulsed ejections of Fluorowillardiine, Iodowillardiine and NMDA. Cell firing in response to the ejected agonists returned to baseline levels within 2-5 minutes after UBP 302 microiontophoresis ceased. B. Comparison of the effect of UBP 302 (80 nA) on Iodowillardiine, Fluorowillardiine and NMDA evoked responses. Cell firing in response to Iodowillardiine ($n = 5$), but not in response to Fluorowillardiine ($n = 5$) and NMDA ($n = 5$) was significantly inhibited by microiontophoretically administered UBP 302. * $P < 0.05$

5.3.3 Dose effects of UBP 302 ejected at currents 20, 40 and 80 nA on Iodowillardiine evoked firing

UBP 302 reversibly suppressed the post-synaptic response to Iodowillardiine (n = 26; $F_{4,40}$ = 12.18; P < 0.001; figure 58). This inhibition was proportionate to the magnitude of the ejecting current and by inference dose dependent, though the sensitivity to UBP 302 did vary between a small number of neurons. In the majority of neurons recovery was immediate upon termination of UBP 302 ejection, though in some neurons it varied between 2-5 minutes after UBP 302 microiontophoresis ceased. Ejection of control (Cl^- and OH^-) at the same currents and duration had no effect ($F_{4,40}$ = 1.55; P = 0.23; figure 58).

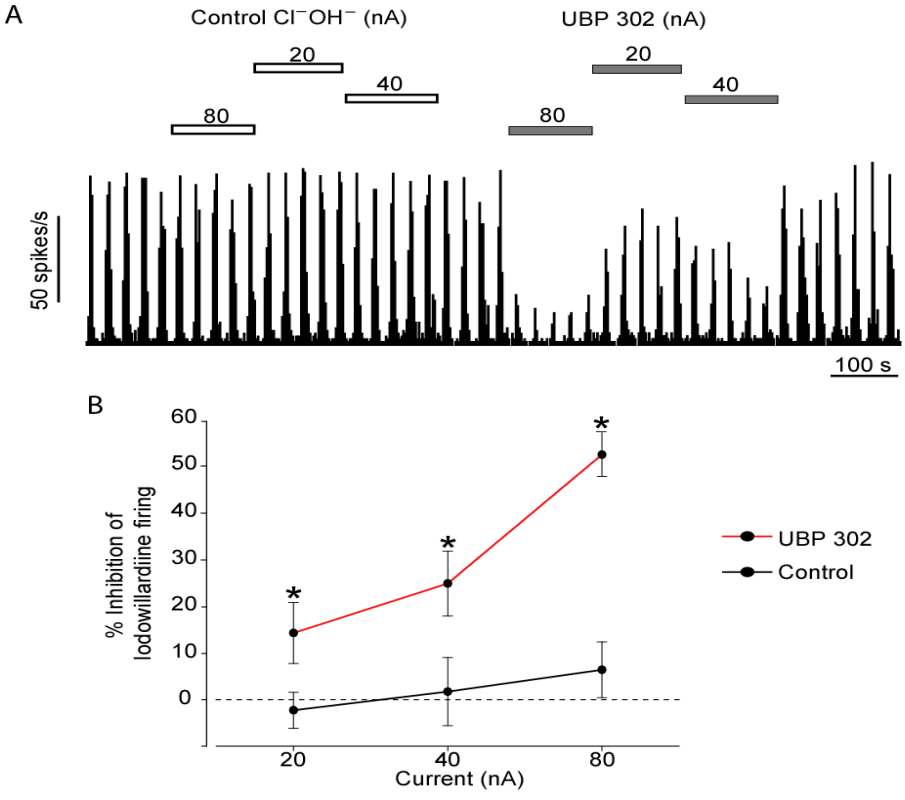


Figure 58: Effects of microiontophoretically delivered UBP 302 at currents 20, 40 and 80 nA on the firing rates of third order neurons to pulsed ejection of Iodowillardiine

A. Example of a representative neuron of the effects of UBP 302 at currents 20, 40 and 80 nA, applied in a random order over 5 consequent epochs of Iodowillardiine evoked firing. B. Current response curves for UBP 302 and control (Cl^- and OH^-) on Iodowillardiine evoked firing. * P < 0.05

5.3.4 Effect of UBP 302 on superior sagittal sinus-evoked stimulation

Ejection of UBP 302 significantly inhibited the response to SSS stimulation in comparison to the control ($n = 26$; $t_{25} = 3.47$, $P < 0.005$; figure 59) in all neurons tested in a reversible manner. The drug was ejected for 3-4 minutes. In all cells full recovery was recorded 10-15 minutes after UBP 302 microiontophoresis ceased, in contrast to the almost immediate recovery seen on post-synaptic firing to Iodowillardiine ejection. Ejection of control (Cl^- and OH^-) had no effect in comparison to baseline ($t_{25} = 1.74$, $P = 0.10$).

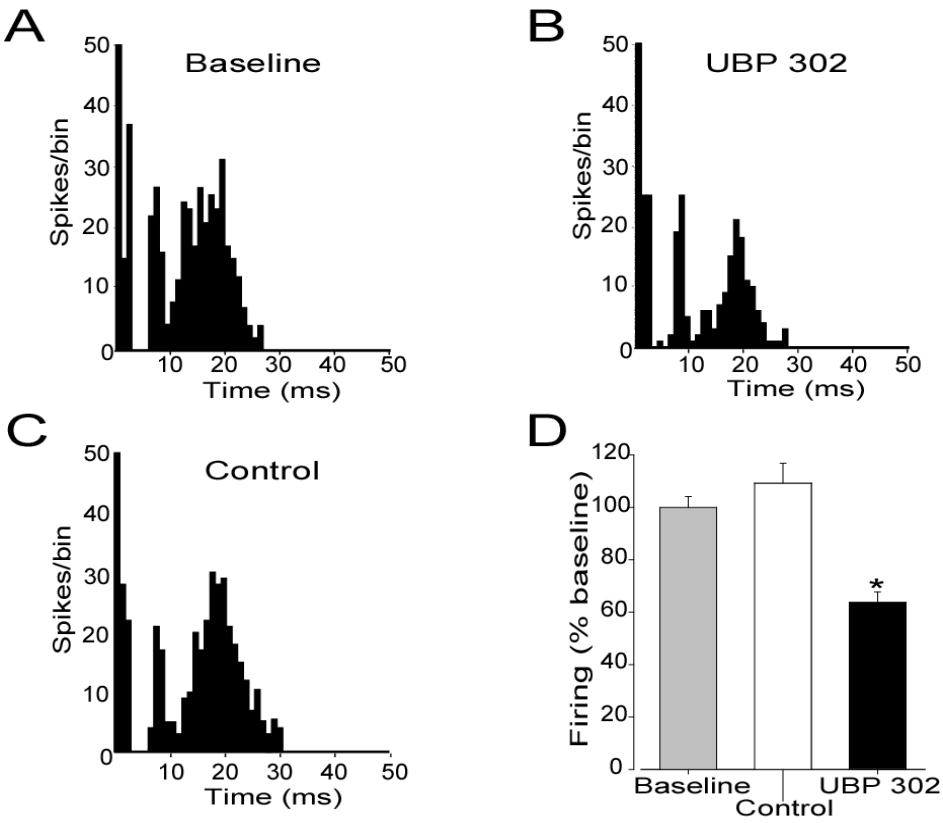


Figure 59: Effect of microiontophoresis of UBP 302 on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-C. Example of post-stimulus histograms from a representative neuron, recorded during baseline conditions (A), during ejection of UBP 302 (B) and during ejection of control Cl and OH anions (C). D. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$

5.3.5 Effect of UBP 302 during blockade of GABAergic transmission

The inhibitory action of UBP 302 (80 nA) on the Iodowillardiine response could be antagonised by the ejection of the selective $GABA_A$ antagonist bicuculline (80 nA) (UBP 302 vs. UBP 302 & Bicuculline: $n = 6$; $t_5 = 4.34$, $P < 0.05$; figure 60). Iontophoresis of bicuculline significantly increased cell firing in response to Iodowillardiine compared to baseline levels ($t_5 = 3.36$, $P < 0.05$). However, this was not significant when compared to its control ($t_5 = 1.81$, $P = 0.13$; figure 60), making it unlikely that the GABAergic antagonist had any direct effect on neuronal excitability. Control ejection (Na^+) had no significant effect compared to baseline recordings ($n = 6$; $t_5 = 0.03$, $P = 0.98$; figure 60).

Similarly bicuculline could fully reverse the inhibitory action of UBP 302 on SSS stimulation response (UBP 302 vs. UBP 302 & bicuculline: $t_5 = 3.77$, $P < 0.05$; figure 56), whereas individual ejection of bicuculline or Na^+ control had no effect ($P \geq 0.46$; figure 61).

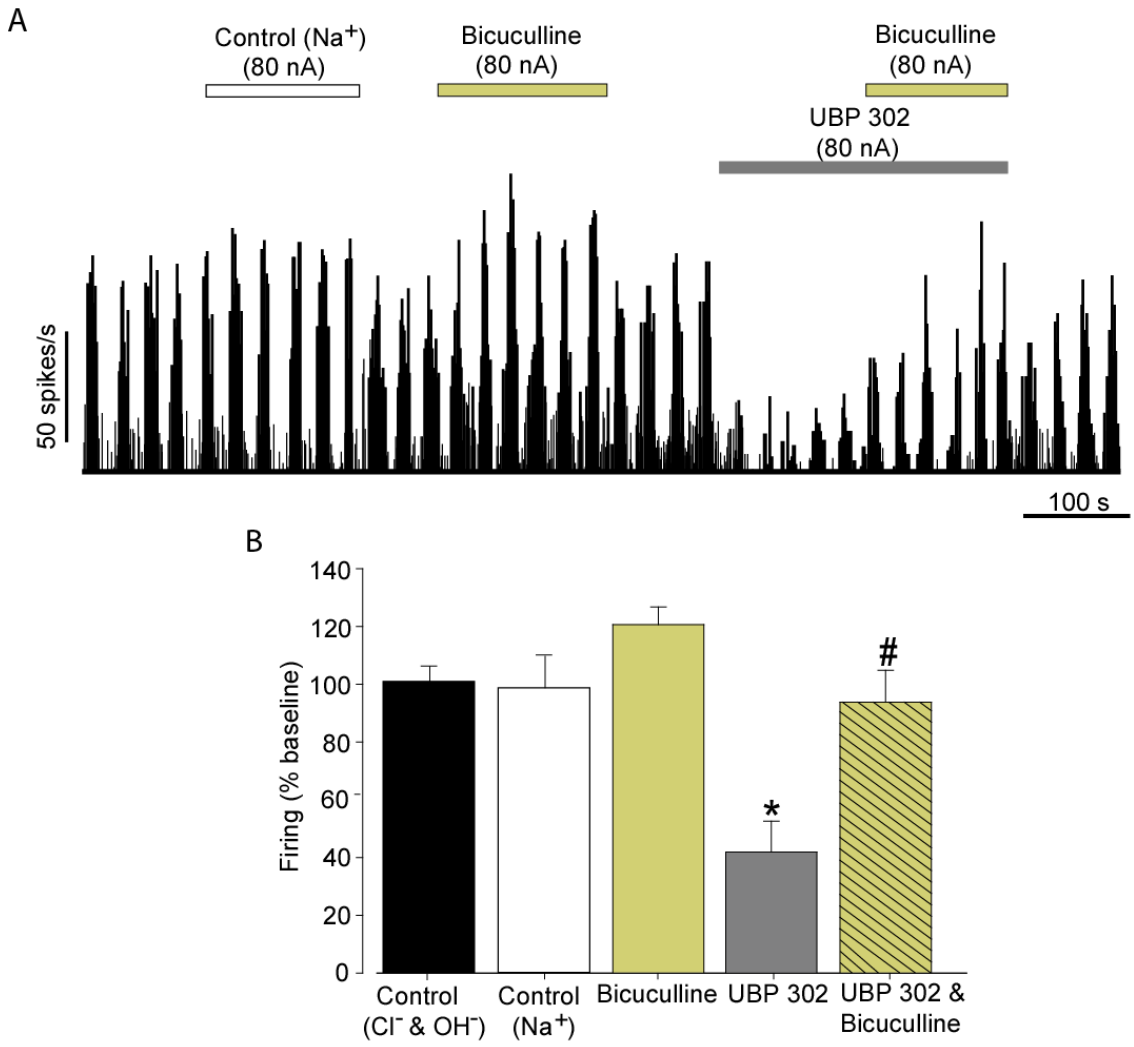


Figure 60: Effect of microiontophoresis of UBP 302 and bicuculline on responses of thalamocortical neurons to Iodowillardiine ejection

A. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs and their controls are shown with the solid bars. Ejection of control (Na^+) and bicuculline alone had no effect, while ejection of UBP 302 had a potent inhibitory effect that was reversed by the $GABA_A$ receptor antagonist bicuculline. B. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to UBP 302.

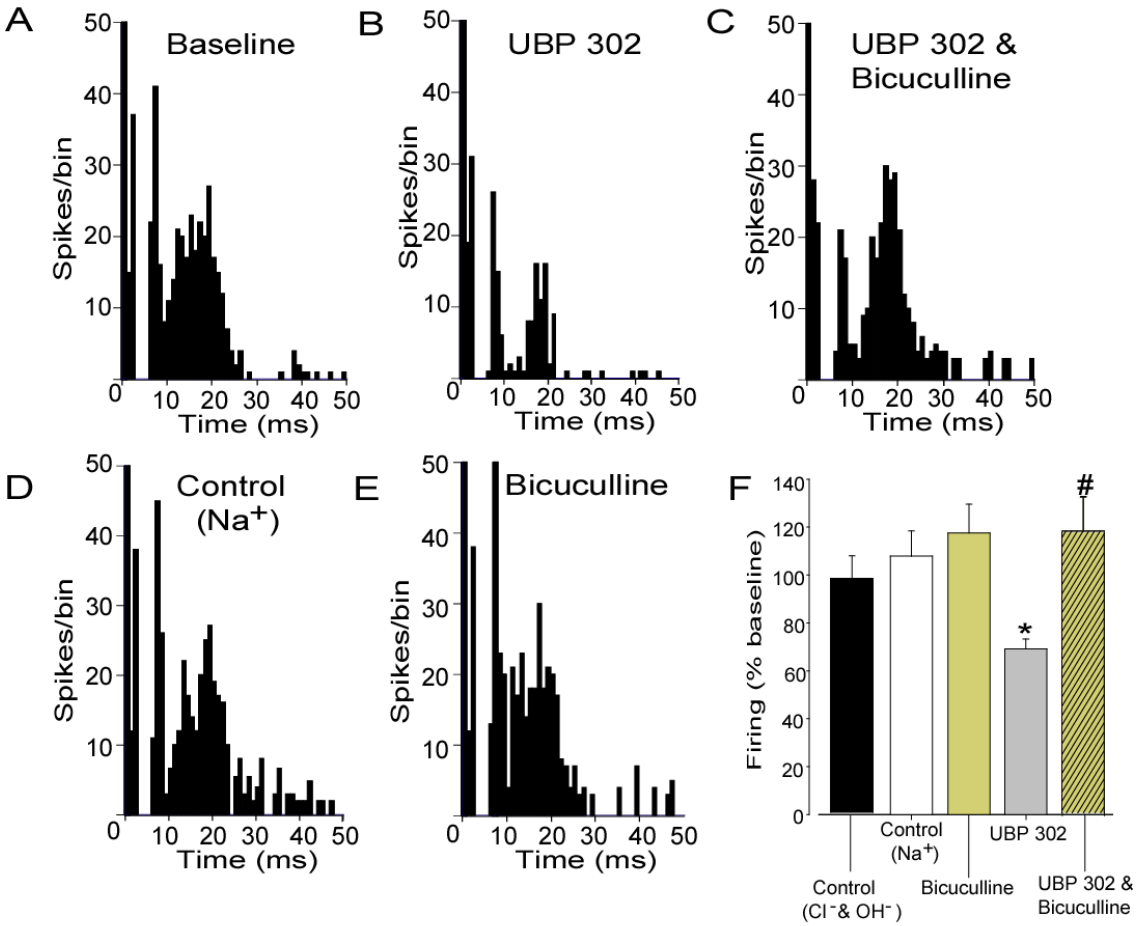


Figure 61: Effect of microiontophoresis of UBP 302 and bicuculline on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-E: Example of post-stimulus histograms generated from a representative thalamocortical neuron following electrical stimulation of the SSS during baseline (A), ejection of UBP 302 (B), co-ejection of UBP 302 with bicuculline (C), ejection of Na cations (D) and during ejection of bicuculline alone (E). F. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to UBP 302.

5.3.6 Effect of UBP 302 during modulation of serotonergic transmission

Effect of naratriptan (5-HT$_{1B/1D/F}$ receptors agonist)

Ejection of UBP 302 (80 nA) or naratriptan (60 nA) individually significantly reduced SSS stimulation responses to a similar extend (UBP 302 vs. control: $n = 9$; $t_8 = 5.24$, $P < 0.001$; naratriptan vs. control: $t_8 = 7.52$, $P < 0.001$; figure 62A-B). Co-ejection of the two drugs inhibited SSS stimulation responses even further to 53% ± 5 ($t_8 = 12.44$, $P < 0.001$) and this was significantly bigger than the inhibition induced by UBP 302 alone, (UBP 302 vs. UBP 302 & naratriptan: $t_8 = 2.34$, $P < 0.05$; figure 62A-B).

Ejection of UBP 302 or naratriptan individually significantly reduced responses to Iodowillardiine (UBP 302 vs. control: $n = 7$; $t_6 = 6.73$, $P < 0.001$; naratriptan vs control: $n = 7$; $t_6 = 8.57$, $P < 0.001$). Similarly to responses to SSS stimulation, co-ejection of the two drugs further inhibited Iodowillardiine responses by 20% ($n = 7$; $t_6 = 12.97$, $P < 0.001$) and this response was significantly different than the inhibition induced by UBP 302 alone (UBP 302 vs. UBP 302 & naratriptan: $t_6 = 3.00$, $P < 0.05$; figure 62C-D).

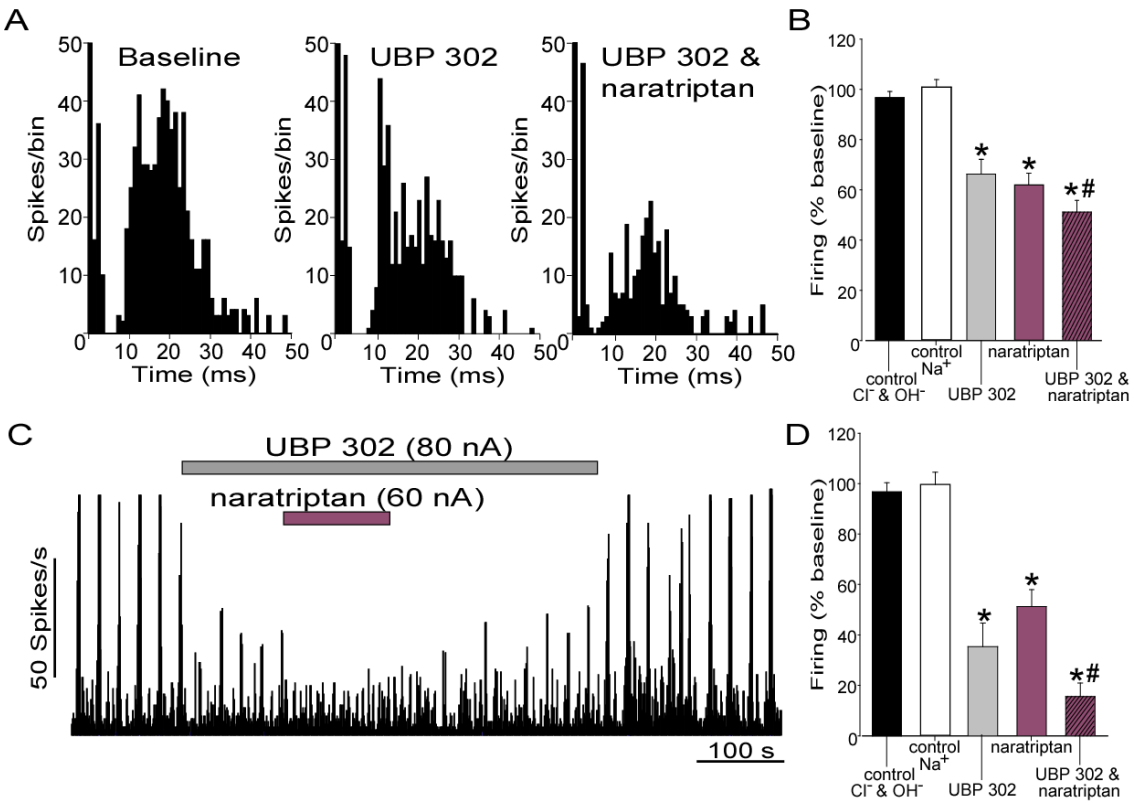


Figure 62: Effect of co-microiontophoresis of UBP 302 and naratriptan on responses of thalamocortical neurons to superior sagittal sinus stimulation and to Iodowillardiine evoked firing

A. Example of post-stimulus histograms generated from a representative thalamocortical neuron following electrical stimulation of the SSS during baseline, ejection of UBP 302 and co-ejection of UBP 302 with naratriptan. B. Comparison of the SSS stimulation evoked firing under each condition. C. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs is shown with solid bars. Ejection of UBP 302 has a potent inhibitory effect that is further reduced with the 5-$HT_{1B/1D}$ agonist naratriptan. D. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to UBP 302.

Effect of GR127935 (5-HT$_{1B/1D}$ receptors antagonist)

Ejection of the 5-HT$_{1B/1D}$ receptor antagonist GR127935 (60 nA) alone increased neuronal firing in response to Iodowillardiine to 33% but this was not significant compared to control Na^+ (n = 13; t_{12} = 1.33, P = 0.21). Co-ejection of UBP 302 with GR127935 failed to reverse the inhibition induced by UBP 302 on post-synaptic firing in response to Iodowillardiine pulses (UBP 302 vs. control: n = 13, t_{12} = 6.12, P < 0.001; UBP 302 vs. UBP 302 & GR127935: n = 13; t_{12} = 1.29, P = 0.22; figure 63).

In contrast, at the same duration and current GR127935 could significantly antagonise the inhibitory action of naratriptan on Iodowillardiine evoked firing (n = 5; naratriptan vs. control: t_4 = 4.82, P < 0.05; naratriptan vs. naratriptan & GR127935: t_4 = 6.26, P < 0.05; figure 64).

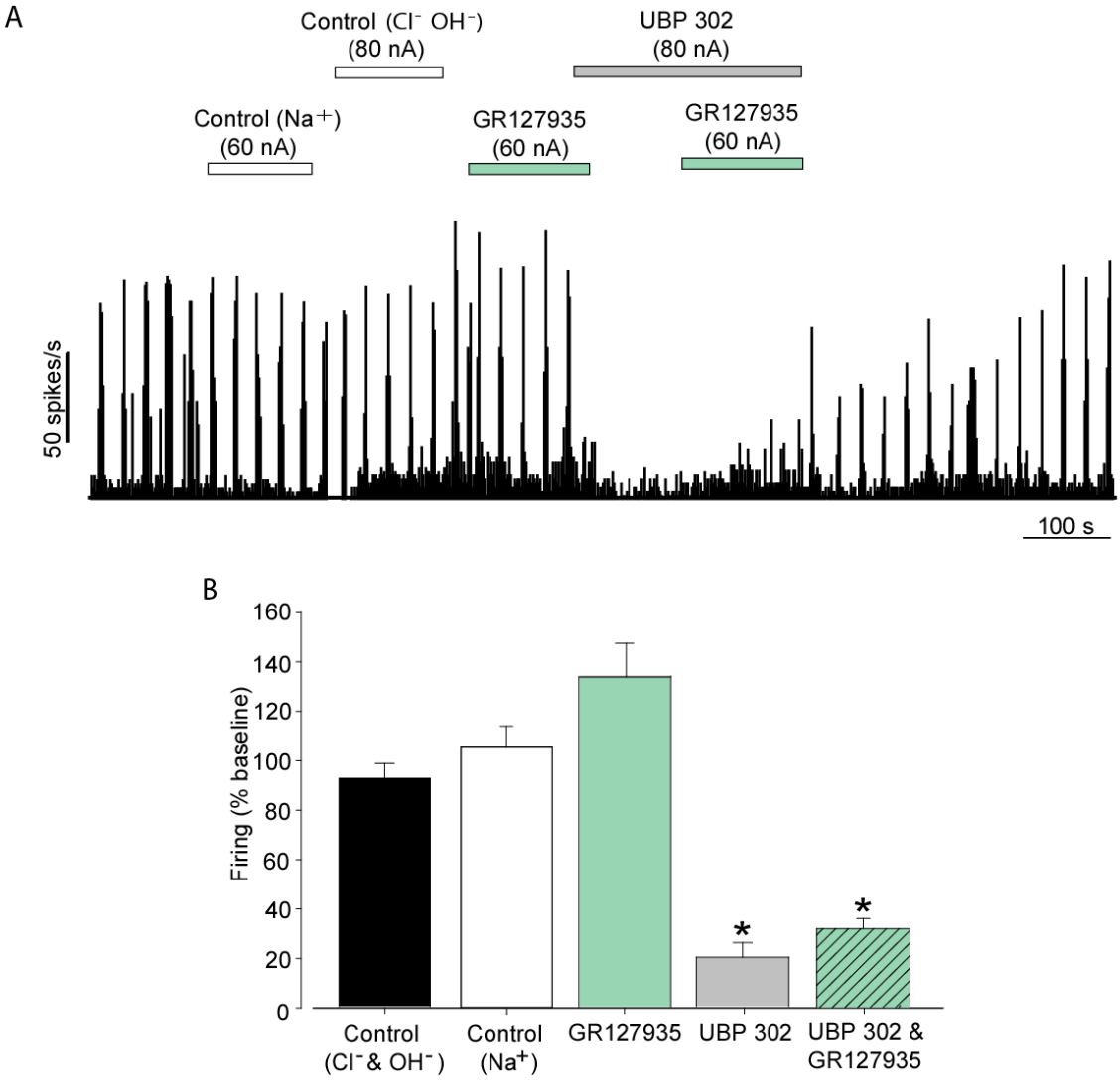


Figure 63: Effect of microiontophoresis of UBP 302 and GR127935 on responses of thalamocortical neurons to Iodowillardiine ejection

A. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs and their controls are shown with the solid bars. Ejection of neither Na cations nor Cl and OH anions or GR127935 alone had any effect. Ejection of UBP 302 had a potent inhibitory effect that was not reversed by the 5-$HT_{1B/D}$ receptor antagonist GR127935. B. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control

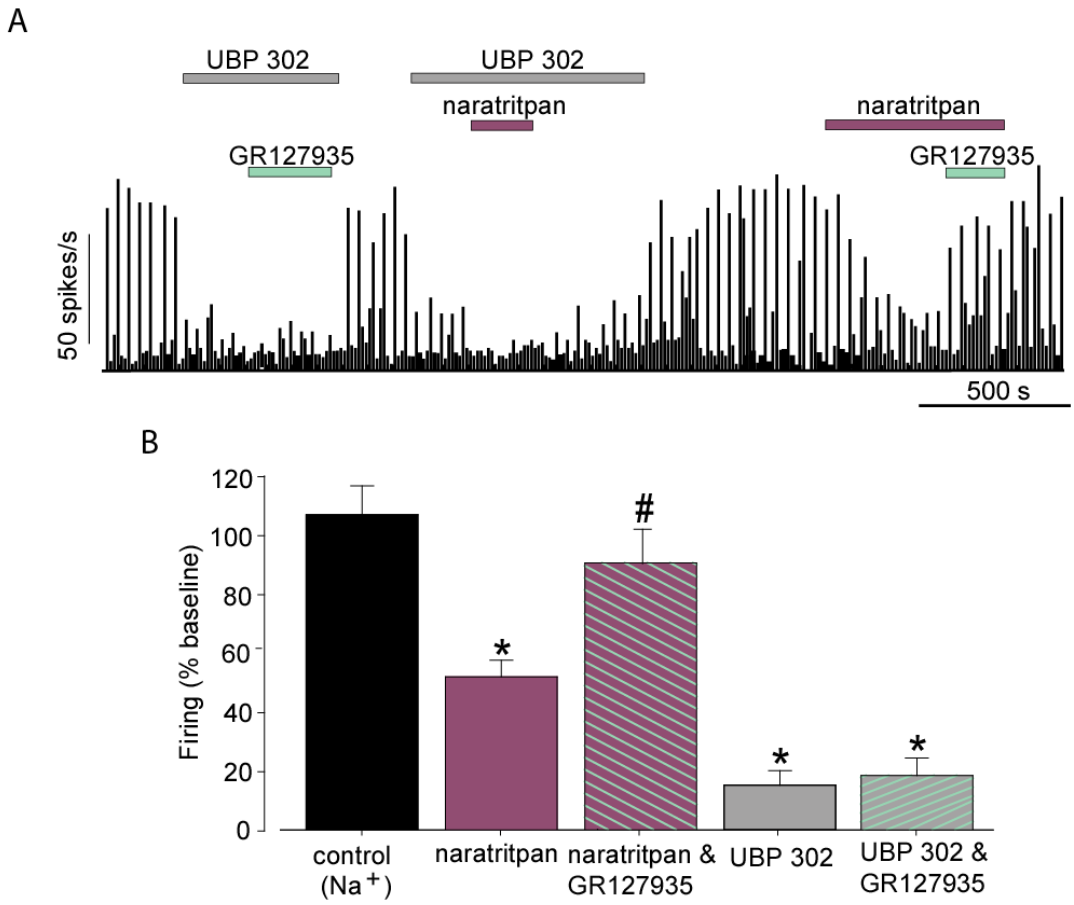


Figure 64: Effect of co-application of UBP 302 with GR127935 and of naratriptan with GR127935 on responses of thalamocortical neurons to Iodowillardiine ejection

A. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs is shown with the solid bars. Ejection of UBP 302 (80 nA) or naratriptan (60 nA) alone inhibited post-synaptic firing, which was further inhibited when the two drugs were co-ejected. Administration of GR127935 (60 nA) failed to reverse the potent inhibitory effect of UBP 302 while was able to reverse the inhibitory actions of naratriptan. B. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to naratriptan

Additionally, co-ejection of UBP 302 with GR127935 did not change the inhibitory actions of UBP 302 on firing in response to SSS stimulation responses (UBP 302 vs. control Cl^- and OH^-: $n = 13$, $t_{12} = 9.09$, $P < 0.001$; UBP 302 vs. UBP 302 & GR127935: $t_{12} = 1.97$, $P = 0.07$; figure 65). Ejection of the 5-$HT_{1B/1D}$ receptor antagonist GR127935 (60 nA) alone significantly increased neuronal firing in response to superior sagittal sinus stimulation compared to its control Na^+ ($n = 13$; $t_{12} = 2.25$, $P < 0.05$).

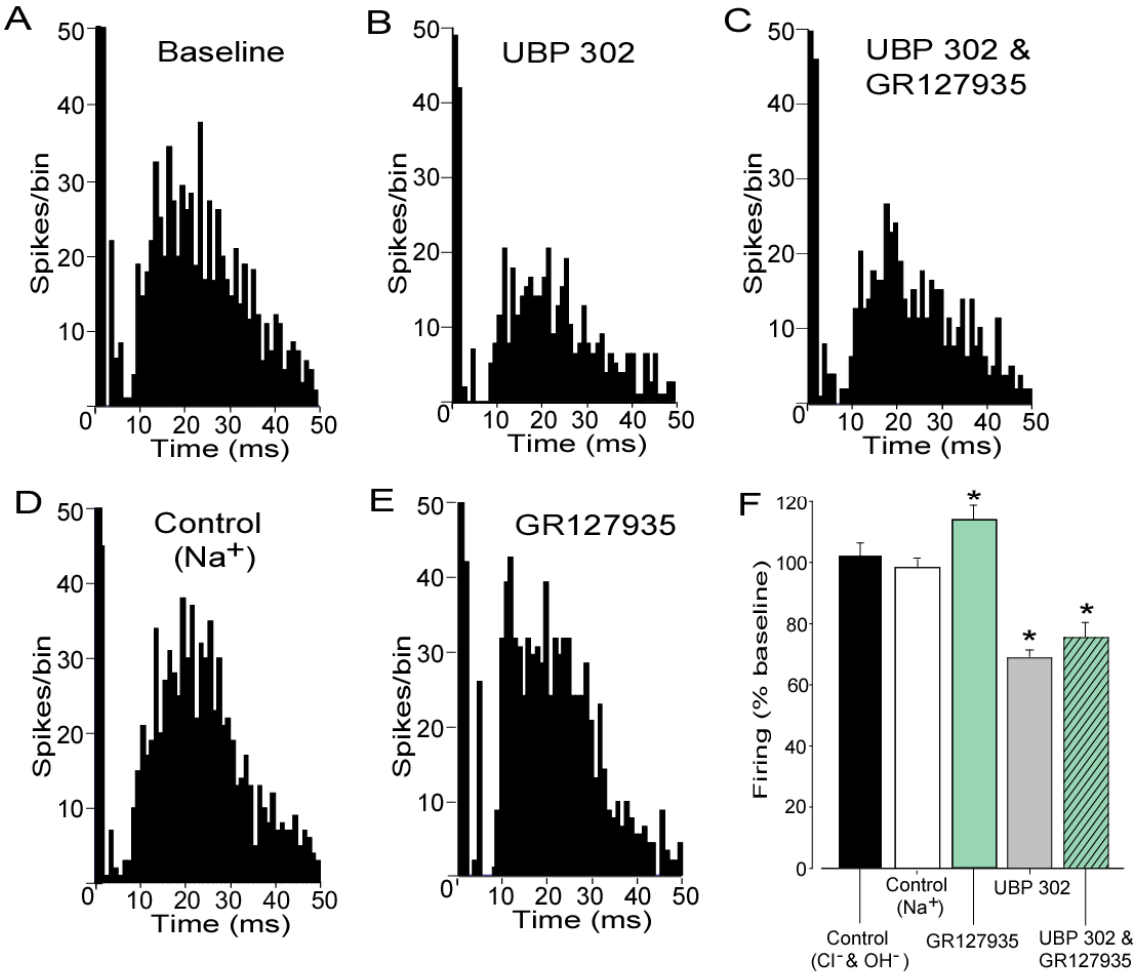


Figure 65: Effect of microiontophoresis of UBP 302 and GR127935 on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-E. Example of post-stimulus histograms generated from a representative thalamocortical neuron following electrical stimulation of the SSS, during baseline conditions (A), ejection of UBP 302 (B), co-ejection of UBP 302 with GR127935 (C), and during ejection of control Na cations (D) and GR127935 alone (E). There was a reproducible inhibition of thalamic responses to SSS stimulation during iontophoresis of UBP 302 which was not reversed by co-administration of UBP 302 with the 5-$HT_{1B/1D}$ receptor antagonist. F. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$ compared to control

Effects of WAY100135 (5-HT_{1A} receptor antagonist)

Ejection of WAY100135 alone neither affected neuronal firing in response to Iodowillardiine ($n = 9$; $t_8 = 1.13$, $P = 0.29$; figure 66) nor to SSS stimulation ($t_8 = 0.01$, $P = 0.99$; figure 67) compared to control. Ejection of Na^+ control did not affect neuronal firing compared to baseline (Iodowillardiine evoked firing: $t_8 = 0.73$, $P = 0.49$; SSS stimulation evoked firing: $t_8 = 1.37$, $P = 0.21$).

Co-ejection of UBP 302 with WAY100135 did not alter the inhibition induced by UBP 302 on post-synaptic firing in response to Iodowillardiine (UBP 302 vs. control: $t_8 = 3.21$, $P < 0.05$; UBP 302 vs. UBP 302 & WAY100135: $t_8 = 1.28$, $P = 0.24$; figure 66).

Additionally, co-ejection of UBP 302 with WAY100135 did not change the UBP 302 induced inhibition on firing in response to SSS stimulation responses (UBP 302 vs. control: $t_8 = 4.86$, $P < 0.005$; UBP 302 vs. UBP 302 & WAY100135: $t_8 = 0.95$, $P = 0.37$; figure 67).

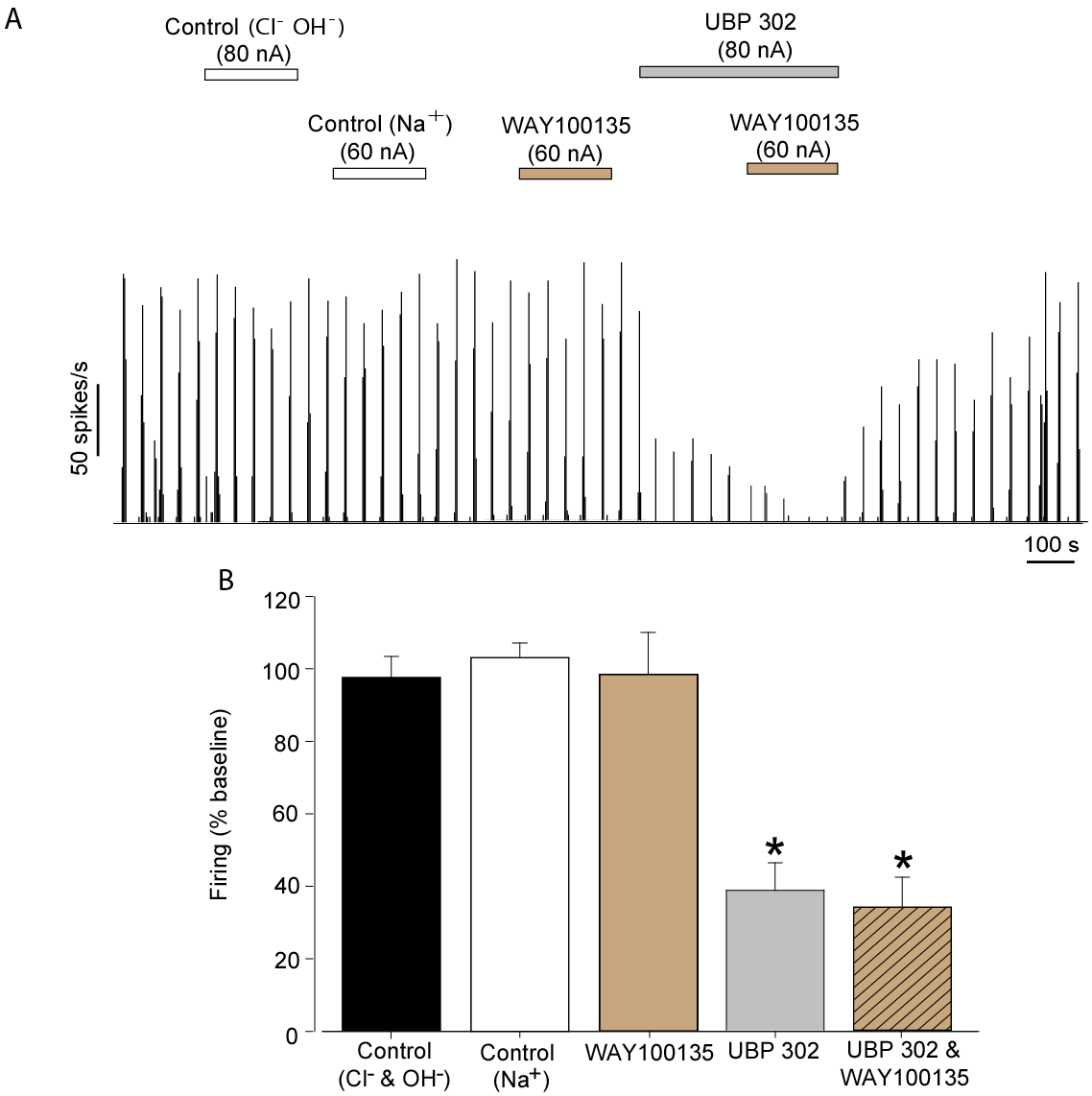


Figure 66: Effect of microiontophoresis of UBP 302 and WAY100135 on responses of thalamocortical neurons to Iodowillardiine ejection

A. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs and their controls are shown with solid bars. Ejection of neither Na^+ cations nor Cl^- and OH^- ions or WAY100135 alone had any effect. Ejection of UBP 302 had a potent inhibitory effect that was not reversed by the 5-HT_{1A} receptor antagonist WAY100135. B. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control

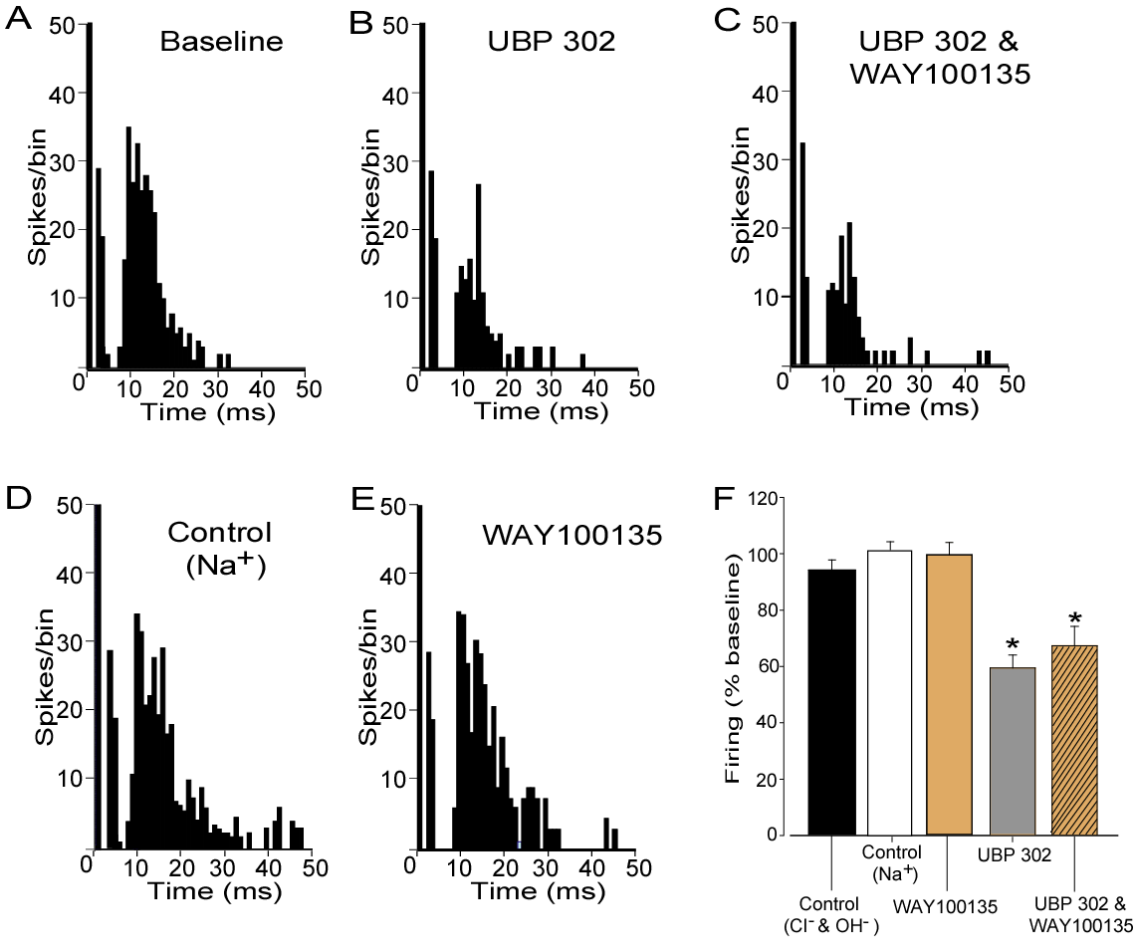


Figure 67: Effect of microiontophoresis of UBP 302 and WAY100135 on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-E. Example of post-stimulus histograms generated from a representative thalamocortical neuron following electrical stimulation of the SSS, during baseline conditions (A), ejection of UBP 302 (B), co-ejection of UBP 302 with WAY100135 (C), and during ejection of control (D) and WAY100135 alone (E). There was a reproducible inhibition of thalamic responses to SSS stimulation during iontophoresis of UBP 302 which was not reversed by co-administration of UBP 302 with the 5-HT_{1A} receptor antagonist. F. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$ compared to control

Effects of NAS-181 (rat 5-HT_{1B} receptor antagonist)

Ejection of NAS-181 alone increased neuronal firing in response to Iodowillardiine by 18% but this was not significant compared to control ions ejection ($n = 10$; $t_9 = 1.99$, $P = 0.08$). Co-ejection of UBP 302 with NAS-181 significantly reversed the inhibition induced by UBP 302 when ejected alone on Iodowillardiine evoked firing (UBP 302 vs. control: $t_9 = 14.43$, $P < 0.001$; UBP 302 vs. UBP 302 & NAS-181: $t_9 = 6.37$, $P < 0.001$; figure 68).

Similarly, co-ejection of UBP 302 with NAS-181 significantly reversed the inhibition induced by UBP 302 when ejected alone on cell firing in response to SSS electrical stimulation (UBP 302 vs. control: $t_9 = 5.91$, $P < 0.001$; UBP 302 vs. UBP 302 & NAS-181: $t_9 = 6.26$, $P < 0.001$; figure 69). Ejection of either NAS-181 alone or Na^+ had any effect on neuronal firing in response to SSS stimulation ($P \geq 0.41$).

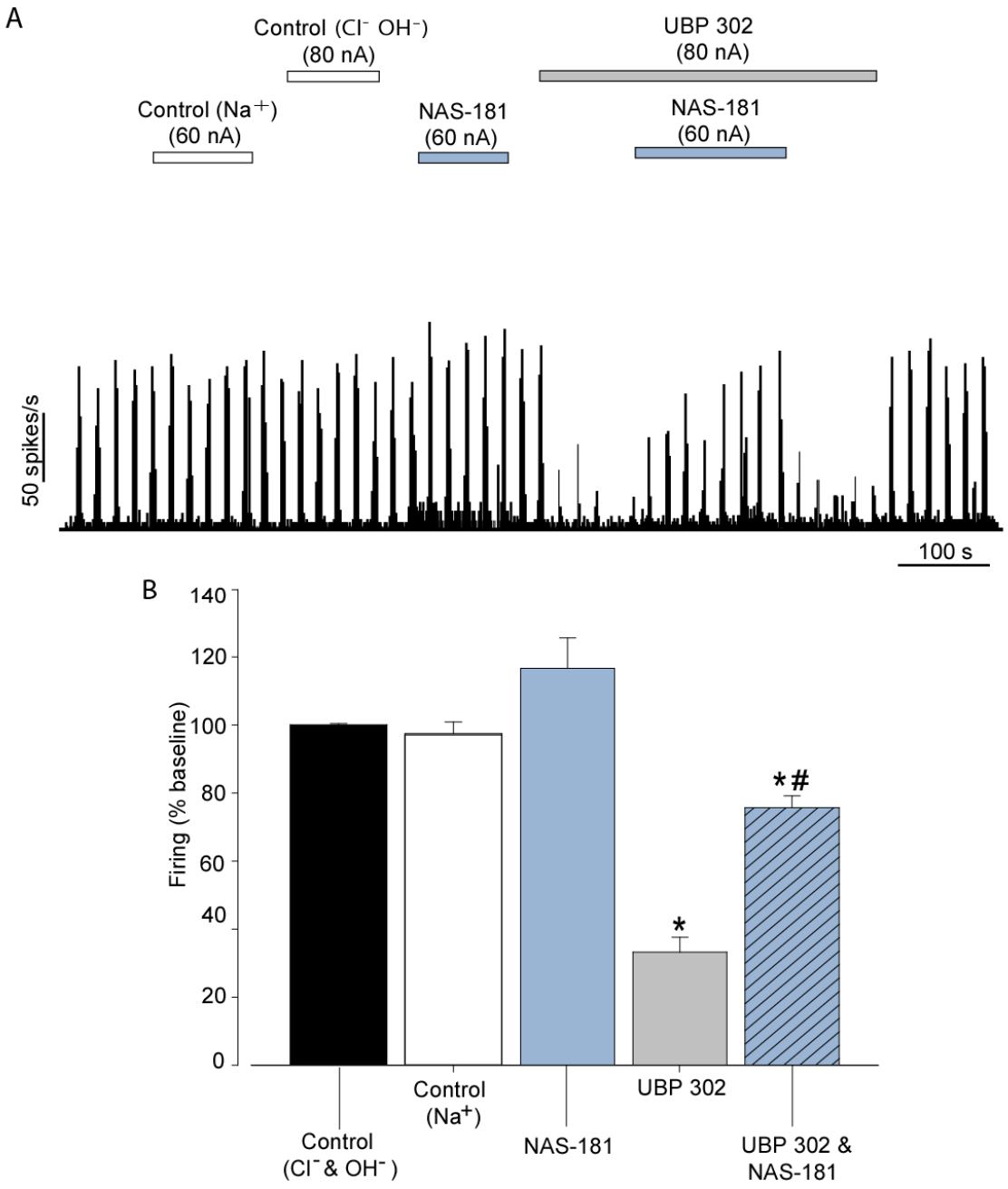


Figure 68: Effect of microiontophoresis of UBP 302 and NAS-181 on responses of thalamocortical neurons to Iodowillardiine ejection

A. Example of the response of a neuron to pulsed Iodowillardiine. Ejection of drugs and their controls are shown with the solid bars. Ejection of neither Na^+ cations nor Cl^- and OH^- ions or NAS-181 alone had any effect. Ejection of UBP 302 had a potent inhibitory effect that was significantly reversed by the 5-HT_{1B} receptor antagonist NAS-181. Comparison of the Iodowillardiine evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to UBP 302

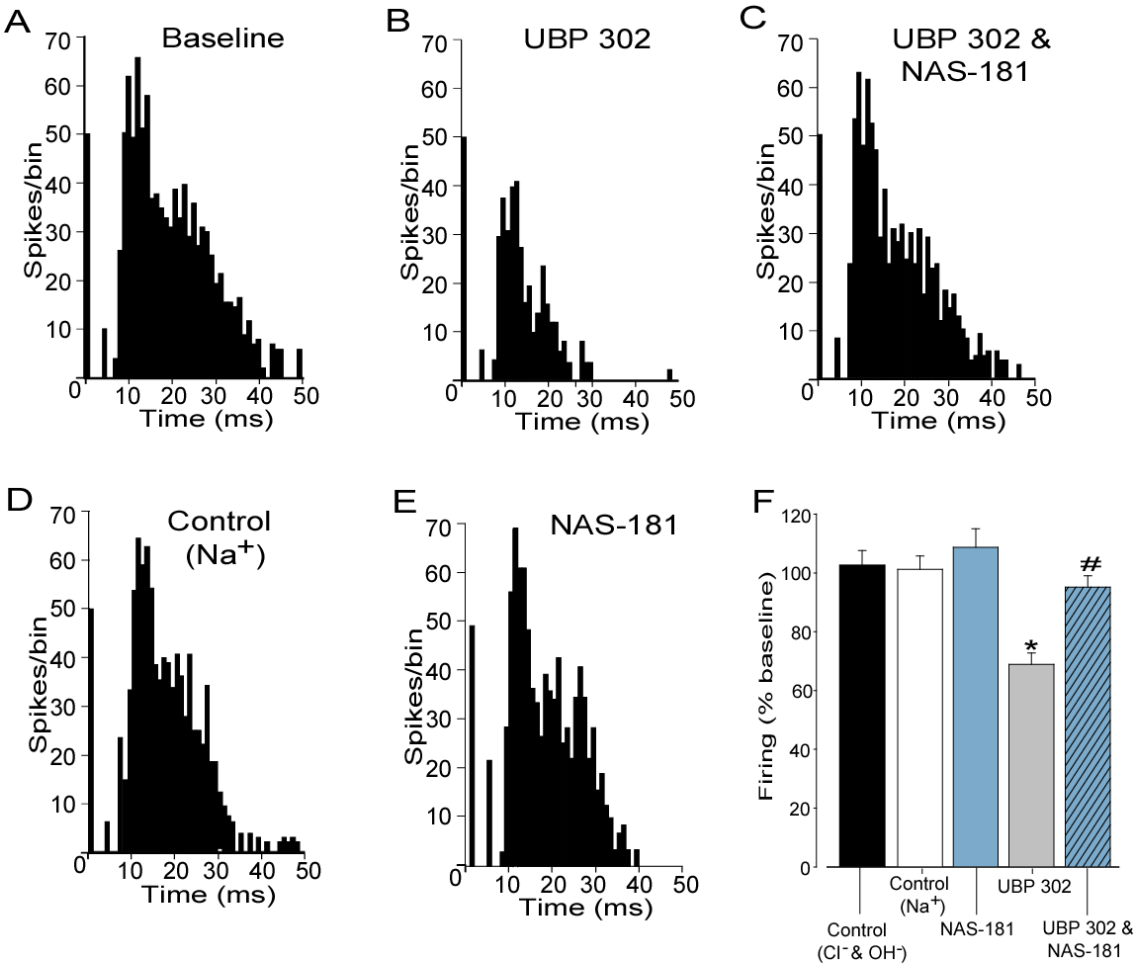


Figure 69: Effect of microiontophoresis of UBP 302 and NAS-181 on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-E. Example of post-stimulus histograms generated from a representative thalamocortical neuron following electrical stimulation of the SSS, during baseline conditions (A), ejection of UBP 302 (B), co-ejection of UBP 302 with NAS-181 (C), and during ejection of control (D) and NAS-181 alone (E). There was a reproducible inhibition of thalamic responses to SSS stimulation during iontophoresis of UBP 302 which was significantly reversed by co-administration of UBP 302 with the 5-HT_{1B} receptor antagonist NAS-181. F. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$ compared to control, # $P < 0.05$ compared to UBP 302

5. 4 Conclusions

This study demonstrates that glutamatergic neurotransmission selectively through iGluR5 carrying kainate receptors at the level of VPM can potently modulate trigeminovascular nociception. This modulation can be through post-synaptic iGluR5 kainate receptors on third order neurons, since the selective iGluR5 receptor antagonist used, further to its inhibitory actions on trigeminovascular activation, could also antagonise post-synaptic firing in response to kainate receptors' activation. Also evidence from this study suggests an interaction between iGluR5 receptor, GABAergic and serotonergic transmission at the level of VPM (figure 70).

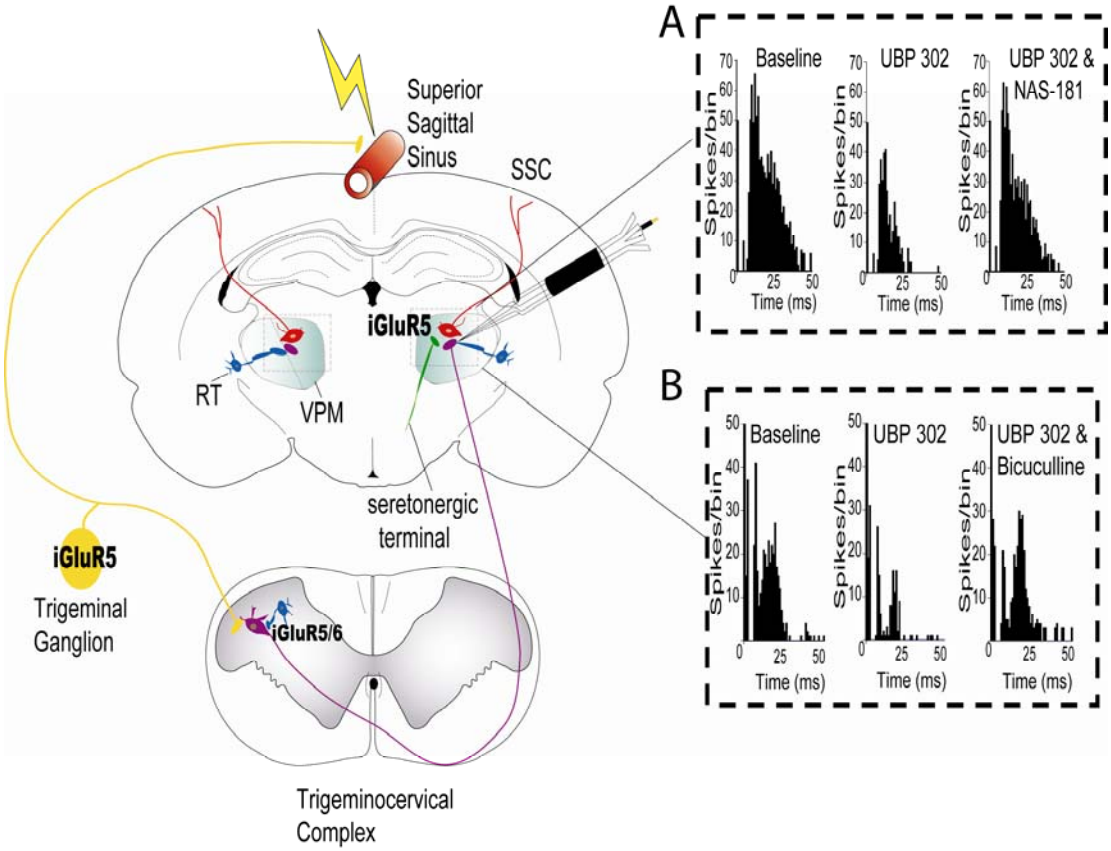


Figure 70: Overview of the effects of microintophorised UBP 302 on third order trigeminovascular neurons

Selective antagonism of iGluR5 receptors was shown to result in inhibition of trigeminovascular processing and this effect was reversed by 5-HT_{1B} (A) and $GABA_A$ (B) receptors antagonists.

RT, reticular nucleus; SSC, somatosensory cortex; VPM, ventroposteromedial thalamic nucleus

The thalamus is a potentially exciting target for migraine research as nearly all afferent sensory information to the cerebral cortex is relayed through it. The thalamus receives large modulatory inputs from brainstem monoaminergic centres (Peschanski and Besson, 1984; Westlund et al., 1990; Simpson et al., 1997) and these areas have been implicated by brain imaging and other studies to be pivotal in migraine pathophysiology (Goadsby et al., 2002; Goadsby, 2007). From a translational neurosciences perspective VPM has proved a site of action of triptans, β-blockers and of valproate and has correlated with the efficacy of anti-migraine therapies in clinical practice (Shields et al., 2003; Shields and Goadsby, 2005, 2006). The current study further supports that the VPM nucleus is a potential site of action of new kainate anti-migraine antagonists.

Neurons identified in this study were mostly found along the shell region of the VPM. This is in agreement with previous studies in the VPM demonstrating that the neurons activated in response to trigeminovascular activation are located along the shell region of VPM with the posterior complex and the ventroposterolateral thalamus (Perl and Whitlock, 1961; Honda et al., 1983; Vahle-Hinz and Gottschaldt, 1983; Yokota et al., 1985; Shields and Goadsby, 2005, 2006). All neurons identified in this study, were selectively activated by the iGluR5 receptors agonist Iodowillardiine, indicating the presence of functional kainate receptors in the VPM in agreement with electrophysiological and anatomical studies (Ibrahim et al., 2000a; Ibrahim et al., 2000b; Binns et al., 2003).

Local ejection by microiontophoresis of the iGluR5 antagonist UBP 302 inhibited both cell firing in response to trigeminovascular activation and in response to an exogenously applied kainate receptor agonist. Since post-synaptic firing was inhibited, this suggests that blockade of post-synaptic kainate receptors can at least partly account for the observed reduction of trigeminovascular nociception. Using microiontophoresis and similar probes for kainate receptors, Binns and colleagues (2003) demonstrated that post-synaptic kainate receptors were present on neurons responding to whisker stimulation and be activated by selective receptor agonists. However, they could not be inhibited by the kainate receptor antagonist LY382884 and thus concluded that post-synaptic kainate receptors do not participate in sensory modulation. It is possible that the antagonist used in the current study is more selective for kainate receptors and furthermore the currents used to eject Iodowillardiine (maximum 25 nA) were sufficient

to avoid high drug concentrations delivered to very small areas so that even when selective agonists are used, inappropriate receptors can be activated.

In the same set of experiments Binns et al. (2003) showed that application of the kainate receptor antagonist LY382884 could inhibit sensory responses, without blocking post-synaptic firing, and thus concluded that under *in vivo* physiological conditions activation of pre-synaptic kainate receptors on GABAergic terminals arising from the thalamic relay nucleus occurs during sensory stimulation. It has been suggested that blockade of these pre-synaptic kainate receptors results in an enhancement of the GABAergic inhibition evoked during sensory stimulation (Salt, 2002; Binns et al., 2003). Recurrent GABAergic inhibition is known to occur during sensory activation (Houser et al., 1980; Ross et al., 1993; Lee et al., 1994a, b); it is not clear though whether recurrent GABAergic inhibition also occurs during trigeminovascular activation. The modulatory action of GABA on sensory transmission in the thalamus is well documented (Salt, 1989; Spreafico et al., 1993; Lee et al., 1994a, b; Hartings and Simons, 2000; Shields et al., 2003). In the experiments reported here application of the $GABA_A$ antagonist alone caused a non-significant increase of firing in response to SSS stimulation and this is in agreement with a previous microiontophoretic study of GABA receptors in the VPM (Shields et al., 2003). A significant effect of bicuculline could have been observed if a higher ejection current was used. Due to limitations of the technique however (higher current resulted in barrel blockage), this was not possible and remains unclear whether recurrent GABAergic inhibition also occurs during trigeminovascular activation. The inhibitory effects of UBP 302 were antagonised by bicuculline in a similar fashion shown by Binns et al. (2003) and this may additionally suggest that blockade of pre-synaptic kainate receptors on GABAergic terminals might occur and result in an enhancement of the GABAergic inhibition. Furthermore, it is unlikely that UBP 302 achieved its effects by a direct agonistic interaction with GABA receptors, since the direct application of a GABA receptor agonist would be expected to inhibit all glutamate agonists evoked firing and not selectively firing in response to kainate receptors activation (Kaneko and Hicks, 1990; Shields et al., 2003; Storer et al., 2004b).

This study further investigated any possible pharmacological mechanisms by which UBP 302 exerted this effect through actions that involve serotonergic modulation. As triptans have agonist activity at multiple 5-HT_1 receptor subtypes (Shields and Goadsby, 2006), antagonists of 5-HT_{1A}, 5-$HT_{1B/1D}$ and 5-HT_{1B} receptors were co-ejected with

UBP 302. Antagonism of UBP 302 with a selective 5-HT_{1B} antagonist reversed the inhibitory actions of UBP 302 in a similar fashion as bicuculline. This might suggest the possible presence of pre-synaptic kainate receptors on serotonergic terminals in the VPM and further indicate a possible role of these receptors in modulating serotonergic release in the sensory thalamus, in a similar fashion as on GABAergic terminals. Such a role for kainate pre-synaptic kainate receptors has been shown in other brain structures (Ohta et al., 1994). Again, it is unlikely that UBP 302 achieved its effects by a direct agonistic interaction with 5-HT receptors, since application of a serotonin receptor agonist would be expected to modify all evoked post-synaptic responses to ejected glutamate receptor agonists and not selectively firing in response to kainate receptors activation (Prieto-Gomez et al., 1989; Shields and Goadsby, 2006). In support with this is the fact that co-ejection of UBP 302 with naratriptan was able to further inhibit cell firing, suggesting that the two compounds were acting on separate neuronal receptors.

Whether serotonergic transmission at the level of the VPM is modulated through kainate receptors under normal conditions is not known. In a microdialysis study it was shown that activation of kainate but not AMPA receptors on DRN and median raphe nucleus neurons can modulate extracellular serotonin levels (Tao et al., 1997) and additionally modulate serotonin release in the amygdale and PAG (Viana et al., 1997). That might suggest that kainate receptors on serotonergic terminals arising from the DRN could have an analogous modulatory role in the thalamus as shown on other central serotonergic terminals (Ohta et al., 1994), but this cannot directly concluded from our studies. A future study might involve serotonergic inhibition of the thalamus through stimulation of the DRN (Prieto-Gomez et al., 1989), and combined local ejection of selective kainate agents in the thalamus. The fact that the 5-HT_{1B} antagonist could reverse the inhibitory action of the kainate receptor antagonist on post-synaptic firing in response to kainate receptors activation, might additionally suggest a post-synaptic modulatory interactions between kainate and 5-HT_{1B} receptors. However, no further evidence exists for such post-synaptic receptor interactions in the thalamus and elsewhere in the brain.

Why the effects of UBP 302 were reversed only by the 5-HT_{1B} receptors antagonist and not by the 5-$HT_{1B/1D}$ receptor antagonist which, as shown here and elsewhere (Shields and Goadsby, 2006) can reverse the effects of naratriptan, is not known. This might suggest that triptans at the level of VPM mainly act through 5-HT_{1B} receptors and may

reflect that in the rat brain 5-HT_{1B} receptors outnumber 5-HT_{1D} receptors (Bruinvels et al., 1993; Bruinvels et al., 1994; Varnas et al., 2001; Varnas et al., 2005). These effects may have also arisen through a combination of factors. Firstly, the 5-HT_{1B} antagonist NAS-181 is twice as potent than the 5-$HT_{1B/1D}$ antagonist GR127935 (NAS-181: Ki for r5-HT_{1B} receptors ~ 47 nM; GR127935: Ki for r5-HT_{1B} receptors ~ 100 nM). Additionally as a technical limitation of the iontophoretic technique, we may not have been able to eject enough of the GR127935 compound to have an effect in the same way NAS-181 had an effect (though with the same current and duration GR127935 was able to reverse the effects of naratriptan), and thus it is not possible to know the concentration of any drug that each cell is exposed to.

In conclusion, we propose that the VPM nucleus is likely involved in the transmission of painful sensory information to the cortex in conditions in which the trigeminovascular nociceptive system is active. Glutamatergic neurotransmission through kainate receptors in the VPM nucleus may potently modulate trigeminovascular nociception and this pharmacological action may explain some part of the efficacy of the kainate receptor antagonist LY466195 in migraine.

Chapter 6: Effects of LY466195 and topiramate on trigeminovascular nociception– potential actions in the treatment of migraine via kainate receptors

6.1 Introduction

The previous studies of trigeminovascular nociception reported in this thesis (chapter 4 and 6) examined the effects of a kainate receptor antagonist following acute administration, directly on neurons firing in response to trigeminovascular stimulation. The antagonist UBP 302 used in the previous studies is one of the most potent iGluR5 kainate receptor antagonists described to date (More et al., 2004). However there is a lack of evidence on its lipophylicity and its ability to cross the blood-brain-barrier and to our knowledge the antagonist has not been previously described in an *in vivo* model. Although chemical analysis of the molecule was not conducted the existence of a carboxylic group on the compound might suggest decreased lipophylicity of the molecule. In order to examine the systemic effects of proposed antagonists for kainate receptors we utilised the iGluR5 kainate antagonist LY466195 and the anti-convulsant topiramate.

LY466195 has been described as the one of the most potent kainate receptor antagonists to date (Weiss et al., 2006). In animals it has been shown to be effective in reducing Fos expression and PPE following stimulation of the trigeminal ganglion (Weiss et al., 2006). Important clinical correlates also exist with a double-blinded randomized study, at which LY466195 was significantly efficacious in relieving head pain in 50% of migraine patients (Johnson, KW, International Headache Research Seminar, Copenhagen, 25 March 2007).

Moreover topiramate is a clinically effective migraine preventive (Bussone et al., 2006) and has been effective in animal models of trigeminovascular activation relevant to migraine (Storer and Goadsby, 2004; Akerman and Goadsby, 2005a, b). Topiramate is a widely used anti-convulsant (Perucca, 1997; Rosenfeld, 1997). It's mechanisms of action are as yet poorly understood and a variety of sites have been proposed, including certain voltage-activated Na^+ and Ca^{2+} channels, $GABA_A$ receptors, and AMPA/kainate glutamate receptors (Shank et al., 1994; Dodgson et al., 2000; Silberstein and Tfelt-Hansen, 2006). It seems that the common mechanism of action on these different receptors is allosteric modulation of the channels through protein phosphorylation mainly by protein kinases A and C (Silberstein and Tfelt-Hansen, 2006). Recently it has been suggested that the site of action of topiramate in anti-epileptic treatment is the iGluR5 subunit of the kainate receptor (Gryder and Rogawski, 2003; Dudek and

Bramley, 2004), since it has been shown that topiramate can inhibit iGluR5-mediated currents in the amygdala and also inhibit kainate-induced clonic seizures but not AMPA or NMDA induced seizures (Gryder and Rogawski, 2003; Kaminski et al., 2004).

In the present study, we used electrophysiology in combination with microiontophoresis to investigate the potential *in vivo* effects of LY466195 on second order neurons in the TCC as well as on third order neurons in the VPM. In addition LY466195 was given intravenously and recordings were made from the VPM and TCC. Due to experimental limitations, topiramate was only given intravenously and recordings were made from third order neurons in the VPM (figure 71).

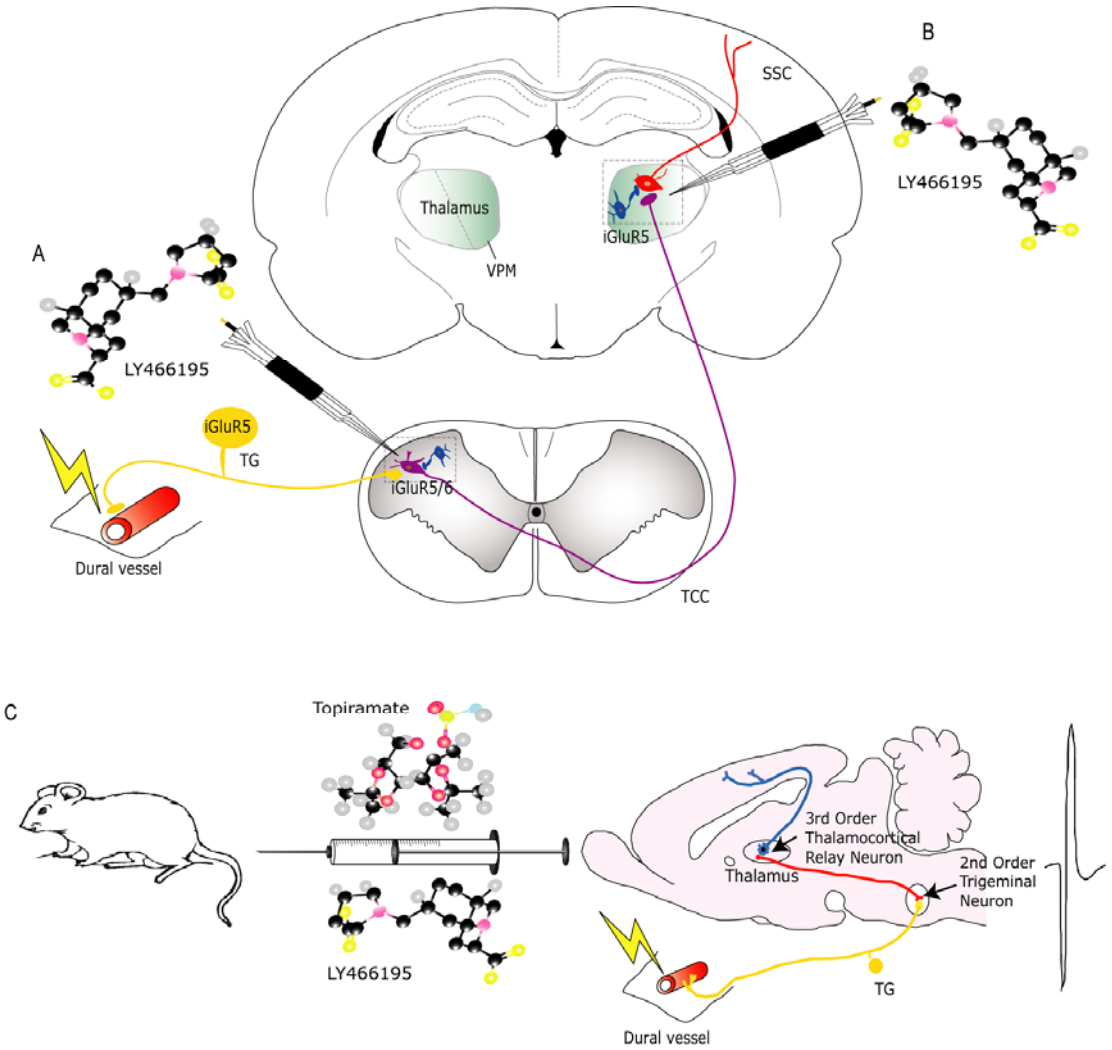


Figure 71: Electrophysiology of neurons in the ventroposteromedial thalamic nucleus and in the trigeminocervical complex

Electrophysiological recordings were made from second order neurons in the trigeminocervical complex (TCC) and from third order neurons in the ventroposteromedial thalamus (VPM). LY466195 was administered by microiontophoresis at both areas (A and B). In addition LY466195 was administered intravenously and recordings were made from neurons that received convergent inputs from dural vessels in both the VPM and the TCC (C). Topiramate was administered intravenously and recordings were made from neurons that received convergent inputs from dural vessels in the VPM (C).
TG; trigeminal ganglion

6.2 Methods

6.2.1 Animals

Sixty-four male Sprague-Dawley rats (280–380 g) were anesthetized with 60 $mgkg^{-1}$ pentobarbital intraperitoneally and then maintained with 25-35 $mgkg^{-1}h^{-1}$ infusion of pentobarbital (section 2.2). Surgical procedures, stimulation of the dural vessels and recordings were as described in sections 2.3.4-5 and 2.4.2-3.

6.2.2 Drugs

Microiontophoretic application

Micropipette barrels were filled with 200 mM NaCl for automated current balance, pontamine sky blue, Iodowillardiine, NMDA, Fluorowillardiine, LY466195 and dH_2O at pH 8 which was used as control (OH ions were ejected as control). In some experiments in the TCC serine was also included in one of the barrels, and it was used as a ligand of the glycine site of NMDA receptors in the presence of LY466195. As LY466195 displayed some activity on NMDA receptors (Weiss et al., 2006), serine was used in order to study any possible effects of the antagonist on the glycine site of NMDA receptors. All tested compounds were chosen according to their pharmacological activity (table 24), and were all ejected as anions as shown in table 24. Microiontophoretic barrels had resistances of 25-65 MΩ.

Intravenous application

Intravenous injections of LY466195 and topiramate were given as described in section 2.9.2. The doses selected for each drug were based on previous *in vivo* applications of these compounds in rats (table 25).

Table 24: Characteristics of test compounds used by microiontophoresis

	Polarity	Ejection current (nA)	pH	Pharmacological activity
Fluorowillardiine	(-)	5 - 25	8.0	iGluR1,2 and 4 AMPA receptor agonist (Jane et al., 1997)
Iodowillardiine	(-)	5 - 25	8.0	iGluR5 kainate receptor agonist (Jane et al., 1997; Swanson et al., 1998)
NMDA	(-)	10 - 50	8.0	NMDA receptor agonist (Watkins and Jane, 2006)
D-Serine	(-)	80	8.0	Ligand of the glycine site of NMDA receptor (Mothet et al., 2000)
LY466195	(-)	5, 10, 20	8.0	iGluR5 kainate receptor antagonist (Weiss et al., 2006)

NMDA, *N*–methyl–D–aspartic acid; LY466195, (3S,4aR,6S,8aR)-6-[[(2S)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]de cahydro-3-isoquinolinecarboxylic acid

Table 25: Characteristics of test compounds administered intravenously

Drug	Area of recording	Doses	Reference
LY466195	VPM	10, 50, 100 μgkg^{-1}	(Weiss et al., 2006)
	TCC	50, 100, 200 μgkg^{-1}	
Topiramate	VPM	30 $mgkg^{-1}$	(Akerman and Goadsby, 2005a, b)

LY466195, (3S,4aR,6S,8aR)-6-[[(2S)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]de cahydro-3 isoquinolinecarboxylic acid

6.2.3 Experimental protocol

Recordings in VPM and TCC– microiontophoresis of LY466195

Neurons were identified as previously described (section 2.11.5) in VPM and TCC. All neurons displayed baseline responses to dural vessel stimulation and five stable baseline cycles of glutamate receptor agonist were recorded. When more than one agonist was tested, the agonists tested for each experiment were ejected in a random order. LY466195 or vehicle control was ejected at currents of 5, 10 and 20 nA each current applied during 5 cycles of glutamate receptor agonists. A post-stimulus histogram was collected at the maximum ejecting current of LY466195 (20 nA) and during recovery period. The effects of LY466195 on second order neurons in the TCC were also tested fielding relation to nociceptive and non-nociceptive activation of the cutaneous facial receptive field.

To test any possible effects of LY466195 on NMDA receptor's glycine site, serine (NMDA receptor's glycine site ligand) was co-ejected with NMDA on second order neurons in the TCC responding to stimulation of the MMA. Serine was then co-ejected with LY466195 (20 nA), and their effects on NMDA-evoked firing were compared to that of serine and control when ejected alone. Post-stimulus histograms were recorded during each condition. Upon termination of the experiment, the tissue was fixed in order to identify the location of the recording site (section 2.11.6).

Recordings in VPM and TCC– intravenous administration of LY466195 and topiramate

Once a neuron with convergent inputs from the dura mater and facial receptive field was identified responses were tested using the following protocol. (1) Three baseline collections of dural stimulation (2) intravenous administration of LY466195 (at doses as shown in table 25) or topiramate (30 $mgkg^{-1}$ for VPM recordings) or control vehicle intervention (3) Dural stimulation collections at 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 and 90 minutes.

The effects of 100 μgkg^{-1} of LY466195 or control vehicle were also assessed on the post-synaptic firing in response to microintophorised Iodowillardiine, NMDA and Fluorowillardiine in four neurons with convergent inputs from the dura mater and facial

receptive field in the VPM. Responses were tested using the following protocol: (1) baseline collections of epochs representing firing in response to microintophorised Iodowillardiine, NMDA and Fluorowillardiine for at least 30 minutes (2) intravenous administration of LY466195 (100 μgkg^{-1}) or control vehicle intervention (3) collections of epochs representing firing in response to microintophorised Iodowillardiine, NMDA and Fluorowillardiine for 90 minutes.

6.3 Results

6.3.1 Effects of LY466195 on neuronal firing in the ventroposteromedial thalamic nucleus

A total of 36 cells were studied in the VPM (figure 72), and each displayed convergent trigeminal viscerosomatic inputs classified by cutaneous receptive fields as shown in table 26. Cells responded with an increased probability of firing to electrical SSS stimulation with an average latency of 11 ± 2 ms to the onset. To be included in the study, a probability of firing of greater than 30% was required. Only cell bodies were recorded, which were characterized by their biphasic action potential morphology and increased firing response to glutamate receptor agonist(s).

Table 26: Receptive field characteristics

Receptive field characteristics	Number of cells
Low threshold mechanosensitive (LTM)	13
Nociceptive specific (NS)	0
Wide dynamic range (WDR)	6
Vibrissal (# of vibrissae activated/cell: median)	17 (2)

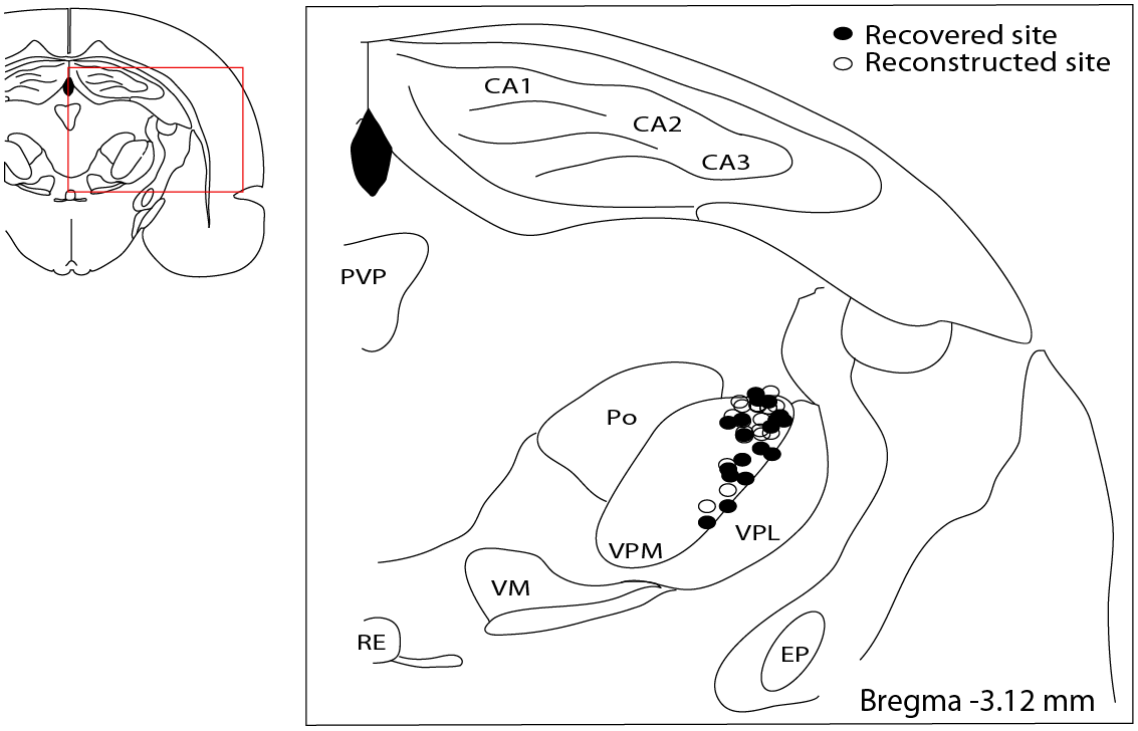


Figure 72: Localisation of recording sites within the ventroposteromedial thalamus

Reconstruction of recording sites within the ventroposteromedial thalamus (VPM) projected onto a representative section from Paxinos and Watson (1997) atlas. Sites directly identified from the ejection of pontamine sky blue are marked with solid circles (●) and sites reconstructed from the microdrive recordings with open circles (○).
CA1, CA2, CA3, CA1-3 fields of Ammon's horn; EP, Entopeduncular nucleus; Po, Posterior complex; PVP, Paraventricular nucleus; RE, Reunions nucleus; VM, Ventromedial nucleus; VPL, Ventroposterolateral nucleus; VPM, Ventroposteromedial nucleus

Effects of microiontophoretic application of LY466195 on post-synaptic firing evoked by glutamate agonists

LY466195 was applied by microiontophoresis on cells firing in response to the agonists NMDA, Fluorowillardiine and Iodowillardiine ($n = 9$). For all three agonists tested there was no difference across the mean firing of the five repeated epochs recorded during baseline (Fluorowillardiine: $F_{1,9} = 1.45$, $P = 0.26$; Iodowillardiine: $F_{1,10} = 1.46$, $P = 0.26$; NMDA: $F_{1,9} = 1.42$, $P = 0.27$) and all responses were reliable (Cronbach's $\alpha \geq 0.86$).

LY466195 potently inhibited responses to Iodowillardiine ($F_{1,11} = 5.57$, $P < 0.05$) and NMDA ($F_{2,20} = 33.29$, $P < 0.001$) evoked-firing in a dose response manner (figure 73). Although a small effect of LY466195 was seen on Fluorowillardiine evoked firing this was not significant ($F_{1,8} = 3.25$, $P = 0.11$; figure 73). Cell firing in response to the ejected agonists returned to baseline levels with Iodowillardiine-evoked firing displaying an almost immediate recovery upon LY466195 microiontophoretic cessation, whereas recovery of cell firing in response to NMDA ejection was recorded within 2-5 minutes after LY466195 microiontophoretic cessation.

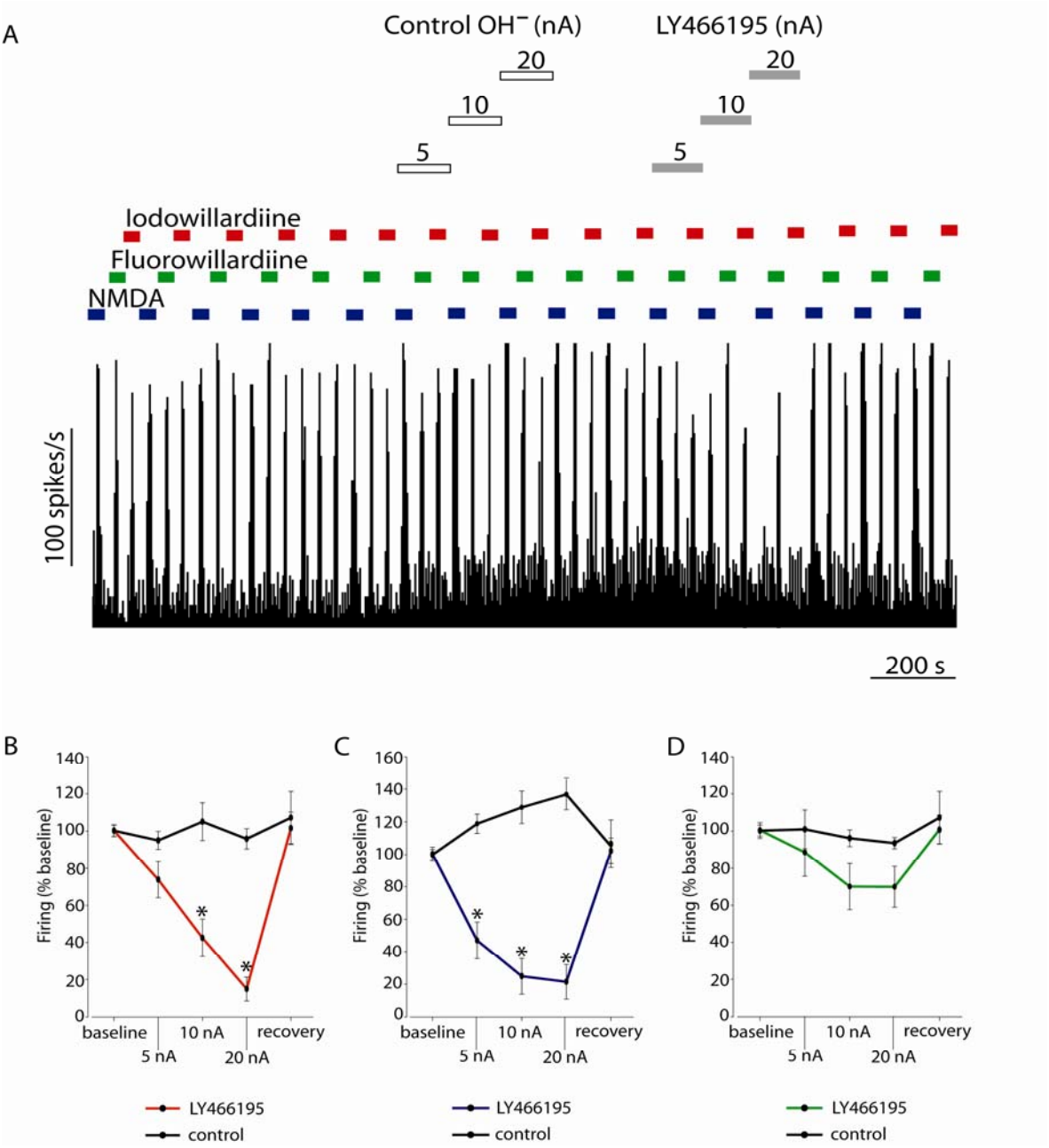


Figure 73: Effects of microiontophoretically delivered LY466195 on the firing rates of third order neurons to pulsed ejections of the receptor agonists Iodowillardiine, Fluorowillardiine and NMDA

A. Example of the effects of LY466195 and control (OH^-) on the firing rates to pulsed ejections of Fluorowillardiine, Iodowillardiine and NMDA. Cell firing in response to the ejected agonists returned to baseline levels immediately or within 2–5 minutes after LY466195 microiontophoresis ceased. B–C. Comparison of the effect of LY466195 and control OH^- on Iodowillardiine (B), NMDA (C) and Fluorowillardiine (D) evoked responses. Cell firing in response to Iodowillardiine and NMDA, but not in response to Fluorowillardiine was significantly inhibited by microiontophoretically administered LY466195. * $P < 0.05$

Effects of microiontophoretic application of LY466195 on responses to dural stimulation

Ejection of LY466195 significantly inhibited the response to SSS stimulation in comparison to control ($n = 9$; $t_8 = 6.64$, $P < 0.001$; figure 74) in a reversible manner. The drug was ejected for 3-4 minutes at 20 nA. In all cells full recovery was recorded 5-10 minutes after LY466195 microiontophoresis ceased. Ejection of control ions (OH^-) had no effect in comparison to baseline ($t_8 = 1.14$, $P = 0.29$).

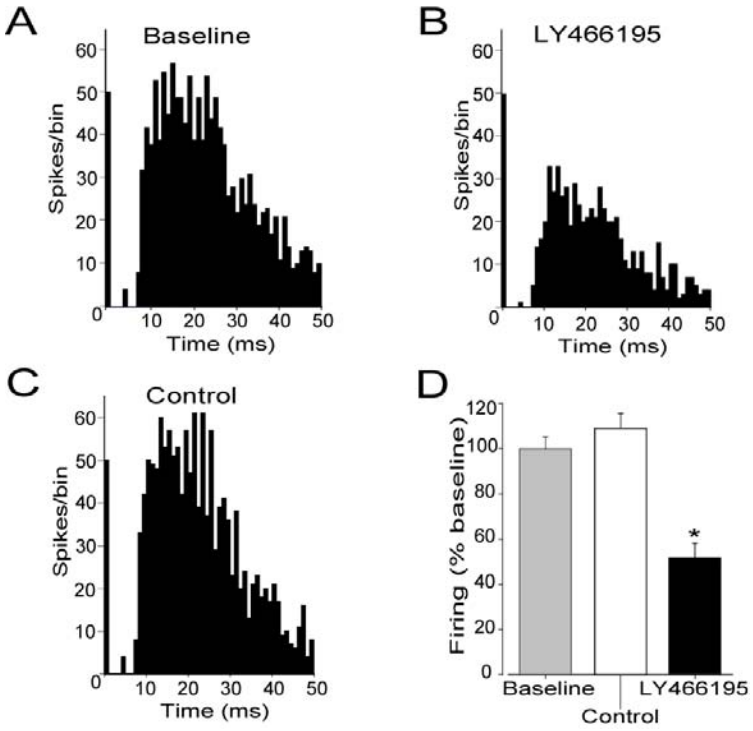


Figure 74: Effect of microiontophoresis of LY466195 on responses of thalamocortical neurons to superior sagittal sinus stimulation

A-C. Example of post-stimulus histograms from a representative neuron, recorded during baseline conditions (A), during ejection of LY466195 (B) and during ejection of control OH^- (C). D. Comparison of the SSS stimulation evoked firing under each condition. * $P < 0.05$

Effects of intravenous administration of LY466195 on responses to dural stimulation

LY466195 administrated intravenously significantly inhibited thalamocortical activity in response to SSS stimulation at 50 (n = 6; $F_{3,14}$ = 4.28, P < 0.05) and 100 μgkg^{-1} (n = 6; $F_{3,11}$ = 8.66, P < 0.005) but not at 10 μgkg^{-1} (n = 5; $F_{2,8}$ = 0.57, P = 0.59; figure 75). LY466195 at 100 μgkg^{-1} significantly decreased responses at 5 minutes and maximally at 20 minutes by 52% (t_{10} = 4.6, P < 0.005) returning to baseline levels after 90 minutes. At the dose of 50 μgkg^{-1} LY466195 displayed a significant effect on firing at 15 minutes (t_7 = 4.08, P < 0.05) by maximum of 22% and responses returned to baseline levels within 90 minutes. Intravenous administration of vehicle control had no effect on firing in response to SSS stimulation (n = 6; $F_{3,15}$ = 0.77, P = 0.53).

In all animals neither intravenous administration of LY466195 or saline control demonstrated any significant changes on blood pressure (table 27).

Table 27: Blood pressure effects of LY466195

	LY466195 (μgkg^{-1})						Control	
	100		50		10			
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Blood pressure ± SEM (mmHg)	123 ± 13	111 ± 10	103 ± 9	100 ± 10	113 ± 15	111 ± 9	98 ± 16	96 ± 15

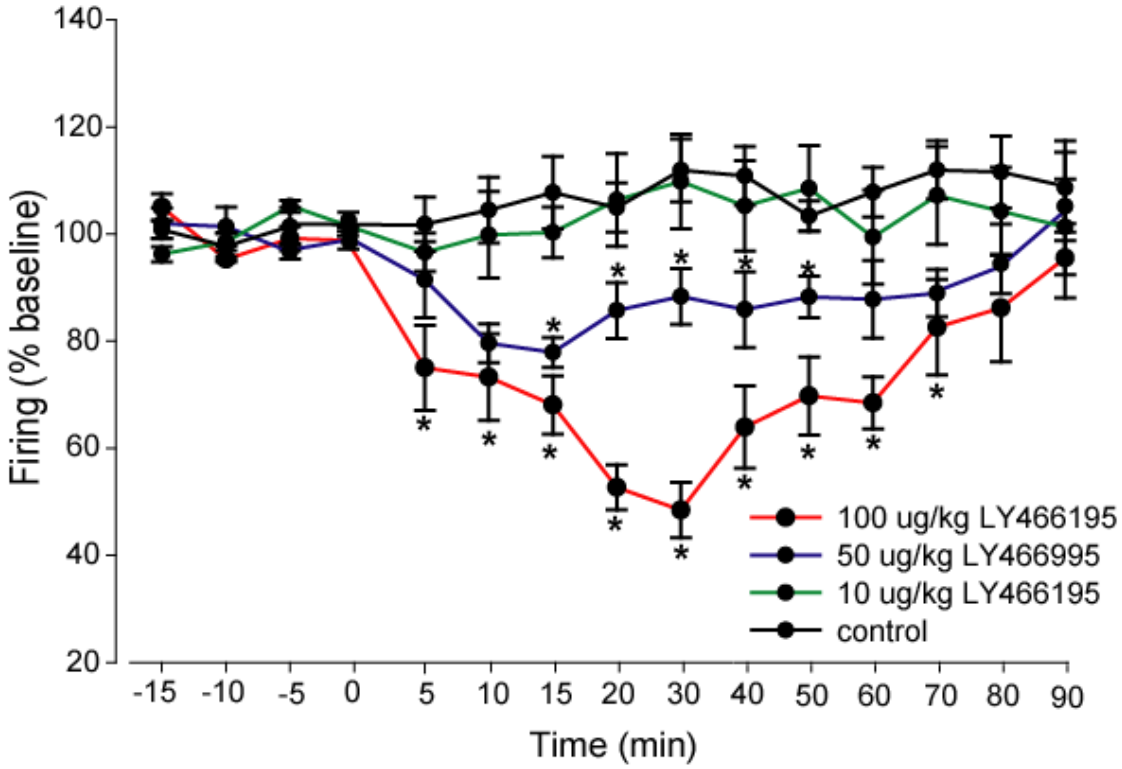


Figure 75: Effect of intravenously administrated LY466195 on responses of thalamocortical neurons to superior sagittal sinus stimulation

LY466195 inhibited firing to dural electrical stimulation at 50 and 100 μgkg^{-1}, but not at 10 μgkg^{-1}. * $P < 0.05$

Effects of intravenous administration of LY466195 on post-synaptic firing evoked by glutamate agonists

LY466195 administrated intravenously at a dose of 100 μgkg^{-1} (n = 4) significantly and reversibly inhibited firing in response to microiontophoretic administration of Iodowillardiine ($F_{2,7}$ = 27.33, P < 0.005) and NMDA ($F_{1,6}$ = 10.72, P < 0.05) but not to Fluorowillardiine ($F_{1,6}$ = 4.3, P = 0.08; figure 76). LY466195 decreased responses 10 minutes following administration and overall there was no significant difference between its effects on Iodowillardiine and NMDA-evoked firing (independent t test NMDA vs. Iodowillardiine; $P \geq 0.56$). Intravenous administration of vehicle control had no effect on firing in response to any of the three agonists tested (n = 4; $P \geq 0.30$).

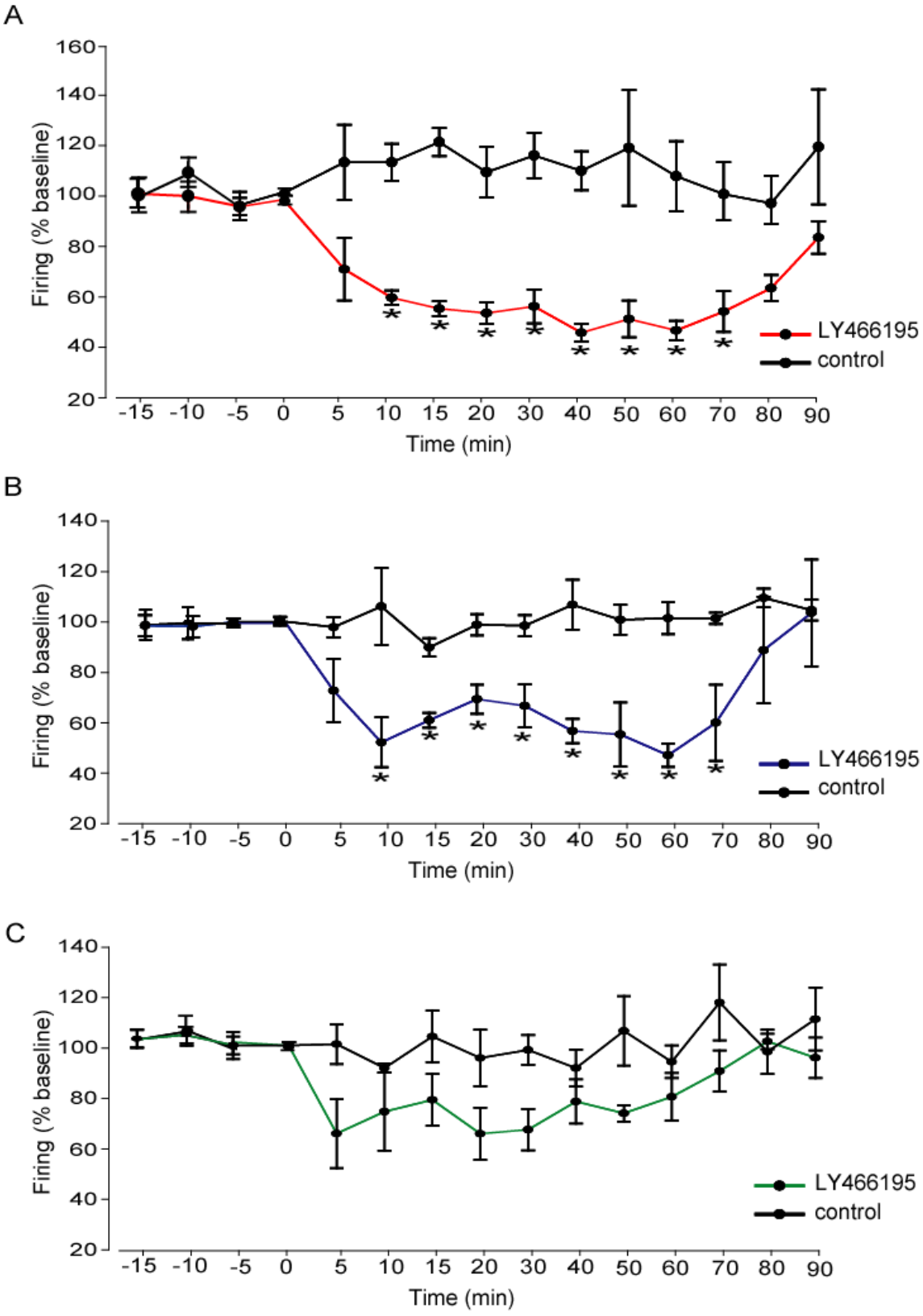


Figure 76: Effect of intravenously administrated LY466195 on post-synaptic firing of thalamocortical neurons to the glutamate receptor agonists NMDA, Fluorowillardiine and Iodowillardiine

LY466195 (100 μgkg^{-1}) inhibited firing to microiontophoretic application of Iodowillardiine (A) and NMDA (B) but not to Fluorowillardiine (C). * $P < 0.05$

6.3.2 Effects of LY466195 on neuronal firing in the trigeminocervical complex

A total of 37 wide dynamic range (WDR) units were studied from the TCC (figure 77) and each displayed convergent trigeminal viscerosomatic inputs of the ophthalmic dermatome. Extracellular recordings were made from neurons responding in a reversible excitatory manner to all glutamate agonists tested and to MMA electrical stimulation with latencies consistent with Aδ fibres (on average 9-12 ms).

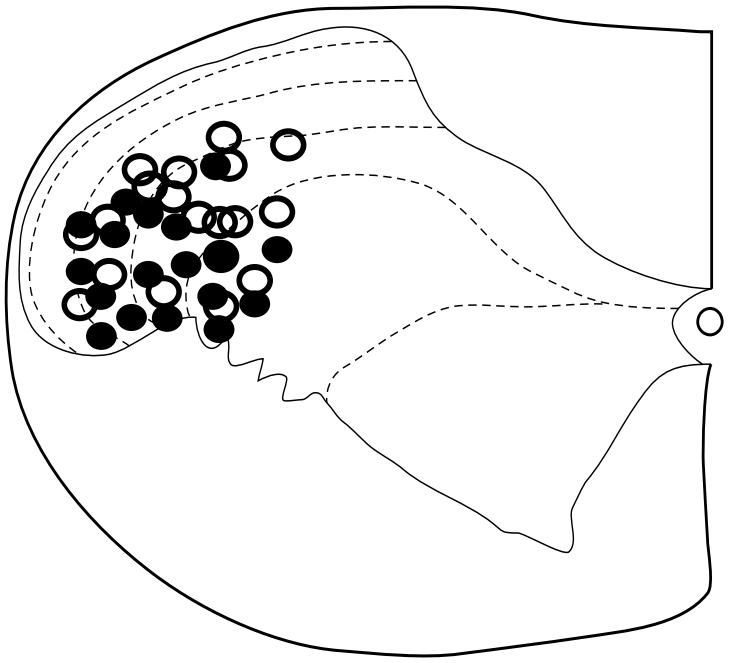


Figure 77: Localisation of recording sites within the trigeminocervical complex
A. Reconstruction of recording sites within the C1 spinal cord level, plotted after Paxinos and Watson (1997) indicating recording sites identified histologically (solid circles represent pontamine sky blue spots) and by microdrive readings (open circles).

Effects of microiontophoretic application of LY466195 on post-synaptic firing evoked by glutamate agonists

LY466195 was applied by microiontophoresis on cells firing in response to the agonists NMDA, Fluorowillardiine and Iodowillardiine ($n = 8$). For all three agonists tested there was no difference across the mean firing of the five repeated epochs recorded during baseline (Fluorowillardiine: $F_{2,12} = 0.99$, $P = 0.39$; Iodowillardiine: $F_{2,13} = 0.43$, $P = 0.64$; NMDA: $F_{2,16} = 2.41$, $P = 0.12$) and all responses were reliable (Cronbach's $\alpha \geq 0.94$).

As in the VPM, LY466195 potently inhibited responses to Iodowillardiine ($F_{4,28} = 26.41$, $P < 0.001$) and NMDA ($F_{2,11} = 12.56$, $P < 0.005$) evoked-firing in a dose response manner (figure 78). A less potent effect of LY466195 was also seen on Fluorowillardiine-evoked firing ($F_{2,13} = 4.46$, $P < 0.05$; figure 78) and this was significant compared to control at the highest current ($t_{11} = 2.78$, $P < 0.05$). Cell firing in response to the ejected agonists returned to baseline levels with Iodowillardiine-evoked firing displaying an almost immediate recovery upon LY466195 microiontophoretic cessation, whereas recovery of cell firing in response to NMDA ejection was recorded within 2-5 minutes after LY466195 microiontophoretic cessation.

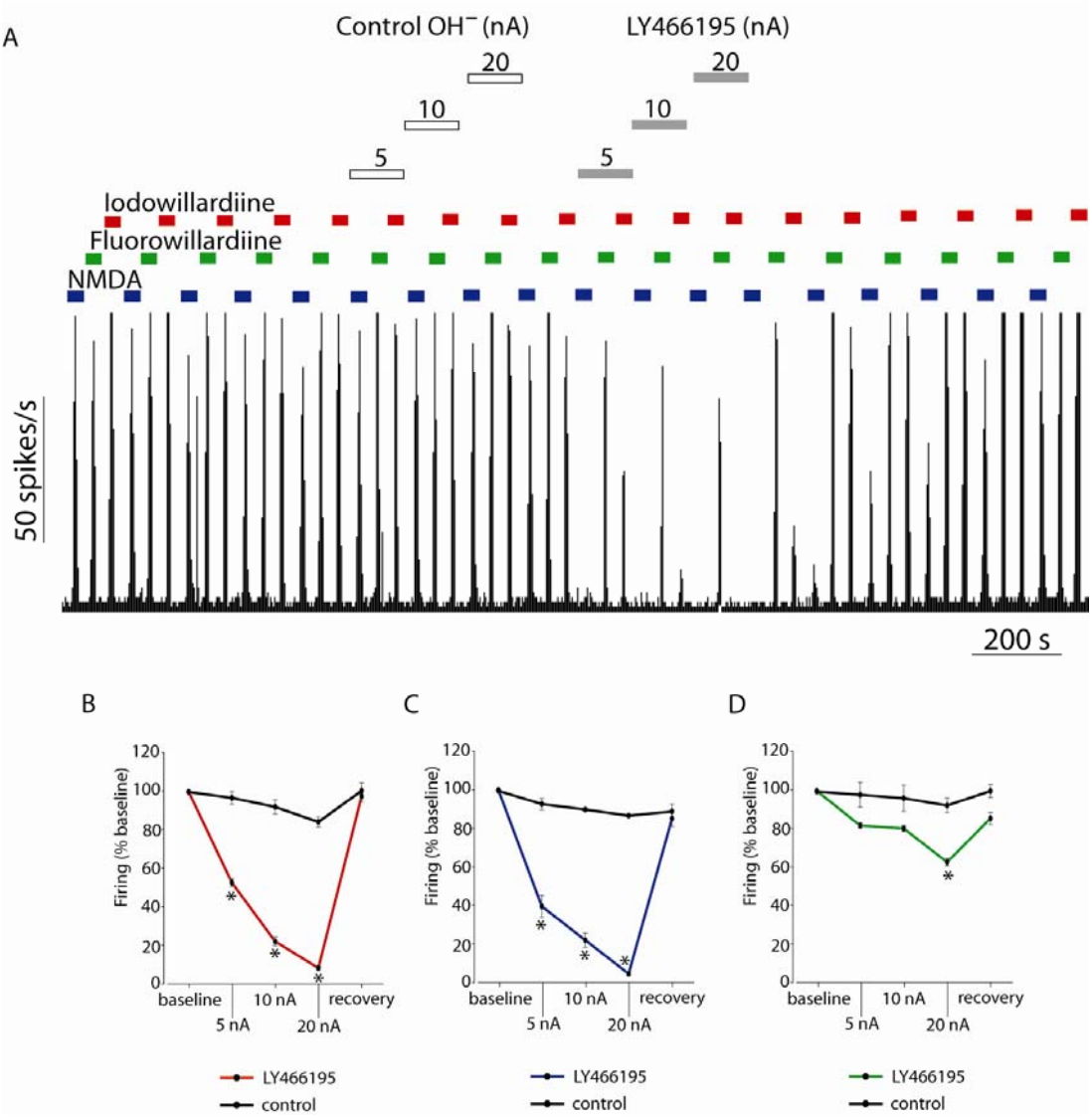


Figure 78: Effects of microiontophoretically delivered LY466195 on the firing rates of second order neurons to pulsed ejections of the glutamate receptor agonists Iodowillardiine, Fluorowillardiine and NMDA

A. Example of the effects of LY466195 on the firing rates to pulsed ejections of Fluorowillardiine, Iodowillardiine and NMDA. Cell firing in response to the ejected agonists returned to baseline levels immediately or within 3-5 minutes after LY466195 microiontophoresis ceased. B–C. Comparison of the effect of LY466195 and control OH^- on Iodowillardiine (B), NMDA (C) and Fluorowillardiine (D) evoked responses. Cell firing in response to Iodowillardiine, NMDA and to Fluorowillardiine was significantly inhibited by microiontophoretically administered LY466195. * $P < 0.05$

Effects of microiontophoretic application of LY466195 on responses to dural stimulation

Ejection of LY466195 significantly inhibited the response to MMA stimulation in comparison to control OH^- ($n = 8$; $t_7 = 4.51$, $P < 0.005$; figure 79) in all neurons tested in a reversible manner. The drug was ejected for 4 minutes at 20 nA. In all cells full recovery was recorded 5-10 minutes after LY466195 microiontophoresis ceased. Ejection of control ions (OH^-) had no effect in comparison to baseline ($t_7 = 1.65$, $P = 0.14$).

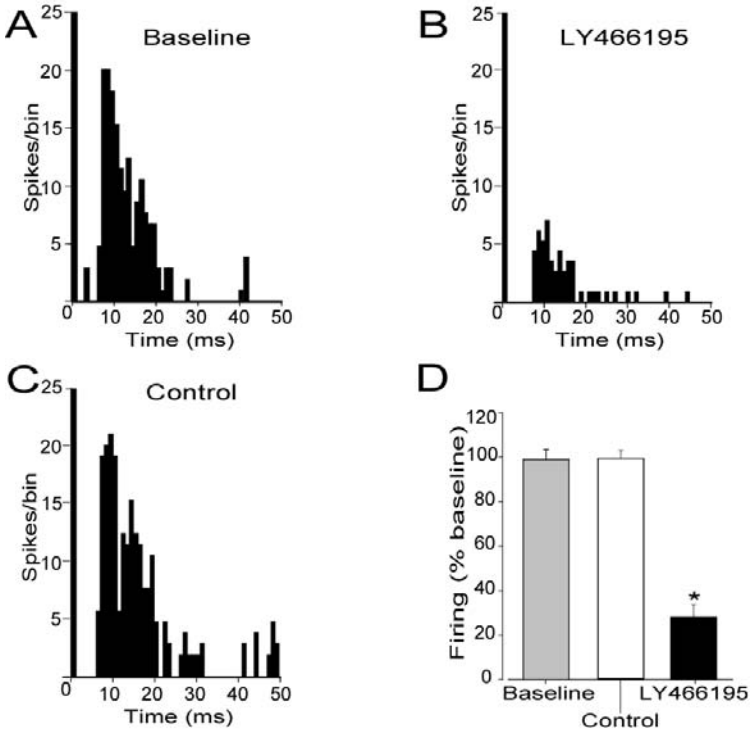


Figure 79: Effect of microiontophoresis of LY466195 on responses of trigeminocervical neurons to middle meningeal artery stimulation

A-C. Example of post-stimulus histograms from a representative neuron, recorded during baseline conditions (A), during ejection of LY466195 (B) and during ejection of control OH^- (C). D. Comparison of the MMA stimulation evoked firing under each condition. * $P < 0.05$

Effects of microiontophoretic application of LY466195 and serine on NMDA-evoked post-synaptic firing

As in both the VPM and TCC studies LY466195 demonstrated a significant action on NMDA receptors we aimed to investigate whether this effect was due to actions of LY466195 on the glycine/serine binding site of the NMDA receptor. Serine is an endogenous co-agonist of glutamate at the NMDA receptor, increasing the affinity of the receptor for the endogenous agonist glutamate. LY466195 (20 nA) was co-ejected with NMDA on second order neurons in the TCC responding to stimulation of the MMA, under continuous presence of serine (80 nA). Post-stimulus histograms were recorded during each condition.

Ejection of serine alone neither significantly affected neuronal firing in response to NMDA ejection (n = 6; t_5 = -1.94, P = 0.11; figure 80) nor to MMA stimulation (t_5 = 0.13, P = 0.91; figure 80) compared to control (OH^-). Co-ejection of serine with LY466195 failed to reverse the inhibitory actions of LY466195 on NMDA evoked post-synaptic firing (LY466195 & serine vs. control: t_5 = 4.71, $P < 0.05$; figure 80). Additionally, co-ejection of LY466195 with serine did not change the LY466195 induced inhibition on firing in response to MMA stimulation (LY466195 & serine vs. control: t_5 = 27.26, $P < 0.001$; figure 80).

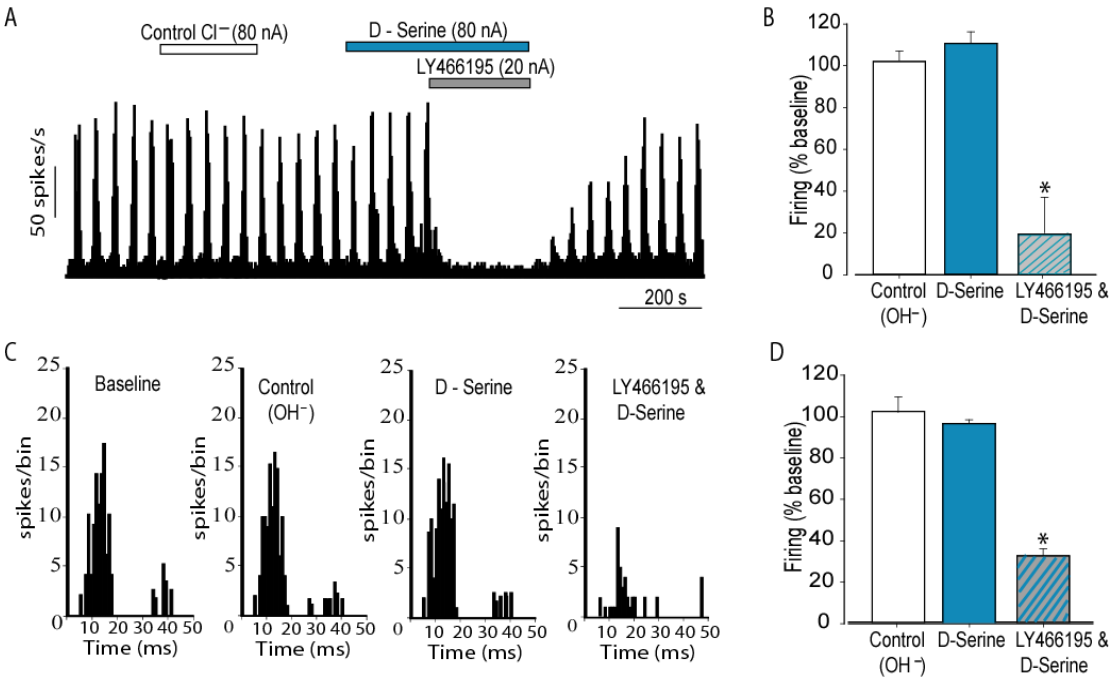


Figure 80: Effect of microiontophoresis of LY466195 and serine on responses of trigeminocervical neurons

A. Example of the response of a neuron to pulsed NMDA. Ejection of serine, LY466195 and control (OH^-) are shown with the solid bars. Ejection of LY466195 had a potent inhibitory effect that was not reversed by pre-treatment with serine. B. Comparison of the NMDA evoked firing under each condition. C. Post-stimulus histograms generated from a representative trigeminocervical neuron following electrical stimulation of the MMA. Microiontophoresis of serine or OH^- control at the same current had no effect on neuronal firing. Co-ejection of LY466195 with serine failed to block the inhibitory actions of LY466195. D. Comparison of the MMA stimulation evoked firing under each condition. * $P < 0.05$ compared to control

Effects of microiontophoretic application of LY466195 on receptive field stimulation

The effects of LY466195 on responses to innocuous (brush) and noxious (pinch and corneal brush) mechanical stimulation of the V1 facial cutaneous receptive field were tested in five WDR neurons (figure 81).

LY466195 significantly reduced in a dose-dependent manner evoked firing to both pinch and corneal brush stimulation to a maximum of 65% and 85% respectively, as well as to innocuous mechanical stimulation of the ophthalmic dermatome by a maximum of 40%, in all neurons tested (brush: $F_{1,6} = 8.75$, $P < 0.05$; pinch: $F_{1,6} = 0.81$, $P < 0.05$; corneal: $F_{1,7} = 7.99$, $P < 0.05$). Firing returned to baseline within 15 minutes after the occlusion of LY466195 ejection.

Vehicle control ejection at the same currents and duration did not change the firing rate evoked by innocuous ($F_{2,5} = 0.04$; $P = 0.90$) and noxious (pinch: $F_{1,5} = 0.04$; $P = 0.87$; corneal: $F_{2,6} = 0.80$; $P = 0.46$) stimulation of the receptive field.

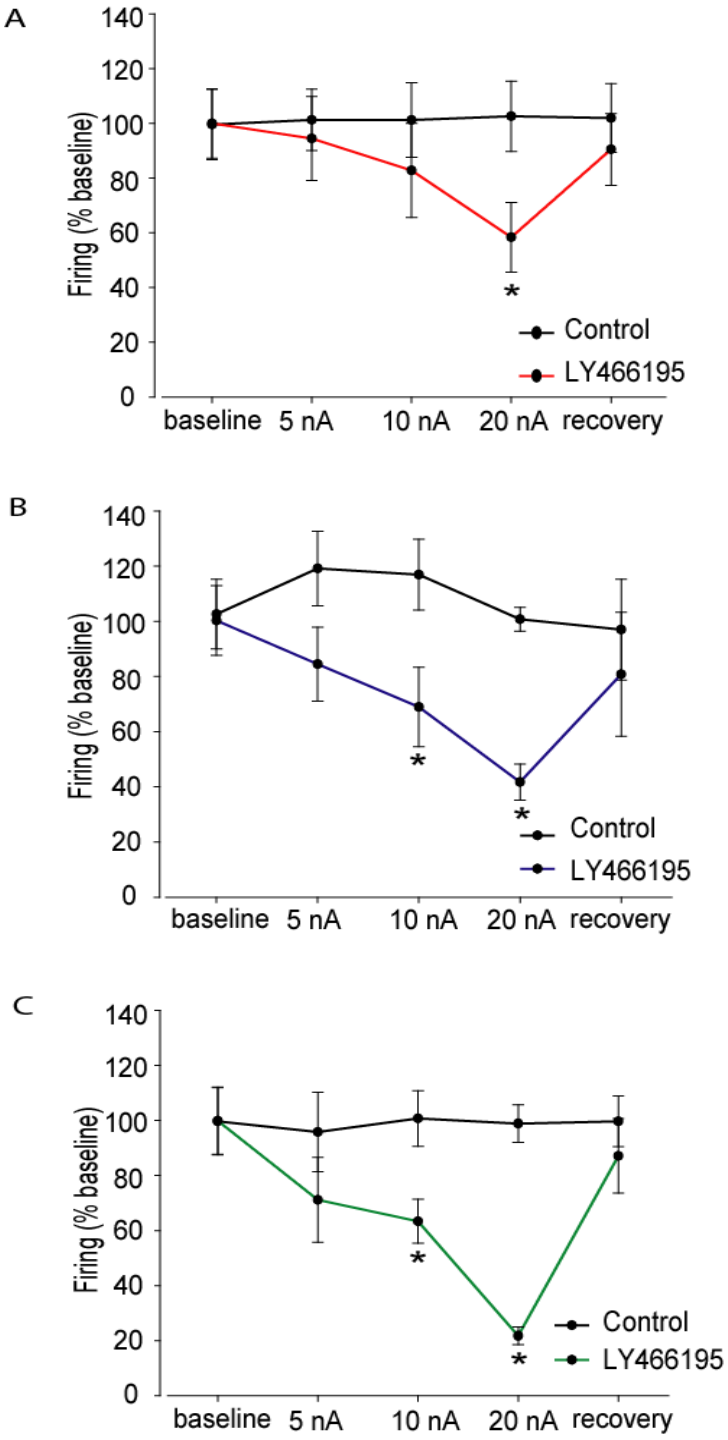


Figure 81: Effects of microiontophoretically delivered LY466195 on the firing rates of second order neurons in response to receptive field characterisation

Microiontophoretic application of LY466195 at currents 5, 10 and 20 nA significantly inhibited firing to both innocuous (brush: A) and nocuous (corneal: B; pinch: C) stimulation of the ophthalmic dermatome. Ejection of vehicle control had no effect on cell firing. * $P < 0.05$

Effects of intravenous LY466195 on middle meningeal artery-stimulation evoked activity

LY466195 administered intravenously significantly inhibited trigeminocervical activity in response to MMA stimulation at a dose of 100 μgkg^{-1} ($n = 7$; $F_{2,10} = 4.22$, $P < 0.05$; figure 82) by a maximum of 33% ($t_{12} = 4.48$, $P < 0.05$; table 28). LY466195 at 200 μgkg^{-1} significantly decreased responses ($n = 5$; $F_{3,11} = 5.28$, $P < 0.05$) at similar levels as the 100 μgkg^{-1} dose by a maximum of 32% ($t_6 = 4.20$, $P < 0.05$; figure 82) and there was no significant difference between the effects induced by the two doses ($P \geq 0.40$). Cell firing returned to baseline levels within 90 minutes. Neither LY466195 at the dose of 50 μgkg^{-1} ($n = 5$; $F_{2,7} = 2.70$, $P = 0.13$) nor saline control ($n = 7$; $F_{2,7} = 2.25$, $P = 0.18$) displayed a significant effect on cell firing in response to MMA stimulation. In all animals neither intravenous administration of LY466195 nor of saline control demonstrated any significant changes on blood pressure (table 29).

Table 28: Percentage of maximum inhibition of cell firing by different LY466195 doses in response to dural stimulation

	LY466195 (μgkg^{-1})							
	10		50		100		200	
	TCC	VPM	TCC	VPM	TCC	VPM	TCC	VPM
% inhibition	–	5 $P = 0.6$	7 $P = 0.1$	22 $P < 0.05$	33 $P < 0.05$	52 $P < 0.05$	32 $P < 0.05$	– $P < 0.05$

Table 29: Blood pressure effects of LY466195

	LY466195 (μgkg^{-1})						Control	
	200		100		50			
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Blood pressure ± SEM (mmHg)	98 ± 17	88 ± 11	108 ± 11	101 ± 10	93 ± 17	91 ± 18	118 ± 17	118 ± 18

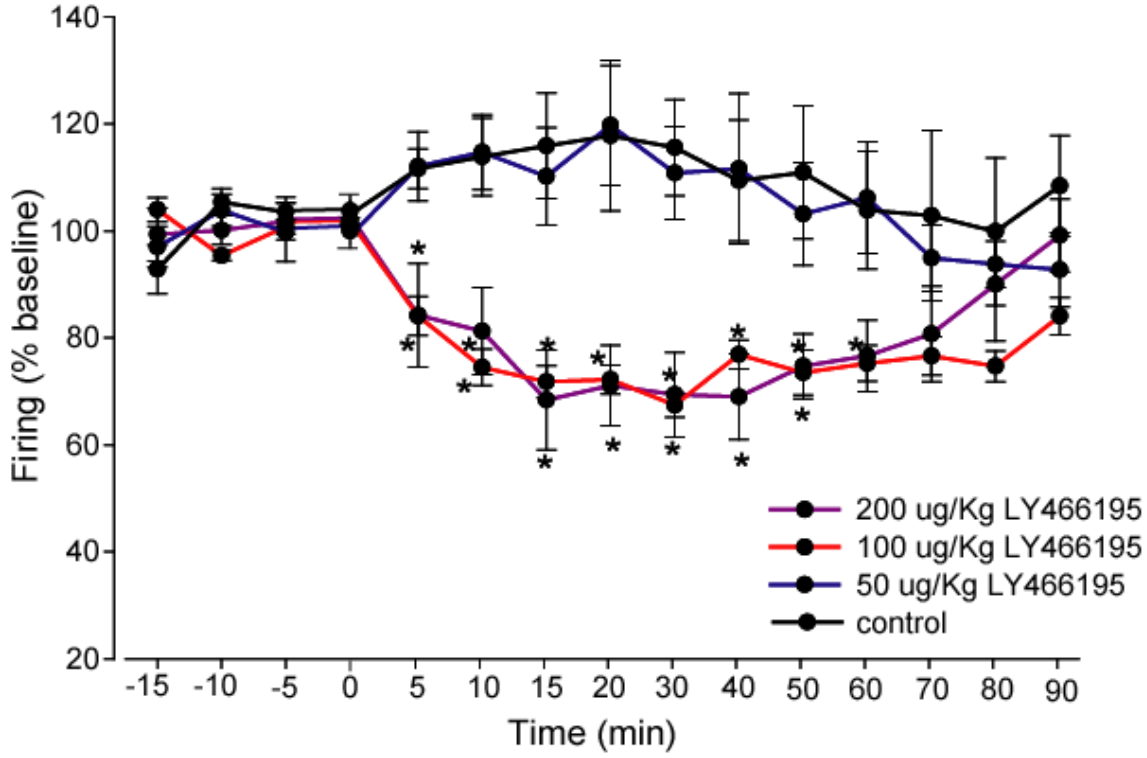


Figure 82: Effect of intravenously administrated LY466195 on responses of trigeminocervical neurons to middle meningeal artery stimulation

LY466195 inhibited firing to dural electrical stimulation at 200 and 100 μgkg^{-1}, but not at 50 μgkg^{-1}. * $P < 0.05$

6.3.3 Effects of intravenous administration of topiramate on responses to dural stimulation in the ventroposteromedial thalamic nucleus

A total of 14 cells were studied in the VPM (figure 83), and each displayed convergent trigeminal viscerosomatic inputs classified by cutaneous receptive fields as shown in table 30. Cells responded with an increased probability of firing to electrical stimulation of the SSS with an average latency of 12 ± 2 ms to the onset. Recordings were made with microiontophoretic electrodes and all cells studied displayed increased firing in response to Iodowillardiine. Only cell bodies were recorded, which were characterised by their biphasic action and the increased firing in response to Iodowillardiine. Topiramate was not tested by microiontophoresis as difficulties in its solubility caused a high degree of barrel blockade.

Table 30: Receptive field characteristics

Receptive field characteristics	Number of cells
Low threshold mechanosensitive (LTM)	4
Nociceptive specific (NS)	0
Wide dynamic range (WDR)	3
Vibrissal (# of vibrissae activated/cell: median)	7 (3)

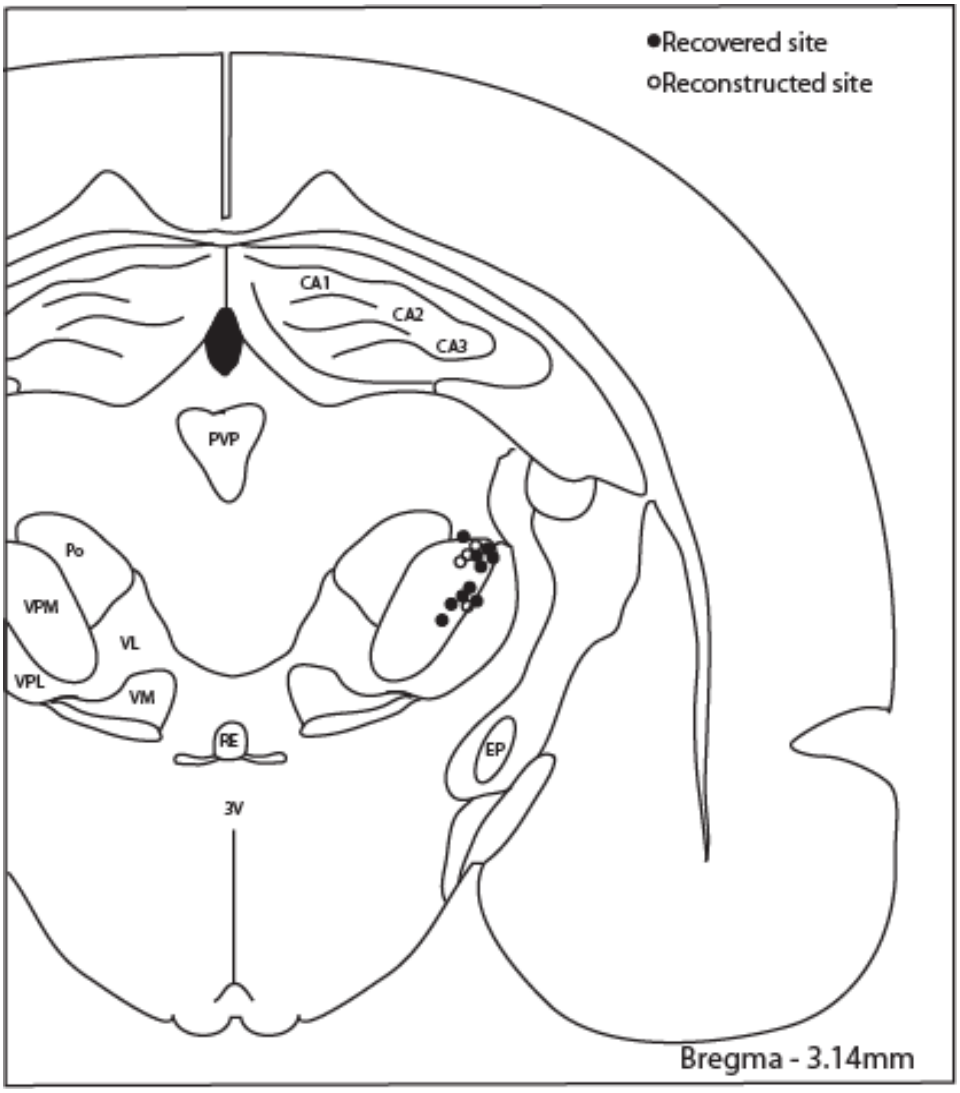


Figure 83: Localisation of recording sites within the ventroposteromedial thalamus

Reconstruction of recording sites within the ventroposteromedial thalamus projected onto representative sections from the Paxinos and Watson (1997) atlas. Sites directly identified from the ejection of pontamine sky blue are marked with solid circles (●) and sites reconstructed from the microdrive recordings with open circles (○).

CA1, CA2, CA3, CA1-3 fields of Ammon's horn; EP, Entopeduncular nucleus; Po, Posterior complex; PVP, Paraventricular nucleus; RE, Reunions nucleus; VM, Ventromedial nucleus; VPL, Ventroposterolateral nucleus; VPM, Ventroposteromedial nucleus; 3V, Third ventricle; RT, reticular nucleus

Topiramate administrated intravenously at 30 $mgkg^{-1}$ significantly inhibited trigeminovascular activity in VPM in response to SSS stimulation ($n = 8$; $F_{2,16} = 5.11$, $P < 0.05$; figure 84) at a maximum of 28% ($t_9 = 4.51$, $P < 0.005$). Ninety minutes following intravenous administration of topiramate, no full recovery of cell firing in response to SSS stimulation was seen. Intravenous administration of dH_2O given as vehicle control had no effect on firing in response to SSS stimulation ($n = 6$; $F_{2,9} = 0.43$, $P = 0.69$; figure 84). In all animals neither intravenous administration of topiramate nor of saline control demonstrated any significant changes on blood pressure (table 31).

Table 31: Blood pressure effects of topiramate 30 $mgkg^{-1}$

	Topiramate (30 $mgkg^{-1}$)		Control	
	Pre-	Post-	Pre-	Post-
Blood pressure (mmHg)	115 ± 16	110 ± 18	115 ± 16	113 ± 14

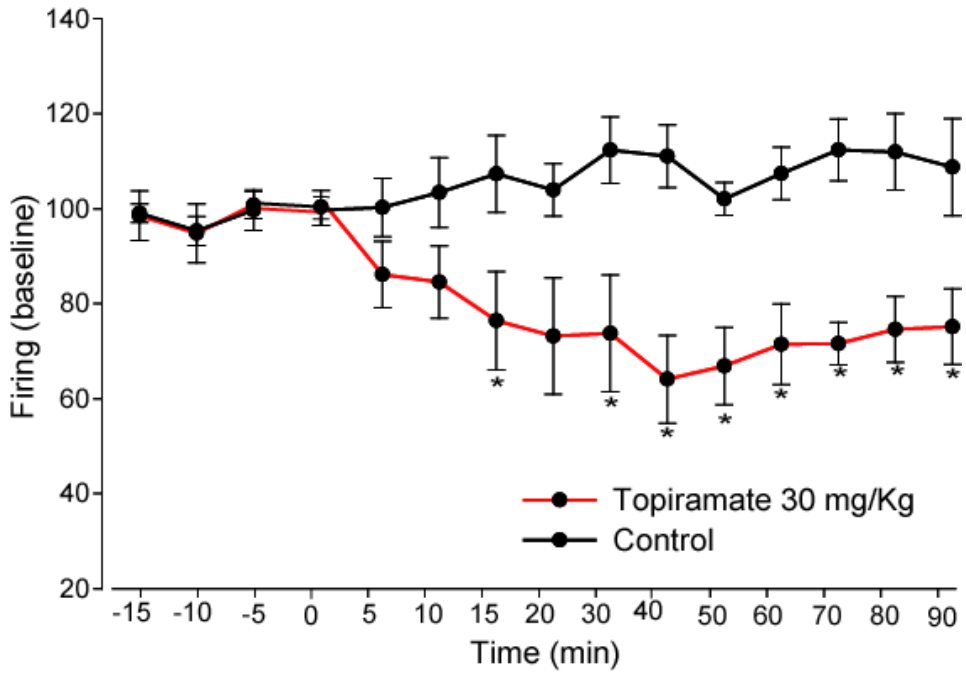


Figure 84: Effect of intravenously administered topiramate on responses of thalamocortical neurons to superior sagittal sinus stimulation

Topiramate at 30 $mgkg^{-1}$ inhibited cell firing to dural electrical stimulation. Firing did not recovered 90 minutes following administration of topiramate. * $P < 0.05$

6.4 Conclusions

The study demonstrated that the LY466195 compound either administrated intravenously or locally on neurons by microiontophoresis can potently inhibit trigeminovascular stimulation, both at the level of TCC and VPM. Furthermore the results obtained from recordings in both the TCC and the VPM following intravenous administration of LY466195 confirm an important role of VPM as a potential target of anti-migraine treatments. The microiontophoretic experiments conducted for this study demonstrated that although the compound has a potent action on iGluR5 receptor activation, it also potently inhibits NMDA-evoked firing. Intravenous administration of topiramate was able to inhibit firing of thalamocortical neurons in response to SSS stimulation; however due to the difficulties seen in using this compound with microiontophoresis further experiments will be needed in order to identify its site of action, as well as to have a better understanding of any interactions of this compound with glutamate receptors.

Microiontophoretic application of LY466195 demonstrated a potent action of this antagonist on iGluR5 kainate receptor-evoked firing, and this is in accordance with its characterisation as described by Weiss and colleagues (2006). However we also observed a significant action on NMDA receptors. Based on *in vitro* binding data and electrophysiological studies on native iGluR5 receptors on DRG and on native NMDA receptors on hippocampal neurons, LY466195 was shown to be 55-fold selective for the iGluR5 receptor over NMDA receptor and this antagonist activity of LY466195 was competitive (Weiss et al., 2006). The compound given at 100 $mgkg^{-1}$ orally was shown to have no ataxia related to NMDA antagonist-like behavioural effects in an automated activity monitoring system in rats (Weiss et al., 2006). A major limitation of microiontophoresis is that we cannot completely control the concentration of the drug ejected (Stone, 1985) and thus it is possible that we ejected enough of the drug to act unselectively on NMDA receptors. However, the NMDA effects of LY466195 were observed at very small currents (5 nA) and at this current the inhibitory action of LY466195 was more potent on NMDA-evoked firing rather than on selective iGluR5 kainate receptors-evoked firing. It is worth mentioning that with this technique the antagonist UBP 302 demonstrated high selectivity for iGluR5 kainate receptors over NMDA and AMPA receptors as shown in chapters 4 and 5.

The kainate receptor subunits share common structural features, although they share less than 20% homology with the NMDA receptors at the amino acid level (Huettner, 2003), and as with most kainate receptor antagonists described to date, it is more common to have some action on AMPA receptors than on NMDA receptors (Ruscheweyh and Sandkuhler, 2002; Dolman et al., 2005). We aimed to further investigate whether the LY466195 compound has any actions on the glycine/serine binding site of the NMDA receptors as seen with the antagonist CNQX (used in studies described in chapter 4) which has an antagonistic action on the glycine/serine site of the NMDA receptor (Lester et al., 1989). Serine, which is derivered from glycine, is a co-agonist of glutamate at the NMDA receptor, increasing the affinity of the receptor for the endogenous agonist glutamate (Mothet et al., 2000; Shleper et al., 2005). Hence antagonism of this ligand binding site will also antagonise NMDA receptor function. Ejection of LY466195 in the presence of serine, failed to prevent the inhibitory actions of LY466195 on NMDA evoked firing. As LY466195 antagonist activity is competitive (Weiss et al., 2006), the excess presence of serine ejected by microiontophoresis could have competed with the actions of LY466195, if the later was acting on the glycine/serine site. The results and the competitive nature of LY466195 could also suggest that LY466195 acts on the glutamate binding site.

An NMDA receptor action of LY466195 was also seen in the VPM when LY466195 was given intravenously, (100 μgkg^{-1}); however these results should be treated carefully due to the small number of units tested. It was proved difficult to find cells with stable responses to all three agonists used for at least 30 min, in order to establish reliable baseline responses before administrating the antagonist. More experiments to better investigate this should be planned in the future. Perhaps a more ideal model to test the proposed NMDA action of the compound at the doses which was effective in reducing trigeminovascular activation is the CSD model. Since we know that NMDA receptor but not AMPA/kainate receptors are involved in the development of CSD (Lauritzen and Hansen, 1992), and thus the results could indicate whether systemic administration of the drug results in an action on NMDA receptor sites.

Nevertheless LY466195 is a potent antagonist of trigeminovascular activation and its action on iGluR5 evoked firing further points to an important role of kainate receptors carrying the iGluR5 subunit in the trigeminovascular ascending pathway. Microiontophoresis of LY466195 was also able to inhibit both noxious and innocuous

sensory responses of the ophthalmic dermatome, with noxious responses being more sensitive to LY466195 when ejected at low currents.

Intravenous administration of LY466195 has further shown that it displays its maximum effect (~ 30% of inhibition) on trigeminocervical neurons at a dose of 100 μgkg^{-1} and administration of a higher dose no longer potentiates the inhibitory actions of the compound. Responses of corticothalamic neurons at the same dose were further inhibited to ~ 50% indicating that at the level of the VPM a sum effect of LY466195 was recorded, likely showing the effects of LY466195 both on second as well as on third order neurons, although an action of LY466195 in other areas is also possible. This further suggests that the VPM nucleus can be a potential target for new anti-migraine treatments.

Topiramate has been suggested to display its anti-epileptic effects via multiple mechanisms: inhibition of voltage-sensitive Na^+ channels, increased GABA-induced chloride influx (Gordey et al., 2000) and blockade of glutamate-related excitatory neurotransmission. Specifically, topiramate inhibited the excitatory responses of hippocampal neurons elicited by selective activation of the iGluR5 kainate receptor subtype which are very important on the development of seizures (Rogawski et al., 2003; Dudek and Bramley, 2004). In our experiments we failed to characterise the possible action of topiramate on ionotropic glutamate receptors with microiontophoresis, due to technical limitations. Intravenous administration of topiramate demonstrated inhibition of trigeminocervical firing in response to dural stimulation in cats (Storer and Goadsby, 2004). Similarly our experiments provided evidence for a significant inhibition of thalamocortical firing in response to dural stimulation. However, whether this reflects an action of the compound in the VPM or its action in the TCC as transmitted in the VPM cannot be dissected out with the systemic administration of the compound. In addition due to the different species used in the two studies, it is more difficult to conclude on the actual site of action of topiramate in the CNS. Systemic administration of drugs does not allow the possible localisation of their effects. This is particularly a problem in thalamic studies, as the drugs may have actions at more distal sites, such as the terminals of primary trigeminal nerves or in the TCC. In future experiments the site of action of topiramate could be shown by combining microinjection of topiramate in the TCC and recordings from third order neurons in the VPM. Failure to record full recovery of cell firing in response to dural stimulation 90

minutes following intravenous administration of topiramate, might reflect a prolonged activity of topiramate as the compound's half life has been shown to be 21 hours (Silberstein and Tfelt-Hansen, 2006).

It is clear that inhibition of trigeminovascular neurons activated by a nociceptive stimulus has been predictive of acute anti-migraine activity (De Vries et al., 1999) and a positive effect of the kainate acting compounds tested further suggests a possible role of kainate receptors in migraine pathophysiology.

Chapter 7: General discussion

The studies reported in this thesis aimed to investigate the potential role of kainate receptors carrying the iGluR5 subunit in areas known to be involved in migraine pathophysiology, and further to explore whether compounds with putative kainate receptor mechanism attenuate trigeminovascular transmission and thus be useful in migraine treatment. By using animal models that have been predictive of acute anti-migraine activity we showed that iGluR5 kainate receptors are present on key structures of the "trigeminovascular ascending pathway" and may modulate, through different mechanisms at each locus, nociceptive trigeminovascular responses (figure 85).

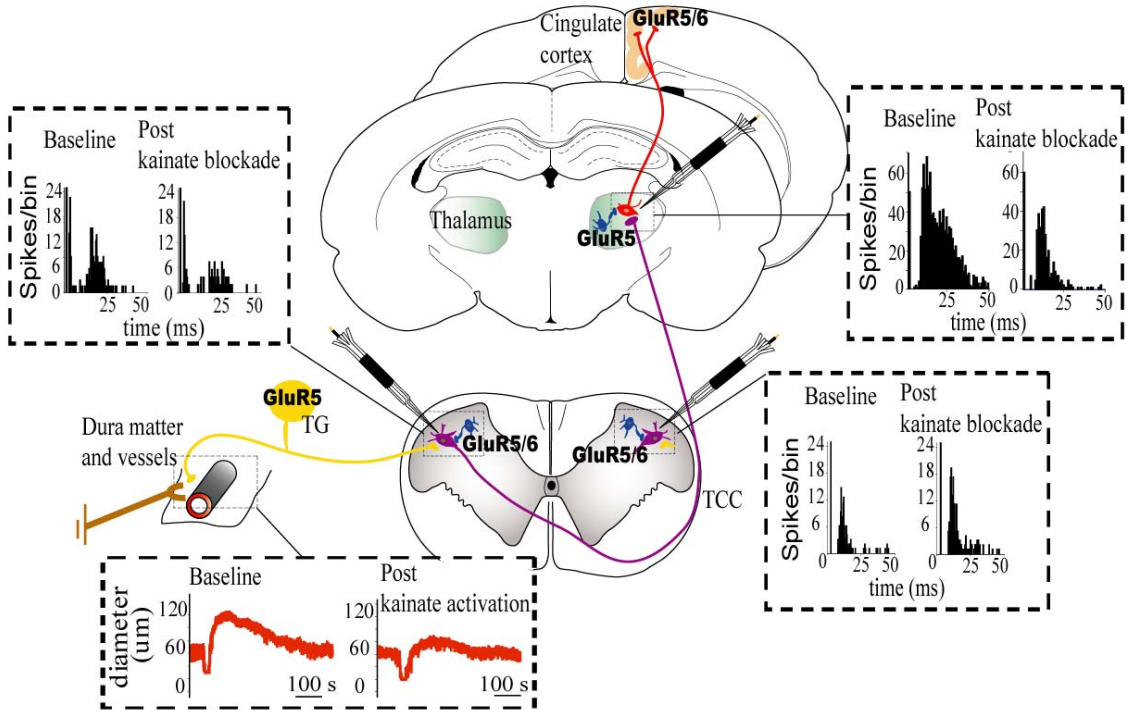


Figure 85: Overview of the role of iGluR5 kainate receptors in the "trigeminovascular ascending pathway"

TG; trigeminal ganglion, TCC; trigeminocervical complex

7.1 Role of iGluR5 kainate receptors in the "trigeminovascular ascending pathway"

The current thesis has demonstrated that iGluR5 kainate receptors are present at the pre-junctional site of the trigeminal afferents. Under normal physiological conditions it is possible that these are silent receptors as their blockade did not show any tonic action. The role of these receptors on trigeminal afferents under normal physiological conditions is not known, although studies of kainate receptors on other peripheral sensory afferents suggest an action of these receptors in detecting normal sensory responses and upon their activation they participate in peripheral nociceptive transduction (Agrawal and Evans, 1986; Ault and Hildebrand, 1993). A similar mechanism might exist on trigeminal afferents. During migraine attacks it has been shown that glutamate and glutamine levels are elevated in the plasma and in the CSF (Ferrari et al., 1990; Martinez et al., 1993; Alam et al., 1998). It is thus likely that peripheral kainate receptors on trigeminal afferents could be activated by the increased levels of glutamate under such abnormal conditions. As shown in the current thesis, activation of these receptors could decrease durovascular afferent activation. This could suggest the presence of a pre-junctional mechanism which acts as a peripheral protective barrier against nociceptive trigeminovascular activation. A failure of this mechanism will result in a decreased threshold for the induction of the nociceptive neurogenic vasodilation.

In the spinal cord and caudal brainstem kainate receptors are present at both pre- and post-synaptic locations (Petralia et al., 1994; Lucifora et al., 2006; Hegarty et al., 2007). Results from our studies show that kainate receptors at the post-synaptic site in the TCC participate in nociceptive transduction; although in some trigeminocervical synapses their post-synaptic inhibition did not alter trigeminovascular activation. On the other hand, at the pre-synaptic site on primary trigeminal afferents it is likely that kainate receptors control glutamate release – as seen in the spinal cord (Kerchner et al., 2001a), acting as a protective mechanism against excess glutamate release as a result of excessive trigeminovascular activation. Such a mechanism is in agreement with the proposed action of kainate receptors at the pre-junctional site, since activation of these receptors results in decreased neurotransmitter release. Failure of this proposed trigeminocervical pre-synaptic kainate receptor-mediated protective mechanism, will

result in a persistent neuronal hyperexcitability (figure 53) and possibly lead to central sensitization as seen in migraine attacks (Burstein et al., 2000; Burstein, 2001).

In the VPM kainate receptors are present on thalamocortical synapses which relay trigeminovascular sensory responses. Their role in the thalamus is still not well understood due to the limited studies conducted to date on the pharmacology of kainate receptors in this structure. Blockade of kainate receptors, with the aid of selective antagonists, probably at the post-synaptic site, is able to inhibit cell firing in response to trigeminovascular stimulation, without predominantly affecting NMDA and AMPA mediated transmission. This indicates that kainate receptors participate in relaying ascending trigeminovascular sensory information to the cortex. Inhibition of trigeminovascular nociception at the level of VPM offers a last line of defence for controlling nociceptive inputs to the somatosensory cortex. Results from these studies suggest that the VPM nucleus is a potential site of action for new kainate receptor antagonists in migraine. VPM neurons may be a target for preventive treatments; certainly they are inhibited by β-adrenoceptor blockers, such as propranolol (Shields and Goadsby, 2005) and GABAergic modulators, such as valproate (Shields et al., 2003). Remarkably they may also be a target for triptans, 5-$HT_{1B/1D}$ receptor agonists, (Shields and Goadsby, 2006), further suggesting that this structure can be a potential target for the development of new anti-migraine therapies. Human imaging studies have confirmed activation of thalamus contralateral to pain in acute migraine (Bahra et al., 2001; Afridi et al., 2005a), and the development of more selective kainate receptor antagonists in the future, will enable further investigation of this thalamic activation.

Further to the possible post-synaptic role of kainate receptors in the VPM, it is likely that iGluR5 receptors are present on serotonergic terminals, in addition to their presence on GABAergic terminals arising from the thalamic reticular nucleus (Salt, 2002; Binns et al., 2003). The kainate receptor mediated inhibition seen in chapter 5 can be modulated by 5-HT_{1B} receptor interactions. Although microiontophoretic application of selective compounds was able to reveal such interactions at the neuronal level, this technique does not provide any further tools for the investigation of the actual mechanism of such interactions. On GABAergic terminals it has been suggested that activation of kainate receptors decreases GABA release (Binns et al., 2003). A widespread finding for kainate receptors, is that they can play a significant role in neuromodulation through effects on presynaptic release of neurotransmitters (Chittajallu

et al., 1999; Lauri et al., 2001; Clarke and Collingridge, 2004). It is hence likely, that this mechanism may account for the kainate receptors on serotonergic terminals in the VPM, arising mostly from the DRN (Consolazione et al., 1984; Prieto-Gomez et al., 1989; Tao et al., 1997). Thus inhibition of the mechanism controlling serotonin release via kainate receptors will result in increased levels of released serotonin which could additionally contribute to the inhibition of thalamocortical firing in response to SSS stimulation, by acting primarily on 5-HT_{1B} receptors. Such a mechanism further suggests a significant role of kainate receptors in modulating neurotransmitter release that will in turn modulate trigeminovascular nociceptive transmission.

Interestingly, the proposed sites of action of kainate acting compounds follow a similar pattern to the proposed action of triptans. Triptans have been shown to act at the presynaptic site of peripheral trigeminal afferents, at pre- and postsynaptic loci in the TCC and in the VPM (Goadsby et al., 2002; Shields and Goadsby, 2006). These structures offer a potential target for novel anti-migraine medications, and the modulation of nociceptive transmission by kainate receptors along this pathway makes kainate receptors good candidates for the development of novel migraine therapeutics. More interestingly, the lack of a vascular function of kainate receptors-in contrast to triptans, and of a peripheral action for kainate antagonists makes a central site of action even more attractive and represents a novel, neuronal, non-vascular, and non-serotonergic approach to the treatment of migraine.

7.2 Targeting kainate receptors

Several neurotransmitters, neuropeptides and receptor families have been implicated in migraine pathophysiology over the years, including serotonin, GABA, dopamine, glutamate, and families of ion channels including voltage- and ligand-gated channels (Williamson et al., 1997c; Mascia et al., 1998; Storer and Goadsby, 1999; Mitsikostas and Sanchez del Rio, 2001; Storer et al., 2004a; Storer et al., 2004b; Holland et al., 2005; Akerman et al., 2007). The failure of triptans to relieve headache in all migraine patients, their increased side effects- including concerns on cardiovascular components, as well as the better understanding of migraine pathophysiology as a brain disorder,

point to the need for the development of new anti-migraine therapeutics that will have a specific action on the trigeminal nerve or on brain areas involved in migraine pathophysiology (Goadsby et al., 2002; Goadsby, 2005a, 2007).

Glutamate is thought to be the major excitatory neurotransmitter for nociceptive information in the nociceptive ascending pathway (Hill and Salt, 1982; Salt and Hill, 1982; Broman and Ottersen, 1992; Salt and Turner, 1998; Vikelis and Mitsikostas, 2007) and thus targeting glutamate receptors is a potential mechanism for the development of anti-migraine therapeutics. Kainate receptors belong to the ionotropic glutamate receptor family and are believed to mediate fast-synaptic transmission although they appear to have a more distinct distribution than AMPA and NMDA receptors. AMPA receptors mediate fast, rapidly desensitizing excitation at most synapses, and are responsible for the initial reaction to glutamate in the synapse. AMPA receptors are usually co-expressed with NMDA, which are responsible for the long term potentiation of excitatory transmission, a mechanism particularly important for the development of memory (Watkins and Jane, 2006). As glutamate is the major excitatory neurotransmitter in the CNS, targeting the widely distributed AMPA and NMDA receptors causes numerous side effects such as memory impairment, psychotomimetic effects, ataxia, sedation, respiratory depression and motor dysfunctions (Koek et al., 1988; O'Neill et al., 1998).

The serious side effects seen with AMPA and NMDA blockers, as well as progress in the pharmacological characterisation of kainate receptor, led during the last decade, to the study of kainate receptors in animal models of nociception. It has been demonstrated that several kainate receptor antagonists have analgesic activity in a variety of animal models of pain, without showing considerable side effects (O'Neill et al., 1998). The distinct presence of kainate receptors at synapses where higher control of excitability is required, such as in the dorsal horn, the hippocampus and the cortex, as well as their involvement in pathological conditions, like pain disorders and epilepsy, suggest a role for these receptors in controlling fast synaptic transmission. This is achieved by regulating neurotransmitter release from pre-synaptic terminals and by mediating post-synaptic transmission. Thus, with the aid of selective kainate agents, synaptic transmission through kainate receptors can be targeted without particularly blocking fast glutamatergic transmission through NMDA and AMPA receptors.

Migraineurs have elevated levels of glutamate (Martinez et al., 1993; Peres et al., 2004) and glutamine (Rothrock et al., 1995) in the cerebrospinal fluid compared to controls, suggesting an excess of neuroexcitatory amino acids in the CNS. A correlation between the glutamate levels and the mean headache scores has been reported (Peres et al., 2004), suggesting a persistent neuronal hyperexcitability that becomes heightened during an attack in migraineurs. In support of this hypothesis, is the finding that migraineurs exhibit cutaneous allodynia during an attack, and thus exhibit signs of the development of central sensitization (Burstein et al., 2000). Evidence from animals support that increased glutamate levels parallel changes in sensory thresholds of facial receptive fields as recorded from secondary neurons in the TCC (Oshinsky and Luo, 2006), thus further supporting the involvement of glutamate in the development of cutaneous allodynia and central sensitization, as seen in migraineurs. The increased glutamate release during central sensitization indicates a prominent role for glutamate receptors on second and third order neurons. The fact that kainate receptor antagonists are better effective in persistent but not in acute pain and iGluR5 knockout mice develop an analgesic phenotype to chronic pain but not to acute pain models (Wu et al., 2007), indicate a role of kainate receptors in states of excess glutamatergic transmission in the nociceptive pathways. Thus kainate receptors could have a prominent role in the development of central sensitization, which is responsible for the prolongation of the migraine attack (Dodick and Silberstein, 2006). As triptans could be less effective in the majority of patients if taken following the development of allodynia (Burstein et al., 2004), novel kainate receptor antagonists could be more efficient in aborting central sensitization of the trigeminovascular pathway and proved advantageous for patients who no longer respond to triptans.

The particular study of the iGluR5 kainate receptors in this thesis, as well as in the variety of studies of the kainate receptors, was chosen due to the deficit of antagonists acting on other kainate subunits, as well as due to the lack of subunit-specific antibodies. To our knowledge the only kainate non-iGluR5 antagonist described to date is the molecule 5-nitro-6,7,8,9-tetrahydrobenzo[g]indole-2,3-dione-3-oxime (NS-102) which was initially proposed as a selective iGluR6 kainate receptor antagonist (Verdoorn et al., 1994). However, functional assays have yielded contradictory results regarding its specificity for kainate over AMPA receptors. Additional problems with the solubility of this molecule have further limited the study of iGluR6 kainate receptors (Lerma et al., 2001). Molecules with combined iGluR5/iGluR6 activity also exist, such as SYM-2081;

their use however as seen in chapter 4 cannot provide direct evidence on the characterisation of iGluR6 carrying receptors. Although with the currently available pharmacological tools we have been able to demonstrate a possible role of iGluR5 subunit in migraine pathophysiology, a role for other kainate subunits might also exists. In agreement with this idea, is the existence of a specific iGluR6 kainate receptor gating mechanism that requires external Na^+ to be operative (Paternain et al., 2003). Given that at least one form of familial hemiplegic migraine is associated with an abnormality in sodium channels (van den Maagdenberg et al., 2007), it behooves us to consider advances in understanding the characteristics of kainate receptors, and their relationship to sodium and other ion channels.

A major hindrance in understanding kainate receptors has been the lack of specific agonists and antagonists. The separation between AMPA and kainate receptors has only been vaguely explored with few exceptions coming from the new willardiine derivatives and of a new series of decahydroxyisoquinoline carboxylates, which again favour the selectivity of iGluR5 carrying kainate receptors (Jane et al., 1997; Lerma et al., 2001; Mayer, 2005). Although a clear pharmacological boundary has been traced between NMDA and the other ionotropic glutamate receptor subfamilies (Watkins and Jane, 2006), as seen in chapter 6 this is not absolutely true, as even antagonists described as highly selective agents for iGluR5 kainate receptors can display a significant action on NMDA receptors.

7.3 Limitations and possible developments of the experimental procedures

Several potential limitations need to be considered in the interpretation of these data. As noted above, electrical stimulation of the dura mater and of dural structures leads in intracranial nociception in humans (Penfield, 1932, 1934; Penfield and McNaughton, 1940; Ray and Wolff, 1940; Wolff, 1948; Wirth and Van Buren, 1971). Experimental and clinical data suggest an activation of the trigeminal innervation of the cranial circulation with involvement of vasoactive neuropeptides such as CGRP (Goadsby et al., 1990; Edvinsson and Goadsby, 1995; Goadsby and Edvinsson, 1998) to explain the peripheral pain mechanisms of the migraine attack. Based on these observations,

electrical stimulation was applied in the animal models used in this thesis, as this is the most consistent method of eliciting intracranial pain (Penfield, 1932, 1934; Penfield and McNaughton, 1940; Ray and Wolff, 1940; Wolff, 1948; Wirth and Van Buren, 1971). Migraine is a complex disorder involving, further to the pain component, sensory and motor disturbances and it is thus quite difficult to actually model migraine. It is therefore important to note that the animal models used are not an exact model for migraine, and are limited in that they provide data on neuronal firing or trigeminal neuronal activation via blood vessel dilation, responsive to trigeminovascular nociceptive stimulation. However, these models are a reasonable surrogate for modelling the intracranial nociception and trigeminovascular activation as seen in migraine attacks. In agreement with this view is that each method utilised in this study has demonstrated a close correlation to the clinical efficacy of acute anti-migraine treatments (Storer and Goadsby, 1997; Williamson et al., 1997a, c; Williamson et al., 2001; Storer et al., 2004a; Storer and Goadsby, 2004; Akerman and Goadsby, 2005b; Shields and Goadsby, 2005, 2006).

Stimulation of dural vessels could have additionally caused cortical activation and this is particularly a problem in VPM recordings, as corticothalamic neurons modulate thalamocortical activity directly through glutaminergic synaptic neurotransmission and indirectly by activation of GABAergic RN neurons. All efforts were concentrated on minimising current spread from the SSS to the somatosensory cortex. A balance had to be achieved between using a voltage of sufficient magnitude to activate trigeminovascular afferents, and not interfering with corticothalamic neurotransmission.

Microiontophoresis has been used extensively to study the pharmacological modulation of trigeminovascular neurotransmission by iGluR5 kainate receptors in the TCC and VPM. There is a strong correlation between an inhibitory action of drugs in this model and clinical efficacy in treating migraine (Storer and Goadsby, 1997; Storer et al., 2004a; Shields and Goadsby, 2005, 2006). Despite the general limitations associated with this technique, which have been discussed in section 2.8 the anatomical precision of drug delivery made it particularly suitable for studying trigeminocervical and thalamocortical pharmacology of kainate receptors in relation to sensory activation. Microiontophoretic ejection is suitable for examining the acute effects of drug administration on neuronal activity. This however can be a limitation when interpreting the effects of prophylactic agents as topiramate, which their effect might not be

observed if the agent displays an indirect-modulating mechanism of action. Microiontophoresis cannot mimic the complex changes that occur in the CNS following chronic administration. This does not invalidate the results of these studies, but it does high-light a limitation of the technique. Nevertheless, the small amount of drugs ejected locally on synapses was in favour of the study of kainate receptors with respect to their physiological property of rapid desensitisation (Jones et al., 1997), which is extensively seen during bath application of kainate agonists on brain slices and cell cultures (Huettner, 1990; Partin et al., 1993; Jones et al., 1997).

Kainate receptors can clearly modulate synaptic neurotransmission and their involvement in trigeminovascular nociception in the thalamus and the caudal brainstem can be extremely complex. It is therefore unlikely to be able to control and study all the possible modulatory inputs that may exist on a neuron with microiontophoresis due to the limited number of barrels that can be used, and the complex networks that occur in these synapses. Thus, although different mechanisms of action of kainate receptors have been suggested, other mechanisms could also be present (Kullmann, 2001; Lerma, 2003). Perhaps the modulation of the kainate receptor mediated inhibition by 5-HT_{1B} receptor interactions, as seen in chapter 5, reflects an example of the complex mechanisms that might exist in these synapses. These experiments are limited in their scope, concentrating on specific questions on the function and pharmacology of kainate receptors with possible therapeutic effects. It should be noted that they were not intended to become an exhaustive examination of kainate mediated modulation of non-glutamate neurotransmitters in the trigeminovascular sensory processing, and only a limited number of serotonergic and GABAergic receptors were studied in the VPM. Rather the studies provide a direction for further exploration of kainate receptor mediated effects on the trigeminovascular system.

The lack of intrinsic interneurons in the rodent VPM allows for a relatively simple interpretation of the results. Though this unique physiology of the rodent VB does not allow us to correlate completely results with the possible effect that the drugs could have in the human thalamus. Since kainate receptors modulate GABAergic neurotransmission, their presence on intrinsic interneurons might further modulate the responses of thalamocortical synapses to trigeminovascular activation. However, as previously mentioned ejected drugs in rat thalamus can act only at one of two sites: either the pre-synaptic terminals of neurons projecting on third order neurons or the cell

bodies of third order neurons. When the modulation of glutamate receptor agonist-driven firing was studied, the effect was localised more specifically to the post-synaptic membrane. Furthermore, as the rodent serotonergic system is fundamentally different to that of humans, it is unknown if the possible effects of the kainate antagonist on serotonergic transmission seen in the VPM (chapter 5) is relevant in humans.

Despite the limitations mentioned above, dissecting out the involvement of kainate receptors in the modulation of nociceptive transmission is a realistic target. If a dysfunction of sensory processing is capable of generating all of these migrainous symptoms, understanding the pharmacologic modulation of nociceptive neurotransmission may offer new insights into the pathogenesis of migraine.

7.4 Conclusions

The pre-clinical observations from this study further contribute to the advancement of our knowledge in the involvement of kainate receptors in the trigeminovascular system and may provide increased understanding of migraine mechanisms, as well as deliver new effective therapies. Glutamate is implicated in trigeminovascular activation, central sensitization and CSD. The pre-clinical and clinical observations argue for a strong link between migraine and the glutamatergic system, and kainate receptors represent a promising target for a valuable therapeutic approach to the migraine treatment. According to this study, the presence of functional kainate receptors illustrates the tactical localisation of these receptors in key structures for the migraine ascending pathway. Results from the current study provide evidence for the regulation of trigeminovascular transmission by both post-synaptic and pre-synaptic kainate receptors and indicate a modulatory role of kainate receptors in trigeminovascular nociceptive processing, at the peripheral level of trigeminal ganglia and at the levels of TCC and VPM. The use of microiontophoresis to study the involvement of the central kainate receptors offers a means of studying the effects of drugs on individual or small numbers of cells *in vivo*. The anatomical precision of delivery makes it ideally suited for studying the pharmacology of kainate receptors in trigeminovascular neurotransmission in the TCC and the thalamus. The results reported in this thesis not only illustrate the complex

neuromodulatory nature of kainate receptors in trigeminovascular nociception, but also further illustrate the complexity of migraine pathophysiology. This study suggests that differential targeting of kainate receptors will provide new impetus in migraine therapeutics and represents a novel, neuronal, non-vascular, and non-serotonergic approach to the treatment of migraine.

Appendix

Table A1. Table of all chemicals used in experiments

NAME	CHEMICAL NAME (IUPAC)	KNOWN BIOLOGICAL ACTIVITY
Bicuculline	[R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodi oxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium iodide	$GABA_A$ receptor antagonist
CGRP	Calcitonin Gene-Related Peptide	Calcitonin Gene-Related Peptide (amino acid)- neuromodulator
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione	AMPA/kainate antagonist
D-Serine	-	Glycine agonist at the NMDA receptor
Fluorowillardiine	(S)-(-)-a-Amino-5-fluoro-3,4-dihydro-2,4-dioxo-1(2H)pyr idinepropanoic acid	AMPA receptor agonist
GR127935	N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methy l-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-ca rboxamide hydrochloride	selective $5\text{-}HT_{1B/1D}$ receptor antagonist
Iodowillarddine	S)-(-)-5-Iodowillardiine	iGluR5-subunit containing kainate receptor agonist
L-Glutamate	(S)-1-Aminopropane-1,3-dicarboxylic acid	glutamate receptor agonist
LY466195	[(3*S*,4*aR*,6*S*,8*aR*)-6-[[(2*S*)-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]decahydro-3-isoquinolinecarboxylic acid)]	competitive GLU_{K5}-selective kainate receptor antagonist
NAS-181	(2R)-2-[[[3-(4-Morpholinylmethyl)-2H-1-benzopyran-8-yl] oxy]methyl]morpholine dimethanesulfonate	antagonist at the rat $5\text{-}HT_{1B}$ receptor
NMDA	N-Methyl-D-aspartic acid	NMDA receptor agonist
SYM-2081	(2S,4R)-4-Methylglutamic acid	kainate receptor modulator
Topiramate	,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate	anticonvulsant; knot well understood biological action; suggestions include: sodium channel blocker; GABAergic channels activator; kainate and AMPA receptors blocker
UBP 302	(S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrim idine-2,4-dione	competitive iGluR5-subunit containing kainate receptor antagonist
WAY-100135	(S)-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl) -2-phenylpropanamide dihydrochloride	$5\text{-}HT_{1A}$ receptor antagonist

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