Identification of regulatory proteins of Acid-Sensing Ion Channels

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ABSTRACT

Acid-sensing ion channels (ASICs) are voltage-independent proton-gated ion channels belonging to the amiloride-sensitive degenerin/epithelial Na^+ channel (DEG/ENaC) family of receptor channels. Six subunits (ASIC1a, 1b, 2a, 2b, 3 and 4) encoded for by four genes, have been identified so far. All ASIC subunits are expressed in dorsal root ganglia (DRG) and have been implicated in physiological sensory processes such as nociception associated with tissue acidosis, cutaneous and visceral mechanosensation, sour taste and cochlear function. However, some ASIC subunits also show a wide distribution throughout the brain, where they are thought to modulate synaptic communication. Supporting this hypothesis several studies demonstrated in mice a role for ASICs in learning, memory and fear behaviour. Recently ASIC1a channels were also shown to make a major contribution to hippocampal neuronal damage in stroke through Ca^{2+} overload. ASIC4 is broadly expressed in the nervous system but is not gated by protons and has no known function.

This thesis describes a genetic analysis carried out to identify interacting partners of ASICs in sensory neurons, in order to shed light on the possible role of ASIC4, and to better understand the functional roles of ASIC1-3 and their regulatory mechanisms. A rat dorsal root ganglion cDNA library was screened in a yeast two-hybrid assay and a number of proteins interacting with the N-terminal domain of rat ASIC1-4 were trapped. Many of these proteins were involved in trafficking of ion channels, G protein pathways, endocytosis, protein ubiquitination, or cell adhesion, suggesting potentially novel roles and regulatory mechanisms for ASIC channels. The annexin II light chain p11 was found to specifically interact with ASIC1a, and to promote its functional expression at the plasma membrane, as shown by immunocytochemistry, cell surface protein biotinylation and electrophysiology. ASIC4 was shown to decrease the protein level of other ASIC subunits, and to downregulate ASIC1a-mediated currents. Preliminary data suggest that this effect may be due to an involvement of ASIC4 in ubiquitination pathways. Overall, this work has led to the identification of interacting proteins that regulate ASICs and suggested novel functions for ASIC4.

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This thesis is dedicated to the memory of my dad.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
5'-RACE	5' rapid amplification of cDNA ends
ABR	auditory-evoked brainstem response
AD	activation domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ASIC	acid-sensing ion channel
BDNF	brain-derived neurotrophic factor
BNC, BNaC	brain sodium channel
bp	base pair
CCV	clathrin-coated vesicle
CFA	complete Freund's ajuvant
CFTR	fibrosis transmembrane conductance regulator
CGRP	calcitonin gene related peptide
CIPP	channel-interacting PDZ-domain protein
CK2	casein kinase 2
CME	clathrin-mediated endocytosis
CNS	central nervous system
DBD	DNA-binding domain
DEG	degenerin
DRG	dorsal root ganglia
ECM	extracellular matrix
ENaC	epithelial sodium channel
EPSC	excitatory postsynaptic current
GABA	γ-amino butyric acid
Gal	galactose
GAP	GTPase-activating protein
GDNF	glial-derived neurotrophic factor
Glc	glucose
GST	glutathione S-transferase
HA	haemagglutinin
his	histidine
IB4	isolectin B4
IPTG	isopropyl-β-D-thiogalactopyranoside
KO	knock-out
LB	Luria broth
leu	leucine
LTP	long-term potentiation
MAGUK	membrane-associated guanylate-kinase protein
MDEG	mammalian degenerin
MW	molecular weight
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NSAID	non steroid anti-inflammatory drug

OD	optical density
ORF	open reading frame
PcTX1	psalmotoxin 1
PKA	protein kinase A, cAMP-dependent protein kinase
PKC	protein kinase C, phospholipase C-dependant protein kinase
PNS	peripheral nervous sytem
Raf	raffinose
RGS	regulator of G protein signaling
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SP	substance P
TF	transcription factor
TM	transmembrane domain
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine
TRP	transient receptor potential
trp	tryptophan
TTX	tetrodotoxin
UAS_G	GAL1 upstream activating sequences
ura	uracil
VR1	vanilloid receptor 1
WT	wild type
Y2H	yeast two-hybrid
X-gal	5-bromo-4-chloro-3-indolyl-βD-galactoside

CHAPTER I

INTRODUCTION

This first chapter provides a general introduction to Acid-Sensing Ion Channels. First, a presentation of the endogenous proton-gated currents originally found in nervous tissue is made. The features of ASICs, one of the ion channel types thought to mediate these H^+-activated currents, is then described in detail; their cloning, the characteristics of their sequence and their expression patterns in the nervous system, crucial information which gives insights into ASIC functions, is reported. The properties of ASIC-mediated currents and the various mechanisms of their regulation is subsequently presented. This is followed by an overview of the current knowledge of the functions of ASICs. Finally an introduction to the yeast two-hybrid technique, which was a major component of this work, is given.

I.1. Proton-gated currents in the nervous system

The extracellular pH in tissues is tightly regulated, but acidification of the extracellular milieu can arise in normal physiological conditions, for example in the synaptic cleft during synaptic transmission, and in pathophysiological situations such as inflammation or ischemia. In the nervous system, external protons are known to modulate the function of several ion channels, e.g. voltage-gated Na^+ channels or N-methyl-D-aspartate (NMDA) receptors, in general causing an inhibition of their function and potentially inducing a decrease in neuronal excitability. For instance, in cultured spinal cord neurons a decrease in extracellular pH depressed neuronal responses to GABA, glutamate or glycine (Gruol *et al*, 1980). However, a direct activation of neurons by a rapid decrease in pH, giving rise to a depolarising sodium current, was also described (Gruol *et al*, 1980; Krishtal and Pidoplichko, 1980). Proton-gated ion channels, supposedly mediating these currents, were later identified (Caterina *et al*, 1997; Waldmann *et al*, 1997a). In this section, an overview of the nervous system and the characteristics of the proton-activated currents described in neuronal tissue are presented. Emphasis is given to primary sensory neurons of the dorsal root ganglia (DRG), which are of particular interest to our lab.

I.1.1. Overview of the nervous system

I.1.1.1. General presentation of the nervous system

The nervous system serves as the body control centre. As a key regulatory and coordinating system, it detects and integrates information (in the form of light, chemical, mechanical, thermal, or noxious stimuli) and generates appropriate reactions by transmitting electrochemical signals through nerves to effector organs such as muscles and glands. The brain and spinal cord form the central nervous system (CNS); the nerves of the somatic and autonomic nervous systems represent the peripheral nervous system (PNS) and connect receptors and effectors to the CNS.

Neurons in the brain receive, analyse, and store information about internal and external conditions. They receive nerve impulses from the spinal cord and 12 pairs of cranial nerves.

The spinal cord carries messages between the CNS and the rest of the body, and mediates numerous spinal reflexes such as the knee-jerk reflex. Spinal nerves, which arise from spinal cord segments, have a dorsal and a ventral root. The dorsal root contains primary afferent fibres that transmit information to the spinal cord from sensory receptors. The ventral root contains efferent fibres that carry messages from the spinal cord to the effectors. Cell bodies of the efferent fibres reside in the ventral horn of the spinal cord grey matter, whereas those of the afferent fibres lie in dorsal root ganglia (DRG) (Fig. I.1). The dorsal horn of the spinal cord grey matter contains soma of interneurons. The white matter contains mainly the axons of ascending and descending pathways.

The somatic nervous system is associated with the reception of external stimuli and voluntary control of body movements through the action of skeletal muscles. It includes 31 pairs of spinal nerves and 12 pairs of cranial nerves, which originate from the spinal cord and brain stem, respectively. Cranial nerves transmit information on the senses of balance, smell, sight, taste, hearing and touch, mostly from the head region. The largest

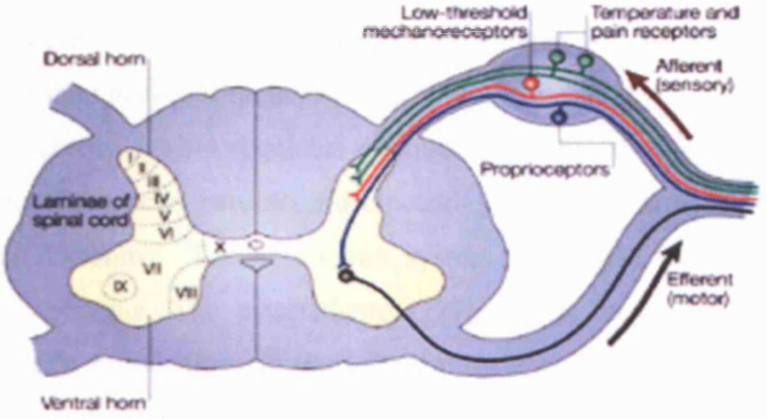

Fig. I.1: **Schematic representation of spinal nerves and their central termination**. Spinal nerves are formed of primary afferent (sensory) and efferent (motor) fibres. The cell bodies of the sensory neurons are located in the dorsal root ganglia and those of motoneurons in ventral horn of the spinal cord (from Patapoutian *et al*, 2003).

is the trigeminal nerve (5^{th} cranial nerve); it comprises both motor and sensory fibres, the cell bodies of the sensory neurons being located in the trigeminal ganglion. Sensory information is processed in the CNS; the resulting orders travel back through the cranial nerves to the skeletal muscles that control movements in the face and throat. In addition, some cranial nerves contain autonomic motor fibres.

The autonomic nervous system regulates internal organs function. The effectors in this system are smooth muscle, cardiac muscle and glands, all structures that function without conscious control. The autonomic system is divided into sympathetic and parasympathetic systems. The sympathetic nerves cause increased blood pressure, breathing rate, and blood flow to muscles in response to stress. Conversely, the parasympathetic nerves have a calming effect; they slow the heartbeat and breathing rate, and promote digestion and elimination. Visceral sensory neurons of the nodose ganglion are part of the major nerve of the parasympathetic system, the vagus nerve (10^{th} cranial nerve). These sensory neurons innervate organs of the thorax and abdomen, and transmit information concerning blood pressure, gastric distension, and blood oxygenation to the CNS.

I.1.1.2. Dorsal root ganglia neurons

The DRG contain different types of primary sensory neurons, each of which can be activated by specific stimuli. The nature and intensity of these stimuli (i.e., mechanical, thermal or chemical; innocuous or noxious), as well as the size of the neurons and their electrical properties are used to classify sensory neurons into three main functional groups (see below, Wood, 2000, Julius and Basbaum, 2001).

Aα/β fibres are large and heavily myelinated and display fast axonal conduction velocity (10-30 m/s). They are involved in mechanosensation and proprioception; they detect innocuous mechanical stimuli from the skin, muscle, joints or viscera. They are generally described as not contributing to the perception of pain, although recently, Aα/β nociceptive neurons have been reported (Lawson, 2002). The peripheral terminals of Aα/β mecanoreceptors are generally associated with peripheral end organs such as Merkel cells.

Aδ and C fibres are nociceptors; they are activated by noxious stimuli (i.e. of intensity sufficient to cause or potentially cause tissue damage). Aδ fibres are thinly myelinated and their conduction velocity is around 1.2-10 m/s. They detect noxious mechanical stimuli, and some are also activated by intense heat. Unmyelinated, slowly-conducting (< 1.2 m/s) C fibres comprise about 60% of all nociceptors. Most of them are polymodal, i.e. they respond to noxious thermal, mechanical and chemical stimuli, while others respond to noxious heat only. Aδ nociceptors mediate the rapid sharp, 'first' pain experienced following an insult whereas C-fibre nociceptors mediate the delayed, more diffuse 'second' pain. Nociceptors in general display bare nerve endings at the peripheral terminal.

Sensory neurons can modulate their response to a given stimulus; for example, following tissue damage, inflammatory mediators such as ATP, bradykinin or prostaglandins can increase their sensitivity. This peripheral sensitisation can be a cause of hyperalgesia, an increased response to a noxious stimulus, allodynia, a sensation of pain caused by an innocuous stimulus, or spontaneous pain occurring in the absence of a stimulus (Woolf and Salter, 2000).

C-fibre nociceptors can also be divided into peptidergic and non-peptidergic populations according to their neurochemical characteristics and neurotrophic requirements. The peptidergic neurons contain the neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP). They express TrkA, the high affinity tyrosine kinase receptor for nerve growth factor (NGF), and require NGF for their survival during embryogenesis and postnatal development. In contrast, the non-peptidergic nociceptors contain no SP and selectively bind to a plant lectin called isolectin B_4 (IB_4). This IB_4-positive population of nociceptors also expresses C-Ret, the tyrosine kinase receptor for glial-derived neurotrophic factor (GDNF). These neurons require NGF during embryogenesis and GDNF postnatally (Molliver *et al*, 1997). The central terminals of these 2 classes of nociceptors project to different areas of the dorsal horn of the spinal cord; IB4-positive neurons project mainly to lamina II and IB4-negative to lamina I. However, their functional specificity remains unclear. Dirajlal *et al* (2003) reported that IB4-negative neurons expressed transient as well as sustained proton-

activated currents, whereas IB4-positive cells only displayed sustained ones, suggesting a possible difference for these 2 types of neurons in their respective role in acid-evoked nociception.

Aα/β neurons express TrkB and TrkC, the high affinity tyrosine kinase receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 respectively. Sensory neurons with myelinated axons can be labelled with antibodies directed to neurofilament 200 (N52 clone).

Roughly, in adult mouse and rat, about half of DRG neurons can be stained with N52, and approximately half of the N52-negative neurons are IB4-positive and half are IB4-negative (Dirajlal *et al*, 2003).

The specific functions of the different classes of sensory neurons are also related to the specificity of expression of various ion channels. For example the voltage-gated sodium channel $Na_v1.8$ is predominantly expressed in small diameter nociceptors and is strongly associated with a nociceptive function (Akopian *et al*, 1996). Low threshold Aα/β mechanoreceptors would be expected to contain ion channels activated by low intensity mechanical stimuli (although the molecular identity of such channels remains unknown). Many DRG neurons respond to acid by giving rise to various types of currents, mediated by 2 classes of ion channels; in nociceptors, these currents are thought to mediate acid-induced nociception whereas in non-nociceptive cells their functional significance is not well understood. The characteristics of these proton-gated currents and the ion channels that mediate them are presented below.

I.1.2. Proton-activated currents in the nervous system

Evidence of a direct activation of cultured neurons by protons giving rise to depolarising currents was first given by Gruol *et al* (1980) on a preparation of neurons from mouse spinal cord and by Krishtal and Pidoplichko (1980) on rat trigeminal and dorsal root ganglia neurons, but the molecular identity of the ion channels mediating these currents was only determined 15-20 years later.

I.1.2.1. CNS neurons

Gruol *et al* (1980) showed that a quick drop in pH could activate transient cation currents in CNS neurons. Protons were applied by iontophoresis, and it is not clear what range of pH changes were used. A gradual decrease of pH did not induce any observable change in membrane potential, due to a rapid and complete desensitisation of the current; indeed slow acidification induces asynchronous activation and desensitisation of proton-gated channels, thus preventing detection of a macroscopic current. 50 ms pulses of H^+ induced currents of constant amplitude when applied at 0.13 Hz but responses entirely desensitised after about 5 s at 4.2 Hz (Gruol *et al*, 1980). Proton-activated currents were later described in cultured neurons from many brain structures, such as cortex, cerebellum, brain stem, striatum, and hippocampus (Varming, 1999; Escoubas *et al*, 2000; Bolshakov *et al*, 2002) (Fig. I.2 and table I.1). In general, neurons from a particular brain area displayed relatively homogenous proton-gated currents but variations were observed between currents from different areas. In most neurons these currents were transient Na^+ currents activated below pH 6.8-7.0. They desensitised rapidly ($\tau_{desensitisation}$ 0.6-2.1 s), and were blocked by the diuretic drug amiloride (IC_{50} 4-9 μM). Bolshakov *et al* (2002) identified in all tested neurons (from cortex, cerebellum, brain stem, striatum, and hippocampus) a sustained component activated below pH 3.5. The amplitude of this current was smaller than that of the peak current, and it was not blocked by amiloride.

I.1.2.2. Somatic PNS neurons

In the peripheral nervous system, proton-activated currents are more heterogeneous, different subsets of cells in a given tissue express different types of currents and in some cases species differences have been observed. On the basis of their pharmacological and kinetics properties, these currents can be divided in two categories; i) slowly activating and inactivating non selective cation currents, blocked by capsazepine and found in cells which also respond to capsaicin (the pungent

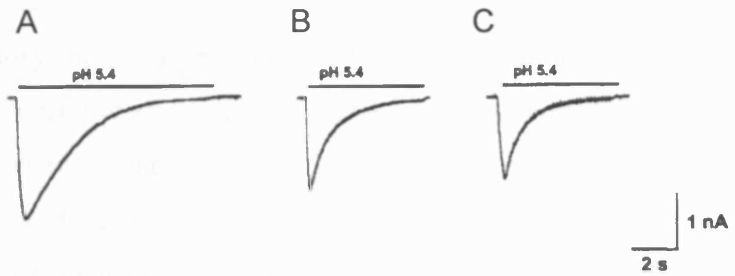

Fig. I.2: **Proton-gated currents recorded in the rat CNS**. A, hypoglossal motoneuron. B, Purkinje cell. C, cortical pyramidal cell (from Bolshakov *et al*, 2002)

Cell type	pH_{50}	$\tau_{inactivation}$ (s)	$\tau_{recovery}$ (s, at pH7.4)	Amiloride block (IC_{50}) (uM)	References
Cerebellar granule cells (rat)	6.6	2.06 ± 0.17 (at pH6)	nd	nd	Escoubas *et al*, 2000
Cortical pyramidal neurons (mouse) (rat)	5.6-6.8 5.5	0.7 ± 0.3 (at pH_{50})	1.5 ± 0.2	6.2 3.7	Varming, 1999 Bolshakov *et al*, 2002
Striatal interneurons (rat)	5.4	0.6 ± 0.4 (at pH_{50})	1.2 ± 0.2	5.0	Bolshakov *et al*, 2002
Hippocampal interneurons (rat)	4.9	0.9 ± 0.1 (at pH_{50})	1.9 ± 0.1	6.0	Bolshakov *et al*, 2002
Hippocampal CA1 pyramidal neurons (rat)	5-6.5	nd	nd	nd	Bolshakov *et al*, 2002
Hypoglossal motor neurons (rat)	6.4	1.3 ± 0.2 (at pH_{50})	2.5 ± 0.5	9	Bolshakov *et al*, 2002

Table I.1: **Characteristics of transient proton-gated currents in brain neurons**

component of chilli peppers), and ii) rapidly activating and inactivating sodium currents, or biphasic currents with a sustained non selective cationic component, both blocked by amiloride.

In rat sensory neurons, Krishtal and Pidoplichko (1980) first observed transient depolarising currents after rapid shifts (0.1 s) in external pH from 7.4 to 6.9 and below. These currents were mainly carried by Na^+ and were originally found in around 10% of the DRG neurons and around 30% of the trigeminal ganglia neurons tested. The time constant of desensitisation was about 0.5 s. In a later study, the same authors examined in more details proton-activated currents in rat trigeminal ganglia neurons (Krishtal and Pidoplichko, 1981). All pH-sensitive currents recorded were rapidly activating sodium currents, but three types of desensitisation kinetics were observed: 78% were rapidly desensitising with a time constant < 0.5 s (Fig. I.3 a), 6.3% were rapidly desensitising with a time constant of 2-3 s (Fig. I.3 b) and 15.7% were characterised by a sustained slowly inactivating component lasting during the whole exposure to acid (Fig. I.3 c). A number of studies have since confirmed the presence of several types of transient proton-activated currents in **rat** DRG neurons (although the most slowly desensitising one is not always recorded) and shown that they are amiloride-sensitive (Escoubas *et al*, 2000; Mamet *et al*, 2002; Dube *et al*, 2005). However, analyses of **mice** DRG neurons seem to identify only one type of transient current, corresponding to the predominant fast kinetics current in rat which has a time constant of desensitisation of around 200 ms (Xie *et al*, 2002; Benson *et al*, 2002; Drew *et al*, 2004).

In addition to the transient, rapidly-inactivating sodium H^+-gated currents, Bevan and Yeats (1991) recorded in around 45% of rat small DRG neurons a slowly activating persistent current, which also responded to capsaicin (Fig. I.3 d), and a biphasic current (Fig. I.3 e). The persistent current was a non selective cation current activated at pH < 6.2 and was encountered mainly in small diameter neurons; this current was later described in many studies and was shown to be blocked by capsazepine (Caterina *et al*, 1997; Drew *et al*, 2004, Dube *et al*, 2005). Mamet *et al* (2002) recorded this current in around 70% of rat DRG neurons.

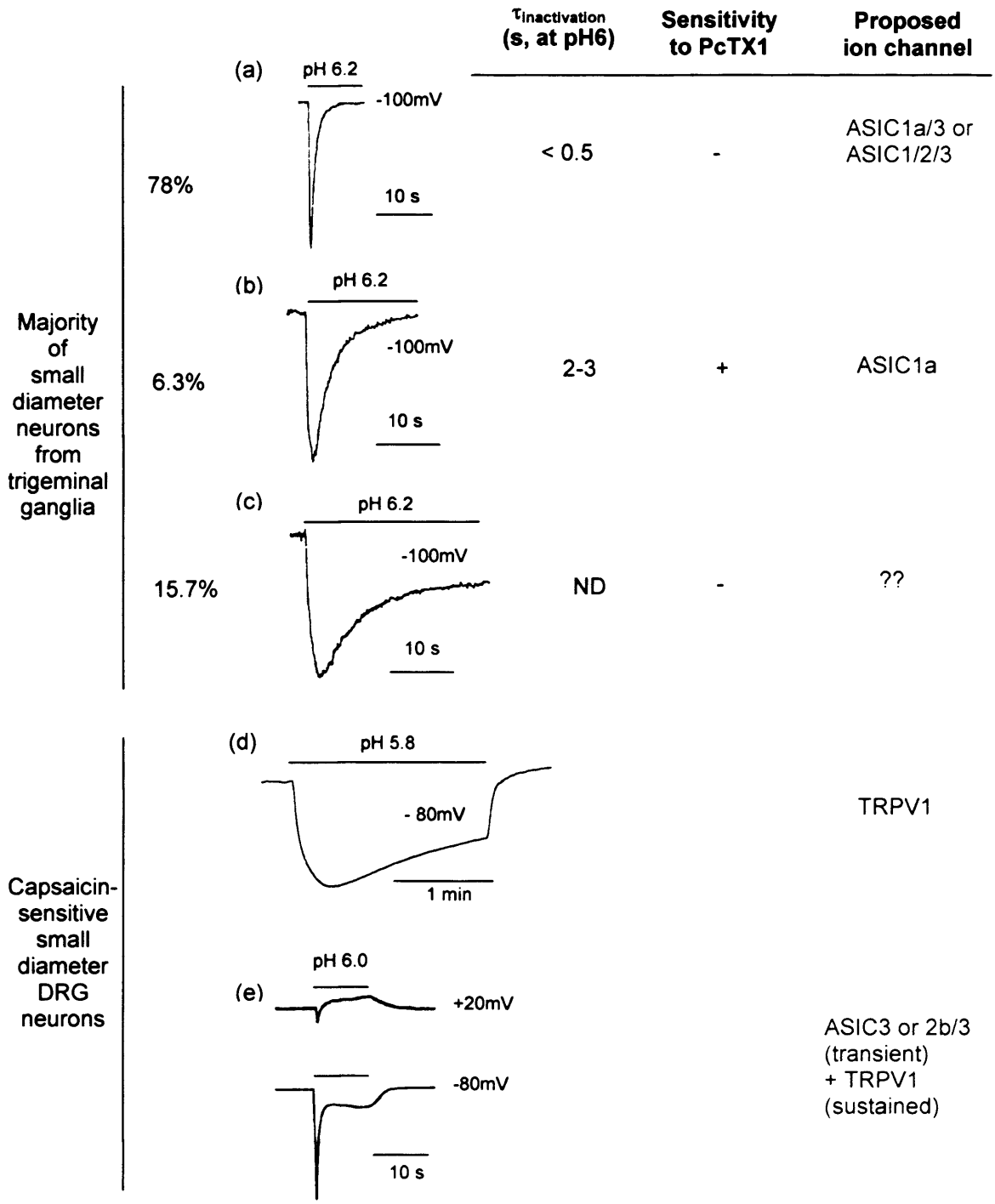

Fig. I.3. **Proton-gated currents recorded in rat sensory neurons** (adapted from a)-c) Krishtal and Pidoplichko, 1981, d)-e) Bevan and Yeats, 1991)

The reported proportions of sensory neurons expressing transient currents in response to protons differ depending on the studies. Krishtal and Pidoplichko (1981) determined that nearly half of rat trigeminal ganglia neurons displayed such currents; the majority of responsive neurons (74%) were small diameter nociceptors. 75% of medium and large diameter neurons were insensitive to drops in pH. Dube *et al* (2005) reported that in rat DRG, around 50% of small-medium and 90% of large diameter neurons displayed ASIC-like currents, and that among transient currents those corresponding to trace a) in fig. I.3 were predominant in DRG neurons of all sizes. In DRG neurons innervating the heart and those innervating the stomach in rat, the proportions are also different (Benson *et al*, 1999; Sugiura *et al*, 2005). Over 95% of cardiac DRG neurons displayed proton sensitivity, and only about 20% of them responded to capsaicin (Benson *et al*, 1999). Rapid decrease in pH to values below 7 induced large transient amiloride-sensitive currents which were followed below pH 6 by a sustained phase insensitive to amiloride. The transient current was selective for Na^+ whereas the sustained one was a non selective cation current. Only two types of kinetics of desensitisation were noted in transient currents; in most cells the time constant of desensitisation was about 200 ms, and in the others $\tau_{desensitisation}$ was around 2-3 s. Nearly all DRG neurons giving rise to gastric afferents, most of which have medium to large diameter cell bodies, express transient amiloride-sensitive acid-gated currents followed by a sustained component (Sugiura *et al*, 2005), and also respond to capsaicin. Surprisingly, neither sustained nor transient currents are blocked by capsazepine. Similarly to cardiac DRG neurons, two types of transient currents could be recorded, one desensitising more rapidly than the other ($\tau_{desensitisation}$ 210 ± 50 ms for the 'fast type' and 2.79 ± 0.31 s for the 'slow type').

I.1.2.3. Autonomic PNS neurons

Protons were also found to elicit depolarising sodium currents in neurons from frog parasympathetic ganglia (Kim *et al*, 1990) and rat sensory neurons of the vagus nerve, which contains a large parasympathetic component (Sugiura *et al*, 2005). Transient amiloride-sensitive inward currents were recorded in 55% of gastric afferents from the

nodose ganglia (Sugiura *et al*, 2005); 83% of these responsive cells desensitised with a time constant of 230 ± 50 ms, and in the 17% left desensitisation was slower ($\tau_{desensitisation}$ 2.63 ± 0.12 s) (Sugiura *et al*, 2005). The remaining 45% of gastric nodose ganglia neurons only displayed a sustained current blocked by capsazepine and in most cases also responded to capsaicin (Fig. I.4).

I.1.2.4. Non neuronal cells

Finally, transient proton-gated sodium currents were recorded in mouse oligodendrocytes, but not in astrocytes or Schwann cells (Sontheimer *et al*, 1989). These currents were activated by rapid drops in pH and desensitised with an average time constant of 1.7 s (Fig. I.5).

I.1.2.5. Expression of proton-gated currents in development

Proton-activated sodium currents were recorded in precursors of DRG neurons from E6 chick embryos (Gottmann *et al*, 1989), in 25% of neurons from the tectum of E12 rats (Grantyn *et al*, 1989) and in all E14-16 mouse oligodendrocyte precursor cells (Sontheimer *et al*, 1989). The currents had the same characteristics as in mature cells. In cultured neuron precursors, these currents were observed before the expression of voltage-gated Na^+ and Ca^{2+} currents. During the differentiation of oligodendrocytes proton-gated sodium currents outlasted voltage-gated Na^+ and Ca^{2+} currents, and their expression was parallel to that of glutamate receptors.

I.1.2.6. Molecular identity of proton-gated ion channels

Two types of ion channels mediating proton-activated currents have been described; ASICs and TRPV1. ASICs mediate rapidly activating and inactivating sodium currents, and biphasic currents with a sustained non selective cationic component when expressed as heteromultimers (Benson *et al*, 2002; Xie *et al*, 2002). ASICs characteristics will be described in details in the next sections. Activation of TRPV1

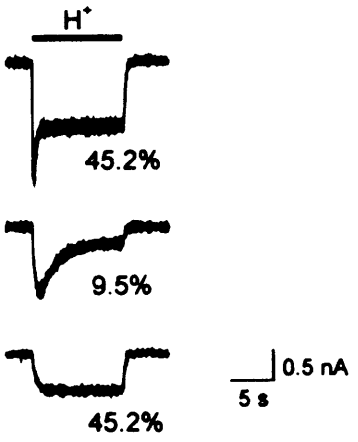

Fig. I.4: **Proton-gated currents recorded in rat nodose ganglia** (from Sugiura *et al*, 2005)

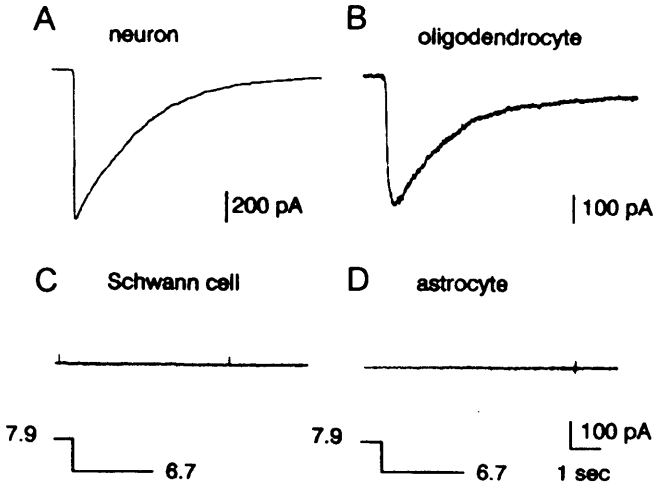

Fig. I.5: **Proton-gated currents recorded in neuronal and non-neuronal cells**. A, rat mesencephalic neuron, B, mouse oligodendrocyte, C, mouse Schwann cell, and D, rat astrocyte (from Sontheimer *et al*, 1989)

gives rise to a slowly activating and inactivating non selective cation current, probably corresponding to the persistent current reported by Bevan and Yeats (1991) (Fig. I.3 d). TRPV1, or vanilloid receptor 1 (VR1), is a member of the Transient Receptor Potential (TRP) channel family. Its structure comprises 6 transmembrane domains. Aside from protons (pH < 6), it can be activated by capsaicin, noxious heat (> 43°C), or endocannabidoid lipids such as anandamide, and is blocked by capsazepine. TRPV1 was first identified in sensory neurons (Caterina *et al*, 1997; Tominaga *et al*, 1998); it was shown to be a major contributor to acid-evoked responses in cutaneous nociceptive neurons (Caterina *et al*, 2000) and to play a crucial role in thermal hyperalgesia in inflammation (Davis *et al*, 2000).

I.2. Acid-Sensing Ion Channels (ASICs)

I.2.1. DEG/ENaC ion channel family

ASICs belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) family of proteins (Fig. I.6). All ion channels in this family are cation channels blocked by amiloride. They share a common structure comprising two transmembrane domains, with intracellular N- and C- termini. They are expressed in various tissues of invertebrate and vertebrate organisms. They exhibit a high degree of heterogeneity in the stimuli that activate them and in the functions they are involved in, such as Na^+ absorption across epithelia, touch transduction, acid-evoked nociception, salt and sour taste and synaptic plasticity.

I.2.1.1. Degenerins

The *C. elegans* degenerins (DEGs) play an important role in mechanosensation. They were called degenerins because certain proteins in this subfamily of DEG/ENaC were found to cause swelling and degeneration of the cells in which they are expressed when affected by specific gain-of-function mutations (reviewed in Mano and Driscoll, 1999).

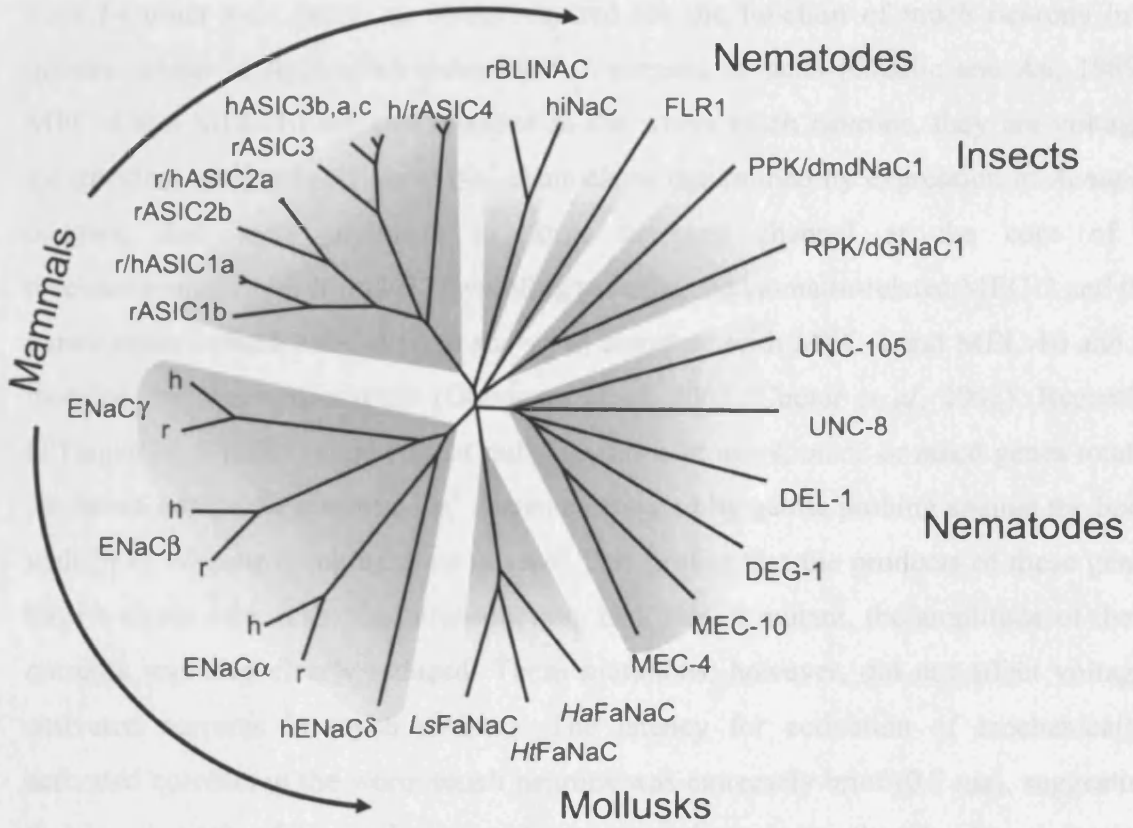

Fig. I.6: **Members of the DEG/ENaC family of proteins**. The protein sequence identity between the different subfamilies is around 15-20%, and the identity within subfamilies is ~30% for degenerins, 38% for the two Drosophila channels, ~65% for the FaNaCh orthologs, ~30% for ENaCs and 45-60% for ASICs (from Kellenberger and Schild, 2002).

Among these proteins, the ion channels MEC-4 (Driscoll and Chalfie, 1991) and MEC-10 (Huang and Chalfie, 1994) are of particular interest. They were identified, along with 14 other *mec* genes, as being required for the function of touch neurons in a genetic screen of light touch-insensitive *C. elegans* mutants (Chalfie and Au, 1989). MEC-4 and MEC-10 are co-expressed in the worm touch neurons, they are voltage-independent amiloride-sensitive Na^+ channels as determined by expression in *Xenopus* oocytes, and were proposed to form the ion channel at the core of a mechanotransduction complex. Two other proteins, the stomatin-related MEC-2 and the paraoxonase-related MEC-6 were shown to associate with MEC-4 and MEC-10 and to increase the channels activity (Goodman *et al*, 2002; Chelur *et al*, 2002). Recently, O'Hagan *et al* (2005) showed that null mutations in *mec4*, *mec2* or *mec6* genes totally abolished amiloride-sensitive Na^+ currents activated by gentle probing against the body wall in *C. elegans* touch neurons *in vivo*. This proves that the products of these genes have a direct role in mechanotransduction. In a *mec10* mutant, the amplitude of these currents was also clearly reduced. These mutations, however, did not affect voltage-activated currents in touch neurons. The latency for activation of mechanically-activated currents in the worm touch neurons was extremely brief (0.7 ms), suggesting that ion channels of the mechanotransduction complex are directly gated by mechanical stimuli, although the precise mechanism of activation remains unknown, and that this activation is the first step in transduction by touch neurons.

Other DEG/ENaC channels have been implicated in mechanotransduction. UNC-8 is expressed in sensory and motor neurons and is thought to be involved in the nematode proprioception (reviewed in Tavernarakis *et al*, 1997). DEL-1 is co-expressed with UNC-8 specifically in stretch-sensing motor neurons.

These data demonstrating a role for DEG/ENaC channels in mechanotransduction were at the origin of many studies examining a potential similar function for ASICs in mammals (see section I.2.5.2).

I.2.1.2. PPK - RPK

Sixteen of the 25 identified DEG/ENaC genes in the fruit fly *Drosophila melanogaster* genome were recently cloned (Adams *et al*, 1998; Liu *et al*, 2003a). These genes seem to be more closely related to each other than to DEG/ENaC channels from other species, implying they may form a distinct subfamily. However, they show a high diversity in the functions they are involved in.

Pickpocket (PPK, now PPK1) and rippedpocket (RPK) were the first of these genes to be characterised functionally (Adams *et al*, 1998). In adults, PPK1 was shown to be expressed exclusively in a subset of peripheral neurons that are involved in mechanosensation. Heterologous expression of the channel in *Xenopus* oocytes did not generate any current, suggesting PPK1 is not constitutively active and may require a specific ligand or mechanical stimulation for activation. Ainsley *et al* (2003) reported that *ppk1* mutants had normal larval touch sensitivity but displayed locomotor abnormalities. As for RPK, its mRNA was only detected in early stage embryos, suggesting it may play a role in early development. When expressed in *Xenopus* oocytes it produced a small constitutive amiloride-sensitive Na^+ current, which was not altered by co-expression with PPK, suggesting these two proteins do not associate to form a single ion channel.

Recently, two other pickpocket genes, PPK11 and PPK19, were shown to be expressed in the salt-sensing terminal organ of the fruit fly (Liu *et al*, 2003a). Behavioural tests of flies in which these genes were downregulated showed a lack of discrimination between water and low NaCl concentration in these mutant flies, as opposed to their wild-type counterparts. Thus, PPK11 and PPK19 were postulated to be involved in salt taste transduction in *Drosophila*.

Finally, 8 PPK genes were detected in the fly tracheal system during late embryogenesis (PPK4, -7, -10, -11, -12, -14, -19, and -28) (Liu *et al*, 2003b). As well as in mammalian lungs, the fly's airways shift from being filled with liquid to air at a specific point in development, and PPK4 and PPK11 channels seemed to play a role in this process. The authors show that both DEG/ENaC blocker amiloride and *PPK4* and

PPK11 downregulation by RNAi lead to defects in liquid clearance from the larvae tracheal system.

I.2.1.3. FaNaCh

FaNaCh was cloned from the snail *Helix aspersa* nervous tissue (Lingueglia *et al*, 1995). It was the first and only member of the DEG/ENaC family to be activated by the peptide FMRFamide (Phe-Met-Arg-Phe-NH2), which is a common neurotransmitter in invertebrates. Since then FMRFamide-gated channels from two other molluscs, *Helisoma trivolvis* and *Lymnaea stagnalis*, were cloned (Jeziorski *et al*, 2000; Perry *et al*, 2001). FaNach is thought to function as a homotetramer (Coscoy *et al*, 1998). FMRFamide has not been detected in mammals and no mammalian ortholog of FaNach has been identified so far, but FMRFamide and related peptides produce various physiological effects in mammals and were found to modulate the function of ASICs (see section I.2.4.2.1).

I.2.1.4. ENaCs

Epithelial Na^+ channels (ENaCs) are constitutively open heteromeric sodium channels formed by the association of three homologous subunits, α, β and γ (Canessa *et al*, 1994). They are expressed on the apical side of most tight epithelia where they mediate Na^+ absorption and so participate in extracellular fluid volume homeostasis, blood pressure and transduction of salt taste. Many factors, such as the hormones aldosterone, insulin and vasopressin have been shown to regulate these channels; clathrin-mediated endocytosis was also identified as a major pathway for downregulation of the channel activity (reviewed in Gormley *et al*, 2003). The function of ENaC is vital; global deletion of each of the three subunits is associated with neonatal death (Hummler *et al*, 1996; McDonald *et al*, 1999; Barker *et al*, 1998). In humans, mutations in ENaC β and γ subunits C-terminal endocytotic PY motif (proline-rich conserved sequence PPPXY) produce a constitutively increased channel activity leading to the appearance of Liddle's syndrome, characterised by hypertension, hypokalemia and metabolic

alkalosis. Loss of function mutations in ENaCs cause salt-wasting disease pseudohypoaldosteronism type I.

All ENaCs transcripts and proteins were detected in rat trigeminal ganglia neurons in conjunction with stomatin, although constitutively active, amiloride-sensitive sodium currents have not been reported in these cells (Fricke *et al*, 2000). β- and γ-ENaC were also localised to medium-large DRG neurons and to a number of mechanosensory nerve endings in the rat footpad (Drummond *et al*, 2000). Based on this localisation and their homology to MEC channels, it was hypothesised that ENaC could be mechanosensitive, but no direct evidence of gating of the channel by mechanical stimuli has been given so far.

Finally, β- and γ-ENaC were shown to be involved in neurite elongation in differentiated PC12 cells, a cell line derived from rat adrenal phaeochromocytoma (Drummond *et al*, 2005). Disruption of ENaC, whether pharmacological or by siRNA, inhibited neurite growth in these cells in a dose-dependent manner. Moreover, expression of β- and γ-ENaC proteins was upregulated by nerve growth factor (NGF), a neurotrophin required in neuronal development and PC12 differentiation into 'neuron-like' cells. The authors suggest this role in neurite growth may be due to putative mechanosensitive properties of ENaC; however, activation of several non-mechanically-activated channels, such as the voltage-gated sodium channel β1 subunit (Davis *et al*, 2004) has also been shown to be important in similar functions.

I.2.1.5. ASICs

ASICs are a subfamily of DEG/ENaC channels originally found in mammals. They were later identified in other distant vertebrates such as lamprey or zebrafish (Paukert *et al*, 2004a; Coric *et al*, 2005). In mammals, all but two subunits, ASIC2b and 4, are activated by the simplest known ligands, protons. They have been implicated in diverse functions, although no roles for ASIC4 have been found yet.

In the next few sections, current data regarding ASICs biology are described, starting with a general introduction to ASICs cloning and a presentation of important regions of their sequence.

I.2.2. ASIC cloning and sequence characteristics

ASIC1a	ASIC, ASICα or BNaC2α
ASIC1b	ASICβ
ASIC2a	MDEG, MDEG1, BNC1, BNaC1a
ASIC2b	MDEG2, BNaC1b
ASIC3	DRASIC, TNaC
ASIC4	SPASIC

Table I.2: **Nomenclature of mammalian ASIC subunits**

The first ASIC subunits cloned were initially called mammalian degenerins (MDEG) or brain sodium channels (BNaC, BNC) because they were found to be distributed in the central nervous system, as their nematode homologs (Price *et al*, 1996; Waldmann *et al*, 1996). Their name was changed to acid-sensing ion channels after Waldmann *et al* (1997a) discovered they were activated by protons (Table I.2). In mammals, 4 genes coding for a total of 6 ASIC subunits have been identified, ASIC1 and 2 each encoding 2 splice variants. ASIC1a was isolated from a rat brain cDNA library (Waldmann *et al*, 1997a) and human brain (Garcia-Anoveros *et al*, 1997). The other ASIC1 splice variant, ASICβ, was originally found to have unique N-terminal 172 amino acids (Chen *et al*, 1998). However, Bässler *et al* (2001) isolated from inner ear a cDNA that encoded a 559 amino acid protein identical to ASICβ, but 46 amino-acid longer at the N-terminus, called ASIC1b. They analysed the ASIC1b N-terminal sequence by 5'-RACE and found that the start codon was conserved in the mouse and human sequences, as opposed to the ASICβ start methionine. They therefore postulated that ASICβ and ASIC1b are not independent splice variants of ASIC1. ASIC2a was cloned from human and rat brain (Price *et al*, 1996; Waldmann *et al*, 1996). Its splice variant ASIC2b was identified by Lingueglia *et al* (1997) from mouse and rat brain. ASIC2b contains 563aa and differs from ASIC2a in its first 236 amino acids. ASIC3 was isolated by Waldmann *et al* (1997b) from rat DRG, and later in humans (Babinski *et al*,

1999). ASIC4 was cloned from rat DRG (Akopian *et al*, 2000) and rat brain (Gründer *et al*, 2000).

In the zebrafish 6 subunits known as zASICs encoded for by 6 genes were recently cloned (Paukert *et al*, 2004a). Three of them, zASIC1.1, 2 and 4.1 are orthologs of mammalian ASIC1a, 2 and 4 respectively. Finally, Coric *et al* (2003; 2005) cloned ASIC1 genes from lamprey, shark, toadfish and chicken, which are highly homologous to their rat ortholog.

Rat ASICs share 40-77 % homology, ASIC4 being the least homologous to the other subunits (Table I.3). Like all other members of the DEG/ENaC ion channel family, ASICs all share a common structure characterised by two transmembrane domains (TMs), a large extracellular loop containing many conserved cysteines, and intracellular N- and C-terminal domains (Fig. I.7).

Rat ASIC sequences bear a range of consensus motifs for N-glycosylation and phosphorylation by various kinases, as predicted by Prosite (see table I.4). The terminal four amino acids of ASIC1 and 2 form a type II PDZ-binding site, whereas ASIC3 carries a type I PDZ-binding sequence elsewhere in its C-terminus. These motifs have been shown to mediate binding with various proteins (see section I.2.4.2.4). ASIC2b contains a cell attachment motif (RGD) in its extracellular loop. RGD sequences are essential cell attachment sites, present for example in fibronectin; they are recognised by integrins and are crucial for cell migration and differentiation. ASIC1a and 2a bear an endocytic YxxL motif in their amino-terminal domain. β- and γ- ENaCs have a similar sequence which is recognised by the adaptor complex 2 (AP-2) and causes clathrin-mediated endocytosis of the channel (Shimkets *et al*, 1997). This suggests that ASIC1a and 2a may undergo similar mechanisms of regulation.

The intracellular parts of ASIC4 have different characteristics to the other ASIC subunits. They do not bear any consensus motifs for protein kinases A (PKA) or C (PKC) phosphorylation, and have only 1 predicted casein kinase 2 (CK2) phosphorylation site in the C-terminal domain. ASIC4 has an extended part in the N-terminus which is not homologous to the other ASIC subunits, and has unique features.

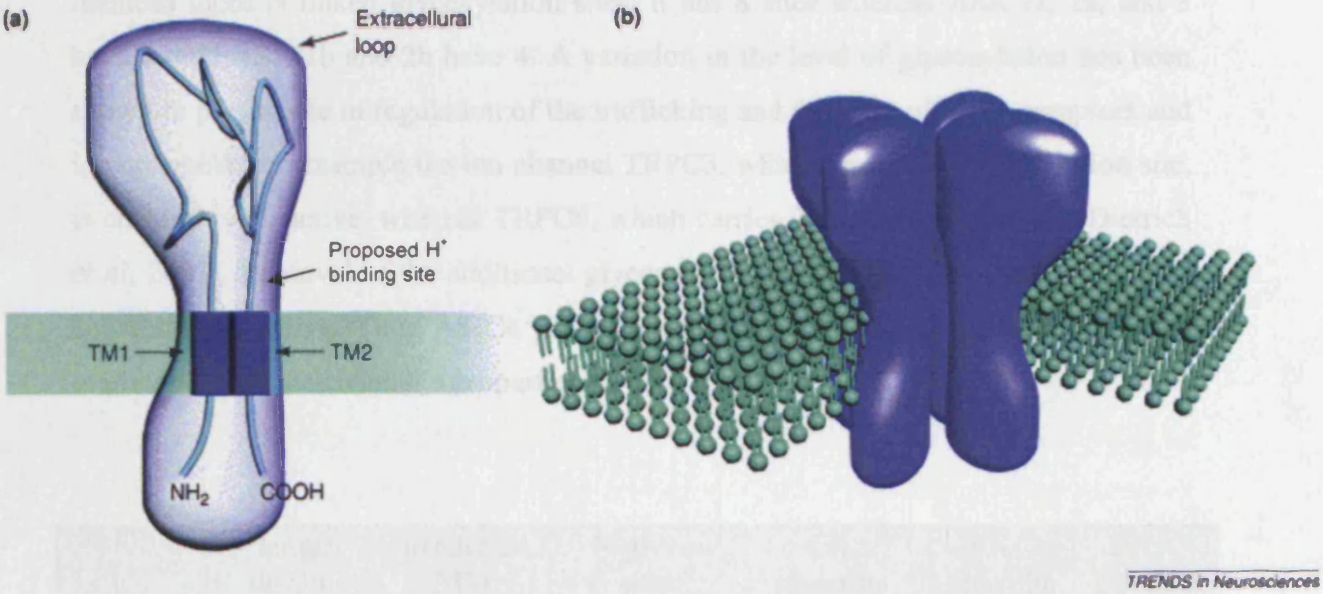

Fig. I.7: **Structure of ASICs**. a) one monomer, b) proposed model of heterotetramer (adapted from Krishtal, 2003)

	ASIC1a	ASIC1b	ASIC2a	ASIC2b	ASIC3	ASIC4
ASIC1a	100					
ASIC1b	77	100				
ASIC2a	65	58	100			
ASIC2b	54	51	76	100		
ASIC3	50	48	48	44	100	
ASIC4	45	45	42	40	43	100

Table I.3: **Sequence identity between ASIC subunits** (%) (by ClustalW analysis)

It contains a type II PDZ-binding domain consensus sequence (EIVC) and 5 lysine residues. Its extracellular loop contains as many cysteines as the other ASICs but includes more N-linked glycosylation sites; it has 8 sites whereas ASIC1a, 2a, and 3 have 2, and ASIC1b and 2b have 4. A variation in the level of glycosylation has been shown to play a role in regulation of the trafficking and function of many receptors and ion channels; for example the ion channel TRPC3, which bears 1 N-glycosylation site, is constitutively active, whereas TRPC6, which carries 2, is tightly regulated (Dietrich *et al*, 2003). Removal of the additional glycosylation site on TRPC6 made the channel constitutively active. Thus ASIC4 glycosylation may also have important functional implications on the channel's properties.

	length (amino-acids)	**predicted MW (kDa)**	**N-glyco. sites**	**CK2 phospho. sites**	**PKC phospho sites**	**PKA phosph sites**
ASIC1a	526	59.6	2 (N366, N393)	4 (T5, S476, S485, T520)	2 (S40, S497)	1 (S477)
ASIC1b	559	62.2	4 (N192, N216, N399, N426)	10 (S12, S13, S20, S37, S39, S51, S59, S509, S518, T553)	2 (S59, S530)	1 (S510)
ASIC2a	512	57.7	2 (N365, N392)	4 (S6, S474, S480, T506)	1 (T39)	0
ASIC2b	563	63.1	4 (N164, N233, N416, N443)	3 (S525, S531, T557)	0	0
ASIC3	533	59.2	2 (N176, N400)	1 (S5)	2 (T40, S523)	0
ASIC4	539	59.4	8 (N138, N167, N181, N191, N243, N341, N376, N405)	1 (S532)	0	0

Table I.4: **Characteristics of ASIC sequences.**
Note: the molecular weight (MW) was predicted from the primary amino-acid sequence. According to Saugstad *et al* (2004) 1 glycosylation adds around 2.5kDa to the MW.

Several studies gave insight into parts of the ASIC sequence involved in proton gating. A region upstream of TM1 and a few amino acids located in the extracellular loop just before TM2 have been implicated in the ion selectivity and the pH dependence of rat ASIC1-3 (Champigny *et al*, 1998; Coscoy *et al*, 1999; Bässler *et al,* 2001). A comparison of rat ASIC1 sequence with those of its orthologs isolated from lamprey and shark, which are not gated by protons although efficiently expressed at the plasma membrane, allowed the identification of several regions of the extracellular loop required for proton sensitivity in the rat (residues 78 to 136, and D351, Q358 and E359) (Coric *et al,* 2003; 2005). In the ASIC1 channels from toadfish and chicken which are activated by protons, the regions with the highest degree of identity to their rat ortholog comprise the extracellular domain and TM2 (Coric *et al,* 2003; 2005). According to these reports, the ion pore is likely to be formed by TM2, as it is for other members of the DEG/ENaC family (for review see Kellenberger and Schild, 2002). Amino-acids 83-85 (SQL) in rat ASIC1 are determinant for the desensitisation properties of the channel, as when replaced by the toadfish sequence (PLM), the channel acquires a much faster rate of inactivation (Coric *et al*, 2003).

Finally, ASICs are modulated by Ca^{2+} and Zn^{2+} ions (see section I.2.4.2.3); the Ca^{2+} binding site is formed by E425 and D432, located immediately upstream of TM2 in rat ASIC1a (Paukert *et al,* 2004b), and the Zn^{2+} binding site was mapped to K133 in ASIC1a (Chu *et al*, 2004). Immke and McCleskey (2003) proposed that H^+ and Ca^{2+} ions compete for the same binding site in the pore of ASIC1a and 3, and that displacement of Ca^{2+} by H^+ allows opening of the channels (Fig. I.7). It is interesting to note that E425 is not conserved in ASIC4, which is not activated by protons. Baron *et al* (2001) also identified the conserved residue H72, positioned just after TM1, as being crucial for proton sensitivity of ASIC2a but not ASIC1a or3, as mutation of this amino acid to alanine abolished the ability of ASIC2a to be activated by protons.

I.2.3. Expression pattern of ASICs

The expression pattern of ASICs in the nervous system has been extensively studied and gives important insights into the ion channels' potential functions. Most studies agree that all ASIC subunits are only expressed in the nervous system, some specifically in sensory neurons, and some in both central and peripheral neurons A summary of these data and the techniques used for the detection of ASICs is presented in table I.5.

I.2.3.1. ASIC1

ASIC1a was originally shown to be expressed at high levels in the brain and small neurons of the dorsal root ganglia (Waldmann *et al*, 1997a). It is also expressed in large DRG fibres innervating the colon and in vagal afferents innervating the stomach and the oesophagus (Page *et al*, 2004; Sugiura *et al*, 2005). ASIC1b mRNA distribution was studied by Northern blot, showing a specific expression of the transcript in DRG. Further analysis by *in situ* hybridisation in DRG established that it was expressed predominantly in large diameter neurons and in a subset of small diameter neurons (Chen *et al*, 1998).

Interesting findings regarding a possible involvement of ASIC1a in modulation of synaptic communication led groups to study more closely the distribution of ASIC1 in different areas of the brain and a possible localisation at the synapse. Garcia-Anoveros *et al* (1997) observed a very broad expression of ASIC1 mRNA in neurons of most regions from human and mouse brain: cerebellum, olfactory bulb, substantia nigra, and most parts of the forebrain. Alvarez de la Rosa *et al* (2002; 2003) examined the expression of rat ASIC1 at the protein level using an antibody directed against a region of the channel common to both splice variants. They confirmed that ASIC1 was present in the DRG and sciatic nerve. In the CNS, ASIC1 protein (supposedly ASIC1a, as ASIC1b is specific for DRG) was also found in most regions of the brain and in the spinal cord. Expression was specific to neurons; immunoreactivity was found on the soma, dendrites and axons of embryonic mouse cultured cortical and hippocampal

	Tissue	Technique	Reference
ASIC1a	Brain, DRG (small neurons)	Northern blot, RT-PCR, *in situ* hybridisation	Waldmann *et al*, 1997a; Chen *et al*, 1998
	Brain (neurons), spinal cord		Alvarez de la Rosa *et al*, 2002; 2003
	DRG (large fibres innervating the colon), nodose ganglia (vagal afferents innervating the stomach and oesophagus)	RT-PCR, Western blot	Page *et al*, 2004; Sugiura *et al*, 2005
ASIC1b	DRG (small and large neurons)	Northern blot, *in situ* hybridisation	Chen *et al*, 1998
ASIC1	DRG (NF200+ and peripherin+ neurons), sciatic nerve	Western blot, Immunohistochemistry	Alvarez de la Rosa *et al*, 2002
	Brain, spinal cord		Alvarez de la Rosa *et al*, 2003
ASIC2a	Brain, spinal cord	Northern blot	Price *et al*, 1996
	Brain (neurons)	Northern blot	Waldmann *et al*, 1996
	DRG (medium-large neurons)	Western blot, Immunohistochemistry	Alvarez de la Rosa *et al*, 2002
	DRG (mainly large, few small neurons), cell bodies and nerve terminals	Immunohistochemistry	Garcia-Anoveros *et al*, 2001
	DRG neurons innervating the stomach	Electrophysiology of labelled dissociated cells	Sugiura *et al*, 2005
ASIC2b	Brain (same areas as ASIC2a), DRG neurons	*In situ* hybridisation	Lingueglia *et al*, 1997
	DRG (medium-large neurons)	Western blot, Immunohistochemistry	Alvarez de la Rosa *et al*, 2002
ASIC3	DRG neurons	Northern blot, *in situ* hybridisation.	Waldmann *et al*, 1997b
	DRG (most small and large neurons)	Immunohistochemistry	Price *et al*, 2001
	DRG (medium-large neurons, few peripherin+ cells)	Western blot, Immunohistochemistry	Alvarez de la Rosa *et al*, 2002
	DRG neurons innervating the heart	Electrophysiology of labelled dissociated cells	Sutherland *et al*, 2001
ASIC4	Brain, spinal cord, DRG (low level)	Northern blot, *in situ* hybridisation	Akopian *et al*, 2000
	DRG, sciatic nerve	Western blot	Alvarez de la Rosa *et al*, 2002

Table I.5: **Tissue distribution of mammalian ASIC subunits**

neurons. In hippocampus ASIC1 was found to be mostly in the dentate gyrus, but also in CA1-4 layers (Wemmie *et al*, 2003). ASIC1 protein was present in synaptic vesicles and in post-synaptic densities fractions. ASIC1 mRNA was detected very early in the developing mouse brain, from embryonic day E11 (Garcia-Anoveros *et al,* 1997), and the protein from embryonic day E12 (Alvarez de la Rosa *et al,* 2003). This is consistent with recordings of proton-gated currents in embryonic rat brain, which could be made from the E12 stage, before voltage-gated Na^+ and Ca^{2+}currents can be recorded (Grantyn *et al*, 1989).

I.2.3.2. ASIC2

ASIC2a mRNA was originally detected only in brain and spinal cord by Northern blot analysis and appeared to be neuron-specific (Price *et al*, 1996; Waldmann *et al*, 1996; Bassilana *et al*, 1997). However, a transcript for ASIC2a was later found in DRG (Price *et al*, 2000), and ASIC2a protein was also detected in DRG and sciatic nerve (Alvarez de la Rosa *et al*, 2002). More precisely Garcia-Anoveros *et al* (2001) localised ASIC2a protein mainly in large DRG neurons expressing the A-fibre marker neurofilament 200 and only in a few small diameter neurons expressing the C-fibre marker peripherin; immunofluorescence was observed in both the cell bodies and the peripheral nerve terminals in the skin. Sugiura *et al* (2005) recorded currents mediated by ASIC2a-containing channels (identifiable by their ability to be upregulated by Zn^{2+}) in cultured DRG neurons that gave rise to afferents innervating the stomach. These neurons were previously labelled by injection of the dicarbocyanine dye DiI in the gastric antrum. Bassilana *et al* (1997) showed that the expression patterns of ASIC2a and ASIC1a in the brain overlap, and that these two subunits can be co-immunoprecipitated after co-expression in SF9 cells. Garcia-Anoveros *et al* (1997) found a very broad expression of ASIC2 transcripts in neurons of most regions from human and mouse brain; ASIC2 mRNA was most abundant in amygdala, caudate nucleus, and hippocampus. In the cerebellum ASIC2a is enriched in the synaptosomal fraction (Jovov *et al*, 2003). This suggests a potential synaptic co-localisation of ASIC2a with ASIC1a. In mouse tectum,

ASIC2a mRNA is detectable even earlier in development than ASIC1, from embryonic day E7 (Garcia-Anoveros *et al*, 1997).

ASIC2b has a very similar distribution to its splice variant in the brain, but it is expressed at higher levels than ASIC2a in sensory neurons of the dorsal root ganglia (Lingueglia *et al*, 1997; Alvarez de la Rosa *et al*, 2002). ASIC2a and 2b co-localise in medium to large DRG neurons (Alvarez de la Rosa *et al*, 2002).

I.2.3.3. ASIC3

ASIC3 expression was originally shown to be specific to sensory neurons in rat (Waldmann *et al,* 1997b). ASIC3 protein was detected in most small and large diameter neurons in mouse DRG and in peripheral nerve terminals in the skin (Price *et al*, 2001). In the rat, Alvarez de la Rosa *et al* (2002) found ASIC3 and both splice variants of ASIC2 co-localised mostly in medium to large DRG neurons. Sutherland *et al* (2001) showed that currents mediated by ASIC3 but no other ASIC subunit have the same characteristics as the proton-gated current found in DRG neurons innervating the heart, implying ASIC3 may be expressed in these cells. Hruska-Hageman *et al* (2004) identified ASIC3 transcript in spinal cord and total brain, and were able to co-immunoprecipitate ASIC3 and PSD-95 from spinal cord. Finally, Babinski *et al* (1999) reported a wide expression of ASIC3 transcript in many organs in human, including lung, kidney, heart, testis, brain and spinal cord.

I.2.3.4. ASIC4

ASIC4 mRNA distribution analysis by Northern blot and *in situ* hybridisation revealed high levels of expression of this subunit in the central nervous system and low levels in DRG (Akopian *et al*, 2000, Gründer *et al*, 2000). It was later confirmed to be expressed in DRG and sciatic nerve by Western blotting by Alvarez de la Rosa *et al* (2002).

I.2.3.5. zASICs

In situ hybridisation revealed that transcripts for all 6 zebrafish zASICs subunits were broadly distributed throughout the central nervous system, suggesting a possible role in neuronal communication (Paukert *et al*, 2004a). They were detectable early in development, between 24 and 48 hours post-fertilisation, indicating a potential function in the immature nervous system. As opposed to the mammalian ASICs which are all expressed in DRG neurons and other neurons of the PNS, only zASIC1.1 was found in peripheral primary sensory neurons of the fish embryo.

I.2.4. ASIC-mediated currents and their regulation

ASICs are ion channels activated by low pH. Determining the features of the currents they mediate, such as their ion selectivity, activation threshold or inactivation rate, is important for the understanding of their physiological significance. In this section, the characteristics of ASIC currents expressed in heterologous systems are described, allowing a comparison between ASIC-mediated currents and endogenous currents of the nervous system. Finally, the various endogenous and exogenous factors that have been so far reported to modulate ASIC function are reported.

I.2.4.1. Characteristics of ASIC currents

When expressed in heterologous systems, most ASIC subunits are activated by low pH and mediate an amiloride-sensitive cation current mainly carried by Na^+, although one subunit (ASIC1a) also displays Ca^{2+} permeability. Most subunits are functional when expressed as homomers and heteromultimers, and the properties of the currents they mediate depend on the combination of subunits in the channel complex. As described earlier, proton-gated currents in the nervous system are heterogeneous (see section I.1.2); different subsets of cells in a given tissue express different types of currents. Some of these endogenous currents are thought to be mediated by ASICs, but assigning

one type of current to one type of ion channel can prove uneasy. Analysis of the properties of heterologously expressed channels, as homo- or heteromers, as well as those of proton-gated currents in neurons from mice lacking ASIC channels can help identify the respective contributions of individual ASIC subunits (Fig. I.8 and table I.6).

ASIC1a expression in *Xenopus* oocytes is associated with a transient rapidly-activating current activated by rapid decreases in pH to values below 6.9. ASIC1a is highly permeable to Na^+, but it is the only ASIC subunit that is also permeable to Ca^{2+}, with a permeability ratio pNa^+/pCa^{2+} of 2.5 (Waldmann *et al*, 1997a). It desensitises rapidly. Recovery from desensitisation, which is slow compared to other ASIC subunits, requires application of neutral pH for about 13 seconds (Benson *et al*, 2002; Sutherland *et al*, 2001). In rat, ASIC1a was proposed to mediate one of the rapidly inactivating H^+-gated cation currents described in rat sensory neurons (Fig. I.3 b) (Waldmann *et al*, 1997a; Escoubas *et al*, 2000; Krishtal and Pidoplichko, 1981). In mice lacking the ASIC1 gene the amplitude of the proton-gated transient currents recorded at pH 6 in small to medium diameter DRG neurons decreased by 65%, showing that ASIC1 contributes an important part to these currents (Benson *et al*, 2002). Deletion of the ASIC1 gene also leads to elimination of proton-gated currents at pH6 in several regions of the brain (hippocampus, cortex) (Wemmie *et al*, 2002; Xiong *et al*, 2004). Finally, in rat, pH-sensitive transient currents recorded in cerebellar granule cells also mimicked ASIC1a-mediated currents (Escoubas *et al,* 2000).
The other splice variant of the ASIC1 gene, **ASIC1b**, also evokes a transient rapidly-activating amiloride-sensitive current when expressed in COS-7 cells and is activated by pH below 6.5 (Chen *et al*, 1998; Bässler *et al*, 2001). The ion channel is permeable to Na^+ and K^+ but not Ca^{2+}.

ASIC2a gives rise to a transient proton-activated Na^+ current when expressed in COS cells. It displays, however, a lower proton affinity and needs a lower pH than the other ASIC subunits to be activated ($pH < 5.5$) (Lingueglia *et al*, 1997). ASIC2a is expressed at low levels in DRG neurons, and seems to make a contribution to proton-gated currents in small or large DRG neurons (Price *et al*, 2000; Benson *et al*, 2002). Deletion

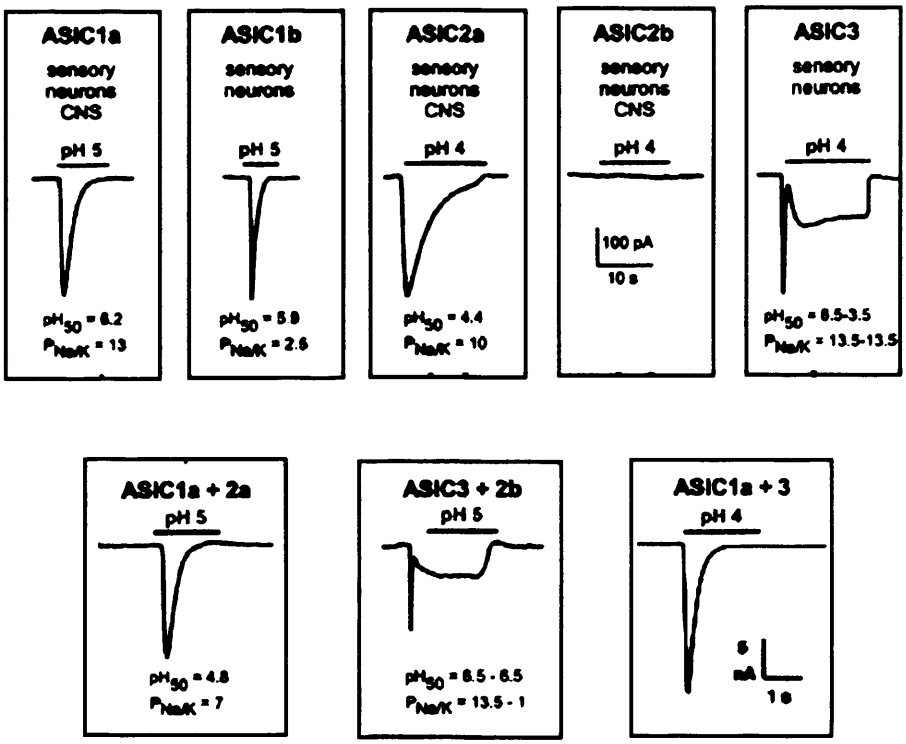

Fig. I.8: **Currents mediated by individual ASIC subunits and ASIC heteromultimers** (adapted from Voilley, 2004 and Escoubas *et al*, 2000). Scale is 100 pA (vertical) and 10 s (horizontal) for all traces except that corresponding to ASIC1a + 3.

	Activation pH	$\tau_{inactivation}$ (s)	$\tau_{recovery}$ (s, at pH7.4)	Permeability	Amiloride block (IC_{50})	Expression system	References
ASIC1a	6.9	1.4 ± 0.05 2.10 ± 0.30 (at pH 6)	13	$Na^+ > Ca^{2+} > K^+$	10 µM	*Xenopus* oocytes COS-7	Waldmann *et al*, 1997 Escoubas *et al*, 2000 Sutherland *et al*, 2001
ASIC1b	6.5	1.7 ± 0.27 (at pH 6)	5.9	$Na^+ > K^+$	21 µM	COS-7 COS-7	Chen *et al*, 1998 Sutherland *et al*, 2001
ASIC2a	5.5	3.15 ± 0.51 (at pH 4)	0.60	Na^+	28 µM	COS-7 COS-7	Lingueglia *et al*, 1997 Benson *et al*, 2002 Champigny *et al*, 1998
ASIC2b	N/A	N/A	N/A	N/A	N/A	COS-7	Lingueglia *et al*, 1997
ASIC3	6.5 (peak) < 4 (sust.) 7.0 (peak) < 5.5 (sust.)	0.32 ± 0.51 (at pH 6)	0.58	$Na^+ > K^+$	63 µM	COS-7 COS-7	Waldmann *et al*, 1997b Sutherland *et al*, 2001
ASIC4	N/A	N/A	N/A	N/A	N/A	COS-7, HEK-293	Akopian *et al*, 2000

Table I.6: **Characteristics of ASIC currents in heterologous expression systems**

of ASIC2 gave rise to a current that was more pH-sensitive and that desensitised more slowly than the wild-type current. Moreover, ASIC2a is thought to mediate in part currents recorded from rat DRG neurons innervating the stomach (Sugiura *et al*, 2005). ASIC2a also participates in proton-activated currents in rat hippocampal neurons (Askwith *et al*, 2004; Chu *et al*, 2004).
Expression of **ASIC2b** as a homomer in COS cells does not yield a proton-gated current but if co-expressed with other ASIC subunits, it has a modulatory function in heteromeric channels (see below) (Lingueglia *et al*, 1997).

ASIC3, when expressed in COS-7 cells, mediates an inward Na^+ current, characterised by an early rapidly inactivating current and a sustained late current (Waldmann *et al*, 1997b). The transient current desensitises more rapidly than that of any other ASIC subunit. The two components of the current have different pH dependences of activation, the sustained current being activated by a rapid decrease in pH from 7.3 to below 5.5 whereas drops to pH 7 are sufficient to induce the transient current (Sutherland *et al*, 2001). The sustained component of ASIC3-mediated current is highly selective for Na^+, and can be activated by a slow decrease in pH, as opposed to currents mediated by the other ASIC subunits, which require rapid drops in pH (Waldmann *et al*, 1997b). Both components are blocked by amiloride. Studies in mice lacking the ASIC3 gene revealed that this subunit does contribute to the proton-gated currents observed in small, medium and large DRG neurons. In isolated neurons from ASIC3 knockout mice the rate of desensitisation of H^+-activated currents was much slower than in wild type cells (Price *et al*, 2001; Benson *et al*, 2002), which is consistent with the fact that ASIC3-mediated transient current is the one that desensitises most rapidly among the ASIC channels. In addition the average peak current amplitude was slightly increased in ASIC3 null neurons (Price *et al*, 2001). ASIC3 is also thought to underlie the proton-gated currents observed in DRG heart afferents (Sutherland *et al*, 2001).

Finally, **ASIC4** does not yield a proton-gated current when expressed in *Xenopus* oocytes (Gründer *et al*, 2000), and its expression in COS-7 and HEK-293 cells does not alter the small endogenous proton-gated currents, suggesting that this subunit is not

functional on its own (Akopian *et al*, 2000). Whether ASIC4 associates with other subunits is currently unknown.

As mentioned above, co-expression of combinations of ASIC subunits in heterologous systems gives rise to currents with properties different from each individual channel, suggesting they can assemble to form functional heteromultimers.

In mice DRG neurons, where only one type of transient pH-activated current was described (Xie *et al*, 2002; Benson *et al*, 2002; Drew *et al*, 2004), Benson *et al* (2002) reported that ASIC1, 2 and 3 all made a contribution to the native pH-sensitive current (see above). Moreover, the native current desensitised more rapidly than any individual ASIC subunit ($\tau_{desens.}$ 0.15 s), suggesting that this current does not represent the sum of currents from homomeric channel but may be mediated by heteromeric channels comprising all 3 subunits. Besides, the native current was very closely mimicked by co-expression of ASIC1, 2 and 3.

In rat sensory neurons, 3 types of transient proton-activated currents were described (Fig. I.3); they cannot all be accounted for by individual ASIC subunits, therefore several studies attempted to identify which combinations of ASICs could mediate them. The most rapidly inactivating current in sensory neurons (Fig. I.3 a), which corresponds to the current observed in mouse DRG neurons, was suggested to be mediated by **ASIC1a/3** heteromeric channels (Escoubas *et al*, 2000; Hesselager *et al*, 2004). Co-expression of ASIC1a and 3 in COS cells yields a transient proton-gated current that inactivates very rapidly at pH 4 ($\tau_{desensitisation}$ 0.16-0.19s) and does not have a sustained component, as opposed to ASIC3 homomers. However, by analogy to the mouse data, one could think this current is mediated by channels containing all three subunits. In HEK293 cells, co-expression of a dominant-negative mutant form of ASIC3 with ASIC1a, 2a or 3 almost completely abolished proton-gated currents in these cells, confirming that ASIC3 can heteromultimerise with ASIC1a and 2a (Mogil *et al*, 2005). ASIC2b is not functional as a homomer but when co-transfected with ASIC2a or ASIC3 (but not ASIC1a) proton-gated Na^+ currents with kinetics different than the ones generated by ASIC2a or ASIC3 alone can be recorded (Lingueglia *et al*, 1997). In particular co-expression of **ASIC2b and ASIC3** yields a biphasic current at pH 4 in

which the sustained late component is nonselective ($pNa^+ = pK^+$). The authors note that this current is comparable to the biphasic proton-gated currents described by Bevan and Yeats (1991) in nociceptors (Fig. I.3 e). However, the latter was activated at pH 6, whereas the sustained phase of ASIC3-mediated current is normally activated at more acidic pH, so it is not clear whether ASIC3 participates in both phases of the current or only in the transient one. The sustained current could also be accounted for by TRPV1, which can be activated at pH 6 and which is known to be responsible for most proton-gated currents in capsaicin-sensitive DRG neurons (Caterina *et al*, 2000). Finally, **ASIC1a and 2a** also seem to be able to assemble in functional heteromultimers as the currents they evoke after co-expression in *Xenopus* oocytes display kinetics of activation and inactivation which can be fitted by single exponentials intermediate between the ones produced by either subunit alone (Bassilana *et al*, 1997). This heteromultimerisation was also demonstrated *in situ* in some hippocampal neurons (Askwith *et al*, 2004). ASIC1a/2a heteromers could mediate proton-activated currents recorded in some hippocampal neurons (Askwith *et al*, 2004).

The most slowly desensitising transient proton-gated currents found in sensory neurons (Fig. I.3 c) is not mimicked by any individual ASIC or any combination of ASIC subunits (Hesselager *et al*, 2004). It may partly be mediated by ASIC2a, which has the slowest rate of desensitisation among ASIC subunits, but may require regulatory subunits which are not present in heterologous expression systems.

I.2.4.2. Modulation of ASIC currents

I.2.4.2.1. Neuropeptides

Neuropeptide FMRFamide (Phe-Met-Arg-Phe amide) is a neurotransmitter found in *C. elegans* and other invertebrates. The gene encoding FMRFamide-related neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe amide, NPFF) is found in mammals (Perry *et al*, 1997). When injected in the spinal cord, NPFF has been found to have an analgesic effect mediated indirectly through opioid receptors; this effect is blocked by anti-opioid

drugs, although the neuropeptides display a poor affinity for all opioid receptors. Two GPCR receptors for NPFF were later identified in the spinal cord dorsal horn and DRG. Although ASICs are not directly gated by neuropeptides, there is evidence that these peptides modulate responses to acid. Askwith *et al* (2000) showed that FMRFamide slowed desensitisation of acid-evoked Na^+ currents in DRG neurons at pH 5 and of currents generated by ASIC1a expressed in *Xenopus* oocytes. Catarsi *et al* (2001) reported that NPFF induced a potentiation of the peak current generated by heteromultimeric channels formed by the association of ASIC2a and ASIC3, as well as a slowing of desensitisation, but not of the corresponding homomeric ion channels. They also found the heteromultimeric channels to be more sensitive to protons in the presence of the neuropeptide. Xie *et al* (2003) studied the effect of FMRFamide-related peptides on proton-gated currents in DRG neurons of ASIC knockout and wild-type mice and concluded that the modulation of these currents was via an effect of the neuropeptides mainly on ASIC3 and to a lesser degree on ASIC1. All these observations suggest that NPFF, which is upregulated during inflammation, may influence nociceptor excitability through modulation of ASIC-mediated currents during inflammatory conditions in mammals.

I.2.4.2.2. Antagonists

ASICs are inhibited by the K^+-sparing diuretic amiloride at relatively high concentrations (IC_{50} 10-60 μM, Waldmann *et al*, 1997a; 1997b). This compound blocks ENaC with a higher affinity, at submicromolar concentration, and it is thought to act by entering the channel pore (Poet *et al*, 2001). It also blocks T-type voltage-dependent Ca^{2+} channels (Tang *et al*, 1988) as well as the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger (Kleyman and Cragoe, 1988). Given the non selectivity of this compound, a number of groups have searched for more antagonists. Several toxins were found to block specifically various ASIC subunits and proved to be useful pharmacological tools. First Escoubas *et al* (2000) identified a 40-amino-acid toxin from tarantula *Psalmopoeus cambridgei* venom, psalmotoxin 1 (PcTX1). In heterologous expression systems PcTX1 potently blocks ASIC1a homomeric channels (IC_{50} 0.9 nM), and has no

effect on other homomeric or heteromultimeric ASIC channels, including ASIC1a-containing heteromers; it does not block ENaC or several voltage-gated potassium channels which are inhibited by related tarantula toxins. In small diameter rat DRG neurons PcTX1 inhibits the subset of transient proton-gated current that has a time constant of desensitisation of around 2 s, corresponding to trace b) in fig.I.3, confirming this trace may correspond to homomeric ASIC1a-mediated current. The toxin does not affect the other types of currents. Finally PcTX1 totally blocks the transient pH-sensitive current recorded in cerebellar granule cells (Escoubas *et al,* 2000).

Another toxin unrelated to PcTX1, APETx2, was later isolated from the sea anemone *Anthopleura elegantissima* venom (Diochot *et al*, 2004). The 42-amino-acid peptide inhibits the transient component of currents mediated by ASIC3 homomeric (IC_{50} 63 nM) and ASIC3-containing heteromeric channels with the exception of ASIC2a/3 heteromers. It does not have any effect on a variety of voltage-gated potassium channels. In rat DRG neurons the toxin reduced the amplitude of transient currents corresponding to trace a) in fig. I.3 by around 50%, confirming a role for ASIC3 in these currents. In DRG neurons from ASIC3 null mice, the remaining non-TRPV1 proton-gated current was resistant to APETx2.

Finally, Dube *et al* (2005) described a small molecule called A-317567 which inhibits all transient proton-gated currents in DRG neurons (IC_{50} 2-30 μM).

I.2.4.2.3. Modulation by extracellular ions

Lactate significantly increases ASIC3-mediated currents and neuronal excitability at pH 7 in rat DRG neurons, at concentrations encountered during muscle ischemia (15 mM) (Immke and McCleskey, 2001). It has similar effects on ASIC1a-mediated currents in transfected cells. The authors demonstrate that this effect is not due to a direct activation of the channel by lactate but rather to a decrease of divalent ions Ca^{2+} and Mg^{2+} in the extracellular media. Consistent with these findings, it was later confirmed that decreasing extracellular Ca^{2+} concentration allowed ASIC1a to be activated at higher pH in injected *Xenopus* oocytes (Babini *et al,* 2002), and induced an increase in ASIC1a-mediated current amplitude in hippocampal neurons (Gao *et al,* 2004).

Following these observations, Immke and McCleskey (2003) proposed this was the actual gating mechanism for ASICs: they showed that Ca^{2+} and H^+ ions compete for the same site in the ion pore of ASIC3 and ASIC1a, and that displacement of Ca^{2+} by H^+ allows opening of the channel. At pH 7.4, decreasing the Ca^{2+} concentration from 1 mM to 30 μM was sufficient to generate an amiloride-sensitive current in ASIC3-transfected cells. Paukert *et al* (2004b) later identified the Ca^{2+} binding sites in ASIC1a as E425 and D432, as mutations of these amino acids totally abolished the Ca^{2+} block of the mutated channel; however these mutations are not sufficient to make the channel constitutively active.

Zn^{2+} ions have different effects on ASIC1a and 2a channels. High concentrations of zinc (100 - 300 μM) greatly increase currents mediated by ASIC2a and ASIC2a-containing channels in injected *Xenopus* oocytes, but do not activate a current alone (Baron *et al*, 2001). This was also observed in mouse cortical neurons (Chu *et al*, 2004). In the latter study, however, chelation of Zn^{2+} ions contaminating solutions at nanomolar concentrations by TPEN dramatically increased currents mediated by ASIC1a homomeric and ASIC1a/2a heteromeric channels in a dose-dependant manner. Using cortical neurons from ASIC1 and ASIC2 knock-out mice the authors demonstrated that ASIC1a was indispensable for the observed zinc inhibition, and mapped the high affinity Zn^{2+} binding site to K133 in the extracellular domain of ASIC1a, a residue which is not conserved in the other ASIC subunits.

I.2.4.2.4. Regulation of ASICs by interacting proteins

Many studies have shown that association of neuronal ion channels and receptors with adapter proteins influences their localisation and regulation (Okuse *et al*, 2002; Sheng *et al*, 1996; Gormley *et al*, 2003). Several groups have found proteins that directly interact with different ASIC subunits, mostly using yeast two-hybrid screens.

Anzai *et al* (2002) identified the PDZ domain-containing protein CIPP (channel-interacting PDZ domain protein) as a partner of ASIC3 in DRG neurons. CIPP is known

to interact via one or several of its four PDZ domains with different ion channels and neuronal cell surface molecules (Kurschner *et al*, 1998); in particular, it binds to the NMDA receptor subunits 2B and 2C, raising the interesting possibility that CIPP may be a scaffold protein that could mediate a functional coupling between ASIC3 and NMDA receptors in sensory neurons. Co-expression of CIPP with ASIC3 in COS cells did not change the biophysical properties of ASIC3 but produced a 5-fold increase of ASIC3 peak current density at pH 5 and 5.5; this appeared to reflect an increase in the number of functional channels in the cell membrane rather than an increase in channel conductance.

Two other groups showed that the protein PICK1 (protein interacting with C kinase 1) interacts via its PDZ domain with the C-terminal last 4 amino-acid residues of ASIC1a, 2a and 2b, in yeast two-hybrid screens (Duggan *et al*, 2002; Hruska-Hageman *et al*, 2002). PICK1 is known to co-localise with the GluR2/3 AMPA receptor subunits and PKCalpha in the post-synaptic density of excitatory synapses, and participates in glutamate receptor trafficking (Xia *et al*, 1999). Likewise, PICK1 and ASIC1a were found to co-localise at synapses after co-transfection of cultured hippocampal neurons and at the sensory terminals of DRG neurons (Duggan *et al*, 2002). Thus, PICK1 may provide a link between ASIC1a and AMPA receptors post-synaptically, which may have important implications for generation and amplitude of EPSCs.

Co-expression of PICK1 and ASIC2b in heterologous systems affected the distribution of the channel, causing the formation of intracellular coaggregates of the two proteins (Duggan *et al,* 2002; Hruska-Hageman *et al,* 2002).

ASIC1a and 1b, but not any other ASIC subunit, have a predicted KRSS consensus sequence for cAMP-dependent protein kinase (PKA) phosphorylation in their intracellular C-terminus. Leonard *et al* (2003) showed that ASIC1a, but not ASIC2a or 3, was indeed phosphorylated by PKA at this site, and that this phosphorylation disrupted the interaction of ASIC1a with PICK-1 and the co-localisation of the two proteins.

PICK-1 was then shown to be a 'mediator' protein essential for ASIC2a PKC-dependent phosphorylation, which upregulates ASIC2a-mediated current by 300%

(Baron *et al*, 2002a). This phosphorylation occurs on the consensus site TIR located just before the start of the first transmembrane domain. A similar site (SLK) is present at the same position on the sequence of ASIC1a, and it is reasonable to think this subunit may be regulated in a comparable fashion. ASIC2b, which is not active by itself but can heteromerise with the ASIC3 subunit (Lingueglia *et al*, 1997), does not bear any PKC phosphorylation motif but its interaction with PICK1 allows PKC-dependant phosphorylation of ASIC3 (Deval *et al*, 2004). This phosphorylation significantly upregulates ASIC2b/3-mediated current.

Hruska-Hageman *et al* (2004) showed that the PDZ domain-containing protein PSD-95 found in post-synaptic densities binds to the C-terminus of ASIC3 on a PDZ-binding sequence. The two proteins were co-immunoprecipitated from spinal cord. PSD-95 was found to decrease ASIC3 cell surface expression after co-transfection in COS-7 cells.

ASIC1a, 2a and 3 also interact with stomatin (Price *et al*, 2004), the mammalian homolog of MEC-2, a protein thought to link the ion channel of the mechanosensitive receptor complex to the cytoskeleton in *C. elegans* (Goodman *et al*, 2002). However, stomatin has opposite effects on ASICs and DEG channels: it increases MEC-4 and MEC-10-associated currents but potently inhibits homomeric ASIC3-mediated current, slightly decreases the time constant of desensitisation of the heteromeric ASIC1a/3 and ASIC2a/3 channels as well as homomeric ASIC2a, and has no effect on homomeric ASIC1a current. Biotinylation studies suggested that the significant effect observed on ASIC3 current amplitude is not due to a decrease in the number of channels at the plasma membrane, but to alteration of its gating properties. Therefore, on these grounds, a parallel between MEC-4 and -10 and ASIC functions does not seem obvious. The authors propose that binding to stomatin may switch ASIC3 from a pH-sensing to a mechanotransducer channel.

Finally, the cystic fibrosis transmembrane conductance regulator (CFTR), which is both a Cl^- channel and an epithelial transport regulator, is known to downregulate ENaC, and Ji *et al* (2002) studied a potential effect of this protein on ASICs. In a *Xenopus* oocyte

expression system heteromeric ASIC1a/2a function was upregulated by CFTR via an increase of the Na^+ ions interaction outside the pore of the complex.

I.2.5. ASIC functions

So far protons are the only identified activator of ASICs. However, as described earlier, not all subunits are directly gated by this ligand. Two mammalian ASIC subunits, 2b and 4, are not gated by low pH, although ASIC2b heteromerises with other subunits and modulates their function. The zebrafish zASIC 1.1, 1.2, 1.3 and 4.1 expressed in *Xenopus laevis* oocytes mediate H^+-gated rapidly activating and desensitising Na^+ currents whereas zASIC2 and 4.2 are not activated by drops in pH (Paukert *et al*, 2004a). Similarly, lamprey and shark ASIC1 orthologs are not gated by protons (Coric *et al,* 2003; 2005). Hence the proton sensitivity of ASICs does not seem to be evolutionarily conserved for all subunits. This suggests ASICs including ASIC2b and 4 could be activated by other types of ligand, and could have other functions than those suggested by their ability to sense drops in pH.

In mammals, all ASICs are expressed in sensory neurons and they are thought to mediate the transient proton-gated currents observed in these cells. The physiological relevance of these transient currents, however, remains unclear. In nociceptors, they could be involved in acid-induced nociception, although the characteristics of ASIC currents may not be consistent with the physiology of acid-evoked pain. Moreover, ASICs are not only expressed in nociceptors but also in large diameter DRG neurons, which peripheral fibres do not respond to acid, but to light touch.

Aside from their property to be activated by protons, ASIC expression in mechanosensory endings in the skin and homology to the nematode mechanosensory channels MEC-4 and MEC-10 led to the hypothesis that they could be involved in mechanotransduction in mammals.

Finally, in the central nervous system, where ASIC1a and 2a are broadly expressed, these subunits have been proposed to play a role in synaptic communication. Post-

synaptic ASICs could be activated by the transient acidification that occurs in the synaptic cleft due to exocytosis of pre-synaptic acidic vesicles, and thus participate in the depolarisation of post-synaptic neurons. In pathophysiological states accompanied by tissue acidification such as brain ischemia, activation of Ca^{2+}-permeable ASIC1a was proposed to contribute to neuronal death through Ca^{2+} overload.
In the following section, the involvement of ASICs in the above functions is discussed.

I.2.5.1. Acid-induced nociception and modulation of pain pathways

Exposure to exogenous acidic solutions evokes pain (Steen *et al*, 1995b; Ugawa *et al*, 2002 ; Jones *et al*, 2004). Acidification of the external milieu is a common feature of many pathophysiological and painful conditions (Reeh and Steen, 1996). The extracellular pH can undergo a shift from 7.4 in physiological conditions to as low as 6 in pathophysiological states such as inflammation, hematomas, edema, fractures, blisters, ischemia or cancer (Clarke *et al.*, 1993; Helmlinger *et al.*, 1997). Tissue acidosis during inflammation can originate from acid degranulation of stimulated mast cells and migrating leukocytes, or lysed cells releasing their acidic content. In addition, tissue acidosis in muscle, heart or brain ischemia results from lactic acid production in the absence of oxygen. Extracellular acidification participates, along with inflammatory mediators such as bradykinin, histamine, and serotonin, in the pain felt in these pathophysiological conditions (Issberner *et al*, 1996; Voilley, 2004; Sugiura *et al*, 2005). Application of acid on to the receptive fields of 30% of small DRG nociceptors induces a sustained action potential discharge in these cells (Steen *et al*, 1992; 1995b). This may be due to initiation of proton-activated depolarising currents in nociceptors innervating the affected regions, which is consistent with an ion channel being located at the fibres' terminals.
As described in section I.1.2.6, low pH can activate two types of ion channels in nociceptors, ASICs and TRPV1. ASICs mediate rapidly activating and desensitising sodium currents, and biphasic currents with a sustained non selective cationic component when expressed as heteromultimers (Benson *et al*, 2002; Xie *et al*, 2002). Activation of TRPV1 requires lower pH than ASIC1 and 3 to be activated ($pH < 6$) and

gives rise to a slowly activating and inactivating non selective cation current. Both types of channels have been proposed to participate in acid-induced nociception, although the kinetics of TRPV1-mediated currents seem to be more compatible with the physiology of acid-evoked pain than ASICs. ASIC currents require a rapid decrease in pH for activation, and it is not clear whether such pH changes occur in tissues innervated by nociceptors. Moreover, it is known that the painful sensation perceived during tissue acidosis is due to a sustained excitation of nociceptors, and persists until pH goes back to neutral values (Steen *et al*, 1995b), which is not consistent with the kinetics of desensitisation of the currents mediated by ASICs. ASIC3-mediated currents do have a sustained component, which can be activated by slow acidification, but the current obtained by heteromerisation of ASICs, which is thought to represent the physiological current, at least in mice, does not comprise a sustained phase. In spite of these observations, some evidence suggests ASICs may play a role in **acid-induced nociception**.

Amiloride, a non specific ASIC blocker, has been shown to reduce pain induced by infusion of acidic solution (pH $\geq$ 6) (Ugawa *et al*, 2002) or by transdermal iontophoresis of protons in the skin of healthy human volunteers (Jones *et al*, 2004). In the latter technique, however, it is difficult to know at what concentration and how fast protons are delivered to the sensory neuron terminals and whether they can actually activate ASICs. Although the inhibition of ASICs by amiloride is not specific, its anti-nociceptive properties could be at least in part due to a blockade of these ion channels. Moreover, Dube *et al* (2005) showed that A-317567, a more potent and selective ASIC antagonist, had clear analgesic effects, in a dose-dependent fashion, in a rat model of post-operative pain. In this model of nociceptive pain, skin and muscle incisions cause a prolonged drop in pH and an increase in lactate concentration at the site of the wound (Woo *et al*, 2004). Amiloride also inhibited acetic acid-induced abdominal constrictions and formalin-induced paw licking (Ferreira *et al*, 1999). However, wild type and ASIC3 KO mice had similar behavioural responses in tests in which paw licking frequency after injection of pH 3 acetic acid in the skin was used as a measurement of acute pain (Price *et al*, 2001). Chen *et al* (2002) observed in ASIC3 KO mice an increased sensitivity compared to wild-type animals in various acute pain models, in

response to high intensity noxious mechanical, thermal and acidic stimuli. Finally, Mogil *et al* (2005) described the generation of a transgenic mouse (FVB strain) expressing a dominant-negative form of ASIC3, in which proton-activated currents mediated by ASICs in DRG neurons were almost abolished. This mouse showed increased baseline response to a number of mechanical and chemical stimuli compared to wild type animals.

As for TRPV1, Jones *et al* (2004) showed that in humans, desensitising the channel by repetitive application of capsaicin on the skin did not lead to a reduction of the sensitivity to acid. However, Ugawa *et al* (2002) reported that capsazepine, an inhibitor of TRPV1, reduced the sensation of pain induced by more extreme acidification (pH 5) in human. Thus, both ASICs and TRPV1 may play a role in acid-induced nociception, although the data showing an increased sensitivity to acid in mice lacking ASIC genes suggest their participation in this process may not involve or be limited to transduction in sensory neurons.

ASIC1, 2 and 3, when co-expressed in the same cells, are thought to heteromerise and to all participate in transient proton-gated currents in many nociceptors, but the relative expression of the various subunits in DRG neurons innervating specific areas may determine their implication in nociception in different organs. For example, ASIC1a and 2a, which were shown to carry proton-gated currents in gastric DRG afferents (Sugiura *et al*, 2005) could play a role in chemonociception in the stomach, as the pH within gastric ulcers reaches 6.6, which would be low enough to activate ASIC1a. As reported earlier, Sutherland *et al* (2001) proposed that ASIC3 alone mediates acid-evoked currents in DRG cardiac afferents, and is involved in the perception of ischemia-related pain in the heart. This would be consistent with the fact that ASIC3-mediated currents in sensory neurons are upregulated by lactate, a compound being released in the anaerobic conditions of ischemia, allowing the channel to be activated at higher pH (Immke and McCleskey, 2001).

ASICs are also thought to play a role in **inflammatory pain**, a pathological condition resulting from peripheral and/or central sensitisation of the nervous system,

characterised by an increased sensation of pain in response to noxious stimuli (hyperalgesia) or non noxious stimuli (allodynia). A potential involvement of ASICs in neuropathic pain has not been examined.

Pro-inflammatory mediators are known to potentiate the direct effect of protons on excitation of sensory neurons (Steen *et al*, 1995a) and non steroid anti-inflammatory drugs (NSAIDs) inhibit acid-induced pain (Steen *et al*, 1996). ASIC1a and 3 mRNA levels and current amplitude are increased during inflammation after injection of CFA, and this effect is reversed in the presence of NSAIDs (Voilley *et al*, 2001). This effect was postulated to increase sensory neurons' excitability during inflammation, but it is important to note that even in this condition no action potential firing was observed during the sustained phase of ASIC3-mediated currents, thus precluding an involvement of ASICs in a prolonged activation of nociceptors by protons. Some NSAIDs seem to directly block heterologously expressed ASIC-mediated currents. Yiangou *et al* (2001) reported an increase in ASIC3 protein expression in the muscle and mucosal layers of the inflamed intestine of patients suffering from Crohn's disease. Mamet *et al* (2002) then showed that the pro-inflammatory mediator NGF was responsible for the increase in transcription of ASIC3 by direct effect on the ASIC3 promoter. Additionally, H^+-gated currents mediated by ASIC2a and 3 are upregulated by PKC (via an interaction with PICK1) (Baron *et al*, 2002a; Deval *et al*, 2004), which can be activated through heterotrimeric G protein pathways by receptors of inflammatory mediators such as serotonin or bradykinin. This could also represent a mechanism for hyperexcitability of sensory neurons in inflammatory conditions.

In rats, Dube *et al* (2005) used injection of complete Freund's ajuvant (CFA) as a model for inflammation and showed that amiloride and A-317567 induced lowered thermal hyperalgesia. This is somewhat surprising as ASICs are not activated by heat, thus these observations may reflect changes that occur in central neurons rather than peripherally, as if blocking ASICs somehow reduced hyperexcitability of spinal cord neurons.

In C-fibre nociceptors from ASIC3 KO mice, action potential discharge in response to acid application (pH 5) to the corresponding receptive fields was reduced by 50% compared to wild type, but was unchanged at pH 4 (Price *et al*, 2001). ASIC3 null mice

exhibited lowered hypersensitivity to mechanical stimulation after one acid injection in skeletal muscle (in the paw on the injected side), but a small increase in sensitivity after induction of mechanical hyperalgesia by injection of carrageenan (Price *et al*, 2001). The same group later developed a model of mechanical hyperalgesia by repeated acid injections in the muscle (Sluka *et al,* 2003); in this model, both the ipsi- and contralateral paws display hyperalgesia, probably resulting from central sensitisation. ASIC3 but not ASIC1 KO mice showed clear deficiencies in the development of secondary mechanical hyperalgesia (Sluka *et al,* 2003).

Chen *et al* (2002) also generated ASIC3 null mutant mice but did not observe any change in development of mechanical and thermal hyperalgesia after injection of carragenan or capsaicin.

The contrasting results of ASIC3 deletion in mice are difficult to explain. The discrepancies observed between the two groups may be due to the fact that the two knock-out mice were of different genetic backgrounds, i.e. C57/BL6 and CD1, respectively. These two strains may respond differently to noxious stimuli; alternatively, inactivation of the ASIC3 gene may result in divergent modifications of gene expression in these mice.

It is also important to note that these studies all involved mice lacking one ASIC gene, and that part of ASIC-mediated currents in sensory neurons still remained. The mouse expressing a dominant-negative form of ASIC3 and displaying no transient proton-gated currents (Mogil *et al*, 2005) showed increased mechanical hypersensitivity in several models of inflammation. No change in thermal sensitivity was observed, whatever the intensity of the stimuli.

Overall, all these data substantiate a role for ASICs in modulation of nociceptive processing in particular during inflammation, not necessarily only as direct transducers of noxious chemical or mechanical stimuli in sensory neurons but possibly as modulators of transmission of nociceptive input to central neurons.

As a conclusion the role of ASICs in pain pathways thus remains enigmatic.

I.2.5.2. Mechanotransduction

Mechanotransduction is crucial to the senses of touch, proprioception, and hearing. It is believed that ion channels are at the centre of the mechanotransduction complex that converts mechanical stimuli into electrical signals; however the molecular identity of these ion channels remains unknown. Among several classes of ion channels ASICs have been proposed as candidate mechanotransducers in mammals (Tavernarakis *et al*, 1997). As reported in section I.2.1.1, this hypothesis was originally based on their sequence homology with several nematode DEG/ENaC channels, such as MEC-4 and -10 and UNC-8 which have been implicated in different aspects of mechanosensation (Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Tavernarakis *et al*, 1997). There is no published record of heterologously expressed ASICs being directly activated by mechanical stimuli, but this may be (by analogy to MEC-4 and -10) because mechanical gating of such channels requires specific tethering to auxiliary proteins only present in sensory neurons. Based on these observations several groups studied putative implications of ASICs in cutaneous and visceral mechanoperception, as well as hearing, but, as discussed below, the data are not always consistent.

ASIC2 and ASIC3 are expressed in specialised cutaneous mechanosensitive structures, i.e. lanceolate nerve endings in hairy skin (ASIC2-3), Meissner corpuscules in glabrous skin (ASIC3), and Merkel disk terminals (ASIC2) (Price *et al*, 2000; 2001; Garcia-Anoveros *et al*, 2001). Price *et al* (2000) reported that ASIC2 was involved in normal detection of light touch, in that the sensitivity of low-threshold rapidly adapting mechanoreceptors, and to a lesser degree that of low-threshold slowly adapting mechanoreceptors, is reduced in ASIC2 knock-out mice. Using the skin-nerve preparation they showed that the frequency of action potential discharge generated by mechanical stimuli applied to the receptive field of the corresponding fibres was significantly reduced in mice lacking the ASIC2 gene. However, no difference in threshold for action potential firing was measured, which does not support a direct role of ASIC2 in mechanotransduction. Moreover, another group showed that responses of low-threshold rapidly-adapting mechanoreceptors in skin-nerve preparation were not

affected by the deletion of the ASIC2 gene (Roza *et al*, 2004). This difference in phenotype may partly be due, however, to mice strain differences.

The putative role of ASIC3 in mechanotransduction was also studied through the means of a global knock-out mouse (Price *et al*, 2001). Surprisingly, ASIC3 deletion had opposite effects on responses of different classes of afferent fibres to mechanical stimuli. In the skin nerve preparation, the sensitivity of myelinated mechano-nociceptors was reduced in null mice whereas the sensitivity of low-threshold rapidly adapting mechanoreceptors was increased by two-fold. After induction of mechanical hyperalgesia by injection of carageenan, a small decrease of the threshold for paw withdrawal evoked by von Frey hairs was observed in ASIC3 knock-out mice, but baseline levels were unchanged. The diverse effects of the loss of ASIC3 in different classes of sensory neurons suggest that in these types of cells ASIC3 may be associated in heteromultimers with different ASIC subunits or interact with different proteins. ASIC3-mediated pH-activated current is potently inhibited by stomatin, the mammalian homolog of MEC-2 which is thought to be part of the mechanotransduction receptor complex in *C. elegans* (Price *et al*, 2004; Goodman *et al*, 2002). The authors propose that stomatin may switch ASIC3 from a pH-activated to a mechanically-activated channel, but no published report showed a direct activation of ASIC3 by mechanical stimuli, in the presence or absence of stomatin. Opposing the idea that ASIC2 and ASIC3 are involved in mechanotransduction, mechanically activated currents from cell bodies of cultured DRG neurons (McCarter *et al*, 1999; Drew *et al*, 2002) have recently been shown not to be mediated or regulated by ASIC2 or 3 (Drew *et al*, 2004).

ASIC1 does not seem to contribute in any way to mechanosensory function in the skin, as judged by analysis of action potential firing by 5 classes of mechanosensitive fibres in response to mechanical stimuli in skin-nerve preparation from wild-type/knock-out mice as well as in behavioural tests (Page *et al*, 2004).

Several published studies demonstrate that some ASIC subunits play a role in visceral mechanosensation. Page *et al* (2004; 2005) showed that ASIC1, 2 and 3 were expressed in nodose ganglia (which contain the cell bodies of vagal afferents that innervate the stomach and oesophagus). They analysed the effect of the loss of ASIC1 on several

aspects of gastrointestinal function and provided evidence that ASIC1 makes a 'negative' contribution to mechanosensation in gastro-intestinal afferents (Page *et al*, 2004). In 'viscera-nerve' preparations from ASIC knockout mice, action potential discharge frequencies were clearly increased in colonic splanchnic afferents (high threshold, rapidly adapting) as well as gastric and oesophageal vagal afferents (low threshold, slowly adapting [= tension receptors] and rapidly adapting [= mucosal receptors]) compared to wild-type. This increased mechanosensitivity resulted in a doubling of the time of gastric emptying of a solid meal in the knockout mice, without affecting the faecal output. Similar studies were conducted in ASIC2 and ASIC3 knockout mice (Page *et al*, 2005). Responses of different afferent fibres to mechanical stimuli were affected in different ways by the loss of these genes: mechanosensitivity of vagal gastro-oesophageal mucosal fibres as well as splanchnic colonic serosal afferents was increased in the ASIC2 knock-out animals, whereas vagal gastro-oesophageal tension receptors had decreased responses to stretch, and splanchnic colonic mesenteric fibres' sensitivity was not affected. In this study ASIC3 seemed to play a more consistent role, as the loss of this gene resulted in decreased mechanosensitivity in all 4 types of fibres, although that observed in gastro-oesophageal mucosal afferents was not statistically significant. In DRG ASIC2 was not found to participate in mechanosensation in CGRP-releasing nociceptive fibres innervating the colon (Roza *et al*, 2004).

The different reported levels of contribution (positive, negative, none) of ASIC1, 2 and 3 in cutaneous and visceral mechanosensation suggest that these subunits, which are present in the same neurons, must somehow assemble in different configurations to form functional ion channels in a cell-type-specific manner, for example as a result of interactions with specific proteins. The fact that certain subunits decrease mechanosensitivity suggests that they may not represent the mechanotransducing ion channel *per se*, but could rather be regulatory subunits in a mechanotransduction complex.

Finally, some evidence indicates that ASIC2 and 3 may be important in hearing. ASIC3 mRNA level was found to be increased by 58-fold in the cochlea compared to brain

(Hildebrand *et al*, 2004) (it is important to note though that ASIC3 is known to be mainly expressed in the DRG and very little in the brain; this study does not provide a comparison of the levels of expression of ASIC3 in the cochlea and in the DRG). In the mouse cochlea ASIC2 and ASIC3 proteins were observed in neurons of the spiral ganglion (which innervate hair cells), where ASIC2 seems to generate the most important part of proton-gated currents (Peng *et al*, 2004; Hildebrand *et al*, 2004). Auditory function was assessed by measurement of auditory-evoked brainstem response (ABR) thresholds from wild-type mice and mice lacking the ASIC2 or ASIC3 gene. ASIC2 knock-out mice had normal hearing thresholds, which confirmed Roza *et al* (2004) findings, but they proved to be more resistant to noise-induced temporary threshold shift: after 1h exposure to a noise of 110 dB ASIC2 null mice did not prove to experience temporary hearing threshold shift as much as wild-type mice. It is thought that ASICs could be involved in modulation of post-synaptic responses, as protons are released in the synapse from synaptic vesicles that could activate ASICs present at the plasma membrane of post-synaptic neurons (see below). The authors showed that ASIC2 was present in neurites of spiral ganglion neurons at the base of inner hair cells, and propose that excessive activation of ASIC2 could cause exicitotoxicity in spiral ganglion neurons and therefore participate to the generation of this threshold shift (Peng *et al*, 2004). As for ASIC3, it was shown to play a role in normal hearing in 4 month-old mice, as ASIC3 knock-out mice were found to have hearing deficits compared to wild-type, although it is worth mentioning that the numbers of mice used in this study were rather small (2 WT, 5 KO).

Thus ASICs seem unlikely to be involved in mechanotransduction in inner ear, although they may participate in some aspects of the auditory function.

I.2.5.3. Modulation of synaptic activity

The broad distribution of ASIC1, 2 and 4 across many regions of the CNS as well as the similarly widespread expression of their orthologs zASICs in the zebrafish CNS, suggests they could be involved in a function shared by most neurons in the CNS. So far, only data implicating ASIC1 in functions in central neurons are available. To be

activated efficiently, ASIC1a requires an environment where substantial drops in pH can occur rapidly and reversibly. For this reason it is thought that the synaptic cleft, where synaptic vesicles release acidic content (pH5.7, Miesenböck *et al*, 1998), could provide an appropriate stimulus for activation of this ion channel. As described thereafter, no direct evidence of activation of ASIC1a post-synaptically has been found, but this ion channel was nevertheless implicated in pathways related to hippocampus, cerebellum and amygdala functions.

Proton-gated currents recorded from the soma of cultured hippocampal neurons are mainly mediated by ASIC1 (Wemmie *et al*, 2002; Alvarez de la Rosa *et al*, 2003). In these same neurons, however, ASIC1 does not seem to be directly activated by a drop in pH occurring during basal synaptic activity: in cultured neurons EPSCs induced by 30 Hz, 1 s stimulus train were not blocked by amiloride (Alvarez de la Rosa *et al*, 2003) and in hippocampal slices fEPSPs, indicators of induced responses in the postsynaptic neuron, were not different in WT and ASIC1 KO (Wemmie *et al*, 2002). Alvarez de la Rosa *et al* (2003) found that high-frequency stimulation of presynaptic neurons in the presence of AMPA and NMDA receptor antagonists for excitatory synapses or $GABA_A$ receptor antagonist for inhibitory synapses, did not evoke any residual postsynaptic currents, hence no current attributable to ASIC1 activation. However, in ASIC1 null mice, Wemmie *et al* (2002) recorded a clear deficiency in the early phase of long-term potentiation (LTP) in hippocampal neurons after high frequency stimulation. This correlated with behavioural analysis of ASIC1 knock-out mice which revealed specific deficits in spatial learning and memory. Indeed it is thought that activity-dependent synaptic plasticity, such as LTP, could be involved in the mechanisms underlying memory mediated by the hippocampus (reviewed in Martin and Morris, 2002). Wemmie *et al* (2002) also showed ASIC1 may participate in cerebellum-dependent learning; ASIC1 KO mice had normal unconditioned eye-blink response but showed clear deficits in eye-blink conditioning (in acquiring eye-blinking in response to a specific sound followed by a noxious periorbital shock) compared to WT. ASIC1 was also found to mediate proton-gated currents in amygdala neurons and to contribute to behavioural response controlled by this structure, in particular fear conditioning, as shown by studies on transgenic mice (Wemmie *et al*, 2003; 2004). ASIC1 KO mice as

well as mice overexpressing ASIC1 had normal sensory and motor functions and basal fear behaviour compared to WT; however ASIC1 KO mice had more difficulty than WT animals in acquiring conditioned fear behaviour to various stimuli whereas mice overexpressing ASIC1 had enhanced fear conditioning ability.

I.2.5.4. ASIC1a and neuronal damage

As mentioned earlier, ASIC1a is the most broadly distributed ASIC subunit in the CNS, and deletion of the ASIC1 gene leads to elimination of proton-gated currents in several regions of the brain (Wemmie *et al*, 2002; Xiong *et al*, 2004). ASIC1a is particularly interesting because it is the only ASIC subunit that forms homomeric ion channels that are permeable to Ca^{2+}. Yermolaieva *et al* (2004) demonstrated in their Ca^{2+} imaging study that heterologously-expressed homomeric ASIC1a but not ASIC2a or heteromeric ASIC1a/2a channels are a major non-voltage-gated pathway for Ca^{2+} entry in cells. Similarly, in mouse hippocampal neurons, a drop in pH to pH 6 evoked an increase in Ca^{2+} that was not seen in ASIC1 knock-out mice, indicating that the Ca^{2+} influx observed was mediated by ASIC1. This may have important implications in stroke, which results in toxic Ca^{2+} overload in neurons. Traditionally, neuronal death during brain ischemia was associated with NMDA function, but ASIC1a was recently implicated as well.

In cultured cortical neurons, under oxygen and glucose deprivation conditions, which model ischemia, the magnitude of ASIC1a-mediated proton-gated current was increased, thus facilitating toxic Ca^{2+} overload of ischemic neurons (Xiong *et al*, 2004). This does not seem compatible with the fact that ASIC1a desensitises very quickly and requires rapid drops in pH for activation, as pH during ischemia is unlikely to decrease in a matter of milliseconds. However, the authors reported that in these ischemic conditions, ASIC1a desensitisation rate was increased by ~2.5-fold, providing a possible answer to this question. The ASIC1-mediated increase in intracellular Ca^{2+} contributed to cell death in cultured hippocampal neurons (Yermolaieva *et al,* 2004). This was confirmed by Xiong *et al* (2004), who reported that in mouse brain, tissue acidosis resulting from experimental stroke activated ASIC1a-dependent Ca^{2+} entry in

neurons, causing neuronal injury. Moreover, in these studies, blockade or deletion of ASIC1a could protect the brain from ischemic injury and constituted a more potent neuroprotective strategy against stroke than NMDA antagonism.
Taken together, all these results suggest that ASIC1a regulation may be a promising strategy to tackle neuronal damage resulting from brain ischemia (Benveniste and Dingledine, 2005).

I.2.5.5. Other functions

Sour taste is essentially mediated by protons. About 25% of taste receptor cells respond to acidic stimuli by a depolarisation which induces an influx of extracellular Ca^{2+} (Richter *et al*, 2003). ASIC2a was identified as a candidate receptor for the gustatory transduction of this taste in rats (Ugawa *et al*, 1998). It was cloned from a rat circumvallate papilla cDNA library and *in situ* hybridisation confirmed its expression in the taste buds of rat tongue. At a subcellular level, the protein was found at the plasma membrane and the apical part of taste bud cells. ASIC2b was later isolated from rat circumvallate papilla and shown to co-precipitate and co-localise with ASIC2a in some taste receptor cells (Ugawa *et al*, 2003). Proton-gated currents evoked by acetic acid were found to be greater than the ones generated by HCl, at equal pH, which is consistent with the fact that acetic acid has a more sour taste than HCl (Ugawa *et al*, 1998). Acid-induced sodium currents recorded from rat vallate taste cells were partly blocked by amiloride, a non-selective ASIC inhibitor (Lin *et al*, 2002).
However ASIC2 mRNA was not found by RT-PCR in mouse taste buds, and Ca^{2+} imaging studies showed no difference in the response of mouse taste buds to acidic stimuli in mice lacking the ASIC2 gene compared to wild-type (Richter *et al*, 2004). The differences observed between the data in rat and mouse suggests ASIC2 is probably not a universal mammalian receptor for sour taste.

Transcripts for ASIC1, 2, 3, and 4 were identified in rabbit retina (Brockway *et al*, 2002), and recently, proton-gated currents that resemble the ones mediated by ASICs were described in rat retinal ganglion cells (Lilley *et al*, 2004). Ettaiche *et al* (2004)

attributed these currents to ASIC2a/2b, which are expressed in photoreceptors and neurons in the retina. Deletion of ASIC2 resulted in enhanced visual transduction compared to wild-type mice, as well as higher light-induced damage to the retina, suggesting a protective role for ASIC2 in light-induced retinal degeneration, although the mechanisms of such a function remain unclear.

Finally, ASICs have been shown to be expressed very early in the developing nervous system, as early as E7 mouse embryos for ASIC2 and E11 for ASIC1 (Garcia-Anoveros *et al,* 1997). Transient proton-gated currents resembling ASIC-mediated currents were detected in DRG cells from E6 chick embryos (Gottmann *et al*, 1989), and in neurons from the tectum of E12 rats (Grantyn *et al*, 1989). The physiological role of such currents remains unclear, but this suggests they may somehow be related to neuronal development. Mice lacking individual ASIC genes, however, do not seem to have major defects in their nervous system.

I.3. The yeast two-hybrid system

As described in section I.2.5, ASICs have been implicated in various physiological or pathophysiological functions associated with their ability to sense drops in pH, such as acid-evoked nociception, or neuronal damage in ischemia. Their role in large diameter DRG neurons, however, remains enigmatic, as a direct involvement in mechanotransduction is not clear. In the CNS, ASICs were shown to be important in functions related to several brain regions, although a direct activation during synaptic activity could not be demonstrated. Thus, it is reasonable to think ASICs may still have new ligands and functions to be discovered.

One way of investigating a protein's function is to identify molecules with which it interacts *in vivo*. Protein-protein interactions, whether permanent or transient, are fundamental in most biological processes, from the formation of multimeric receptors or enzymatic complexes to the regulation of trafficking or signal transduction pathways, and antigen recognition. Proteins interacting with each other may be

expected to be involved in the same cellular process, and finding molecular partners of a given protein can provide valuable information on its subcellular localisation, the molecular mechanisms of its regulation, and ultimately its function. In order to investigate further the roles of ASICs in sensory neurons, we screened a rat DRG cDNA library (Kong *et al*, 2001) using a yeast two-hybrid (Y2H) system to identify proteins that interact with the intracellular N-terminal domains of the channels.

I.3.1. Principle

The yeast two-hybrid assay, or 'interaction trap' is a powerful and the most widely used method to identify novel protein-protein interactions. The system takes advantage of the structure of eukaryotic transcription factors (TFs); they consist of two separate domains, a DNA-binding domain which can bind to promoter regions of certain genes, and a transcription-activation domain, which induces transcription of the genes downstream of these promoter sequences. Very importantly the two modules do not need to be covalently attached to be functional, and they can be exchanged from one transcription factor to another to form functional hybrids (Hope and Struhl, 1986; Brent and Ptashne, 1985). Given these properties, the Y2H system uses transcription of reporter genes to produce specific readily observable biological readout in the yeast *Saccharomyces cerevisiae* in order to identify proteins from a library interacting with a protein of interest (or part of it usually), called the 'bait'. Commonly used reporter genes in this type of assay comprise *LacZ* and *LEU2*, which encode β-galactosidase and an enzyme required for the biosynthesis of leucine, respectively. In the interaction trap strategy (Fig. I.9), the bait is fused to the DNA-binding domain of a transcription factor, and all the proteins encoded for by the cDNAs in the library being screened are fused to an activation domain. Once translated in the cytoplasm, these two fusion proteins are directed to the yeast nucleus thanks to a nuclear localisation signal. Hence, in the nucleus, the bait fusion binds to the promoter region located upstream of the reporter genes, via the DNA-binding domain. When an interaction occurs between the bait and a protein from the library, the activation domain is physically brought in close proximity to the DNA-binding domain. This association reconstitutes a functional

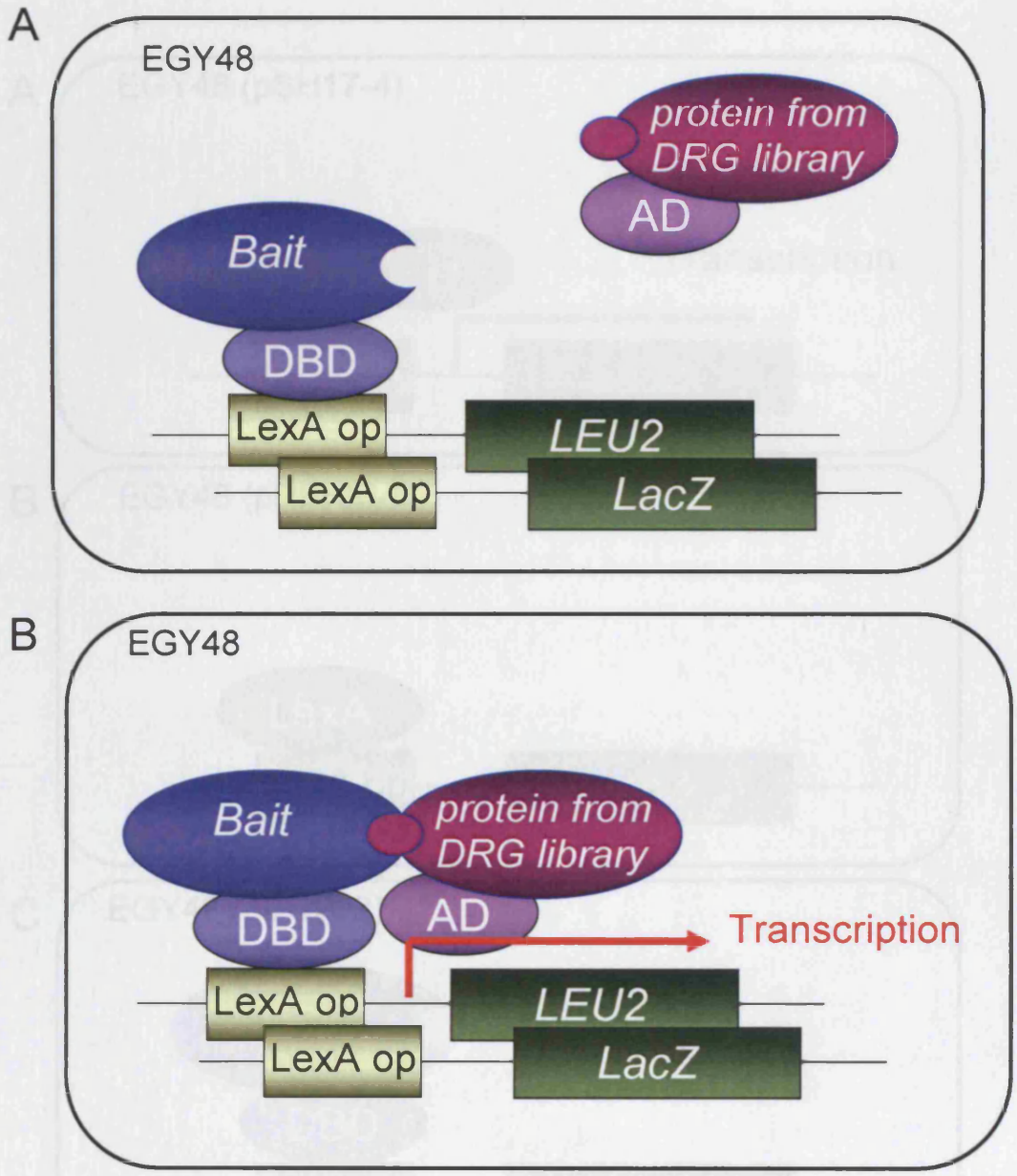

Fig. I.9: **Principle of the interaction trap**. The bait is fused to LexA DNA binding domain (DBD) and proteins from the DRG library are fused to B42 activation domain (AD). *A*, The bait fusion binds to LexA operators (LexA op) upstream of the reporter genes but in the absence of an interacting library protein there is no transcription of the reporter genes. *B*, The interaction between the bait and the library protein allows the reconstitution of a functional hybrid transcription factor, and thereby transcription of the reporter genes.

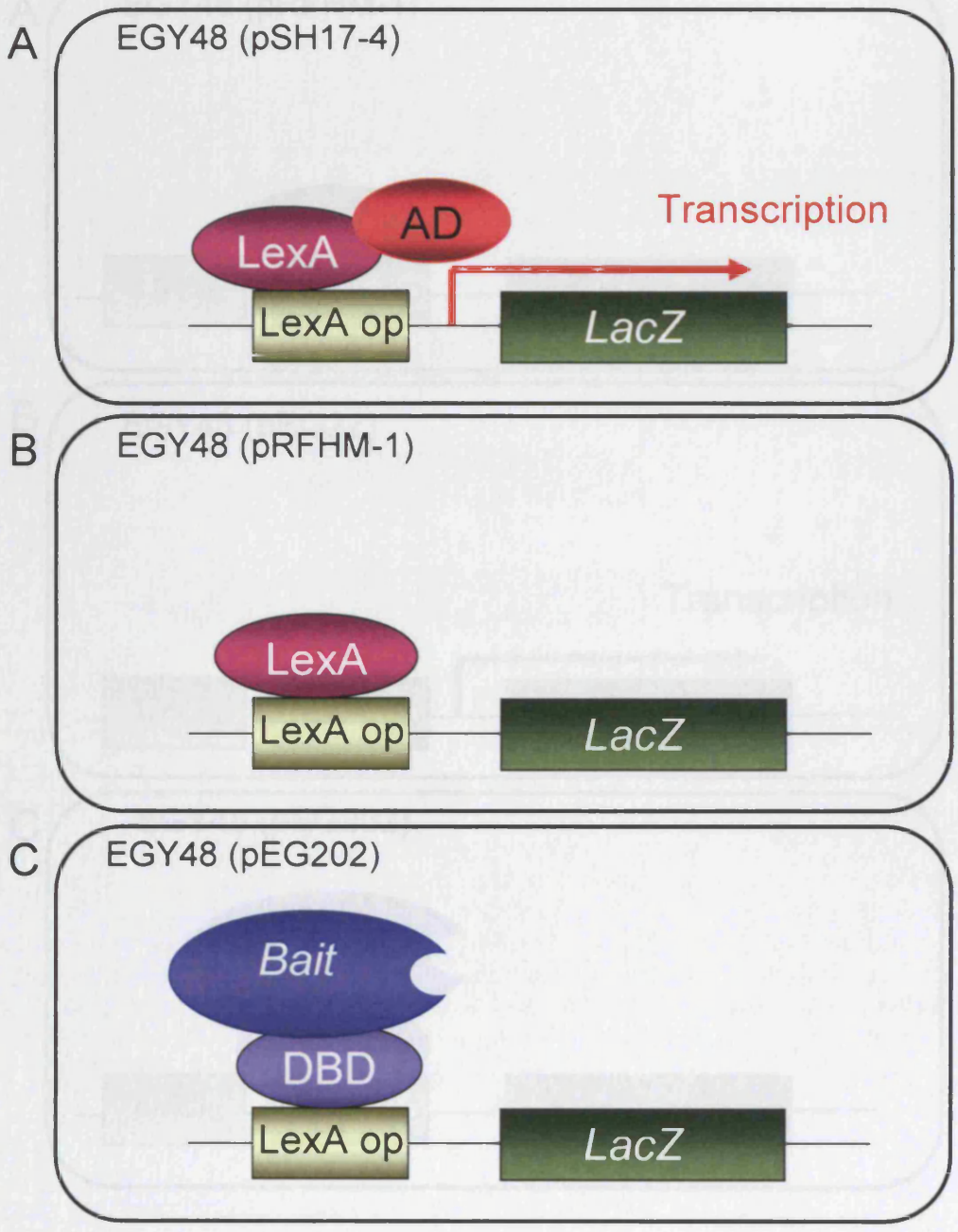

Fig. I.10: **Principle of the activation assay**. The reporter plasmid pSH18-34 carries LexA operators (LexA op) upstream of *LacZ* cDNA. *A*, positive control; the yeast is transformed with plasmid pSH17-4 encoding a LexA protein fused to the GAL4 activation domain (AD) which activates *LacZ* transcription. *B*, negative control; the yeast is transformed with plasmid pRFHM-1 encoding LexA only, and there is no transcription of *LacZ*. *C*, the yeast is transformed with plasmid pEG202 encoding the bait fused to LexA DNA-binding domain (DBD). If the bait does not have intrinsic transcriptional activity, *LacZ* is not transcribed

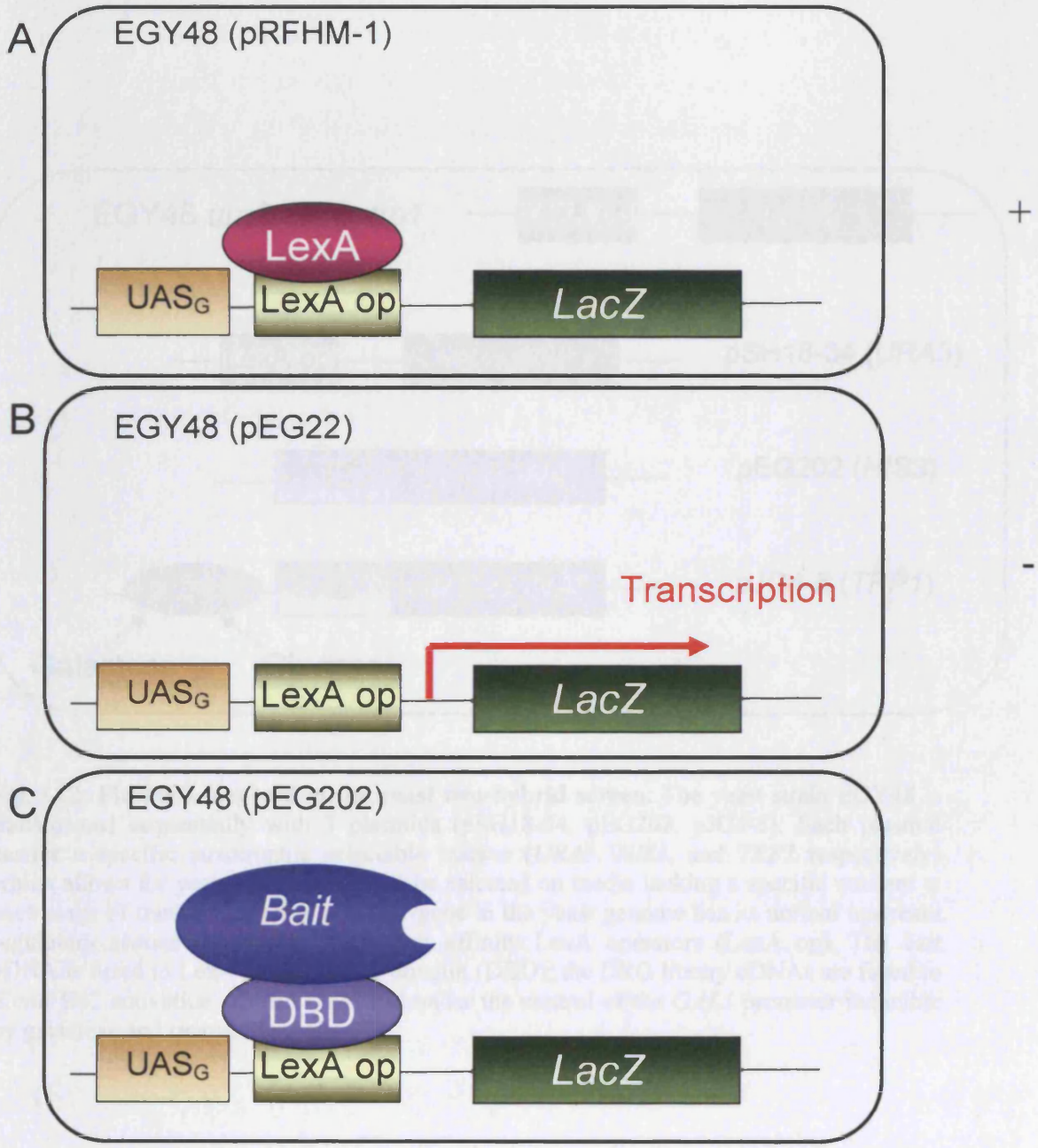

Fig. I.11: **Principle of the repression assay**. The reporter plasmid pJK101 carries LexA operators (LexA op) in the middle of the promoter upstream of *LacZ* cDNA. *A*, positive control; the yeast is transformed with plasmid pRFHM-1 encoding a LexA protein which binds the LexA
operators in the middle of the promoter and blocks *LacZ* transcription. *B*, negative control; the plasmid pEG22 does not encode LexA and the transcription of *LacZ* can be activated. *C*, the yeast is transformed with plasmid pEG202 encoding the bait fused to LexA DNA-binding domain (DBD). If the bait reaches the nucleus and binds to LexA operators, *LacZ* is not transcribed.

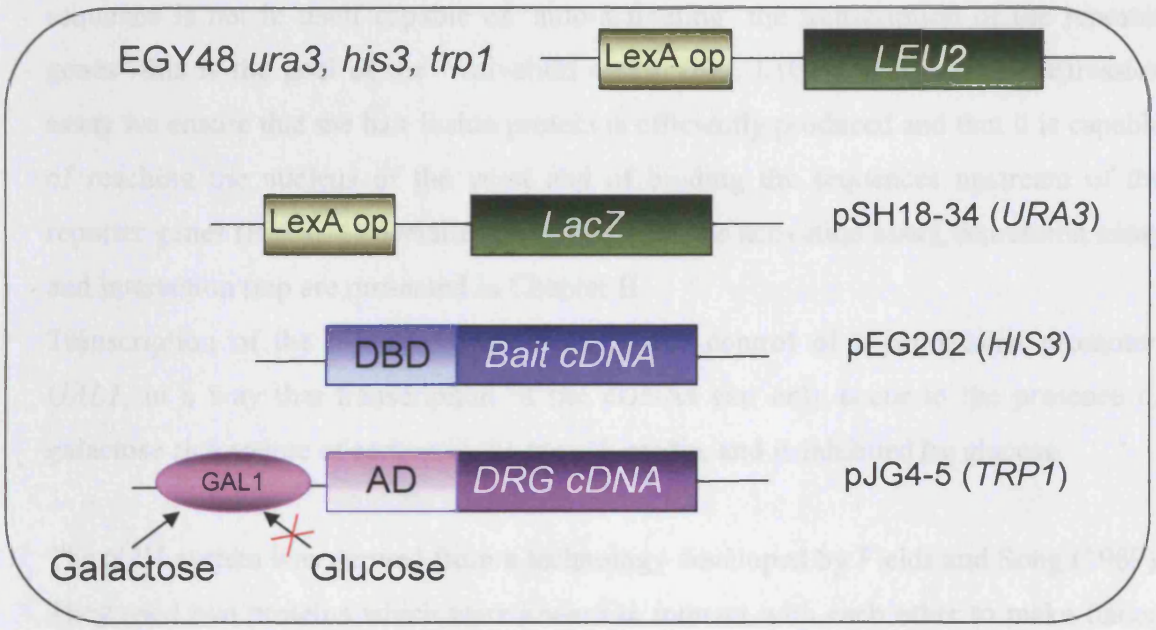

Fig. I.12: **Plasmids involved in the yeast two-hybrid screen**. The yeast strain EGY48 is transformed sequentially with 3 plasmids (pSH18-34, pEG202, pJG4-5). Each plasmid carries a specific auxotrophic selectable marker (*URA3*, *HIS3*, and *TRP1* respectively) which allows for yeast transformants to be selected on media lacking a specific nutrient at each stage of transformation. The *LEU2* gene in the yeast genome has its normal upstream regulatory sequences replaced with high affinity LexA operators (LexA op). The bait cDNA is fused to LexA DNA binding domain (DBD); the DRG library cDNAs are fused to *E.coli* B42 activation domain (AD) and under the control of the *GAL1* promoter inducible by galactose and repressed by glucose.

transcription factor which triggers the transcription of the downstream reporter genes. Cells expressing a protein interacting with the bait are subsequently selected on media lacking leucine and containing X-gal.

Before the start of the interaction trap, it is therefore essential to verify that the bait sequence is not in itself capable of 'auto-activating' the transcription of the reporter genes -this is the goal of the 'activation assay' (Fig. I.10). Finally, in the repression assay we ensure that the bait fusion protein is efficiently produced and that it is capable of reaching the nucleus of the yeast and of binding the sequences upstream of the reporter genes (Fig. I.11). Detailed procedures for the activation assay, repression assay and interaction trap are presented in Chapter II.

Transcription of the cDNA library is under the control of a conditional promoter, *GAL1*, in a way that transcription of the cDNAs can only occur in the presence of galactose as a source of carbon in the growth media, and is inhibited by glucose.

The Y2H system was derived from a technology developed by Fields and Song (1989). They used two proteins which were known to interact with each other to make fusion proteins, one with a DNA-binding domain and the other with an activation domain of a transcription factor. The interaction occurring between the two hybrid proteins in the yeast nucleus was able to activate transcription of the reporter gene *LacZ*. In this study the yeast GAL4 transcription factor which controls the expression of enzymes required for the metabolism of galactose, was used. However GAL4 is an endogenous yeast protein and it has a strong transcriptional activity, therefore this method was likely to give a high background, with high sensitivity. The technique was then modified by Gyuris *et al* (1993), incorporating the DNA-binding domain of bacterial repressor LexA and the bacterial activation domain B42 which has weaker transcriptional activity than GAL4. These proteins of bacterial origin do not interact with or activate transcription of endogenous yeast sequences. Moreover bacterial LexA operators replaced the endogenous promoter region upstream of *LEU2* in the yeast genome, and upstream of *LacZ*, so endogenous yeast transcription factors cannot activate their expression. In this way, the transcription of the reporter genes is much more specific, the method gives a much lower background, and is also less sensitive. In addition to

these transcription factors, the *Herpes simplex* virus protein VP16 has also been used as a transcriptional activation domain in conjunction with GAL4 DNA-binding domain (Fearon *et al*, 1992) or LexA (Vojtek *et al*, 1993). Because of its higher transcriptional activity, the systems which utilize VP16 are likely to have the highest sensitivity among the different yeast two-hybrid systems.

I.3.2. Advantages

Fields and Song's technique (1989) was revolutionary as it permitted a simple screening of a large number of unknown proteins for interactions with a given bait, and it allowed the immediate and easy identification of these 'preys', as the cDNA is cloned at the same time as the interaction between the proteins is detected. This was not the case in conventional biochemical techniques such as cross-linking, co-immunoprecipitation or chromatographic co-purification (for review see Phizicky and Fields, 1995). The Y2H screen studies interactions in a eukaryotic cell, which is a clear advantage compared to *in vitro* techniques or bacterial expression systems when studying mammalian proteins, but at the same time the yeast endogenous proteins are unlikely to bind and potentially disrupt interactions between the mammalian proteins studied. The reporter gene approach allows amplification of the signal induced by the interaction and weak interactions can be detected. This can be modulated by the number of LexA operators placed upstream of the reporter genes and the strength of the transcription activation domain chosen. In other methods, such as co-purification, only strong protein interactions which remain stable through lysis steps which involve detergents can be detected. Finally the method is cheap, does not need any specific equipment, and although labour-intensive is relatively easy to implement.

I.3.3. Limitations

I.3.3.1. False negatives

A number of factors can result in missing some interactions. In the Y2H assay, the proteins screened have to be directed to the yeast nucleus, since the system is based on transcription of reporter genes. This may not provide an appropriate environment for the interaction to occur, especially for membrane proteins which are highly hydrophobic. Moreover, the host being yeast, if mammalian proteins are studied, they may not undergo adequate folding or post-translational modifications, which could be required for these interactions. The cDNA library does not contain exclusively full length cDNAs, and the detection of interaction with the N-terminal part of large proteins may be limited when the cDNA is prepared with oligo(dT) primers. Finally, expression of certain proteins may be toxic to the yeast.

I.3.3.2. False positives

In the Y2H all the proteins represented in the cDNA library are placed in proximity of the bait. Therefore, some proteins may be able to interact without it having any functional significance, for example if these proteins are never found in the same cell type or at the same subcellular level *in vivo*. Finally, some proteins are described as 'sticky', they have been found to interact with many different proteins, simply because of the nature of their sequence (e.g. cytochrome C oxidase).

Therefore, identification of interacting proteins in a Y2H screen has to be followed by a confirmation of the interaction, if possible *in vivo*, and the function of this interaction must be proven to be biologically relevant.

I.3.4. Modifications of the system

Over the years the two-hybrid system has been adapted to a wide range of applications. For example protein-DNA interactions were successfully identified by yeast one-hybrid assay (Wang and Reed, 1993). The yeast three-hybrid system was developed to detect and analyse RNA-protein interactions (Putz *et al*, 1996; SenGupta *et al*, 1996). Another derivative of the Y2H, the reverse two-hybrid, in which transcription of the reporter gene inhibits cell growth, was used to identify mutations that caused disruption of interaction between two proteins (Li and Fields, 1993). Young *et al* (1998) adapted the method to successfully identify a Ca^{2+} channel modulator, which disrupts alpha1B-beta3 subunit interactions and inhibits N-type calcium channel activity. 'Dual-bait' systems enable the identification of proteins interacting specifically with one bait but not another, or the characterisation of mutations affecting specifically the binding to one of those baits (Serebriiskii *et al*, 1999). A bacterial two-hybrid was described (Joung *et al*, 2000) which enabled the screening of very large libraries, and its mammalian version was also developed (reviewed in Lee and Lee, 2004). Finally, in the split-ubiquitin assay, the reconstitution of a protein other than transcription factors to provide a readout for protein-protein interaction enabled to study interactions occurring at the plasma membrane (Johnsson and Varshavsky, 1994; Stagljar *et al*, 1998).

In the last few years, with the rise of proteomics, intensive efforts were made to identify protein-protein interactions on a large scale. The yeast two-hybrid system proved to be a very efficient method in this matter, along with the other standard technique, co-purification of protein complexes coupled with protein identification by mass spectrometry. Several genome-wide two-hybrid strategies were used to analyse protein interactions in *S. cerevisiae* with more than 2500 protein-protein interactions described (Fromont-Racine *et al*, 2000; Uetz *et al*, 2000) and *C. elegans* (Walhout *et al*, 2000), and recently the split-ubiquitin system was also scaled up to identify interactors for membrane proteins of the yeast (Miller *et al*, 2005).

CHAPTER II

MATERIALS AND METHODS

II.1. The yeast two-hybrid screen

II.1.1. Yeast strain

The yeast strain EGY48 was used in which the reporter gene *LEU2* has its normal upstream regulatory sequences replaced with 3 high affinity LexA operators, each of which can potentially bind 2 LexA dimers. EGY48 also carries mutations in the selectable marker genes *his3*, *trp1* and *ura3*.

II.1.2. Yeast plasmids

All yeast plasmids listed below contain the yeast 2μ origin of replication.

- pEG202 is a 10kb multicopy yeast plasmid containing the selectable marker gene *HIS3* (Fig. I.12). The bait DNA is inserted downstream of the full-length LexA coding region (amino acids 1-202, containing a DNA binding domain), flanked by the yeast *ADH1* promoter and terminator.
- The library plasmid pJG4-5 is a 6.4kb plasmid containing the selectable marker *TRP1*. Under the control of the *GAL1* inducible promoter, the cDNAs are expressed as fusion proteins downstream of the 'activation tag', comprising the SV40 nuclear localisation signal (9 amino acids), the B42 activation domain derived from *E. coli* (87 amino acids) and the haemagglutinin (HA) epitope tag (9 amino acids).
- The reporter plasmid pSH18-34 used in the interaction trap carries the *GAL1* coding sequence fused to *LacZ*, but the GAL1 upstream activating sequences (UAS_G) have been replaced by 4 *colE1* LexA operators. It contains the yeast selectable marker *URA3*.
- The reporter plasmid pJK101 is derived from the same plasmid pSH18-34 was made from, but it contains most of the *GAL1* UAS_G, and 1 *colE1* LexA operator between UAS_G and the *GAL1* TATA. It is used to measure repression of *LacZ* transcription by LexA fusions. It contains as well the yeast selectable marker *URA3*.

- pSH17-4 is used as a positive control in the activation assay. It encodes for a LexA protein fused to the GAL4 activation domain. This fusion protein binds to LexA operators upstream of the reporter genes and activates their transcription. The plasmid carries the *HIS3* marker.

-pRFHM-1 derives from pSH17-4 but encodes for the LexA protein only. Thus it is used as a negative control in the activation assay, as the protein made binds to LexA operators upstream of the reporter genes but does not activate their transcription. It is also used as a positive control in the repression assay, where the LexA protein made binds to the LexA operator placed between the UAS_G and the TATA upstream of the reporter gene and blocks its transcription. It also carries the *HIS3* marker.

- pEG22 is used as a negative control in the repression assay, indeed it does not encode for LexA protein and does not block transcription of the *LacZ* reporter gene. It also carries *HIS3* gene.

II.1.3. cDNA library

The rat DRG cDNA library used in the yeast-2-hybrid screen was a kind gift of Dr. M. Chao, NYU, USA. cDNAs were prepared using oligo(dT) oligos and were directionally cloned into a modified version of expression vector pJG4-5 at the BstX1/Not I sites (Kong *et al*, 2001).

II.1.4. Amplification of the cDNA library

Library transformants frozen stock was diluted 1:100 in Luria Broth (LB) containing 100 µg/mL ampicillin (LB + amp), and 3 mL of this suspension was plated on a total of 60 10-cm agar LB + amp dishes. After 24h incubation at 37°C, dishes were cooled down for 2h at 4°C, and all colonies were scraped from the agar plates, washed with LB + amp, and subjected to Maxiprep according to the manufacturer's protocol (Qiagen). A total of 1 mL of DNA at 1mg/mL concentration was obtained.

II.1.5. Baits

The baits were cloned into pEG202 bait plasmid at EcoRI/XhoI sites by Dr. L. Cingolani from a rat DRG cDNA library provided by Dr. M. Chao (ASIC1a, 1b, 3 and 4), or from cDNA prepared by Dr. L. Cingolani from rat DRGs (ASIC2a).

PCR primers were designed to amplify the N-terminal region (NT) of rASIC1a, rASIC1b, rASIC2a, rASIC3 and rASIC4, corresponding to residues 1-43, 1-88, 1-42, 1-43 and 1-67 respectively. Restriction sites EcoRI and XhoI were introduced at the 5' and 3' ends respectively in the PCR products. EcoRI sites are underlined and XhoI sites are boldfaced.

ASIC1aNT (143bp)

N-rASIC1a-f: 5'-GGAATTCATGGAATTGAAGACCGAGG

N-rASIC1a-r: 5'-A**CTCGAG**CCGCTTCAGAGACAGCCG

ASIC1bNT (278bp)

N-rASIC1b-f: 5'- GGAATTCATGCCCATCCAGATCTTTTGTTC

N-rASIC1b-r: 5'- A**CTCGAG**CTGCCTTGGCCCTGGGC

ASIC2aNT (141bp)

N-rASIC2a-f: 5'-GGAATTCATGGACCTCAAGGAGAGCC

N-rASIC2a-r2: 5'-CA**CTCGAG**ACGCCGGATGGTCAGC

ASIC3NT (143bp)

N-rASIC3-f: 5'-TGAATTCATGAAACCTCGCTCCGGACTG

N-rASIC3-r: 5'-A**CTCGAG**TCGGCGCAGGGTCAGGC

ASIC4NT (215bp)

N-rASIC4-f: 5'-GGAATTCATGCCGATCGAGATTGTGTG

N-rASIC4-r: 5'-A**CTCGAG**TCTGCGCAGTCCATGGGG

II.1.6. Yeast Transformation

Yeast was grown in YPD Medium (1% yeast extract, 2% peptone, 2% glucose) overnight at 30°C with shaking. The culture was diluted to an OD_{600} of 0.2 in a final volume of 400 mL and allowed to grow to $OD_{600} = 1$. The culture was centrifuged at

2000g for 5 min, and washed once in sterile water. Cells were then washed twice with 5 mL and 2 mL sterile LiOAc/TE (10 mM Tris-HCL pH 7.5, 1 mM EDTA, 100 mM lithium acetate pH 7.5) respectively.

For each transformation, 2 µg transforming DNA and 60 µg single-stranded salmon sperm DNA were mixed to 100 µL of the competent cells suspension. 600 µL sterile 40% (w/v) PEG-3600 in LiOAc/TE solution was added and the mixture was incubated at 30°C for 30 min, and then at 42°C for 15 min after addition of 30 µL DMSO. The yeast suspension was then centrifuged, resuspended in 50 µl of TE Buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and spread onto agar plates supplemented with 2% glucose and lacking the appropriate amino acid for selection and incubated at 30°C.

II.1.7. Activation assay

The activation assay was performed to ensure that the baits do not have intrinsic transcriptional activity on *LEU2* and *LacZ* reporter genes (Fig. I.10). The yeast strain EGY48 was transformed consecutively with the reporter plasmid pSH18-34 and either the bait plasmid pEG202 or the positive control plasmid pSH17-4 or the negative control plasmid pRFHM-1. Transformants were selected on agar plates containing glucose as source of carbon and lacking uracile and histidine ('Glc ura- his-': 2% glucose, 6.7 g/L yeast nitrogen base without amino acids, 2 g/L amino acid dropout powder lacking the appropriate amino acids, 1.5% bacto agar) after 2 days incubation at 30°C. Four individual colonies from each transformation were grown overnight at 30°C in liquid Glc ura- his- media, diluted the next day to OD_{600} = 0.2 and grown to OD_{600} = 0.5. 10 µL drops of the cultures and of 100- and 1000-fold dilutions were plated onto one Glc ura- his- plate and two agar plates containing galactose and raffinose as carbon source, one lacking uracil, histidine and leucine (Gal/Raf ura- his- leu-) and one lacking uracile and histidine and containing X-Gal (Gal/Raf ura- his- X-Gal: 2% glucose, 6.7 g/L yeast nitrogen base without amino acids, 2 g/L amino acid dropout powder, 1.5% bacto agar, 10% 10x BU salts [70 g/L Na2HPO4, 30 g/L NaH2PO4], 80 mg/L X-Gal). Growth at 30°C was monitored for 3 days.

II.1.8. Repression assay

The baits, which are expressed as fusion proteins with the DNA binding domain of LexA, were tested to ensure they enter the yeast nucleus and bind the LexA operators placed upstream of the reporter genes (Fig. I.11). The yeast strain EGY48 was transformed consecutively with the reporter plasmid pJK101 and either the bait plasmid pEG202 or the positive control plasmid pRFHM-1 or the negative control plasmid pEG22. Transformants were selected on Glc ura- his- agar plates. Four individual colonies from each transformation were streaked onto Glc ura- his- X-Gal and Gal/Raf ura- his- X-Gal dishes and the plates were incubated at 30°C and examined after 1 day.

II.1.9. Interaction trap

The yeast strain EGY48 was transformed consecutively with the reporter plasmid pSH18-34, the bait plasmid pEG202 and the cDNA library plasmid pJG4-5. These plasmids carrying specific selectable markers (*URA3*, *HIS3*, and *TRP1* respectively) (Fig. I.9), the yeast transformants were sequentially selected on media lacking the specific corresponding auxotrophic factor(s). Transformants containing the 3 plasmids were grown until colonies were 1-2 mm in diameter. Colonies were harvested, washed twice in TE buffer, and finally resuspended in one pellet volume of sterile glycerol solution (65% v/v glycerol, 0.1 M MgSO4, 25 mM Tris-HCl pH 7.4). 1 mL aliquots of the cell suspension were frozen at -70°C.

Library transformants (1-2 x 10^7 for ASIC1a, 2a and 3, and 7.5 x 10^7 for ASIC4) were then thawed, diluted 10-fold in Gal/Raf ura- his- liquid media, and grown for 4h at 30°C. After washing the cells with water, transformants expressing a protein from the cDNA library that interact with the baits were selected on Gal/Raf ura- his- trp- leu- dishes. After 2 to 4 days incubation, colonies were streaked onto grided and numbered Gal/Raf ura- his- trp- leu- dishes and left to grow for further 2 days. Library transformants expressing a protein that interact with the baits should exhibit galactose-dependent growth on media lacking leucine, as well as galactose-dependent β-galactosidase activity. For this reason colonies were then patched on Glc ura- his- trp-

master plates to turn off the *GAL1* inducible promoter and stop expression of the activation-tagged cDNA proteins, and grown for 1 day at 30°C, before making replicas onto the following 4 plates: Glc ura- his- trp- X-Gal and Gal/Raf ura- his- trp- X-Gal to test for β-galactosidase activity, and Glc ura- his- trp- leu- and Gal/Raf ura- his- trp- leu- to test for *LEU2* expression. The plates were incubated at 30°C and results were examined after 1, 2 and 3 days.

Colonies that grew on media containing galactose and lacking leucine but not on glucose media lacking leucine, and turned blue on galactose X-Gal plates but remained white on glucose X-Gal plates were subjected to yeast miniprep. The crude DNA from these minipreps was subsequently transformed into *E.coli* strain KC8 by electroporation to isolate the library vector pJG4-5 containing the cDNAs identified as positives.

II.1.10. Yeast Miniprep

Yeast colonies from appropriate selection plates were scraped and washed in TE buffer (10 mM Tris-CL pH 7.5, 1 mM EDTA). Cells were spun briefly, resuspended in 0.5 ml S Buffer (10 mM K_2HPO_4 pH 7.2, 10 mM EDTA, 50 mM β-mercaptoethanol, 50 μg/ml lyticase) and incubated at 37°C for 30 min. After addition of 100 μl lysis solution (25 mM Tris-HCl pH 7.5, 25 mM EDTA, 2.5% SDS) the mixture was incubated at 65°C for 30 min. 166 μl 3 M potassium acetate (pH 5.5) was added to the lysate which was then chilled on ice for 10 min. After a 10 min centrifugation, the supernatant was retained and DNA was precipitated by addition of 0.8 ml cold ethanol 10 min at 4°C. DNA was then washed with 70% ethanol.

II.1.11. Electroporation

Electroporation was performed with a Gene Pulser apparatus (BioRad). For each transformation, 1 μl of crude yeast miniprep was mixed to 40 μl of KC8 cells, and placed in an ice-cold 0.1 cm electroporation cuvette (BioRad). An electric pulse was applied at 1.8 kV, 600 Ω resistance and 10 μF capacitance for 5 ms. Immediately after the pulse, 500 μL of SOC medium (2% Bacto-Tryptone, 0.5% Bacto-yeast extract,

0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) was added to the cells, and after 1h incubation at 37°C with shaking, cells were plated on M9 agar plates (1.4% glucose, 90 mM Na2HPO4.H2O, 22 mM KH2PO4, 8 mM NaCl, 20 mM NH4Cl, 12% agar, 1 mM thiamine, 0.74 g/L aminoacid supplement Trp-), which lacked tryptophan so only transformants containing the plasmid pJG4-5 could be recovered.

II.1.12. Bacterial miniprep

Bacterial transformants were grown overnight at 37°C in Luria Broth (LB) supplemented with 100 µg/mL ampicillin. The bacterial pellet was resuspended in 150 µL of Solution I (50 mM glucose, 25 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/mL RNase A). Cells were lysed by addition of 150 µL of Solution II (200 mM NaOH, 1% SDS) 5 min at room temperature. 150 µL of Solution III (3 M potassium acetate, 5 M glacial acetic acid) were then added and chilled on ice for 15 min. The cell debris were pelleted by centrifugation, 450 µL of phenol/chloroform were added to the supernatant and the mixture was vortexed briefly and centrifuged. DNA was precipitated by addition of 1 mL ethanol to the aqueous phase, incubation at -20°C for 30 min followed by a centrifugation at 14000 rpm for 20 min, and then washed with 70% ethanol. The pellet was resuspended in 20 µL of water.

II.1.13. DNA sequencing

0.5 µg DNA to sequence was added to a mixture containing 3 µL 2.5x sequencing buffer (900 mM Tris-HCl, 5 mM $MgCl_2$, pH 9), 1 µL reaction mix (Big Dye DNA Sequencing Kit, ABI Prism), 3.2 pmol of appropriate primer, and water to 10 µL. The following cycling programme was used: 25 cycles of 96°C 35sec/49°C 45sec/60°C 4min.

DNA was precipitated by addition of 90 µL 75% isopropanol, incubation at room temperature for 1h, and centrifugation 20 min at 14000 rpm, and washed with 250 µL 75% isopropanol. Dried DNA samples were dissolved in 10 µL formamide and loaded

onto the automated capillary electrophoresis system ABI Prism® 3100 Genetic Analyser.

II.2. Functional study of ASIC1a-p11 interaction

II.2.1. Cell culture and transient transfection

Mammalian cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (Sigma) and 250 U/mL penicillin/streptomycin (Invitrogen). Transient transfection was performed by lipofection according to the manufacturer's instructions (Invitrogen), using 1 µg DNA and 3 µL lipofectamine 2000 per 35 mm dish, or 5 µg of DNA and 15 µL of Lipofectamine 2000 per 10 cm dishes. Transfected cells were used 24h after transfection.

II.2.2. Flag-tagging of ASICs

Primers were designed to amplify a fragment comprising the first 444 nucleotides of rASIC1a and introduce a BamHI restriction site at the 5' end, and a Flag sequence, NotI and SalI sites at the 3' end of this segment, as follows;

BamHIkozacrASIC1aF: 5'-CGGGATCCACCATGGAATTGAAGACC

ASIC1a_1flagNotISalI-R:

5'AATAGTCGACATTATAGCGGCCGCcCTTATCGTCGTCATCCTTGTAATCCT

TGAAGCTCCGGAAGTTGGC

The same procedure was carried out to amplify ASIC2a first 441 nucleotides, except an EcoRI site was introduced at the 5' end;

EcoRIkozacrASIC2aF: 5'-CGAATTCACCATGGACCTCAAGGAGAGC

ASIC2a_1flagNotISalI-R:
5'GGTAGTCGACATTATAGCGGCCGCcCTTATCGTCGTCATCCTTGTAATCTT
TGTAGTGTTTGAAGTTGGC
Similarly, ASIC3 first 420 nucleotides were amplified:
EcoRIkozacrASIC3F2: 5'- CGAATTCACCATGAAACCTCGCTCCGGACTG
ASIC3_1flagNotISalI-R2:
5'AATAGTCGACATTATAGCGGCCGCaCTTATCGTCGTCATCCTTGTAATCCA
TGAAGCCAGGTGGTGCG
ASIC4 first 810 nucleotides were also amplified:
EcoRIkozacrASIC4F: 5'-CGAATTCACCATGCCGATCGAGATTGTGTG
ASIC4_1flagNotISalI-R:
5'AATAGTCGACATTATAGCGGCCGCaCTTATCGTCGTCATCCTTGTAATCAC
CGAACCCCAGCTGGTGG

These PCR products were subcloned into pRK7 at the BamHI or EcoRI and SalI sites.

Specific primers were then used to introduce by PCR NotI and SalI sites respectively at the 5' and 3' ends of the second region of rASICs;
rASIC1a_2Not1-F: 5'-TATAATGCGGCCGCCCCAAGCCCTTCAACATGCG
rASIC1astopSalIR: 5'-CACGTCGACTTAGCAGGTAAAGTCCTC
rASIC2a_2Not1-F: 5'- TATAATGCGGCCGCCCGAAGCAGTTCAGCATGCTGG
rASIC2astopSalIR: 5'- CACGTCGACTCAGCAGGCAATCTCCTCCAG
ASIC3_2Not1-F2: 5'- TATAATGCGGCCGCCCCAGTCCGACCTTTGACA
rASIC3stopSalIR2: 5'- GCAGTCGACCTAGAGCCTTGTGACGAGGTAACAG
rASIC4_2Not1-F: 5'-TATAATGCGGCCGCGTGTCCCCAGGCTTCCAGAC
rASIC4stopSalIR: 5'-CACGTCGACCTAGCAAGCAAAGTTTTCAAAGAGGC

These segments were subcloned into the constructs described above at the NotI and SalI sites.

II.2.3. GST pull-down assay

II.2.3.1. Production of GST-fusion proteins

The rASIC1a and rASIC3 N-terminal domains were subcloned into pGEX-5X-1 (Amersham Biosciences) at EcoRI/XhoI sites and transformed into *E. coli* BL-21 for expression as GST fusion proteins. The original pGEX-5X-1 vector was used to produce GST alone. For each construct, one bacterial colony was grown in 20 mL LB containing 100 μg/mL ampicillin overnight at 33°C. The bacterial suspension was diluted to OD_{600nm} = 0.2 in 100 mL LB and grown until it reached OD_{600nm} = 0.6. Protein production was induced with 0.5 mM IPTG for 4h at 33°C. Cells were pelleted and resuspended in 10 mL ice-cold binding buffer (PBS containing 1% Triton X-100 and aprotinin, leupeptin, pepstatin A, benzamidine, iodoacetamide and 0.5 uM PMSF), and sonicated 3 times 20 sec. After centrifugation at 4100 rpm for 10 min the supernatant was incubated 30 min at room temperature with 300 μL 50% glutathione-Sepharose 4B resin (Amersham Biosciences), the beads were washed 3 times with 20 mL ice-cold PBS and resuspended in 300 μL binding buffer.

II.2.3.2. Production of His-tagged p11

p11 full length cDNA was subcloned by PCR into pQE30 (Qiagen Qiaexpressionist system, Qiagen) using primers that introduced a BamHI restriction site (underlined) and HA tag at the 5' end of p11 and a PstI site (boldfaced) at its 3' end, as follows:

BamHA: CG<u>GGATCC</u>CCTACCCTTATGATGTG

1a-80PstI: GCA**CTGCAG**CTACTTCTTCTGCTTCAT

The p11 construct was transformed into *E. coli* M15[pREP4] and expressed as a His-tagged protein. Bacteria were grown as described above, but at 37°C. Cells were pelleted and lysed as described above and the crude supernatant was used in the pull-down assay without further purification.

II.2.3.3. GST pull-down assay

GST- and GST-fusion protein-bound glutathione Sepharose beads were incubated with 6His-p11 for 5h at room temperature with mild agitation. The samples were then washed 4 times with 1 mL ice-cold PBS, resuspended in 40 µL Laemmli SDS-sample buffer (2X: 100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT), heated 5 min at 95°C and run on two SDS-polyacrylamide gels (10% and 14%) in Laemmli buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS) at 200V. One of the gels (10%) was stained with Coomassie blue and the other one (14%) was used for Western blotting.

II.2.3.4. Western blotting

Proteins were transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS, 20% methanol) for 1h at 100V. The membrane was blocked with 5% non fat milk in PBS-Tween (0.1%) for 1h at room temperature and then incubated with rabbit anti-6His antibody (Camlab) diluted 1:1000 in blocking buffer overnight at 4°C. The membrane was then washed 3 times with PBS-Tween and the secondary antibody (horseradish peroxidase HRP-conjugated goat anti-rabbit IgG, Jackson Laboratories) was applied at 1:1000 in PBS-Tween for 2h at room temperature. His-tagged p11 was detected by chemiluminescence using the ECL Detection reagent (Amersham Pharmacia Biotech) and BioMax films (Kodak). In order to roughly determine appropriate exposure times (i.e. that would not lead to saturation of the film), several exposure times were used, typically 30 sec, 1 min, 2 min, 5 min, 10 min, 20 min, and 40 min. After visual examination, when subsequent quantification was necessary, only films presenting band intensities that seemed to fall in the linear range of detection were selected. Linescans were performed in order to make sure that the films were not saturated, and bands intensities were assessed using the ImageJ software (http://rsb.info.nih.gov/ij/). A more accurate quantification could have been obtained with the Phosphorimager detection system.

II.2.4. Co-immunoprecipitation of ASIC1a and p11 from DRG

DRGs were extracted from 4 WT and 4 ASIC1 KO mice, homogenised in 1 mL lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, and aprotinin, leupeptin, pepstatin A, benzamidine, iodoacetamide and 250 uM PMSF) and incubated on ice for 1h with mild agitation. After centrifugation at 14000 rpm for 2 min, the supernatant was pre-cleaned by adding 50 µL protein A-Sepharose beads (Amersham Biosciences) and incubating on ice for 1h. This was carried out twice, and after centrifugation at 10000 rpm for 1 min, the pre-cleaned supernatant was retained and kept on ice. Protein A-Sepharose beads were loaded with rabbit anti-chicken-IgY IgG (Sigma) for 1h at room temperature, and washed 3 times with TBS. Subsequently chicken anti-p11 IgY was added to the beads and incubated for 1h at room temperature. After washing 3 times with TBS, the pre-cleaned DRG lysate was mixed with the antibody-loaded beads, and incubated overnight at 4°C. The beads were washed 3 times with lysis buffer and all supernatant was removed. They were resuspended in Laemmli SDS-sample buffer, heated 5 min at 95°C, and run on a 7% SDS-polyacrylamide gel. Western blotting was carried out as described above, using rabbit anti-ASIC1 antibody at 1:200 dilution (Alomone) and HRP-conjugated goat anti-rabbit IgG at 1:1000 dilution (Jackson Laboratories).

II.2.5. Immunocytochemistry

CHO-K1 cells were cultured on 13mm poly-L-lysine-coated coverslips, transfected with 0.2 µg rASIC1aFlag-pRK7 and either 0.8 µg p11-pBS500 or 0.8 µg pBS500 as negative control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24h post-transfection cells were fixed with 4% PFA for 5 min at room temperature, permeabilised if appropriate with 0.1% Triton X-100 in blocking solution (10% goat serum in PBS) for 15 min at room temperature, and incubated for 2h at room temperature with rabbit anti-Flag antibody (Sigma) diluted 1:2000 in blocking solution (+ or - Triton X-100). The cells were washed 3 times with PBS, incubated in goat anti-rabbit-TRITC antibody (Jackson Immunoresearch) at 1:200

dilution for 1h at room temperature, and washed 3 times with PBS before the coverslips were mounted onto slides.

II.2.6. Biotinylation of cell surface proteins

CHO-K1 cells were cultured in 10-cm dishes and transfected with 1.5 μg rASIC1aFlag-pRK7 and either 3.5 μg p11-pBS500 or 3.5 μg pBS500 as negative control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One day after transfection, cells were lysed with lysis buffer containing protease inhibitors (see above), and the total protein concentration in all samples was measured using a BCA Protein Assay (Pierce). Samples were all diluted to the same protein concentration in lysis buffer and equal volumes of all samples were used for subsequent biotinylation. Biotinylation and recovery of plasma membrane proteins were carried out using the Cell Surface Protein Biotinylation and Purification Kit (Pierce), according to the manufacturer's protocol. Samples were analysed by immunoblotting using a rabbit anti-Flag antibody (Sigma) diluted 1:2000. Band intensities were compared using the ImageJ software (see above).

II.2.7. Whole cell voltage clamp experiments

The patch clamp technique was used in the whole-cell configuration to measure ASIC-mediated currents in the presence and absence of p11.

In this technique, a glass pipette with a tip diameter of about 1 μm, the patch clamp microelectrode, is tightly sealed onto the cell membrane; this seal must display a very high resistance (gigaseal) in order to minimise leak currents occurring between the pipette and the cell. Suction is applied to rupture the patch of membrane situated underneath the pipette in order to gain electrical access to the cell's interior. In voltage-clamp recording, the cell is held at a constant voltage and whenever the cell deviates from this holding voltage, for example when an ionic current passes across the plasma membrane, the operational amplifier generates an 'error signal'. The error signal is the difference between the holding potential specified by the experimenter and the actual

voltage of the cell. The feedback circuit of the voltage clamp injects current into the cell as needed to reduce the error signal to zero. This current can be measured, giving an accurate reproduction of the currents flowing across the entire cell membrane. Thus it is not only possible to maintain the membrane potential at a desired voltage, it is also possible to record the amount of current that is necessary to keep it at that level.
Currents corresponding to the activity of ASIC channels in the cell membrane can be recorded during rapid application of acidic solutions near the surface of the cell.

CHO-K1 cells were transfected with equimolar amounts of ASIC1a and p11-GFP, or GFP as a negative control, and used for electrophysiology 24h after incubation. Successfully co-transfected cells were identified using an epi-fluorescence inverted microscope. The H^+-gated ASIC currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Pipettes were pulled from borosilicate glass capillaries with a P-97 puller (Sutter Instruments) and had resistances of 1-3 MΩ. Currents were digitised with a Digidata 1322A data acquisition system (Axon Instruments). Data were recorded and stored using Clampex 8.1 (Axon Instruments). Recordings were performed at room temperature. Off-line analysis and fits were performed using Clampfit 9.0 (Axon Instruments) and SigmaPlot 8 (Systat Software). pH-current density relationships were fitted with a Hill equation of the form: $I(H^+) = I_{max} [H^+]^n (K^n + [H^+]^n)^{-1}$, where I_{max} is the maximal ASIC1a current density at a given holding potential, $[H^+]$ is the concentration of protons, n is the Hill coefficient that estimates the degree of co-operativity in ligand binding and K is the pH value that produces a current density that is 50% of I_{max}. The time constants (τ) of desensitisation of ASIC currents were derived from single exponential fits of the decaying phase of the currents. Values are expressed as means ± S.E.M. The statistical significance of differences between sets of data was tested using Student's t-test. The pipette solution contained (in mM): 126 CsCl, 14 CsF, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 3 Na_2EGTA, 4 MgATP, 10 HEPES (pH 7.3). The bath solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5.10^{-2} capsazepine (pH 7.4). For acidic solutions, HEPES was replaced by MES. CHO-K1 cells do not express an endogenous ASIC current. Nevertheless, capsazepine was added to extracellular solutions because CHO-K1 cells expressed a small endogenous

capsaicin-insensitive slowly desensitising H^+-gated current which was blocked by capsazepine (not shown). Rapid changes in extracellular pH were achieved in 20 ms by a RSC 160 rapid solution changer (BioLogic) commanding a 6-outlet micro-perfusion system.

All electrophysiology recordings and analysis were performed by Dr. F. Rugiero.

II.3. Study of ASIC4 potential novel roles

II.3.1. Co-immunoprecipitation of ASIC1a and 4 from transfected CHO-K1 cells

CHO-K1 cells were transfected with 2.5 µg ASIC1a-pRK7 and 2.5 µg ASIC4Flag-pRK7 in 10-cm dishes (see above). Untransfected cells were used as a control. One day post-transfection cells were lysed with 1 mL lysis buffer containing protease inhibitors. Samples were incubated for 1h on ice with mild agitation. Cellular debris were removed by centrifugation 2 min at 14000 rpm. The supernatant was pre-cleaned by adding 50 µL protein A-Sepharose beads (Amersham Biosciences) and incubating on ice for 1h. After centrifugation at 10000 rpm for 1 min at 4°C, the pre-cleaned supernatant was retained and kept on ice. Protein A-Sepharose beads were loaded with about 2.5 µg mouse anti-Flag IgG (Sigma) for 1h at room temperature, and washed 3 times with TBS. Subsequently about half of the total pre-cleaned cell lysate was mixed with the antibody-loaded beads, and incubated for 3h at RT. The beads were washed 3 times with lysis buffer and all supernatant was removed. They were resuspended in Laemmli SDS-sample buffer, heated 5 min at 95°C, and run on a 7% SDS-polyacrylamide gel. Western blotting was carried out as described above, using rabbit anti-ASIC1 antibody at 1:300 dilution (Alomone) and HRP-conjugated goat anti-rabbit IgG at 1:1000 dilution (Jackson Laboratories).

II.3.2. Downregulation of ASIC1a protein by ASIC4

CHO-K1 cells were transfected with Flag-tagged ASIC1a-, 2a-, 3-pRK7, in conjunction with ASIC4-pRK7 or pRK7 alone, in 35mm dishes. The same total amount of DNA was used in each transfection (i.e., 0.5 μg ASICFlag + 0.5 μg ASIC4 or PRK7). After 1 day, cells were lysed. Samples were diluted to the same total protein concentration, and equal amounts of proteins were loaded on SDS-polyacrylamide gel for analysis by Western blotting using an anti-Flag antibody, as described above. An antibody directed to GAPDH (1:5000) was used on the same membrane as a control for protein loading.

II.3.3. Whole cell voltage clamp experiments

CHO-K1 cells were co-transfected with equimolar amounts of the following constructs:
ASIC1a-pIRES2-DsRed-Express and ASIC4-pIRES2-EGFP (BD Biosciences),
ASIC1a-pIRES2-DsRed-Express and ASIC4delta-pIRES2-EGFP,
ASIC1a-pIRES2-DsRed-Express and pIRES2-EGFP as a negative control,
ASIC3-pIRES2-DsRed-Express and ASIC4-pIRES2-EGFP,
ASIC3-pIRES2-DsRed-Express and pIRES2-EGFP as a negative control.
In all individual transfections the same total amount of DNA was used (i.e., 0.5 μg of each construct). Cells were used for electrophysiology 24h after transfection. Experiments were carried out using the materials and method described above.
All electrophysiology recordings and analysis were performed by Dr. F. Rugiero.

II.3.4. Ubiquitination studies

The sequence corresponding to ASIC4Flag first 21 amino-acids was deleted from the ASIC4Flag sequence by PCR, using the following primers:
EcoRIkozacrASIC4DeltaF: 5'-CGAATTCACCATGGAGGCAGGGGATGAGC
rASIC4stopSalIR: 5'-CACGTCGACCTAGCAAGCAAAGTTTTCAAAGAGGC
The PCR product, referred to as ASIC4deltaFlag, was cloned into pRK7, pIRES2-DsRed-Express and pIRES2-EGFP at EcoRI and SalI sites.

Ubiquitin or HA-tagged ubiquitin were overexpressed with ASIC4Flag-pRK7 or ASIC4deltaFlag-pRK7 in CHO-K1 cells (using equimolar amounts of all constructs). After 24h incubation, cells were lysed. Samples were diluted to the same total protein concentration, and equal amounts of proteins were loaded on SDS-polyacrylamide gel for analysis by Western blotting using a rabbit anti-Flag antibody, as described above. A mouse anti-HA antibody (1:400) and a mouse anti-GAPDH antibody (1:5000) were used on the same membrane as controls for protein ubiquitination and protein loading, respectively.

II.3.5. Proteasome-dependent degradation

CHO-K1 cells were transfected with 1 µg ASIC1aFlag-pRK7 in 8 individual 35mm dishes. 24h post transfection growth media was replaced in all dishes with media containing 50 µg/mL cycloheximide (Sigma); in 3 dishes the media was supplemented with 10 µM lactacystin (Sigma) prepared in DMSO and in other 3 dishes with DMSO alone. The 2 remaining dishes were used as negative control (t_0). After 1h, 3h and 5h incubation at 37°C 5% CO_2, as well as prior to any incubation (t_0), cells from individual dishes were washed and lysed; equal amounts of proteins were loaded on SDS-polyacrylamide gel for analysis by Western blotting, as described above. GAPDH immunoreactivity was used as a control for protein loading.

II.3.6. Neurite outgrowth in differentiated ND-C cells

ND-C cells were grown on coverslips and transfected with 1 µg ASIC4Flag-pRK7 or 1 µg ASIC4deltaFlag-pRK7. 3h after transfection, the transfection media was replaced with differentiation media (DMEM, 0.2% FBS, 1% penicillin/streptomycin, 1 mM dibutyryl cAMP). After 3 days incubation at 37°C 5% CO_2, cells were subjected to immunocytochemistry as described above, using a mouse anti-Flag antibody (Sigma) at 1:2000 dilution, and anti-mouse IgG conjugated to Alexa Fluor 488 at 1:1000.

CHAPTER III

RESULTS AND DISCUSSION

III.1. Identification of proteins interacting with ASICs N-terminal domains in rat dorsal root ganglia by yeast two-hybrid screen

III.1.1. Results

A yeast two-hybrid screen was used to identify proteins interacting with the N-terminal intracellular domains of ASICs. The actual interaction trap was preceded by an activation assay and a repression assay, in order to verify the suitability of the baits in the Y2H screening.

III.1.1.1. Activation assay

Before starting the Y2H screening the ASIC N-terminal domains chosen as baits were tested for any intrinsic transcriptional properties on the reporter genes *LacZ* and *LEU2* in an activation assay (Fig. I.10, table III.1). The plasmid pSH17-4 encoding a functional hybrid transcription factor was used as a positive control. Yeast cells transformed with this plasmid were capable of growing on Gal/Raf ura- his- leu- media and turned blue on Gal/Raf ura- his- X-Gal. The negative control, pRFHM-1 plasmid encoding only the LexA DNA binding domain, did not induce transcription of the reporter genes and thereby did not allow the yeast to grow or turn blue on these selective media. ASIC1b N-terminal domain alone was found to activate the transcription of both *LacZ* and *LEU2*, and for this reason could not be used in the Y2H screen. In the sequence which is not homologous to its splice variant ASIC1a, ASIC1b N-terminus contains an acidic blob containing 8 glutamic acid residues which could be responsible for this property. Therefore this sequence was split in two parts (a and b) and each of them was tested separately in the activation assay. Both segments were still found to activate transcription of the reporter genes on their own and could not be used in the screen. None of the other baits had intrinsic transcriptional activity, and they could be subsequently tested in the repression assay.

	Glc ura- his-	Gal/Raf ura- his- leu-	Gal/Raf ura- his- X-Gal
pSH17-4 (+)	+	+	blue
pRFHM-1 (-)	+	-	white
ASIC1aNT	+	-	white
ASIC1bNT	+	+	blue
ASIC1bNT-a	+	+	blue
ASIC1bNT-b	+	+	blue
ASIC2aNT	+	-	white
ASIC3NT	+	-	white
ASIC4NT	+	-	white

Table III.1: **Activation assay results.** + indicates growth and - no growth on the corresponding media (note: baits are cloned in pEG202 vector; all vectors contain the selectable marker *HIS3*)

III.1.1.2. Repression assay

To ensure that the bait fusion proteins were synthesised efficiently, reached the nucleus of the yeast and bound the sequences upstream of the reporter genes, a repression assay was performed (Fig. I.11, table III.2). The reporter plasmid used in this assay, pJK101, is derived from that used in the activation assay and the interaction trap, pSH18-34, but it bears a LexA operator in the middle of the promoter controlling *LacZ* expression, between the UAS_G and the *GAL1* TATA. In this manner, LexA-bait fusions, when binding to the LexA operator, block *LacZ* transcription, and the yeast colonies remain white. The plasmid pRFHM-1, which encodes the LexA protein, was used as a positive control for repression of the transcription of *LacZ*. Yeast transformed with this plasmid remained white after 1 day incubation at 30°C. The plasmid pEG22, which does not encode for LexA protein and therefore does not block transcription of the *LacZ* reporter gene, was used as a negative control. In the yeast containing this plasmid, transcription of *LacZ* was not repressed, and the colonies turned blue. The fusion proteins containing ASIC2a and 4 N-termini induced the same level of repression of the transcription of *LacZ* as the LexA protein itself, and those containing ASIC1a and 3 N-termini were

slightly less efficient, as a low level of transcription of *LacZ* was detected after 1 day incubation. Overall these 4 baits were found suitable to be used in the yeast two-hybrid screen.

	Gal/Raf ura- his- X-Gal
pRFHM-1 (+)	white
pEG22 (-)	blue
ASIC1aNT	very light blue
ASIC2aNT	white
ASIC3NT	very light blue
ASIC4NT	white

Table III.2: **Repression assay results**

III.1.1.3. Interaction trap

Once the chosen baits were confirmed to be suitable, an interaction trap was carried out. The yeast EGY48 was transformed with all appropriate plasmids (Fig. I.9). Each plasmid carries an auxotrophic selectable marker which ensures that only yeast clones actually transformed with these plasmids can grow on media lacking the corresponding nutrients. The ability to synthesise leucine and β-galactosidase is only conferred to yeast cells in which a protein from the DRG library interacts with the bait, and this property is galactose-dependent. Therefore positive interactors were identified as yeast colonies growing on media lacking leucine and producing a blue pigment on media containing X-Gal, and showing galactose-dependency for these phenotypes.

From 1-7.5 x 10^7 transformants screened, a total of 329 positive clones were selected. Only clones that activated both reporter genes were picked. These 329 cDNAs encoded a total of 86 different proteins. 53 positive clones encoding 22 different proteins were identified as interacting with ASIC1a N-terminus (NT), 156 positive clones encoding 36 different proteins for ASIC2aNT, 110 positive clones encoding 34 different proteins for ASIC3NT, and 10 positive clones coding for 3 different proteins for ASIC4NT

(Table III.3). Some of the selected clones were not found to code for any protein as they were either in the wrong reading frame or sequence from the 3' untranslated region (UTR) (8 for ASIC1a -or 15%, 16 for ASIC2a -or 10%, 23 for ASIC3 -or 21%, and 3 for ASIC4 -or 30%). On average the protein fragments corresponding to the cDNAs cloned comprised around 300 amino acids. Most of the proteins selected are specific for one ASIC subunit. A few ones interact with both ASIC1a and 2a, which show the highest degree of homology within ASICs (with the exception of splice variants) (Table III.3) and overlapping expression pattern in the nervous system (Bassilana *et al,* 1997).

Given the high number of interactors obtained, a simple classification of the clones was established according to their function or the type of regulatory pathway in which they are involved (Table III.3). Some proteins found to interact with ASICs are involved in trafficking of ion channels, G protein pathways, endocytosis, ubiquitination, cell adhesion and motility; some are associated with the cytoskeleton, others with the extracellular matrix; some are mitochondrial proteins, transcription factors, or enzymes. Finally a few clones are proteins of unknown function, or even novel proteins which have not been described in any database.

In order to validate results from a Y2H screen, it is necessary to confirm the interaction between the bait and the prey, to make sure both proteins are expressed in the same type of cells/organelles, and finally determine the functional significance of the interaction. For obvious timing reasons we were not able to carry out these experiments for all interactors identified. Several clones were selected for further investigation: 1) p11, a protein involved in ion channel trafficking which was found to interact with ASIC1aNT, and 2) ubiquitin which was identified as binding to ASIC4NT. A detailed study of the role of p11 on ASIC1a plasma membrane expression (Donier *et al*, 2005) is presented in section III.2, followed by a report on preliminary experiments suggesting novel roles for ASIC4 in section III.3.

Table III.3: **Clones interacting with ASIC N-termini in the yeast two-hybrid assay**

Clone name	Protein, accession number	Cloned/ Full length (aa)	ASIC 1a	ASIC 2a	ASIC 3	ASIC 4	References
G protein pathways							
1a-22, 1a-44, 1a-58, 1a-94, 1a-138, 1a-152, 2a-3, 2a-6, 2a-13, 2a-16, 2a-25, 2a-70, 2a-74, 2a-75, 2a-82, 2a-84, 2a-86, 2a-98, 2a-101, 2a-117, 2a-133, 2a-136, 2a-140, 2a-141, 2a-178	GIPC (RGS-GAIP interacting protein C terminus, regulates vesicular trafficking and clathrin-mediated endocytosis), BC070505	333/333	6	19			de Vries *et al*, 1998
1a-121	RACK-1 (receptor for activated C kinase, guanine nucleotide binding protein, beta polypeptide 2-like 1, involved in functional modulation of $GABA_A$ by GPCRs), NM_130734	257/317	1				Brandon *et al*, 2002
2a-10	Heterotrimeric G protein β 2 (involved in GPCR signaling), NM_031037	340/340		1			
3-58	Heterotrimeric G protein β 1 (involved in GPCR signaling), NM_030987	329/340			1		
3-48	RAB GDP dissociation inhibitor alpha (controls membrane/cytosolic partitioning of Rab GTPases), NM_017088	300/447			1		Schimmoller *et al*, 1998
3-121	JLP (JNK-interacting leucine-zipper protein, a scaffolding protein that binds to $G_{\alpha 13}$ and components of the JNK/p38MAPK signaling complex), AF327451	140/1307			1		Kashef *et al*, 2005
Clathrin-coated vesicles / endocytosis							
1a-20, 1a-69	Clathrin, light chain (component of coat in coated pits), NM_031974	248/248	2				
1a-100	Adaptor protein complex 2, μ2 subunit (recruits receptors for clathrin-mediated endocytosis), NM_053837	324/435	1				Ohno *et al*, 1995
3-5	Vacuolar ATPase, 14 kD subunit (associated with the catalytic sector of clathrin-coated vesicle H^+-ATPase, ATP-	119/119			1		Nelson, 2003

	dependent proton pump), NM_053884						
Ubiquitination / proteasome							
4-2, 4-3, 4-8, 4-12, 4-37	Polyubiquitin, NM_138895					5	
1a-113	Proteasome 26S subunit, ATPase subunit (ATP-dependent degradation of ubiquitinated proteins), BC062019	179/442	1				
2a-155	Proteasome subunit alpha type 1, NM_017278	142/263		1			
1a-151	Ubiquitin and ribosomal protein S27a, NM_031113	118/156	1				
1a-1, 1a-10, 1a-103, 1a-109	Ubiquitin conjugating enzyme UBC9 (SUMO E2-conjugating enzyme), NM_0130050	158/158	4				Mo and Moschos, 2005
Trafficking							
1a-54, 1a-55, 1a-56, 1a-59, 1a-80, 1a-105, 1a-108, 1a-129, 1a-132, 1a-164, 1a-167, 1a-192, 1a-202, 1a-207	p11 (S100 Ca^{2+} binding protein A10, promotes ion channels membrane expression), NM_031114	95/95	14				Okuse *et al*, 2002
2a-14	Contactin 1 (enhances the density of voltage-gated sodium channels at the plasma membrane), NM_057118	416/1021		1			Shah *et al*, 2004
2a-123	DnaJ-like protein (Hsj2, or hsp40, assists hsp70 proteins in protein folding and targeting), NM_022934	232/397		1			Hartl, 1996
Cytoskeletal / cytoskeleton-associated proteins							
3-2, 3-10	β actin (cytoskeleton), NM_031144	231/375			2		
2a-53	Microtubule-associated protein 1 A MAP1A (regulates microtubule shape and dynamics and mediates interactions between actin microfilaments and microtubules), NM_030995	649/2774		1			Nunez and Fischer, 1997
2a-8, 2a-73, 2a-119, 2a-126, 2a-158, 2a-182, 2a-189, 2a-195	OSM (osmosensing scaffold for MEKK3, actin-binding scaffold protein required for p38 activation in response to sorbitol-induced hyperosmolarity, ortholog of human malcavernin, or CCM2, involved in cerebral cavernous malformations), AY442689	354/453		8			Uhlik *et al*, 2003 Zawistowski *et al*, 2005
2a-1, 2a-26, 2a-27, 2a-38, 2a-40, 2a-42, 2a-68, 2a-77, 2a-79, 2a-83, 2a-94, 2a-100, 2a-124, 2a-135, 2a-137, 2a-138, 2a-139, 2a-144, 2a-149, 2a-150, 2a-153, 2a-160, 2a-164,	CLP-36 (PDZ and LIM domain 1, localises to actin stress fibres in association with alpha-actinin, recruits kinase Click1 to actin stress fibres), NM_017365	327/327		29	2		Wang *et al*, 1995; Vallenius and Makela, 2002

2a-168, 2a-173, 2a-186, 2a-191, 2a-192, 2a-197, 2a-199, 3-151, 3-155							
1a-149, 2a-78	TCTP (translationally-controlled tumor protein 1, microtubule-stabilizing Ca^{2+}-binding protein), NM_053867	137/172	1	1			Bommer and Thiele, 2004
2a-193	Kelch-like 7 (Drosophila) (actin-binding protein of unknown function), XM_575329	283/790		1			
2a-60, 2a-67, 2a-127	Dynein, cytoplasmic, light chain 2A, NM_131910	96/96		3			Ye *et al*, 2000
1a-19	Tctex-1(light chain of cytoplasmic dynein, microtubule-associated motor complex, involved in actin dynamics during neurite outgrowth), NM_031318	113/113	1				Chuang *et al*, 2005
4-14	Moesin (Msn, actin-binding protein involved in linking cytoskeleton/membrane, cell growth, migration and differentiation), NM_030863					1	Bretscher *et al*, 2002
Cell/cell contact, cell motility							
1a-2, 1a-6	Tspan-6, transmembrane 4 (tetraspanin) superfamily, member 6 (tetraspanins are membrane-associated proteins which regulate cell adhesion, migration), XM_217563	242/245	2				Todd *et al*, 1998
2a-59, 4-46	PAPIN (plakophilin-related armadillo repeat protein-interacting) or PDZD2 (binds delta catenin, localised at cell-cell junction; secreted after proteolytic cleavage, unknown function), NM_022940	210/2767		1		1	Deguchi *et al*, 2000; Yeung *et al*, 2003
2a-44, 2a-143	Ilkap (integrin-linked kinase-associated serine/threonine phosphatase 2C, modulates cell adhesion), NM_022606	225/392		2			Kumar *et al*, 2004
1a-107	Zyxin (focal adhesion protein found to play a role in the regulation of the actin cytoskeleton and in cell motility), XM_216124	179/667	1				Yoshigi *et al*, 2005
2a-194	Zonula occludens 2 protein (ZO-2) (MAGUK scaffold protein of the tight junction, links proteins of the tight junction to the actin cytoskeleton), U75916	624/1164		1			Gonzalez-Mariscal *et al*, 2000
Basement membranes / extracellular matrix proteins							
3-7, 3-17	Nidogen 2 protein (laminin-binding basement membrane protein), XM_573694	187/1585			2		Bader *et al*, 2005

3-104, 3-112, 3-124, 3-158	Matrilin 2 (a filament-forming protein widely distributed in extracellular matrices), XM_216941	398/1250			4		Deak, *et al*, 1997
3-85, 3-99	Procollagen, type VI, alpha 1, XM_215375	745/1223			2		
3-117	Procollagen, type IV, alpha 2 (important for basement membrane stability), XM_225043	159/1906			1		Poschl *et al*, 2004
2a-31, 2a-107, 2a-122, 2a-134	Bone morphogenetic protein 1 (Bmp1, metalloproteinase that converts procollagens into the major fibrous components of mammalian extracellular matrix). Different N term, XM_573814	284/990		4			Gopalakrishnan *et al*, 2004
3-138, 3-147	Fibulin 5 (RGD motif-containing scaffold for elastin fibre assembly, involved in endothelial cell adhesion through integrin binding, regulates cell growth and motility), NM_019153	448/448			2		Schiemann *et al*, 2002; Lee *et al*, 2004
Nervous system-specific proteins							
3-28	Neuroblastoma, suppression of tumorigenicity 1 Nbl1, or Dan (involved in inflammatory pain), NM_031609	178/178			1		Ohtori *et al*, 2004
2a-159	Latexin (+20 different aa) (carboxypeptidase A inhibitor, expressed in small diameter DRG neurons + marker of a subset of glutamatergic neurons in lateral neocortex), NM_031655	166/223		1			Takiguchi-Hayashi *et al*, 1998; Arimatsu *et al*, 1999
1a-26	Amyloid beta (A4) precursor protein (APP, involved in neurite growth in hippocampal neurons, and in memory formation; its processing leads to Abeta accumulation in Alzheimer's disease), BC062082	2201 /2201	1				Salinero *et al*, 2000; Mileusnic *et al*, 2000
2a-37, 2a-112	Calsyntenin 1 (a postsynaptic membrane protein containing a Ca^{2+}-binding domain), XM_243040	887/981		2			Vogt *et al*, 2001
1a-75	Periaxin (role in the stabilization of myelin), NM_023976	597/1384	1				Gillespie *et al*, 1994
2a-49, 2a-51, 2a-55, 2a-57, 2a-61, 2a-62, 2a-81, 2a-93, 2a-105, 2a-113, 2a-167, 2a-172, 2a-177, 2a-190	Microtubule and syntrophin associated serine/threonine kinase (Sast170, brain-specific syntrophin-binding protein), NM_181089	784/1570		14			Lumeng *et al*, 1999
Mitochondrial proteins							
3-35, 3-40, 3-41, 3-46, 3-53,	Mitochondrial processing peptidase beta (cleaves off	449/489			27		Gakh *et al*,

3-55, 3-60, 3-69, 3-70, 3-71, 3-77, 3-81, 3-82, 3-92, 3-96, 3-98, 3-102, 3-118, 3-122, 3-125, 3-128, 3-133, 3-139, 3-143, 3-146, 3-149, 3-161	targeting sequence of nuclear-encoded mitochondrial proteins imported from the cytoplasm), NM_022395						2002
2a-180, 3-176	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, XM_215044	156/156		1	1		
2a-66, 2a-163, 2a-183	ATP synthase, H^+ transporting, mitochondrial F1 complex, delta subunit, NM_139106	116/168		3			
2a-36	Mitochondrial ribosomal protein L53, XM_342712	119/119		1			
Transcription factors / nuclear proteins							
3-25, 3-30, 3-73	Islet-2 (insulin related protein 2 , LIM-homeodomain-type transcription factor, regulates neuronal differentiation), NM_020471	360/360			3		Tsuchida *et al*, 1994
1a-4	Nuclear localized protein 1, U94988	625/625	1				
3-136	Small nuclear ribonucleoprotein E (snRNP-E, component of the mRNA splicing apparatus), XM_341120	92/92			1		
Other proteins							
3-29	Eukaryotic translation elongation factor 2 (Eef2) (involved in regulation of peptide elongation; NMDAR activation was shown to induce its phosphorylation), NM_017245	148/858			1		Scheetz *et al*, 2000
3-68	Prosaposin (sulfated glycoprotein 1, precursor of saposins involved in degradation of sphingolipids, role in organisation of membrane phospholipid bilayer), NM_013013	180/554			1		Fu *et al*, 1994; Vaccaro *et al*, 1999
3-74	MORG1 (mitogen-activated protein kinase organizer 1, scaffold protein of the extracellular signal-regulated kinase (ERK) pathway), AY365434	161/315			1		Vomastek *et al*, 2004
1a-21	Ribosomal protein L31 (Rpl31) (homologous to 60S), NM_022506	125/125	1				
3-160	Meiotic recombination protein REC14, BC098059	305/305			1		
2a-30	p32-subunit of replication protein A (Rpa2) (Transcriptional gene silencing protein), NM_021582	182/270		1			Elmayan *et al*, 2005

Enzymes							
2a-5, 2a-9, 2a-22, 2a-23, 2a-28	Peroxiredoxin 1 (Prdx1, peroxidase), NM_057114	108/199		5			
1a-62	Similar to UMP-CMP kinase, BC098727	166/227	1				
2a-99, 2a-121, 2a-151	Guanylate kinase 1, NM_001013115	188/198		3			
3-180	aarF domain containing kinase 5, NM_172960	460/460			1		
3-36	Protein phosphatase type 1A (formely 2C), Mg-dependent, alpha isoform (Ppm1a), NM_017038	152/382			1		
3-15	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform, NM_057140	486/589			1		
3-64	Enolase 1, alpha (Eno1), NM_012554	423/434			1		
2a-87, 2a-131	Glucosidase, alpha (lysosomal enzyme that degrades glycogen, involved in Pompe disease, glycogen storage disease type II), NM_199118	195/953		2			
3-130	Sulfite oxidase (Suox), NM_031127	303/546			1		
3-43	S-adenosylhomocysteine hydrolase-like 1 (Ahcyl1), XM_342312	148/636			1		
2a-50, 2a-52, 2a-65, 2a-181	Methionine adenosyltransferase II, alpha (Mat2a), NM_134351	288/395		4			
2a-102, 2a-114, 2a-175	Hypoxanthine guanine phosphoribosyl transferase, NM_012583	218/218		3			
2a-161	Methyltransferase like 5, transcript variant 2 (Mettl5), XM_892172	136/209		1			
2a-33, 2a-35, 2a-128, 2a-166, 2a-169, 2a-174, 2a-185, 3-141	Dehydrogenase/reductase (SDR family) member 4, BC070961	259/279		7	1		
3-110, 3-127	Glutaminyl-tRNA synthetase, BC082002	775/775			2		
2a-198	RNA polymerase 1-3, NM_009087	133/133		1			
2a-132, 3-86, 3-119	Dipeptidylpeptidase III (Dpp3), NM_053748	525/738		1	2		
2a-64, 2a-72, 2a-85	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble (Xpnpep1), NM_131913	129/623		3			
Proteins of unknown function							
3-3, 3-19, 3-27, 3-32, 3-61, 3-89, 3-91, 3-109, 3-111, 3-116, 3-131, 3-134	LOC288451 (-then removed from database, located 465bp in 3'UTR downstream of ubiquitin-like 3 ORF),	123/123			12		

	XM_237860						
3-16, 3-18, 3-90, 3-97	BTB/POZ domain containing protein 3, XM_230616	273/676			4		
3-114	Tetratricopeptide repeat protein 19, XM_577102	388/433			1		
1a-178, 1a-182	No match		2				
3-93	Hypothetical protein FLJ14800, XM_235711	383/519			1		
1a-74, 1a-145	Similar to hypothetical protein MGC2744, NM_001034109	401/412	2				
1a-205, 2a-21, 2a-89, 2a-111, 2a-118, 2a-129, 2a-171	Similar to hypothetical protein MGC18257, XM_146970	250/271	1	6			
3-170	COMM domain containing 9 (may be functionally similar to MURR domain proteins, which regulate transcription factors), BC101873	197/198			1		Burstein *et al*, 2005
2a-157	DEP domain containing 6 (+23aa after aa39) (DEP (disheveled, egl-10, pleckstrin) domains are present in RGS proteins, unknown function), XM_235306	170/283		1			Ponting and Bork, 1996
1a-90, 2a-120, 2a-188	Zinc finger protein 100 (Zfp100), NM_001012105	277/801	1	2			
2a-18, 2a-130	Zinc finger protein 219, BC082017	470/726		2			
1a-24	Zinc finger protein 623, NM_030199	207/499	1				
Unidentified cDNAs							
2a-58	16 days neonate cerebellum cDNA, RIKEN full-length enriched library, clone:9630025M18, AK035997			1			
3-1	BAC CH230-261E4, AC108321				1		
2a-32	BAC clone RP23-180G16 from chromosome 5, AC127339			1			
1a-5, 1a-11, 1a-13, 1a-17, 1a-23, 1a-119, 2a-2, 2a-4, 2a-7, 2a-12, 2a-15, 2a-17, 2a-20, 2a-24, 2a-39, 2a-46, 2a-54, 2a-95, 2a-115, 2a-170, 2a-195, 2a-200, 3-14, 3-20, 3-24, 3-62, 3-78, 3-83, 3-135, 3-153	Not in frame		6	16	8	3	
1a-25, 1a-158, 3-4, 3-12, 3-21, 3-31, 3-39, 3-44, 3-76, 3-84, 3-87, 3-100, 3-101, 3-105, 3-107, 3-123, 3-157	3'UTR		2		15		

III.1.2. Discussion

III.1.2.1. The yeast two-hybrid screen

A yeast two-hybrid screen was performed to identify molecular partners for ASICs. Indeed, although many studies give evidence about various functions they may be involved in, it is generally admitted that ASICs, which are widely expressed in the nervous system, may have yet undiscovered ligands and roles. Discovering proteins they interact with could give insight into their regulation and putative novel functions. Several groups already identified ASIC-interacting proteins, mostly in yeast two-hybrid screens using the ASIC C-terminal domains as baits (see section I.2.4.2.4). Here we selected proteins interacting with ASIC N-termini.

The sensitivity and specificity of a yeast two-hybrid system are described by the weakest interaction between a bait and a prey that can be detected, and whether the transcription of the reporter genes is only and specifically activated when this interaction occurs. These characteristics depend on a number of factors, which have to be set according to the level required. The assay described by Fields and Song (1989) used transcription factor GAL4, an endogenous yeast protein with a strong transcriptional activity; this method was likely to give high sensitivity, but with a high background (low specificity). In order to minimise the selection of false positives, we chose a less sensitive system comprising the DNA-binding domain of bacterial repressor LexA and the bacterial activation domain B42 which has weaker transcriptional activity than GAL4. These bacterial proteins also do not interact with or activate transcription of endogenous yeast sequences. The sensitivity of the Y2H assay also depends on the reporter genes chosen and the number of operators they bear in their promoter region. We used an auxotrophic reporter, *LEU2*, and the colorimetric marker *LacZ* encoding β-galactosidase. In the yeast EGY48 genome *LEU2* has its normal upstream regulatory sequences replaced with 3 high affinity LexA operators, each of which can bind 2 LexA dimers, and in the reporter plasmid pSH18-34 4 LexA operators are placed upstream of *LacZ*. The advantage of using the LexA system is that endogenous yeast transcription factors cannot activate the reporter genes expression,

hence higher specificity. Other *LacZ* reporter plasmids, such as pJK103 or pRB1840, are less sensitive than pSH18-34 as they carry only 2 and 1 LexA operators, respectively. Therefore in our Y2H assay, we used more sensitive reporter genes driven by weaker transcription factors, these being of bacterial origin. In this manner we should obtain specific transcription of the reporter genes, low background, with reasonably high sensitivity.

Using the yeast two-hybrid approach, we identified 86 different proteins, from the 1-7.5 x 10^7 initial clones screened, as interactors for ASIC1a, 2a, 3 and/or 4 from rat dorsal root ganglia cDNA library. ASIC1b N-terminus could not be used in the assay as it displayed intrinsic transcriptional activity. Around 10-20% of the selected positive clones were not in the correct reading frame and did not encode a protein. Statistically it can be assumed that two-thirds of the cDNAs in the library are in a wrong reading frame, but still this proportion of wrong ORF clones is relatively high. It could be explained by the fact that in order to avoid missing positive clones we decided to select not only fast-growing clones that appeared after 1-2 days incubation but also some clones that displayed a slower growth rate, because these could prove to be positive interactors with a low affinity for the bait. In the Y2H system, yeast transformants which do not produce a protein interacting with the bait should not be able to grow on selective media, but actually we observed that after more than 3 days incubation at 30°C, some negative clones start growing, possibly due to a leak transcription of the reporter genes or by feeding off nutrients from lysed cells.
Among the 329 clones that encoded proteins, 117 were full length cDNAs and the average length of the inserts was well over 1kb, which confirms the good quality of the DRG cDNA library. Most of the clones were selected specifically as interacting with one ASIC subunit. Specific expression patterns and current properties, as well as many functional studies imply ASIC subunits do not all serve the same role, therefore it is understandable they may not be in contact with the same molecules in the cell. It is nevertheless striking to observe that sequences with such a high degree of identity specifically interact with one or another protein. This is a good indicator of the specificity of our assay. Some of the clones are novel proteins which have not been

described in any database and characterising them would be of interest. Although some ASIC subunits, such as ASIC1a and 2a have been shown to interact by co-immunoprecipitation (Bassilana *et al*, 1997), no ASIC subunit was identified in our Y2H assay, suggesting that in heteromeric channels the interaction between subunits may not be mediated by their N-termini. No report suggests such interactions occurring via C-terminal domains either. ASICs may interact via other parts of the channel, or may require an adaptor protein to heteromultimerise.

The assortment of proteins found to be associated with ASICs is very varied, ranging from proteins involved in ion channel trafficking, clathrin-mediated endocytosis or ubiquitination, to G proteins or proteins of the cytoskeleton, and many others. Experiments were performed to study the role of annexin II light chain p11 on ASIC1a, as well as ubiquitin, which interacts with ASIC4 and points towards novel functions for this subunit (see sections III.2-3, Donier *et al*, 2005). However, the roles of the remainder of the proteins selected in the Y2H screen are yet to be determined and the potential implications on ASIC functions can only be speculated. The most relevant categories of interactors are discussed below; we mainly concentrate on proteins involved in endocytosis, G protein pathways, and cell adhesion, and discuss the potential false positives selected. Overall these proteins suggest a range of novel functions and regulatory mechanisms for ASICs.

III.1.2.2. Clathrin-coated vesicle (CCV)-mediated endocytosis

Clathrin-mediated endocytosis (CME) is one of the most important routes of receptor and channel internalisation in eukaryotic cells. It leads to receptor downregulation -for example it is essential for synaptic vesicles recycling in neurons, but is also involved in signaling and nutrient uptake. The rate of plasma membrane uptake through CME was estimated to be 1%–5% per minute (Bretscher, 1984; Steinman *et al*, 1983). During CME, specific transmembrane receptors and their bound ligands concentrate into coated pits, formed by the assembly of cytosolic coat proteins such as clathrin. The adaptor protein complex 2 (AP-2) is responsible for binding a tyrosine-based internalisation motif on the receptor as well as recruiting clathrin. Geometric changes

driven by protein/lipid interaction (Farsad and De Camilli, 2003) lead to membrane invagination and ultimately fission of the coated pits to form endocytic vesicles. The large GTPase dynamin in combination with accessory proteins is thought to self-assemble into a 'collar' at the neck of the invaginated coated pits and to help them detach from the plasma membrane (reviewed in Hinshaw, 2000). The clathrin coat is then removed and the vesicles fuse with early endosomes. The contents of these vesicles can be recycled to the plasma membrane via late endosomes or be degraded in the lysosomes, bearing in mind that they can continue signaling along the endocytic pathway.

ASIC-related β and γ ENaCs contain the YXXL endocytic consensus sequence in their C-terminal domain and were shown to undergo CME (Shimkets *et al*, 1997). ASIC1a and 2a, but not any other ASIC subunit, bear this YXXL motif in their N-terminal domain. Several proteins involved in CCV-mediated endocytosis were found in our Y2H screen to bind to these ASIC subunits; in particular clathrin light chain, as well as the medium (μ) chain of the AP-2 complex which is responsible for recruiting receptors for CME were both found to interact with ASIC1a. Dynein light chain (Tctex-1), which also binds to ASIC1a is part of the cytoplasmic microtubule-associated motor complex which transports vesicles and molecules along microtubules. Tctex-1 has been shown to bind to a number of membrane receptors and channels, such as rhodopsin, NGF receptor TrkA, voltage-gated sodium channel $Na_v1.8$ (Tai *et al*, 1999; Malik-Hall *et al*, 2003) and through this interaction often to participate in sorting the proteins via microtubule-based vesicular transport systems.

This strongly suggests a possible regulation of ASIC1a- and/or 2a-containing channels by endocytosis, which has not been reported yet in the literature. It would be interesting to determine whether ASICs internalisation, if it does occur, is constitutive or whether it is regulated by pH or other ligands.

In section III.3 we give evidence that ASIC1a may undergo ubiquitination. It is worth mentioning that ubiquitination can also be a targeting signal for CCV-mediated endocytosis, for degradation by the endosomal/lysosomal pathway. Therefore these two mechanisms could be parallel or complementary options for channel regulation, in a similar fashion to ENaCs (Schild *et al,* 1996; Staub *et al*, 2000).

Finally, it is possible to imagine that endocytosis of ASICs could not only lead to degradation or recycling of the channel to the plasma membrane, but that they could actually have a function in these vesicles. Indeed vesicles along the endocytic pathway have an acidic internal pH, and ASICs might play a role in their pH regulation, in conjunction with other pumps and ion channels. A detailed analysis of ASICs subcellular localisation is required, for example by co-locolisation studies with various vesicle markers, or immunoblotting of different cellular fractions.

III.1.2.3. Guanine nucleotide binding proteins

Heterotrimeric G protein β1 and β2 subunits were isolated as potential interactors for ASIC3 and ASIC2a respectively in our Y2H assay. Heterotrimeric G proteins have been extensively studied; they consist of three subunits, α, β and γ, and are central components of signal transduction pathways associated with G-protein-coupled receptors (GPCRs). In the inactive state, the α subunit binds to GDP and the three subunits are attached together. Upon activation GTP replaces GDP on the α subunit and the affinity of this subunit for the βγ subunits is decreased, resulting in their dissociation. Regulators of G protein signaling (RGS) act as GTPase-activating proteins (GAPs), i.e. they regulate the rate of GTP hydrolysis via interactions with G_α subunits. The separated α and/or βγ subunits can then interact with their effectors. Effectors of the α subunit, such as adenylate cyclase or phospholipase C and the associated downstream signaling events are very well characterised (Hamm, 1998), but the βγ subunits have also been shown to have effectors in their own right. They were first shown to directly activate inwardly rectifying muscarinic K^+ channel GIRK1 in the heart (Logothetis *et al*, 1987). Since then, voltage-gated Ca^{2+} channels, phospholipase A2 and C, adenylate cyclase, and many other proteins have been described to be regulated positively or negatively by βγ subunits (reviewed in Clapham and Neer, 1997). Therefore it would be worth investigating whether βγ subunits regulate ASIC-mediated currents, which would imply, if any effect was observed, that ASIC function

could be controlled by GPCRs and would give exciting new perspectives about their function.

Among the additional G protein-related proteins found in the Y2H screen, RACK-1 (receptor for activated C kinase 1), a WD repeat-containing receptor and anchoring protein for activated PKC β, homologue of G protein β subunit, was identified as a partner for ASIC1a. RACK-1 was previously shown to interact with $GABA_A$ receptor β subunit and enhance PKC-dependent phosphorylation of the receptor (Brandon *et al*, 2002). Moreover blocking this interaction led to inhibition of the functional modulation of $GABA_A$ by muscarinic M1 receptor. Feng *et al* (2001) also reported that blocking the interaction between RACK-1 and PKC abolishes modulation of $GABA_A$ receptors by serotonergic receptors, implying that RACK-1 may be the link that brings PKC close to the ion channel after activation by a GPCR. Baron *et al* (2002b) demonstrated that ASIC2a was significantly upregulated by PKC phosphorylation, in a PICK-1-dependant fashion. This phosphorylation occurs on a consensus site located just upstream of the first TM domain. This motif is conserved in ASIC1a sequence, therefore it is reasonable to think ASIC1a may also undergo PKC phosphorylation, and RACK-1 may be implicated in this process. Following the $GABA_A$ receptor model, it is appealing to imagine that ASICs could be regulated by GPCRs via an interaction with heteromeric G protein β subunit (see above) in a process involving RACK-1 and PKC-dependant phosphorylation. ASICs are assembled as heteromultimers and therefore different subunits could have different functions by mediating interactions with different proteins.

We also detected a strong interaction between ASIC subunits 1a and 2a and PDZ domain-containing protein GIPC (RGS-GAIP-interacting protein, C terminus). GIPC was first described as interacting with the regulator of G protein signaling RGS19/GAIP (De Vries *et al,* 1998) but was later shown to bind to a variety of other membrane proteins such as tyrosine kinase receptors TrkA and TrkB (Lou *et al,* 2001), β1 adrenergic receptor (Hu *et al,* 2003), or dopamine D2 and D3 receptors (Jeanneteau *et al*, 2004). The RGS19-GIPC complex was implicated in the regulation of vesicular

trafficking and clathrin-mediated endocytosis, and may be important in the regulation of GPCRs internalisation (de Vries *et al*, 1998). It is also thought to act as a scaffold that links GPCRs to other signaling molecules. For example, Hu *et al* (2003) showed that GIPC is able to decrease β1 adrenergic receptor-mediated ERK (extracellular signal regulated kinase) activation through a specific interaction with this subunit. Therefore the interaction between ASIC1a and 2a and GIPC may provide another interesting link between the ion channel and aspects of G protein-mediated signaling, and possibly GPCR trafficking via clathrin-mediated endocytosis.

Finally, ASIC3 N-terminal domain binds to the small -or monomeric G protein Rab GDP dissociation inhibitor alpha (Rab GDI). Small GTP-binding proteins of the Rab family are involved in the regulation of membrane trafficking along the endocytic and exocytic pathways (Schimmoller *et al*, 1998). As their heterotrimeric counterparts they bind GDP and GTP, resulting in inactive or active forms, respectively. Rab GDI controls the membrane versus cytosolic portioning of Rab GTPases, which is crucial to their function as regulators of vesicular trafficking.

In summary, in a yeast-based assay ASIC1a, 2a and 3 bind to several proteins involved in G protein pathways and endocytocis control. This suggests that ASIC heteromultimers could be regulated by GPCRs, and/or by endocytosis, although again this is speculation. These ASIC subunits are all present in DRG, but only ASIC1a and 2a are found in the central nervous system, implying that these mechanisms of regulation could differ in different types of neurons. In the CNS, ASICs are reported to have a post-synaptic localisation, whereas clathrin-mediated endocytosis is generally described as a pre-synaptic mechanism implicated in recycling of synaptic vesicles. Post-synaptic ASICs are thought to modulate synaptic communication, by a direct activation by protons released in conjunction with neurotransmitters (although this has not been clearly demonstrated yet). They have been shown to play a role in long-term potentiation. A putative activation of ASICs by GPCRs such as mGluRs may provide another means of amplifying EPSCs. In the PNS, ASICs have been identified at nerve

terminals, where endocytosis of ASICs -and possibly of associated GPCRs could decrease neuronal excitability in response to a yet unidentified ligand.

III.1.2.4. Proteins associated with the cytoskeleton and/or cell adhesion sites

Many cytoskeletal and cytoskeleton-associated proteins, as well as proteins regulating cell adhesion, bind to ASICs (mainly ASIC1a, 2a and 4) in our Y2H assay: e.g. β-actin itself, focal adhesion protein zyxin which promotes actin filament assembly (Fradelizi *et al*, 2001), moesin which serves as a link between actin microfilaments and the plasma membrane (Bretscher *et al*, 2002), a member of the tetraspanin family of proteins which regulate cell adhesion, Ilkap, a phosphatase associated with integrins, or Tctex-1, the dynein light chain which is also found independently of dynein and in that case plays a role in actin remodeling during neurite outgrowth (Chuang *et al*, 2005).

Proteins of the cytosketeton, and proteins modulating them, carry various essential functions in the cell. They control cell shape, they are involved in cell movements, both 'external' (cell migration, muscle contraction) and 'internal' (movement of organelles or chromosomes), and regulate membrane trafficking (reviewed in Suetsugu *et al*, 2003; Etienne-Manneville, 2004). The cytoskeleton was shown in particular to regulate in different ways the function of many ion channels, directly or through accessory proteins acting as cross-linkers. For example cytochalasin D, which prevents polymerisation of actin monomers, has been shown to activate the Na^+ channel ENaC (Ismailov *et al,* 1991), and to inhibit large conductance calcium-activated K^+ channels from rat hippocampal neurons (Huang *et al*, 2002). Mechanically activated channels MEC-4 and MEC-10 involved in touch sensation in *C. elegans* are thought to rely on cytoskeletal proteins for activation, via interactions with MEC-2 (Goodman *et al*, 2002).

Aside from sensory neurons transducing touch, organs and tissues also respond to mechanical forces, in part through cell-cell or cell-extracellular matrix (ECM) contacts, which are crucial for the development and function of many tissues (Ingber, 2005). In the nervous system, mechanical tension was shown to induce axonal growth in chick sensory and forebrain neurons (Zheng *et al*, 1991; Chada *et al*, 1997). The molecular

mechanisms underlying such cellular responses to mechanical stress have not been elucidated, but two types of molecules are thought to activate signaling cascades which lead to remodelling of the cytoskeleton and thereby modification in cell movements: stretch-activated ion channels (Calabrese *et al*, 1999) and components of cell adhesion sites which link the cytoskeleton to proteins of the ECM, e.g. integrins (Katsumi *et al*, 2005), or zyxin (Yoshigi *et al*, 2005). Several ion channels which have not been shown to be directly mechanically gated have been however implicated in this process, although no molecular mechanisms to explain it were proposed. Davis *et al* (2004) demonstrated that β subunits of voltage-gated sodium channels affect neurite growth in postnatal cerebellar granule cells (P14-P21). Overexpression of the β1 subunit in fibroblasts induced a 50% increase in the neurite length of cerebellar granule neurons plated on top of the fibroblast monolayer. Recently, β and γENaCs were shown to be required for NGF-induced neurite growth in PC12 cells (Drummond *et al*, 2005). Pharmacological blockade or protein downregulation of ENaCs both prevented neurite formation in NGF-differentiated PC12 cells. There is no evidence for a direct mechanical activation of these channels, but their function may be modulated by proteins which sense mechanical forces, such as cytoskeletal-associated proteins and cell adhesion proteins. It is worth noting that ASIC2b bears in its extracellular loop an RGD motif, an essential cell attachment site present for example in fibronectin; it is recognised by integrins and is crucial for cell migration. Although this is speculation, the association of ASICs with such molecules, their early expression in the nervous system, and their homology to ENaCs suggest the channels might play a role in neuronal growth. Preliminary data presented in section III.3 suggest a putative role for ASIC4 in regulation of neurite outgrowth in a neuronal cell line. However, mice lacking one ASIC gene do not display major neuronal abnormalities, implying the role of ASICs in neurogenesis, if any, may not be crucial; alternatively compensation mechanisms may occur and balance the lack of a specific ASIC subunit.

III.1.2.5. False positives

Some proteins have been reported in many Y2H screens as not being real interactors of the various baits tested. The most frequently described ones are heat shock proteins and ribosomal proteins; mitochondrial proteins such as cytochrome c oxidase are also often cited, as well as collagen-related proteins (Hengen, 1997). It is striking to see that in our Y2H screen, 6 proteins which are components of the extracellular matrix have been selected, mostly as interacting with ASIC3 N-terminus. This does not make sense as this domain of the channel is located intracellularly and probably does not come in contact with these proteins *in vivo*. Many enzymes such as polymerases and tRNA synthase are also likely to be false positives.

III.2. Annexin II light chain p11 promotes functional expression of ASIC1a

III.2.1. Results

III.2.1.1. ASIC1a N-terminus binds to p11 in the Y2H assay

A yeast two-hybrid assay was used to screen a rat DRG cDNA library in order to identify proteins interacting with the intracellular N-terminal domain of ASICs (section III.1). From approximately 1-2 x 10^7 yeast transformants 53 positive clones were identified as interacting with ASIC1a N-terminus (NT). Of these, 14 clones encoding the full length p11 protein were identified. In the overall screen of ASIC interactors, p11 was identified as interacting only with ASIC1a, suggesting the interaction of p11 with ASIC channels may be subunit-specific. In order to check the specificity of this interaction the yeast EGY48 containing the reporter plasmid pSH18-34 was transformed with plasmids encoding the N-terminal domains of rASIC2a, rASIC3 or rASIC4, and p11, and plated on media containing galactose and lacking uracil, histidine, tryptophan, and leucine (gal/raf ura- his- trp- leu-) and on media containing X-Gal (gal/raf ura- his- trp- X-Gal). After incubation for one day at 30°C only colonies expressing the ASIC1a N-terminus grew efficiently on media lacking leucine and turned blue on media containing X-Gal (Fig. III.1 A). A much lower growth rate was observed with ASIC3, an insignificant level of growth was observed with ASIC2a and no growth at all was detected with ASIC4. Similarly, yeast clones expressing ASIC3 had a very light blue colour and those expressing ASIC2a and 4 remained white. This strongly suggests that the interaction between p11 and ASIC1a is specific for this ion channel subunit.

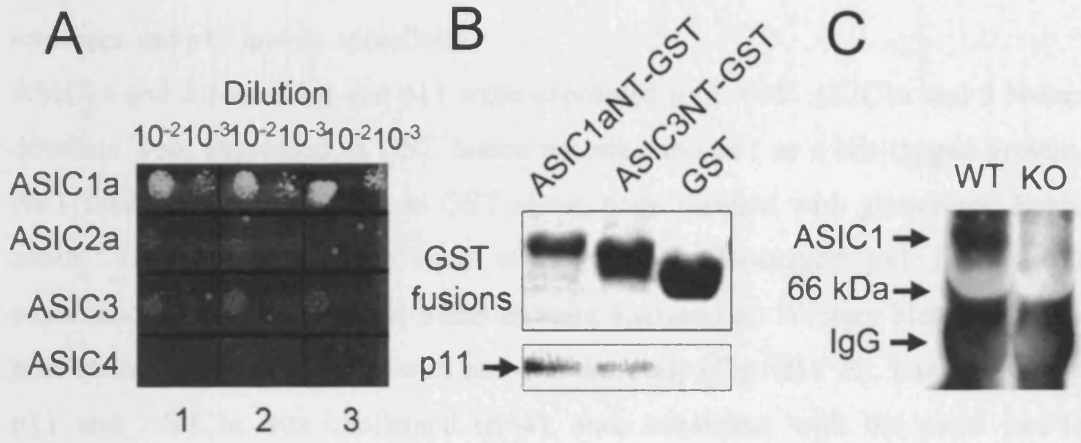

Fig. III.1: **p11 interacts with ASIC1a**. A, the yeast two-hybrid system uses the reporter gene *LEU2* such that only yeast expressing a protein that interacts with the bait can grow on media lacking leucine. Three transformants (lanes 1-3) of each condition were grown in liquid Glc/Ura-/His-/Trp- medium until they all reached Abs = 1, and 10-µl drops of 10^{-2} and 10^{-3} dilutions of the cultures were plated on Gal/Raf/Ura-/His-/Trp-/Leu- medium. B, ASIC1aNT-GST can pull-down His-tagged p11. Pull-down products were analysed by SDS-PAGE, followed by Coomassie Blue staining (upper panel) and Western blotting using anti-His antibody (lower panel). GST was used as a negative control. C, ASIC1 and p11 interact in mouse DRGs. Anti-p11 antibody was used to pull down p11 and its interacting partners from wild-type (WT) and ASIC1 knock-out (KO) mouse DRGs; the Western blot was probed with anti-ASIC1 antibody. Rabbit IgG was used in the co-immunoprecipitation and was detected on the immunoblot by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (heavy chain of 55 kDa).

III.2.1.2. ASIC1a N-terminus binds to p11 in a GST pull-down assay

A GST pull-down assay was used to confirm the interaction between ASIC1a N-terminus and p11 and its specificity.

ASIC1a and 3 N-termini and p11 were expressed in *E. coli*. ASIC1a and 3 N-terminal domains were expressed as GST fusion proteins and p11 as a His-tagged protein. The GST fusion proteins, as well as GST alone, were purified with glutathione-Sepharose beads. These proteins were used to pull down His-tagged p11 from the crude supernatant of bacterial lysate. Samples were analysed on Western blots by Coomassie blue staining and by probing with anti-His antibody (Fig. III.1 B). Interaction between p11 and ASIC1a was confirmed (n=4), and, consistent with the yeast two-hybrid results, an ImageJ software (http://rsb.info.nih.gov/ij/) analysis found an approximately 5 times lower level of interaction with ASIC3. No binding was detected between p11 and the negative control GST alone.

III.2.1.3. ASIC1a binds to p11 in mouse dorsal root ganglia

In order to demonstrate that ASIC1a and p11 interact *in vivo*, the two proteins were co-immunoprecipitated from mouse DRG (n=3). Dorsal root ganglia were extracted from 4 wild-type mice, as well as 4 ASIC1 knock-out mice as a negative control. An antibody (chicken IgY) directed to p11 was used to pull down p11 and its interacting partners from the mouse DRG pre-cleaned lysates. Western blotting analysis of these proteins using an anti-ASIC1 antibody revealed a ~70kDa band which was not present in the sample from ASIC1 knock-out mice DRGs, confirming that this band corresponds to ASIC1 (Fig. III.1 C). This suggests that ASIC1a and p11 interact in mouse DRG.

III.2.1.4. p11 promotes ASIC1a plasma membrane expression

p11 was reported to have a role in the trafficking of several ion channels and receptors. It was shown to interact with $Na_v1.8$, TASK-1, TRPV5/6 and $5HT_{1B}$ receptor and to increase their plasma membrane expression (Okuse *et al*, 2002; van de Graaf *et al*,

2003; Girard *et al*, 2002; Svenningsson *et al*, 2006). A putative similar role of p11 on ASIC1a trafficking was investigated by immunocytochemistry. ASIC sequences were tagged with a Flag epitope (DYKDDDDK). The functional characteristics of the Flag-tagged channels were confirmed to be identical to non-tagged channels by electrophysiology. Flag-tagged ASIC1a or Flag-tagged ASIC3 were transfected with either GFP or p11-GFP fusion in CHO-K1 cells and the ion channels subcellular localisation was observed using an anti-Flag antibody. p11-associated immunoreactivity (green) was localised at the plasma membrane. On average the intensity of the plasma membrane fluorescence corresponding to ASIC1a (red) was approximately two-fold higher when the channel was expressed in conjunction with p11 than in the control (Fig. III.2). This suggests that p11 facilitates transport of ASIC1a to the plasma membrane.

III.2.1.5. Surface protein biotinylation

A distinct approach to immunocytochemistry exploits the availability of cell surface proteins to be biotinylated *in vivo*. Detection of biotinylated proteins can then give a quantitative estimate of membrane-associated protein expression. Surface proteins of CHO-K1 cells co-expressing rASIC1aFlag and either p11-GFP or GFP were biotinylated and purified with streptavidin-conjugated agarose beads. Samples were analysed by immunoblotting using an anti-Flag antibody (n=4) (Fig. III.3). Consistent with the results described above, we observed that the fraction of rASIC1aFlag protein located at the cell surface is increased in the presence of p11. Using the ImageJ software, the intensity of the band corresponding to cell surface ASIC1a in the presence of p11 was found to be about 2.5 times higher than the control. The total amount of ASIC1a was unaffected.

III.2.1.6. p11 increases ASIC1a current density

To study a potential functional effect of p11 on ASIC1a, whole cell patch clamp experiments were carried out in CHO-K1 cells co-expressing ASIC1a and p11-GFP or

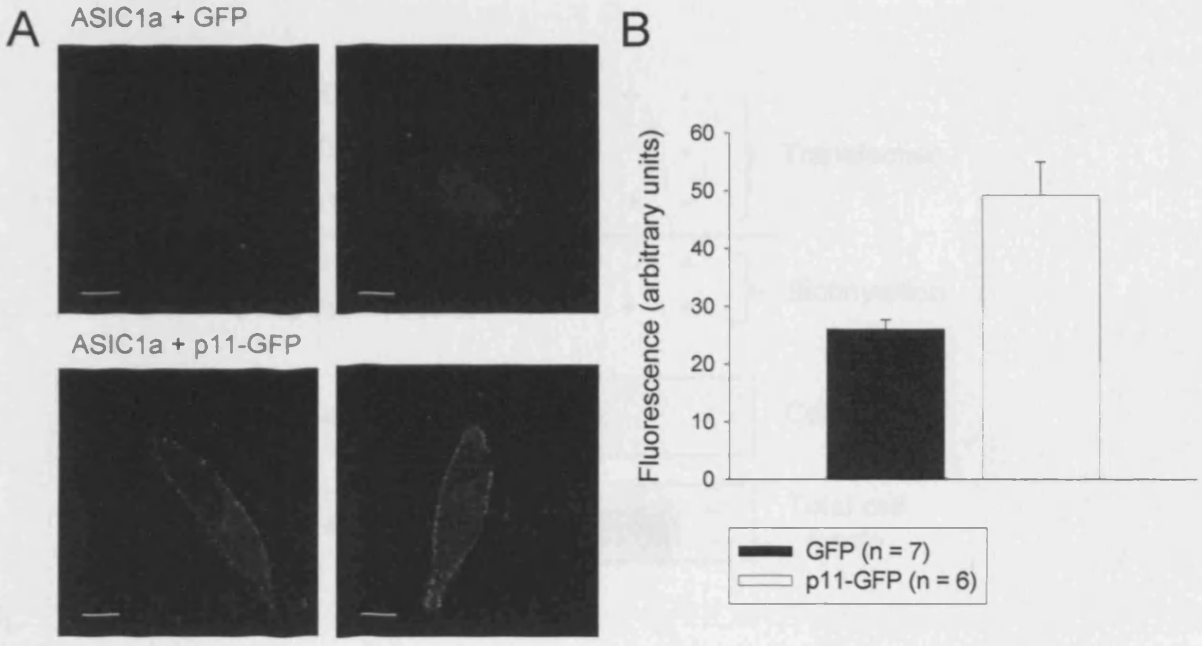

Fig. III.2: **Detection of ASIC1a expression at the plasma membrane by immunocytochemistry**. A, immunocytochemistry was performed on CHO-K1 cells transfected with ASIC1aFLAG (red) and either GFP or p11GFP (green). Surface expression of ASIC1aFLAG was detected using anti-FLAG antibody on fixed and non-permeabilised cells. Scale bar = 10 µm. B, ASIC1aFLAG-specific immunofluorescence was measured along the whole plasma membrane using the confocal microscope driving software. p11 approximately doubled ASIC1a surface expression compared with the GFP control ($p < 0.002$).

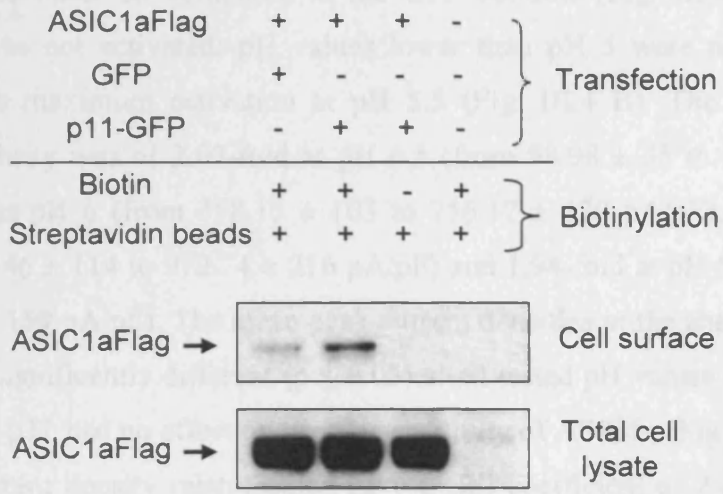

Fig. III.3: **Detection of ASIC1a expression at the plasma membrane by Western blotting after biotinylation of cell-surface proteins**. CHO-K1 cells expressing ASIC1aFLAG and either GFP or p11GFP were subjected to biotinylation of plasma membrane proteins and lysed. A small fraction of the cell lysate was set aside as a control (lower panel), and the remaining lysate was incubated with streptavidin-conjugated beads to recover cell surface-biotinylated ASIC1aFLAG (upper panel). Samples were analysed by Western blotting using anti-FLAG antibody. In the presence of p11, cell-surface expression of ASIC1a-FLAG was increased.

GFP (by Dr. François Rugiero). In whole cell recording, cells were held at -60 mV at pH 7.4 and were then submitted to a drop in external pH (pH 6.5-5) for 5 s before return to pH 7.4. In the presence of p11 a roughly 2-fold increase in ASIC1a current density was observed compared to the GFP controls (Fig. III.4 A). Above pH 6.5 ASIC1a was not activated; pH values lower than pH 5 were not tested as ASIC1a reached its maximum activation at pH 5.5 (Fig. III.4 B). The increase in ASIC1a current density was of 2.07-fold at pH 6.5 (from 98.98 ± 35 to 204.93 ± 27 pA/pF), 2.11-fold at pH 6 (from 358.15 ± 103 to 756.12 ± 170 pA/pF), 1.96-fold at pH 5.5 (from 495.46 ± 114 to 972.74 ± 216 pA/pF) and 1.94-fold at pH 5 (from 411.04 ± 107 to 797.4 ± 159 pA/pF). The mean peak current densities in the absence and presence of p11 were significantly different ($p \leq 0.05$) at all tested pH values (Fig. III.4 B). On the other hand p11 had no effect on the pH sensitivity of ASIC1a (Fig. III.4 C). Analysis of the pH/current density relationships gave a Hill coefficient of 2.42 and a pH of half-activation of 6.26 in absence of p11 and a Hill coefficient of 2.72 and a pH of half-activation of 6.3 in presence of p11 (Fig. III.4 B,C). These figures are not significantly different (Fig. III.4 C), meaning that p11 had no effect on the activation of ASIC1a. We next checked whether p11 affected the desensitisation of ASIC1a by measuring the desensitisation time constants resulting from the fit of the decaying phase of the currents with single exponentials. At all tested pH values the desensitisation time constants were almost identical with and without p11 (Fig. III.4 D). Therefore, p11 alters ASIC1a current density (increases the number of channels being delivered to the cell surface) while leaving the intrinsic properties of the channel (activation and desensitisation) unchanged.

To assess the specificity of the functional effect of p11 on ASIC1a, the same experiments were carried out with ASIC3. Cells were held at -60 mV and ASIC3 was activated by an acid challenge (Fig. III.5 A). ASIC3 peak current density was not changed by over-expression of p11 (Fig. III.5 A,B) nor did p11 have any effect on the desensitisation kinetics (Fig. III.5 C). The ASIC3 current is characterised by two distinct phases: a transient current activated by a slightly acidic pH is followed by a sustained current activated at lower pH. In our conditions (pH 6) the activation level of the transient current was nearly maximal. The sustained current, though much less, was

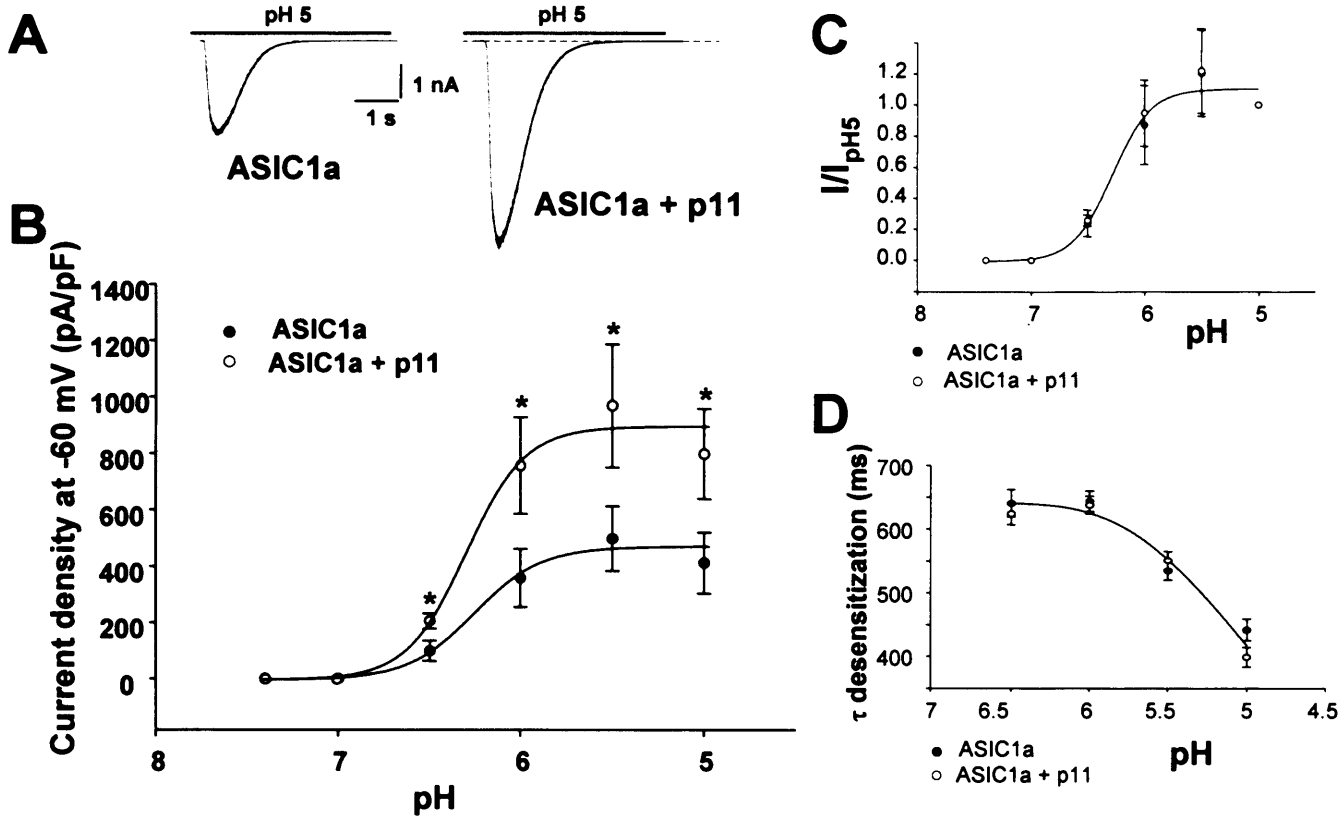

Fig. III.4: **p11 increases ASIC1a current density.** A, representative ASIC1a current traces recorded from CHO-K1 cells co-expressing ASIC1a and GFP (left) or ASIC1a and p11-GFP (right), held at - 60 mV. The currents were activated by a drop in pH from 7.4 to 5 for 5 s. Dashed lines, I = 0. B, mean peak ASIC1a current density at -60 mV as a function of extracellular pH in the absence (n = 4, 11, 15, 12, and 21 from pH 7 to 5) and presence (n = 4, 10, 13, 10, and 20 from pH 7 to 5) of p11. *, $p<0.05$. C, relative peak ASIC1a current densities normalised to the peak ASIC1a current density at pH 5 in the absence and presence of transfected p11. The holding potential was -60 mV. D, mean time constants of ASIC1a current desensitisation derived from single exponential fits of the decaying phase of the currents as a function of extracellular pH in the absence and presence of transfected p11.

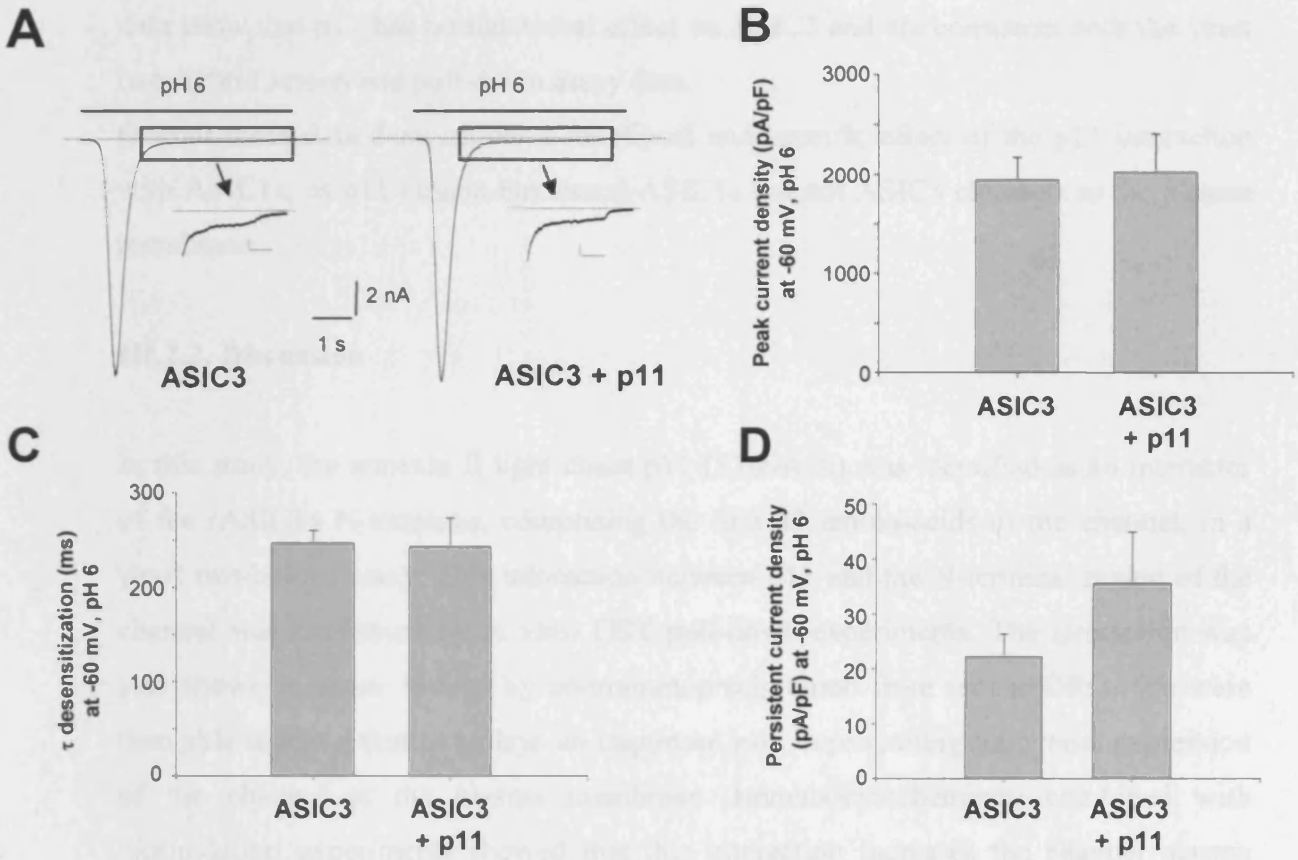

Fig. III.5: **p11 does not affect ASIC3 activity**. A, representative ASIC3 current traces recorded from CHO-K1 cells held at -60 mV and co-expressing ASIC3 and GFP (left) or ASIC3 and p11-GFP (right). The currents were activated by a drop in pH from 7.4 to 6 for 5 s. Insets, close-ups on the persistent phases of the currents. Dashed lines, I = 0. Scale bars = 100 pA (vertical) and 1 s (horizontal). B, mean peak ASIC3 current densities in the absence (left bar) and presence (right bar) of p11. C, mean time constants of ASIC3 current desensitisation derived from single exponential fits of the decaying phase of the currents in absence (left bar) and presence (right bar) of p11. D, mean current densities of the persistent phase of the ASIC3 current in the absence (left bar) and presence (right bar) of p11. In B-D, the recordings were performed at -60 mV and pH 6 (n = 6 and 5 for ASIC3 and ASIC3 + p11 recordings, respectively). There is no statistical difference between the data sets for ASIC3 and ASIC3 + p11.

readily activated and large enough to allow the observation of any change in its amplitude. Again no statistically significant change was observed in the current density of the sustained phase of ASIC3 in presence of p11 (Fig. III.5 D). Taken together, these data show that p11 has no functional effect on ASIC3 and are consistent with the yeast two-hybrid screen and pull-down assay data.
Overall these data demonstrate a functional and specific effect of the p11 interaction with ASIC1a, as p11 targets functional ASIC1a but not ASIC3 channels to the plasma membrane.

III.2.2. Discussion

In this study, the annexin II light chain p11 (S100A10) was identified as an interactor of the rASIC1a N-terminus, comprising the first 43 amino-acids of the channel, in a yeast two-hybrid assay. This interaction between p11 and the N-terminal region of the channel was confirmed by *in vitro* GST pull-down experiments. The interaction was also shown to occur *in vivo* by co-immunoprecipitation from mouse DRG. We were then able to show that p11 plays an important role in promoting functional expression of the channel at the plasma membrane. Immunocytochemistry combined with biotinylation experiments showed that this interaction increases the channel plasma membrane expression. Finally, whole cell patch clamp recordings demonstrated that p11 specifically targets functional ASIC1a channels to the plasma membrane. However p11 does not seem to be an obligatory requirement for expression of ASIC1a at the cell surface, as transfection of the channel alone in CHO cells, which do not express p11 (Okuse *et al*, 2002), leads to expression of functional channels at the plasma membrane. The ASIC1a-p11 interaction is physiologically relevant as p11 is expressed in nerve tissues expressing ASIC1a, i.e., in somatic and visceral sensory neurons (Okuse *et al*, 2002; Gonzalez-Martinez *et al*, 2003) and in the brain (Zhao *et al*, 2004).

p11 is a member of the S100 family of small EF hand-type (helix-loop-helix) Ca^{2+}-binding proteins. S100 proteins are generally found as homodimers and are involved in many cellular processes such as Ca^{2+} homeostasis, phosphorylation, transcription factor

regulation, cytoskeletal dynamics, cell proliferation and differentiation (Donato, 2003). These effects are mediated by interactions with and regulation of other proteins (reviewed in Zimmer *et al*, 2003). Most S100 proteins undergo Ca^{2+}-dependent conformational changes which allow recognition of their target proteins. p11, however, does not bind Ca^{2+}; mutations in both EF hands produce a conformation that resembles that of Ca^{2+}-loaded EF hands, making the protein constitutively 'active'. p11 is generally found tightly associated by hydrophobic interactions with annexin II (also known as p36, annexin A2 or calpactin I heavy chain), to form a tetrameric complex $(p11)_2(p36)_2$, in a Ca^{2+}-independent manner (Waisman, 1995). An oxidised disulfide-linked tetrameric form of p11 has also been described in the extracellular space (Rety *et al*, 1999).

Annexins form a class of non-EF-hand Ca^{2+}-binding proteins. They have the ability to bind to certain membrane phospholipids in their Ca^{2+}-bound conformation (Rescher *et al*, 2004), and have been implicated in many membrane-related functions, such as membrane-cytoskeleton bridging, organisation of membrane microdomains, and regulation of endocytosis and exocytosis (reviewed in Gerke *et al*, 2005). The presence of p11 in the $(p11)_2(p36)_2$ complex dramatically lowers the Ca^{2+} concentration required for annexin II binding to phospholipids (Powell and Glenney, 1987). Annexin II, both alone or as a heterotetramer with p11, also binds to F-actin in the ratio of 2 actin monomers per annexin II molecule (Gerke and Weber, 1984), and is recruited to sites of actin polymerisation. Its ability to bind certain phospholipids and actin suggested that annexin II could have a function in the assembly of cytoskeletal structures at specialised plasma membrane domains such as lipid rafts (reviewed in Hayes *et al*, 2004). Annexin II has been localised at sites of cell-cell contact in epithelial cells (Yamada *et al*, 2005). It was also implicated in the regulation of phagocytosis and exocytosis (König *et al*, 1998).

In this thesis p11 was shown to associate with ASIC1a and to increase its plasma membrane expression. An involvement of annexin II in this process, as a heterotetrameric complex with p11, was not examined but is probable; indeed, intracellularly p11 is mainly found as a heterotetramer, and the role of annexin II in

p11-mediated trafficking of other ion channels was recently demonstrated (van de Graaf *et al*, 2003). The mechanism by which ASIC1a incorporation to the plasma membrane is increased is not known but the involvement of annexin II in exocytosis suggests ASIC1a could be regulated by this process.

p11 is known to increase the cell surface expression of several ion channels. Okuse *et al* (2002) first reported that p11 regulates TTX-resistant sodium channel $Na_v1.8$ in sensory neurons. The binding of p11 to $Na_V1.8$ occurs over a sequence consisting of amino-acids 74-103 of the channel. Nevertheless, a comparison of the sequence 74-103 of $Na_V1.8$ with ASIC1a N-terminal sequence does not show any similarity, suggesting that the interaction of p11 with these channels may occur on different regions of p11. p11 was also shown to be involved in the translocation to the plasma membrane of TASK-1 background K^+ channel (Girard *et al*, 2002), as well as epithelial Ca^{2+} channels TRPV5 and 6 channels (van de Graaf *et al*, 2003) via an interaction with a type I PDZ binding sequence on the C-terminus of all three channels. In the case of TASK-1, binding of p11 causes an endoplasmic reticulum (ER) retention signal to be masked, leading to an increase in translocation of the channel from the ER to the plasma membrane. Thus, binding of different ion channels seems to be able to take place on different regions of p11. This raises the interesting possibility that more than one channel type may interact with the same p11 molecule, and that p11 may serve as a scaffold protein, linking various ion channels depending on the cell type and targeting them to specific membrane domains.

The specific distribution of the annexin II/p11 complex in dynamic actin-rich membrane structures suggests a possible role for ASIC1a in such structures in neurons. This is consistent with the results from the yeast two-hybrid screen presented in section III.1.2.4 which suggest a potential involvement of several ASIC subunits in nerve cell adhesion, growth and/or migration. Indeed these processes all require dynamic cytoskeleton rearrangements, at focal adhesions or cell-cell contact sites for example. As ASICs are known to heteromultimerise, it would be interesting to examine the putative role of p11 in the translocation of ASIC1a-containing heteromers, in addition to the demonstrated effect on ASIC1a homomers. This would represent a way to gather

different ASIC subunits to specific membrane microdomains where they could exert various functions via interactions with specific molecules.

Studies in ASIC1 knock-out mice show that this channel seems to contribute a major part of the amplitude of the transient proton-gated current in mouse DRG at pH 5 (Benson *et al*, 2002). Therefore it would be interesting to determine whether this is due to an increase in the translocation of heteromeric channels by p11 through a specific interaction with ASIC1a. A comparison of the amplitudes of these transient proton-gated currents in sensory neurons from p11 and ASIC1 knock-out mice would be useful. As ASICs are thought to be to some extent involved in the development of hyperalgesia during inflammation, disrupting the interaction between p11 and ASIC1a may prove a useful strategy in the treatment of inflammatory pain. The interaction between ASIC1a and p11 suggests that mediators known to regulate p11 expression may also modulate the expression of functional ASIC1a channels.

p11 was shown to be up-regulated by pro-inflammatory molecules NGF (Okuse *et al*, 2002) and interferon γ (Huang *et al*, 2003), in DRG neurons and epithelial cell lines, respectively. These observations suggest that an increase in the levels of inflammatory molecules in response to tissue damage may lead to an up-regulation of p11, which in turn may enhance the functional expression of ASIC1a (or potentially ASIC1a-containing heteromeric ASIC channels). This may be complementary to the increase of ASIC transcripts observed in animal models of inflammation (Voilley *et al*, 2001) and contribute to an increase in sensory neurons excitability in inflammatory conditions. On the other hand, p11 was also shown to be up-regulated by anti-inflammatory molecules such as glucocorticoid dexamethasone (Yao *et al*, 1999). Besides, p11 directly binds to and inhibits cytoplasmic phospholipase A2 activity (Wu *et al*, 1997); this enzyme is involved in the release of arachidonic acid from membrane lipids, which is subsequently converted into leukotrienes, prostaglandins, and other eicosanoids which play an important role in inflammation (reviewed in Liscovitch and Cantley, 1994). In summary p11 expression seems to be increased by both pro- and anti-inflammatory molecules, suggesting both types of molecules may also regulate ASIC expression in DRG neurons during pathophysiological condition. The role of p11 in inflammation may thus be complex, on one hand increasing the excitability of sensory neurons in

areas of tissue damage by increasing expression of nociceptive channels, and on the other hand causing inhibition of eicosanoid production by blockade of cytosolic phospholipase A2.

Finally, nitric oxide (NO) up-regulates p11 expression (Pawliczak *et al*, 2001). Reactive oxygen/nitrogen species (ROS) including NO are produced during oxygen-glucose deprivation (OGD) conditions. OGD occurs in ischemia where ASIC1a-mediated cell damage has been demonstrated (Xiong *et al*, 2004). Hence, NO might be responsible for up-regulating ASIC1a via p11 during brain ischemia which would lead to an increase in neuronal damage.

Pharmacologically disrupting the association between p11 and ASIC1a could potentially lead to significant progress in the search for a treatment of brain ischemia.

III.3. Novel roles for ASIC4 suggested by Y2H screen

ASIC4 does not mediate a proton-gated current when expressed heterologously and to date no function has been ascribed to it. Characterisation of proteins with which it interacts could be of great value as this may give insight to its function. In our Y2H screen, we found that the ASIC4 N-terminus bound to polyubiquitin, PAPIN, and moesin. Preliminary experiments suggest putative functional implications to the link between ASIC4 and these 3 proteins, although the data presented below is tentative and requires further investigations.

III.3.1. Results

III.3.1.1. ASIC4 binds to ASIC1a in CHO-K1 cells

ASIC1a and ASIC4Flag were co-expressed in CHO-K1 cells. ASIC4Flag and its interacting proteins were pulled down using an antibody directed to the Flag epitope. Samples were analysed by Western blotting, using an anti-ASIC1 antibody. ASIC1a was efficiently co-immunoprecipitated with ASIC4Flag (n=3) (Fig. III.6). Untransfected CHO-K1 cells were used as a negative control. This represents the first evidence of a putative association between ASIC4 and another ASIC subunit, although the method used relies on overexpression meaning that confirmation of binding *in vivo* is required. Moreover, an additional negative control showing an absence of signal in cells transfected with ASIC1a alone is necessary to confirm the validity of this result.

III.3.1.2. ASIC4 downregulates ASIC1a total protein level

Flag-tagged ASIC1a-3 were co-transfected in CHO-K1 cells with either ASIC4-pRK7 or the empty pRK7 vector. Total cell lysates were subjected to Western blotting and analysed with anti-Flag antibody (Fig. III.7 A). In the presence of ASIC4 the amount of

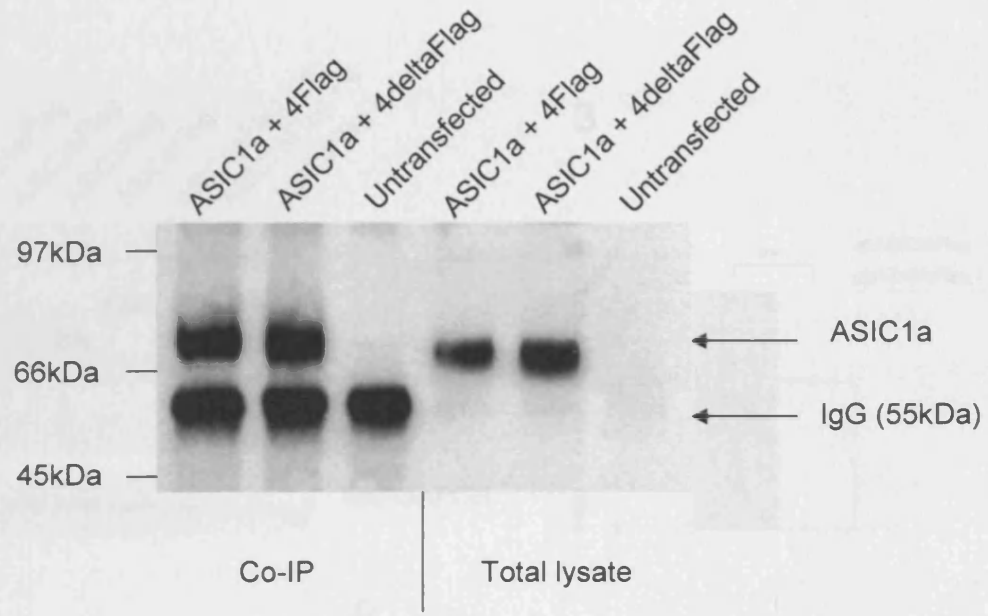

Fig. III.6: **ASIC1a and ASIC4 co-immunoprecipitate in CHO-K1 cells.** Cells were co-transfected with ASIC1a and ASIC4Flag or ASIC4deltaFlag (in which the 21 N-terminal amino-acids are deleted). Following cell lysis, a fraction of the cell lysate was kept aside as a control (total lysate). An anti-Flag antibody was used to precipitate ASIC4Flag and the proteins it interacts with (co-IP). The immunoblot was probed with anti-ASIC1 antibody. Both ASIC4Flag and ASIC4deltaFlag were able to pull-down ASIC1a.

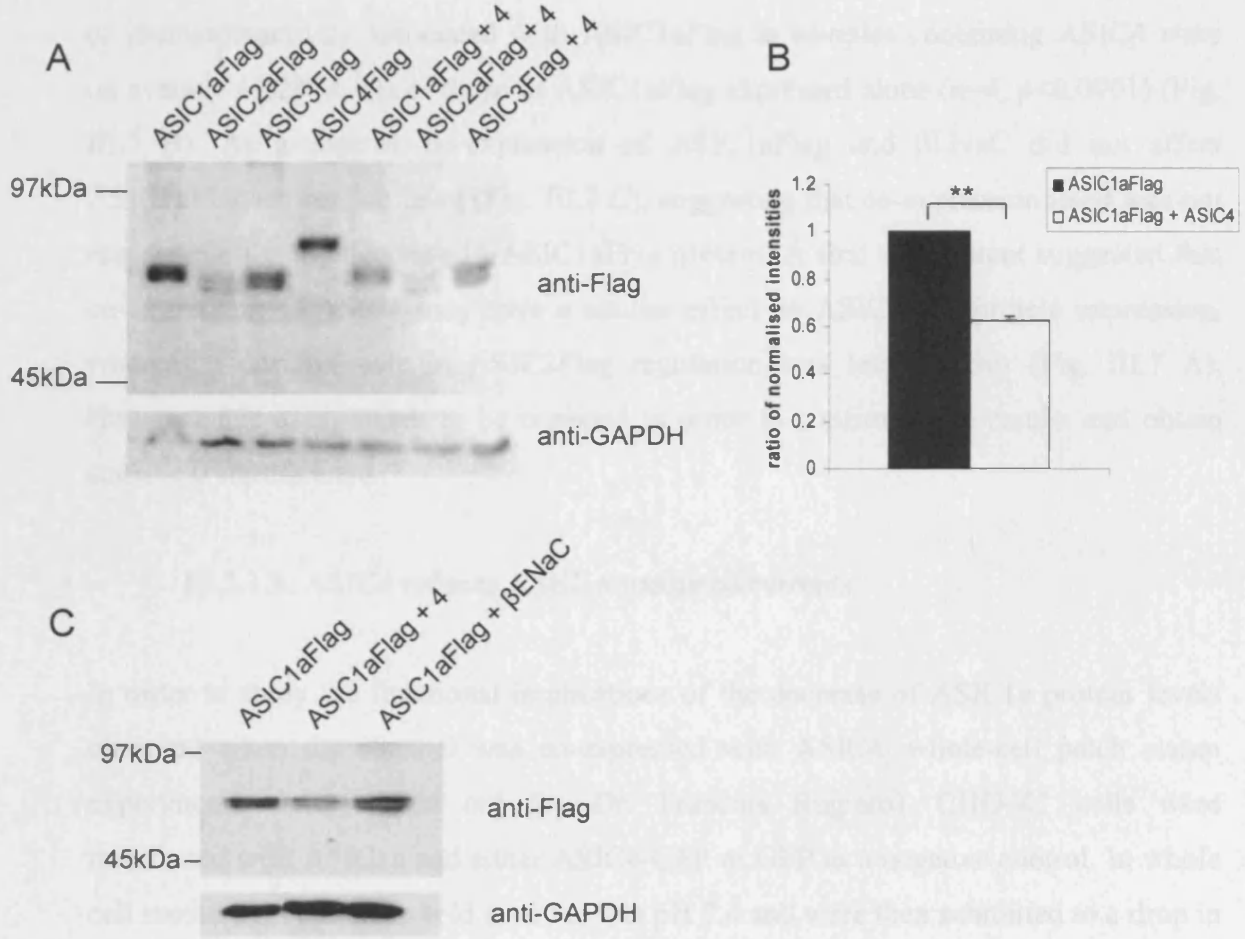

Fig. III.7: **ASIC4 reduces ASIC1a total protein expression**. A, CHO-K1 cells were transfected with ASICFlag constructs, with or without ASIC4. Total cell lysates were subjected to immunoblotting, and Western blot was probed with anti-Flag antibody. B, intensities of ASIC1aFlag bands (in the absence or presence of ASIC4) from 4 independent immunoblots were measured using the ImageJ software and were normalised to intensities of the corresponding GAPDH bands. Co-expression of ASIC4 reduces ASIC1aFlag total protein level by around 40% ($n=4$, $p<0.0001$). C, as a control, βENaC was co-expressed with ASIC1aFlag, which did not result in a decrease of ASIC1a total protein ($n=2$).

ASIC1aFlag total protein was consistently found to be around 40% lower than ASIC1aFlag transfected alone. Using the ImageJ software, intensities of ASIC1aFlag bands were measured and normalised to those of GAPDH bands; the normalised values of immunoreactivity associated with ASIC1aFlag in samples containing ASIC4 were on average 62.2% ± 2% of those of ASIC1aFlag expressed alone (n=4, $p<0.0001$) (Fig. III.7 B). As a control, co-expression of ASIC1aFlag and βENaC did not affect ASIC1aFlag expression level (Fig. III.7 C), suggesting that co-expression itself was not responsible for the decrease in ASIC1aFlag protein. A first experiment suggested that co-expression of ASIC4 may have a similar effect on ASIC3Flag protein expression, whereas a putative role in ASIC2Flag regulation was less obvious (Fig. III.7 A). However this assay needs to be repeated in order to confirm these results and obtain statistical significance.

III.3.1.3. ASIC4 reduces ASIC1a-mediated currents

In order to study the functional implications of the decrease of ASIC1a protein levels observed when the channel was co-expressed with ASIC4, whole-cell patch clamp experiments were carried out (by Dr. François Rugiero). CHO-K1 cells were transfected with ASIC1a and either ASIC4-GFP or GFP as a negative control. In whole cell recording, cells were held at -60 mV at pH 7.4 and were then submitted to a drop in external pH (pH 5) for 5 s before return to pH 7.4. In the presence of ASIC4 a 3.2-fold decrease in ASIC1a current density was observed compared to the negative controls (from 427.5 ± 105 to 134.1 ± 34 pA/pF) (Fig. III.8 A). The mean peak current densities in the absence and presence of ASIC4 were significantly different ($p \leq 0.05$). Other pH values have not been tested yet, therefore it is not clear whether ASIC4 alters the pH sensitivity of ASIC1a. Likewise, kinetics of activation and desensitisation of currents mediated by ASIC1a in the presence and absence of ASIC4 need to be determined.

ASIC4 was also shown to reduce ASIC3-associated currents at pH 6 in CHO-K1 cells (Fig. III.8 B). The reduction in ASIC3 current density in the presence of ASIC4 was of 2.0-fold at pH 5 (from 2025.9 ± 232 to 991.2 ± 188 pA/pF). The mean peak current densities in the absence and presence of ASIC4 were significantly different ($p \leq 0.005$).

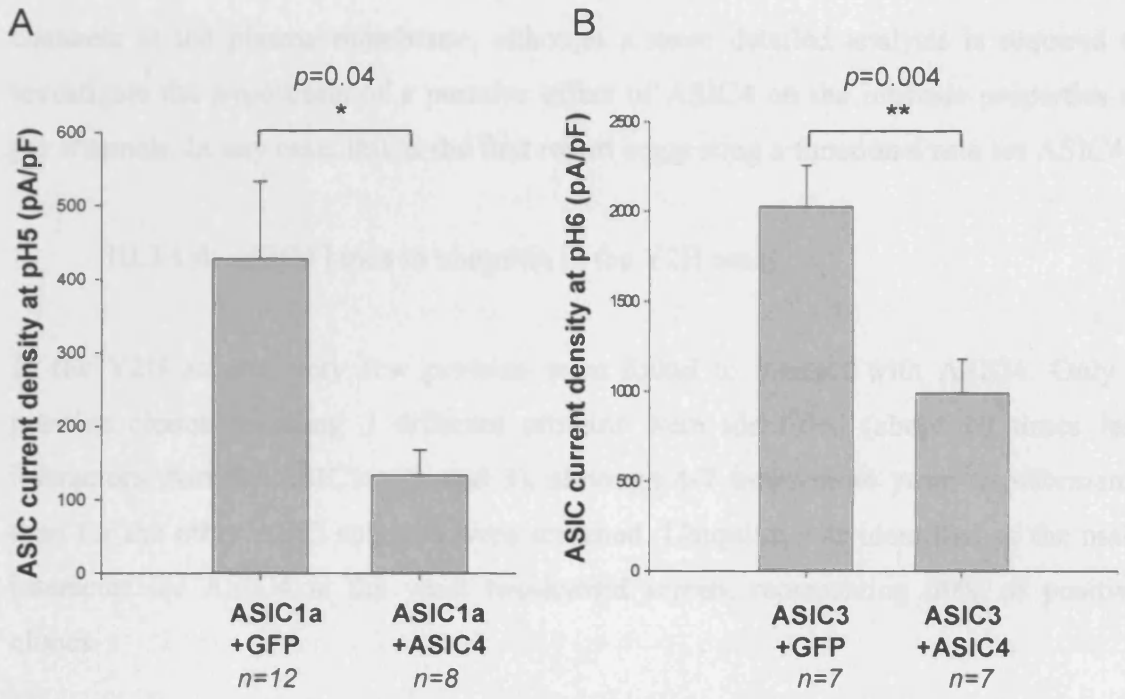

Fig. III.8: **ASIC4 reduces ASIC1a and ASIC3 current densities**. A, mean peak ASIC1a current densities in the absence (left bar) and presence (right bar) of ASIC4. B, mean peak ASIC3 current densities in the absence (left bar) and presence (right bar) of ASIC4.

These data suggest that the reduction of ASIC1a (and possibly of ASIC3) total protein levels in the presence of ASIC4 translates into a decrease in proton-gated currents mediated by these channels; this may be due a decrease in the number of functional channels at the plasma membrane, although a more detailed analysis is required to investigate the hypothesis of a putative effect of ASIC4 on the intrinsic properties of the channels. In any case, this is the first report suggesting a functional role for ASIC4.

III.3.1.4. ASIC4 binds to ubiquitin in the Y2H assay

In the Y2H screen, very few proteins were found to interact with ASIC4. Only 7 positive clones encoding 3 different proteins were identified (about 10 times less interactors than for ASIC1a, 2a and 3), although 4-7 times more yeast transformants than for the other ASIC subunits were screened. Ubiquitin was identified as the main interactor for ASIC4 in the yeast two-hybrid screen, representing 60% of positive clones.

III.3.1.5. ASIC4 does not appear to undergo ubiquitination

As mentioned above, ASIC4 displayed a very specific binding pattern in the Y2H screen, compared to the other ASICs. ASIC4 has an extended N-terminus compared to the other baits; this extra-sequence contains specific features such as 5 lysine residues and a type II PDZ binding site, which could potentially account for the specific pattern of interactions observed in the Y2H assay. In order to evaluate a potential role for this sequence in ASIC4 function, amino-acids 1-21 which include the lysine residues and the PDZ binding site, were deleted by PCR. This construct is referred to as ASIC4delta. Because N-terminal lysine residues are known to be substrates for ubiquitin covalent attachment, we first tested the hypothesis that ASIC4, but not ASIC4delta, might undergo ubiquitination. Preliminary results suggest this does not seem to be the case (n=1). Ubiquitin was overexpressed with ASIC4Flag or ASIC4deltaFlag in CHO-K1 cells, and samples were analysed by Western blotting. Detection of multiple bands of higher molecular weight than ASIC4 and ASIC4delta would indicate polyubiquitination

of the channels. However, when using an anti-Flag antibody, the only bands observed had the size of ASIC4 or ASIC4delta proteins alone, suggesting neither ASIC4 nor ASIC4delta undergo ubiquitination (Fig. III.9).

To ensure the system did allow detection of ubiquitinated proteins, an HA-tagged ubiquitin was also expressed in conjunction with ASIC4Flag or ASIC4deltaFlag, and an antibody directed to the HA epitope was used to visualise ubiquitinated proteins. A smear of high molecular weight ubiquitinated proteins was detected in the samples containing the HA-tagged ubiquitin only.

ASIC4delta retained its ability to bind to and downregulate ASIC1a-mediated currents (Fig. III.6 lane 2 and III.10).

III.3.1.6. ASIC1a undergoes proteasome-dependent degradation

The two major pathways for protein degradation in the cell involve the 26S proteasome or lysosomes. Polyubiquitination generally leads to degradation of target proteins in the proteasome. In order to investigate the mechanisms involved in ASIC1a degradation, CHO-K1 cells expressing ASIC1aFlag were grown in the presence of lactacystin, a proteasome blocker, or DMSO as a negative control, and the total protein level of ASIC1a was monitored over several hours (n=1) (Fig. III.11). Cycloheximide was added to all samples to prevent *de novo* protein expression. Over 5 hours, levels of ASIC1a protein significantly decreased, indicating a relatively quick turnover of the channel. When the proteasome was blocked with lactacystin, ASIC1a protein was also reduced over time, but at a slower rate. This indicates that ASIC1a is at least in part degraded in the proteasome, which suggests the channel may undergo ubiquitination.

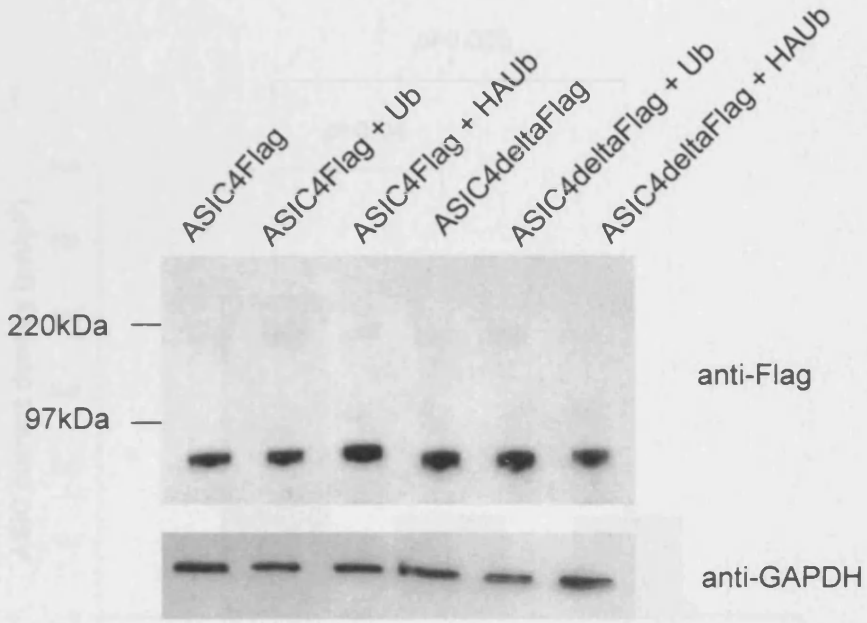

Fig. III.9: **ASIC4 may not be regulated by ubiquitination.** CHO-K1 cells were transfected with ASIC4Flag or ASIC4deltaFlag in the presence or absence of ubiquitin (Ub) or HA-tagged ubiquitin (HAUb). Western blot was probed with anti-Flag (top pannel) or anti-GAPDH (bottom pannel) antibodies. Only bands corresponding to ASIC4Flag and ASIC4deltaFlag molecular weights were detected with the anti-Flag antibody, suggesting these channels do not undergo ubiquitination.

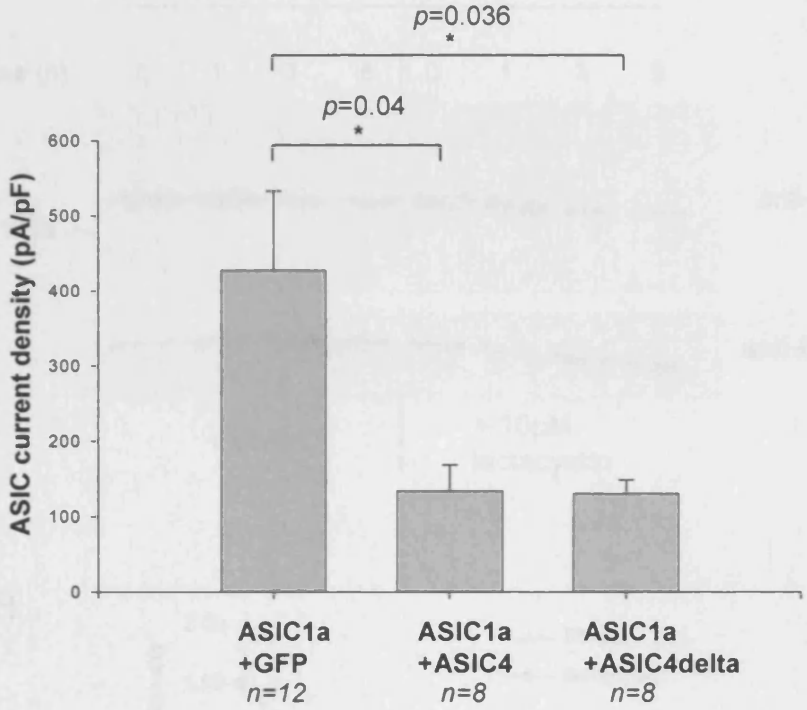

Fig. III.10: **ASIC4delta reduces ASIC1a current density in a similar manner as ASIC4**. Mean peak ASIC1a current densities in the absence (left bar) and presence of ASIC4 (middle) or ASIC4delta (right).

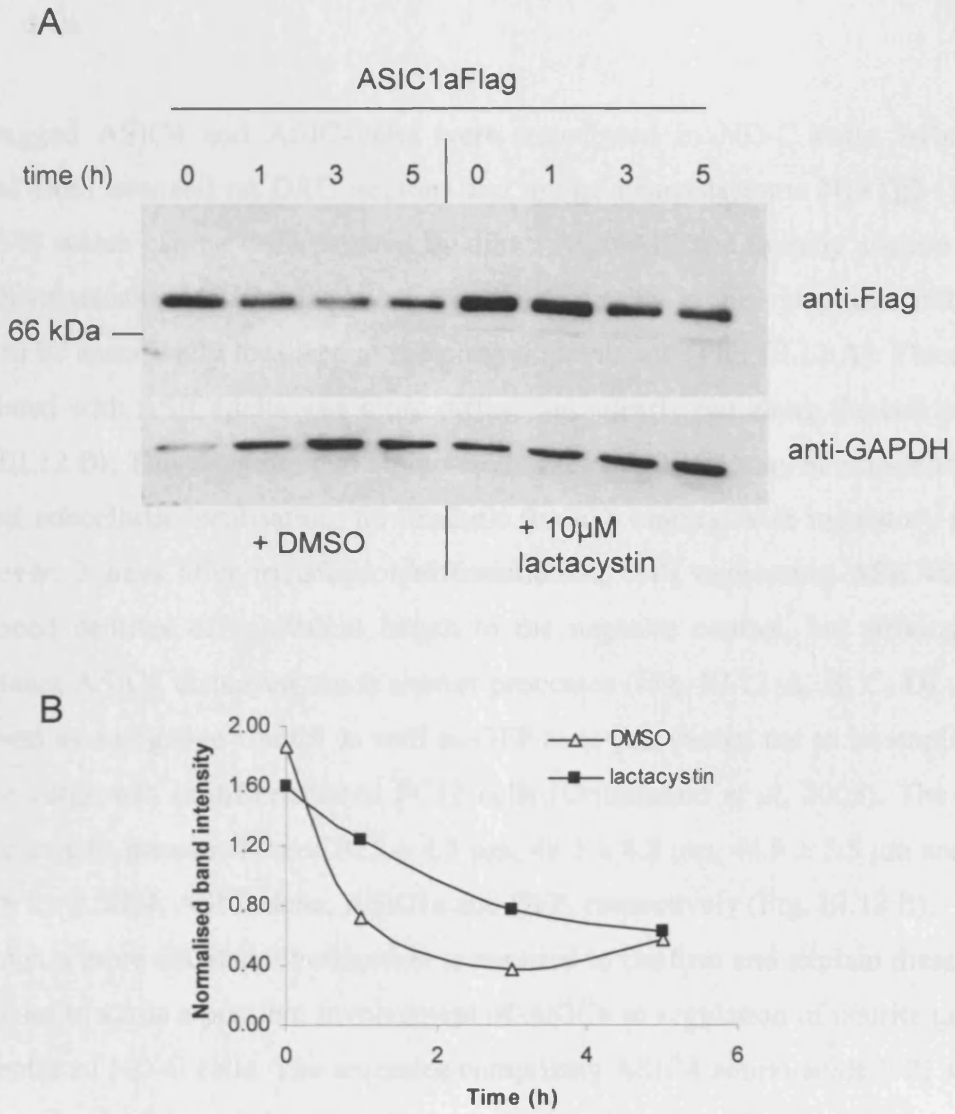

Fig. III.11: **ASIC1a may be degraded in the proteasome**. A, CHO-K1 cells expressing ASIC1aFlag were lysed and subjected to immunoblotting after incubation with or without lactacystin for 1, 3 or 5h (n=1). Cycloheximide prevented *de novo* protein expression in all samples. B, ASIC1aFlag band intensities were normalised to those of GAPDH. Degradation of ASIC1aFlag appears to be slower in the presence of the proteasome blocker, suggesting ASIC1a may undergo proteasomal-dependent degradation.

III.3.1.7. ASIC4 may be involved in regulation of neurite outgrowth in ND-C cells

Flag-tagged ASIC4 and ASIC4delta were transfected in ND-C cells, hybrid cells derived from neonatal rat DRG neurons and mouse neuroblastoma N18Tg2 (Wood *et al*, 1990) which can be differentiated by dibutyryl cAMP and thereby acquire neuron-like characteristics. ASIC4 displayed a punctate pattern in the cytoplasm and did not seem to be specifically localised at the plasma membrane (Fig. III.12 A). Fluorescence associated with ASIC4delta was more diffuse and clearly ran along the cell processes (Fig. III.12 B). This suggests that amino-acids 1-21 of ASIC4 may be important for the channel subcellular localisation, for example through binding with regulatory proteins. Moreover, 3 days after transfection/differentiation, cells expressing ASIC4delta had developed neurites of equivalent length to the negative control, but strikingly cells expressing ASIC4 displayed much shorter processes (Fig. III.12 A, B, C, D). ASIC1a was used as a negative control as well as GFP as it was shown not to be implicated in neurite outgrowth in differentiated PC12 cells (Drummond *et al*, 2005). The average neurite lengths measured were 20.5 ± 4.5 μm, 48.3 ± 4.8 μm, 44.9 ± 5.5 μm and 39.9 ± 3.8 μm for ASIC4, ASIC4delta, ASIC1a and GFP, respectively (Fig. III.12 E).
Although a more detailed investigation is required to confirm and explain these results, they point towards a possible involvement of ASIC4 in regulation of neurite growth in differentiated ND-C cells. The sequence comprising ASIC4 amino-acids 1-21 seems to play a major inhibitory role, possibly through ubiquitination of various proteins and/or interaction with regulatory proteins via its PDZ-binding site.

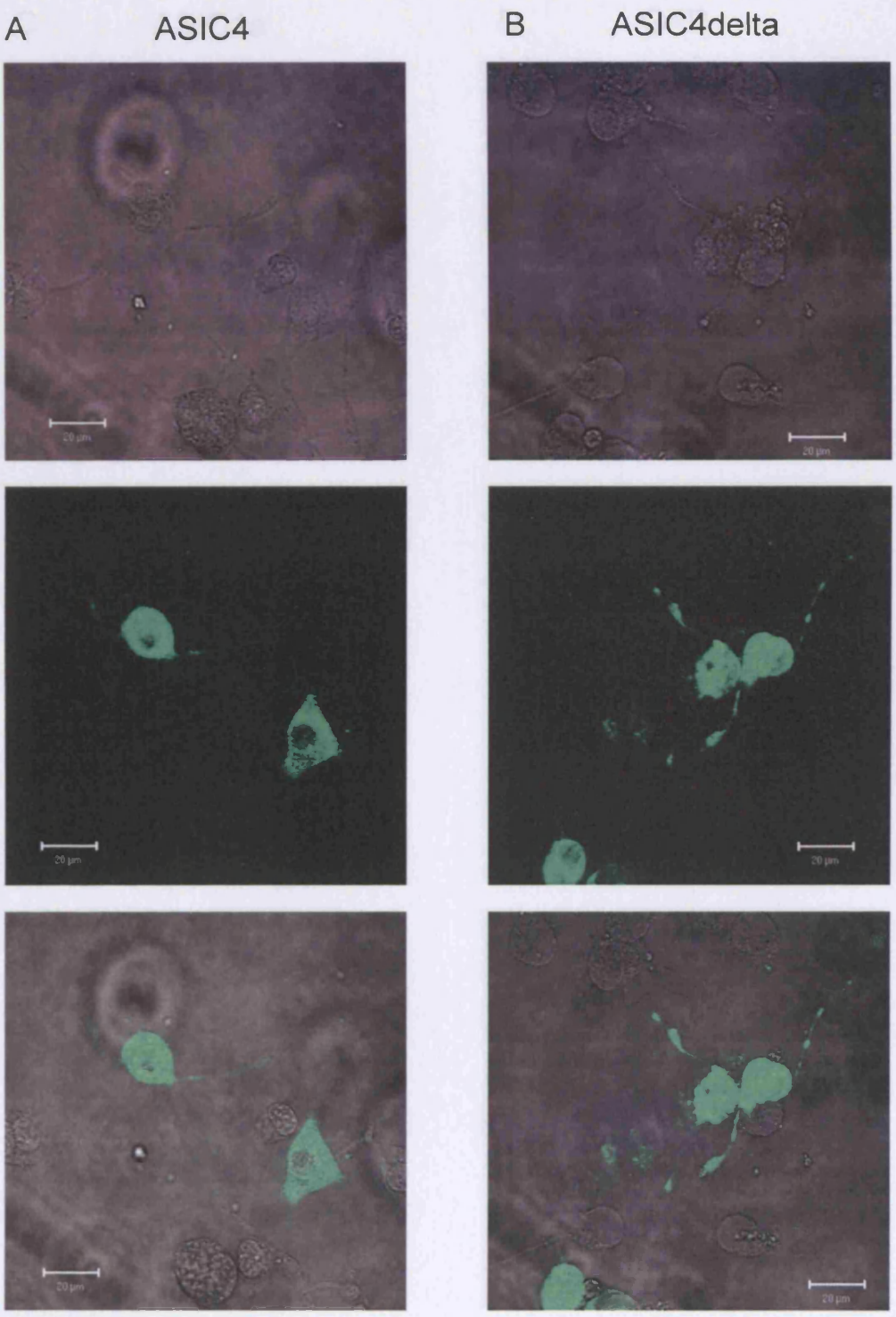

Fig. III.12: **Overexpression of ASIC4Flag and ASIC4deltaFlag in differentiated ND-C cells**. Top panel: bright field; middle panel: cells expressing ASIC4Flag (A) or ASIC4deltaFlag (B); bottom panel: overlay.

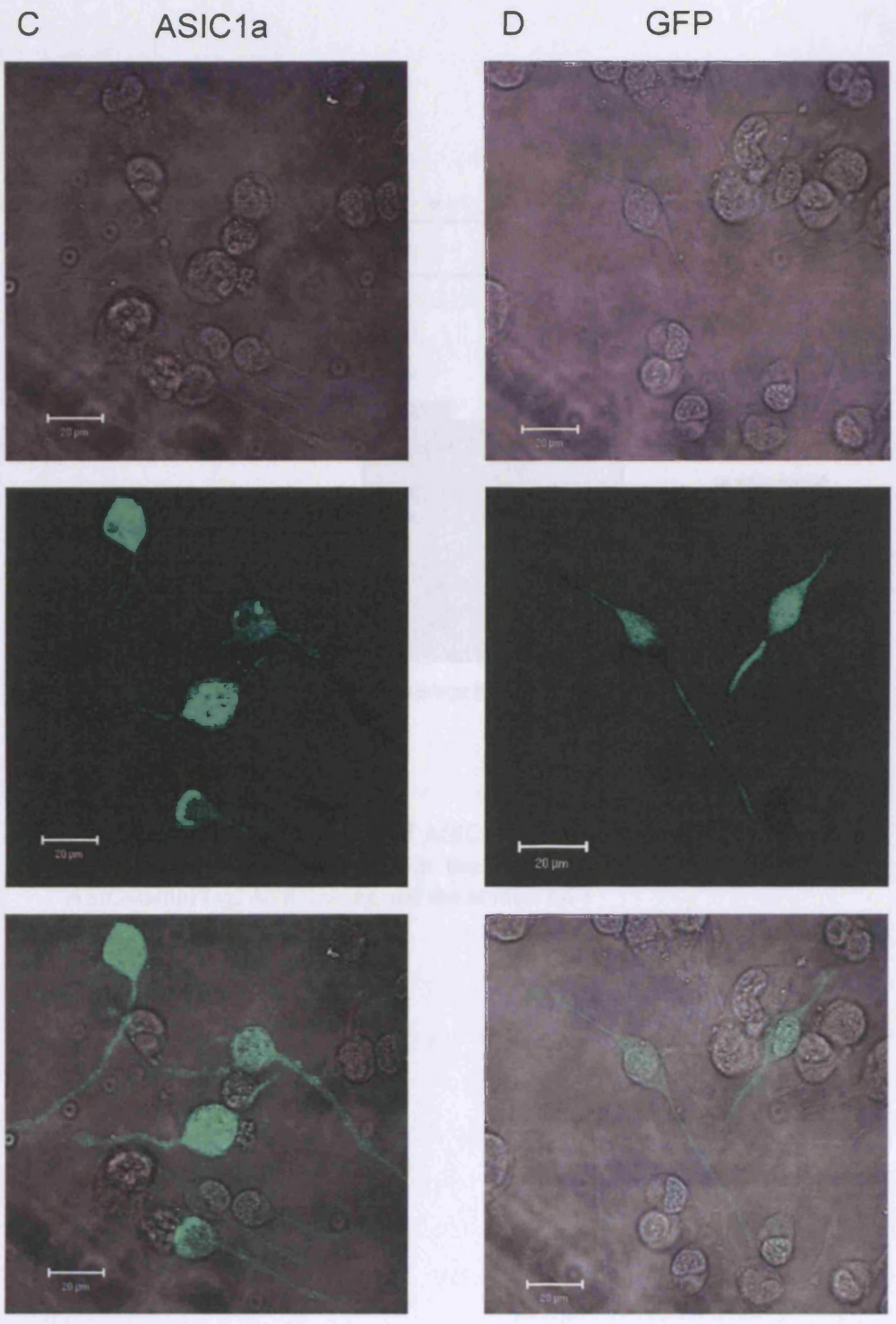

Fig. III.12: Top panel: bright field; middle panel: cells expressing ASIC1aFlag (C) or GFP (D); bottom panel: overlay.

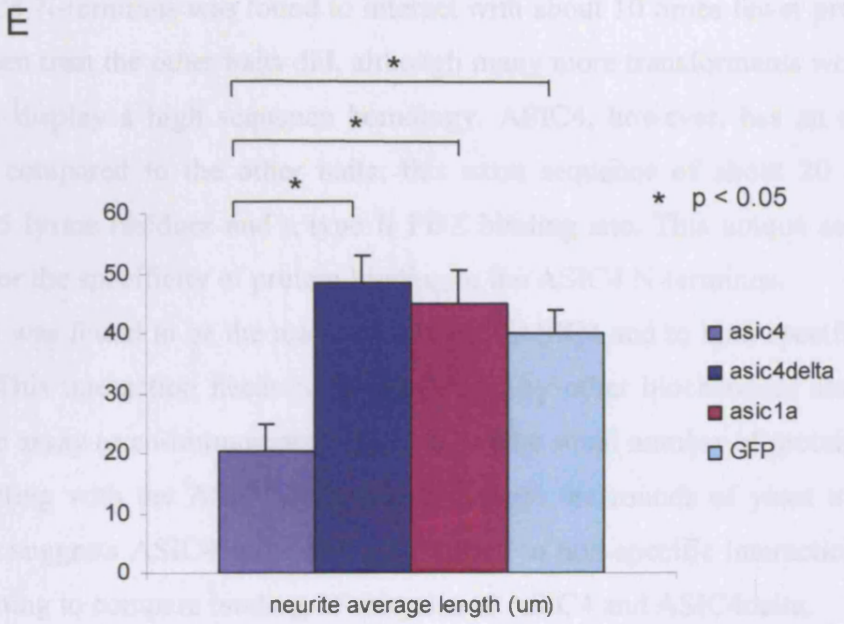

Fig. III.12: E, overexpression of ASIC4Flag in differentiated ND-C cells results in ~2-2.5-fold decrease in the length of processes compared to ASIC4deltaFlag, ASIC1aFlag and the control GFP

III.3.2. Discussion

The ASIC4 N-terminus was found to interact with about 10 times fewer proteins in our Y2H screen than the other baits did, although many more transformants were screened. The baits display a high sequence homology. ASIC4, however, has an extended N-terminus compared to the other baits; this extra sequence of about 20 amino-acids contains 5 lysine residues and a type II PDZ binding site. This unique sequence may account for the specificity of protein binding to the ASIC4 N-terminus.
Ubiquitin was found to be the main interactor of ASIC4 and to bind specifically to this subunit. This interaction needs to be confirmed by other biochemical assays such as pull-down assay or co-immunoprecipitation, but the small number of proteins identified as interacting with the ASIC4 N-terminus through the rounds of yeast transformants screening suggests ASIC4 sequence is not prone to non-specific interactions. It would be interesting to compare binding of ubiquitin to ASIC4 and ASIC4delta.

Ubiquitination is a posttranslational modification which involves covalent attachment of ubiquitin to lysine residues of target proteins. Lysine residues in ubiquitin can in turn serve as modification sites, resulting in the formation of polyubiquitin chains on the substrate protein. Covalent attachment of ubiquitin(s) serves as a localisation signal; monoubiquitination is generally described as being a signal for endocytosis followed by recycling, whereas polyubiquitination involving Lys48 leads to proteasomal degradation, but ubiquitination has also been implicated in other functions (Wilkinson *et al*, 2005; Fig. III.13). The molecular mechanisms which allow recognition of the various types of ubiquitination signals are not well understood. Covalent binding of ubiquitin moieties generally requires 3 enzymes; an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligase (E3) (reviewed in Ciechanover, 2005).
Many receptors and ion channels are regulated by ubiquitination. For example, Staub *et al* (1997) showed that ENaC subunits are ubiquitinated *in vivo* on lysine residues at the N-terminus and that mutating these residues leads to an increase in channel retention at the plasma membrane and channel activity.

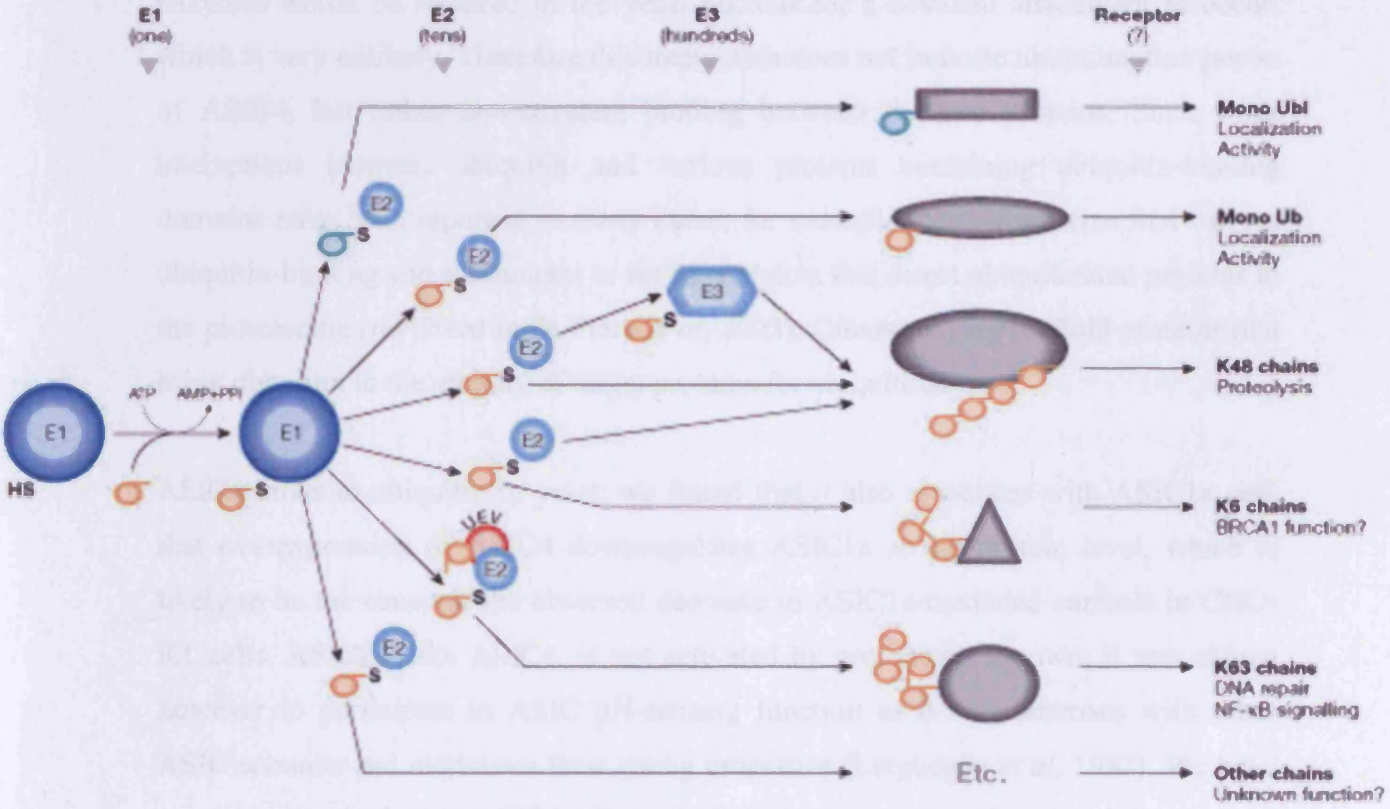

Fig. III.13: **Schematic representation of ubiquitination modifications and their functions** (from Wilkinson *et al*, 2005).

ASIC1a, 2a and 3 all contain a single lysine residue in their N-terminal domains, and ASIC4 contains five; these residues may be potential substrates for ubiquitination. However, the interaction between ASIC4 and ubiquitin detected in the Y2H assay probably represents a non-covalent interaction, as the presence of E1, E2 and E3 enzymes would be required in the yeast nucleus for a covalent attachment to occur, which is very unlikely. Therefore this interaction does not indicate ubiquitination *per se* of ASIC4, but rather non-covalent binding between the two proteins. Such weak interactions between ubiquitin and various proteins containing ubiquitin-binding domains have been reported in many cases; for example, certain proteins that carry a ubiquitin-binding site are thought to act as receptors that direct ubiquitinated proteins to the proteasome (reviewed in Di Fiore *et al*, 2003). Others may be scaffold proteins that bring ubiquitin to the vicinity of target proteins for ubiquitination.

ASIC4 binds to ubiquitin in yeast; we found that it also associates with ASIC1a, and that overexpression of ASIC4 downregulates ASIC1a at the protein level, which is likely to be the cause of the observed decrease in ASIC1a-mediated currents in CHO-K1 cells. ASIC2b, like ASIC4, is not activated by protons on its own; it was shown however to participate in ASIC pH-sensing function as it heteromerises with other ASIC subunits and modulates their gating properties (Lingueglia *et al*, 1997). We have not determined whether ASIC4 affects ASIC1a intrinsic properties, thus it is not clear whether ASIC4 actually heteromultimerises with ASIC1a. However, as discussed below, the downregulation of ASIC1a total protein by ASIC4 points towards a different function for ASIC4 than simply that of an ion channel subunit.

We found indications that ASIC1a may undergo ubiquitination as this channel appears to be degraded in the proteasome. The fact that in the presence of ASIC4 the total amount of ASIC1a protein in the cell is reduced suggests ASIC4 somehow promotes ASIC1a degradation or inhibits ASIC1a expression, at the transcriptional or translational level. Indeed, if ASIC4 was simply involved in retention of ASIC1a in the ER, the total pool of ASIC1a would probably not vary in the presence or absence of ASIC4. We found evidence that ASIC4 may be involved in protein ubiquitination, and these preliminary data raise the hypothesis that ASIC4 may promote ASIC1a

degradation through ubiquitination. The first 21 amino-acids of ASIC4 sequence may be important in this function as overexpression of ASIC4delta, which lacks these amino-acids, may result in impaired ubiquitination. After a closer analysis of the ASIC4 sequence, a consensus site for ubiquitin non-covalent binding, known as CUE domain (Shih *et al*, 2003) was identified at its N-terminus. This motif comprises a proline residue and a di-leucine motif separated by a stretch of around 20 amino-acids. Neither of these features are present in the sequences of the other ASIC subunits. ASIC4delta still contains the 2 leucines but not the proline residue, which may result in partial binding of ubiquitin and thereby less efficient ubiquitination of target proteins. However, this is not compatible with the fact that ASIC4delta retains completely its ability to downregulate ASIC1a-mediated current. Another possibility is that ASIC4 could bind to ubiquitinated ASIC1a and target the channel to the proteasome for degradation. Alternatively, ubiquitin may bind to a different motif in ASIC4 downstream of amino acids 1-21.

In summary, although further experiments are required, the data presented in this section raise the interesting possibility that ASIC4, through its non-covalent binding to ubiquitin and to ASIC1a, might serve as a scaffold which brings ubiquitin in close proximity of ASIC1a for ubiquitination. Alternatively, ASIC4 may bind to ubiquitinated ASIC1a and somehow facilitate its targeting to the proteasome.

ASIC4 seems to be able to inhibit neurite outgrowth in differentiated ND-C cells. The sequence comprising the first 21 amino-acids of the channel appears to play a crucial role in this process, as deletion of this sequence results in elongation of processes equivalent to the control.

As described in section III.1.2.4, two main types of molecules are thought to be involved in remodeling of the cytoskeleton leading to modulation of cell movements: stretch-activated ion channels and components of cell adhesion sites. Stretch-activated cation channels in leech neurons were shown to play a role in neurite growth (Calabrese *et al*, 1999). Therefore it would be interesting to determine whether ASIC4 or ASIC4delta could be activated by mechanical stimulation. Alternatively, as discussed below, ASIC4 may regulate neurite outgrowth through interactions with an accessory

protein involved in cell adhesion, for example via ASIC4 PDZ-binding site, which is deleted in the ASIC4delta construct.

PDZ (PSD95, Disks-large, ZO-1 homologous) domains are ubiquitous sequences of approximately 80 to 100 amino-acids which are the most common mediator for protein-protein interaction. They generally recognise short motifs, PDZ binding sites, often located at the C-terminus of target proteins, often receptors or ion channels; besides PDZ domains can also bind to other structures such as other PDZ domains, or phosphatidylinositol moieties (Harris and Lim, 2001; Jelen *et al*, 2003). PDZ domain proteins are generally associated with the plasma membrane, and often bear several PDZ domains or other signaling domains such as SH3 or LIM domains which allow them to bind to several proteins at the same time. They are indeed thought to have the general role of scaffolding proteins that help assembly of signaling complexes and targeting of their interacting proteins to specific subcellular localisations.

One of the proteins found to interact specifically with ASIC4NT in our Y2H assay is the PDZ domain protein PAPIN (plakophilin-related armadillo repeat protein-interacting, C terminus) or PDZD2. The PAPIN cDNA that was cloned in our screen encodes for the C-terminal 210 amino acids from the 2767 amino acids of the total protein. This fragment contains 2 of the 6 PDZ domains of PAPIN. If the interaction is real it is therefore likely to be mediated by one of these motifs and ASIC4 PDZ binding site. PAPIN's function has not been determined yet but it was detected at cell-cell contact points and several lines of evidence presented below suggest it may inhibit formation of cellular processes and cell motility. Thus, it would be tempting to imagine that ASIC4 could also downregulate neurite outgrowth through interactions with PAPIN.

PAPIN was first identified as a binding partner for delta catenin, a neuron-specific protein of the p120 family of proteins associated with adherens junctions (Deguchi *et al*, 2000). Delta catenin was also located at post-synaptic densities, where it interacts with PSD-95, a scaffolding protein binding to NMDA receptors and to various signaling molecules involved in their regulation. Deletion of delta catenin leads to severe impairment of cognitive functions and synaptic plasticity (Israely *et al*, 2004).

PAPIN contains 6 PDZ domains and is known to bind to delta catenin via the second one. The interaction with ASIC4 is likely to be mediated by one of the last two PDZ domains. PAPIN was first localised at sites of cell-cell contact, supposedly associated with delta catenin (Deguchi *et al*, 2000). Proteins of the p120 family are found at adherens junctions, but also in the cytoplasm where they can bind to actin and tubulin, and in the nucleus where their function is currently unknown; they appear to have different roles at these different subcellular locations (reviewed in Hatzfeld, 2005). When in the cytoplasm, p120 is implicated in the formation of cellular processes, and increase in cell motility, in various cell types, through an inhibition of RhoA GTPase. Similarly, cytoplasmic delta catenin upregulates elongation and branching of neurites in neurons. When at the plasma membrane, p120 is associated with and stabilises cadherins, adhesion molecules that mediate intercellular interactions. These two roles are mutually exclusive; once sequestered near the plasma membrane, p120 stabilises cell-cell adhesion but cannot inhibit RhoA and thereby does not promote growth of processes and cell motility anymore. Delta catenin contains PDZ-binding motifs at its C-terminus and interactions with PDZ domain proteins such as PAPIN are thought to help recruit signaling molecules at sites of cell-cell contact and thereby to regulate adherens junction formation and localisation (Kosik *et al*, 2005).

In transfected ND-C cells, ASIC4 does not seem to be localised at the plasma membrane but follows a punctate, vesicular pattern in the cytoplasm. Fluorescence associated with ASIC4delta seems more diffuse and runs along the cell processes. Following the model where PAPIN may maintain delta catenin at the plasma membrane and thereby inhibit neurite outgrowth, it is possible to imagine the PAPIN/ASIC4 interactions may have a comparable outcome by modifying ASIC4 subcellular localisation. PAPIN is expressed in PC12 cells, which can be differentiated into neuron-like cells, so it is reasonable to assume it could also be present in ND-C cells.

Moesin was also found to interact with ASIC4NT in our Y2H screen. Moesin is a ubiquitous actin-binding protein that links the plasma membrane to the cytoskeleton and is also implicated in cell growth, migration and differentiation (reviewed in Bretscher *et al*, 2002). In cultured neurons, it was shown to play a major role in promoting neurite outgrowth and axonal growth cone formation (Paglini *et al*, 1998).

Therefore a role for ASIC4 in neurite elongation, positively and negatively regulated by an association with moesin and PAPIN respectively, seems to be a very appealing hypothesis.

As mentioned earlier, ASIC1-3 have been shown to be expressed early during embryogenesis. Akopian *et al* (2000) showed that ASIC4 mRNA expression increases during postnatal development in parts of the brain, and in DRG ASIC4 transcript was only observed in adult rats. In contrast, in the spinal cord ASIC4 mRNA levels were higher in newborn than adult rats. These observations suggest that, if ASIC4 is involved in neuronal development, it may play different roles in various parts of the nervous system. Its expression pattern in the brain and DRG suggests it could contribute to inhibition of neurite elongation in adult brain and sensory neurons.
It would be interesting to determine whether ASIC4 expression varies during LTP. One can imagine that a putative downregulation of ASIC4 during LTP could affect this function in two ways: (i) it could result in an upregulation of ASIC1a protein, thus enhancing the channel's impact in this process, and (ii) it could trigger expansion of the synapse, contributing to further strengthening of synaptic transmission during high frequency stimulation. Similarly, downregulation of ASIC4 in neuromas would indicate that ASIC4 could be indirectly involved in axon sprouting after nerve injury.

As described in section III.1.2.4, β subunits of voltage-gated sodium channels (Davis *et al,* 2004) and β and γENaCs (Drummond *et al*, 2005) have been implicated in neurite outgrowth in neurons and PC12 cells respectively. No molecular mechanisms have been proposed to explain these observations, but the ion channel function of ENaC is likely to be important, since its pharmacological blockade leads to an inhibition of neurite elongation (Drummond *et al*, 2005). It is not clear whether an ion channel function is required for ASIC4-dependent regulation of neurite growth, since ASIC4 has not been found to act as an ion channel. The observed inhibition of neurite elongation in ND-C cells is unlikely to be a consequence of ASIC1a downregulation by ASIC4, as ASIC4 and ASIC4delta were found to equally reduce ASIC1a expression.

Moreover Drummond *et al* (2005) showed that deletion of ASIC1a by siRNA in PC12 cells did not affect neurite growth.

III.3.3. Future work

III.3.3.1 Downregulation of ASIC expression by ASIC4

In order to test the theory that ASIC4 may enhance ubiquitination of ASIC1a or may act as a 'receptor' for ubiquitinated ASIC1a and facilitate its access to the proteasome, many experiments still need to be performed.

First, the hypothesis of a potential downregulation of ASIC1a by ASIC4 at the transcriptional level, although unlikely, should be eliminated by quantitative RT-PCR studies.

A confirmation of the interaction between ASIC4 N-terminus and ubiquitin is necessary. Mutation of the putative CUE motif, if it indeed represents the ubiquitin-binding site on ASIC4, would abolish this interaction.

It is also required to show in a direct manner ubiquitination of ASIC1a, for example by co-expressing the channel and ubiquitin and monitoring the presence of high molecular weight proteins bearing ASIC1a-associated immunoreactivity. Mutating the lysine residue present at the N-terminus of ASIC1a should prevent ubiquitination.

It would then be necessary to determine whether, in the presence of ASIC4, ubiquitination of ASIC1a is upregulated as well as ASIC1a proteasomal degradation. This would indicate that ASIC4, by binding ASIC1a and ubiquitin, may be a scaffold that promotes ASIC1a ubiquitination by bringing the two molecules together. If on the other hand ASIC4 is a 'receptor' for ubiquitinated ASIC1a, ASIC1a degradation in the proteasome should increase in the presence of ASIC4 and mutating the ubiquitin binding site on ASIC4 should abolish ASIC4-dependent downregulation of ASIC1a, but an increase in ubiquitination of ASIC1a itself should not be observed.

We showed that a cell line derived from DRG neurons, ND-C, expresses ASIC1a and ASIC4. It would be interesting to downregulate ASIC4 in this cell line by siRNA and study the effect this has on ASIC1a ubiquitination and on ASIC1a-mediated current.

This system offers the advantage of not depending on over-expression, and provides an easier system of study than neurons. Ultimately, if this approach is successful we will study the effect of the downregulation of ASIC4 on transient proton-gated currents in neurons. It will then be interesting to identify factors that regulate ASIC4 expression, either acutely or in long term during development, and in this way modulate proton-gated currents in neurons.

Finally, a potential role of ASIC4 in regulating the other ASIC subunits, other ion channels or various other proteins expression would be appealing and could be the next step of the study.

III.3.3.2. Neurite outgrowth in ND-C cells

Downregulation of ASIC4 by siRNA in differentiated ND-C cells would provide direct answers regarding the putative role of ASIC4 in neurite elongation. To study a potential involvement of PAPIN and moesin, the binding sites for these proteins on ASIC4 will have to be determined; mutations in these sites should abolish binding to ASIC4 and its putative effect on ASIC4-dependent regulation of neurite elongation. A putative activation of ASIC4 and ASIC4delta by mechanical stimuli would also be worth investigating.

Finally, tissue-specific deletion of ASIC4 in mice, for example in DRG neurons, would provide crucial information about the putative role of ASIC4 in the development of sensory neurons and in regulation of other ASIC subunits or potentially other proteins *in vivo*.

CHAPTER IV

CONCLUSIONS AND PERSPECTIVES

In this project, a genetic screen has been implemented in order to identify molecular partners of ASIC channels in sensory neurons. Several studies had previously reported the identification of proteins interacting with ASIC intracellular C-terminal domains, whereas this work describes molecules binding to the N-terminus of the channels. A total of 86 different proteins were selected as potential interactors for one or more ASIC subunits. The diversity of the pathways in which these proteins are involved suggests a wide range of new regulatory mechanisms and new functions for ASICs; they indicate for example a potential regulation of ASICs by endocytosis and ubiquitination, a possible functional association between ASICs and G-protein-coupled receptors, and direct and indirect links between ASICs and the actin cytoskeleton.

Functional studies showed that the annexin II light chain p11 is a key element in ASIC1a targeting to the plasma membrane. On the basis of these results, a p11 null mouse could be exploited to examine the implications of this regulation on transient proton-gated currents in neurons. However, behavioural effects in inflammation or in CNS functions in these mice would be difficult to attribute to the sole downregulation of ASICs. p11 has been shown to promote cell surface targeting of other ion channels and receptors, such as $Na_v1.8$ or $5\text{-}HT_{1B}$, which are also involved in these functions, thus a p11 knock-out mouse would be expected to have a confounded phenotype resulting from disruption of these various interactions.

A role for ASIC4 was determined in the regulation of expression of other ASIC subunits; preliminary experiments suggested this may result from an involvement of ASIC4 in protein ubiquitination or in targeting of ubiquitinated proteins to the 26S proteasome. Additionally, our results indicated a possible role for ASIC4 in the inhibition of neurite outgrowth in a neuronal cell line. Although very exciting, as no function had been previously reported for ASIC4, these data require further investigation (presented in section III.3.3). The downregulation of ASIC currents by ASIC4 could have important functional implications *in vivo*. In DRG, ASIC4 transcript is found only in adults, and in the brain, its levels increase during postnatal development. In contrast, the other ASIC subunits are expressed early during

embryogenesis. A decrease in ASIC1-3 transcript expression during development has not been reported; however, as ASIC4-dependent downregulation of ASICs is likely to occur post-translationally, it would be interesting to investigate a potential complementary pattern in ASIC4 and ASIC1-3 protein expression during development. As discussed in section III.1.2.4, the association between ASIC1-3 and various proteins involved in actin-binding or actin remodelling suggests a possible role for ASIC1-3 in neuronal growth. On the other hand, preliminary results indicate that ASIC4 may have an inhibitory effect on neurite elongation. Therefore it would be tempting to imagine positive and negative roles in these processes for ASIC1-3 and 4 respectively, the action of ASIC4 resulting from ASIC1-3 downregulation. However, the observation that ASIC1a does not have an effect on neurite outgrowth in PC12 cells does not support this hypothesis, although ASIC2 and 3 could still be involved. Alternatively, ASIC4-dependent inhibition of neurite elongation may result from a totally different mechanism, for example from interactions with accessory proteins such as PAPIN or moesin. If our *in vitro* data are confirmed, deletion of the ASIC4 gene could prove a useful tool to study the role of this protein in neuronal development *in vivo*, as well as the implications of the downregulation of ASIC-mediated currents, for example in models of inflammation.

The functional implications of the interactions between ASICs and the remainder of the proteins selected in the Y2H screen have yet to be determined. The large number of clones renders the task difficult, but in some cases key experiments could rapidly open new perspectives concerning ASIC functions. For example, as ASICs were found to interact with G protein β subunits, a putative link between ASICs and GPCRs could be examined by studying the effect of overexpression of these β subunits, or the effect of G proteins activators on ASIC-mediated currents in neurons. Putative endocytosis of ASICs could be monitored by means of an antibody directed to the extracellular domain of the channels, in response to various ligands, such as protons, neurotransmitters or inflammatory mediators. These studies provide exciting and challenging prospects for the future of research on ASICs.

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