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EARLY FUNCTION OF KCC2 AND WNT GENES

Cytoskeletal effects in neural stem cells

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To my family

ABSTRACT

From closure of the neural tube until formation of neuronal networks, cells undergo several crucial changes which depend on the expression of many important molecules. GABA, the principal inhibitory neurotransmitter in the adult nervous system, acts as an excitatory signal during embryonic development. This is a necessary neurotrophic signal resulting from a high level of chloride in neural cells. During neural maturation, the potassium-chloride co-transporter, KCC2, lowers the intracellular chloride level and switches the GABA response to hyperpolarizing. Studies have shown a role for KCC2 in the formation of neuronal dendrites but earlier functions in mammals have not yet been revealed. Among the most well-known factors regulating early nervous system development are the Wnt proteins, which act as extracellular ligands. This thesis describes the action of KCC2 and Wnt genes on the cytoskeleton of neural progenitors and demonstrates how a change in their expression can alter neural cell behaviour.

To correlate the expression pattern of KCC2 with functionality, we recorded the GABA response in fetal rat respiratory neurons. The developmental switch from depolarizing to hyperpolarizing was shown to be around embryonic day (E) 20. The KCC2 expression pattern in the main respiratory-related nuclei – preBötzinger complex and parafacial respiratory group – changed drastically between E18 and P0. From being essentially cytoplasmic at E18, KCC2 appeared to be translocated to the plasma membrane at E20. At P0, the KCC2 protein was localized predominantly at the plasma membrane and maintained this expression pattern postnatally.

Overexpression of KCC2 in the neural tube of transgenic mouse embryos had a deleterious effect on the nervous system development. Transgenic embryos at E9.5-E13.5 displayed a reduced neuronal differentiation and impaired neural crest migration. Similar results were obtained with a truncated form of KCC2, lacking the sequence for ion transport, implying that the effects were not GABA-dependent. Interestingly, the neural tube of transgenic embryos had an aberrant distribution of the cytoskeletal protein actin, suggesting an interaction between KCC2 and the cytoskeleton.

In a related study, we examined transgenic embryos with neural-specific overexpression of Wnt7a. These embryos have been shown to exhibit cytoskeletal defects discernible as impaired adherens junctions in the rostral neural tube. Analysis of E9.5 and E10.5 transgenic embryos showed a reduced neuronal differentiation, indicating a role for Wnt7a in the control of neuronal progenitor maturation. Moreover, the downstream signalling gene Vangl2 was upregulated in the neural tube. We therefore studied transgenic embryos overexpressing Vangl2 in neural progenitors and loop-tail embryos with a natural mutation in the Vangl2 gene. Both Vangl2 gain-of-function and loss-of-function resulted in cytoskeletal defects in the neural tube, characterized by aberrant distribution of actin and other cytoskeletal components. Moreover, the small GTPase Rac1 was redistributed in the cells of the neural tube, indicating an interaction between Vangl2 and Rac1. Cell studies using HEK293T, MDCK and C17.2 cell lines showed similar effects of Vangl2 on the cytoskeleton as well as altered cell adhesion and motility. Interestingly, these effects could be blocked by a Rac1 knock-down, verifying the interaction between Vangl2 and Rac1 observed in vivo. Taken together, these results demonstrate the significance of a coordinated expression of cytoskeletal-interacting proteins during nervous system development.

LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following original articles, referred to in the text by their Roman numerals.

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

II. **Zachi Horn**, Panagiotis Papachristou, Maria Shariatmadari, Julie Peyronnet, Beatrice Eriksson and Thomas Ringstedt (2007). Wnt7a overexpression delays beta-tubulin III expression in transgenic mouse embryos. *Brain Research*, 1130: 67-72.

III. Maria Lindqvist, **Zachi Horn**, Vitezslav Bryja, Panagiotis Papachristou, Rieko Ajima, Ernest Arenas, Terry P. Yamaguchi, Hugo Lagercrantz and Thomas Ringstedt (2008). Regulation of cell adhesion and adherens junctions by Vangl2. *Submitted*.

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LIST OF ABBREVIATIONS

aCSF	Artificical cerebrospinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	Brain-derived neurotrophic factor
CE	Convergent extension
CNS	Central nervous system
Dvl	Dishevelled
E	Embryonic day
E_{GABA}	Reversal potential for GABA
FITC	Fluorescein isothiocyanate
Fz	Frizzled
GABA	Gamma-aminobutyric acid
HEK	Human embryonic kidney
JNK	Jun N-terminal kinase
KCC2	Potassium chloride cotransporter 2
MDCK	Madin-Darby canine kidney
NK1R	Neurokinin-1 receptor
NKCC1	Sodium potassium chloride cotransporter 1
NMDA	N-methyl-D-aspartic acid
ORF	Open reading frame
P	Postnatal day
PCP	Planar cell polarity
PCR	Polymerase chain reaction
pFRG	Parafacial respiratory group
preBötC	PreBötzinger complex
Vangl2	Van Gogh-like 2
VLM	Ventro-lateral medulla
wt	Wild type

1 INTRODUCTION

1.1 DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

1.1.1 Neurulation

The formation of the central nervous system (CNS) is a program of incredible precision, which requires a highly coordinated progression of events. Prior to CNS formation, three main cell layers must be generated. The upper and lower germ 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 undergo an epithelial-to-mesenchymal transition and move inward at the primitive streak, generating the third germ layer, mesoderm. This is the middle layer that gives rise to connective tissues, muscles and the vascular system (Kinder *et al.*, 1999). The innermost layer, 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, 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 at 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

1.1.2.1 Cell fate

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 throughout the body, developing 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). The forebrain becomes further 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.

1.1.2.2 Inductive signals

Control of CNS formation and patterning involves the interplay of multiple signalling systems, which interact to mediate the transcriptional events that specify cell fate. The neural tube is patterned by various signalling 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 signalling 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 signalling is also believed to function along the entire length of the neuraxis regulating antero-posterior neural fates (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). Wnts will be described in more detail in section 1.3.

1.1.3 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 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).

1.1.3.1 Neurotransmitters and receptors

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. Gamma-aminobutyric acid (GABA) and glycine are inhibitory transmitters in the adult CNS, but act as excitatory transmitters in immature neurons (see section 1.2.1). The interplay between the ionotropic NMDA, AMPA and $GABA_A$ receptors during development is important for neuronal interaction 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).

1.1.3.2 Emergence of the respiratory rhythm

The most studied neuronal network formation might be the visual system. The respiratory network, which is investigated in this thesis, has also received a lot of attention but is still not fully understood. Respiratory rhythms are initiated at E15 in mouse (Thoby-Brisson *et al.*, 2005) and E17 in rat (Greer *et al.*, 1992). Neurons with bursting pacemaker properties that depend on a persistent Na^+ current have been identified as the source (Smith *et al.*, 1991). GABA, glycine and glutamate promote the emergence of respiratory rhythms (Ren & Greer, 2003). The respiratory center comprises the preBötzinger complex (preBötC) and parafacial respiratory group (pFRG), localized in the ventrolateral medulla of the brainstem. It is still under debate whether these regions are the exact sites for respiratory rhythm generation. Moreover, the function of these neuronal groups for controlling the inspiratory or expiratory drive has not yet been agreed on (Feldman & Del Negro, 2006). Specific markers that identify preBötC are the neurokinin-1 receptor (NK1R) and somatostatin (Stornetta *et al.*, 2003). NK1R-positive preBötC neurons are born in the ventricular zone at E12-13 and reach the preBötC area at E14-15 in mouse (Thoby-Brisson *et al.*, 2005) and E16-17 in rat (Pagliardini *et al.*, 2003). PreBötC neurons expressing NK1R are crucial for breathing control as targeted destruction of these neurons leads to ataxic respiration (Gray *et al.*, 2001). NK1R is also expressed in surrounding areas, including pFRG (Nattie & Li, 2002). A recent study showed that pFRG is also critical for respiratory rhythm generation. Mice bearing a mutation in Phox2b specifically lacked glutamatergic neurons in the pFRG region and displayed an irregular breathing pattern. These mice died soon after birth (Dubreuil *et al.*, 2008).

1.2 NEURONAL CHLORIDE HOMEOSTASIS DURING DEVELOPMENT

1.2.1 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^-, and $GABA_B$ 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, GABA has depolarizing actions. This is due to the difference in the intracellular chloride concentration ($[Cl^-]_i$), which depends on the expression of chloride transporters (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 excitatory action of GABA has been proposed to be crucial for the maturation and differentiation of neurons and neuronal networks 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 excitation and Ca^{2+} influx act as crucial triggers for plasticity of synaptic connections and for 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 signalling 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.

1.2.2 Cation Chloride Cotransporters

Regulation of the cellular chloride concentration is important for controlling cell volume and pH. The chloride concentration 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 only expressed in neural cells, whereas KCC3 and KCC4 are found both in CNS and other tissues (Delpire & Mount, 2002; Payne *et al.*, 2003).

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 neural 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 switch of the GABA response to hyperpolarizing (Fig 1) (Plotkin *et al.*, 1997; Rivera *et al.*, 1999).

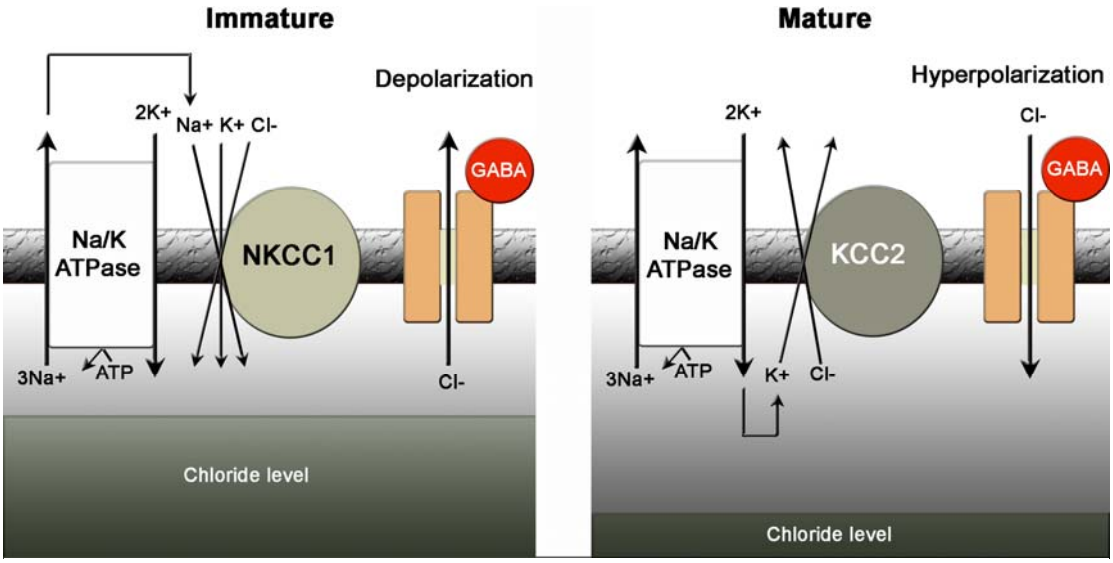


Fig 1. The developmental GABA switch results from KCC2-mediated extrusion of chloride in mature neurons.

1.2.3 KCC2

KCC2 is a neuron-specific isoform of the KCC-family. It is a glycosylated protein with 12 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, dorsal root ganglion, 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 low expression levels which begin in the spinal cord (E11.5 in mice) and increase in relation to the phylogenetic developmental view: spinal cord – brainstem – hippocampus – cortex (Rivera *et al.*, 1999; Stein *et al.*, 2004; Delpy *et al.*, 2008).

1.2.3.1 Role in the GABA switch

The electrochemical gradient for Cl^- sets the reversal potential for GABA resonses (E_{GABA}). As described above, immature neurons contain high $[Cl^-]_i$. Therefore, E_{GABA} is higher than the resting membrane potential and results in a depolarization when $GABA_A$ receptors are activated. When KCC2 is developmentally upregulated, it lowers $[Cl^-]_i$ and shifts E_{GABA} to more negative levels than the resting membrane potential. This generates the hyperpolarizing response to GABA in mature neurons (Rivera *et al.*, 1999).

It has been shown in various studies that KCC2 is critical for lowering $[Cl^-]_i$ in the CNS. Cortical neurons lacking KCC2 fail to regulate $[Cl^-]_i$ (Zhu *et al.*, 2005). In addition, $KCC2^{-/-}$ mutant mice die at birth due to respiratory failure. These mice lacked inhibitory responses to GABA in motoneurons, leading to severe defects in motor control (Hubner *et al.*, 2001). Moreover, hypomorphic KCC2-deficient mice with only 5-10% expression of KCC2 displayed frequent generalized seizures and died shortly after birth (Woo *et al.*, 2002), whereas mice retaining 15-20% of normal KCC2 protein levels were viable but displayed increased anxiety-like behaviour and impaired learning

in several tests, and were 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 switched 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. This resulted in impaired morphological maturation of cortical neurons, pronounced as a reduced dendritic length and branch number (Cancedda *et al.*, 2007). Furthermore, overexpressing KCC2 from the onset of development in zebrafish impaired neuronal development in the brain and spinal cord. The embryos displayed a perturbed neuronal differentiation and axonal growth, and fewer motoneurons and interneurons (Reynolds *et al.*, 2008). These results show the importance of an excitatory GABA response for neuronal development and demonstrate that a developmental delay of KCC2 upregulation is essential for the effects of GABA to occur.

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 a prerequisite for the re-establishment of connectivity in damaged brain tissue (Rivera *et al.*, 2004).

1.2.3.2 Is there an earlier role of KCC2?

Studies have shown that KCC2 appears to be expressed early in development when GABA is still excitatory. Phosphorylated (active) KCC2 protein was present at E13 in mice and preceded the decline of E_{GABA} in the hippocampus and spinal cord (Stein *et al.*, 2004). Moreover, KCC2 displayed functional Cl^- transport already at E11.5-13.5 in the mouse spinal cord although the GABA response did not shift until late fetal age (Delpy *et al.*, 2008). KCC2 was present at the depolarizing period also in the brainstem lateral superior olive, and became integrated into the plasma membrane only with increasing postnatal age (Balakrishnan *et al.*, 2003). However, the potential role of KCC2 before the developmental GABA switch was not investigated in these studies.

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 and functional excitatory synapses. In the absence of KCC2, neurons developed long dendritic protrusions paralleled by a reduction of 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, indicating that the effects were not mediated through ion transport. Intriguingly, expression of the KCC2 C-terminal only yielded a phenotype similar to $KCC2^{-/-}$ neurons, and in addition, interacted with the cytoskeletal protein 4.1N. Thus, a novel role for KCC2 has been shown during neuronal maturation. The interaction between KCC2 and the cytoskeleton during spine morphogenesis may be crucial for synchronizing excitatory and inhibitory transmission in cortical networks.

1.3 WNT GENES

The Wnt genes encode a large family of secreted glycoproteins that regulate many neural cell behaviours, including proliferation, differentiation, survival, polarity and movements. Wnts are also important for the remodelling of axons. The name is derived from the proto-oncogene Integration factor-1 (Int-1) and Drosophila Wingless, which were found to be orthologous genes. In mammals, 19 Wnt genes have been identified and several roles have been ascribed to many of them using knockout and overexpression models (Miller, 2002; He, 2003).

1.3.1 Wnt signalling

Reception and transduction of Wnt signals involves binding of Wnt proteins to Frizzled (Fz) receptors. There are ten known Fz genes in mammals, which bind Wnts with varying specificity. The same Fz can bind several Wnts and opposite, which makes Wnt/Fz signalling highly complex. Wnt signalling also requires coreceptors, several of which have been identified. In addition, cell-surface proteoglycans appear to have a role as they bind and concentrate Wnt proteins on the cell (Nusse, 2003).

Fz signals through at least three different intracellular pathways (Fig 2). The canonical pathway is the best described of these and involves stabilization of the transcription cofactor β-catenin. In the absence of Wnt signalling, the level of β-catenin is kept low through phosphorylation by glycogen synthase kinase-3β (GSK-3β) bound to a scaffolding complex of Axin and adenomatous polyposis coli (APC) protein, which targets β-catenin for degradation in proteasomes. Activation of Wnt signalling leads to phosphorylation of Dishevelled (Dvl), which in interaction with Axin antagonizes GSK-3β, resulting in accumulation of β-catenin. The excess β-catenin becomes available for binding to transcription factors, and induces expression of target genes. In addition, β-catenin binds to cadherins and links them through α-catenin to the actin cytoskeleton (Huelsken & Behrens, 2002; Nelson & Nusse, 2004).

The non-canonical pathway is less established and involves two pathways that may be overlapping. One of these is the planar cell polarity (PCP) pathway, which regulates the polarity of cells through cytoskeletal organization. This is believed to be achieved through the activation of Jun N-terminal kinase (JNK) via Dvl, but the exact mechanism is not known. Other components of this pathway include Scrb1 (Scribble), Vangl (Van Gogh-like), Celsr1 (Flamingo), Pk (Prickle) and the small GTPases Rho and Rac (Montcouquiol *et al.*, 2003; Fanto & McNeill, 2004; Li *et al.*, 2005; Rosso *et al.*, 2005; Ybot-Gonzalez *et al.*, 2007).

The other subdivision of the non-canonical pathway, known as the Wnt/Ca^{2+} pathway, involves an increase in intracellular Ca^{2+} and activation of protein kinase C (PKC) and calcium/calmodulin-regulated kinase II (CamKII). This is mediated via heterotrimeric G-proteins and Dvl (Sheldahl *et al.*, 2003).

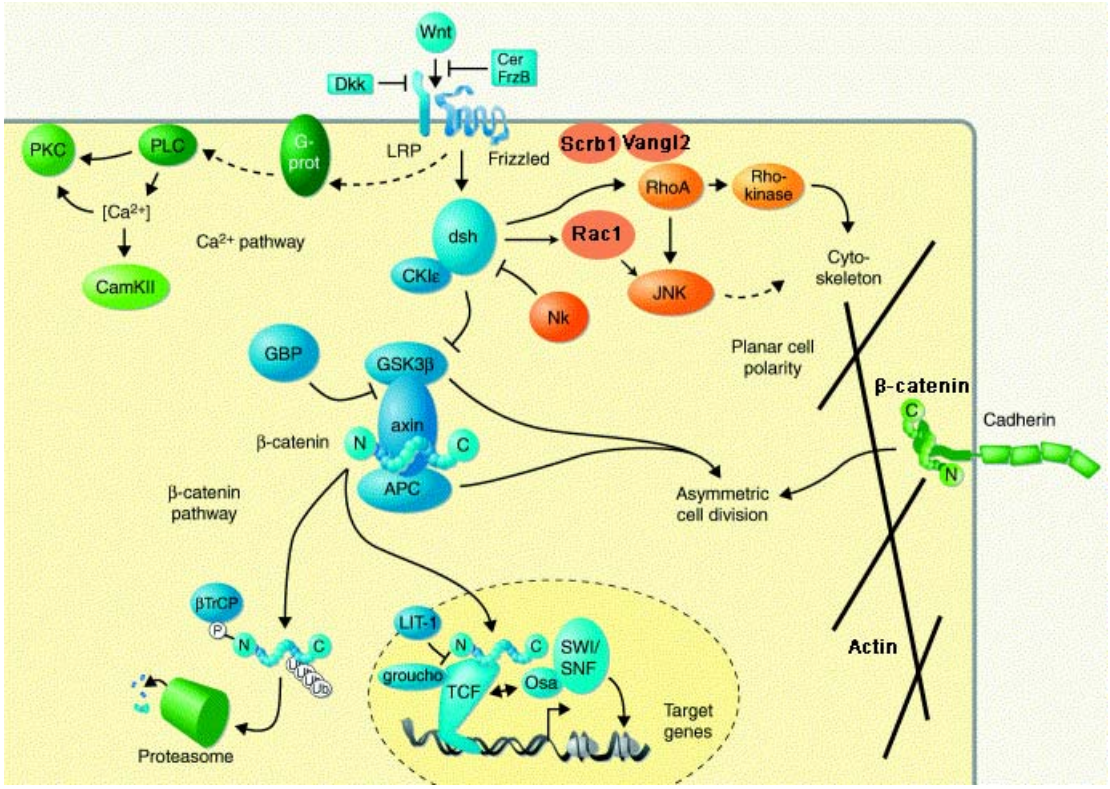


Fig 2. The Wnt signalling pathways.
Adapted from Current Opinion in Genetics & Development, 2001, vol 11, J. Huelsken and W. Birchmeier, *New aspects of Wnt signalling pathways in higher vertebrates*, pp 547-553. Copyright (2001), with permission from Elsevier.

1.3.2 Regulation of neural development

It is believed that the same Wnt can signal through all three pathways depending on the embryonic stage. This may be determined by which Fz member and coreceptor that is expressed at a certain stage.

The β-catenin pathway primarily regulates cell proliferation and cadherin-mediated cell adhesion. The cytoplasmic pool of β-catenin appears to depend on cadherin expression, as overexpression of cadherins sequesters β-catenin at the plasma membrane and thereby makes it unavailable for signalling to the nucleus (Sanson *et al.*, 1996). However, expression of constitutively active β-catenin leads to an enlarged brain and spinal cord in mice, whereas the tissue mass of the brain and spinal cord is reduced in β-catenin mutant mice (Zechner *et al.*, 2003). Thus, β-catenin is essential for the maintenance of proliferation in neural progenitors, and impinges on the decision to proliferate or differentiate. Moreover, ectopic expression of Wnts can lead to induction of a secondary body axis. This was shown to be mediated through the β-catenin pathway, hence demonstrating its role in cell proliferation (Sokol *et al.*, 1991). In addition, alterations in the Wnt/β-catenin pathway are involved in cancer induction and progression. Some key events in cancer are activating mutations in β-catenin, or loss-of-function mutations in APC, which predisposes to colorectal cancer (Nelson & Nusse, 2004).

The PCP pathway controls polarized cell movements during gastrulation and neurulation. PCP is a property shown by cells to become polarized within the plane of the epithelium, along an axis perpendicular to the apical-basal axis of the cell. Examples of PCP include the coordinate organization of scales in fish, feathers in birds and hairs in mammals. Vangl2, Celsr1 and Scrb1 have been demonstrated to regulate PCP in the mammalian inner ear (Montcouquiol *et al.*, 2006). PCP genes play a key role in convergent extension (CE), the process in which mesodermal cells intercalate and thus elongate the body axis. Alterations in the expression of PCP genes cause defective CE movements and failures in neurulation, resulting in a shortened body axis and neural tube defects (NTDs), as shown for example in JNK, Scrb1, Vangl, Fz and Pk mutants (Yamanaka *et al.*, 2002; Jenny *et al.*, 2003; Murdoch *et al.*, 2003; Ueno & Greene, 2003; Ciruna *et al.*, 2006; Wang *et al.*, 2006; Torban *et al.*, 2008).

The Wnt/Ca^{2+} pathway has also been implicated in the regulation of cell movements during gastrulation (Choi & Han, 2002). How this pathway overlaps with the PCP pathway is unclear, but it has been shown that the PCP components Rho and Pk can stimulate Ca^{2+} signalling (Choi & Han, 2002; Veeman *et al.*, 2003). Wnt/Ca^{2+} activity also functions in promoting ventral cell fate and antagonizing dorsal cell fate (Saneyoshi *et al.*, 2002). Moreover, the downstream effector CamKII has well established roles in synaptogenesis and long term potentiation (Menegon *et al.*, 2002).

1.3.3 Regulation of the cytoskeleton

Controlled cell behaviour is essential for embryonic development and requires a coordinated regulation of the cytoskeleton and cell adhesion. Wnts have been shown to regulate the microtubule and actin cytoskeleton via both the canonical and non-canonical pathways (Torres & Nelson, 2000; Ciani & Salinas, 2007). The key protein in the canonical pathway, β-catenin, controls cadherin-mediated cell adhesion and mediates the interaction of adherens junction molecules with the actin cytoskeleton (Brembeck *et al.*, 2006). The PCP components Rac1 and RhoA have also been implicated in cell adhesion and movements through cytoskeletal regulation (Braga *et al.*, 1997; Nobes & Hall, 1999).

Our group has previously shown that Wnt7a overexpression in the neural tube disrupts adherens junctions and results in impaired cranial neurulation in mouse embryos (Shariatmadari *et al.*, 2005). Adherens junctions, situated on the apical end of the neural progenitors near the lumen, impose rigidity to the neural tube and are essential for cranial neurulation (Ybot-Gonzalez & Copp, 1999). Mouse embryos overexpressing Wnt7a displayed reduced levels of actin, β-catenin and N-cadherin at the adherens junctions. In addition, mRNA levels of Vangl2 were increased in the neural tube, indicating that the effects were mediated in part through PCP signalling.

2 HYPOTHESES / AIMS

Study I: The role of KCC2 for respiratory rhythm generation

Hypothesis: KCC2 is initially expressed in the cytoplasm of neural cells in the brainstem and requires activation in order to be transported to the plasma membrane.

Aims:
- To examine the KCC2 expression pattern in the brainstem neural networks.
- To investigate a possible correlation of KCC2 membrane expression and the developmental GABA switch in the respiratory center.

Study II: Cytoskeletal interaction of KCC2 and Wnt genes in neural progenitors

Hypothesis: Early KCC2-expression and Wnt overexpression will interfere with neural cell behaviours such as differentiation and migration, and patterning of the nervous system.

Aims:
- To overexpress KCC2 in neural progenitors and study associated changes in the development of the nervous system. This may involve a premature switch of the GABA response and/or an interaction with the cytoskeleton of neural cells.
- To overexpress Wnt genes in the immature nervous system and examine the effects on downstream signalling molecules and the cytoskeleton, as well as on neural development.
- To compare the roles of KCC2 and Wnt genes on cytoskeletal interactions and subsequent effects on neural cell development.

3 MATERIALS AND METHODS

The methods applied in this thesis are presented below. The methodology is also described in detail in papers I-III.

In vitro preparations and solutions
Experiments were performed on Wistar rats from fetal age E16 to E21 and postnatal age P0 to P3. Fetal rats were removed from pentobarbital anesthetized pregnant dams and the brainstem-spinal cords were quickly isolated under dissection microscope. The brainstem was rostrally decerebrated between the sixth cranial nerve roots and the lower border of trapezoid body. Motor nerve activity was monitored from ventral roots of cervical nerves (C4).

Intracellular recordings
For whole-cell recordings from respiratory neurons, the 'blind' patch clamp technique was employed. In brief, electrodes were inserted into the ventrolateral medulla. When the electrode approached a respiratory neuron, which was identified by monitoring extracellular action potential discharges, positive pressure was released and negative pressure was applied for seal formation. Afterwards, the whole-cell configuration was established by gentle suction, which was combined with injection of a single-shot hyperpolarizing current pulse. Membrane potential was recorded with a single-electrode voltage clamp amplifier. Current-voltage (I-V) relationships were obtained by administration of de- or hyperpolarizing current steps (0.5 s, 5-80 pA). Input membrane resistance was measured at resting membrane potential (or at a holding potential of –50 mV) from the I-V relationships. Gramicidin-perforated patch recordings (5-10 μg/ml in pipette solution) were performed in a subgroup of respiration-related neurons using standard procedures. Neuronal activity and C4 activity were stored on magnetic tape in a DAT recorder for subsequent data analysis using Axoscope, OriginPro 8 (Originlab Corporation) and JMP (SAS Institute).

Drugs
GABA was used in all experiments (50-500 μM) and in some experiments synaptic activity was blocked by tetrodotoxin (TTX, 0.2 μM). The $GABA_A$ antagonist Bicuculline (0.1 mM) and the $GABA_A$ agonist Muscimol (2 μM) were used to validate the response to GABA (all drugs obtained from Sigma-Aldrich). In a subgroup of the E16-E18 preparations, respiratory rhythmic activity was increased by temporary addition of Adrenaline (0.2 μM) to the aCSF while searching for respiration-related neurons. A period of at least 5 min stable intracellular recording was performed during control conditions before addition of drugs in the aCSF followed by a washout period with control aCSF.

Immunohistochemistry on brainstem slices
This method was employed to analyze the developmental expression pattern of KCC2 in the respiratory center. Brainstem sections from rat embryos and pups (Sprague Dawley) E16 – P5 were studied using a modified protocol of the immunohistochemistry method. An NK1R antibody was used to localize the respiratory-related centres in ventrolateral medulla. The staining pattern of sagittal sections is shown in Fig 3. Since the precise location of preBötC is difficult to assess

with current markers, we determined its approximate area by its relative distance to nearby nuclei which are also positive for NK1R. A modified protocol was necessary as both the NK1R and KCC2 antibodies were of the same source (rabbit). The immunostaining was done in several steps. Briefly, a highly diluted NK1R antibody (1:30 000) was applied first. The extremely high dilution ensured that no cross-reaction occurred. The NK1R-staining was enhanced by the biotin-avidin complex conjugated with a peroxidase, followed by the peroxidase substrate, FITC-conjugated Tyramide signal amplification (TSA). Next, the KCC2 antibody was applied at a 1:500 dilution followed by the secondary Cy3-antibody. It was evident that only KCC2 was labelled with the Cy3-antibody since the highly diluted NK1R antibody could only be detected through amplification.

Generation of the nestin-KCC2, nestin-KCC2-ΔNTD, Vangl2-HA and nestin-Vangl2 constructs

The human nestin (hnestin) 1852 vector was generated from the hnestin 1852/LacZ plasmid (Lothian and Lendahl, 1997). For the KCC2 study, a 3348 bp fragment spanning the open reading frame (ORF) of KCC2 and flanked by XhoI and HindIII sites was generated by PCR from a cDNA clone (I.M.A.G.E. Consortium [LLNL] cDNA CloneID 6838880). The upstream primer was 5'-TAA CTC GAG *ATG* CTC AAC AAC CTG ACG and the downstream primer was 5'-GAC AAG CTT TCA GGA GTA GAT GGT GAT G (the XhoI respectively HindIII sites are underlined and the start codon is indicated in italics). The KCC2-ΔNTD sequence (N-terminal deletion, aa 1-100) in a pIRES-EGFP vector was kindly given to us by Dr. Claudio Rivera. The KCC2 and KCC2-ΔNTD fragments were subcloned into pBluescript SK- (Stratagene, La Jolla, CA) and sequenced. The fragments were then cut out and subcloned into the XhoI and SmaI sites of the hnestin 1852 vector containing an IRES-EGFP sequence. For the Vangl2 study, a 1566 bp fragment spanning the ORF of Vangl2 and flanked by XhoI and HindIII sites was generated by PCR from a cDNA clone containing the Vangl2 coding sequence (I.M.A.G.E. Consortium [LLNL] cDNA CloneID 6509008). The cDNA clones were purchased from RZPD (www.rzpd.de). The upstream primer was 5´-TAA CTC GAG *ATG* GAC ACC GAG TCC CAG TA and the downstream primer was 5´-GAC AAG CTT TCA CAC AGA GGT CTC CGA (the XhoI respectively HindIII sites are underlined and the start codon is indicated in italics). The Vangl2 fragment was subcloned into pBluescript SK- and sequenced. It was then cut out and subcloned into the XhoI/HindIII site of the pcDNA3-HA expression vector or the NotI site of the hnestin 1852 vector.

Creation of transgenic embryos, identification and in situ hybridization

The expression cassettes, hnestin 1852/tk promoter with KCC2 (or KCC2-ΔNTD) ORF and IRES-EGFP, and hnestin 1852/tk promoter with Vangl2 ORF, were excised from the vector backbone, purified, and used for pronuclear injection of fertilized mouse (B6D2F1) oocytes. Pronuclear injection and implantation of oocytes into pseudopregnant mice was employed to generate transgenic mice at Karolinska Center for Transgene Technologies (KCTT). Pregnant dams with embryos at E8.5 – 15.5 were sacrificed by spinal dislocation, and the embryos were rapidly dissected out. The transgenic embryos were identified by PCR, using yolk sac DNA as a template. A sense primer complementary to human nestin intron 2 was combined with an antisense primer complementary to the KCC2 or Vangl2 ORF. KCC2 protein expression assayed by immunohistochemistry (se below) verified the level of overexpression. For identification of nestin-Wnt7a embryos, see Shariatmadari et al. (2005). Vangl2 mRNA expression was investigated by digoxigenin (DIG) in situ hybridization since no antibody to Vangl2 was available at the time of the study. Yolk sac DNA was used to

genotype Loop-tail embryos. PCR was performed with primers against a microsatellite sequence for the lpt locus. The wild type sequence generates a 95 bp product, the lpt sequence a 140 bp product. Animals were treated according to European Communities Council guidelines (directive 86/609/EEC).

Immunohistochemistry

This method was employed to immuno-label sections of transgenic mouse embryos and their wild type littermates. Monoclonal anti-β-catenin (BD Biosciences, San Jose, CA) was used at a 1:200 dilution, monoclonal anti-β-tubulin III/TuJ1 (Covance, Denver, PA) at a 1:500 dilution, rabbit anti-SOX10 (Affinity Bioreagents, Golden, CO) at a 1:200 dilution, rabbit anti-KCC2 at 1:500, rabbit anti-Phospho-histone 3 at 1:200 (both from Upstate Biotechnology, Lake Placid, NY), rabbit anti-phospho-SAPK/JNK (Cell Signaling Technology, Danvers, MA) at a 1:500 dilution, monoclonal anti-AP-2α at 1:50, rabbit anti-Rac1 at 1:200, monoclonal anti-RhoA at 1:50, monoclonal anti-Connexin 43 at 1:50, rabbit anti-N-cadherin at 1:100, and goat anti-Scrb1 at a 1:50 dilution (all from Santa Cruz Biotechnology, Santa Cruz, CA). The 4.1N antibody (rabbit) was a kind gift from Dr. Claudio Rivera. Secondary Cy3- or FITC-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used at a 1:400 dilution. When the distribution of actin microfibers was investigated, 50 µg/ml of FITC-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO) was added to the solution. TuJ1 immunolabelling was visualized with a Mouse Horseradish Peroxidase Extravidin Staining kit (Sigma-Aldrich).

Cell cultures and transfection experiments

Human embryonic kidney (HEK) 293T cells, Madin-Darby Canine Kidney (MDCK) cells and the pluripotent neural stem cell line C17.2 were cultured in their respective mediums. Cells were transfected with the Vangl2-HA expression vector, control vectors pcDNA, pDsRed or pEGFP, and dominant negative (dn) Rac1 (Rac1N17), constitutive active (ca) Rac1 (Rac1V12), dn RhoA (RhoAN19) or ca RhoA (RhoAV14) expression vectors, using Lipofectamine 2000 (Invitrogen). RNAi hairpins (Stealth^{TM} siRNA; Invitrogen) complementary to human Rac1 mRNA were used in some experiments.

Immunocytochemistry

Cultured cells were stained at appropriate times after transfection. Endogenous actin was visualized with 50 µg/ml FITC-Phalloidin (Sigma-Aldrich, St. Louis, MO). Primary antibodies used were anti-β-catenin (BD Biosciences, San Jose, CA) at a 1:200 dilution, anti-N-cadherin at a 1:100 dilution, anti-Rac1 at a 1:200 dilution, anti-RhoA at a 1:50 dilution, anti-Scrb1 at a 1:10 dilution (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-SAPK/JNK (Cell Signaling Technology, Danvers, MA) at a 1:500 dilution, and anti-HA (Sigma-Aldrich, St. Louis, MO) at a 1:300 dilution. The Scrb1 immunofluorescence was enhanced by Vectastain ABC (Vector Laboratories, Burlingame, CA) at a 1:100 dilution, followed by FITC-conjugated TSA (PerkinElmer, Boston, MA) at a 1:50 dilution.

Aggregation assay

This method enabled analysis of cell adhesion. MDCK cells were used because of their ability to adhere tightly to each other. Cells were cultured until 70% confluency and then transfected with the above mentioned plasmids. After 4 hours the cells were carefully scraped off the culture dishes and counted. The cells were then transferred in drops of 30 µl (1000 cells/µl) to the inside of a 24-well plate-lid. The hanging drops

were incubated for 24-48 hours to allow the cells to aggregate. The drops were then pipetted up and down 10 times and analyzed in a light microscope.

Wound assay

To study cell migration, subconfluent C17.2 cells were transfected as above and then allowed to reach 100% confluency. The cells were then treated with 10µM Mitomycin C (Sigma-Aldrich) for 3 hours to arrest the cell cycle. Thereafter, a wound was made through the cells using a 200 µl pipet tip. Medium was changed to serum-reduced (1% FBS) to keep the cells from dividing, and a line was drawn underneath the culture dish perpendicular to the scratch. Pictures were taken just above or below the line under a light phase-contrast microscope, immediately and after 18 hours. For quantification of the results, a representative area was chosen in micrographs taken at T=0 h, and the distance between the edges of the wound were measured. The same area in micrographs from T=18 h was identified. The measured distance was used as a baseline, which combined with a fixed height yielded a rectangular field. The number of cells within the field was calculated, and then divided by the area.

Immunoprecipitation

This was done to investigate potential protein-binding partners to Vangl2. Cells were extracted in ice-cold lysis buffer and the extracts were incubated with an anti-HA antibody (Abcam). Immunoprecipitates were collected on Protein G Sepharose Fast flow beads (Amersham Biosciences) by overnight rotation, washed with lysis buffer, resuspended in 2x Laemmli sample buffer and subjected to SDS-PAGE followed by Western blot analysis using anti-Rac1 (Upstate) and anti-RhoA (Santa Cruz Biotechnology) antibodies.

Pull down assay

To examine whether Vangl2 changed the active state of Rac1 and RhoA, pull down assays were performed using Glutathione-*S*-transferase (GST)-p21-activated kinase (PAK), and GST-RHOtekin recombinant proteins, which bind to active Rac1 and RhoA respectively. Active Rac1 and RhoA are coupled to GTP, which is hydrolyzed to GDP when inactive. Therefore, quick performance at 4^{o}C is necessary to keep the proteins in the active state. Western blotting using anti-Rac1 and anti-RhoA antibodies was then done to detect the relative fraction of active protein.

RESULTS AND DISCUSSION

3.1 THE GABA SWITCH IS CORRELATED WITH MEMBRANE EXPRESSION OF KCC2 IN THE RESPIRATORY CENTER

The switch in polarity of the GABA response is a prerequisite for the propagation of respiratory rhythms from the brainstem – and consequently – for breathing (Hubner *et al.*, 2001). The time-point for the GABA switch in respiratory neurons has shown to be around E19 in rat using extracellular recordings on medullary slice and brainstem-spinal cord preparations (Ren & Greer, 2006). The same study also showed that a KCC2-blocker, furosemide, diminished the effects of the GABA-agonist muscimol at postnatal day (P) 1. However, the developmental influence of GABA on respiratory neurons using intracellular recordings, and the developmental expression of KCC2 in the respiratory center has not previously been shown. We therefore examined the response of fetal rat respiratory neurons to GABA using intracellular recordings and investigated a possible correlation with the KCC2 expression pattern in the respiratory center.

3.1.1 The GABA response becomes hyperpolarizing in late fetal respiratory neurons (Paper I)

We recorded respiratory neuron activity in rat ventrolateral medulla (VLM) from E16 to P2 using 'conventional whole-cell' and 'gramicidin-perforated' patch clamp. The response to bath application of GABA was predominantly depolarizing in E16-E20 neurons. A small fraction of neurons hyperpolarized at late fetal age (E18-E21). In preparations from postnatal rats (P0-P2) hyperpolarization was the dominant response. The effect of GABA was blocked with 100 μM bicuculline, a $GABA_A$ receptor antagonist. Based on these data, it is apparent that the response of respiratory neurons to GABA is in transition around birth. This is in agreement with Ren and Greer's data showing the switch at E19 in rat (Ren & Greer, 2006) and another study showing a hyperpolarizing response in VLM at E18.5 in mice (Ikeda *et al.*, 2004), although it has also been reported that the switch occurs at P3 in mice (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 (Woodin *et al.*, 2003), probably as a result of ongoing changes in expression of chloride transporters at late fetal age (Farrant & Kaila, 2007). It has been shown that, during development, E_{GABA} shifts to hyperpolarizing values earlier in the soma than in dendrites (Romo-Parra *et al.*, 2008). 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.

A recent study proposed that the switch in action of GABA in the hippocampus is not associated with a switch 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). However, our results clearly demonstrate that hyperpolarization occurs in respiratory neurons at late fetal age.

3.1.2 KCC2 changes its intracellular localization during the GABA switch (Paper I)

In order to correlate the GABA switch with $[Cl^-]_i$ regulation, we analyzed the developmental KCC2 expression pattern in the rat respiratory center, comprising the preBötzinger complex (preBötC) and the parafacial respiratory group (pFRG), located in the VLM (Fig 3). We used an antibody to NK1R to localize the respiratory center in brainstem sections from E16 to P5 rats. Double-staining with a KCC2-antibody revealed a diffuse cytoplasmic expression of the KCC2 protein at E16 and E18. The distribution of KCC2 was still somewhat diffuse at E20 but small clusters of labelled protein were detected in (or near) the plasma membrane. The diffuse pattern was more discernible in preBötC than in pFRG at this stage. At P0, KCC2 clearly delineated the membrane of NK1R-positive cells in both preBötC and pFRG. No evident cytoplasmic staining could be observed. The membrane staining of KCC2 was maintained postnatally (P3 and P5). Quantification of the KCC2 staining intensity in the membrane and cytoplasm of cells in the studied areas showed an increase of KCC2 in the membrane and decrease of KCC2 in the cytoplasm from E18 to P3. Interestingly, pFRG had significantly

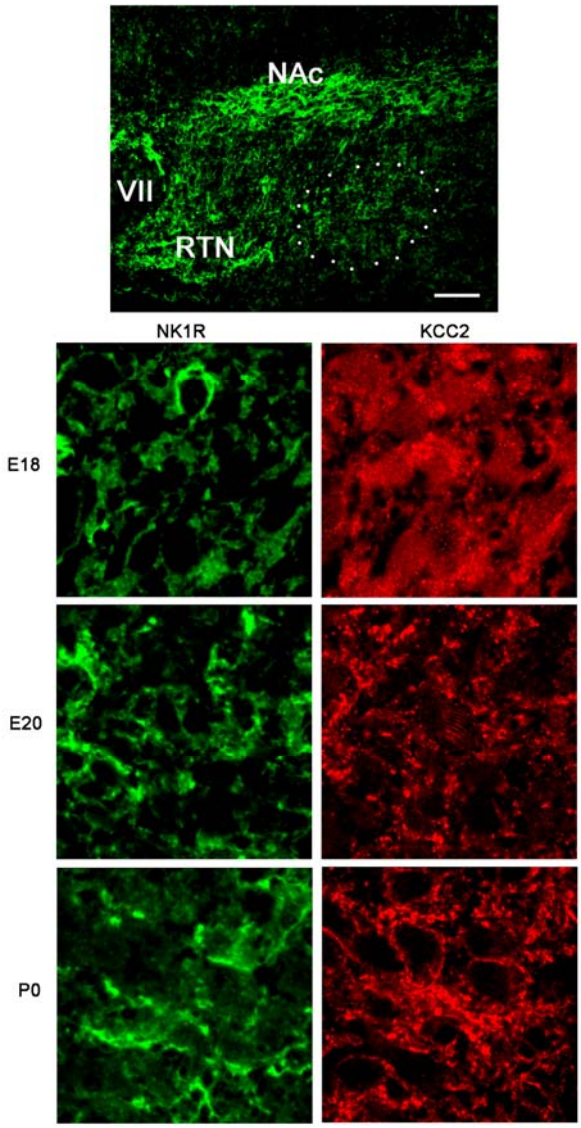


Fig 3. NK1R-staining in the ventrolateral medulla (top). The dashed circle indicates the approximate area of preBötC. KCC2-staining in preBötC is shown at E18 – P0. See main text for details.

higher staining intensity in the membrane than preBötC at E20, but not postnatally.

Though this does not prove that a hyperpolarizing response is needed earlier in pFRG than preBötC, it contributes to the debate regarding the role of pFRG in respiratory rhythm generation. Some investigators claim pFRG to be a master inspiratory oscillator (Onimaru & Homma, 2003; Onimaru *et al.*, 2006) whereas others assert that pFRG is an expiratory rhythm generator (Feldman & Del Negro, 2006; Janczewski & Feldman, 2006; Onimaru *et al.*, 2006). Our finding that KCC2 appears to be upregulated in the plasma membrane earlier in pFRG than in preBötC implies that these two regions differ in response to GABA at E20 and perhaps also in the modulation of respiratory rhythm at perinatal age. A recent study revealed the importance of pFRG in regular breathing at birth, as mice with disrupted pFRG neurons died from central apnea (Dubreuil *et al.*, 2008).

The role of KCC2 for respiratory function has been demonstrated before (Hubner *et al.*, 2001). It has also been shown that a decrease in the intracellular K^+ concentration impairs the function of KCC2 and results in an absence of the GABA switch in respiratory neurons (Ikeda *et al.*, 2004). Several factors have been reported to control the upregulation of KCC2, such as BDNF (Aguado *et al.*, 2003) and tyrosine kinase-dependent phosphorylation (Kelsch *et al.*, 2001). Moreover, maternal oxytocin, an essential hormone for labour, triggers a transient inhibitory switch in GABA during delivery (Tyzio *et al.*, 2006). KCC2 mRNA levels were however not changed upon blockade of oxytocin receptors. Rather, it was suggested that oxytocin downregulates NKCC1 activity, although mRNA levels of NKCC1 did not significantly differ either. However, the study did not analyze the KCC2 protein levels. It is likely that oxytocin activates the transition of the KCC2 protein from the cytoplasm to the plasma membrane.

3.1.3 Early KCC2 expression can interact with the cytoskeleton of neural cells (unpublished results)

While the data presented above point to a correlation between the GABA switch and membrane localization of KCC2, they also show that expression of the KCC2 protein exists before the switch occurs. The cytoplasmic appearance of KCC2 in early neuronal development has recieved little attention and it is yet unclear if the protein has a functional role intracellularly before the integration into the plasma membrane. We therefore overexpressed KCC2 exclusively in the developing neural tube of transgenic mouse embryos using regulatory sequences in the nestin gene that direct the expression to neural progenitors (Lothian & Lendahl, 1997). Compared to wild type littermates, transgenic embryos at E9.5 had smaller brains and spinal cords, and frequently exhibited improperly flexed bodies. At later stages (E11.5-E15.5) transgenic embryos died and displayed a number of abnormalities such as underdeveloped brains, lack of blood circulation and craniofacial defects.

Studies of the transgenic embryos revealed a reduced neuronal differentiation, assayed by β-tubulin III-labelling, and impaired neural crest migration, assayed by AP-2α/SOX10-labelling. This may explain the phenotypes since neural crest cells contribute to the developing heart and face. Interestingly, the neural tube of transgenic embryos had aberrant levels and distribution of the cytoskeletal protein actin,

suggesting that the effects were mediated by a cytoskeletal interaction (Fig 4). This is in agreement with a previous study where it was shown that KCC2 can interact with the actin cytoskeleton to promote dendrite formation independently of the ion transport function (Li *et al.*, 2007). It is possible that the effects were partly mediated by a premature switch in GABA action. However, similar results were obtained with a truncated form of KCC2, lacking the sequence for ion transport (KCC2-ΔNTD), suggesting that the effects were not GABA-dependent.

Other studies have also employed KCC2 overexpression models and demonstrated altered neuronal development (Cancedda *et al.*, 2007; Reynolds *et al.*, 2008). However, the use of an inactive KCC2 construct as control has not produced similar effects and the authors therefore concluded that a premature switch of GABA was the cause of the impaired development. Nevertheless, it is tempting to speculate whether the results from these studies are accurate. Cancedda et al. (2007) showed a reduction in dendritic length and branching when KCC2 was overexpressed and a "normal" phenotype using a mutated form of KCC2. The published figure did however not demonstrate this clearly as the phenotype appeared to differ also with inactive KCC2. In addition, Reynolds and coworkers (2008) did not show any data regarding the expression of KCC2 in their inactive control, which raises the question of whether the inactive KCC2 protein was actually expressed at all. There is however a possibility that a species difference accounts for the discrepancy between their results in zebrafish and our results in mice.

The mechanism leading to the cytoskeletal effects and subsequent disruption of neural cell differentiation and migration is unclear. KCC2 can interact with the cytoskeletal protein 4.1N (Li *et al.*, 2007). We therefore immunostained for 4.1N and observed an aberrant pattern of this protein in transgenic embryos, similar to the actin staining. Moreover, labelling for the gap junction subunit Connexin43 in wildtype embryos displayed a focused expression at extension processes of migrating neural crest cells, whereas the protein had a more even distribution around the plasma membrane of a large fraction of the neural crest cells in transgenic embryos. Indeed, Connexin43 has been reported to be expressed in contact points between radial fibres and migrating cortical neurons, in interaction with the internal cytoskeleton (Elias *et al.*, 2007). Thus, we suggest that KCC2 overexpression alters Connexin43 distribution through cytoskeletal effects, and this may in turn perturb neural crest migration.

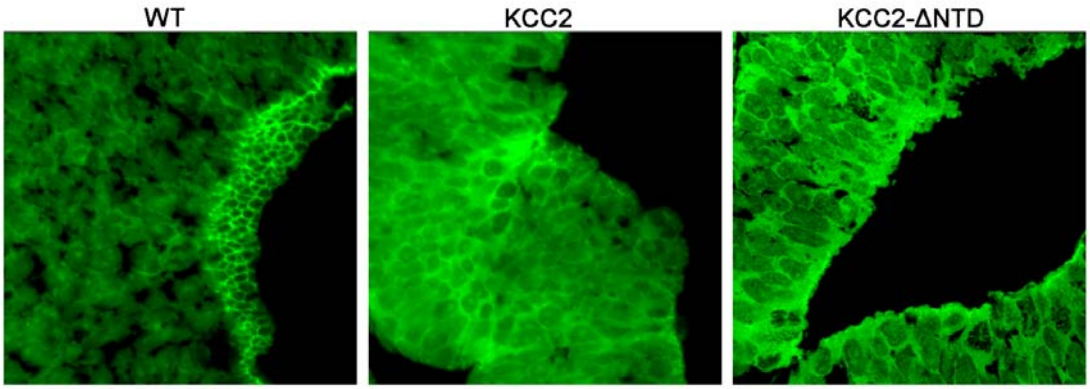


Fig 4. The neural tube of wild type and transgenic (KCC2 and KCC2-ΔNTD) embryos stained with FITC-Phalloidin to show the actin distribution.

3.2 WNT SIGNALLING REGULATES NEURONAL DIFFERENTIATION AND CYTOSKELETAL FUNCTIONS

A previous study by our group showed that Wnt7a overexpression had a severe effect on the neural tube development in mice. Already during neurulation, the most affected embryos failed to close the rostral neural tube. Less affected embryos displayed a successful closure but defective formation of telencephalic vesicles due to the loss of neural tube rigidity. This was found to be the result of disrupted adherens junctions, marked by a misdistribution of cytoskeletal components responsible for stability and rigidity in the neural tube (Shariatmadari *et al.*, 2005). Next, we extended this study and investigated a possible effect of Wnt7a on neural cell fate. Moreover, we examined downstream signalling molecules and their role in cell adhesion and migration.

3.2.1 Wnt7a delays neuronal differentiation (Paper II)

To assess the effect of Wnt7a on neuronal differentiation and patterning we employed the same transgenic mouse embryos used in our previous study (Shariatmadari *et al.*, 2005). These mice overexpress Wnt7a in the developing neural tube under control of an enhancer sequence from the nestin second intron, similar to the KCC2-overexpressing mice described above. Depending on the copy number and insertion sites of the transgene construct, the phenotypes of the embryos differed in severity. We studied neuronal differentiation at E9.5 and E10.5 in these embryos by labelling for β-tubulin III, an early neuronal marker. The neural tube of transgenic embryos at E9.5 displayed a significantly lower ratio of labelled cells (63% of wt, $p<0.05$) compared to wild type littermates. This was also evident at E10.5 where wild type embryos exhibited a high number of β-tubulin III positive cells in an ordered pattern in the periphery of the neural tube, while transgenic embryos displayed a lower ratio of labelled cells (43% of wt, $p<0.01$) in a more disordered pattern. The pattern of β-tubulin III positive cells in E10.5 transgenic embryos closely resembled that of E9.5 wild type embryos, suggesting a delay in neuronal differentiation. The extent of Wnt7a's effect on differentiation was correlated with the degree of overexpression, being more apparent in embryos with severe neurulation defects.

Wnt7a has been reported to both promote and delay neuronal differentiation. Explant cultures from E10.5 embryos transfected with HA-tagged Wnt7a showed a reduced frequency of β-tubulin III positive cells (Viti *et al.*, 2003). Another study revealed that HA-tagged Wnt7a decreased, while untagged Wnt7a increased neuronal differentiation in explant cultures from E11.5 embryos (Hirabayashi *et al.*, 2004), indicating that the HA-tag might have interfered with the activity of Wnt7a. However, we used untagged Wnt7a in our study and observed a similar effect to that where HA-tagged Wnt7a was used. Thus, it is likely that the role of Wnt7a in regulating neuronal differentiation is also dependent on the embryonic stage, suppressing at E9.5-10.5 and promoting at E11.5.

It is unclear whether the delay in neuronal differentiation is coupled to an increase in glial differentiation, altered proliferation or increased apoptosis. We therefore analyzed

cell proliferation using an antibody for phospho-histone 3, an M-phase marker. The frequency of labelled cells was significantly lower in transgenic embryos at E9.5 but not at E10.5. This suggests that the delay in neuronal differentiation at E9.5 and E10.5 may be due to a reduced proliferation at stages earlier than E10.5. However, assaying proliferation by BrdU incorporation did not reveal any significant reduction in E8.5 transgenic embryos (Shariatmadari *et al.*, 2005). Thus, it is possible that Wnt7a can delay neuronal differentiation without affecting proliferation. Our previous study also showed a significant increase in apoptosis in the neural tube of transgenic embryos, but since the number of apoptotic cells was still moderate and largely scattered, it does not seem to be a likely cause of the reduction in β-tubulin III positive cells.

Since cell fate depends on the environment, altered differentiation properties could be due to a change in the relative position of the cells. Therefore, we examined a potential antero-posterior shift of the neural tube in transgenic embryos by labelling for the telencephalic markers Otx1 and Otx2, and the midbrain marker En-2. However, we did not detect any differences between wild type and transgenic embryos, indicating that a positional shift was not the cause of the delayed neuronal differentiation.

3.2.2 The effects of Wnt7a are partly mediated by Vangl2 (Papers II and III)

An interesting finding in the Wnt7a-overexpressing embryos was that mRNA for the PCP gene Vangl2 was upregulated in the neural tube, as assayed by quantitative in situ hybridization (162% of wt, $p<0.05$). This indicates that Wnt7a had activated the PCP pathway and thereby mediated its effect on neuronal differentiation. Wnt7a has previously been shown to activate the PCP pathway in the mammalian cochlea, where it mediates the orientation of stereociliary bundles (Dabdoub *et al.*, 2003). However, in our study, it cannot be ruled out that an interaction with the canonical pathway had occurred. The defective neurulation in the transgenic embryos appeared to be a result of disrupted adherens junction components such as β-catenin, the key protein in the canonical pathway (Shariatmadari *et al.*, 2005). Indeed, the PCP and canonical pathways have been shown to interact both in an agonistic (Ciani & Salinas, 2007) and antagonistic (Weidinger & Moon, 2003) manner. We were however unable to detect active β-catenin in the nucleus of cells in the neural tube. In this context, provided that Wnt7a has been shown to promote neuronal differentiation through activation of the canonical pathway at E11.5 (Hirabayashi *et al.*, 2004), we suggest that Wnt7a stimulates the PCP pathway before E11.5, thereby counteracting the canonical pathway and neuronal differentiation.

To investigate this further, we created transgenic embryos overexpressing Vangl2 under control of the nestin second intron. In line with our previous results, embryos overexpressing Vangl2 phenocopied those overexpressing Wnt7a. Transgenic embryos at E8.5 and E9.5 displayed similar defects in convergent extension and cranial neurulation. This appeared also to be due to disrupted adherens junctions, as a patchy expression of actin, β-catenin, and N-cadherin was observed. Furthermore, cellular distribution of the PCP-components Scrb1, JNK and Rac1 was altered in transgenic embryos, suggesting an interaction of these proteins with Vangl2 signalling. The misdistribution was manifested by a loss of Scrb1 and Rac1 enrichment at the adherens junctions and a more diffuse cytoplasmic staining in the neural tube. JNK was observed

as intracellular granules in the neural tube cells of wild type embryos, while these granules were lost in transgenic embryos. No evident effects could be detected on the expression of RhoA, ROCK and β-pix, which all have been reported to interact with Rac1 (Flaiz *et al.*, 2008a).

To compare these effects with Vangl2 loss-of-function, we employed loop-tail embryos ($lp^{-/-}$) with a point mutation in Vangl2. These embryos exhibit craniorachischisis, a failure to close the neural tube from the hindbrain and caudally (Torban *et al.*, 2007). We examined embryos at E9.5 and detected a disruption of adherens junction components comparable to Vangl2-overexpressing embryos. Moreover, Scrb1 and Rac1 were similarly misdistributed. Interestingly, the JNK granules appeared to be increased in the neural cells of loop-tail embryos, as opposed to the transgenic embryos, indicating that JNK might be suppressed by Vangl2 signalling.

Taken together, Wnt7a appears to signal via Vangl2 to regulate convergent extension, neurulation and neuronal differentiation. This is further supported by a reduction in β-tubulin III expression in Vangl2-overexpressing embryos (unpublished data). Moreover, Vangl2 signalling affects Scrb1, Rac1 and JNK expression in the neural tube. Both Vangl2 overexpression and loss-of-function results in a similar phenotype, which has also been reported before (Darken *et al.*, 2002; Park & Moon, 2002) and can be explained by the idea that the PCP signal must be fine-tuned to an appropriate level that is essential for proper cell movements during gastrulation and neurulation.

3.2.3 Vangl2 regulates cell adhesion and migration in interaction with Rac1 (Paper III)

To further analyze the cytoskeletal effects of Vangl2 in vitro, we used the epithelial cell lines HEK293T and MDCK. These cell lines were chosen as they grow in monolayers, closely attached to each other, thereby suitable for studying cell adhesion. Cells were transfected with either a control plasmid or a Vangl2-plasmid where the expression is driven by a CMV promoter. The actin cytoskeleton was analyzed after 24-48 hours, stained with FITC-phalloidin. While control-transfected cells grew in large monolayers and with circumferential actin connecting cells to each other, Vangl2-transfected cells often grew in smaller layers, more loosely attached to each other, and had a more discontinuous pattern of actin. Co-transfection with a GFP-actin plasmid to assess the effect on newly produced actin revealed a disrupted formation of the cytoskeleton in cells overexpressing Vangl2. Intracellular clusters of actin were often observed in these cells. In addition, labelling for β-catenin and N-cadherin showed a more diffuse pattern in Vangl2-transfected cells, indicating that other cytoskeletal components had also been altered.

Since PCP signalling has been shown to involve the small GTPases RhoA and Rac1, we employed DNA plasmids coding for constitutive active (RhoV14, RacV12) or dominant negative (RhoN19, RacN17) proteins, which 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. Interestingly, co-transfecting Vangl2 with RacN17 rescued the disrupted actin localization back to normal. The same result was obtained when RacN17 was

substituted with Rac1 RNAi. Moreover, co-transfecting Vangl2 with RacV12 potentiated the effects on the cytoskeleton. However, no interactions could be seen when co-transfecting Vangl2 with RhoV14 or RhoN19. This suggests that Vangl2 affects the actin cytoskeleton in interaction with Rac1, but not with RhoA. The finding that the actin distribution was not affected with Vangl2 and RacN17 (or Rac1 RNAi) together, implies that Vangl2 mediates its effects via Rac1 or in parallel with Rac1. This was further supported by immunostainings of transfected cells, where Vangl2 altered the distribution of Rac1, but not RhoA. However, immunoprecipitation assays showed that Vangl2 binds to both Rac1 and RhoA but does not affect their activity. Thus, we suggest that Vangl2 acts by distributing Rac1 within the cell.

Since Vangl2 could disrupt the actin cytoskeleton we investigated whether cell adhesion was also affected. For this purpose we used MDCK cells because they adhere firmly to each other. We performed aggregation assays where transfected cells were grown in hanging drop cultures and were then exposed to mechanical dispersion. This revealed that Vangl2 significantly reduced cell aggregation compared to control-transfected cells (59% of control, $p<0.05$). Co-transfecting Vangl2 with RacN17 restored the normal ability to aggregate, whereas co-transfection with RacV12 potentiated the reduction in aggregated cells. Again, no interaction was observed with Vangl2 and RhoV14 or RhoN19. These results support the above findings on actin distribution, suggesting that Vangl2 can alter cell adhesion by cytoskeletal modulation.

Loss of cell adhesion can often promote migration (Fischer & Quinlan, 1998). We therefore examined the effect of Vangl2 on cell migration in wound assays (Fig 5). The neural stem cell line C17.2 was chosen for this purpose since they do not adhere to each other as firmly as the epithelial cell lines, thereby enabling a straight cut to be scratched through the cell sheet. A significantly higher number of cells transfected with Vangl2 invaded the open "wound" area compared to control-transfected cells (140% of control, $p<0.05$). Co-transfection with RacN17 blocked the effects of Vangl2, in line with our previous findings.

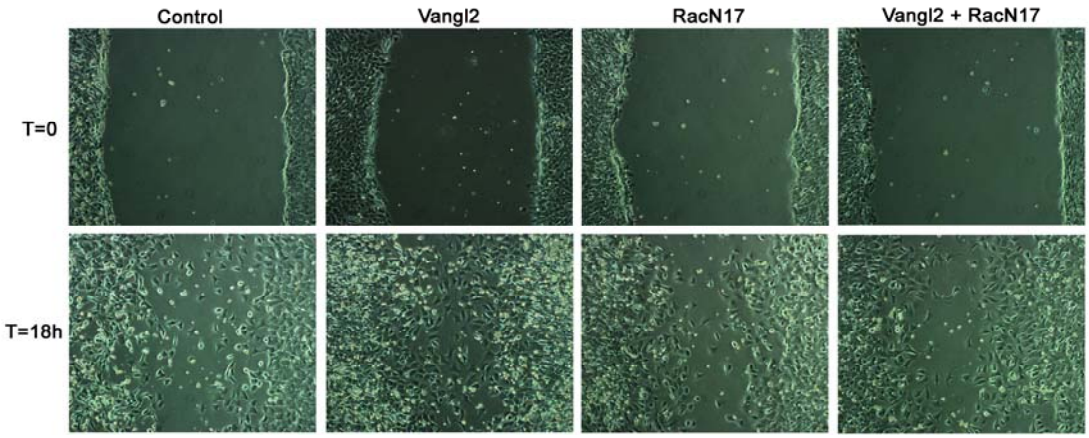
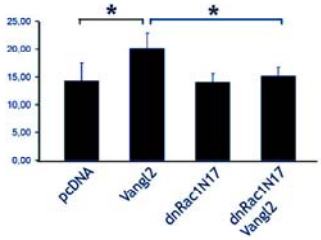


Fig 5. Cell migration analyzed in wound assay. The diagram shows the quantification of cells invading the "wound" after 18 hours. Increased cell migration was observed in Vangl2-transfected cells, and this was restored by co-transfecting with RacN17.



The interaction of Vangl2 with Rac1 found in vitro is consistent with the in vivo finding that Rac1 is redistributed in the neural tube of Vangl2-overexpressing embryos. The other PCP-components affected in vivo, Scrb1 and JNK, could also be part of the effects seen in vitro. Vangl2 has already been shown to immunoprecipitate with Scrb1 (Montcouquiol *et al.*, 2006). Moreover, both JNK and Scrb1 have been shown to regulate cell migration (Yamanaka *et al.*, 2002; Dow *et al.*, 2007). We therefore assessed whether the distribution of these proteins were affected in Vangl2-transfected HEK293T cells. Indeed, the expression of JNK correlated with that observed in the transgenic embryos; Vangl2 transfection decreased intracellular JNK "granules". Moreover, whereas control-transfected cells displayed a clear Scrb1-labelling close to the plasma membrane, Vangl2 transfection resulted in a more diffuse labelling of Scrb1. Intriguingly, co-transfecting Vangl2 with Rac1 RNAi further reduced Scrb1 around the plasma membrane and produced an even more diffuse intracellular staining, which was almost undetectable. This implies that an interplay exists between Vangl2, Rac1 and Scrb1. It is possible that a reduction in Rac1 levels makes Vangl2 more accessible for binding to Scrb1.

In sum, Vangl2 regulates the actin cytoskeleton and thereby cell adhesion in epithelial cell lines and rigidity in the neural tube. We also showed that Vangl2 mediates these effects in interaction with Rac1. This supports previous reports that Rac1 is involved in regulating the stability of adherens junctions (Quinlan, 1999; Flaiz *et al.*, 2008b). Moreover, JNK and Scrb1 may also play a role in the observed effects and signify the importance of a balanced expression of PCP molecules for correct cell behaviour.

3.3 SIMILARITIES AND DIFFERENCES IN CYTOSKELETAL EFFECTS BETWEEN KCC2 AND WNT SIGNALLING GENES

The effects of both KCC2 and Wnt7a/Vangl2 overexpression involved an interaction with the cytoskeleton. In both cases, the cytoskeletal effects were observed early in development during critical periods of neural tube formation and patterning. Both KCC2 and Wnt7a/Vangl2 reduced the ratio of differentiating neurons. Whether this was due to the cytoskeletal effects is still unclear. However, Wnt7a/Vangl2 disrupted the adherens junctions leading to impaired neurulation, while this was unaffected by KCC2. Instead, KCC2 overexpression resulted in aberrant levels and/or distribution of actin in the cells of the neural tube, thereby affecting body flexure. In addition, KCC2 perturbed neural crest migration, whereas Wnt7a/Vangl2 affected cell movements already during convergent extension.

Vangl2 was shown to disrupt the actin cytoskeleton in epithelial cell lines, in interaction with Rac1. This included intracellular aggregates of actin in HEK293T and stress fiber induction in MDCK. Stress fibres are common in cultured cells and play a role in cell adhesion, contractility, morphogenesis and migration (Pellegrin & Mellor, 2007). Interestingly, cultured C17.2 cells displayed stress fibres, which were disrupted by KCC2 transfection (preliminary data). Cells overexpressing KCC2 were more round in shape and often exhibited intracellular clusters of actin, similar to HEK293T cells overexpressing Vangl2. The effect of Vangl2 on the actin formation in C17.2 has not yet been tested. However, Vangl2 transfection increased migration of C17.2 cells, implying that the cytoskeleton in these cells was also affected by Vangl2.

4 CONCLUSIONS

- The appearance of KCC2 in the plasma membrane parallels the developmental GABA switch in the respiratory centres. KCC2 is present in the cytoplasm before the functional switch.

- Neural-specific overexpression of KCC2 at early embryonic stages interferes with neuronal differentiation and neural crest migration by an ion-independent mechanism. This appears to be due to an interaction with the cytoskeleton.

- Wnt7a delays neuronal differentiation and upregulates Vangl2 in the neural tube of transgenic mouse embryos.

- Overexpression of Vangl2 in neural progenitors causes defective neural tube formation and altered components of the adherens junctions in mouse embryos.

- Vangl2 acts together with Rac1 to regulate cell adhesion and migration in epithelial and neural stem cell lines.

5 RELEVANCE AND FUTURE STUDIES

This thesis describes cytoskeletal effects of two different developmental systems. One of these, the Wnt signalling system, has a well established role in regulation of the cytoskeleton and related cell functions already early in development when the neural tube forms. The other one, KCC2, has only recently been ascribed to a novel role in cytoskeletal organization. However, previous studies have not described KCC2 in interaction with the cytoskeleton prior to the formation of dendritic spines. Thus, the results presented in this thesis are the first showing that KCC2 can interact with the cytoskeleton of neural cells as early as during neural tube patterning and neural crest migration.

Further studies of the KCC2-overexpressing embryos include additional pronuclear injections with a newly created DNA-construct, based on the same form of inactive KCC2 (KCC2-C568A) used in the study by Reynolds et al. (2008). This will be done in order to assess the validity of their results as they did not report any abnormal effects with this mutation. It is possible, although unlikely, that this point mutation produces a protein that is unable to interact with the actin cytoskeleton.

Moreover, this thesis shows that the developmental GABA switch and KCC2 membrane expression correlate in the respiratory center. This demonstrates the importance of KCC2 for respiratory development. Further investigation of KCC2 activation in the brainstem can be done using slice cultures according to the "Stoppini method" (Stoppini *et al.*, 1991). Studies of the respiratory center in VLM (preBötC and pFRG) at stages around birth (E18 – P3) will then reveal a better understanding of the role of KCC2 for respiratory function. This may be achieved by application of substances (eg. oxytocin, BDNF) to the medium and examination of subsequent effects on the expression pattern of KCC2. In addition, this method also enables transfection of cells in brainstem cultures using gene gun to overexpress genes (eg. BDNF, KCC2) exclusively in the respiratory center.

Wnt signalling is highly complex and this thesis lays another small piece to the gigantic Wnt puzzle. We demonstrated that Wnt7a regulates neuronal differentiation, possibly via the PCP pathway, in which Vangl2, Rac1, Scrb1 and JNK were shown to interact. This increases our understanding of how planar cell polarity genes are regulated in mammals. In addition, our results show that altered PCP signalling leads to neurulation defects and thus contributes to the research on NTDs. In fact, NTDs, including spina bifida and anencephaly, are among the most common birth defects with an incidence that ranges between 1 and 8 per 1000 births depending on the geographical location. Furthermore, the effect of Vangl2 on cell adhesion and migration does not only implicate a role in convergent extension movements, but may also be involved in tumour progression.

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