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Systematic Studies of Kir and TRP Channel mRNAs in the Norepinephrenergic Neurons of the Locus Coeruleus

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SYSTEMATIC STUDIES OF KIR AND TRP CHANNEL MRNAS IN THE

NOREPINEPHRENERGIC NEURONS OF THE LOCUS COERULEUS

by

SAKUNTALA JYOTHIRMAYEE TADEPALLI

Under the Direction of Chun Jiang

ABSTRACT

Neurons in the Locus coeruleus (LC) play an important role in the central CO_2 chemosensitivity. However, the molecular mechanisms for neuronal CO_2 chemosensitivity remain unclear. To demonstrate the expression of pH/CO_2 sensitive ion channels, we screened the inward rectifier K^+ channels (Kir) and transient receptor protein (TRP) channels, as parallel studies in this lab suggested that certain Kir and TRP channels are involved in neuronal responses to high levels of CO_2. Our results showed that several members of the Kir and TRP channel families were robustly expressed in the LC neurons at the mRNA level. Of particular interest are TRPC5, Kir4.1 and Kir5.1 channels that are all pH-sensitive. The rich expression of various pH-sensitive Kir and TRP channels suggests that these ion channels are likely to play a role in the chemosensitivity of LC neurons.

INDEX WORDS: Norepinephrine, Dopamine β-hydroxylase, Brainstem, Locus coeruleus, Intrinsic membrane properties, Breathing rhythm, CO_2 chemosensitivity.

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ABBREVIATIONS

cDNA   complementary DNA

CO_2 carbon dioxide

DMSO   dimethyl sulfoxide

GAPDH   glyceraldehyde 3-phosphate dehydrogenase

PCR   polymerase chain reaction

qPCR   quantitative real-time PCR

RQ   relative quantitation

RT-PCR   reverse transcriptase polymerase chain reaction

scPCR   single cell PCR

TM   transmembrane segment

TMD   transmembrane domain

TRP   transient receptor potential channels

WT   wild type

Kir   Inward rectifier potassium channels

LC   locus coeruleus

PBC   preBotzinger complex

NK1R   neurokinin 1 receptor

PPE   preproenkephalin

LFT   long term facilitation

FN   facial nucleus

NTS   Nucleus of solitary tract

rVRG   rostral ventral respiratory group

NA   nucleus ambiguus

LRN   lateral reticular nucleus

RTN   retrotrapezoid nucleus

NE   norepinephrine

DAG   diacylglycerol

1. INTRODUCTION

Breathing is a vital function controlled autonomically and voluntarily by several brain areas. Central breathing activity is a continuous process starting from the stage of utero. The control and regulation of breathing relies on the neural networks especially those in the brainstem. The connections in the neural networks are changed according to the age and maturity. Such a complex behavior is responsible for the maintenance of stable levels of CO_2, O_2 and pH as well as the regulation of speech, singing, body postures, etc.

There are three critical aspects of breathing, i.e., rhythmicity, plasticity and chemosensitivity. Rhythmicity is the regular pattern of breathing. Plasticity is the adaptive behavior of breathing according to the changes in the environment like altitude, pregnancy and disease. Chemosensitivity is the regulation of the breathing in the neural networks according to the changes in the levels of CO_2, O_2 and pH (Feldman et al., 2003).

1.1. Rhythmicity

The breathing rhythm generation relies on neural networks and the pacemaker properties of individual neurons. The brain stem neurons are crucial for the rhythm generation. The neural kernel for the rhythmicity is located in the rostral ventrolateral medulla in an area called preBotzinger complex (PBC)(Smith et al., 1991). The lesioning of the PBC region in vitro resulted in abolishing the rhythmicity. The disruption of the local synaptic transmission of PBC also disrupts the activity of rhythm generation (Bongianni et al., 2002; Fung et al., 1994; Gray et al., 1999; Solomon et al., 1999). The

distinct patterns of discharge in the PBC neurons are important for the rhythm generation for breathing. The absence of clear making for the PBC hindered the clear understanding of its involvement in the rhythmicity. The expression of neurokinin 1 receptor (NK1R) in the ventral respiratory column in the rodent proposed the identification of PBC neurons (Gray et al., 1999). These neurons in the region are further divided depending upon preproenkephalin (PPE) expression. The neurons with PPE are bulbospinal cells which are larger than the neurons without PPE which are propriobulbar and more rostral (Guyenet et al., 2002). The bulbospinal neurons are not involved in the rhythm generation, indicating that the PBC region contains mostly propriobulbar neurons with neurokinin 1 receptors (Makeham et al., 2001). The pacemaker neurons in the PBC region do not affect the normal rhythm generation (Del Negro et al., 2002). The overlapping of different patterns of neural networks in the PBC region is responsible for different patterns of respiration like eupnea, sighing, gasping, etc. (Lieske et al., 2000; Ramirez et al., 2002). These different respiratory patterns are produced because of articulation of synaptic and intrinsic properties in PBC region (Rekling et al., 2000).

1.2. Plasticity

Plasticity is the property involved during the conditions like changes in the levels of O_2, hypoxia, hypercapnia, conditioning, neural injury (Cheng et al., 2002; Gozal and Gozal, 2001; Mitchell and Johnson, 2003; Powell et al., 1998). The changes in the environmental conditions, aging, and disease are the conditions where the

neuroplasticity plays an important role. Neuroplasticity requires the activation of different receptor complexes like serotonin, dopamine and norepinephrine (Baker et al., 2001; Huey et al., 2000a; Huey et al., 2000b; Kinkead et al., 2001; Mitchell et al., 2001). Serotonin is involved in neuroplasticity in different conditions like hypercapnic exercise, intermittent hypoxia, chemoafferent denervation (Forster, 2003; Forster et al., 2000; Golder et al., 2001; Ling et al., 2001; Mitchell and Johnson, 2003; Prabhakar, 2001). Serotonin receptor complex is involved in long term facilitation (LFT) of respiration (Bach and Mitchell, 1996). This involvement depends upon different conditions like age, previous experience and gender. The prior experience shows the property of metaplasticity in breathing (Kinkead et al., 1998). The response of LFT also depends upon the arterial CO_2 levels (Janssen and Fregosi, 2000).

1.3. Chemosensitivity

The chemosensitivity of breathing is regulated by chemoreceptors which sense the changes in O_2, CO_2, and pH (Ballantyne and Scheid, 2001). The O_2 chemosensitivity is regulated in carotid bodies that are located at the fork of the carotid arteries. Although the CO_2/pH chemoreceptors are found in the carotid bodies, most are located in the brain stem known as central chemoreceptors. The central chemoreceptors are extremely sensitive to the minor changes in the levels of CO_2/pH. The changes are indicated by the changes in the acid-base levels in the blood and the brain (Nattie and Li, 2008). There are various locations in the brain which regulate the chemoreception. Some of sites include fastigial nucleus (FN), locus coeruleus (LC), Nucleus of solitary tract (NTS), rostral ventral respiratory group (rVRG), nucleus

ambiguus (NA), lateral reticular nucleus (LRN), and retrotrapezoid nucleus (RTN).

The changes in intracellular and extracellular pH are the key aspects of central chemoreception. Some motor neurons that are modulated by respiration are sensitive to the extracellular pH (Bayliss et al., 2001) whereas the neurons located in LC and medullary raphe nuclei are involved in sensing intracellular levels of pH (Filosa et al., 2002; Wang et al., 2001b). Amino acids like histidine present in the proteins are involved in sensing the pH (Jiang et al., 2001). There are many locations that sense the levels of pH like low resistance gap junctions with TASK channels and inward rectifying K^+ channels and pH sensitive ion transporter proteins (Bayliss et al., 2001; Dean et al., 2001; Jiang et al., 2001; Solomon, 2003; Spengler et al., 2001). These various locations sense the changes in the pH simultaneously. The lower brain is the major location for the central chemoreception (Ballantyne and Scheid, 2001; Li et al., 1999; Nattie and Li, 2002; Solomon, 2003). The response of the neurons to the increased levels of CO_2 indicates the role of chemosensitive neurons. The chemoreceptor sites work together to produce increased ventilation with the increase in the levels of CO_2. The increase in the ventilation by 120% with the increase in the levels of CO_2 at low intensity show that most of the chemoreceptor sites work simultaneously which produced overall high sensitivity to CO_2 levels (Li and Nattie, 2002). Some of the neurons in the PBC site are also involved in chemoreception (Gray et al., 2001). The chemoreception is coupled to the breathing rhythmicity. The serotonergic and noradrenergic medullary neurons are involved in different functions like neuroplasticity and chemoreception. The serotonergic neurons play a role in the development of motor neurons involved in respiration and neuronal growth.

1.4. LC neurons as chemosensitive site in the brainstem
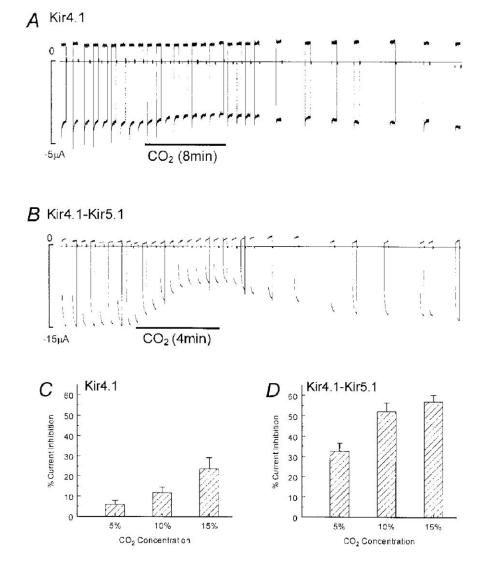
The noradrenergic neurons present in the LC expressing c-fos, increases breathing by focal acidosis and the neurons are excited by CO_2 (Haxhiu et al., 2001). The LC is the major source of noradrenergic neurons located in the dorsal pontine region of brain stem adjacent to the fourth ventricle. The LC neurons are involved in regulating the cardiovascular function during hypercapnic conditions which causes the increase in the arterial blood pressure. The noradrenergic neurons present in the LC form the highest group of neurons that are excited by high levels of CO_2/pH. Among the 80% of the noradrenergic neurons present in the LC region 64% of them responded to levels of CO_2. This indicates the involvement of LC neurons in breathing. The change in the levels of intracellular pH increases the firing rate of the LC neurons. The change in the extracellular pH along with the intracellular pH modulates the firing activity in the LC neurons. There are many signals that cause the chemosensitivity of LC neurons along with the change in the pH. The chemosensitivity of LC neurons in turn affect multiple targets. This will cause the increase in the firing activity in the LC neurons. The norepinephrenergic neurons present in the LC region extend their projections to other parts of the brain like frontal cortex, cerebellum, hippocampus, spinal cord and brainstem (Berridge and Waterhouse, 2003; Dunn et al., 2004). The disruption of the intrinsic properties of the LC neurons during diseased conditions is caused due to changes in NE levels (Zhang et al., 2010). The firing activity of LC neurons during hypercapnic conditions is because of the presence of different ions channels and their responses.

1.5. Inhibition of Inward rectifier K$^+$ channels

The inhibition of K$^+$ channels produce depolarization. There are various categories of K$^+$ channel members that are present in the brain. The different categories of K$^+$ channel members include calcium activated K$^+$ channels, inwardly rectifying K$^+$ channels (Walder et al.), voltage gated K$^+$ channels and tandem pore domain K$^+$ channels. A group of K$^+$ channels that are mostly present in the neurons are the inwardly rectifying K$^+$ channels (Walder et al.). The Kir channels preferably move K$^+$ into the cell rather than out of the cell in the absence of extracellular Na$^+$. The name inwardly rectification indicates that the channel passes positive charge more easily in the inward direction. These channels have a role in establishing the resting membrane potential of the cell. Kir channels exist as tetramers and align to form a pore region to allow the passage of ions. Each subunit of the tetramer consists of two transmembrane domains (TM1, TM2). The subunits can form either homodimers or heterodimers. The constitutively active K$^+$ channels (Kir2.x), the G-protein coupled receptor Kir channels (Kir3.x), kir channels (Kir6.x) that are involved in cellular metabolism and sensitive to ATP and K$^+$ transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x). The members of each subfamily have identical amino acid sequence. Kir channels are commonly found to be present in the endothelial cells, neurons, cardiac myocytes and kidneys. Their roles vary according to the cell type in which they are present. Disruption of the channel function due to mutations or blockers can lead to severe pathologies. Kir5.1 is often co-expressed with Kir4.1 as Kir4.1/Kir5.1 heterodimers. These heterodimers are present in the glomeruli and neocortex of the olfactory bulb. Because of the varied channel

properties, each member of the Kir channel has a different physiological role. There are different members of Kir channels that are expressed in the LC neurons. These Kir channels are expressed in different locations of brainstem including LC.
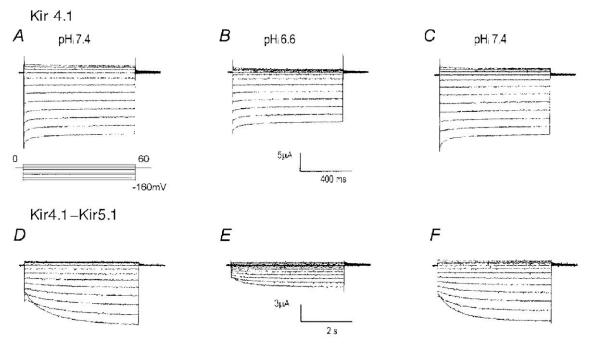
The previous studies show the inhibition of Kir4.1/Kir5.1 at high levels of CO_2. The Kir4.1 and Kir5.1 were microinjected into the Xenopus oocytes and were incubated for 2-3days for the channel expression. The different levels of CO_2 show the inhibition of channel members at high levels of CO_2 (figure 1) (Xu et al., 2000).

A Kir4.1

0

$-5\mu A$

CO$_2$ (8min)

B Kir4.1-Kir5.1

0

$-15\mu A$

CO$_2$ (4min)

C Kir4.1

% Current Inhibition

5%   10%   15%

CO$_2$ Concentration

D Kir4.1-Kir5.1

% Current Inhibition

5%   10%   15%

CO$_2$ Concentration

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Figure 1: The expression levels of Kir4.1 and Kir5.1 channel currents.
   The A and B show the inhibition of Kir 4.1 and Kir4.1-Kir5.1 channel currents at high levels of CO$_2$ respectively. The C, D shows the % current inhibition to that of CO$_2$ concentration of Kir4.1 and Kir4.1-Kir5.1 respectively.
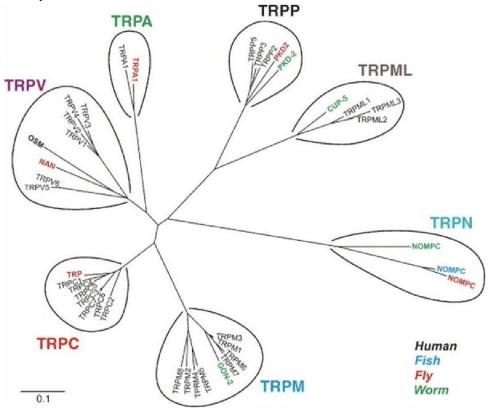
Figure 2: The figure shows the inhibition of Kir4.1 and Kir4.1-Kir5.1 channel currents at low levels of pH.

The decrease in the intracellular pH produced by hypercapnic conditions cause the inhibition of various Kir channels like homomeric Kir 4.1 channels and heteromeric Kir4.1-Kir5.1 channels (figure 2) (Xu et al., 2000).

1.6. Activation of Transient Receptor Potential channels (TRP)

The activation of cationic channels also produces depolarization. One of the most interesting diversified superfamily of cationic channels are Transient Receptor Potential (TRP) channels. The TRP channels are divided into seven families depending upon the homology of their sequences (Clapham, 2003; Corey, 2003; Montell et al., 2002) (Corey, 2003) (Clapham, 2003).The TRP super family is divided into two groups, group 1 and group 2 (Palmer et al., 2001) (Denis and Cyert, 2002). The group 1 of TRP superfamily consists of TRPA, TRPC, TRPM, TRPN, TRPV channels whereas the group 2 consists of TRPML and TRPP channels. The cation selective ion channels are formed by the homo or hetero-tetramer assembly of different channels. The selectivity and permeability of different cations by the ion channels depends on the assembly of the channels. The TRP channels are recognized to be important because of their role in responses to external stimuli like light, chemicals, temperature and sounds. These TRP channels are expressed in almost all multicellular organisms including yeast. The TRP like protein expressed in yeast vacuole is recognized as TRPY which is also called *yvc1* (Palmer et al., 2001). The families of TRP channels share some common features like the six putative transmembrane domains and a pore region in between fifth and sixth segments of transmembrane domain. The study of mutated Drosophila in which the

transient response to light was observed which led to the discovery of *trp* gene. The study of the trp gene in different multicellular organisms led to the formation of TRP superfamily.

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Figure 3. The figure shows the phylogenetic tree of TRP superfamily.

TRPA family has only one member -TRPA1. The locations of TRPA channel are hair cells (Corey et al., 2004), dorsal root ganglion and trigeminal ganglion. The TRPA1 protein is characterized by the presence of 14 N-terminal ankyrin repeats because of which it is called ANKTM1 protein (Story et al., 2003). These proteins are mostly located in the stereocilia of hair cells and are found to be involved in mechanosensing. These channels are Ca^{2+} dependent and are voltage dependent to some extent. The TRPA1 gene has some similarity with TRPM8 gene. The TRPA1 channels are also involved in sensation of cold along with TRPM8 channels (McKemy, 2005).

The TRP channel family members that are closely related to Drosophila TRP channels are TRPC channel members. There are seven TRPC channels which are grouped into 4 categories. TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5 are the categories of TRPC channels. They share a common feature of TRP box EWKFAR sequence near the C-terminal and 3-4 ankyrin repeats near the N-terminal (Zimmer et al., 2000). The TRP channels are non-selective to cations and are highly permeable to Ca^{2+} compared to Na^+. The permeability depends upon the complexity of the ion channels where they form heterotetramers or homotetramers (Hofmann et al., 2002) (Strubing et al., 2001). The TRPC heterotetramers are mostly formed by TRPC1 and TRPC4/ TRPC5, TRPC4 and TRPC5 subfamilies. Some channels of TRPC are activated by phospholipase C (Hofmann et al., 1999) and some are found to be activated by diacylglycerol (DAG) (Venkatachalam et al., 2002). The TRPC1 is expressed ubiquitously in multicellular organisms. It is found to be activated by different mechanisms and it is the channel which is mostly activated by store operated Ca^{2+} entry channels. The TRPC1 channel is insensitive to thapsigargin (Lintschinger et al.,

2000) and diacylglycerol (Yuan et al., 2003). TRPC3 channel is highly expressed in brain and is also present in smooth and cardiac muscles (Clapham, 2003). It is one of the channels which is stimulated by DAG and is also activated by T-cell receptor (Wedel et al., 2003). The TRPC3 channel is present in Na^+/Ca^{2+} exchanger (Rosker et al., 2004). TRPC4 and TRPC5 channels are expressed in brain and are activated by phospholipase C (Schaefer et al., 2000). High expression of TRPC6 is found in brain (Jia et al., 2007; Li et al., 2005; Zhou et al., 2008b) and lungs whereas the TRPC7 channel is expressed in kidney and pituitary gland (Berg et al., 2007). The TRPC6 and TRPC7 channels are closely related to each other.

The TRPV channels also called vanilloid channels are found to be mostly involved in nociception. There are six TRPV channels TRPV1, TRPV2, TRPV3, TRPV4, TRPV5, and TRPV6. The expression levels of TRPV1 and TRPV2 channels are high expressed in spinal and peripheral nerve terminals and they are found to be sensitive to temperature and non-selective to cations (Planells-Cases et al., 2005). The vanilloid compounds like capsaicin and higher temperatures activate TRPV1 whereas TRPV2 is only activated by harmful temperatures (Caterina et al., 1999). The TRPV1 and TRPV2 channels are present in the brain (Caterina et al., 1999; Kowase et al., 2002; Steenland et al., 2006). The translocation of TRPV2 to the plasma membrane occurs when the channel is activated (Iwata et al., 2003; Kanzaki et al., 1999; Nagasawa et al., 2007). The TRPV3 channels are Ca^{2+} activated cationic channels which are sensitive to warm temperatures (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). The repeated activation of TRPV3 channels will cause sensitization. TRPV4 channels are also activated by warm temperatures. TRPV4 channels are modulated by

CaM and ATP. The TRPV4 channels present in the hypothalamus, skin and primary sensory neurons are involved in sensing warm temperatures. The TRPV4 knockout mice express damaged bladder function. The other functions regulated by TRPV4 include vascular tone, bone deposition and remodeling.

The abundance of TRPV6 is much higher than TRPV5. These two members of TRPV family are inwardly rectifying cationic channels and are insensitive to heat and the highly permeable to Ca^{2+}. These channels are sensitive to various secondary messengers like CaM, ATP, Mg^{2+} and protein kinases. The TRPV family members are expressed in kidneys. TRPV5 channels are crucial in active reabsorption and transcellular transport of Ca^{2+} in the distal convoluted loop and connecting tubules of kidneys. The TRPV6 reabsorbs Ca^{2+} in various areas of kidneys like cortical and medullary ducts of nephron and convoluted tubules.

TRPM family consists of eight channel members which are grouped according to their amino acid sequence similarities. TRPM1 and TRPM3 belong to a group where both of these channel members are outwardly rectifying channels. These two channel members are expressed in brain whereas TRPM1 is also present in melanocytes and retina and TRPM3 is also expressed in pituitary and kidney (Oancea et al., 2009). TRPM2 channel is expressed in neurons and mutations in this channel causes neurodegenerative disorders (Kaneko et al., 2006; Olah et al., 2009). The levels of intracellular Ca^{2+} and oxidative stress activate TRPM2 channel. TRPM2 channel has a considerable role in monocytes chemotaxis (Hara et al., 2002). The only channel members of TRPM family that are selective to monovalent ions are TRPM4 and TRPM5 channels (Launay et al., 2002). These channels produce outwardly rectifying

current. The decrease in the intracellular pH inhibits TRPM5 channel whereas the increase in the levels of ATP inhibits TRPM4 channels. The expression levels of TRPM4 channels are considerable in brain and kidney whereas TRPM5 channels are mostly involved in taste reception (Perez et al., 2002; Reading and Brayden, 2007). TRPM6 and TRPM7 channels have protein kinase activity along with ion channel activity. These are outwardly rectifying channels inhibited by Mg^{2+} (Schlingmann and Gudermann, 2005). The extracellular acidic pH potentiates these channel members. The TRPM6 channels are highly expressed in kidneys and intestine where they play a considerable role in the reabsorption of Mg^{2+} in these areas. The role of TRPM6 in the closure of neural tube during development is critical (Walder et al., 2009). TRPM7 is mostly expressed in vascular smooth muscles and in sympathetic neurons where they have a considerable role in the release of neurotransmitters (Li et al., 2007). TRPM8 channels are activated by cold temperatures and are expressed in many tissues (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). The TRPML channels are involved in compartment trafficking and are expressed intracellularly (Kim et al., 2009). Most of the TRP channel currents are modulated at acidic pH conditions.

1.7. Significance

The current study was performed to test the expression of the pH/CO_2-sensitive Kir and TRP channels in the LC neurons. Our results showed that several members of these two ion channel families were expressed in the LC neurons. The presence of these pH sensitive ion channels in the cells suggests their contributions to CO_2 chemosensitivity.

2. MATERIALS AND METHODS

2.1. Acute Dissociation of LC Neurons

LC-containing brain slices were obtained from C57BL/6 mice at age of 2-4 weeks as described above (Zhang et al. 2010). The slices were then digested at $35^{o}C$ for 30min-45min with papain (0.25%, type XI, Sigma) in oxygenated dissociation buffer containing (in mM) 140 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 25 D-glucose, and 10 HEPES, pH 7.40. The slices were transferred to oxygenated dissociation buffer containing 1 mg/ml papain inhibitor and washed twice with dissociation buffer. The LC area was micropunched and gently triturated in dissociation buffer with fire-polished Pasteur pipettes. The dissociation buffer containing triturated LC was dropped into 35-mm petri dishes and kept at room temperature for 10 min before being observed with Hoffman modulation optics. Individual neurons were harvested with patch pipettes and put into eppendorf tubes with solution containing 10× RT buffer, RNase free water and RNase OUT (4.5:4.5:1). The neurons were immediately frozen with liquid nitrogen and kept at -$80^{o}C$ for further experiments.

2.2. Reverse transcription PCR and quantitative real-time PCR

The LC regions were obtained by micro puncture of the pontine sections from WT mice. The tissue obtained from two mice was homogenized for 2min in solution provided in the RNeasy mini Kit (Qiagen). The total mRNAs from the tissue was extracted according to the manufacturer's instructions (Qiagen). The concentration of the mRNA obtained was determined using spectrophotometer (absorption at 260 nm and 280 nm). The first strand of cDNA from the total mRNA was synthesized with

random hexamers as primers. The cDNAs obtained were used either for quantitative

PCR (qPCR) or regular PCR. The qPCR primers were designed for the target genes

using Primer3.0 software (Applied Biosystems, Warrington, UK). The qPCR was

performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on the ABI

PRISM 7500 (Applied Biosystems) in the fast mode for the amplification of cDNAs

according to the manufacturer's instructions with 500nM prime. The endogenous

control used in the quantitation of target genes was a house-keeping gene, GAPDH.

The endogenous reference gene was run parallel with the targeted genes. Each gene

was performed in quadruplets obtained from WT animal. The ΔCT method (where CT is

threshold cycle) and GeneAmp 5700 SDS software were used for obtaining the data.

The expression levels of the targeted genes were normalized to that of GAPDH (ΔCT).

The same set of primers was used for both qPCR and regular PCR with the difference

in concentrations (Zhang et al. 2010). The other set of qPCR experiments were

performed with specific primers.

Table 1. List of RT-PCR primers.

Target gene	Primer Sequence	Accession No.
TRPC1	Fw: TGCAGATTTCAATGGGACAGAT	NM_011643
	Re: CGTGAAGGAAAACAGAACAAGGA	
TRPC3	Fw: GAAAGACACGGGCACAAAGC	NM_019510
	Re: GGGTGAGCCACAAACTTTTTG	
TRPC4	Fw: TTTCCACGTTATTCGAGACACTACA	NM_016984
	Re: CGTGCTGGGCTTTGACATT	
TRPC5	Fw: AAGGACCTCCCCCAACTGTT	NM_009428
	Re: CACCATCCCACATTTCCTTTATC	
TRPC6	Fw: TTCAGTAACCCTCCAAGACAATATCA	NM_013838
	Re: TTATCAATCTGGGCCTGCAAT	
TRPM2	Fw: CCGGAGAGGCTTGGTCAA	NM_138301
	Re: CTGCTTCATCACGCCTGTGT	
TRPM4	Fw:GCGCCTGCTGCACATCTT	NM_175130
	Re:CAAGCCACACGCAGAGGAA	
TRPM5	Fw: GGAGCCAGACAGCTTGGATATG	NM_020277
	Re: CTTGGTGCCTCTGTTAGCTTCTC	
TRPM6	Fw: TCAAGAGCCTCACCACCATTATT	NM_153417
	Re: TTGGTGTAAACGGTATGGTCTGA	
TRPM7	Fw: GACGATGGAGGATAGTGAAAACAA	NM_021450
	Re: CCATCACTGCTGTCCAAAATCTT	
TRPM8	Fw: CCCTGGCCAAAGTTAAGAATGAT	NM_134252
	Re: CACTGCTCGGGTCTCATATTCA	
TRPV1	Fw: GGCGACCATCCCTCAAGAG	NM_001001445
	Re: GTACCACAGACACCAGCATGAAC	
TRPV2	Fw:CCAGCCATTCCCTCATCAAA	NM_011706
	Re: ATACCCCCAAGCAGGATCAGA	
TRPV3	Fw: GAGACACCCCTGGCTTTGG	NM_145099
	Re: GGAAGTGATGTCTGTCTGCTCATT	
TRPV4	Fw: GAGAAAGGTCGTGGAGAAGCA	NM_022017
	Re: GCCGATTGAAGACTTTGAGGAT	
TRPV5	Fw: GAACACCACCAGGAGCAGAATC	NM_001007572
	Re: TAGCTGCTCTTGTACTTCCTCTTTGT	
Kir 2.2	Fw: CATTACAGCCCCAAGCCTCA	NM_010603
	Re: GCCCGTCCTCCTCTGATGA	
Kir 4.1	Fw: TGCCCCGCGATTTATCA	NM_001039484
	Re: GGGCGGCTCTCTGTCTGA	
Kir 5.1	Fw: GCTTCAGCTATTTTGCCCTCAT	NM_010604
	Re: ACCGTGCCCTCTACCACATG	
Kir 6.2	Fw: CGGAGAGGGCACCAATGT	NM_010602
	Re: AAAGGAAGGCAGATGAAAAGGA	

2.3. Polymerase Chain Reaction

The polymerase chain reaction was performed to study the expression levels of the different TRP channels. The PCR reaction mixture contained 1.25μl dNTP, 2.5μl of DMSO (Dimethyl sulphoxide), 4μl MgCl2, 10μl of 5X green GoTaq Flexi byffer, 0.25μl Taq Polymerase enzyme (5U/μl), 2μl primer mix (1.0μg/μl), 2μl of template, 28μl of ddH2O for two tubes. The thermal cycling included initial activation at 95°C for 2min followed by denature, annealing and extension at 95°C for 30sec, 60°C for 30 sec and 72°C for 2 sec respectively. The final extension was at 70°C for 10min and the number of cycles was 36.

2.4. Single cell PCR

scPCR was performed for LC neurons obtained by acute dissociation as described above. Two sets of primers were designed for the targeted genes using Primer3.0 software. The first PCR was performed using the One Step RT-PCR kit (Qiagen) for obtaining the cDNAs from the LC neuron cells. The second PCR was performed using Hotstar Taq DNA polymerase (Qiagen). The One Step RT-PCR reaction mix contained 10μl of 5X OneStep RT-PCR Buffer (pH 8.7), 10 μl of 5X Q-solution, 2 μl of dNTP mix (containing 10mM of each dNTP), 1μl of primer mix (1.0μg/μl), 2 μl of OneStep RT-PCR Enzyme Mix (pH 9.0), 15μl of RNase Free H_2O and finally 10μl of template was added to the mixture. The thermal cycling conditions included 30min of reverse transcription at 50°C followed by initial PCR activation at 95°C for 15min. The three step cycling included denature and annealing at 94°C for 45

sec and 53°C for 45 sec respectively followed by extension at 72°C for 1 min and the

final extension at 72°C for 10 min. The number of cycles performed was 30. The

primers used were specific for the targeted genes. The Hotstar PCR reaction mixture

contained 5µl of 10× PCR buffer, 1 µl of dNTP mix (containing 10mM of each dNTP),

0.5µl of HotStar Taq DNA Polymerase, 10µl of 5X Q-solution, 1µl primer mix

(1.0µg/µl), 0.5µl of template (cDNA) and made to 50µl with ddH2O. The thermal

cycling included 40 cycles with initial PCR activation at 95°C for 15min followed by

denature, annealing and extension steps at 94°C for 45sec, 53°C for 45sec and 72°C

for 1min respectively. The final extension was at 72°C for 10min.

Table 2. List of single-cell PCR primers.

Target gene	Primer Sequence	Accession No.
TH	Fw: TTGGAGGCTGTGGTATTC	NM_009377
	Re: GAAGTGAGACACATCCTCC	
TH_NEST	Fw: GAAGCCAAAATCCACC	NM_009377
	Re: CCAGGTGGTGACACTTATC	
Kir4.1	Fw: TAAGAAGAGGGCCGAGAC	NM_001039484
	Re: CAGACGTTGCTGATGC	
Kir4.1_NEST	Fw: GTGGCTTCCCATAACG	NM_001039484
	Re: TTTAAGGGGCTGGTCTC	
Kir5.1	Fw: AAGAGAGCCCAGACCATAC	NM_010604
	Re: CTCACAGCTGCTCACCA	
Kir5.1_NEST	Fw: CGATGGCGTTTAAAGACC	NM_010604
	Re: CATCATGAAACCTGTGGC	
TRPC4	Fw: AGGCTGGAGGAGAAGACACT	NM_016984
	Re: TAGCAGCACGAGGCAGTAGA	
TRPC4_NEST	Fw: CACACAGCCTCCTATCTGAC	NM_016984
	Re: TCAAGGAGATTGTTGCCAGA	
TRPC5	Fw: TCCCAGCAATGTAAGCTC	NM_009428
	Re: CTTGACATAGGCCACGAT	
TRPC5_NEST	Fw: CAGAAGTATCATGACCTGGC	NM_009428
	Re: GGTTGCTTCTGGGTGAG	
GFAP2	Fw: CAAGCACGAAGCTAACG	NM_010277
	Re: CCCTTCCAATTCTAACCC	
GFAP2_NEST	Fw: GCCACCTACAGGAAATTG	NM_010277
	Re: CACACCTCACATCACCAC	

3. SPECIFIC AIM: To demonstrate the mRNA expression of various Kir and TRP channels in the LC neurons.

A series of studies was performed to address this aim. First, the presence of specific Kir and TRP mRNAs in the LC tissue was determined using regular PCR. Second, the expression levels of the channels were quantified using qPCR. Third, sc PCR analysis was carried out to show that the channel mRNAs identified above were indeed located in catecholaminergic neurons in the LC, as specific antibodies are still unavailable for most to these K+ channels.

4. RESULTS

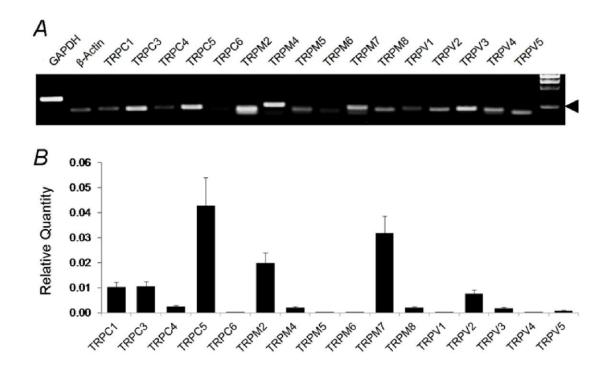## 4.1. Expression levels of various TRP channel mRNAs in LC using PCR and qPCR

Figure 4. Expression levels of various TRP channels in LC neurons using random primers in RT-PCR.

   A. All representative members in the TRPC, TRPM and TRPV families were included in the RT-PCR analysis. Several TRP channels were clearly detected in the mRNA level. Arrow head indicates 100bp. B. Analysis of TRP mRNA expression in the LC tissue with quantitative PCR (qPCR) indicated that the expression of TRPC5, TRPV2 and TRPV5 were several folds higher than other TRP channels. Data are presented as means ± s.e. (n = 16-20 samples from 5 experiments).

   Previous studies have shown the presence of TRP channels in the central nervous system (Kunert-Keil et al., 2006). However, the expression of specific members of the TRP channels in the LC is still elusive. To investigate the expression of specific TRP channels in the LC area, we systematically studied all representative TRP channels in terms of the presence of mRNAs of these TRP channels in the LC tissue using regular PCR. Brainstem slices were firstly obtained. After the LC was

identified based on its pontine location with the IV ventricle as a landmark, the LC area was isolated using micro-puncture. The LC tissue was then used for the mRNA extraction. The mRNAs obtained were subjected to RT- PCR to obtain cDNAs using random primers. The cDNAs produced were used as templates to detect the expression of TRP channels. To prove that the tissue contained LC neurons, a specific enzyme (tyrosine hydroxylase or TH) of these catecholaminergic neurons was examined in each sample obtained. Only those that showed a clear expression of TH were accepted for further studies. With such an approach, we found that several TRP channel mRNAs were highly expressed in the LC (Figure 8).

Subsequently, the expression levels of various TRP channels were quantified using qPCR on the basis of the ΔΔCT method (Figure 4) .Consistent with our regular PCR tests, the TRPC5 mRNA was readily detected in the qPCR, whose expression level was highest among all TRP channels. The TRPM7 and TRPM2 mRNAs were found to be expressed at a moderate level in the LC tissue. The expression levels of TRPC1, TRPC3 and TRPV2 mRNAs were also detectable at low levels, whereas the expression levels of other TRP channel mRNAs were insignificant.

4.2. Confirmation of TRP channel expressions using specific primers in RT-PCR

To further analyze of the TRP expression in the LC neurons, the expression of various TRP channel mRNAs was determined using primers specific for each TRP channel mRNA (Table 1). These studies also showed the highest expression of TRPC5 mRNA in the LC. The expression levels of several TRPV channels were remarkable followed by TRPC1 and TRPC3 mRNAs. The expression levels of TRPM2

and TRPM4 mRNAs were low, while the expression levels of other TRP channel

mRNAs were undetectable. These results thus were consistent with our data in

Section 4.1
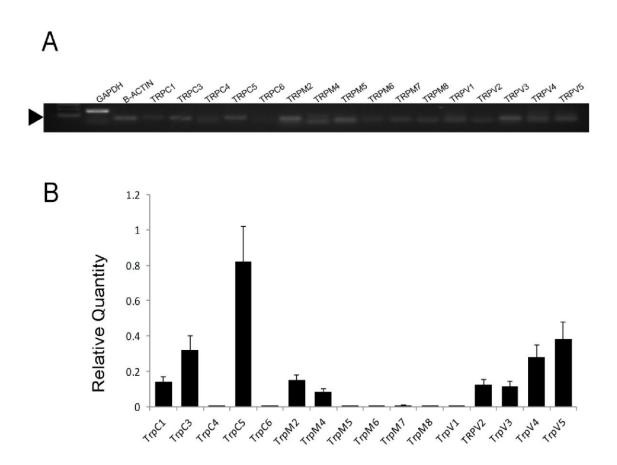
Figure 5. Expression levels of various TRP channels using specific primers in RT-PCR
        A. All representative members in the TRPC, TRPM and TRPV families were included
in the RT-PCR analysis. Several TRP channels were clearly detected in the mRNA level.
Arrow head indicates 100bp. B. Analysis of TRP mRNA expression in the LC tissue with
quantitative PCR (qPCR) indicated that the expression of TRPC5 were several folds higher
than other TRP channels. Data are presented as means ± s.e. (n = 16 samples from 4
experiments).

4.3. Expression levels of various Kir channel mRNAs in LC neurons

The expression levels of various Kir channel mRNAs were similarly studied

using regular PCR and q PCR. Figure 6 shows the expression of Kir mRNAs in the LC

tissue. The expression levels of Kir4.1 and Kir5.1 mRNAs in the LC neurons were high,

especially the Kir4.1, whereas the expression levels of other Kir channel mRNAs were

very low (Figure 6).
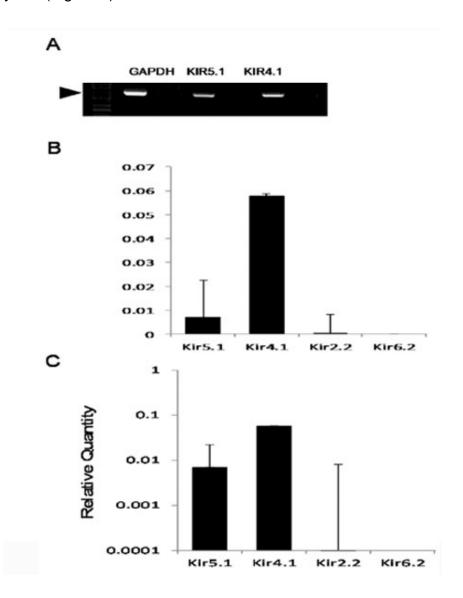
Figure 6. Expression levels of various Kir channels in the LC neurons
A. The members of Kir channel family included for the study were Kir4.1, Kir5.1 due to their significant expression levels in the qPCR. Arrow head indicates 650bp. B. Analysis of various Kir channel mRNA expression in the LC tissue with quantitative PCR (qPCR) indicated that the expression of Kir4.1 were several folds higher than other Kir channels. Data are presented as means $\pm$ s.e. (n = 6-28 samples from 2-8 experiments).

4.4. Dissociation of LC neurons

Since antibodies for the channels are still not available, the scPCR was performed to prove the presence of the expression of the Kir channels and TRP channels in the LC neurons. The LC neurons were dissociated from the LC tissue from mice. The LC tissue was digested with papain for 30min-45min at 37˚C and the inhibitor was then added to suppress the papain. The LC neurons were isolated in the dissociation buffer. After morphological studies with the Hoffman modulation optics (Figure 7), single LC neurons were harvested with patch pipettes and stored at -80˚C for further scPCR analysis.

Figure 7. The dissociated LC neurons
The LC tissue was dissociated using Papain from WT mice. The dissociation buffer containing triturated LC was dropped into 35-mm petri dishes and kept at room temperature for 10 min before being observed with Hoffman modulation optics. Individual neurons were harvested with patch pipettes and stored at -80°C for further experiments.

4.5. Expression levels of various TRP and Kir channels in LC neurons using scPCR
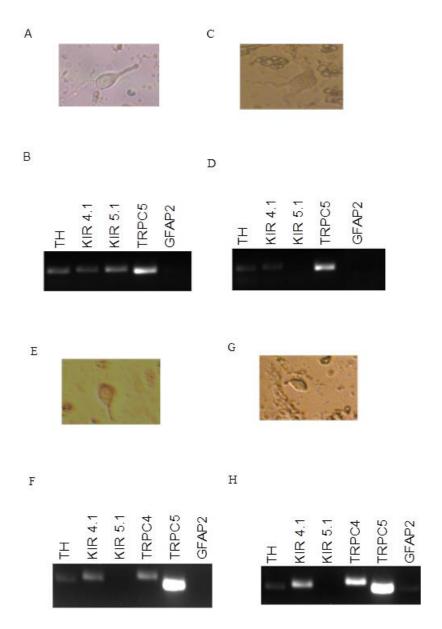

Figure 8. Expression levels of various Kir and TRP channels in single LC neurons.

       A, C, E, G. A cell acutely dissociated from the LC tissue. Calibration: 50 µ. B, D, F, H. scPCR analysis showed that this cell was a TH-positive neuron, and expressed the TRPC5 mRNA. Kir4.1 and Kir5.1 mRNAs were also seen in the cell.

Each single neuron was subjected to scPCR analysis for the expression levels

of the Kir and TRP channel mRNAs with high levels of expression in the LC as shown

in Sections 4.1 and 4.2. We found the high expression of TRPC5 mRNAs in the LC

neurons. The expression levels of Kir4.1 and Kir5.1 mRNAs were rather heterogenous

in LC neurons, with some cells showing the expression of Kir4.1 mRNA alone, Kir5.1

mRNA alone, or both Kir4.1 and Kir5.1 mRNAs. The expression of TRPC4 mRNAs was

much lower than TRPC5 (Figure 8). The high expression of TRPC5 mRNA was

consistently seen in all LC neurons.

4.6. Negative controls for the PCR experiments
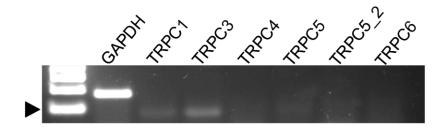
**Figure 9. Expression levels of various TRPC channel mRNAs in cardiac
ventricular muscle tissue.**
All representative members in the TRPC families were included in RT-PCR. Only
TRPC3 and TRPC1 mRNAs were detected in the cardiac ventricular muscle tissue. Arrow head
indicates 100bp. (n = 3 experiments).

The control experiments were performed to assure the conditions of the PCR.

The previous studies have shown that the expression levels of various TRP channels in

various tissues. The lack of expression of TRPC5 was found in the cardiac muscle cells

(Kunert-Keil et al., 2006). Therefore, RT-PCR experiments were performed to assure

the conditions of the PCR for the negative expression of TRPC5 in the cardiac muscle

tissue. Consistent with previous reports, our results did not produce positive bands in

the cardiac ventricular muscles for TRPC5. The cardiac ventricular muscle tissue

expressed TRPC3 mRNAs at a relatively high level followed by TRPC1 mRNAs.
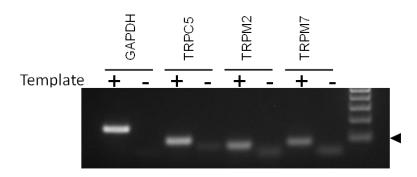
Figure 10. Negative control PCR experiments for highly expressed TRP channels.
The negative control experiments were performed for three most abundant TRPs in LC. In the absence of template cDNAs, a weak and fuzzy band was found. In the presence of cDNA templates, a strong band was produced in each TRP, which was clearly larger than the primer band with expected size. Arrow head indicates 100bp. (n = 3 experiments).

The second set of control experiment was performed to test whether the

contaminations from primers affect the PCR reactions. The PCR experiments were

performed without cDNA template in parallel to that of with cDNA template. In the

absence of cDNA template, weak primer bands were observed. These fussy bands

were greater than primers, suggesting the primer multimerization. In the presence of

cDNA templates, strong bands were found. These bands with expected sizes for each

PCR product cannot be missed with the primer bands, because they were clearly larger

than the primers bands and none of the primer bands were seen in the presence of

cDNA templates.

5. DISCUSSION

The breathing is a complex behavior with critical aspects like plasticity, rhythmicity and chemosensitivity. The chemosensitivity is important for the breathing process as it controls the levels of O_2, CO_2 and pH. According to the previous studies the LC neurons were involved in sensing the intracellular pH (Wang et al., 2001b). The pH sensing property of LC neurons indicates the involvement of LC neurons in controlling the chemosensitivity. The noradrenergic neurons present in the LC form the highest group of neurons that are excited by high levels of CO_2/pH. Among the 80% of the noradrenergic neurons present in the LC region 64% of them responded to levels of CO_2. This indicates the involvement of LC neurons in breathing as one of the chemosensitive sites. There are many signals that cause the chemosensitivity of LC neurons along with the change in the pH and levels of CO_2. The hypercapnic conditions cause the increase in the firing activity of LC neurons. The sensing of the pH and the levels of CO_2 is mostly due to the presence of various ion channels like inward rectifying K^+ channels (Jiang et al., 2001) and TRP channels. The Kir channels, Kir 4.1 and Kir 5.1 are found to be highly expressed in the brain (Xu et al., 2000). The Kir 4.1 channel member and Kir4.1-Kir5.1 heterodimers are inhibited at the high levels of CO_2 (Xu et al., 2000). So the presence of various Kir channels was studied in the LC neurons. The expression of Kir channel members in the LC neurons indicates their role in the chemosensitivity. The experiments performed included qPCR and scPCR which shows the expression of mRNAs in the LC neurons. The results indicated the presence of Kir4.1 and Kir5.1 in the LC neurons which demonstrates their role in the

chemosensitivity. The activation of cationic channels also causes the firing activity of the LC neurons. The major cationic channels include the TRP channel members. Most of the TRP channel members are expressed in the brain (Strubing et al., 2001; Zhou et al., 2008a). Some of the TRP channels respond to the changes in the levels of pH (Andersson et al., 2004; Kim et al., 2008; Semtner et al., 2007). So the experiments were performed to study the expression of various TRP channel members. The data obtained shows the expression of most of the representative members of TRP channels in the LC neurons with the high expression levels of TRPC5 mRNA.

The control experiments in the cardiac ventricular muscle tissue with the negative expression of TRPC5 mRNA confirmed the conditions of the PCR. The expression of TRPC3 and TRPC1 mRNAs without the presence of TRPC5 mRNAs in the cardiac ventricular muscle tissue according to the previous studies assure the high expression of TRPC5 mRNAs in the LC tissue. The negative expression of TRPC5 mRNAs in the cardiac ventricular muscle tissue even with the second set of TRPC5 primers assures the accuracy of the primers used in the PCR. The absence of strong bands without cDNA template in the PCR reactions assures the absence of contamination from primers. The fuzzy bands with sizes greater than that of primers show the multimerization of primers. The previous studies show the ability of Taq polymerase for the reverse transcription and direct amplification of mRNA (Jones, 1993; Tse and Forget, 1990). Therefore, the negative control experiments cannot be produced without the reverse transcriptase in RT-PCR reactions.

The high expression levels of TRPC5 in the LC neurons were confirmed with scPCR experiments. The expression levels of TRPC5 showed consistency in qPCR

and scPCR which indicates the presence of TRPC5 in LC neurons. So the expression of various TRP channel members and Kir channel members in the LC neurons indicates their role in the chemosensitive property of LC neurons.

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