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and DEPARTMENT OF BIOSCIENCES
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# CYTOSKELETON-INTERACTING PROTEINS IN BRAINSTEM DEVELOPMENT

## *Roles of KCC2 and Vangl2*

Zachi Horn


Karolinska Institutet

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*Dedicated to my family*

# ABSTRACT

The brainstem is the most evolutionary conserved division of the brain. It develops from the hindbrain and midbrain regions of the neural tube and forms neural networks that regulate vital functions of the body. One of the most critical roles is to generate respiratory rhythm for the regulation of oxygen, carbon dioxide and pH levels. This is achieved by pacemaker neurons and neural networks in the medulla oblongata, controlled by different modulatory systems. The mechanisms whereby the respiratory rhythm is generated and regulated are not fully understood and have only recently started to be unveiled.

This thesis describes the importance of two different gene products, KCC2 and Vangl2, for proper development of the brainstem. We show that, while these genes act in separate phases of development, they share the common feature of regulating the integrity of the neuronal cytoskeleton necessary for maturation of the brainstem.

KCC2 is a neuronal $K^+/Cl^-$ cotransporter that is responsible for the developmental shift in the postsynaptic response to GABA. A fundamental premise for this thesis is that we found KCC2 protein expression in the hindbrain region of mice already at embryonic day 9.5, although its ion transport activity does not become functional until late fetal age. We show that the depolarizing effect of GABA elicits increased activity of fetal respiration-related neurons. In addition, the developmental GABA shift is associated with plasma membrane targeting of KCC2 in respiration-related regions of rats around birth.

Overexpression of KCC2 in the mouse neural tube resulted in altered neuronal differentiation and neural crest migration. These effects were independent of the ion transport function of KCC2 and were shown to rely on a structural interaction with the cytoskeleton-associated protein 4.1N. Thus, transport-inactive KCC2 may regulate neuronal differentiation and migration during early development. We assessed the early importance of KCC2 further in mice knockout for this gene, which die at birth from respiratory failure. Brainstem organotypic cultures of these mice displayed a lower correlated network activity in the preBötzinger region. In addition, characterization of the respiration-related regions showed less glutamatergic synapses in the parafacial respiratory group of KCC2-deficient mice. This indicates that KCC2 is essential for the maturation of respiratory neural networks.

Finally, we show that the planar cell polarity gene Vangl2 regulates neural tube closure in the hindbrain region by promoting the formation of adherens junctions. Vangl2 was found to structurally interact with Rac1. Moreover, disruption of adherens junctions by a partial blockade of Rac1 could be rescued by Vangl2. This suggests that Vangl2 plays a critical role in the recruitment of Rac1 to the adherens junctions.

In conclusion, the results presented in this thesis increase our knowledge of brainstem development, from closure of the neural tube until the formation of functional neural networks. Our findings have potential implications for research and understanding of neural tube defects as well as breathing disorders, such as congenital central hypoventilation syndrome, that arise from aberrant formation of the neural networks constituting the central pattern generator for breathing.

# LIST OF PUBLICATIONS

I. Eric Herlenius, **Zachi Horn**, Ikuo Homma, Hiroshi Onimaru (2010). Brainstem KCC2 and GABA during perinatal development of the central respiratory rhythm. *Manuscript*.

II. **Zachi Horn**, Thomas Ringstedt, Peter Blaesse, Kai Kaila, Eric Herlenius (2010). Premature expression of KCC2 in embryonic mice perturbs neural development by an ion transport-independent mechanism. *European Journal of Neuroscience*, 31: 2142-2155.

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# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AGM | Aorta-gonad-mesonephros |
| BDNF | Brain-derived neurotrophic factor |
| BMP | Bone morphogenetic protein |
| BötC | Bötzinger complex |
| C568A | Cysteine to alanine substitution in amino acid 568 |
| CCC | Cation chloride cotransporter |
| cDNA | Complementary DNA |
| $[Cl^-]_i$ | Intracellular chloride concentration |
| CNS | Central nervous system |
| CPG | Central pattern generator |
| Dcx | Doublecortin |
| DIV | Days in vitro |
| DLHP | Dorso-lateral hinge point |
| DNA | Deoxyribonucleic acid |
| ΔNTD | N-terminal deletion |
| DRG | Dorsal respiratory group |
| E | Embryonic day |
| $E_{GABA}$ | Reversal potential for GABA responses |
| EGFP | Enhanced green fluorescent protein |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| FL | Full length |
| GABA | γ-aminobutyric acid |
| GDP / GTP | Guanosine di/tri-phosphate |
| HEK | Human embryonic kidney |
| KCC | $K^+/Cl^-$ cotransporter |
| MAP | Microtubule associated protein |
| MDCK | Madin Darby canine kidney |
| MHP | Medial hinge point |
| mRNA | Messenger RNA |
| NA | Nucleus ambiguus |
| NK1R | Neurokinin-1 receptor |

| | |
|---|---|
| NKCC | $Na^+/K^+/Cl^-$ cotransporter |
| PBC | preBötzinger complex |
| PCP | Planar cell polarity |
| PDZ | Postsynaptic density protein 95 / Drosophila disc large tumor suppressor / Zonula occludens-1 protein |
| pFRG | Parafacial respiratory group |
| $PGE_2$ | Prostaglandin $E_2$ |
| PRG | Pontine respiratory group |
| PSA-NCAM | Polysialylated neural cell adhesion molecule |
| RA | Retinoic acid |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RTN | Retrotrapezoid nucleus |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SST | Somatostatin |
| TMR-SP | Tetramethylrhodamine-conjugated substance P |
| Vangl2 | Van Gogh-like 2 |
| VGlut2 | Vesicular glutamate transporter 2 |
| VRG | Ventral respiratory group |

# 1 BACKGROUND

Having evolved hundreds of millions of years ago, the brainstem is the most ancient part of the brain. Emerging from the spinal cord as a thickening of the rostral end, it joins at the base of the brain and forms a gateway between higher order centers and the rest of the body. Although relatively small compared to the brain as a whole, the brainstem carries the machinery for the most fundamental functions of vertebrates. Resembling the entire brain of the present-day reptiles, it is sometimes also called the *reptilian brain*. It appeared already in non-air-breathing fish and developed into a more advanced stage during the evolution of terrestriality, when tetrapods were encountered with the challenge of eliminating $CO_2$ in air. Breathing is one of the many life-maintaining functions controlled by the brainstem, which also include cardiovascular regulation, consciousness, alertness, reflexive actions and pain sensitivity.

The brainstem develops from the anterior region of the neural tube, which forms all the brain structures. Prior to this regionalization, the neural tube develops similarly along the entire body axis. Before reviewing brainstem development it may therefore be useful to highlight some general features of the initial phases of neural tube development.

## 1.1 OVERVIEW OF CENTRAL NERVOUS SYSTEM DEVELOPMENT

### 1.1.1 Gastrulation and neurulation

The formation of the central nervous system (CNS) is a process of incredible precision, which requires a highly coordinated progression of events. Prior to CNS formation, three main germ layers must be generated. The initial upper and lower cell layers, termed epiblast and hypoblast, are formed by segregation of the inner cell mass. This is followed by gastrulation, during which cells undergo several dramatic morphological transitions and movements whereby they change their positions relative to each other (Tam & Loebel, 2007). A raised groove forms in the dorsal surface of the epiblast, called the *primitive streak*. Epiblast cells move inward at the primitive streak and displace the hypoblast cells to generate the innermost germ layer, the endoderm. Thereafter, intercalating epiblast cells undergo an epithelial-to-mesenchymal transition and create the middle layer, the mesoderm. This layer gives rise to connective tissues,

muscles and the vascular system (Kinder *et al.*, 1999). The endoderm gives rise to the gut and associated visceral organs (Lewis & Tam, 2006), whereas the outermost layer, ectoderm, gives rise to the nervous system and skin (Tam, 1989). An important structure, called the *notochord*, is formed in the mesoderm. The notochord defines the midline of the body and induces the formation of the neural tube.

During the first step, known as neural induction, the neural plate forms as a uniform sheet of neural progenitors at the dorsal midline of the ectoderm. The neural plate is reshaped by a process called *convergent extension*, during which the embryo is extended along the antero-posterior axis, while narrowed along the medio-lateral axis. This is followed by neurulation, the process in which the neural plate buckles at its midline to generate neural folds. The dorsal tips of the folds then fuse to form the neural tube. Neurulation takes place during embryonic day (E) 7 – 9 in mice and 3 – 4 postovulatory weeks in humans. It is accompanied by regionalization of the neural tube rostro-caudally into the future brain and spinal cord, and dorso-ventrally into sensory and motor neuron precursors (Smith & Schoenwolf, 1997; Copp *et al.*, 2003).

### 1.1.2 Cell specialization and regionalization

Neural stem cells are born in the ventricular zone of the neural tube and give rise to neurons, astrocytes and oligodendrocytes. Stem cells initially divide symmetrically, generating daughter cells with self-renewal properties, before they gradually acquire differentiation properties and divide asymmetrically, generating a specialized cell and a stem cell in each division. Specialized cells migrate to their final positions and create a properly patterned CNS (Morrison, 2002). Neural crest cells migrate from the dorsal region of the neural tube to various locations and develop into the peripheral nervous system as well as contributing to different tissues and organs such as the facial bones and the heart (Tucker, 2004).

The rostral part of the neural tube initially forms three brain vesicles: the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). These vesicles are then further regionalized (Fig. 1). The forebrain becomes regionalized anteriorly into the telencephalon and posteriorly into the diencephalon. The telencephalon develops into the cerebral hemispheres and the diencephalon gives rise to the thalamic and hypothalamic regions (Marin & Rubenstein, 2002). The hindbrain becomes subdivided into metencephalon which forms the pons and cerebellum, and myelencephalon which forms the medulla oblongata (Melton *et al.*, 2004). The midbrain is not subdivided further.

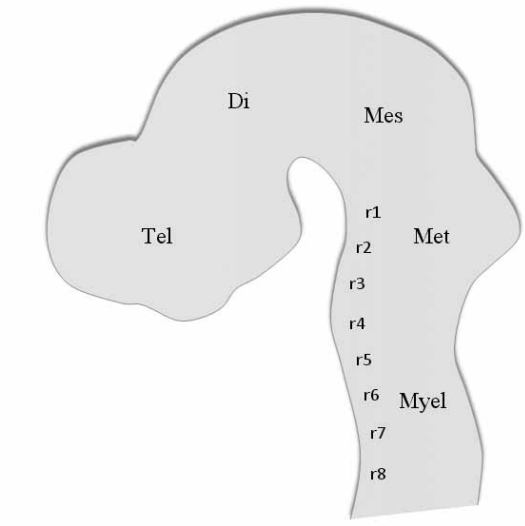


Figure 1 | **Regionalization of the brain vesicles.** The neural tube becomes subdivided in the antero-posterior direction into the telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), metencephalon (Met) and myel-encephalon (Myel). The rhombo-meric segments r1 – r8 are indicated in the hindbrain region.

### 1.1.3 Inductive signals

Control of CNS formation and patterning involves the interplay of multiple signaling systems, which interact to mediate the transcriptional events that specify cell fate. The neural tube is patterned by various signaling systems both along the dorso-ventral and rostro-caudal axis (Joyner, 2002). As cells in different positions in the embryo are exposed to different signalling factors, the position that a cell occupies early in development is of critical importance in determining its fate.

Some of the key factors in the signaling systems are the bone morphogenetic proteins (BMPs), retinoic acid (RA), fibroblast growth factors (FGFs), Hedgehogs and Wnts. In general terms, BMPs, FGFs and Wnts seem to be required for dorsal neuron specification, whereas Sonic Hedgehog promotes ventral cell fate. Graded Wnt signaling is also believed to function along the entire length of the neuraxis regulating antero-posterior neural fates (Lee & Jessell, 1999; Megason & McMahon, 2002; Gunhaga *et al.*, 2003). Along the same axis, opposing gradients of RA and FGFs regulate the regionalization of the hindbrain and spinal cord, respectively (Melton *et al.*, 2004).

## 1.2 BRAINSTEM DEVELOPMENT

The brainstem consists of three parts: midbrain, pons and medulla oblongata. As described above, these regions arise from two of the three brain vesicles, the mesencephalon and rhombencephalon.

### 1.2.1 Midbrain

The midbrain develops from the middle brain vesicle (mesencephalon), and is the brain structure that is the least differentiated from its developmental form. The midbrain is the rostralmost part of the brainstem. It comprises the tectum, involved in auditory and visual processing, and tegmentum, which develops visual-motor control and becomes a part of the basal ganglia through a dopaminergic neuron-rich structure called substantia nigra (Nakamura, 2001; von Bohlen und Halbach & Unsicker, 2009). The midbrain lumen develops into the cerebral aqueduct.

### 1.2.2 Hindbrain

The hindbrain becomes segmented into rhombomeres, which can be described as separate transient compartments that specify where the cranial nerves originate (Fig. 1). The neural crest from each rhombomere develops into ganglia of neuronal cells whose axons form a nerve. The first nerves appear from the even-numbered rhombomeres r2 (trigeminal), r4 (facial and vestibulocochlear) and r6 (glossopharyngeal) (Lumsden & Keynes, 1989). The rhombomeric patterning also regulates spatial and temporal appearance of central pattern generators (Borday *et al.*, 2006). The boundaries between the rhombomeres are correlated with the expression boundaries of highly evolutionary conserved transcription factors known as the homeotic (Hox) genes. Expression of the Hox genes precedes rhombomere formation and plays an important role in the establishment of rhombomere-specific neuronal patterns. Each rhombomere expresses a unique combination of transcription factors, which is thought to specify each region. Mutation studies have shown that a disrupted Hox gene expression can alter the location from where cranial motor nerves emerge or even cranial nerve formation (Melton *et al.*, 2004; Gray, 2008). Duplications or deletions of specific Hox gene clusters may have caused evolutionary changes in species over time.

Similar to the patterning along the anterior-posterior axis, the dorso-ventral axis of the developing hindbrain is specified by many evolutionary conserved genes. These are further divided into different rhombomeric segments. For example, Phox2b is

expressed in several neuronal groups along the dorso-ventral axis but only in the segments r4 – r7 (Gray, 2008).

The hindbrain rhombomeres mature into the anterior pons (r1 – r3) and the posterior medulla oblongata (r4 – r8). The pons comprises several cranial nerve nuclei such as the trigeminal (V), abducens (VI), facial (VII) and vestibulocochlear (VIII) nuclei. In addition, it carries nerve tracts that conduct sensory signals to the thalamus, and signals from the forebrain to the cerebellum and medulla (Angeles Fernandez-Gil *et al.*, 2010). Within the pons is also the pontine respiratory group, also known as the pneumotaxic center, which is involved in the control of the respiratory rhythm and airway muscles (von Euler, 1977).

The medulla oblongata connects directly to the spinal cord and contains the cranial nerve nuclei solitarius (VII, IX, X), ambiguus (IX, X, XI), vagus motor (X) and hypoglossus (XII). The reticular formation runs from here through the whole brainstem and is one of the most evolutionary conserved portions of the brain, with functions in somatic motor control, locomotion, cardiovascular control, pain regulation, consciousness and sleep. The central pattern generator for breathing resides in medulla and will be described in more detail below. Moreover, the medulla contains chemoreceptors and baroreceptors for regulating respiration and vasoconstriction, respectively, and various reflex centers for vomiting, coughing, sneezing and swallowing (Wang, 2009; Angeles Fernandez-Gil *et al.*, 2010).

### 1.2.3 Establishment of neural networks

In 1873, the Italian neuroanatomist Camillo Golgi was the first to visualize the structure of single neurons. The observation was that each neuron consists of a cell body with a long process called the axon and a complex tree-like structure called the dendritic tree. We now know that the shape of dendritic trees is crucial for neuronal signal integration and firing patterns (Mainen & Sejnowski, 1996; Vetter *et al.*, 2001).

The neurons in the brainstem are organized in three layers, which are retained throughout development. The birth of neural cells takes place in the ventricular zone and as the cells divide, migrating cells form the second layer, the intermediate (or mantle) zone, which becomes progressively thicker as more cells are added to it. Here, the neural cells differentiate into neurons and glia. The neurons establish connections among themselves and send axons to the opposite side of the ventricular lumen, thereby creating the third layer, the marginal zone. This is the white matter of the brainstem, which is composed of the axons of multiple pathways, covered with myelin sheaths

formed by glial cells. The gray matter contains the neuronal cell bodies grouped together in clusters. As the brainstem matures it becomes divided into a dorsal portion that receives sensory input, and a ventral portion that control motor functions (Angeles Fernandez-Gil *et al.*, 2010).

The function of the CNS critically depends on the establishment of synaptic connections. Synapses are formed when axons contact dendrites or soma of their target neurons. Some synapses are stabilized and others are eliminated, i.e. "those that fire together wire together" (Goodman & Shatz, 1993). Physiological communications at a synapse occur mainly through the release of neurotransmitters from the presynaptic neuron, which bind to receptors at the postsynaptic cell (Cohen-Cory, 2002).

Expression of neurotransmitters and receptors is essential for synapse formation and neuronal wiring. The expression depends on the specific developmental time window and environment. Glutamate is the principal excitatory transmitter acting on several receptors, both ionotropic and metabotropic. The ionotropic NMDA receptors dominate in the immature brain and are involved in long-term potentiation and synaptic plasticity and refinement. During maturation, NMDA receptors are substituted by AMPA and kainate receptors, which acquire the role of fast neuronal transmission. γ-aminobutyric acid (GABA) and glycine are inhibitory transmitters in the adult CNS, but act as excitatory transmitters in immature neurons (see section 1.3). The interplay between the ionotropic NMDA, AMPA and $GABA_A$ receptors during development is important for neuronal communication, as NMDA receptors are 'silent' at resting membrane potential due to voltage-dependent $Mg^{2+}$ block. $GABA_A$-mediated depolarization removes the blockage of NMDA receptors and thus has a crucial role early in development, which is taken over by AMPA receptors later on (Ben-Ari *et al.*, 1997; Herlenius & Lagercrantz, 2004; Wang & Kriegstein, 2008).

### 1.2.4 Central pattern generators

A central pattern generator (CPG) can be described as a group of neurons exhibiting pacemaker properties that produces a rhythmic pattern of signals. This rhythmic activity generates cycles of movements with repetitive increasing and decreasing periods. CPGs control a variety of motor behaviours such as breathing, locomotion, scratching, chewing and swallowing (Hooper, 2000; Grillner, 2006). The CPG for certain behaviours is usually not confined to only one group of neurons, but rather to several groups that may be distanced from each other. For instance, the CPG for locomotion relies on neuronal cell groups both in the spinal cord and hindbrain

(Hagglund *et al.*, 2010). The neurons within a CPG may not have rhythmogenic abilities individually but generate rhythmic activity only when coupled to each other. Thus, their individual activities are synchronized into a specific pattern of bursts, which depends on a highly coordinated interplay between the neurons. This activity pattern, in turn, generates a rhythmic motor output that defines the CPG behaviour.

CPGs appear to be much more conserved than the peripheral structures they control. Amphibians inflate their lungs through a positive-pressure buccal force pump while mammals, birds and reptiles use a negative-pressure lung aspiration. The nature of this difference lies in the innervation of muscles responsible for breathing. In mammals, both cranial and spinal nerves are involved in respiratory muscle control whereas in amphibians, cranial nerves are the only source for the respiratory drive. Despite this difference in mechanical features, the CPG for breathing in the brainstem has been shown to be preserved (Hedrick *et al.*, 2001). Moreover, as animals mature their rhythmic patterns may change. For example, tadpoles swim while frogs hop, and humans crawl before walking. Similarly, the normal breathing rhythm acquires additional patterns such as sighs and gasps. Thus, CPGs exhibit fundamental properties that are innately established and become multifunctional as new synaptic connections are formed (Hooper, 2000; Lieske *et al.*, 2000). The different behavioural outputs depend on neuromodulatory inputs and sensory feedbacks (Katz & Harris-Warrick, 1999).

### 1.2.5 Emergence of the respiratory rhythm

> *Every time a certain portion is destroyed, be it of the brain or of the spinal cord, a function is compelled to cease suddenly, and before the time known beforehand when it would stop naturally, it is certain that this function depends upon the area destroyed. It is in this way that I have recognized that the prime motive power of respiration has its seat in that part of the medulla oblongata that gives rise to the nerves of the eighth pair; and it is by this method that up to a certain point it will be possible to discover the use of certain parts of the brain.*
>
> \- Julien Jean César Le Gallois (1770 - 1814)

The origin of breathing was the first description of a specific region in the brain exhibiting a certain function (Le Gallois, 1812). During the last decades, various studies

have further shown that distinct neuronal populations in the brainstem are responsible for the generation and modulation of the respiratory rhythm (for review see Smith *et al.*, 2009). These include the pontine respiratory group (PRG), the dorsal respiratory group (DRG) and the ventral respiratory group (VRG). The respiratory pattern is transmitted to bulbospinal pre-motor neurons, which relay the breathing output to cranial and spinal motor neurons controlling respiratory muscles.

The PRG is located in the pons and consists of the parabrachial and the Köllicker-Fuse nuclei. This region is thought to mediate the phase-switch from inspiration to expiration via afferent activity from pulmonary stretch receptors. Inspiration is kept inhibited from this region during the whole phase of expiration, and thus, the PRG is a critical control point for a functional breathing system. It is however not involved in the generation of the respiratory rhythm (von Euler, 1977).

The DRG lies in the dorsal medulla and comprises the nucleus tractus solitarii, which is also involved in taste sensation and cardiovascular, gastrointestinal and cough reflexes (Andresen & Mendelowitz, 1996; Canning, 2007). This nucleus receives afferent input from pulmonary stretch receptors as well as peripheral baro- and chemoreceptors. Thus, it integrates information from the cardiovascular and respiratory system to modulate the breathing output (Bonham *et al.*, 2006; Guyenet *et al.*, 2010). Similar to the PRG, the DRG is not involved in rhythm generation.

The VRG is located in the ventrolateral region of medulla oblongata and consists of several neuronal cell groups, including the Bötzinger complex (BötC), preBötzinger complex (PBC), parafacial respiratory group (pFRG) and retrotrapezoid nucleus (RTN). This region has received much attention during the last decades and is considered the primary center for respiratory rhythm generation. Our understanding of the VRG originates from experiments on isolated brainstem-spinal cord preparations of neonatal rodents (Suzue, 1984). We now know that the PBC and the pFRG, located ventral to the nucleus ambiguus (NA) and facial nucleus, respectively, are the main regions constituting the CPG for breathing (Fig. 2) (Feldman & Del Negro, 2006).

Inception of the respiratory rhythm takes place in the third trimester and more specifically at E15 in mouse (Thoby-Brisson *et al.*, 2005) and E17 in rat (Greer *et al.*, 1992). Neurons with intrinsic bursting pacemaker properties that depend on a persistent $Na^+$ current have been identified as the source (Smith *et al.*, 1991). The respiratory rhythm was first reported to emerge from the PBC (Smith *et al.*, 1991; Rekling & Feldman, 1998), but more recent studies have highlighted the role of pFRG in rhythmogenesis (Onimaru & Homma, 2003; Dubreuil *et al.*, 2008; Thoby-Brisson *et*

*al.*, 2009). The main site for rhythm generation and the function of the two neuronal groups for controlling the inspiratory or expiratory drive has not yet been agreed on (Feldman & Janczewski, 2006; Onimaru & Homma, 2006). Indeed, the network system controlling the different phases and subphases of the respiratory cycle is complex and the most widely recognized model proposes at least six different types of respiratory neurons (Hilaire & Pasaro, 2003). Neurons within the pFRG have been shown to rhythmically discharge both before and after inspiration, suggesting that this region controls more than one phase of the respiratory cycle (Onimaru & Homma, 2003; Onimaru *et al.*, 2006). However, other investigators propose that the pFRG generates expiratory activity whereas the PBC is the main inspiratory rhythm generator (Feldman & Del Negro, 2006; Janczewski & Feldman, 2006). Nevertheless, there is growing evidence that the PBC and pFRG do not act independently but rather interact to form a coupled oscillatory system during development. This theory is based on the finding that the pFRG oscillator emerges before the PBC (at E14.5 in mice), then couples with, and thereby increases the frequency of motor bursts from, the PBC (Thoby-Brisson *et al.*, 2009). Notably, this coupling relies on the formation of electrical contacts between the neurons through gap junctions. Rhythmogenesis is blocked by the gap junction antagonist carbenoxolone, indicating a critical role of electrical synapses prior to the maturation of chemical synapses (Thoby-Brisson *et al.*, 2009; Jaderstad *et al.*, 2010).

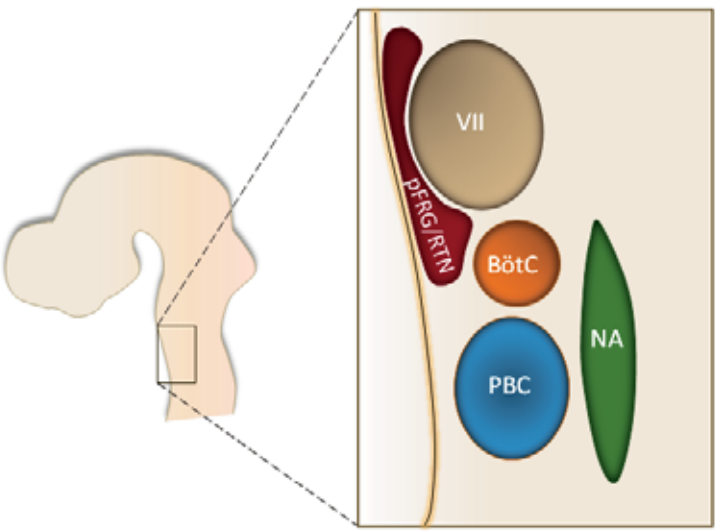


Figure 2 | **The ventral respiratory group.** The key site for respiratory rhythmogenesis develops from the rhombomeric segments r4 – r7. This region includes the parafacial respiratory group/retrotrapeziod nucleus (pFRG/RTN), facial nucleus (VII), Bötzinger complex (BötC), preBötzinger complex (PBC) and nucleus ambiguus (NA).

Immunohistochemical markers that identify PBC neurons are the substance P receptor, also known as the neurokinin-1 receptor (NK1R), and somatostatin (SST) (Stornetta *et al.*, 2003). PBC neurons are born in the ventricular zone at E9.5 – E11.5 (Gray *et al.*, 2010) and reach the PBC area at E14 – E15 in mice (Thoby-Brisson *et al.*, 2005) or E16 – E17 in rats (Pagliardini *et al.*, 2003). NK1R is also expressed in surrounding areas, including the pFRG (Onimaru *et al.*, 2009). While GABA, glycine, acetylcholine and glutamate activity is thought to promote the neural network formation and the emergence of the respiratory rhythm (Ren & Greer, 2003), other neuromodulators have also been shown to play a crucial role in the regulation of the breathing pattern (Rekling & Feldman, 1998; Herlenius *et al.*, 2002). The importance of the PBC and pFRG neurons for the respiratory rhythm has been demonstrated in several studies. Targeted disruption of NK1R-expressing neurons in the PBC (Gray *et al.*, 2001) and pFRG (Nattie & Li, 2002) leads to ataxic respiration in rats. Moreover, bilateral destruction of SST-expressing neurons in the PBC produces persistent apnea in rats (Tan *et al.*, 2008). The pFRG neurons also express the transcription factor Phox2b, which is mutated in congenital central hypoventilation syndrome in humans. In addition, mice mutant for Phox2b display an irregular breathing pattern and die at birth (Dubreuil *et al.*, 2008; Dubreuil *et al.*, 2009). Other transcription factor mutations that affect rhombomeric patterning and interneuron specification have also been shown to cause respiratory failure, indicating a complex genetic system for the formation of the CPG for breathing (Blanchi *et al.*, 2003; Rhee *et al.*, 2004; Pagliardini *et al.*, 2008; Rose *et al.*, 2009; Bouvier *et al.*, 2010; Gray *et al.*, 2010). Furthermore, there appears to be a high potential for plasticity within the respiration-related regions. Notably, the number of pFRG neurons has been found to increase in response to incremental PBC destruction in goats, thereby rescuing the respiratory rhythmogenesis (Neumueller *et al.*, 2010). This may reflect a compensatory mechanism in which neurogenesis or migration is involved (Bordey, 2010).

In addition to its rhythm-generating properties, the VRG also contains neurons that modulate the rhythm in response to afferent and chemoreceptive information. The RTN is located ventral to the facial nucleus and is anatomically indistinguishable from the pFRG region. The neurons within the RTN are involved in chemoreception, such as responses to hypercapnia, and regulates breathing following changes in $CO_2$ and pH (Nattie & Li, 2002). Moreover, astrocytes in the RTN region are also important for chemoreception and regulation of breathing through pH-dependent release of ATP (Gourine *et al.*, 2010). While RTN neurons have not been found to generate pre-

inspiratory activity, and the pFRG has not been identified in adult mammals, a recent study suggests that the nearby region BötC adopts a pre-inspiratory role during hypoxic conditions in adults (Fortuna *et al.*, 2008). The BötC is found rostral to the PBC and is also involved in modulation of the respiratory rhythm through inhibition of inspiratory and expiratory neurons during the late phase of expiration (Tian *et al.*, 1999).

In summary, generation of the respiratory rhythm is a highly complex system and involves several regions in the brainstem, including the pacemaker sites PBC and pFRG, the activity of which is balanced by integration of chemical and mechanical information from the whole body.

## 1.3 NEURONAL CHLORIDE HOMEOSTASIS DURING DEVELOPMENT

### 1.3.1 GABA signalling and neuronal maturation

GABA binds to two types of receptors, $GABA_A$ and $GABA_B$. $GABA_A$ is a ligand-gated ion channel permeable to $Cl^-$ and $HCO_3^-$, whereas $GABA_B$ is a metabotropic receptor coupled to $K^+$ and $Ca^{2+}$ channels via G proteins (Kaila, 1994). In the adult brain, GABA is the principal inhibitory neurotransmitter, regulating many functions such as muscle control, memory and anxiety. In immature neurons, however, the postsynaptic GABAergic response is depolarizing. This is due to a high intracellular chloride concentration ($[Cl^-]_i$), which in turn is caused by the age-dependent expression of cation-chloride cotransporters (see below). The early importance of GABA is evident as establishment of GABAergic synapses precedes the appearance of glutamatergic synapses (Ben-Ari *et al.*, 1997). The depolarizing action of GABA has been proposed to be crucial for the differentiation and maturation of neurons within the CNS. Activation of voltage-dependent $Ca^{2+}$ channels, induced by $GABA_A$-mediated depolarization, increases the intracellular $Ca^{2+}$ concentration and activates a wide range of intracellular cascades involved in neuronal migration, growth and differentiation. Furthermore, $GABA_A$-mediated depolarization and $Ca^{2+}$ influx act as crucial triggers for plasticity of synaptic connections and for the establishment and patterning of neural networks (Yuste & Katz, 1991; Owens & Kriegstein, 2002; Bolteus & Bordey, 2004; Cancedda *et al.*, 2007; Farrant & Kaila, 2007).

Despite its excitatory role in the immature CNS, GABA can also exert suppression of glutamatergic signaling by “shunting inhibition”. This occurs when the conductance is increased (i.e. resistance decreased) due to opening of ion channels.

According to Ohm's law, more current is required to change the voltage when the resistance is lower. In other words, a glutamate signal that would normally depolarize the postsynaptic membrane is inhibited due to GABA-mediated opening of $Cl^-$ channels. This has been shown in several brain regions, including the hippocampus early in development (Lamsa *et al.*, 2000; Banke & McBain, 2006), and is thought to be important for postsynaptic stabilization when hyperpolarization does not occur.

### 1.3.2 Cation-chloride cotransporters

Regulation of the cellular $[Cl^-]_i$ is important for controlling cell volume and pH. The $[Cl^-]_i$ also determines the neuronal response to GABA and glycine (Misgeld *et al.*, 1986). Members of the cation-chloride cotransporter (CCC) family regulate ion homeostasis through inward or outward directed flux of ions, which depends on electrochemical gradients set by active transporters. The gradient of $K^+$ is used by the $K^+/Cl^-$ cotransporters (KCC) to extrude $Cl^-$ out of the cell, whereas the $Na^+$ gradient is used by $Na^+/K^+/Cl^-$ cotransporters (NKCC) to accumulate $Cl^-$ within the cell. So far, four $K^+/Cl^-$ cotransporters (KCC1-4) and two $Na^+/K^+/Cl^-$ cotransporters (NKCC1-2) have been discovered. NKCC1 and KCC1 are generally expressed in all tissues, and NKCC2 is restricted to the kidney. KCC2 is expressed in central neurons only, whereas KCC3 and KCC4 are found both in CNS and other tissues (Delpire & Mount, 2002; Payne *et al.*, 2003). Expression of the CCCs has a highly regulated spatio-temporal pattern. In the embryonic CNS, KCC2 and KCC3 are found in more mature regions, while KCC4 and NKCC1 are expressed in undifferentiated regions (Li *et al.*, 2002).

In immature neurons, high level expression of the chloride loader NKCC1 and low level expression of the chloride extruder KCC2 results in elevated $[Cl^-]_i$. This produces the depolarizing response to GABA in neuronal cells. Progressive loss of NKCC1 and increased membrane expression of KCC2 are the two major factors determining the $[Cl^-]_i$ reduction in maturing neurons and the subsequent shift of the GABAergic response to hyperpolarizing (Fig. 3) (Plotkin *et al.*, 1997; Rivera *et al.*, 1999; for review, see Blaesse *et al.*, 2009).

### 1.3.3 KCC2

KCC2 is a neuron-specific isoform of the KCC-family. It is a glycosylated protein with 12 predicted transmembrane domains and has a size of ~145 kDa (Payne *et al.*, 1996). In the mature CNS, KCC2 is widely expressed in the cortex, hippocampus, retina, cerebellum, brainstem and spinal cord (Payne *et al.*, 1996; Lu *et al.*, 1999; Vu *et al.*,

2000; Coull *et al.*, 2003). During development, however, KCC2 displays lower expression levels, which increase spatio-temporally in relation to the ontogenetic developmental patterns: spinal cord – brainstem – hippocampus – cortex (Rivera *et al.*, 1999; Stein *et al.*, 2004; Delpy *et al.*, 2008). By alternative splicing, two isoforms termed KCC2a and KCC2b are generated from the mammalian KCC2 (Slc12a5) gene. These isoforms constitute around 50% each of the total KCC2 levels during embryonic development. KCC2b is the isoform that is strongly upregulated postnatally (Uvarov *et al.*, 2007). As most studies so far have used antibodies that detect both KCC2a and KCC2b, the term "KCC2" here refers to both isoforms.

The electrochemical gradient for $Cl^-$ sets the reversal potential for GABA responses ($E_{GABA}$). As described above, immature neurons contain high $[Cl^-]_i$. Therefore, $E_{GABA}$ is higher than the resting membrane potential and results in depolarization when $GABA_A$ receptors are activated. When KCC2 becomes transport-active, it lowers $[Cl^-]_i$ via $Cl^-$ extrusion and shifts $E_{GABA}$ to more negative levels than the resting membrane potential (Fig. 3). This generates the hyperpolarizing response to GABA in mature neurons (Rivera *et al.*, 1999). A similar function has been ascribed to KCC2 homologues in *C. elegans* and *Drosophila*, indicating an early evolutionary origin of this gene (Hekmat-Scafe *et al.*, 2006; Tanis *et al.*, 2009). The functional activation of KCC2 cotransport relies on several factors, such as afferent input, phosphorylation, membrane trafficking, intracellular localization and formation of oligomers (Sernagor *et al.*, 2003; Shibata *et al.*, 2004; Blaesse *et al.*, 2006; Kanold & Shatz, 2006; Lee *et al.*, 2007; Hartmann *et al.*, 2009).

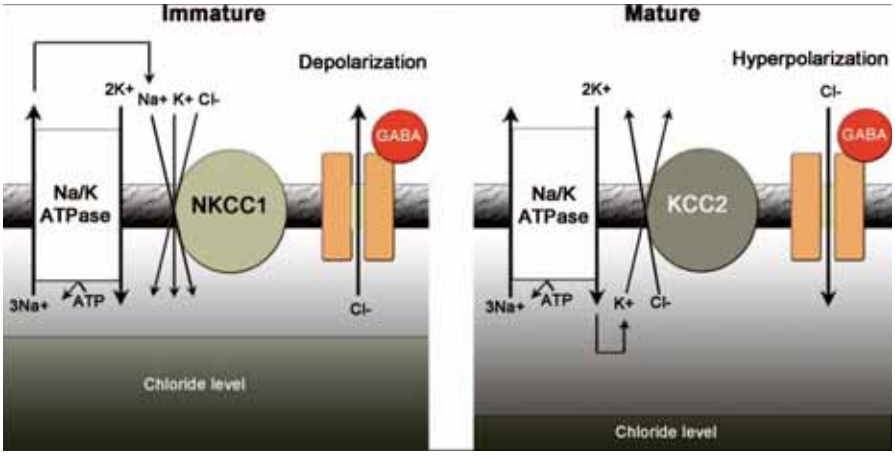


Figure 3 | **The developmental GABA shift.** The postsynaptic response to GABA shifts from depolarizing to hyperpolarizing following KCC2-mediated $Cl^-$ extrusion in maturing neurons.

It has been shown in various studies that KCC2 is critical for lowering $[Cl^-]_i$ in the CNS. For instance, cortical neurons lacking KCC2 fail to regulate $[Cl^-]_i$ (Zhu *et al.*, 2005). $KCC2^{-/-}$ mice, which are devoid of both isoforms, have severe defects in motor control and die at birth due to respiratory failure. These mice lack of inhibitory responses to GABA in motor neurons (Hubner *et al.*, 2001). Moreover, KCC2-deficient mice with only 5-10% of postnatal KCC2 expression display frequent generalized seizures and die within two weeks after birth (Woo *et al.*, 2002). It was recently found that these mice have a specific deletion of the KCC2b isoform (Uvarov *et al.*, 2007). In addition, hypomorphic mice retaining 15-20% of normal KCC2 protein levels are viable but display increased anxiety-like behaviour and impaired learning in several tests and are more susceptible to seizures (Tornberg *et al.*, 2005). This indicates an important role for KCC2 in controlling CNS excitability.

Two different studies in 2005 showed that overexpression of KCC2 in immature neurons shifts $E_{GABA}$ prematurely. Additional effects reported were decreased GABA-elicited calcium responses (Lee *et al.*, 2005) and increased density of $GABA_A$ receptors and synapses (Chudotvorova *et al.*, 2005). In another study, KCC2 was electroporated in utero in rat ventricular progenitors at E17, which resulted in impaired morphological maturation of cortical neurons, seen as a reduced dendritic length and branch number (Cancedda *et al.*, 2007). Furthermore, overexpressing KCC2 from the onset of development in zebrafish impairs neuronal development in the brain and spinal cord. The embryos display a perturbed neuronal differentiation and axonal growth, and fewer motoneurons and interneurons (Reynolds *et al.*, 2008). These studies highlight the importance of a spatio-temporally regulated KCC2 expression for neuronal development. It is possible that a premature $E_{GABA}$ shift abolishes the neurotrophic function of depolarizing GABA.

Another situation illustrating the neurotrophic role of GABA is in the adult CNS where KCC2 is downregulated under pathophysiological conditions (e.g. epilepsy, injury). This seems to reflect a recapitulation of early developmental mechanisms, which may be necessary for the re-establishment of connectivity in damaged brain tissue (Rivera *et al.*, 2004).

Interestingly, studies have shown that KCC2 is expressed early in development when GABA is still depolarizing. KCC2 mRNA is present at E10.5 in mice and precedes the decline of $E_{GABA}$ in the hippocampus and spinal cord (Stein *et al.*, 2004). Moreover, KCC2 displays functional $Cl^-$ transport already at E13.5 in the mouse spinal cord although the GABAergic response does not shift until late fetal age (Delpy *et al.*,

2008). KCC2 is present at the depolarizing period also in the brainstem lateral superior olive, and becomes integrated into the plasma membrane only with increasing postnatal age (Balakrishnan *et al.*, 2003). These studies did not address the potential role of KCC2 before the developmental $E_{GABA}$ shift, which is one of the aims of this thesis.

The formation of dendritic spines is closely paralleled by upregulation of KCC2 in cortical regions. KCC2 is accumulated in the vicinity of excitatory synapses in dendritic spines (Gulyas *et al.*, 2001), suggesting a role in spine development. This was further investigated by Li and coworkers (Li *et al.*, 2007). Interestingly, KCC2 was shown to play a crucial role in the formation of mature dendritic spines. In the absence of KCC2, neurons developed long dendritic protrusions paralleled by a reduction in active synapses. Ectopic expression of both full-length KCC2 and a truncated form of KCC2, lacking the sequence for functional cotransport in the N-terminal, restored the normal spine morphology in $KCC2^{-/-}$ neurons, indicating that the morphogenic effects were not mediated through ion transport. Intriguingly, ectopic expression of only the C-terminal of KCC2 in wild type neurons yielded a phenotype similar to $KCC2^{-/-}$ neurons, and in addition, interacted with the cytoskeleton-associated protein 4.1N. Thus, a novel structural role for KCC2 has been shown during dendritic maturation, which may be crucial for synchronizing excitatory and inhibitory transmission in cortical networks.

## 1.4 THE NEURONAL CYTOSKELETON

During morphogenesis, neurons become polarized and extend neurites and dendrites, which relies on coordinated dynamics and organization of the cytoskeleton. The cytoskeleton of neurons comprises three distinct structural complexes: microtubules, neurofilaments and microfilaments.

### 1.4.1 Microtubules

Neuronal microtubules are structurally similar to those found in other eukaryotic cells. They consist of heterodimers of α- and β-tubulin aligned to protofilaments that form a hollow tube. The tubulins are further divided into various isotypes with different post-translational modifications and microtubule-stabilizing proteins. Tubulin isotypes differ mainly in the carboxy terminus, which is the region where most post-translational modifications and interactions with stabilizing proteins occur. Neuron-specific isotypes are the class III and IVa β-tubulins. The composition of microtubules depends on the

neuronal environment and varies between the soma, dendrites and axons. Microtubules regulate neurite extension and establishment, and maintain the integrity of intracellular compartments. They also play a role in mitosis, differentiation and intracellular transport (Luduena, 1993; Heidemann, 1996; Poulain & Sobel, 2010).

The stabilizing proteins include several categories of molecules with different locations and functions. The microtubule-associated proteins (MAPs) can be divided into three families: MAP1, MAP2 and tau. MAPs regulate neurite and axonal outgrowth through microtubule stabilization and organization, and provide functional interactions with other cytoskeletal components. Moreover, temporal changes in MAP expression during CNS development indicate a highly coordinated function for neuronal maturation. Another stabilizing protein is Doublecortin (Dcx), which is mutated in X-linked lissencephaly, a severe malformation of the cerebral cortex. Dcx has an essential role in neuronal migration, as well as in neurite and dendrite elongation and branching (Poulain & Sobel, 2010).

### 1.4.2 Neurofilaments

Neurofilaments are the neuronal type of intermediate filaments. They consist of a core rod domain with multiple α-helical domains that form coiled coils. The CNS contains a diverse set of neurofilaments with distinctive cellular distributions and developmental expression patterns. They provide mechanical strength and a stable cytoskeletal structure for maintenance of neuronal morphology. In addition, they are the most abundant structural components in axons and play an important role in the establishment and maintenance of axonal diameter (Cleveland *et al.*, 1991). Other intermediate filaments found in the developing CNS include vimentin, glial fibrillary acidic protein, peripherin and nestin, which are expressed in neuronal and glial precursors. As nestin is found in multipotent cells and downregulated during subsequent maturation, it can be used as a marker for immature neural cells (Yamaguchi *et al.*, 2000).

### 1.4.3 Microfilaments

Actin is the core subunit of microfilaments and is organized in strings of monomers (G-actin), which are further intertwined into fibrils (F-actin). Actin microfilaments is found throughout neurons but enriched at the plasma membrane, growth cones and dendritic spines. The membrane actin plays important roles in cell adhesion, establishment of cell morphology, and maintenance of membrane protein distribution. Microfilaments are

also the basis of filopodia and lamellipodia that control growth cone motility and guidance, as well as cell migration (Luo, 2002).

Many cytoskeleton-associated proteins are found in neurons. For instance, spectrin and ankyrin have roles in cross-linking the microfilaments to each other and to the plasma membrane (Cunha & Mohler, 2009). Moreover, N-cadherin and catenins are critical for intercellular connections, mechanical strength and spatial segregation. Cadherins interact with other cadherins on adjacent cells, and are anchored into the cell by a complex of α-, β- and p120-catenin. The catenins, in turn, bind to the actin cytoskeleton, thereby creating a mechanical unit called adherens junctions that connect cells together (Harris & Tepass, 2010).

### 1.4.4 Regulation of the cytoskeleton

Coordinated control of the cytoskeleton and cell adhesion is essential for morphological changes during embryonic development. A wide variety of factors have been shown to interact directly or indirectly with the cytoskeleton and thereby influence the behaviour of the cell. One category of regulatory factors is ion transport proteins, which were described in section 1.3. Ion transporters anchor the actin cytoskeleton to the plasma membrane by binding to ankyrin and members in the 4.1 family. While this interaction is important for the localization, clustering and functional activity of the ion transporters, it can also play a structural role in cytoskeletal organization within the cell (Denker & Barber, 2002). A transport-independent structural role of KCC2 was described above and will be further detailed in this thesis. In addition, NKCC1 has recently been shown to induce a secondary body axis, also in the absence of transport-activity, when overexpressed in Xenopus embryos (Walters *et al.*, 2009). Moreover, gap junctions, which are traditionally known for their roles in intercellular coupling and transport of ions, metabolites and other molecules between cells, have in recent studies been suggested to act as adhesive contacts that mediate neuronal migration. This novel function was shown to depend on an interaction with the actin cytoskeleton (Elias *et al.*, 2007).

The cytoskeleton is also regulated by extracellular signals that are interpreted by cell surface receptors and downstream signalling pathways. Among the most important paracrine factors during early development are the Wnt proteins, which regulate many aspects of neural cell development, including proliferation, differentiation, survival, polarity and migration (Miller, 2002). Wnts act via different signaling pathways, all of which can influence the microtubule and actin cytoskeleton (Brembeck *et al.*, 2006;

Ciani & Salinas, 2007). One of the key players, β-catenin, is continuously degraded in the cytoplasm but is stabilized by Wnt signaling. This allows it to act as a transcription factor or take part in the formation of adherens junctions (Nelson & Nusse, 2004). Other signaling proteins that regulate neuronal morphogenesis are the planar cell polarity molecules (described below) and the Rho GTPases, which include Cdc42, Rac1 and RhoA. These GTPases act as intracellular molecular switches that exist in an active GTP-bound form and an inactive GDP-bound form. The GTP-bound form binds to downstream effectors that participate in the regulation of actin polymerization and organization, which is essential for cell adhesion and migration (Braga *et al.*, 1997; Nobes & Hall, 1999), as well as neurite growth, guidance and branching (Luo, 2002). A cooperative action of different GTPases has been demonstrated in several studies. For example, selective inhibition of Rac1 or RhoA perturbs adherens junctions, and expression of a constitutively active form of Rac1 is not sufficient to stabilize the junctional complex when endogenous RhoA is blocked (Braga *et al.*, 1997).

### 1.4.5 Planar cell polarity and Vangl2

The planar cell polarity (PCP) proteins participate in Wnt signaling and control polarized cell movements during gastrulation and neurulation via cytoskeletal organization. PCP is cell polarity within the plane of epithelium, along an axis perpendicular to the apical-basal axis of the cell. Examples of PCP include the coordinated organization of scales in fish, feathers in birds and hairs in mammals. PCP genes play a key role in convergent extension, the process in which mesodermal cells intercalate and thus elongate the body axis. Alterations in the expression of PCP genes cause defective convergent extension movements and impaired neurulation, resulting in a shortened body axis and neural tube defects, as shown in many PCP mutants (Curtin *et al.*, 2003; Murdoch *et al.*, 2003; Ueno & Greene, 2003; Wang *et al.*, 2006; Torban *et al.*, 2008).

The naturally occurring mouse mutant *Looptail* displays a severe neural tube defect known as craniorachischisis that is characterized by a completely open neural tube from the midbrain-hindbrain boundary to the most caudal region. The *Looptail* mouse has a missense mutation in a gene called Van Gogh-like 2 (Vangl2), also known in the litterature as Strabismus (the *Drosophila* homolog) and *Looptail* associated protein (Ltap). Vangl2 is a membrane protein with four transmembrane domains and a large intracellular PDZ-binding domain (Kibar *et al.*, 2001). Vangl2 mutations in *Drosophila* disrupt the polarity of hairs on wing cells and orientation of eye ommatidia

(Taylor *et al.*, 1998; Wolff & Rubin, 1998). In the mammalian inner ear, Vangl2 has been demonstrated to regulate the polarity of hair cell stereociliary bundles in interaction with the PCP proteins Scrb1, Fz3 and Celsr1 (Montcouquiol *et al.*, 2003; Montcouquiol *et al.*, 2006). Vangl2 has a crucial role in convergent extension and neural tube closure in several organisms, including mouse, zebrafish and xenopus (Kibar *et al.*, 2001; Goto & Keller, 2002; Jessen *et al.*, 2002). The mechanism whereby these processes are regulated is still unclear, but several studies have suggested that Vangl2 recruits other PCP proteins to the plasma membrane to form an active signaling complex, which stimulates Rho GTPases and influences the cytoskeleton (Torban *et al.*, 2004).

Mutations in Vangl2 have recently been found also in human cases of neural tube defects, both in cranial and spinal variants of the disease (Kibar *et al.*, 2010; Lei *et al.*, 2010). While a related protein, Vangl1, sharing 68% sequence identity and a similar overall structure, has been found to be mutated in patients with both familial and sporadic types of spinal neural tube defects (Kibar *et al.*, 2007), Vangl2 mutations have so far been found only in sporadic cases with no reported family history (Kibar *et al.*, 2010).

# 2 AIMS

The main objective of this thesis was to identify specific roles of the cytoskeleton-interacting proteins KCC2 and Vangl2 in different aspects of brainstem development.

*Specific aims of the studies:*

- To investigate the developmental expression and membrane targeting of KCC2 and its correlation with the $E_{GABA}$ shift in respiration-related neurons.

- To elucidate the role of the early expressed, transport-inactive, KCC2 protein in the developing central nervous system.

- To characterize the importance of KCC2 for functional and structural maturation of the central pattern generator for breathing.

- To determine mechanisms whereby Vangl2 regulates neural tube closure in the hindbrain region.

# 3 METHODOLOGY

In this section, aspects of methodology will be discussed and detailed information about specific methods used can be found in the papers as listed below.

- In vitro brainstem-spinal cord preparations *Papers I, III*
- Brainstem organotypic cultures *Paper III*
- Generation of DNA expression constructs *Papers II, IV*
- Generation of transgenic and mutant embryos *Papers II – IV*
- Phenotyping and genotyping *Papers II – IV*
- Immunohistochemistry *Papers I – IV*
- In situ hybridization *Paper IV*
- Cell culturing and transfections *Papers II, IV*
- Immunocytochemistry *Papers II, IV*
- Aggregation and wound assays *Papers II, IV*
- Immunoprecipitation and GTPase activity assay *Papers II, IV*
- Western blot *Papers II, IV*
- Fluorescence and confocal microscopy *Papers I – IV*
- Time-lapse imaging using calcium dyes *Paper III*

## 3.1 IN VIVO MODELS

### 3.1.1 Transgenic mouse embryos

Generation of transgenic mice by pronuclear injection is an expensive and time-consuming process, but still widely used as a complement to knockout models. The advantage of overexpressing a gene is that the use of spatio-temporally regulated enhancers can easily produce a tissue- and time-specific expression. The resulting phenotype can then be ascribed to a specific function of the protein, and functional or structural interactions with other proteins, in a given time frame and cell type. A disadvantage is that the integration of the transgene into the genome is random and could generate an insertional mutation that interferes with the function of an existing gene.

For this thesis, transgenic mouse embryos overexpressing KCC2 or Vangl2 were generated by Karolinska Center for Transgene Technologies (KCTT), using DNA constructs made by us. Briefly, female B6D2F1 mice (F1 of strains C57Bl/6 x DBA2) were superovulated, mated to fertile B6D2F1 males, and then sacrificed the next day. Fertilized oocytes were recovered from excised oviducts and the DNA construct was microinjected into the male pronucleus. Injected zygotes were then reimplanted into the oviduct of foster females of the NMRI strain, which had been made pseudopregnant by mating with vasectomized males. Transgenic embryos then started to develop and could be collected at desired time points.

The DNA constructs contained cDNA of the gene of interest (three different variants of KCC2 in *Study II*, and Vangl2 in *Study IV*), a thymidine kinase promoter, and a tissue-specific enhancer upstream of the sequence. We chose a 1852 bp sequence in the second intron of the human nestin gene as enhancer. This enhancer sequence has previously been shown to direct the expression exclusively to neural progenitor cells from around E7.5 when neurulation takes place (Lothian & Lendahl, 1997). Thus, a neural-specific overexpression beginning in the ventricular zone could be studied at different time points of CNS development. Transgenic embryos were identified by PCR using a sense primer complementary to the nestin sequence and an antisense primer complementary to the KCC2 or Vangl2 sequence.

### 3.1.2 Mutant mouse embryos

Deletion of a gene can be obtained in different ways. While KCC2 knockouts have been generated by a gene targeting technique, *Looptail* mice have a natural mutation in the Vangl2 gene. However, the homozygous genotype of both of these mice completely lacks functional expression of the relevant gene and is lethal perinatally.

The $KCC2^{-/-}$ mouse was first described in 2001 (Hubner *et al.*, 2001). They do not move or breathe spontaneously and die within minutes after birth. In addition to the motor deficits they also display lung atelectasis and omphaloceles. There are no reported KCC2 mutations in any human syndromes, suggesting that a mutation may result in early mortality, similar to what has been observed in mice. However, KCC2 has been found to be downregulated in neuronal trauma, neuropathic pain and epilepsy, although this may be a consequence rather than an inducing factor (see Blaesse *et al.*, 2009). This is where one of the limitations of the knockout technique applies, as a gene mutation can often be lethal early in development and later functions of the gene are therefore not possible to study. While this can be overcome by the use of conditional

knockouts, it has not yet been employed for KCC2. However, specific deletion of the KCC2b isoform causes early seizures in mice (Woo *et al.*, 2002; Uvarov *et al.*, 2007), and mice retaining 15 – 20% of the KCC2 expression show impaired learning, and increased anxiety-like behaviour and seizure susceptibility (Tornberg *et al.*, 2005). As this thesis focuses on the role of KCC2 during embryonic development, the neonatal lethality is an object of elucidation rather than a concern. We only used $KCC2^{-/-}$ and wild type genotypes in our study since heterozygous ($KCC2^{+/-}$) mice do not differ from wild type mice (Hubner *et al.*, 2001). $KCC2^{+/-}$ mice (C57Bl/6N) were maintained and bred to generate the desired homozygous genotypes, which were collected at the latest possible time point (E17.5 – E18.5) to study neural network formation in the brainstem respiratory centers.

The *Looptail* mouse was described already in 1949 but it was not until 2001 it was found to have a mutation in the Vangl2 gene (Kibar *et al.*, 2001). This mouse provides a model for the most severe neural tube defect known as craniorachischisis (described in section 1.4.5), which comprises 10 – 20 % of human cases, but also for other forms of neural tube defects that are related to Vangl2 mutations (Kibar *et al.*, 2010). *Looptail* heterozygotes may display the more common defect spina bifida, although at a lower frequency. In craniorachischisis, the onset of neural tube closure in the hindbrain-cervical boundary fails and hence the brainstem and spinal cord regions do not form. This thesis focuses on an early stage of the defective closure and thus employs *Looptail* embryos at E9.5.

All animals in these studies were treated according to European Communities Council guidelines (directive 86/609/EEC) and the experiments were approved by the local Animal Ethics Committees of Karolinska Institutet and the University of Helsinki.

## 3.2 IN VITRO MODELS

### 3.2.1 Brainstem-spinal cord preparations

For electrophysiological recordings, the brainstem-spinal cord preparation is a widely recognized model due to its easily accessible nerve roots and preserved physiological functions such as the respiratory rhythm (Suzue, 1984). This in vitro set up enables modifications of the perfused physiological solution to study the effect of different drugs, neurotransmitters, neuromodulators, and pH, $O_2$ and $CO_2$ variations. The

respiratory output involves activity of different nerves, including cranial (VII and XII), phrenic, and ventral cervical nerves.

We employed brainstem-spinal cord preparations from perinatal rats to study the nature of GABA in respiratory rhythm generation in *Study I*. Motor nerve activity was recorded from cervical (C4) ventral roots, and intracellular whole cell and gramicidin-perforated patch recordings were made from ventrolateral medulla. The substances and drugs used were GABA and its agonist Muscimol and antagonist Bicuculline, and the $Na^+$ channel blocker Tetrodotoxin.

### 3.2.2 Brainstem organotypic cultures

The use of organotypic cultures is a well established technique to study functional networks and motor neuron activity in vitro. Indeed, brainstem slices at the level of medulla oblongata are widely used for analysis of the respiratory rhythm by calcium imaging and/or electrophysiology (Smith *et al.*, 1991; Feldman & Del Negro, 2006; Ruangkittisakul *et al.*, 2006; Hartelt *et al.*, 2008; Thoby-Brisson *et al.*, 2009). However, the in vitro rhythm may differ from the in vivo rhythm due to the lack of afferent input (Richter & Spyer, 2001) and the common use of high [$K^+$] levels to maintain rhythmic activity (Ren & Greer, 2006). Nevertheless, medullary slices containing the PBC have been shown to generate long-lasting rhythm with a high sensitivity to neuromodulators also at physiological [$K^+$] (3 mM) (Ruangkittisakul *et al.*, 2006).

We employed medullary slice cultures of wild type and $KCC2^{-/-}$ E17.5 – E18.5 embryos in *Study III*. Transversal slices were cut 300 µm thick and placed onto semi-permeable membranes with culture medium underneath, according to the Stoppini interface method (Stoppini *et al.*, 1991). This method preserves the cytoarchitectural organization of the slice for extended time periods and allows for live imaging studies as well as electrophysiological recordings from individual neurons or nerve roots. Slices containing the PBC were selected as described by Ruangkittisakul *et al.*, 2006. Anatomical landmarks that were used as reference constituted the shape of the whole slice and of the pyramids and $4^{th}$ ventricle, the presence of the hypoglossal nuclei as darker regions dorso-medially, and if visible, also the hypoglossal nerve root. For further verification, tetramethylrhodamine-conjugated substance P (TMR-SP) was used to label NK1R-positive cells (see below).

### 3.2.3 Neural and epithelial cell lines

Different cell lines were employed for this thesis depending on the specific purpose of the study. The mouse neural stem cell line C17.2 was used to assess cytoskeletal interactions and migration of neural cells, whereas the epithelial cell lines HEK293 and MDCK were used to study adherens junctions and cell adhesion.

The C17.2 clone is derived from the mouse cerebellum at postnatal day (P) 4. This cell line is maintained multipotent and undifferentiated by v-myc immortalization, and is capable of generating both neurons and glia (Snyder *et al.*, 1992). The cells have been used in various studies to replace lost cells in the CNS of injured or mutant mice, and integrate well in the host tissue via gap junctional couplings (Jaderstad *et al.*, 2010). Moreover, C17.2 cells have been shown to exhibit a remarkable migrating capacity in vivo when implanted to the mouse midbrain (Ourednik *et al.*, 2002).

HEK (human embryonic kidney) 293 and MDCK (Madin-Darby canine kidney) are derived from the kidney of a human embryo and adult cocker spaniel, respectively. They are commonly used as a general model for epithelial cells, characterized by their tight organization into sheets, and thus enable studies of adherens junctions and cell adhesion. There are some controversies around the origin of HEK293 cells, and they have been reported to express neurofilaments and other neuronal proteins (Shaw *et al.*, 2002). While this would make HEK293 cells even more suitable for this thesis, the structure of adherens junctions is essentially similar in all epithelial cells, and cell characterization is therefore of less importance here.

Cells were transfected either with DNA constructs to overexpress proteins or with RNA interference (RNAi) to inhibit protein expression. The DNA constructs were created using a pcDNA vector with cDNA of the gene of interest (different variants of KCC2 in *Study II*, and Vangl2 in *Study IV*). Constructs containing constitutive active and dominant negative Rac1 and RhoA were also used in *Study IV*. RNAi is a short double-stranded RNA sequence, corresponding to a sequence in the gene of interest, which is broken down to smaller fragments by the enzyme Dicer. These fragments are separated to single strands that bind to and induce cleavage of the target mRNA. For *Study IV*, we used RNAi against Vangl2 and Rac1.

## 3.3 CELL COMMUNICATION

### 3.3.1 Calcium imaging

Time-lapse imaging with calcium indicators is a widely used technique to study the activity of living cells (Uhlen & Fritz, 2010). This technique employs indicators based on the $Ca^{2+}$ chelators EGTA or BAPTA with fluorescent properties that are activated upon $Ca^{2+}$ binding. We used the calcium indicator Fluo-4, which is excited at 488 nm and gives a bright fluorescence emission (Gee *et al.*, 2000). To facilitate entrance into the cells in a preserved neutral form, it is initially coupled to a lipophilic acetoxymethyl ester group that is cleaved off by intracellular esterases to give free fluorescent Fluo-4.

We used calcium imaging to assess the cellular activity and correlation within the PBC of wild type and $KCC2^{-/-}$ medullary slice cultures. Before Fluo-4 loading, we incubated the slices in medium containing TMR-SP to verify the localization of NK1R-positive neurons. TMR-SP is internalized after binding to NK1R and even increases the frequency of respiratory bursts measured from the hypoglossal nerve in medullary slices (Pagliardini *et al.*, 2005). As TMR-SP is excited at 565 nm it is suitable for co-labelling with Fluo-4. During image acquisition, the slices were constantly perfused with a standard physiological solution, which contained 3 mM $[K^+]$ similarly to Ruangkittisakul *et al.*, 2006. In some experiments, responses to 100 µM glutamate or 10 nM prostaglandin $E_2$ ($PGE_2$) were measured. Calcium oscillations were recorded using a fluorescent microscope (Zeiss) for 20 – 30 minutes with a 2 second interval. In the initial experiments, phototoxicity was often observed already after 10 minutes, and was found to be due to a high exposure time (1 second). By decreasing the exposure time to 200 ms, the image sharpness was slightly decreased although the cells were still clearly visible, but phototoxicity could hence be avoided for the duration of the experiment.

### 3.3.2 Correlation analysis

Despite the use of calcium imaging to determine PBC cellular activity and response to modulators (Hartelt *et al.*, 2008; Bouvier *et al.*, 2010), how cells coordinate their activity in this region to produce a behaviour has not previously been shown. Network structures within the PBC have only been estimated according to the distance between the neurons (Hartelt *et al.*, 2008), and not in relation to how their oscillating activities are related and act in concert to elicit functional output. We have employed a script for MATLAB (from Erik Nilsson and Per Uhlén, see Uhlen, 2004) that uses the extracted

mean intensity values and x- and y-coordinates from time-lapse imaging series to calculate correlation coefficients for each cell pair within the studied region. This coefficient (Pearson's correlation) is determined by a linear similarity between two signals based on the time lag from one burst to the other. We plotted the coefficients in graphs showing 1) the degree of correlation as a function of distance between the cells, and 2) the degree of correlation in relation to the x- and y-coordinates of the cells in the image series. This is shown to be a valuable method for determining network-like structures based on the synchronization of oscillating activity (Uhlén *et al.*, unpublished data).

## 3.4 CELL AND TISSUE LABELLING

### 3.4.1 Immunohistochemistry

Immunohistochemical stainings on fixed whole embryo or brainstem cryosections were performed in all studies of this thesis. For *Studies I* and *III*, which involved examination of the medullary respiratory regions, embryos or brainstem preparations were sectioned sagittally to obtain a view over the whole medulla with pFRG and PBC in the same sections. For *Studies II* and *IV*, we sectioned embryos transversally for several reasons. First, these studies included smaller embryos at early stages and thus, transversal sectioning provided more sections for the experiments. Second, transversal sectioning gives a better view when investigating potential neurulation deficits, and third, facilitates visualization and quantification of neural cells distributed medio-laterally.

A large number of antibodies were employed for these studies, all of which are described in the four papers. They include neuronal differentiation, specification, migration and maturation markers (β-tubulin III, Dcx, PSA-NCAM, MAP2, NK1R, SST, Phox2b), neural crest markers (AP-2α, SOX-10), proliferation and apoptosis markers (phospho-histone-3, Caspase-3), cytoskeleton-associated proteins (4.1N, β-catenin, p120-catenin, N-cadherin, Rac1, RhoA), ion transporters and gap junctions (KCC2, connexin-43), planar cell polarity proteins (Vangl2, Scrb1) and a synaptic marker (VGlut2). After the antibodies had bound to their targets they were visualized with fluorescent secondary antibodies. The actin cytoskeleton was visualized with FITC- or TRITC-conjugated Phalloidin, a death cap mushroom toxin that binds to F-actin and prevents its depolymerization. All stainings were performed according to standard immunohistochemical protocols, except the NK1R/KCC2 labelling in *Study I*,

in which some modifications were done due to the same species origin of these antibodies. Here, the sequential labelling steps included 1) NK1R antibody at a dilution high enough (1:30,000) to avoid detection by the secondary antibody for KCC2, 2) amplification of the NK1R signal by a biotin/avidin complex conjugated with horseradish peroxidase, 3) detection of NK1R by a fluorescent substrate (FITC-TSA), 4) KCC2 antibody at normal dilution (1:500), and 5) detection of KCC2 by a fluorescent secondary Cy3-antibody at normal dilution. While this modified protocol did not produce optimal NK1R stainings, it was still a valuable alternative as the NK1R and KCC2 antibodies available from other species turned out to be considerably poor.

To prevent cross-reactions, the antibodies were titrated to determine the optimal dilutions and control experiments were done with the respective primary antibody omitted. Analysis was performed by fluorescent (Zeiss) and confocal (Leica) microscopy. Quantifications were done either by counting positive cells manually or by measuring the staining intensity in ImageJ.

### 3.4.2 Immunocytochemistry

Immunocytochemical stainings were performed on fixed cell cultures to determine the integrity of the cytoskeleton in *Studies II* and *IV*. For this we used FITC- or TRITC-conjugated Phalloidin and antibodies for 4.1N, KCC2, Vangl2, Rac1, RhoA, β-catenin and N-cadherin. In some experiments, Vangl2 and constitutive active or dominant negative Rac1 and RhoA were detected by antibodies to their respective HA- and Myc-tags. Control cells were visualized by transfection of an EGFP or DsRed expression plasmid, and RNAi by co-transfection of a fluorescent oligo. Analysis was done by fluorescent (Zeiss) and confocal (Leica or Zeiss) microscopy.

### 3.4.3 In situ hybridization

To assess gene expression at the mRNA level, in situ hybridization provides a detailed map of the expression pattern in sections of an organ or whole embryo. Here, an antisense RNA probe is hybridized with the mRNA encoded by a particular gene. This probe is labelled by incorporation of nucleotides that are either radioactive or conjugated with a dye, and thus, enables visualization of only those cells that have mRNA from the gene of interest.

We used in situ hybridization to compare Vangl2 expression between wild type and transgenic embryos in *Study IV*. The disadvantage with this method is that it is more complicated and time-consuming than protein labelling, and very sensitive to

contamination by ribonucleases that degrade RNA. However, it is a valuable option when an antibody against the protein is not available, which was the case at the time of our Vangl2 study. We generated an antisense probe from the Vangl2 coding sequence by PCR with digoxigenin-conjugated UTP as one of the nucleotides. Digoxigenin is a compound made by plants and not found in animal cells, which makes it easy to distinguish probe-bound cells from other cells. After the probe had bound to the tissue sections, a digoxigenin antibody conjugated with alkaline phosphatase was applied. This enzyme converted the substrate NBT/BCIP into a purple precipitating product that was visible in a light microscope, and hence, enabled detection of Vangl2 expressing cells.

## 3.5 CELL ADHESION AND MIGRATION

### 3.5.1 Aggregation assay

To assess cell adhesion in *Study IV*, we grew the cells in aggregates, dispersed them mechanically, and analyzed the number and size of remaining aggregates. For this, we used MDCK cells due to their ability to form tight connections. We also tried this method with HEK293 cells, but these cells did not bind as tightly to each other as MDCK cells did, and were to a larger extent dispersed into single cells by mechanical disruption. Transfected MDCK cells were grown in hanging drops, then pipetted up and down 10 times and analyzed in a light microscope (Nikon). For reliable results, it was of critical importance that all drops contained an equal number of cells, and therefore, careful counting of cells and proper resuspension to get the same cell dilution was done. Moreover, pipetting was applied with identical force in all drops.

To analyze the results, a square grid was applied over each image and the number of squares containing aggregates (> 4 cells) were counted and divided by the number of squares with no aggregates. While this method is rather semi-quantitative, it still provides a valuable estimate of the relative effect of different over- or under-expressed proteins on the adherens junctions.

### 3.5.2 Wound assay

Wound assay (also known as “wound healing” or “in vitro scratch” assay) is an economical, easy and fast method to assess cell migration in vitro. This method is based on the properties of cells to migrate and replace an area of lost cells. For this, C17.2

cells are highly suitable due to their migration capacity (Ourednik *et al.*, 2002), and were employed successfully in *Studies II* and *IV*. We also tried this method with MDCK and HEK293 cells but observed that scratching a confluent cell layer often removed larger sheets and did not leave a straight scratch.

Briefly, subconfluent cells were transfected and allowed to reach 100% confluency. They were then treated with Mitomycin C, which is a DNA cross-linker that inhibits DNA synthesis and cell division. A scratch was made through the cell layer using a pipett tip and the medium was changed to serum-reduced medium. The migration of cells through the scratch could then be observed in a light phase-contrast microscope (Nikon). We photographed the cells immediately (T = 0) and after 18 hours, which was found to be a suitable time point to see differences in the proportion of migrating cells. As cell proliferation was inhibited both by Mitomycin C and the minimal serum concentration, the cells covering the area were most likely migrating. This could also be observed by the distance between the cells and their somewhat elongated shape. To be sure that the identical area was photographed at the different time points, a line was drawn underneath the culture dish and images were captured just above or below the line. The results were quantified both by a calculation of the recolonized area percentage based on processed binary images in ImageJ, and manually by counting the proportion of cells in the scratch.

## 3.6 PROTEIN ACTIVITY AND INTERACTION

### 3.6.1 Immunoprecipitation

Protein-protein interactions can be determined by the immunoprecipitation method, whereby a cell lysate is incubated together with an antibody for the protein of interest and then pulled down by Sepharose beads with high affinity for the antibody. We employed this method to study interactions of different variants of KCC2 with 4.1N in *Study II*, and different variants of Vangl2 with Rac1 in *Study IV*. Control experiments were done both by transfection of a control plasmid and by precipitating with an unspecific IgG antibody. The bead-antibody-protein complexes were purified and run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and interacting proteins were visualized by immunoblotting. As the degree of interaction was quantified based on the band size, Western blot had to be performed also against the precipitated protein for normalization. This method does not determine whether the

interacting partner binds directly or indirectly to the protein of interest. However, for the specific aims of this thesis, it was sufficient to assess if the proteins were part of the same interacting complex.

### 3.6.2 Rac and Rho activation assays

This method was employed in *Study IV* to determine the activity, i.e. the GTP- or GDP-bound state, of Rho and Rac in cells with Vangl2 overexpression. Here, a modified version of the immunoprecipitation technique uses recombinant proteins, with high affinity for the GTP-bound Rho or Rac and coupled to Sepharose beads, to pull down the active molecule. The recombinant proteins include Glutathione-S-transferase (GST)-RHOtekin and GST-p21-activated kinase with affinity for Rho and Rac, respectively. The bead-protein complexes were run on SDS-PAGE and visualized by blotting with Rho and Rac antibodies. Although the method is straightforward, the activity state of the GTPases is highly temperature sensitive and GTP is quickly hydrolyzed to GDP if the temperature is not kept cold enough. As only the GTP-bound form is pulled down by the beads, the temperature is critical until the washed bead-protein complexes are resuspended in loading buffer. The gel band corresponding to the active form of the GTPase was measured and compared between control and Vangl2 transfected cells.

## 3.7 STATISTICAL METHODS

In all four studies, statistical differences between groups were determined using Student's *t*-test for equal variances or a variant known as Welch's unpaired *t*-test for unequal variances. Differences were considered to be statistical significant at $P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ *** (two-sided). Data are presented as mean ± SD. Pearson's correlation coefficients were calculated to determine the linear relationship between oscillating cells in *Study III* (see section 3.3.2). For the transgenic and mutant embryos, only littermates at a specific age were compared between groups.

# 4 RESULTS AND DISCUSSION

## 4.1 DEVELOPMENTAL INCREASE IN KCC2 EXPRESSION

Expression of the KCC2 protein during development was investigated in mouse embryos between E9.5 – E18.5 (*Studies II* and *III*) and in the brainstem of perinatal rats between E16 – P3 (*Study I*). The KCC2 protein was detected in the hindbrain and spinal cord regions already at E9.5 in mice and was co-expressed with β-tubulin III. Not all cells expressing β-tubulin III were positive for KCC2, indicating that KCC2 expression does not precede differentiation. The proportion of β-tubulin III positive cells expressing KCC2 increased with age and was also spread rostrally to the diencephalon at E13.5 and the basal telencephalic plate and olfactory bulb at E15.5. Expression could however not be detected in the neocortex even at E18.5. These results are in accordance with previous studies of KCC2 mRNA expression (Li *et al.*, 2002; Stein *et al.*, 2004). The novelty in our findings was an earlier time point for the inception of KCC2 expression. Moreover, presence of mRNA transcripts does not always reflect presence of the corresponding protein as gene expression can be controlled by many means also at the level of translation (Groisman *et al.*, 2000). Thus, our results demonstrate that KCC2 is expressed also at the protein level in neuronal progenitors of the early mouse hindbrain and spinal cord.

### 4.1.1 KCC2 expression in brainstem and respiratory regions

The developmental expression pattern of KCC2, beginning in the spinal cord and hindbrain, indicates that the expression follows neuronal maturation. Interestingly, we found the KCC2 protein also in a subset of neural crest cells emerging from the hindbrain at E9.5. While the significance of this feature is unclear, it might reflect an involvement in cell migration (see section 4.2.1). At E13.5, we observed that the KCC2-positive neural crest had formed ganglia for the trigeminal and facial nerves. Presence of KCC2 in ganglia has been reported before (Lu *et al.*, 1999), although it is somewhat controversial due to the static depolarizing action of GABA in these neurons (Sung *et al.*, 2000). However, a mosaic pattern of $[Cl^-]_i$ has recently been shown in the dorsal root ganglia and was suggested to result from a differential expression of KCC2 and NKCC1 (Gilbert *et al.*, 2007).

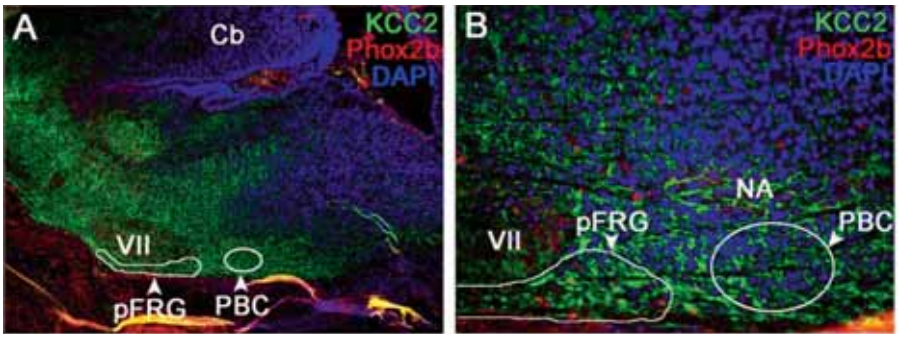


Figure 4 | **KCC2 protein expression at E15.5.** During inception of the respiratory rhythm in mice, the KCC2 protein is highly expressed in the medulla oblongata, including the respiration-related regions. Overview of the lateral medulla and part of the cerebellum (Cb), labelled with antibodies for KCC2 (green) and Phox2b (red), is shown in (A), and higher magnification of the ventral region in (B). Cell nuclei are stained with DAPI (blue).

At E11.5, the KCC2 protein was detected throughout the marginal zone of the neural tube in the hindbrain region. Thus, the newly born respiration-related neurons at this stage (Gray *et al.*, 2010) are presumably KCC2-positive. By E15.5, when the respiratory rhythm emerges in mice (Thoby-Brisson *et al.*, 2005), we found a high KCC2 expression in the ventrolateral medulla, including the PBC and pFRG regions (Fig. 4). KCC2 was co-expressed with SST and Phox2b in the PBC and pFRG, respectively. Similarly, we observed KCC2 protein expression in the rat ventrolateral medulla from E16. As rats generally mature somewhat more slowly than mice, E17 is the approximate time of respiratory rhythm inception in rats (Pagliardini *et al.*, 2003). By co-labelling with NK1R, we showed KCC2 expression in the respiration-related regions.

An interesting finding was that the KCC2 protein appeared in the cytoplasm of respiration-related neurons and nearby regions at E16 and E18 in rats. The intracellular distribution of KCC2 was still somewhat diffuse at E20 but small clusters of labelled protein were detected at the plasma membrane, while a remarkably lower amount of protein could be seen in the cytoplasm. At P0, KCC2 outlined the membrane of NK1R-positive cells in the PBC and pFRG. Only scarce cytoplasmic staining could be observed. The membrane staining of KCC2 was maintained at P3.

Quantification of the KCC2 staining intensity in the studied regions showed an increase at the plasma membrane and decrease in the cytoplasm from E18 to P3. It is however important to note that the term 'plasma membrane' here should be regarded with caution as proteins can stay close to the membrane without incorporating. While NK1R is a membrane receptor, it is internalized when bound to substance P (Pagliardini *et al.*, 2005) and therefore not fully reliable as a membrane marker. To more closely define the plasma membrane, we performed co-staining of KCC2 or NK1R with the previously characterized plasma membrane protein CD90 (Kemshead *et al.*, 1982). Triple-staining was unfortunately not successful in this application. However, both NK1R staining at all ages and KCC2 staining at postnatal ages co-localized with CD90, and although still semi-quantitative, NK1R was hence shown to be valid as plasma membrane marker in this study.

### 4.1.2 KCC2 expression precedes GABA inhibition

We recorded respiratory neuron activity in the rat ventrolateral medulla from E16 to P2 using extracellular recordings from C4 ventral roots, as well as 'conventional whole-cell' and 'gramicidin-perforated' patch clamp, in *Study I*. The response to bath application of GABA was predominantly depolarizing in E16 – E20 neurons. A small fraction of neurons were inhibited at late fetal age (E18 – E21). In preparations from postnatal rats (P0 – P2), inhibition was the dominant response. The effect of GABA was blocked with the $GABA_A$ receptor antagonist bicuculline. Based on these data, it is apparent that the response of respiration-related neurons to GABA is in transition around birth. This is in agreement with previous studies showing the hyperpolarizing shift at E19 in rat (Ren & Greer, 2006) and E18.5 in mice (Ikeda *et al.*, 2004), although it has also been reported that the shift occurs at P3 in the mouse PBC (Ritter & Zhang, 2000).

The differential response from neurons of the same age could be due to that $E_{GABA}$ may differ in various parts of the cell (Khirug *et al.*, 2008; Romo-Parra *et al.*, 2008), probably as a result of ongoing changes in expression of chloride transporters during development. Moreover, $GABA_A$-channels are also permeable to bicarbonate ions (Kaila *et al.*, 1993; Kulik *et al.*, 2000). We therefore measured the effect of removal of bicarbonate ions using HEPES-buffered, bicarbonate-free extracellular solution. Indeed, this resulted in a decrease in GABA-induced depolarization by 30% at late fetal age, suggesting that bicarbonate contributed to the observed effects of GABA. Nevertheless, the response to GABA was still depolarizing.

A recent study proposed that the excitatory to inhibitory switch in action of GABA in the hippocampus is not associated with a shift in the polarity of GABA responses to hyperpolarizing, but rather with shunting inhibition (Tyzio *et al.*, 2008). Shunting inhibition has also been observed in respiratory neurons (Tonkovic-Capin *et al.*, 2001), as well as in optic neurons (Ariel & Kogo, 2005), neocortical neurons (Ulrich, 2003), olfactory neurons (Smith & Jahr, 2002), and spinal cord neurons (Tapia *et al.*, 2001). Thus, the GABA-mediated inhibition of the rhythm postnatally may not be the result of hyperpolarization, but of an increased conductance and hence shunting of the glutamate-mediated currents.

Taken together, these data show that the KCC2 protein is present before the hyperpolarizing shift in $E_{GABA}$ in the respiration-related regions, and relocates to the plasma membrane when the shift takes place. Interestingly, the pFRG had significantly higher membrane staining compared to the PBC at E20, but not postnatally. Though this does not provide evidence of an earlier hyperpolarizing shift in the pFRG, it may point to a functional difference between these regions. Indeed, the pFRG becomes rhythmically active before the PBC and increases the rhythmic activity of the PBC by coupling to it (Thoby-Brisson *et al.*, 2009). The mechanism controlling the plasma membrane targeting of the KCC2 protein is not fully understood, but studies have implicated phosphorylation as an important factor (Lee *et al.*, 2007; Lee *et al.*, 2010).

## 4.2 A TRANSPORT-INDEPENDENT ROLE OF KCC2 IN DEVELOPMENT

As described above, we found KCC2 protein expression in the brainstem before the developmental $E_{GABA}$ shift in respiration-related neurons. The potential role of this early expressed protein has not previously been shown. Moreover, apart from the perturbed motor control, $KCC2^{-/-}$ mice had no reported developmental defects in the CNS. However, one of the limitations with knockout studies is developmental and physiological compensation, which may take over the function of the deleted gene. Indeed, other KCCs are also expressed in the CNS (Li *et al.*, 2002).

Using a nestin-driven KCC2 expression, we generated transgenic mouse embryos overexpressing KCC2 in the neural tube in *Study II*. By this technique we attempted to address two questions: 1) What effect will a possible prematurely shifted $E_{GABA}$ have on neural development? 2) Is there a second role of KCC2 prior to the $E_{GABA}$ shift?

### 4.2.1 KCC2 influences neuronal differentiation and migration

We collected transgenic embryos between E9.5 – E15.5. KCC2 overexpression severely impaired neural tube development and was lethal to the embryo at E13.5 – E15.5. No transgenic embryos were found at later stages, further supporting the lethal effect. Moreover, we generated additional transgenic litters overexpressing KCC2 variants that have previously been shown to lack the co-transport function (Li *et al.*, 2007; Reynolds *et al.*, 2008). These variants included an N-terminal deleted (ΔNTD) and a single amino acid substituted (C568A) KCC2 sequence. Importantly, all KCC2-ΔNTD, but not KCC2-C568A, transgenic embryos displayed phenotypes similar to those overexpressing the full length (KCC2-FL) variant. This indicated that 1) the effect of the transgene was not dependent on KCC2's transport function, and 2) the KCC2-ΔNTD and KCC2-C568A variants differed in their mode of action.

The phenotypes of the KCC2-FL and KCC2-ΔNTD embryos were characterized by a smaller size, underdeveloped brain vesicles, defective body curvature and facial abnormalities (Fig. 5). Although not all embryos had every feature, they still showed closely similar phenotypes, indicating that the effects were due to the transgene and not to insertional mutations. The KCC2-C568A embryos displayed mostly normal phenotypes but two out of six embryos at E9.5 had milder abnormalities in brain size and body flexure, suggesting a dose-dependent effect. At E13.5 and P0, no developmental defects could be detected, and thus, KCC2-C568A mice were the only transgenic variant that survived until birth.

We found a significantly lower proportion of cells expressing the early neuronal markers β-tubulin III, Dcx, and PSA-NCAM in the neural tube of KCC2-FL and KCC2-ΔNTD, but not KCC2-C568A, embryos (Table 1). As the neural cells mature earlier in the hindbrain and spinal cord, the differences were most evident in these regions. No changes were observed in proliferation or apoptosis, assessed with phospho-histone-3 and Caspase-3 antibodies, respectively. This indicates that the effect on neuronal differentiation was not secondary to an induced cell death or interference with the cell cycle, and corroborates previous reports on zebrafish (Reynolds *et al.*, 2008). The reduction in neuronal progenitors could however be due to slowed radial migration. KCC2 has been reported to have no effect on radial migration of pyramidal neurons (Cancedda *et al.*, 2007) but inhibits interneuron migration in the cortex (Bortone & Polleux, 2009). Interestingly, PSA-NCAM labelling showed a greater proportion of migrating cells in the ventricular/intermediate zones relative to the

marginal zone in the neural tube of KCC2-FL and KCC2-ΔNTD, but not KCC2-C568A, embryos. Thus, radial migration of neuronal progenitors may have been affected in the first two groups of transgenic embryos. The previous studies in the zebrafish embryo and murine cortex, mentioned above, all concluded that the effects on neuronal development was dependent on the transport-function of KCC2. However, we found similar effects of KCC2-FL and KCC2-ΔNTD embryos, indicating a transport-independent role of KCC2.

The phenotypes of the E13.5 transgenic embryos, particularly the cleft palate and lack of blood circulation (Fig. 5), suggested an impaired neural crest migration. Indeed, facial abnormalities combined with a potential circulatory defect have a well-known association with neural crest migration failure (Clouthier *et al.*, 1998). We used the neural crest markers AP-2α and SOX-10, which are expressed at different migratory stages. AP-2α is expressed in the cranial migratory neural crest and in post-migratory crest in the pharyngeal arches, whereas SOX-10 is mainly expressed in the neural crest emerging from the hindbrain (Ishii *et al.*, 2005). We found a reduced proportion of transversal embryo sections expressing AP-2α and SOX-10 in E9.5 KCC2-FL and KCC2-ΔNTD, but not KCC2-C568A, embryos (Table 1). In addition, the lower proportion of neural crest was accompanied with smaller groups of scattered neural crest cells. Thus, neural crest migration was perturbed in KCC2-FL and KCC2-ΔNTD embryos. The reduced migration was corroborated in an in vitro wound assay, indicating that age or environment is of less importance for the migratory effects.

| | **KCC2-FL / WT** | **KCC2-ΔNTD / WT** | **KCC2-C568A / WT** |
|---|---|---|---|
| **β-tubulin III** | 77% ** | 66% * | 92% n.s. |
| **Dcx** | 42% * | 31% * | 83% n.s. |
| **PSA-NCAM** | 66% * | 62% * | 107% n.s. |
| **AP-2α / SOX-10** | 63% * | 70% * | 95% n.s. |

Table 1 | **Proportion of neuronal and neural crest markers in transgenic relative to wild type embryos.** ** $P < 0.01$, * $P < 0.05$, n.s.: not significant.

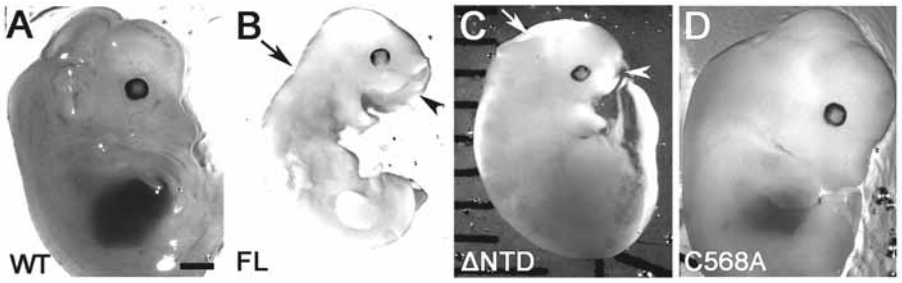


Figure 5 | **Phenotypes of E13.5 transgenic embryos.** A) Wild type embryo. B) KCC2-FL embryo with a small hindbrain (arrow) and cleft palate (arrowhead). C) KCC2-ΔNTD embryo with an underdeveloped midbrain (arrow) and cleft palate (arrowhead). D) KCC2-C568A embryo with no aberrant features.

Neural crest migration at later stages was not possible to determine due to the necrotic tissue of E13.5 transgenic embryos. It is therefore not clear what had caused the death of these embryos. The speculation in *Paper II* that the neural crest contribution to the bone marrow could be altered is most likely invalid, as hematopoietic stem cells are initially formed in a mesodermal structure known as the aorta-gonad-mesonephros (AGM) and do not inhabitate the bone marrow until after birth (Medvinsky & Dzierzak, 1996). Nevertheless, disruption of the cardiac neural crest results in abnormal aortic arch arteries and cardiac outflow septum (Brown & Baldwin, 2006), suggesting that the AGM region could also be affected in severe cases. While there is a risk of promoter leakage if the transgene integrates in the vicinity of other enhancer elements (Lothian & Lendahl, 1997), our results are presumably not due to ectopic expression in non-neural cells as all transgenic KCC2-FL and KCC2-ΔNTD embryos had similar phenotypes, and therefore, random insertion close to a particular enhancer in all cases is unlikely.

### 4.2.2 Interaction of KCC2 with the neuronal cytoskeleton

As KCC2-ΔNTD had effects similar to KCC2-FL, we assessed whether an interaction with the cytoskeleton, as previously reported (Li *et al.*, 2007), could be part of the mechanism for a potential transport-independent role in neuronal differentiation and migration. We labelled embryo sections and C17.2 cells transfected with the KCC2-FL, KCC2-ΔNTD and KCC2-C568A expression plasmids with Phalloidin and a 4.1N antibody. In wild type embryos, actin and 4.1N were enriched at the adherens junctions of the neural tube. In addition, cells in the neural tube had a circumferential expression

of these proteins. C17.2 control cultures displayed stress fibres of actin, which is a normal feature of cells grown in a dish, and 4.1N was found mainly close to the plasma membrane. Interestingly, embryos and C17.2 cells overexpressing KCC2-FL and KCC2-ΔNTD had higher cytoplasmic levels of actin and 4.1N. In the cell cultures, actin was partially seen as smaller aggregates, indicating a defective assembly of microfilament subunits. Western blot did not show any overall increase in actin protein levels in C17.2 cells. Unfortunately, due to the low number of transgenic embryos we had none left for Western blot. However, we do not propose an upregulation of actin but suggest a cytoplasmic redistribution, resulting from an overexpressed KCC2 with low levels of membrane targeting signals at early stages. It is possible that KCC2 links to 4.1N and actin already in the cytoplasm to regulate cell behaviours in neuronal development.

In contrast to the other two variants, KCC2-C568A had no obvious effects on the actin cytoskeleton or 4.1N distribution. This implied that the C568A mutation rendered KCC2 less able to interact with the microfilaments. By immunoprecipitating the three variants of KCC2, we showed that the interaction with 4.1N was significantly reduced for KCC2-C568A compared to KCC2-FL and KCC2-ΔNTD. This result provided further support for our finding that the effects of KCC2-FL and KCC2-ΔNTD on neural development were due to a structural interaction with the actin cytoskeleton. Hence, as previous studies have employed KCC2-C568A as control (Cancedda *et al.*, 2007; Reynolds *et al.*, 2008), the role of KCC2 in neuronal maturation shown in zebrafish and rats may also be transport-independent. It is however important to note that KCC2-FL, in contrast to KCC2-ΔNTD, induced migratory arrest of cortical interneurons in mouse explants (Bortone & Polleux, 2009), indicating a dual effect of KCC2 on migration.

The mechanism leading to the interference with neuronal differentiation and migration is unclear. However, by staining for the gap junction subunit Connexin-43, which has been shown to be expressed in contact points between radial fibres and migrating cortical neurons (Elias *et al.*, 2007), we observed that a large fraction of neural cells in KCC2-FL and KCC2-ΔNTD embryos had lost their polarized Connexin-43 expression in extension processes normally seen in wild type and KCC2-C568A embryos. Interestingly, Connexin-43 has been shown to mediate adhesive contacts in interaction with the actin cytoskeleton (Elias *et al.*, 2007). While this does not fully explain the mechanism of action, KCC2 overexpression may alter cytoskeleton-mediated cell polarization and hence associated neural cell behaviours.

## 4.3 KCC2 IS ESSENTIAL FOR RESPIRATORY RHYTHM GENERATION

As described above, KCC2 can influence neuronal differentiation and migration when overexpressed in vivo. Moreover, KCC2 is endogenously expressed in the marginal zone of the hindbrain at early stages (E9.5 – E11.5). Notably, SST and NK1R positive PBC neurons are derived from Dbx1-expressing progenitors that arise in the hindbrain at the same time point (Gray *et al.*, 2010). This indicates that the respiration-related neuronal progenitors may express KCC2 before the formation of the CPG for breathing. As no previous studies have shown any characterization of the neural networks for rhythmogenesis in mice lacking KCC2, we employed $KCC2^{-/-}$ mouse embryos at E17.5 – E18.5 to examine the structure and function of the respiration-related regions in *Study III*.

### 4.3.1 $KCC2^{-/-}$ mice lack synchronized network activity

We cultured rhythmically active medullary slices at the PBC level on semi-permeable membranes according to the Stoppini interface method (Stoppini *et al.*, 1991). Wild type and $KCC2^{-/-}$ slices were cultured for 5 – 19 days in vitro (DIV) and then used for calcium imaging to study cell communication within the PBC. During imaging, slices were perfused with a standard physiological solution containing 3 mM $[K^+]$. This showed to be sufficient for spontaneous rhythmic activity in accordance with Ruangkittisakul *et al.*, 2006. It must be considered, however, that we used TMR-SP to localize the PBC region, and this substance can stimulate the respiratory rhythm (Pagliardini *et al.*, 2005). Nevertheless, the use of physiological $[K^+]$ ensured that the function of KCC2 in wild type slices was not altered. Notably, high $[K^+]$, which has been commonly used in other studies, would reasonably lead to GABA-mediated efflux rather than influx of $Cl^-$, and hence, an absence of hyperpolarizing GABA responses even in the presence KCC2.

Wild type and $KCC2^{-/-}$ slices displayed oscillating activity for the duration of the experiments (< 1 hour per slice). They both responded to the substances glutamate and $PGE_2$ with an increased and decreased activity, respectively. However, the PBC of $KCC2^{-/-}$ slices had weaker and less regular bursts compared to wild type slices. We then calculated correlation coefficients for each pair of cells in the PBC region using a script for MATLAB. Interestingly, the correlation analysis demonstrated a decreased number of correlated cells in the PBC of $KCC2^{-/-}$ slices. Moreover, the correlation coefficients had lower values in $KCC2^{-/-}$ compared to wild type slices. This was observed for all

stages in vitro examined. The PBC of wild type slices displayed small clusters of highly correlated cells, which are thought to interact to produce a network-like structure of distinct cell groups within the PBC (Hartelt *et al.*, 2008). Furthermore, while the PBC of wild type cultures appeared to attain higher correlations with longer distances when the slices were cultured longer (> 15 DIV), the $KCC2^{-/-}$ PBC did not show any signs of network structure formation.

These results indicate that KCC2 expression is necessary for the formation of functional neural networks in the PBC. Interestingly, it has previously been reported that BDNF overexpression upregulates KCC2 and produces an increased spontaneous activity with higher correlations in the hippocampus (Aguado *et al.*, 2003). Although this effect was not shown to be attributable to KCC2, it is possible that KCC2 plays a role in synaptogenesis in neural networks. Our results show that the PBC is still active in $KCC2^{-/-}$ slices, suggesting that the PBC progenitors can integrate to the correct region without KCC2 expression. However, the low activity correlation points to a perturbed regulation of the rhythm, hence leading to an impaired respiratory motor output from this region.

### 4.3.2 Maturation of respiration-related neurons requires KCC2

To further assess the structural maturation of respiration-related neural networks, we studied fixed cryosections of wild type and $KCC2^{-/-}$ embryos. The PBC was analyzed by NK1R and SST labelling, and the pFRG by NK1R and Phox2b labelling. We found that the anatomical integrity of the PBC appeared normal in $KCC2^{-/-}$ embryos. This was in line with our finding that the pacemaker activity of PBC neurons was not absent. Hence, PBC neurons do not depend on KCC2 expression for migration to their final destination. However, the altered activity correlation in the PBC indicated a defective rhythm modulation. Indeed, respiratory failure has previously been shown without any significant changes in the anatomical structure or pacemaker function of the PBC (Wallen-Mackenzie *et al.*, 2006; Pagliardini *et al.*, 2008; Rose *et al.*, 2009). Interestingly, we found that the pFRG region had a lower NK1R expression but retained Phox2b expression in $KCC2^{-/-}$ embryos. This suggested that the segmental patterning of the hindbrain was not altered and that pFRG progenitors had also reached their destination, but indicated a perturbed maturation of these neurons.

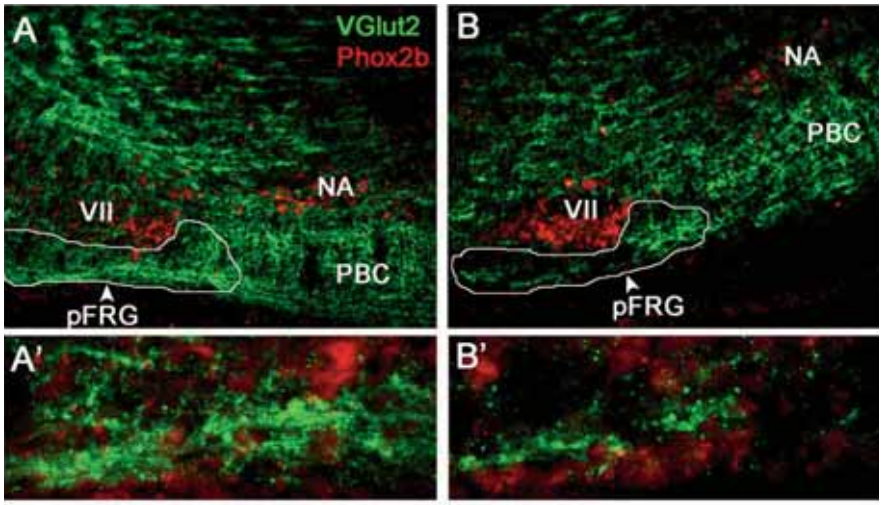


Figure 6 | **Loss of glutamatergic synapses in the pFRG of KCC2$^{-/-}$ embryos.** Wild type embryos display high VGlut2 expression (green) in the pFRG, outlined in (A). Phox2b expression (red) marks the facial nucleus (VII), nucleus ambiguus (NA) and a subset of neurons in the pFRG. KCC2$^{-/-}$ embryos show a distinctive reduction in VGlut2 expression in the pFRG, outlined in (B). Higher magnifications of the pFRG are shown in (A') and (B'). VGlut2 expression in the PBC is not significantly different.

We labelled sections with antibodies for MAP2 and vesicular glutamate transporter 2 (VGlut2) to analyze neuronal, dendritic and synaptic maturation. While wild type embryos displayed a gradual increase of MAP2 at the ventral side of medulla, KCC2$^{-/-}$ embryos had remarkably lower MAP2 expression throughout the medulla. Moreover, the pFRG, but not the PBC, of KCC2$^{-/-}$ embryos had a significantly lower expression of VGlut2 (Fig. 6). This indicates that the absence of KCC2 alters MAP2 expression in hindbrain neurons, and glutamatergic synapses distinctively in the pFRG. In this context, it is important to note that mice lacking MAP2 are viable and fertile although they have shorter and thinner dendrites (Harada *et al.*, 2002). Thus, the lower MAP2 expression is most likely not a cause of the impaired rhythmogenesis by itself. However, similar to KCC2$^{-/-}$ mice, VGlut2 null mutant mice die at birth from respiratory failure (Wallen-Mackenzie *et al.*, 2006). Notably, mice lacking VGlut2 have a preserved PBC region with pacemaker properties, indicating an impaired rhythmogenesis through the lack of afferent modulations. This is of considerable

interest for our findings, as we have shown that KCC2$^{-/-}$ mice display a reduced VGlut2 expression by almost 50% in the pFRG (Fig. 6). Moreover, recent studies have demonstrated a critical role of the pFRG for respiratory rhythmogenesis that is necessary for survival (Dubreuil *et al.*, 2008; Thoby-Brisson *et al.*, 2009). Thus, our results suggest that KCC2 deficiency impairs synaptogenesis in pFRG neurons, which leads to an altered synchronization of the respiratory rhythm.

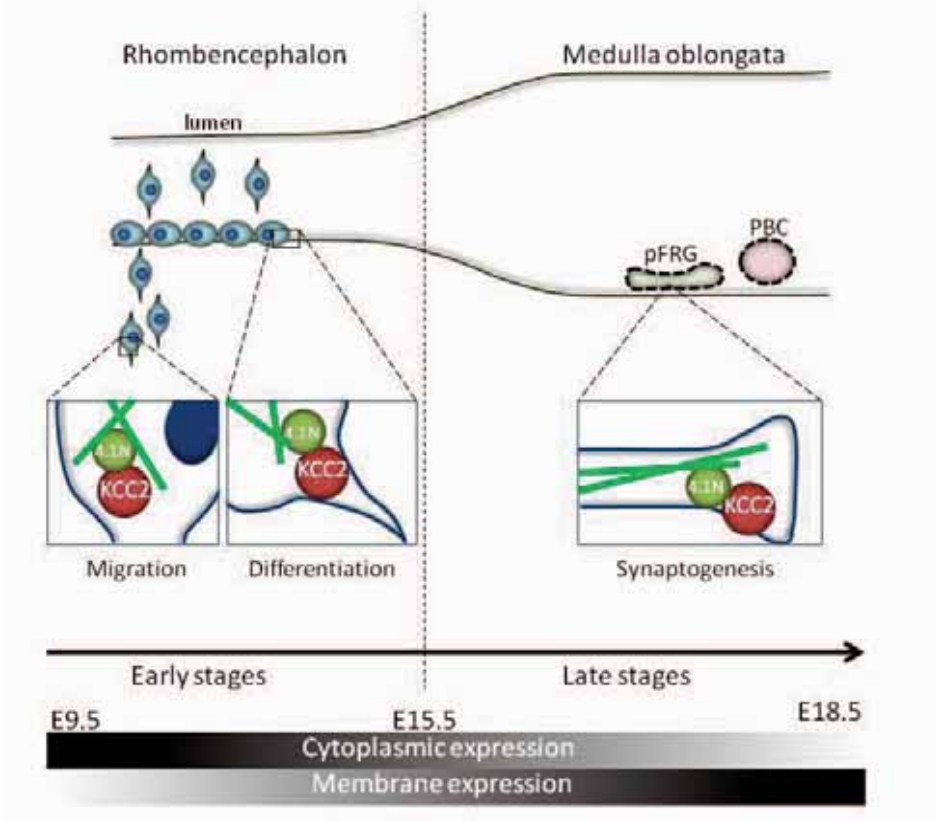


Figure 7 | **Potential early functions of transport-inactive KCC2.** Development of the neural tube region, from the rhombencephalon to the medulla oblongata, is shown at early (E9.5 – E15.5) and late (E15.5 – E18.5) stages of the mouse embryo. Hypothetical models of interaction between KCC2, 4.1N and actin microfilaments illustrate our results of KCC2's effect on migration, differentiation and synaptogenesis. The interaction of KCC2 with 4.1N in the synapse is still unclear. The decrease in cytoplasmic and increase in membrane expression of KCC2 over time is indicated at bottom.

The KCC2$^{-/-}$ mice have previously been shown to lack inhibitory responses to GABA in respiratory motor neurons (Hubner *et al.*, 2001). However, the respiratory rhythm initiates already at E15.5 in mice (Thoby-Brisson *et al.*, 2005), when GABA is still depolarizing. Hence, rhythmogenesis is not dependent on the hyperpolarizing shift. This is further supported by other studies reporting that mice mutant for the KCC2b isoform breathe spontaneously (Woo *et al.*, 2002; Uvarov *et al.*, 2007). It is likely that KCC2a is the essential isoform for the maturation of respiration-related neural circuits. Indeed, KCC2a is downregulated after birth (Uvarov *et al.*, 2007), suggesting a prenatal role for this isoform in neuronal development.

Taken together, this study shows that KCC2 expression is critical for the maturation of glutamatergic synapses needed for respiratory rhythm generation. This role is presumably transport-independent and indicates a cytoskeleton-regulating function in synaptogenesis. Thus, we suggest a novel structural role of KCC2 in the development of excitatory synapses, in addition to its role in dendritic spine formation. Based on the results from this thesis we propose a model for the transport-independent role of KCC2 in neural migration, differentiation and synaptogenesis (Fig. 7).

## 4.4 THE ROLE OF VANGL2 IN HINDBRAIN FORMATION

As described above, KCC2 is a cytoskeleton-interacting protein that plays a role in the maturation of neurons in the brainstem. However, the cytoskeleton is regulated by many means also before the neural tube is even formed. Indeed, convergent extension and following closure of the neural tube requires substantial mechanical strength that is achieved by neural progenitor cells in the neural plate. Neurulation is initiated at the hindbrain – cervical boundary (closure 1) before the tube "zips up" caudally and other closures in the cranial region take place. Failure of closure 1 results in craniorachischisis, characterized by impaired formation of the hindbrain and spinal cord. It has previously been shown that a mouse model for this neural tube defect (*Looptail*) carries a mutation in Vangl2 (Kibar *et al.*, 2001). In *Study IV*, we have further investigated the role of Vangl2 in the regulation of neural tube closure by employing *Looptail* and Vangl2 overexpressing embryos.

### 4.4.1 Vangl2 is crucial for the formation of adherens junctions

Similar to KCC2 transgenic embryos, we used the nestin enhancer to overexpress Vangl2 exclusively in the neural tube. Transgenic embryos were studied at E8.5 – E9.5, when neurulation occurs. We found that transgenic embryos were smaller than their wild type littermates and had an impaired cranial neurulation. Hence, the increased Vangl2 expression impaired both convergent extension and neurulation in the cephalic region. This is in line with previous results showing that Wnt7a overexpression increases Vangl2 expression in the neural tube and impairs cranial neurulation (Shariatmadari *et al.*, 2005; Horn *et al.*, 2007). Our findings also indicate that Vangl2 takes part in the intracellular pathway of Wnt7a signaling.

Bending of the neural plate involves the formation of hinge regions; one medial hinge point (MHP) causing the neural folds to elevate, and two dorso-lateral hinge points (DLHPs) that converges the neural folds inwards (Smith & Schoenwolf, 1997). We found that both *Looptail* and Vangl2 transgenic embryos had elevated folds, and hence had a preserved MHP. However, the neural folds were splayed wide apart instead of bending inwards. Thus, the function of the DLHPs had failed in these embryos.

Elongation of cells in the DLHP is dependent on microtubules, and constriction is achieved by microfilaments and associated proteins that accumulate and form adherens junctions at the apical end of these cells (Nyholm *et al.*, 2009). Adherens junctions have been shown to exert a critical function in cranial neurulation and its disruption impairs the rigidity and mechanical strength of the neural tube (Ybot-Gonzalez & Copp, 1999; Shariatmadari *et al.*, 2005). We therefore analyzed the neural tube adherens junctions in the *Looptail* and Vangl2 transgenic embryos. Indeed, both Vangl2 gain- and loss-of-function altered the expression of the adherens junction components actin, N-cadherin, β-catenin, and p120-catenin in the neural tube. These proteins were partly absent at the apical side and particularly in the DLHP regions. In addition, Rac1 expression was redistributed from the adherens junctions to cell somas scattered in the whole neural tube, indicating that Vangl2 regulates Rac1 localization.

Our findings demonstrate that Vangl2 is essential for the formation of adherens junctions required for neural tube closure. Both gain- and loss-of-function produced similar phenotypes, which has also been observed in xenopus and zebrafish (Darken *et al.*, 2002; Park & Moon, 2002), and can be explained by the theory that PCP signals must be fine-tuned to an appropriate level that is critical for proper cell behaviour during gastrulation and neurulation. However, *Looptail* embryos have impaired neurulation from the hindbrain and caudally, whereas Vangl2 overexpressing embryos

displayed this defect from the hindbrain and rostrally. Thus, they differed in completion of closures, which may be due to that cranial neurulation is more sensitive to cytoskeletal disruption (Ybot-Gonzalez & Copp, 1999). The integrity of adherens junctions in the cranial part of *Looptail* embryos was intact, suggesting that Vangl2 is mainly required for closure 1 in the hindbrain region. Nevertheless, both gain-and loss-of-function result in shorter body length, indicating perturbed convergent extension movements. Notably, Vangl2 has recently been shown to regulate convergent extension via antero-posterior cell polarization (Ciruna *et al.*, 2006).

### 4.4.2 The regulation of adherens junctions is Rac1-dependent

To obtain a more mechanistic view of Vangl2's effect on adherens junctions we studied the epithelial cell lines HEK293 and MDCK. We showed that these cell lines expressed Vangl2 mRNA and had the Vangl2 protein co-localized with the adherens junctions. As expected, Vangl2 overexpression and knockdown disrupted the adherens junction components actin, cadherin and β-catenin. In addition, the cells often grew in smaller clusters and were more loosely attached to each other. Moreover, as Vangl2 has been shown to contain a PDZ-binding domain that binds other PCP molecules (Kallay *et al.*, 2006), we assessed whether the expression of a Vangl2 construct lacking this sequence (Vangl2-Δ4) would have similar effects. Interestingly, Vangl2-Δ4 expressing cells had normal adherens junctions, indicating that the PDZ-binding domain is essential for the regulatory effect of Vangl2 on the cytoskeleton.

Our in vivo data suggested that Rac1 may have a role in the same pathway as Vangl2. We found that Vangl2 co-localized with Rac1 at the adherens junctions in the cell cultures. In addition, both overexpression and knockdown of Vangl2 resulted in altered cellular distribution of Rac1. Moreover, co-immunoprecipitation showed that Vangl2 is part of the same binding complex as Rac1 via the PDZ-binding domain. Notably, we also found that Vangl2 binds to RhoA but does not alter its cellular distribution in vitro or in vivo. The active state of Rac1 and RhoA was not changed by Vangl2 overexpression, suggesting that Vangl2 can bind to these GTPases without affecting their activity.

To analyze whether Rac1 and/or RhoA mediate the effect of Vangl2 on the actin cytoskeleton, we employed DNA constructs coding for constitutive active (Rac1-V12, RhoA-V14) and dominant negative (Rac1-N17, RhoA-N19) GTPases. These variants have been shown to regulate actin organization in epithelial cells (Jou & Nelson, 1998). Indeed, transfection of either of these plasmids alone altered the

distribution of the actin cytoskeleton in our experiments. Intriguingly, co-transfecting Rac1-N17 with Vangl2 rescued the disruption of the actin localization. Similar results were obtained when Rac1-N17 was substituted with Rac1 RNAi. Moreover, co-transfecting Rac1-V12 with Vangl2 potentiated the effects on the cytoskeleton. However, no interplay could be seen when co-transfecting Vangl2 with RhoA-V14 or RhoA-N19. This indicates that Vangl2 mediates its role in interaction with Rac1, but not with RhoA. Our results also suggest that Vangl2 overexpression and Rac1 knockdown balanced each other. This was supported by the finding that Vangl2-Δ4 did not rescue the effect of Rac1-N17.

For a more functional analysis of the adherens junctions, we assessed cell adhesion by aggregation and wound assays. The former method showed that Vangl2 overexpression significantly reduced cell aggregation (59% of control, $P < 0.05$). Similar reduction was observed with Vangl2 RNAi but not with Vangl2-Δ4. Co-transfection of Vangl2 with Rac1-N17 restored the normal ability to aggregate, while co-transfection with Rac1-V12 potentiated the reduction in cell aggregates. Again, no interplay was observed between Vangl2 and the RhoA variants. Furthermore, the wound assays demonstrated that Vangl2 transfection significantly increased the number of cells migrating into the open area (140% of control, $P < 0.05$). Co-transfection with Rac1-N17 blocked the effects of Vangl2 overexpression, in line with our previous findings. These results are consistent with the view that loss of cell adhesion can often promote migration (Fischer & Quinlan, 1998).

Taken together, our results show that an increased or decreased expression of Vangl2 alters the actin cytoskeleton and adherens junctions. We demonstrate that an interaction of Rac1 with the PDZ-binding domain of Vangl2 is essential for the regulation of adherens junction integrity. This corroborates previous reports that Rac1 is involved in regulating the stability of adherens junctions (Jou & Nelson, 1998; Quinlan, 1999; Flaiz *et al.*, 2008). The interplay between Vangl2 and Rac1 was evident from protein rescue experiments. Notably, Rac1-N17 is believed to compete with endogenous Rac1 for the membrane target sites (Feig & Cooper, 1988), and RNAi does not produce a complete knockdown of the Rac1 expression (Kim *et al.*, 2007). Therefore, the balancing effects of Vangl2 overexpression and Rac1 blockade can be explained by a model in which Vangl2 recruits endogenous Rac1 to the adherens junctions, as illustrated in Fig. 8. Thus, Vangl2 appears to be critical for the recruitment of Rac1 to functional sites and subsequent formation of adherens junctions necessary for neural tube closure in the hindbrain region.

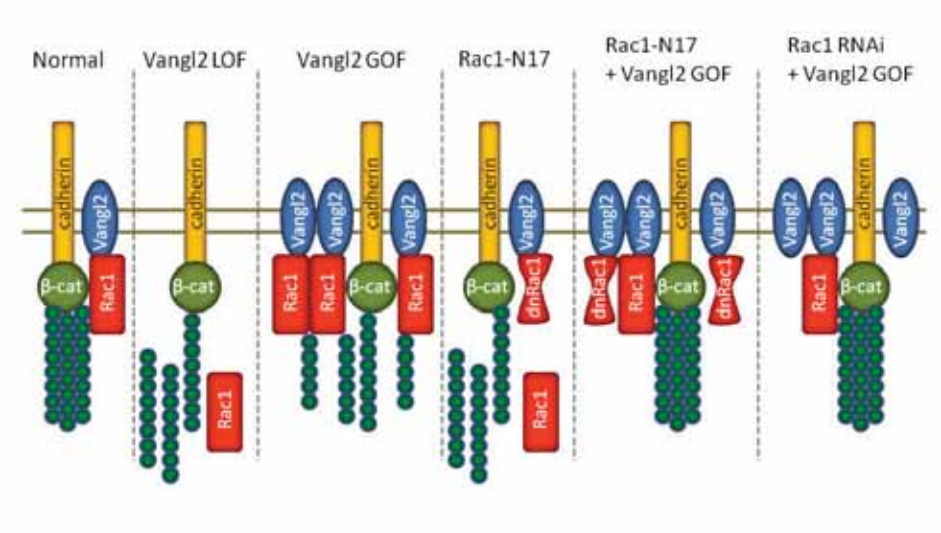


Figure 8 | **Model of interaction between Vangl2 and Rac1.** The model shows six different conditions of Vangl2 and Rac1 expression with our proposed mechanism of interaction. Actin microfilaments are displayed as strings of pearls (green). In the normal condition, Vangl2 targets Rac1 to the plasma membrane and promotes the formation of adherens junctions, in which microfilaments link to cadherin via catenins. In Vangl2 loss-of-function (LOF), Rac1 lacks a membrane targeting signal and the adherens junctions fail to form. In Vangl2 gain-of-function (GOF), excess Vangl2 and Rac1 at the membrane results in disordered microfilaments. When Rac-N17 is expressed, the dominant negative Rac1 (dnRac1) competes with endogenous Rac1 for Vangl2's binding site and inhibits the formation of adherens junctions. Vangl2 GOF rescues Rac1 blockade (Rac1-N17 or Rac1 RNAi) by recruiting remaining endogenous Rac1 to the adherens junctions.

# 5 CONCLUSIONS

This thesis describes the roles of KCC2 and Vangl2 in different aspects of brainstem development, from closure of the neural tube until the formation of functional neural networks.

- The KCC2 protein is expressed in the mouse hindbrain already at E9.5 – E11.5, which correlates with the birth of respiration-related neurons.

- KCC2 expression precedes the developmental $E_{GABA}$ shift in respiration-related regions, and membrane targeting of KCC2 is associated with an inhibitory response to GABA.

- Overexpression of KCC2 in the neural tube results in decreased neuronal differentiation and perturbed neural crest migration, which is lethal for embryos before E15.5.

- The effects on neural development are dependent on a structural interaction of KCC2 with the actin cytoskeleton via 4.1N.

- KCC2 expression is essential for the formation of brainstem neural networks that generate respiratory rhythm, possibly via its transport-independent role.

- Disruption of KCC2 leads to altered development of glutamatergic synapses in the parafacial respiratory group.

- Vangl2 expression is crucial for the formation of adherens junctions that mediate neural tube closure in the hindbrain region.

- The formation and regulation of adherens junctions depend on a structural interaction between Vangl2 and Rac1.

# 6 RELEVANCE AND FUTURE PERSPECTIVES

Uncovering the role of vital genes for embryonic development is important for our understanding of the complex genetic system that controls the emergence of different physiological functions. KCC2 and Vangl2 are both essential for neural development, which in animal models is manifested by a defective formation of the brainstem.

The results presented in this thesis are the first showing that KCC2 has a crucial function in vivo prior to the hyperpolarizing shift in GABA responses. Our findings indicate that KCC2 regulates the maturation of neurons and the formation of functional neural networks through a structural interaction with actin microfilaments. This corroborates recent reports that ion transporters and other membrane proteins with well-known functions in cellular homeostasis can also provide structural support for different neural cell behaviours (Elias *et al.*, 2007; Walters *et al.*, 2009).

In addition, our results provides an increased knowledge of how breathing arises, and thereby, how life is maintained. Importantly, the thesis contributes to the ongoing debate on the involvement of different brainstem regions in respiratory rhythm generation, and highlights the significance of the pFRG. It also shows that KCC2 expression is critical for the formation of neuronal connections within this vital region. Loss of Phox2b-expressing glutamatergic neurons in the pFRG causes congenital central hypoventilation syndrome, also known as *Ondine's curse*. This syndrome is manifested by episodes of apnea during sleep, and affects about 1 in 200,000 live born children. Interestingly, a subset of these patients develops neural crest-derived tumours (Berry-Kravis *et al.*, 2006) and display distinctive facial features (Todd *et al.*, 2006), suggesting that an altered migratory behaviour, possibly by a dysregulation of the neural cytoskeleton, may be involved.

Further studies are needed to elucidate the full mechanism of KCC2 in the transport-independent regulation of the actin cytoskeleton. In particular, it will be important to reveal the role of KCC2 in synaptogenesis, and if this is a characteristic of only the pFRG or of additional neural networks in the CNS. Notably, $KCC2^{-/-}$ mice also lack spontaneous movements (Hubner *et al.*, 2001), indicating a perturbed CPG for locomotion in addition to the impaired breathing. Thus, KCC2 may be one of the key molecules for neural network development.

A disruption in Vangl2 expression leads to impaired neurulation, and hence, this thesis contributes to the research on neural tube defects. In fact, neural tube defects,

including spina bifida, craniorachischisis and anencephaly, are the most common birth defects with an incidence that ranges between 1 and 8 per 1000 births depending on the geographical location. Importantly, Vangl2 mutations have recently been found in human cases with cranial and spinal neural tube defects (Kibar *et al.*, 2010; Lei *et al.*, 2010). Anencephaly may be due to a disruption of both the Vangl1 and Vangl2 genes, as has been observed in mice that are double heterozygous for Vangl1 and Vangl2 (Torban *et al.*, 2008). However, numerous genes are known to cause neural tube defects in animal models, and hence, other patterns of genetic interactions are possible.

Moreover, we have identified an interplay between Vangl2 and Rac1 that is critical for the formation of adherens junctions in the hindbrain region of the neural tube. This increases our understanding of how PCP proteins act in mammals. Further studies are necessary to determine the exact mechanism of this interaction and if other PCP proteins play a role. Furthermore, the effect of Vangl2 on cell adhesion and migration does not only implicate its roles in convergent extension and neural tube closure, but may also be involved in tumour progression.

Taken together, our findings show that KCC2 and Vangl2 are essential genes for the formation and maturation of the brainstem. We have revealed functions of these gene products that structurally regulate the neuronal cytoskeleton, and thus, the stage may now be set for future investigations to further identify important roles of these genes in neuronal development.

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# 8 REFERENCES

Aguado, F., Carmona, M.A., Pozas, E., Aguilo, A., Martinez-Guijarro, F.J., Alcantara, S., Borrell, V., Yuste, R., Ibanez, C.F. & Soriano, E. (2003) BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2. *Development*, **130**, 1267-1280.

Andresen, M.C. & Mendelowitz, D. (1996) Sensory afferent neurotransmission in caudal nucleus tractus solitarius--common denominators. *Chem Senses*, **21**, 387-395.

Angeles Fernandez-Gil, M., Palacios-Bote, R., Leo-Barahona, M. & Mora-Encinas, J.P. (2010) Anatomy of the brainstem: a gaze into the stem of life. *Semin Ultrasound CT MR*, **31**, 196-219.

Ariel, M. & Kogo, N. (2005) Shunting inhibition in accessory optic system neurons. *J Neurophysiol*, **93**, 1959-1969.

Balakrishnan, V., Becker, M., Lohrke, S., Nothwang, H.G., Guresir, E. & Friauf, E. (2003) Expression and function of chloride transporters during development of inhibitory neurotransmission in the auditory brainstem. *J Neurosci*, **23**, 4134-4145.

Banke, T.G. & McBain, C.J. (2006) GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development. *J Neurosci*, **26**, 11720-11725.

Ben-Ari, Y., Khazipov, R., Leinekugel, X., Caillard, O. & Gaiarsa, J.L. (1997) GABAA, NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. *Trends Neurosci*, **20**, 523-529.

Berry-Kravis, E.M., Zhou, L., Rand, C.M. & Weese-Mayer, D.E. (2006) Congenital central hypoventilation syndrome: PHOX2B mutations and phenotype. *Am J Respir Crit Care Med*, **174**, 1139-1144.

Blaesse, P., Airaksinen, M.S., Rivera, C. & Kaila, K. (2009) Cation-chloride cotransporters and neuronal function. *Neuron*, **61**, 820-838.

Blaesse, P., Guillemin, I., Schindler, J., Schweizer, M., Delpire, E., Khiroug, L., Friauf, E. & Nothwang, H.G. (2006) Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. *J Neurosci*, **26**, 10407-10419.

Blanchi, B., Kelly, L.M., Viemari, J.C., Lafon, I., Burnet, H., Bevengut, M., Tillmanns, S., Daniel, L., Graf, T., Hilaire, G. & Sieweke, M.H. (2003) MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth. *Nat Neurosci*, **6**, 1091-1100.

Bolteus, A.J. & Bordey, A. (2004) GABA release and uptake regulate neuronal precursor migration in the postnatal subventricular zone. *J Neurosci*, **24**, 7623-7631.

Bonham, A.C., Chen, C.Y., Sekizawa, S. & Joad, J.P. (2006) Plasticity in the nucleus tractus solitarius and its influence on lung and airway reflexes. *J Appl Physiol*, **101**, 322-327.

Borday, C., Coutinho, A., Germon, I., Champagnat, J. & Fortin, G. (2006) Pre-/post-otic rhombomeric interactions control the emergence of a fetal-like respiratory rhythm in the mouse embryo. *J Neurobiol*, **66**, 1285-1301.

Bordey, A. (2010) More neurons for respiratory adaptation: is neurogenesis at work? *Respir Physiol Neurobiol*, **173**, 118-119.

Bortone, D. & Polleux, F. (2009) KCC2 expression promotes the termination of cortical interneuron migration in a voltage-sensitive calcium-dependent manner. *Neuron*, **62**, 53-71.

Bouvier, J., Thoby-Brisson, M., Renier, N., Dubreuil, V., Ericson, J., Champagnat, J., Pierani, A., Chedotal, A. & Fortin, G. (2010) Hindbrain interneurons and axon guidance signaling critical for breathing. *Nat Neurosci*, **13**, 1066-1074.

Braga, V.M., Machesky, L.M., Hall, A. & Hotchin, N.A. (1997) The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J Cell Biol*, **137**, 1421-1431.

Brembeck, F.H., Rosario, M. & Birchmeier, W. (2006) Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev*, **16**, 51-59.

Brown, C.B. & Baldwin, H.S. (2006) Neural crest contribution to the cardiovascular system. *Adv Exp Med Biol*, **589**, 134-154.

Cancedda, L., Fiumelli, H., Chen, K. & Poo, M.M. (2007) Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo. *J Neurosci*, **27**, 5224-5235.

Canning, B.J. (2007) Encoding of the cough reflex. *Pulm Pharmacol Ther*, **20**, 396-401.

Chudotvorova, I., Ivanov, A., Rama, S., Hubner, C.A., Pellegrino, C., Ben-Ari, Y. & Medina, I. (2005) Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses. *J Physiol*, **566**, 671-679.

Ciani, L. & Salinas, P.C. (2007) c-Jun N-terminal kinase (JNK) cooperates with Gsk3beta to regulate Dishevelled-mediated microtubule stability. *BMC Cell Biol*, **8**, 27.

Ciruna, B., Jenny, A., Lee, D., Mlodzik, M. & Schier, A.F. (2006) Planar cell polarity signalling couples cell division and morphogenesis during neurulation. *Nature*, **439**, 220-224.

Cleveland, D.W., Monteiro, M.J., Wong, P.C., Gill, S.R., Gearhart, J.D. & Hoffman, P.N. (1991) Involvement of neurofilaments in the radial growth of axons. *J Cell Sci Suppl*, **15**, 85-95.

Clouthier, D.E., Hosoda, K., Richardson, J.A., Williams, S.C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R.E. & Yanagisawa, M. (1998) Cranial and cardiac neural crest defects in endothelin-A receptor- deficient mice. *Development*, **125**, 813-824.

Cohen-Cory, S. (2002) The developing synapse: construction and modulation of synaptic structures and circuits. *Science*, **298**, 770-776.

Copp, A.J., Greene, N.D. & Murdoch, J.N. (2003) Dishevelled: linking convergent extension with neural tube closure. *Trends Neurosci*, **26**, 453-455.

Coull, J.A., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A., De Koninck, P. & De Koninck, Y. (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*, **424**, 938-942.

Cunha, S.R. & Mohler, P.J. (2009) Ankyrin protein networks in membrane formation and stabilization. *J Cell Mol Med*, **13**, 4364-4376.

Curtin, J.A., Quint, E., Tsipouri, V., Arkell, R.M., Cattanach, B., Copp, A.J., Henderson, D.J., Spurr, N., Stanier, P., Fisher, E.M., Nolan, P.M., Steel, K.P., Brown, S.D., Gray, I.C. & Murdoch, J.N. (2003) Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. *Curr Biol*, **13**, 1129-1133.

Darken, R.S., Scola, A.M., Rakeman, A.S., Das, G., Mlodzik, M. & Wilson, P.A. (2002) The planar polarity gene strabismus regulates convergent extension movements in Xenopus. *EMBO J*, **21**, 976-985.

Delpire, E. & Mount, D.B. (2002) Human and murine phenotypes associated with defects in cation-chloride cotransport. *Annu Rev Physiol*, **64**, 803-843.

Delpy, A., Allain, A.E., Meyrand, P. & Branchereau, P. (2008) NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. *J Physiol*, **586**, 1059-1075.

Denker, S.P. & Barber, D.L. (2002) Ion transport proteins anchor and regulate the cytoskeleton. *Curr Opin Cell Biol*, **14**, 214-220.

Dubreuil, V., Ramanantsoa, N., Trochet, D., Vaubourg, V., Amiel, J., Gallego, J., Brunet, J.F. & Goridis, C. (2008) A human mutation in Phox2b causes lack of CO2 chemosensitivity, fatal central apnea, and specific loss of parafacial neurons. *Proc Natl Acad Sci U S A*, **105**, 1067-1072.

Dubreuil, V., Thoby-Brisson, M., Rallu, M., Persson, K., Pattyn, A., Birchmeier, C., Brunet, J.F., Fortin, G. & Goridis, C. (2009) Defective respiratory rhythmogenesis and loss of central chemosensitivity in Phox2b mutants targeting retrotrapezoid nucleus neurons. *J Neurosci*, **29**, 14836-14846.

Elias, L.A., Wang, D.D. & Kriegstein, A.R. (2007) Gap junction adhesion is necessary for radial migration in the neocortex. *Nature*, **448**, 901-907.

Farrant, M. & Kaila, K. (2007) The cellular, molecular and ionic basis of GABA(A) receptor signalling. *Prog Brain Res*, **160**, 59-87.

Feig, L.A. & Cooper, G.M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol Cell Biol*, **8**, 3235-3243.

Feldman, J.L. & Del Negro, C.A. (2006) Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci*, **7**, 232-242.

Feldman, J.L. & Janczewski, W.A. (2006) Point:Counterpoint: The parafacial respiratory group (pFRG)/pre-Botzinger complex (preBotC) is the primary site of respiratory rhythm generation in the mammal. Counterpoint: the preBotC is the primary site of respiratory rhythm generation in the mammal. *J Appl Physiol*, **100**, 2096-2097; discussion 2097-2098, 2103-2098.

Fischer, R.S. & Quinlan, M.P. (1998) Identification of a novel mechanism of regulation of the adherens junction by E1A, Rac1, and cortical actin filaments that contributes to tumor progression. *Cell Growth Differ*, **9**, 905-918.

Flaiz, C., Utermark, T., Parkinson, D.B., Poetsch, A. & Hanemann, C.O. (2008) Impaired intercellular adhesion and immature adherens junctions in merlin-deficient human primary schwannoma cells. *Glia*, **56**, 506-515.

Fortuna, M.G., West, G.H., Stornetta, R.L. & Guyenet, P.G. (2008) Botzinger expiratory-augmenting neurons and the parafacial respiratory group. *J Neurosci*, **28**, 2506-2515.

Gee, K.R., Brown, K.A., Chen, W.N., Bishop-Stewart, J., Gray, D. & Johnson, I. (2000) Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium*, **27**, 97-106.

Gilbert, D., Franjic-Wurtz, C., Funk, K., Gensch, T., Frings, S. & Mohrlen, F. (2007) Differential maturation of chloride homeostasis in primary afferent neurons of the somatosensory system. *Int J Dev Neurosci*, **25**, 479-489.

Goodman, C.S. & Shatz, C.J. (1993) Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell*, **72 Suppl**, 77-98.

Goto, T. & Keller, R. (2002) The planar cell polarity gene strabismus regulates convergence and extension and neural fold closure in Xenopus. *Dev Biol*, **247**, 165-181.

Gourine, A.V., Kasymov, V., Marina, N., Tang, F., Figueiredo, M.F., Lane, S., Teschemacher, A.G., Spyer, K.M., Deisseroth, K. & Kasparov, S. (2010) Astrocytes control breathing through pH-dependent release of ATP. *Science*, **329**, 571-575.

Gray, P.A. (2008) Transcription factors and the genetic organization of brain stem respiratory neurons. *J Appl Physiol*, **104**, 1513-1521.

Gray, P.A., Hayes, J.A., Ling, G.Y., Llona, I., Tupal, S., Picardo, M.C., Ross, S.E., Hirata, T., Corbin, J.G., Eugenin, J. & Del Negro, C.A. (2010) Developmental Origin of PreBotzinger Complex Respiratory Neurons. *J Neurosci*, **30**, 14883-14895.

Gray, P.A., Janczewski, W.A., Mellen, N., McCrimmon, D.R. & Feldman, J.L. (2001) Normal breathing requires preBotzinger complex neurokinin-1 receptor-expressing neurons. *Nat Neurosci*, **4**, 927-930.

Greer, J.J., Smith, J.C. & Feldman, J.L. (1992) Respiratory and locomotor patterns generated in the fetal rat brain stem-spinal cord in vitro. *J Neurophysiol*, **67**, 996-999.

Grillner, S. (2006) Biological pattern generation: the cellular and computational logic of networks in motion. *Neuron*, **52**, 751-766.

Groisman, I., Huang, Y.S., Mendez, R., Cao, Q., Theurkauf, W. & Richter, J.D. (2000) CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell*, **103**, 435-447.

Gulyas, A.I., Sik, A., Payne, J.A., Kaila, K. & Freund, T.F. (2001) The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur J Neurosci*, **13**, 2205-2217.

Gunhaga, L., Marklund, M., Sjodal, M., Hsieh, J.C., Jessell, T.M. & Edlund, T. (2003) Specification of dorsal telencephalic character by sequential Wnt and FGF signaling. *Nat Neurosci*, **6**, 701-707.

Guyenet, P.G., Stornetta, R.L., Abbott, S.B., Depuy, S.D., Fortuna, M.G. & Kanbar, R. (2010) Central CO2 chemoreception and integrated neural mechanisms of cardiovascular and respiratory control. *J Appl Physiol*, **108**, 995-1002.

Hagglund, M., Borgius, L., Dougherty, K.J. & Kiehn, O. (2010) Activation of groups of excitatory neurons in the mammalian spinal cord or hindbrain evokes locomotion. *Nat Neurosci*, **13**, 246-252.

Harada, A., Teng, J., Takei, Y., Oguchi, K. & Hirokawa, N. (2002) MAP2 is required for dendrite elongation, PKA anchoring in dendrites, and proper PKA signal transduction. *J Cell Biol*, **158**, 541-549.

Harris, T.J. & Tepass, U. (2010) Adherens junctions: from molecules to morphogenesis. *Nat Rev Mol Cell Biol*, **11**, 502-514.

Hartelt, N., Skorova, E., Manzke, T., Suhr, M., Mironova, L., Kugler, S. & Mironov, S.L. (2008) Imaging of respiratory network topology in living brainstem slices. *Mol Cell Neurosci*, **37**, 425-431.

Hartmann, A.M., Blaesse, P., Kranz, T., Wenz, M., Schindler, J., Kaila, K., Friauf, E. & Nothwang, H.G. (2009) Opposite effect of membrane raft perturbation on transport activity of KCC2 and NKCC1. *J Neurochem*, **111**, 321-331.

Hedrick, M.S., Broch, L., Martinez, M., Powell, J.L. & Wade, R.E. (2001) Is the vertebrate respiratory central pattern generator conserved? Insights from in-vitro and in-vivo amphibian models. *Adv Exp Med Biol*, **499**, 127-132.

Heidemann, S.R. (1996) Cytoplasmic mechanisms of axonal and dendritic growth in neurons. *Int Rev Cytol*, **165**, 235-296.

Hekmat-Scafe, D.S., Lundy, M.Y., Ranga, R. & Tanouye, M.A. (2006) Mutations in the K+/Cl- cotransporter gene kazachoc (kcc) increase seizure susceptibility in Drosophila. *J Neurosci*, **26**, 8943-8954.

Herlenius, E., Aden, U., Tang, L.Q. & Lagercrantz, H. (2002) Perinatal respiratory control and its modulation by adenosine and caffeine in the rat. *Pediatr Res*, **51**, 4-12.

Herlenius, E. & Lagercrantz, H. (2004) Development of neurotransmitter systems during critical periods. *Exp Neurol*, **190 Suppl 1**, S8-21.

Hilaire, G. & Pasaro, R. (2003) Genesis and control of the respiratory rhythm in adult mammals. *News Physiol Sci*, **18**, 23-28.

Hooper, S.L. (2000) Central pattern generators. *Curr Biol*, **10**, R176.

Horn, Z., Papachristou, P., Shariatmadari, M., Peyronnet, J., Eriksson, B. & Ringstedt, T. (2007) Wnt7a overexpression delays beta-tubulin III expression in transgenic mouse embryos. *Brain Res*, **1130**, 67-72.

Hubner, C.A., Stein, V., Hermans-Borgmeyer, I., Meyer, T., Ballanyi, K. & Jentsch, T.J. (2001) Disruption of KCC2 Reveals an Essential Role of K-Cl Cotransport Already in Early Synaptic Inhibition. *Neuron*, **30**, 515-524.

Ikeda, K., Onimaru, H., Yamada, J., Inoue, K., Ueno, S., Onaka, T., Toyoda, H., Arata, A., Ishikawa, T.O., Taketo, M.M., Fukuda, A. & Kawakami, K. (2004) Malfunction of respiratory-related neuronal activity in Na+, K+-ATPase alpha2 subunit-deficient mice is attributable to abnormal Cl- homeostasis in brainstem neurons. *J Neurosci*, **24**, 10693-10701.

Ishii, M., Han, J., Yen, H.Y., Sucov, H.M., Chai, Y. & Maxson, R.E., Jr. (2005) Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. *Development*, **132**, 4937-4950.

Jaderstad, J., Jaderstad, L.M., Li, J., Chintawar, S., Salto, C., Pandolfo, M., Ourednik, V., Teng, Y.D., Sidman, R.L., Arenas, E., Snyder, E.Y. & Herlenius, E. (2010) Communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host. *Proc Natl Acad Sci U S A*, **107**, 5184-5189.

Janczewski, W.A. & Feldman, J.L. (2006) Distinct rhythm generators for inspiration and expiration in the juvenile rat. *J Physiol*, **570**, 407-420.

Jessen, J.R., Topczewski, J., Bingham, S., Sepich, D.S., Marlow, F., Chandrasekhar, A. & Solnica-Krezel, L. (2002) Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol*, **4**, 610-615.

Jou, T.S. & Nelson, W.J. (1998) Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J Cell Biol*, **142**, 85-100.

Joyner, A.L. (2002) Establishment of anterior-posterior and dorsal-ventral pattern in the early central nervous system. In Rossant, J., Tam, P.P.L. (ed) *Mouse Development – Patterning, Morphogenesis and Organogenesis.* Academic Press, pp. 107-126.

Kaila, K. (1994) Ionic basis of GABAA receptor channel function in the nervous system. *Prog Neurobiol*, **42**, 489-537.

Kaila, K., Voipio, J., Paalasmaa, P., Pasternack, M. & Deisz, R.A. (1993) The role of bicarbonate in GABAA receptor-mediated IPSPs of rat neocortical neurones. *J Physiol*, **464**, 273-289.

Kallay, L.M., McNickle, A., Brennwald, P.J., Hubbard, A.L. & Braiterman, L.T. (2006) Scribble associates with two polarity proteins, Lgl2 and Vangl2, via distinct molecular domains. *J Cell Biochem*, **99**, 647-664.

Kanold, P.O. & Shatz, C.J. (2006) Subplate neurons regulate maturation of cortical inhibition and outcome of ocular dominance plasticity. *Neuron*, **51**, 627-638.

Katz, P.S. & Harris-Warrick, R.M. (1999) The evolution of neuronal circuits underlying species-specific behavior. *Curr Opin Neurobiol*, **9**, 628-633.

Kemshead, J.T., Ritter, M.A., Cotmore, S.F. & Greaves, M.F. (1982) Human Thy-1: expression on the cell surface of neuronal and glial cells. *Brain Res*, **236**, 451-461.

Khirug, S., Yamada, J., Afzalov, R., Voipio, J., Khiroug, L. & Kaila, K. (2008) GABAergic depolarization of the axon initial segment in cortical principal neurons is caused by the Na-K-2Cl cotransporter NKCC1. *J Neurosci*, **28**, 4635-4639.

Kibar, Z., Salem, S., Bosoi, C.M., Pauwels, E., De Marco, P., Merello, E., Bassuk, A.G., Capra, V. & Gros, P. (2010) Contribution of VANGL2 mutations to isolated neural tube defects. *Clin Genet*.

Kibar, Z., Torban, E., McDearmid, J.R., Reynolds, A., Berghout, J., Mathieu, M., Kirillova, I., De Marco, P., Merello, E., Hayes, J.M., Wallingford, J.B., Drapeau, P., Capra, V. & Gros, P. (2007) Mutations in VANGL1 associated with neural-tube defects. *N Engl J Med*, **356**, 1432-1437.

Kibar, Z., Vogan, K.J., Groulx, N., Justice, M.J., Underhill, D.A. & Gros, P. (2001) Ltap, a mammalian homolog of Drosophila Strabismus/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. *Nat Genet*, **28**, 251-255.

Kim, M.J., Futai, K., Jo, J., Hayashi, Y., Cho, K. & Sheng, M. (2007) Synaptic accumulation of PSD-95 and synaptic function regulated by phosphorylation of serine-295 of PSD-95. *Neuron*, **56**, 488-502.

Kinder, S.J., Tsang, T.E., Quinlan, G.A., Hadjantonakis, A.K., Nagy, A. & Tam, P.P. (1999) The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development*, **126**, 4691-4701.

Kulik, A., Nishimaru, H. & Ballanyi, K. (2000) Role of Bicarbonate and Chloride in GABA- and Glycine-Induced Depolarization and [Ca2+]i Rise in Fetal Rat Motoneurons In Situ. *J. Neurosci.*, **20**, 7905-7913.

Lamsa, K., Palva, J.M., Ruusuvuori, E., Kaila, K. & Taira, T. (2000) Synaptic GABA(A) activation inhibits AMPA-kainate receptor-mediated bursting in the newborn (P0-P2) rat hippocampus. *J Neurophysiol*, **83**, 359-366.

Le Gallois, J.J.C. (1812) *Expériences sur le principe de la vie*. D´Hautel, Paris.

Lee, H., Chen, C.X., Liu, Y.J., Aizenman, E. & Kandler, K. (2005) KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. *Eur J Neurosci*, **21**, 2593-2599.

Lee, H.H., Jurd, R. & Moss, S.J. (2010) Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2. *Mol Cell Neurosci*, **45**, 173-179.

Lee, H.H., Walker, J.A., Williams, J.R., Goodier, R.J., Payne, J.A. & Moss, S.J. (2007) Direct protein kinase C-dependent phosphorylation regulates the cell surface stability and activity of the potassium chloride cotransporter KCC2. *J Biol Chem*, **282**, 29777-29784.

Lee, K.J. & Jessell, T.M. (1999) The specification of dorsal cell fates in the vertebrate central nervous system. *Annu Rev Neurosci*, **22**, 261-294.

Lei, Y.P., Zhang, T., Li, H., Wu, B.L., Jin, L. & Wang, H.Y. (2010) VANGL2 mutations in human cranial neural-tube defects. *N Engl J Med*, **362**, 2232-2235.

Lewis, S.L. & Tam, P.P. (2006) Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. *Dev Dyn*, **235**, 2315-2329.

Li, H., Khirug, S., Cai, C., Ludwig, A., Blaesse, P., Kolikova, J., Afzalov, R., Coleman, S.K., Lauri, S., Airaksinen, M.S., Keinanen, K., Khiroug, L., Saarma, M., Kaila, K. & Rivera, C. (2007) KCC2 interacts with the dendritic cytoskeleton to promote spine development. *Neuron*, **56**, 1019-1033.

Li, H., Tornberg, J., Kaila, K., Airaksinen, M.S. & Rivera, C. (2002) Patterns of cation-chloride cotransporter expression during embryonic rodent CNS development. *Eur J Neurosci*, **16**, 2358-2370.

Lieske, S.P., Thoby-Brisson, M., Telgkamp, P. & Ramirez, J.M. (2000) Reconfiguration of the neural network controlling multiple breathing patterns: eupnea, sighs and gasps. *Nat Neurosci*, **3**, 600-607.

Lothian, C. & Lendahl, U. (1997) An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. *Eur J Neurosci*, **9**, 452-462.

Lu, J., Karadsheh, M. & Delpire, E. (1999) Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J Neurobiol*, **39**, 558-568.

Luduena, R.F. (1993) Are tubulin isotypes functionally significant. *Mol Biol Cell*, **4**, 445-457.

Lumsden, A. & Keynes, R. (1989) Segmental patterns of neuronal development in the chick hindbrain. *Nature*, **337**, 424-428.

Luo, L. (2002) Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu Rev Cell Dev Biol*, **18**, 601-635.

Mainen, Z.F. & Sejnowski, T.J. (1996) Influence of dendritic structure on firing pattern in model neocortical neurons. *Nature*, **382**, 363-366.

Marin, O. & Rubenstein, J.L.R. (2002) Patterning, regionalization, and cell differentiation in the forebrain. In Rossant, J., Tam, P.P.L. (eds) *Mouse Development – Patterning, Morphogenesis and Organogenesis.* Academic Press., pp. 75-106.

Medvinsky, A. & Dzierzak, E. (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*, **86**, 897-906.

Megason, S.G. & McMahon, A.P. (2002) A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development*, **129**, 2087-2098.

Melton, K.R., Iulianella, A. & Trainor, P.A. (2004) Gene expression and regulation of hindbrain and spinal cord development. *Front Biosci*, **9**, 117-138.

Miller, J.R. (2002) The Wnts. *Genome Biol*, **3**, REVIEWS3001.

Misgeld, U., Deisz, R.A., Dodt, H.U. & Lux, H.D. (1986) The role of chloride transport in postsynaptic inhibition of hippocampal neurons. *Science*, **232**, 1413-1415.

Montcouquiol, M., Rachel, R.A., Lanford, P.J., Copeland, N.G., Jenkins, N.A. & Kelley, M.W. (2003) Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. *Nature*, **423**, 173-177.

Montcouquiol, M., Sans, N., Huss, D., Kach, J., Dickman, J.D., Forge, A., Rachel, R.A., Copeland, N.G., Jenkins, N.A., Bogani, D., Murdoch, J., Warchol, M.E., Wenthold, R.J. & Kelley, M.W. (2006) Asymmetric localization of Vangl2 and Fz3 indicate novel mechanisms for planar cell polarity in mammals. *J Neurosci*, **26**, 5265-5275.

Morrison, S.J. (2002) Stem cells of the nervous system. In Rossant, J., Tam, P.P.L. (ed) *Mouse Development – Patterning, Morphogenesis and Organogenesis.* Academic Press, pp. 235-250.

Murdoch, J.N., Henderson, D.J., Doudney, K., Gaston-Massuet, C., Phillips, H.M., Paternotte, C., Arkell, R., Stanier, P. & Copp, A.J. (2003) Disruption of scribble (Scrb1) causes severe neural tube defects in the circletail mouse. *Hum Mol Genet*, **12**, 87-98.

Nakamura, H. (2001) Regionalization of the optic tectum: combinations of gene expression that define the tectum. *Trends Neurosci*, **24**, 32-39.

Nattie, E.E. & Li, A. (2002) Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity. *J Physiol*, **544**, 603-616.

Nelson, W.J. & Nusse, R. (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*, **303**, 1483-1487.

Neumueller, S., Hodges, M.R., Krause, K., Marshall, B., Bonis, J., Qian, B., Pan, L.G. & Forster, H.V. (2010) Anatomic changes in multiple brainstem nuclei after incremental, near-complete neurotoxic destruction of the pre-Botzinger Complex in adult goats. *Respir Physiol Neurobiol*.

Nobes, C.D. & Hall, A. (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol*, **144**, 1235-1244.

Nyholm, M.K., Abdelilah-Seyfried, S. & Grinblat, Y. (2009) A novel genetic mechanism regulates dorsolateral hinge-point formation during zebrafish cranial neurulation. *J Cell Sci*, **122**, 2137-2148.

Onimaru, H. & Homma, I. (2003) A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci*, **23**, 1478-1486.

Onimaru, H. & Homma, I. (2006) Point:Counterpoint: The parafacial respiratory group (pFRG)/pre-Botzinger complex (preBotC) is the primary site of respiratory rhythm generation in the mammal. Point: the PFRG is the primary site of respiratory rhythm generation in the mammal. *J Appl Physiol*, **100**, 2094-2095.

Onimaru, H., Ikeda, K. & Kawakami, K. (2009) Phox2b, RTN/pFRG neurons and respiratory rhythmogenesis. *Respir Physiol Neurobiol*, **168**, 13-18.

Onimaru, H., Kumagawa, Y. & Homma, I. (2006) Respiration-related rhythmic activity in the rostral medulla of newborn rats. *J Neurophysiol*, **96**, 55-61.

Ourednik, J., Ourednik, V., Lynch, W.P., Schachner, M. & Snyder, E.Y. (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat Biotechnol*, **20**, 1103-1110.

Owens, D.F. & Kriegstein, A.R. (2002) Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci*, **3**, 715-727.

Pagliardini, S., Adachi, T., Ren, J., Funk, G.D. & Greer, J.J. (2005) Fluorescent tagging of rhythmically active respiratory neurons within the pre-Botzinger complex of rat medullary slice preparations. *J Neurosci*, **25**, 2591-2596.

Pagliardini, S., Ren, J., Gray, P.A., Vandunk, C., Gross, M., Goulding, M. & Greer, J.J. (2008) Central respiratory rhythmogenesis is abnormal in lbx1- deficient mice. *J Neurosci*, **28**, 11030-11041.

Pagliardini, S., Ren, J. & Greer, J.J. (2003) Ontogeny of the pre-Botzinger complex in perinatal rats. *J Neurosci*, **23**, 9575-9584.

Park, M. & Moon, R.T. (2002) The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos. *Nat Cell Biol*, **4**, 20-25.

Payne, J.A., Rivera, C., Voipio, J. & Kaila, K. (2003) Cation-chloride co-transporters in neuronal communication, development and trauma. *Trends Neurosci*, **26**, 199-206.

Payne, J.A., Stevenson, T.J. & Donaldson, L.F. (1996) Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem*, **271**, 16245-16252.

Plotkin, M.D., Snyder, E.Y., Hebert, S.C. & Delpire, E. (1997) Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: a possible mechanism underlying GABA's excitatory role in immature brain. *J Neurobiol*, **33**, 781-795.

Poulain, F.E. & Sobel, A. (2010) The microtubule network and neuronal morphogenesis: Dynamic and coordinated orchestration through multiple players. *Mol Cell Neurosci*, **43**, 15-32.

Quinlan, M.P. (1999) Rac regulates the stability of the adherens junction and its components, thus affecting epithelial cell differentiation and transformation. *Oncogene*, **18**, 6434-6442.

Rekling, J.C. & Feldman, J.L. (1998) Prebötzinger complex and pacemaker neurons - hypotesized site and kernel for respiratory rhythm generation. *Annual Review Physiology*, **60**, 385-405.

Ren, J. & Greer, J.J. (2003) Ontogeny of rhythmic motor patterns generated in the embryonic rat spinal cord. *J Neurophysiol*, **89**, 1187-1195.

Ren, J. & Greer, J.J. (2006) Modulation of Respiratory Rhythmogenesis by Chloride-Mediated Conductances during the Perinatal Period. *J. Neurosci.*, **26**, 3721-3730.

Reynolds, A., Brustein, E., Liao, M., Mercado, A., Babilonia, E., Mount, D.B. & Drapeau, P. (2008) Neurogenic role of the depolarizing chloride gradient revealed by global overexpression of KCC2 from the onset of development. *J Neurosci*, **28**, 1588-1597.

Rhee, J.W., Arata, A., Selleri, L., Jacobs, Y., Arata, S., Onimaru, H. & Cleary, M.L. (2004) Pbx3 deficiency results in central hypoventilation. *Am J Pathol*, **165**, 1343-1350.

Richter, D.W. & Spyer, K.M. (2001) Studying rhythmogenesis of breathing: comparison of in vivo and in vitro models. *Trends Neurosci*, **24**, 464-472.

Ritter, B. & Zhang, W. (2000) Early postnatal maturation of GABAA-mediated inhibition in the brainstem respiratory rhythm-generating network of the mouse. *Eur J Neurosci*, **12**, 2975-2984.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. & Kaila, K. (1999) The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature*, **397**, 251-255.

Rivera, C., Voipio, J., Thomas-Crusells, J., Li, H., Emri, Z., Sipila, S., Payne, J.A., Minichiello, L., Saarma, M. & Kaila, K. (2004) Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. *J Neurosci*, **24**, 4683-4691.

Romo-Parra, H., Trevino, M., Heinemann, U. & Gutierrez, R. (2008) GABA actions in hippocampal area CA3 during postnatal development: differential shift from depolarizing to hyperpolarizing in somatic and dendritic compartments. *J Neurophysiol*, **99**, 1523-1534.

Rose, M.F., Ren, J., Ahmad, K.A., Chao, H.T., Klisch, T.J., Flora, A., Greer, J.J. & Zoghbi, H.Y. (2009) Math1 is essential for the development of hindbrain neurons critical for perinatal breathing. *Neuron*, **64**, 341-354.

Ruangkittisakul, A., Schwarzacher, S.W., Secchia, L., Poon, B.Y., Ma, Y., Funk, G.D. & Ballanyi, K. (2006) High sensitivity to neuromodulator-activated signaling pathways at physiological [K+] of confocally imaged respiratory center neurons in on-line-calibrated newborn rat brainstem slices. *J Neurosci*, **26**, 11870-11880.

Sernagor, E., Young, C. & Eglen, S.J. (2003) Developmental modulation of retinal wave dynamics: shedding light on the GABA saga. *J Neurosci*, **23**, 7621-7629.

Shariatmadari, M., Peyronnet, J., Papachristou, P., Horn, Z., Sousa, K.M., Arenas, E. & Ringstedt, T. (2005) Increased Wnt levels in the neural tube impair the function of adherens junctions during neurulation. *Mol Cell Neurosci*, **30**, 437-451.

Shaw, G., Morse, S., Ararat, M. & Graham, F.L. (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *Faseb J*, **16**, 869-871.

Shibata, S., Kakazu, Y., Okabe, A., Fukuda, A. & Nabekura, J. (2004) Experience-dependent changes in intracellular Cl- regulation in developing auditory neurons. *Neurosci Res*, **48**, 211-220.

Smith, J.C., Abdala, A.P., Rybak, I.A. & Paton, J.F. (2009) Structural and functional architecture of respiratory networks in the mammalian brainstem. *Philos Trans R Soc Lond B Biol Sci*, **364**, 2577-2587.

Smith, J.C., Ellenberger, H.H., Ballanyi, K., Richter, D.W. & Feldman, J.L. (1991) Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science*, **254**, 726-729.

Smith, J.L. & Schoenwolf, G.C. (1997) Neurulation: coming to closure. *Trends Neurosci*, **20**, 510-517.

Smith, T.C. & Jahr, C.E. (2002) Self-inhibition of olfactory bulb neurons. *Nat Neurosci*, **5**, 760-766.

Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartwieg, E.A. & Cepko, C.L. (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell*, **68**, 33-51.

Stein, V., Hermans-Borgmeyer, I., Jentsch, T.J. & Hubner, C.A. (2004) Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. *J Comp Neurol*, **468**, 57-64.

Stoppini, L., Buchs, P.A. & Muller, D. (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods*, **37**, 173-182.

Stornetta, R.L., Rosin, D.L., Wang, H., Sevigny, C.P., Weston, M.C. & Guyenet, P.G. (2003) A group of glutamatergic interneurons expressing high levels of both neurokinin-1 receptors and somatostatin identifies the region of the pre-Botzinger complex. *J Comp Neurol*, **455**, 499-512.

Sung, K.W., Kirby, M., McDonald, M.P., Lovinger, D.M. & Delpire, E. (2000) Abnormal GABAA receptor-mediated currents in dorsal root ganglion neurons isolated from Na-K-2Cl cotransporter null mice. *J Neurosci*, **20**, 7531-7538.

Suzue, T. (1984) Respiratory rhythm generation in the *in vitro* brainstem-spinal cord preparation of the neonatal rat. *Journal of Physiology*, **354**, 173-183.

Tam, P.P. (1989) Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development*, **107**, 55-67.

Tam, P.P. & Loebel, D.A. (2007) Gene function in mouse embryogenesis: get set for gastrulation. *Nat Rev Genet*, **8**, 368-381.

Tan, W., Janczewski, W.A., Yang, P., Shao, X.M., Callaway, E.M. & Feldman, J.L. (2008) Silencing preBotzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. *Nat Neurosci*, **11**, 538-540.

Tanis, J.E., Bellemer, A., Moresco, J.J., Forbush, B. & Koelle, M.R. (2009) The potassium chloride cotransporter KCC-2 coordinates development of inhibitory neurotransmission and synapse structure in Caenorhabditis elegans. *J Neurosci*, **29**, 9943-9954.

Tapia, J.C., Mentis, G.Z., Navarrete, R., Nualart, F., Figueroa, E., Sanchez, A. & Aguayo, L.G. (2001) Early expression of glycine and GABA(A) receptors in developing spinal cord neurons. Effects on neurite outgrowth. *Neuroscience*, **108**, 493-506.

Taylor, J., Abramova, N., Charlton, J. & Adler, P.N. (1998) Van Gogh: a new Drosophila tissue polarity gene. *Genetics*, **150**, 199-210.

Thoby-Brisson, M., Karlen, M., Wu, N., Charnay, P., Champagnat, J. & Fortin, G. (2009) Genetic identification of an embryonic parafacial oscillator coupling to the preBotzinger complex. *Nat Neurosci*, **12**, 1028-1035.

Thoby-Brisson, M., Trinh, J.B., Champagnat, J. & Fortin, G. (2005) Emergence of the pre-Botzinger respiratory rhythm generator in the mouse embryo. *J Neurosci*, **25**, 4307-4318.

Tian, G.F., Peever, J.H. & Duffin, J. (1999) Botzinger-complex, bulbospinal expiratory neurones monosynaptically inhibit ventral-group respiratory neurones in the decerebrate rat. *Exp Brain Res*, **124**, 173-180.

Todd, E.S., Weinberg, S.M., Berry-Kravis, E.M., Silvestri, J.M., Kenny, A.S., Rand, C.M., Zhou, L., Maher, B.S., Marazita, M.L. & Weese-Mayer, D.E. (2006) Facial phenotype in children and young adults with PHOX2B-determined congenital central hypoventilation syndrome: quantitative pattern of dysmorphology. *Pediatr Res*, **59**, 39-45.

Tonkovic-Capin, V., Stucke, A.G., Stuth, E.A., Tonkovic-Capin, M., Krolo, M., Hopp, F.A., McCrimmon, D.R. & Zuperku, E.J. (2001) Differential modulation of respiratory neuronal discharge patterns by GABA(A) receptor and apamin-sensitive K(+) channel antagonism. *J Neurophysiol*, **86**, 2363-2373.

Torban, E., Kor, C. & Gros, P. (2004) Van Gogh-like2 (Strabismus) and its role in planar cell polarity and convergent extension in vertebrates. *Trends Genet*, **20**, 570-577.

Torban, E., Patenaude, A.M., Leclerc, S., Rakowiecki, S., Gauthier, S., Andelfinger, G., Epstein, D.J. & Gros, P. (2008) Genetic interaction between members of the Vangl family causes neural tube defects in mice. *Proc Natl Acad Sci U S A*, **105**, 3449-3454.

Tornberg, J., Voikar, V., Savilahti, H., Rauvala, H. & Airaksinen, M.S. (2005) Behavioural phenotypes of hypomorphic KCC2-deficient mice. *Eur J Neurosci*, **21**, 1327-1337.

Tucker, R.P. (2004) Neural crest cells: a model for invasive behavior. *Int J Biochem Cell Biol*, **36**, 173-177.

Tyzio, R., Minlebaev, M., Rheims, S., Ivanov, A., Jorquera, I., Holmes, G.L., Zilberter, Y., Ben-Ari, Y. & Khazipov, R. (2008) Postnatal changes in somatic gamma-aminobutyric acid signalling in the rat hippocampus. *Eur J Neurosci*, **27**, 2515-2528.

Ueno, N. & Greene, N.D. (2003) Planar cell polarity genes and neural tube closure. *Birth Defects Res C Embryo Today*, **69**, 318-324.

Uhlen, P. (2004) Spectral analysis of calcium oscillations. *Sci STKE*, **2004**, pl15.

Uhlen, P. & Fritz, N. (2010) Biochemistry of calcium oscillations. *Biochem Biophys Res Commun*, **396**, 28-32.

Ulrich, D. (2003) Differential arithmetic of shunting inhibition for voltage and spike rate in neocortical pyramidal cells. *Eur J Neurosci*, **18**, 2159-2165.

Uvarov, P., Ludwig, A., Markkanen, M., Pruunsild, P., Kaila, K., Delpire, E., Timmusk, T., Rivera, C. & Airaksinen, M.S. (2007) A novel N-terminal isoform of the neuron-specific K-Cl cotransporter KCC2. *J Biol Chem*, **282**, 30570-30576.

Wallen-Mackenzie, A., Gezelius, H., Thoby-Brisson, M., Nygard, A., Enjin, A., Fujiyama, F., Fortin, G. & Kullander, K. (2006) Vesicular glutamate transporter 2 is required for central respiratory rhythm generation but not for locomotor central pattern generation. *J Neurosci*, **26**, 12294-12307.

Walters, Z.S., Haworth, K.E. & Latinkic, B.V. (2009) NKCC1 (SLC12a2) induces a secondary axis in Xenopus laevis embryos independently of its co-transporter function. *J Physiol*, **587**, 521-529.

Wang, D. (2009) Reticular formation and spinal cord injury. *Spinal Cord*, **47**, 204-212.

Wang, D.D. & Kriegstein, A.R. (2008) GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. *J Neurosci*, **28**, 5547-5558.

Wang, Y., Guo, N. & Nathans, J. (2006) The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. *J Neurosci*, **26**, 2147-2156.

Vetter, P., Roth, A. & Hausser, M. (2001) Propagation of action potentials in dendrites depends on dendritic morphology. *J Neurophysiol*, **85**, 926-937.

Wolff, T. & Rubin, G.M. (1998) Strabismus, a novel gene that regulates tissue polarity and cell fate decisions in Drosophila. *Development*, **125**, 1149-1159.

von Bohlen und Halbach, O. & Unsicker, K. (2009) Neurotrophic support of midbrain dopaminergic neurons. *Adv Exp Med Biol*, **651**, 73-80.

von Euler, C. (1977) The functional organization of the respiratory phase-switching mechanisms. *Fed Proc*, **36**, 2375-2380.

Woo, N.S., Lu, J., England, R., McClellan, R., Dufour, S., Mount, D.B., Deutch, A.Y., Lovinger, D.M. & Delpire, E. (2002) Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. *Hippocampus*, **12**, 258-268.

Vu, T.Q., Payne, J.A. & Copenhagen, D.R. (2000) Localization and developmental expression patterns of the neuronal K-Cl cotransporter (KCC2) in the rat retina. *J Neurosci*, **20**, 1414-1423.

Yamaguchi, M., Saito, H., Suzuki, M. & Mori, K. (2000) Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. *Neuroreport*, **11**, 1991-1996.

Ybot-Gonzalez, P. & Copp, A.J. (1999) Bending of the neural plate during mouse spinal neurulation is independent of actin microfilaments. *Dev Dyn*, **215**, 273-283.

Yuste, R. & Katz, L.C. (1991) Control of postsynaptic Ca2+ influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron*, **6**, 333-344.

Zhu, L., Lovinger, D. & Delpire, E. (2005) Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. *J Neurophysiol*, **93**, 1557-1568.