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Review

Molecular responses to acidosis of central chemosensitive neurons in brain

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Abstract

Significant advances have been made in understanding how neurons sense and respond to acidosis at the cellular level. Decrease in pH of the cerebrospinal fluid followed by hypercapnia (increased arterial CO_2) is monitored by the chemosensory neurons of the medulla oblongata. Then the intracellular signalling pathways are activated to regulate specific gene expression, which leads to a hyperventilatory response. However, little is known about molecular details of such cellular responses. Recent studies have identified several transcription factors such as c-Jun, Fos and small Maf proteins that may play critical roles in the brain adaptation to hypercapnia. Hypercapnic stimulation also activates c-Jun NH_2-terminal kinase (JNK) cascade via influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. In addition, several transmembrane proteins including Rhombex-29 (rhombencephalic expression protein-29 kDa) and Past-A (proton-associated sugar transporter-A) have been implicated in regulation of H^+ sensitivity and brain acidosis-mediated energy metabolism, respectively. This review discusses current knowledge on the signalling mechanisms and molecular basis of neuronal adaptation during acidosis.

Keywords: H^+-sensitivity; Ventral medullary surface of the medulla oblongata; Hypercapnia-induced genes; Nuclear transcription factor; c-Jun NH_2-terminal kinase; Ca^{2+} channels; Differential display; Glucose homeostasis

Contents

Abbreviations: ASIC, acid-sensing ionic channel; AP-1, activator protein 1; ATF-2, activating transcription factor-2; bZIP, basic leucine zipper; Ca^{2+}/CaM, Ca^{2+}/calmodulin; CRE, cyclic AMP response element; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter; IP_3, inositol triphosphate; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; OGR1, ovarian cancer G-protein-coupled receptor 1; Past-A, proton associated sugar transporter-A; PKC, protein kinase C; PLCβ, phospholipase C-β; VMS, ventral medullary surface.

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1. Introduction

The main components of the respiratory control responsible for autonomic respiration are located in the medulla oblongata [1]. Two defined groups of respiratory neurons are known, the dorsal and ventral medulla. The dorsal group of neurons is located in and near the nucleus of the tractus solitarius (NTS). A change in the arterial partial pressure of CO_2 (P_{CO_2}), O_2 (P_{O_2}) or H^+ regulates the activity of the dorsal group of neurons. The ventral group is a long column of neurons that extends through the nucleus ambiguous and retroambiguous in the ventrolateral medulla. In addition to reacting to peripheral stimuli, the ventral neurons detect changes in the H^+ and CO_2 concentrations in the cerebrospinal fluid (CSF) and brain interstitial fluid. The capacity to detect these changes is called central chemosensitivity.

The H^+ concentration seems to be a stimulant of central chemosensitivity, because the discharge frequency of ventral medullary surface (VMS) neurons in cats increased with lowered pH of CSF [2–5]. Furthermore, according to a review by Ganong [6], the chemoreceptors monitor the H^+ concentration of CSF, including the brain interstitial fluid. Carbon dioxide readily penetrates membranes, whereas H^+ and HCO_3^- penetrate slowly. The CO_2 that enters the brain and CSF is promptly hydrated. The H_2CO_3 dissociates, so that the local H^+ concentration rises. Therefore, the effects of CO_2 on respiration are mainly due to CO_2 movement into the CSF, where it increases the H^+ concentration and stimulates receptors/sensors for H^+. Thus, the direct stimulant of the central chemosensitive neurons may be H^+ rather than CO_2 [7,8].

Whether there are H^+-sensitive neurons in the brainstem is not known. In the last 40 years, many attempts have been made to clarify the localization of H^+-sensitive neurons. The central chemosensitive neurons responsible for respiratory regulation are distributed over the VMS, which is bathed in CSF, and that these neurons are stimulated by excess H^+ and CO_2 to induce a hyperpneic or tachypneic response [5,8–10]. Mitchell et al. [8] initially reported that respiration was stimulated when pledgets soaked with fluid containing high concentrations of CO_2 and H^+ were placed on circumscribed areas in the VMS. Then, many investigators tried to show that the VMS neurons are sensitive to CO_2 and H^+ [5,8,9]. The application of acid or electrical stimulation to the VMS increased ventilation [10]. The firing rate of the VMS neurons was increased by reducing the extracellular fluid pH [2]. On the other hand, many investigators have shown that H^+-sensitive neurons also exist in the extra-VMS regions, specifically in the deep ventrolateral medulla [11,12], NTS [13–15], the vicinity of the NTS [12,16], nucleus raphe [17,18], nucleus locus caeruleus (LC) [19], the vicinity of the LC [15], and the retrotrapezoid nucleus [20]. The discoveries of chemosensitive neurons in many nuclei disproved that the VMS was the unique site of central chemosensitive neurons.

It is still not clear how H^+ excites the H^+-sensitive (chemosensitive) neurons in the VMS. There is some evidence for H^+-sensing ionic channels in sensory neurons: H^+ activates Na^+ conductance in small neurons of the rat trigeminal ganglion [21]; H^+ activates Ca^{2+} channel in rat sensory neurons [22]; a stepwise reduction in extracellular pH induced an increase in Na^+ current in small dorsal root ganglion cells of the frog [23]; and H^+ and capsaicin share a common mechanism of neuronal activation in rat dorsal root ganglion cells [24]. The H^+-sensitive neurons in the VMS may also have H^+-sensing ionic channels/sensors or similar mechanisms for reacting to extracellular H^+ changes. Few studies have investigated the identification of chemosensitive molecules responsible for respiratory regulation in the VMS. Recently, Waldmann et al. succeeded in cloning the H^+-gated cation channel (ASIC, for acid-sensing ionic channel) that belongs to the amiloride-sensitive Na^+ channel/degenerin family of ion channels [25]. ASIC is expressed in dorsal root ganglia and is also distributed widely throughout the brain. The H^+-gated cation cannel is activated transiently by rapid extracellular acidification and induces cation (Na^+, Ca^{2+}, K^+) influx. More recently, it has been shown that ovarian cancer G-protein-coupled receptor 1 (OGR1), previously described as a receptor for sphingosylphosphorylcholine, acts as an H^+-sensing receptor stimulating inositol phosphate (IP) formation [26]. The receptor is stabilized in an inactive state at pH 7.8 and fully activated at pH 6.8. Pertussis toxin did not inhibit IP formation measured at pH 7.0, indicating that OGR1 acts through Gq. Ovarian cancer G-protein-coupled receptor 4 (OGR4) also responds to pH changes, the receptor promotes cAMP formation through Gs. ASIC, OGR1 and 4 are candidates for chemosensitive molecules responsible for respiratory regulation in the VMS. However, it has been no evidence that these channel/receptors are involved in the central chemosensitivity for respiratory regulation.

Until now, detailed mechanisms responsible for central chemosensitivity in the ventral medulla has been an exceedingly difficult task. In this review, to clarify the H^+-sensitive mechanism of respiratory regulation, we will present our results and discuss the following points:

1. Detection of H^+-sensitive neurons.
2. Analysis of intracellular signalling pathway for H^+-induced c-Jun expression.
3. Profiling of H^+-induced genes.

4. Characterization of a novel H^+-associated sugar transporter.

2. Identification of c-Fos as a marker of neuronal activation

At the end of the 1980s, investigators have shown that the *c-fos* proto-oncogene expression is rapidly and transiently activated after various stimuli [27–29]. The *c-fos* gene, transcript and protein product Fos (c-Fos), have been used to identify activated neurons within the central nervous system. Because the c-Fos protein appears 30 min after the stimulus and its expression lasts a few hours depending on the type and strength of the stimulation, c-Fos was found to serve as a marker for activated neurons. With the c-Fos expression method, researchers have been able to detect H^+-sensitive neurons, characterize their neurotransmitters, and identify how signal transduction pathways are involved in H^+-induced cell activation. Sato et al. [30] have applied this technique to identify chemosensitive neurons in the medulla. They found that the c-Fos protein was expressed in the neurons of the VMS and NTS in rats after the animals inhaled 10–15% CO_2. Since then, increasing evidence has shown that the c-Fos protein is expressed in neurons of the VMS and putative chemosensitive sites in response to hypercapnic stimulation [31–33]. Miura et al. have reported a topological map of H^+-sensitive neurons in the VMS and the morphological and immunochemical properties of these neurons by the c-Fos immunohistochemistry [34]. They first examined the most effective concentration of CO_2 for stimulating the desired responses upon inhalation. Rats inhaled air containing 7% CO_2 for 5 min to stimulate the medullary chemosensitive neurons and to induce respiratory responses. At 2.5 min after the onset of inhalation, both the respiratory frequency and tidal volume reached a plateau, and recovered promptly when CO_2 inhalation ceased. During the inhalation of CO_2, the pH and arterial P_{CO_2} changed significantly, but the arterial P_{O_2} did not change. From these observations, inhalation of 7% CO_2 was effective in stimulating the central chemoreceptors. The c-Fos immunoreactive neurons were found bilaterally not only in the VMS (between the caudal end of the nucleus corporis trapezoidei and the caudal end of the area postrema), but also in the rostral and caudal parts of the medial subnucleus of the NTS, the ventral subnucleus of the ambiguous in the caudal ventrolateral medulla, and the medial reticular regions very near to the VMS, which we termed the rostroventromedial medulla (RVMM) [35]. After the CO_2 inhalation, glutamate- and glutamic acid decarboxylase (GAD)-immunoreactive neurons were found in the VMS and RVMM. The topology of these neurons was extremely similar to that of the c-Fos-immunoreactive neurons. The respiratory rhythm is modulated by the glutamatergic and GABAergic (GABA: γ-aminobutyric acid) neurons in the RVMM [35]. These data indicate that both glutamatergic and GABAergic neurons in the VMS and RVMM are involved in H^+-sensitivity and the subsequent modulation of respiratory rhythm. This conclusion is consistent with the experimental results of respiratory responses: respiration was increased in tidal volume and frequency by the application of NMDA (*N*-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid) to the rostral VMS of rats [36]; respiration was suppressed by the application of GABA/muscimol to the intermediate VMS (Schlaefke's area) of cats [37,38] and rats [36]; and respiration was suppressed by the application of taurine/glycine to the intermediate VMS [39].

3. Detection of H^+-sensitive neurons with fluorescent H^+-indicator

Although c-Fos expression is often used as an indicator of H^+-sensitive neurons, many aspects of the H^+-sensing mechanism in the H^+-sensitive neurons remain to be clarified. To better understand the relevance between H^+ and H^+-sensitive neurons, we hypothesized that H^+-sensitive neurons have a significant influx of H^+ from the outside of the neurons when the extracellular pH decreased. If our hypothesis is true, intracellular pH responses during hypercapnic stimulation should be exhibited in the central chemosensitive neurons in the VMS. To test this hypothesis, changes in the intracellular pH of the cultured VMS neurons at various extracellular pHs were evaluated with a fluorescent H^+ indicator 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4- or 5-carboxyfluorescein, diacetoxymethyl ester (BCECF-AM) [40]. The fluorescent intensity decreases when BCECF binds to intracellular H^+. When the VMS neurons labeled BCECF-AM were exposed to perfusates of various extracellular pHs, the fluorescent intensity of the only a few VMS neurons (6%) decreased when the extracellular pH was shifted from 7.40 to 7.20. The frequency of the VMS neurons with lower fluorescent intensity was significantly higher than that of the VMS neurons against extracellular pH 7.4. The subpopulation of neurons showing lower fluorescence intensity was 14 times higher in the VMS neurons than in the medial dorsal medulla as a control. This phenomenon is surprising because low pH affects the maintenance of normal neuronal activity. Only a transient increase in intracellular H^+ seems necessary to trigger the activation of H^+-sensitive neurons. The H^+-sensitive neurons detected that fluorescent H^+-indicator showed immunoreactivity to glutamate (57%) and GAD (23%), indicating that these neurons are glutamatergic or GABAergic. On the other hand, these neurons were not immunoreactive to choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), phenylethanolamine *N*-methyltransferase (PNMT), somatostatin, or substance P. These findings were consistent with our above-mentioned in vivo studies, in which hypercapnic stimulation induced c-Fos expression in glutamate and GAD containing neurons in the VMS [34,35]. Rigatto et

al. [41] also reported that fetal rat medullary neurons that were responsive to low pH did not stain positive for ChAT.

Although ChAT-immunoreactive neurons were not detected in H^+-sensitive neurons, the VMS neurons may be excited by the application of acetylcholine [42] and cholinergic transmission might be involved in the chemosensitive responses in the VMS [43]. On the other hand, Haxhiu et al. [31] showed Fos protein was appeared in TH immunoreactive neurons in the ventrolateral medullary reticular formation and locus ceruleus of the brainstem following exposure to hypercapnic stress. These results suggest that the brainstem catecholaminergic neurons are part of the neuronal network that reacts to hypercapnic exposure. Richerson et al. [18], by using the patch technique on brain slices, found that many of the chemosensitive neurons indicated in these experiments were located within the medullary raphe nuclei. With immunohistochemistry for tryptophan hydroxylase, they found acidosis-stimulated raphe cells were serotoninergic neurons. They proposed that acidosis-induced raphe neurons were not sensitive to H^+ in the CSF but to CO_2 in the blood, because these neurons are located proximally within the vascular tree and lie close to large blood vessels. These findings are highly suggestive of the existence of different mechanisms by different type of neurons in the chemosensitivity involved in respiratory regulation.

4. Signal transduction for H^+-induced c-Jun expression

An increase of the H^+ concentration in the cerebrospinal fluid and brain interstitial fluid by hypercapnic stimulation causes expression of several bZIP transcription factors, such as c-Jun, Fos and small Maf proteins, in specific nuclei in the central nervous system [31,32,34,35,44]. Among such proteins, c-Jun immunoreactive neurons are distributed in the respiration-related motor nuclei of the medulla oblongata and spinal cord, and in the central chemoreceptive area of the ventral medullary surface of the medulla after hypercapnic stimulation. This observation was also confirmed by an in vitro study in which an increase in the concentration of extracellular H^+ of cultured PC-12 cells led to the induction of *c-jun* mRNA [45]. We found significant increase in c-jun mRNA with decrease in extracellular pH from 7.40 to 7.20. The H^+-induced c-jun mRNA expression was inhibited with Ca^{2+}/calmodulin inhibitor trifluoperazine, indicating that the expression of c-jun mRNA by an increase in extracellular H^+ is mediated partly by this system [45]. Calmodulin, a ubiquitous intracellular Ca^{2+} receptor, binds to short peptide sequences of many target proteins upon binding Ca^{2+}. Such interaction is thought to induce a conformational change on the target, resulting in its activation, e.g. phosphorylation. Targets include the CaM kinases and Calcineurin [46]. These studies may clarify the mechanisms of our findings that the hyperventilatory response to the CO_2 inhalation is abolished when the intercellular Ca^{2+} chelator BAPTA-AM (1, 2-bis [2-amino-4-fluorophenoxy] ethane-*N*, *N*, *N′*, *N′*-tetraacetic acid, tetraacetoxymethyl ester) is applied to the VMS [36]. On the other hand, Kuo et al. [47] reported that protein kinase C_α (PKC_α) is a possible mediator in H^+-induced c-Fos expression, and that PKC_α may be activated by Ca^{2+}. Activated PKC_α could lead to an increase in the phosphorylation of Raf-1 kinase, which in turn activates mitogen-activated protein (MAP) kinases to stimulate leading to the enhanced expression of *c-fos* mRNA. Taken together, it is highly probable that an increase in concentration of extracellular H^+ induces c-Jun/Fos through Ca^{2+}/calmodulin and MAP kinase pathways.

The nuclear transcription factor c-Jun is a major stress-activated protein in cells and is likely to coordinate transcription programs in response to stress. c-Jun must be phosphorylated at Ser63 and Ser73 on its NH_2-terminal transactivation domain for augmentation of the transcriptional activity [48–50]. This phosphorylation is mediated by JNK, which itself need to be phosphorylated at Thr183 and Tyr185 by the upstream dual specificity kinases for the activation of its kinase domain [51–53]. This phosphorylation events can be induced by a variety of extra- and intracellular stress signals including UV radiation [54,55], osmotic [56] and oxidative stress [57], alkalinization [58] and cytokines (tumor necrosis factor-α) [59]. Once activated, JNK translocates to the nucleus where it regulates the activity by phosphorylation of several transcription factors such as the Jun family proteins, ATF-2 (activating transcription factor-2) and p53 [60]. Therefore, JNK plays key roles to regulate stress-induced c-Jun activity.

In recent study, we found another intracellular signalling pathway for extracellular H^+-induced c-Jun expression in several cell lines (HEK293, COS-7 and PC-12) [61]. When cells were incubated with low pH medium, the phosphorylation of JNK and expression of c-Jun were clearly observed in cells in an extracellular pH- and time-dependent manner. We also demonstrated that the phosphorylated JNK accumulated in the nucleus via phosphorylation of JNK in cytoplasm due to an increase in the concentration of extracellular pH. Many tumor cells have relatively acidic extracellular pH and are killed by intracellular acid-induced injury. The acid-induced cell death depends on bax, a proapoptotic binding partner of bcl-2, and on JNK signalling pathways [62]. Recently, Yamamoto et al. reported that acidification of the cytoplasm using cycloprodigiosin hydrochloride (cPrG · HCl), a novel H^+/Cl^- symport drug, leads to apoptosis in cancer cells through up-regulation of Fas ligand, JNK and caspase [63]. Taken together, an increase in concentration of extracellular H^+ leads to phosphorylation of JNK, and then the phosphorylated JNK translocates to the nucleus to augment the transcriptional activity of c-Jun.

On the other hand, electrophysiological studies showed that a rapid shift in extracellular pH from 7.4 to 6.9 caused an inward current, probably due to an increase in Na^+ and K^+ permeability across the membrane [64], and that an

increase in concentration of extracellular H^+ induced H^+-gated Na^+ current in the hypothalamic neurons [84]. H^+-induced Na^+ current produces depolarization of the membrane and then provokes influx of extracellular Ca^{2+} via voltage-gated Ca^{2+} channels [65,66]. From these observations, we examined whether the Ca^{2+} influx is involved in extracellular H^+-induced JNK phosphorylation and c-Jun expression using nimodipine, pharmacological agent known as the blocker of voltage-gated Ca^{2+} channels [61]. Nimodipine prevented partly phosphorylation of JNK and expression of c-Jun. This result indicates that extracellular H^+-induced JNK phosphorylation and c-Jun expression are mediated partly by the increase of intracellular Ca^{2+} concentration. Taken together, our results suggest a novel pathway to H^+-induced c-Jun expression: an increase of extracellular H^+ provokes Ca^{2+} influx by depolarization through Na^+ influx, and the increase of intracellular Ca^{2+} concentration induces c-Jun expression through phosphorylation of JNK.

In addition, Kanazawa et al. found that cAMP-immunoreactive neurons in the VMS and injection of IBMX (3-isobutyl-1-methylxanthine), an enhancer of intracellular cAMP, into the intermediate VMS induced hypercapnic responses [36]. Although cAMP has not been shown to function in the central chemosensitive neurons, Chang et al. reported that hypercapnic loading increases the cAMP level, but not cGMP concentrations, in PC12 cells [47,67]. Evidence is accumulating that various molecules are involved in signal transduction to extracellular H^+-induced gene expression.

5. Screening of H^+-induced (hypercapnia-induced) genes

To elucidate the cellular response at molecular level in the extracellular H^+ of neuronal cells, we screened genes that were stimulated by low pH after hypercapnic stimulation. We applied the differential display technique to the adult rat brain and compared gene expression under normocapnic and hypercapnic conditions. Differential display is an established technique that provides the microanalysis of transcriptional changes occurring in a given cell or tissue [68]. The technique has been verified to be effective for identifying novel and differentially expressed genes at various physiological aspects in CNS neural cells, such as RNA-binding peptide RA310 induced by hypoxia-reoxygenation [69], seizure-induced synaptotagmin gene *syt* X [70], zinc transporter ZnT-1 cloned after forebrain ischemia [71] and membrane glycoprotein Pal expressed specifically in photoreceptor cells of the retina [72].

Rats inhaled either air (normocapnic stimulation, 0.04% CO_2) or air containing 7% CO_2 (hypercapnic stimulation) for 5 min to stimulate the medullary chemosensitive neurons [34]. The RNA derived from the VMS after either air or 7% CO_2 inhalation was used for polymerase chain reaction (PCR) amplification. Nine oligo-dT primers anchored downstream were combined with 24 different upstream primers, each composed of 10 nucleotides. In all, 216 different combinations were used for PCR amplification. Differentially amplified PCR fragments were visualized as mRNA fingerprints. Over 11,500 PCR products were generated, and 14 (0.12%) of the observed bands exhibited gene profiles of high expression as a result of hypercapnic stimulation. We found that the sequences of eight clones were novel and that six other clones had already been reported to be genes readily induced by hypercapnia (Table 1). Several representative clones are also shown below.

5.1. Fos/Jun

As mentioned above, the number of Fos/Jun-immunoreactive neurons increased significantly in the VMS after hypercapnic stimulation in vivo. Alternatively, expression of Fos/Jun in the VMS neurons induces when the concentration of H^+ in CSF is increased. We also detected genes for Fos/Jun by differential display technique for the identification of high-expressed genes at hypercapnic stimulation in the VMS and therefore, the technique indicates reliable for screening of extracellular H^+-induced genes.

5.2. Maf protein family

We found that hypercapnic stimulation induced the gene expression of *maf*G [44], a member of the Maf protein family of nuclear basic leucine zipper (bZIP) transcription factors. It has been reported that MafG forms heterodimers with c-Fos at each leucine zipper structure and that the Maf complex recognizes two palindromic DNA sequences, TGCTGACTCAGCA and TGCTGACGTCAGCA [73]. The middle parts of the two consensus binding sequences for Maf are identical with two binding sequences for Fos/Jun (TGACTCA and TGACGTCA) [73]. MafG may be involved in the signal transduction of H^+-sensitivity and respiration with c-Fos protein, either competing for binding sites or interacting directly with c-Fos. MafG-2 is a novel splice variant of MafG and differs from MafG by an insertion of 27 amino acids [74]. Sequence analysis of the protein has shown that the basic domain for DNA binding and the leucine zipper structure are conserved in MafG-2.

Table 1
Hypercapnia-induced protein

Protein	Characteristic	Reference
Fos/Jun	Transcriptional regulator of genes having AP-1 sequence	[30–35,45,47,67]
MafG	Basic leucine zipper transcription factor	[44]
MafG-2	Novel splice variant of MafG	[74]
Rhombex-29	New member of PLP/DM20-M6 family	[81]
Past-A	pH-dependent sugar transporter	[87]

Because expression of *maf*G-2 mRNA increases when extracellular pH is decreased gradually from 7.40 to 7.20, both MafG and MafG-2 may be involved in signal transduction of extracellular pH change.

MafB also belongs to the family of Maf proteins, a subgroup of AP-1-type bZIP transcription factors and is the avian homologue of the murine *kreisler* gene product [75]. Recently, Blanchi et al. reported that a critical function of MafB on respiratory rhythmogenesis [76]. They have identified MafB as a marker of a specific subpopulation of neurons in the preBötzinger complex, one of the principal sites of respiratory rhythmogenesis in the brainstem. Mice deficient for MafB (*Mafb*$^{-/-}$) die from central apnea at birth, and in vitro preparations of *Mafb*$^{-/-}$ brainstems failed to generate a normal rhythmic discharge pattern. Together these observations indicate that Maf proteins play critical roles in the adaptation to hypercapnia and respiratory rhythmogenesis. Several genes have been linked to human syndromes with various respiratory distresses such as Prader-Willi syndrome [77], sudden infant death syndrome (SIDS) [78] and congenital central hypoventilation syndrome [79,80]. Maf proteins may thus provide a new insight to understanding of mechanisms and drug design for these diseases.

5.3. Rhombex-29

One of the novel genes that induced hypercapnic stimulation is Rhombex-29 (*rhomb*encephalic *ex*pression protein-*29* kDa) [81]. Rhombex-29 mRNA is expressed highly in the VMS, moderately in the cerebral cortex and cerebellum, poorly in the lungs and kidneys. The encoded polypeptide was predicted to be 265 residues in length and the relative molecular weight of the residues was calculated as 28,972 Da. From the phylogenic tree that the primary structure of rat Rhombex-29 protein is clearly homologous to that of the major myelin protein family PLP/DM20-M6 of the central nervous system. The hydrophobicity profile of the deduced amino acid sequence of rat Rhombex-29 protein showed that the protein has the four-transmembrane-domain arrangement typical of PLP/DM20-M6 family members.

PLP/DM20-M6 products are capable to transport alkali and alkaline-earth metal ions such as Na^+, K^+, and Ca^{2+} [82–86]. On the other hand, because gene expression of rat Rhombex-29 in VMS neurons increased with the increase in the H^+ concentration in the CSF by hypercapnic stimulation, rat Rhombex-29 may be implicated in regulation of H^+ sensitivity. To test our hypothesis, functional expression and patch clamp studies for Rhombex-29 are under investigation.

6. Characterization of a novel H^+-associated sugar transporter, Past-A

Recently, we cloned a new sugar transporter and named as Past-A (*p*roton-*a*ssociated *s*ugar *t*ransporter-*A*), which is induced in the brain after hypercapnic stimulation [87]. Past-A mRNA is expressed highly in the VMS, moderately in the cerebral cortex and cerebellum, extremely poorly in the heart and kidneys, and not at all in lungs, skeletal muscle, liver, stomach, or intestine. Expression of Past-A transcript start by embryonic day 20 in rats. Then it constantly express in the brain. Past-A immunoreactive neurons were found primarily in the VMS. In response to hypercapnic stimulation, the number of Past-A immunoreactive neurons in the VMS was about four times greater than that of after air inhalation.

The cDNA of Past-A contains an open reading frame encoding a sequence of 751 amino acids, and the relative molecular weight of the residues was calculated as approximately 82 kDa. Analysis of the predicted amino acid sequence suggested the presence of 12 putative membrane-spanning helices with a long cytoplasmic loop between transmembrane (TM) helices TM6 and TM7, and with cytoplasmically oriented NH_2 and COOH termini (Fig. 1A). Primary structure analysis indicated that the Past-A protein belongs to a sugar transporter family in the major facilitator superfamily (MFS) [88]. These proteins have 12 transmembrane-spanning helices. Substrates transported by the sugar transporter family members include pentoses (arabinose and xylose), hexoses (glucose, fructose, and galactose), disaccharides (sucrose, maltose, and lactose), inositols, quinate, and cations. In transfected cells, Past-A mediates glucose uptake. In addition, Past-A has the same membrane topology as the glucose transporters that have 12 membrane-spanning helices with a long cytoplasmic loop between TM6 and TM7, and with cytoplasmically oriented NH_2 and COOH termini. Furthermore, several motifs important for glucose transport activity are conserved: the RXGRR motif (where X denotes any amino acid), the PESP, and the QLS motif. A recent study has identified the QLS sequence of glucose transporter (GLUT) 1, GLUT3, and GLUT4 as a critical motif for hexose selection [89]. Introduction of this QLS motif into the fructose/glucose transporter GLUT2 abrogated fructose transport. Conversely, replacing this sequence with that of GLUT2 led to the transport of fructose in addition to glucose. Past-A has the WLS motif at this position instead of the QLS motif, which suggests that it may transport other hexoses beside glucose. Further studies are needed to define the substrate specificity of Past-A. Past-A shows the highest similarity with membrane-associated transporter protein B of *oryzias latipes* (medaka) and its human and mouse homologues, AIM-1s (antigen isolated from immunoselected melanoma 1s). A previous report suggested that AIM-1 transports substances including galactose and certain saccharides that are required for melanin biosynthesis in the medaka (a small, freshwater teleost) [90]. Because Past-A has a completely conserved sucrose-H^+ transport motif of plants, Past-A may be a sugar-H^+ symporter.

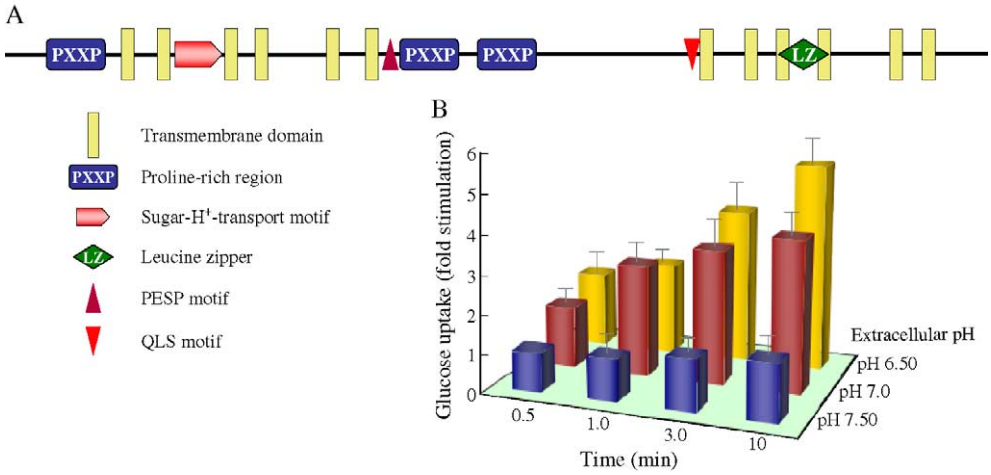

Fig. 1. Structure and function of Past-A. (A) Schematic representation of the domain/motif structure of novel hypercapnia-induced protein Past-A. Past-A contains three proline-rich region, a leucine zipper motif and three motifs (sugar-H^+ transport motif, PESP motif and QLS motif) that have been shown to be critical for the sugar transport. Both N and C termini are intracellular orientation. (B) Characterization of sugar transport activity of Past-A. COS-7 transfected cells with Past-A cDNA were incubated at different pH values for glucose uptake. Sugar uptake was determined by incubation with ^{3}H-labeled glucose. Data are expressed as fold stimulation relative to the extent of uptake observed with COS-7 transfected Past-A cDNA at a pH of 7.50 in 0.5 min.

The Past-A molecule has two unique features that may be relevant to its intracellular functions. The first is the presence of proline-rich regions in the long intracellular loop between TM6 and TM7 and in the amino terminus of Past-A; these regions are not found in other sugar transporters such as mammalian GLUTs or in the related sucrose-H^+ transporters. A poly-proline motif (core sequence PXXP, where X denotes any amino acid) is thought to be involved in specific protein–protein interactions [91]. Past-A may therefore serve as a binding partner for proteins containing Src homology 3 (SH3) domains independent of glucose transporter function. A recent study has indicated that glucose transported via the GLUT1 transporter stimulates a tyrosine kinase-dependent cascade that leads to the activation of the MAP kinase pathway [92]. Some studies have shown that acidification of extracellular pH by hypercapnia triggers intracellular signal transduction, including induction of c-fos mRNA expression through PKC, Ca^{2+}/calmodulin, and MAP kinase pathways [45,47]. The other unique feature of Past-A is the leucine zipper structure in the TM9 and TM10 domains. The leucine zipper consists of a periodic repetition of leucine residues and has been implicated in the facilitation of protein dimerization [93]. The leucine zipper structure is present in many nuclear transcription factors, such as CCATT-box/enhancer binding protein (C/EBP), cAMP response element binding protein (CREB), and Fos/Jun, and has been shown to facilitate protein dimerization. In addition, rat GABA transporter and human dopamine transporter have leucine zipper structures in the transmembrane helices. Leucine zipper structures in the transmembrane helix may be involved in the formation of transmembrane topology by interacting with other proteins or by forming dimers with itself.

Past-A expression is relatively high in the VMS in the brainstem. Here, glucose-sensing neurons respond to hyperglycemia or hypoglycemia by increasing or decreasing their firing rates [94–96]. In the medulla oblongata, the area postrema and the NTS have glucose sensors related to feeding and reproduction [97]. Furthermore, particular raphe nuclei in the medulla oblongata are related to glucose sensing [98,99]. Recently, glucokinase-like immunoreactivity was located in serotonergic neurons in the raphe [100]. These findings emphasize the importance of the medulla oblongata as a glucose-sensing area. The roles of glucose-sensing neurons are critical in both physiological and pathophysiological conditions.

Expression of Past-A is significantly increased in neurons of the VMS in response to decreasing extracellular pH following hypercapnia. Our results also indicate that Past-A has a functional role in controlling glucose uptake along the pH gradient (Fig. 1B), suggesting that hypercapnia may stimulate the uptake of glucose, the primary energy source, into acidosis-stressed neurons of VMS. Signal transduction of Past-A-related sugar homeostasis in neuronal cells after hypercapnia is under investigation. AMP-activated protein kinase (AMPK) is a metabolic stress sensing protein kinase plays a key role in regulation of energy homeostasis [101], more specifically glucose uptake. The kinase is activated by an elevated AMP:ATP ratio due to cellular and environmental stress, such as hypoxia, ischemia and heat shock [102]. It is intriguing to speculate that the promotion of the gene expression and translocation to membrane of Past-A is regulated through the AMPK signalling pathway. Future functional analysis of Past-A may provide new insights into the biochemical regulation of glucose-sensing mechanisms in the brain. Furthermore, type 2 diabetes mellitus exhibits metabolic deficiencies that could be attributed to the malfunction or inactivity of the signalling pathway involved in glucose homeostasis, therefore, presenting a

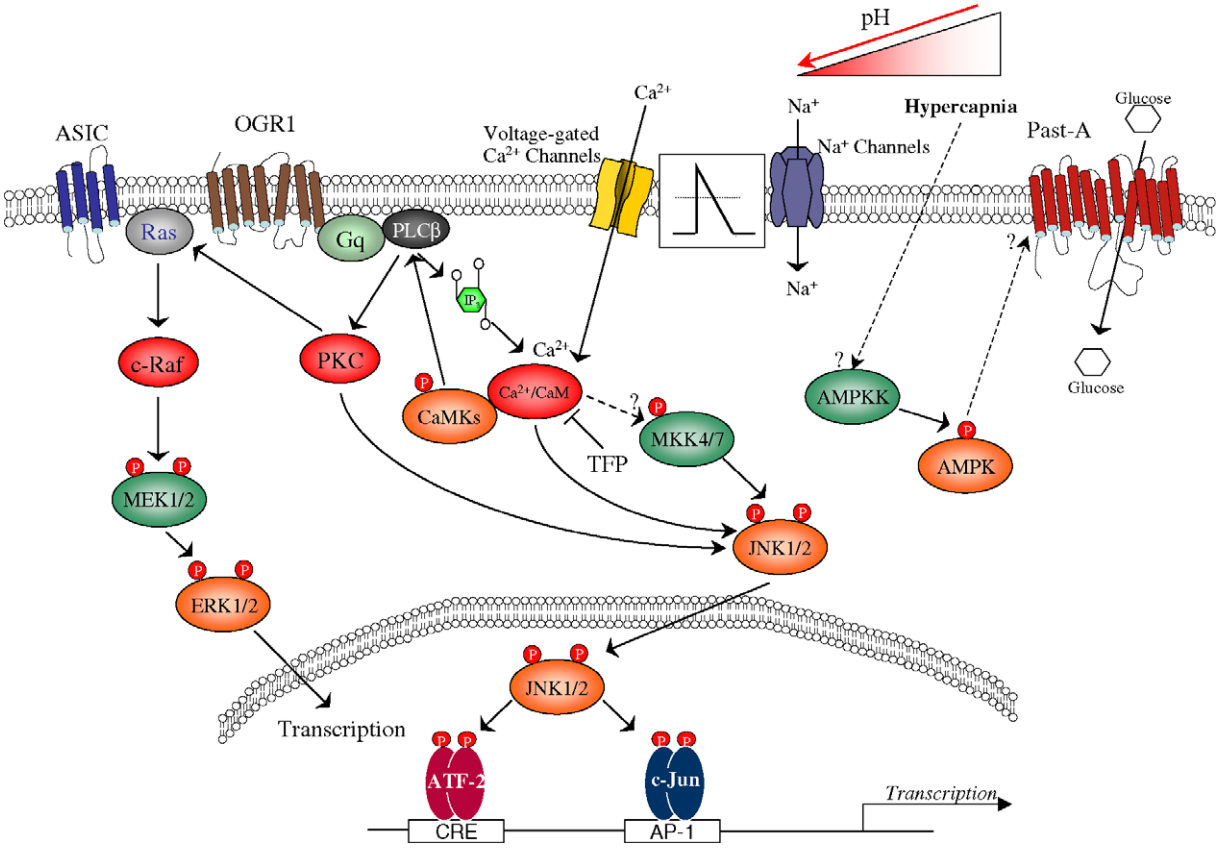

Fig. 2. Signalling pathways in extracellular acidification. Increase of extracellular H^+ induces JNK phosphorylation and c-Jun expression via partly extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels. Elevation of intracellular Ca^{2+} concentration after IgE- and antigen-dependent stimulation in rat basophilic leukemia mast cells increases JNK activity, possibly through the calmodulin pathway [103]. Activation of the CaMK pathway increased JNK kinase activity through regulating the PKC pathway [104]. AMPK is plays a key role in regulation of glucose homeostasis. AMPK phosphorylation at Thr172 by the upstream kinase AMPKK is required for activation of AMPK [105].

possible link and therapeutic target for this pathology (Fig. 2).

7. Conclusions and future perspectives

How neuronal cells sense and respond to changes in pH in their environment is one of fundamental questions in neurophysiology. Recent findings indicate to the existence of different mechanisms elicited by different types of neurons involved in respiratory regulation. It is also becoming clear that several intracellular signal transduction pathways are implicated in the regulation of gene transcription that provides the appropriate set of early responsive genes to acidosis. The deregulation of such pathways has very serious consequences for patients ranging from neuronal damage till death.

On the other hand, recent finding has shown the existence of H^+-induced sugar transporter. Increased expression of Past-A in hypercapnia may protect neurons from acidosis-induced cell damage by supplying extra energy source for recovery. Without such protection mechanism in the brain may be more fragile for metabolic acidosis followed by brain ischemia, trauma and infection. With this regard, Past-A may play crucial role in maintaining homeostasis of brain.

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